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# OUTPUT 1. Improved characterization of the genetic diversity of wild and cultivated species and associated organism

# Activity 1.1. Characterization of Genetic Diversity

## 1.1.1 Using SNP haplotypes to study gene flow in a *P. vulgaris* wild-weedy-crop complex from Colombia: A pilot study.

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### Introduction

Chloroplast haplotypes have been successfully used to determine gene flow direction in *P. vulgaris* populations from Costa Rica (González-Torres *et al,* 2003, 2004). The study of "wild-weedy-cultivated" complexes along common bean range of distribution in the Americas show no preferential gene flow direction in Colombia, Ecuador and Peru (Chacón *et al*, 2006). We conducted a pilot study in the wild-weedy-crop complex G50879 from Colombia (19, wild; 32 weedy and 34 cultivated), previously evaluated with biochemical markers and SSRs, to test the potential of nuclear SNP markers as an alternate tool for the identification of gene flow events and inference about their direction.

### Materials and methods

85 individuals from G50879 wild-weedy-crop complex were genotyped with 20 SNPs located in linkage groups B01 and B07 (where most of the genes and markers related to the domestication syndrome are located according to Koinange *et al*, 1996). The single base extension method on a Luminex$^{100}$ platform (Quintero *et al*, 2004) was used for SNP allele scoring. As codominant markers, SNPs were useful in identifying heterozygotes. SNP blocks and haplotypes were inferred using the HAP software (Halperin & Eskin 2004; URL: http://research.calit2.net/hap/WebServer.htm).

### Results

We addressed first, some characteristics of population structure and we found that percent polymorphic loci, heterozygosity and diversity were considerably higher in biological forms of the complex than in wild populations used as checks (Table 1), weedy forms having the highest values (except for diversity index).

Table 1. Polymorphic SNPs, heterozygosity and diversity of biological forms in the wild-weedy-cultivated complex G50879.

| Gnumber | Status | Polymorphic Loci (%) | h[1] | I[2] |
|---|---|---|---|---|
| G21117 | Wild check | 10 | 0.010 | 0.041 |
| G23996 | Wild check | 20 | 0.045 | 0.096 |
| G50879 | Wild | 80 | 0.129 | 0.413 |
| | Weedy | 95 | 0.182 | 0.445 |
| | Domesticated | 80 | 0.148 | 0.455 |

[1]Observed heterozygosity.
[2]Shannon (1949) diversity index.

Block partition and haplotype prediction within each block was done using HAP software. Both linkage groups (B01 and B07), each having 10 SNPs, were divided in three blocks of limited diversity. Total number of inferred haplotypes was 43, block 3 (B07) having the least number of haplotype variants and block 2 (B01) having the highest diversity of haplotypes (Table 2).

Polymorphism information content (PIC) for each block was calculated and three were found to have values higher than 0.7 (Table 2), similar to the SSRs evaluated by González (2004), when studying gene flow in Costa Rica.

Table 2. Haplotype blocks inferred in linkage groups B01 and B07.

| LG | Block | SNPs (#) | Haplotypes (#) | PIC |
|---|---|---|---|---|
| B01 | 1 | 3 | 8 | 0,702 |
| B01 | 2 | 5 | 12 | 0,756 |
| B01 | 3 | 2 | 4 | 0,310 |
| B07 | 1 | 6 | 11 | 0,731 |
| B07 | 2 | 3 | 6 | 0,498 |
| B07 | 3 | 1 | 2 | 0,288 |

Common and rare variants of haplotypes were found. Wild checks G21117 and G23996 had no more than two haplotypes per block, which agrees with their uniformity in seed size and color. In contrast, all 43 SNP haplotypes were observed in the wild-weedy–crop complex: 84% in weedy forms, 77% in cultivated forms and 70% wild forms.

With the admixture model proposed by Bertorelle & Excoffier (1998) and used by Papa & Gepts (2003), we estimated the relative contribution of both wild and cultivated parental forms, to the weedy types, with the ADMIX1_0 software developed by G. Bertorelle (California University, Berkeley, URL: http://web.unife.it/progetti/genetica/Giorgio/giorgio_soft.html). Then the admixture coefficient $M_Y$ with 1000 resampling events, based on haplotype frequencies and the molecular distances between haplotypes. We named *Mw*, the relative contribution of wild forms and *Mc* that of the cultivated forms. The admixture coefficient for wild forms (0.58 ± 0.12) was slightly higher than that of the cultivated (0.42 ± 0.16), which meant *a priori* that the contribution of wild forms to the weedy types would be 1.34 times greater.

The standard deviation of the calculated coefficients was considerably higher than that reported by Papa & Gepts (2003) when studying wild-weedy-crop complexes from Mexico. According to Hurles *et al* (2003), the confidence of the estimated coefficient relies on the ability to identify clearly distinct parental populations that have been involved in the admixture process. Then, we decided to look at the distribution of SNP haplotypes within and between biological forms, to refine parental populations. The most

informative haplotype blocks (PIC values higher than 0.7) were selected: blocks 1 and 2 in linkage group B01, and block 1 in linkage group B07 (Table 2).

Differences between haplotype frequencies allowed us to identify those haplotypes that better described each of the parental biological forms. In most cases, haplotypes were classified as wild types when their frequency was at least twice the observed in cultivated forms, and vice versa (Figure 1).

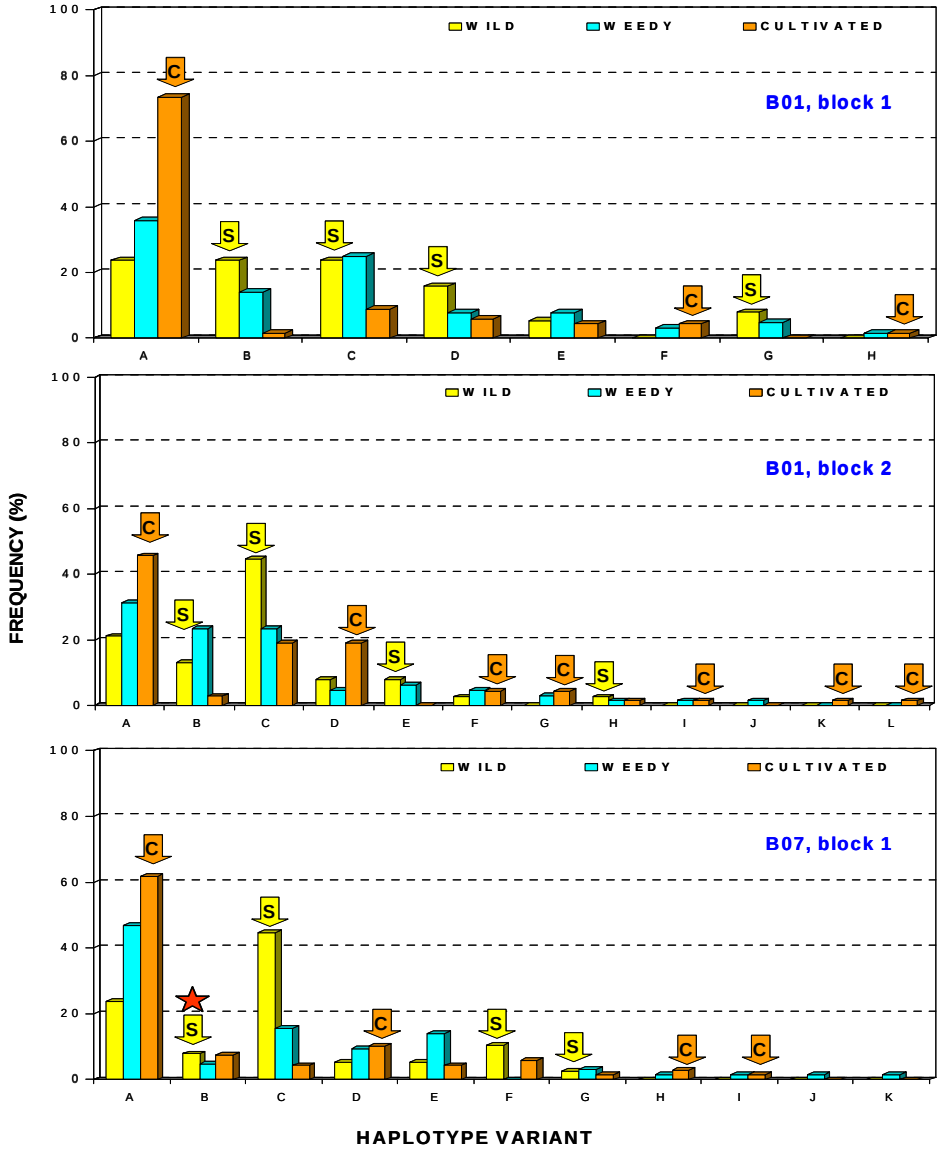


Figure 1. Haplotype frequency in the wild-weedy-crop complex G50879.

Inside wild and cultivated forms, not all the individuals coincide in phenotype (seed weight) and genotype (SNP haplotypes). We defined as truly wild, those individuals that had wild type haplotypes in the three blocks, together with low seed weight (less than 14g/100seeds). Similar criteria were applied to the cultivated materials and then a truly cultivated parental population was defined. When we looked at weedy forms, some materials were added to the corresponding parental population if they were close in phenotype and coincide in genotype. We proposed that parental truly-wild and truly-cultivated selected as above, could give rise to three hybrid subpopulations in this wild-weedy-crop complex: a wild-hybrid, a

cultivated-hybrid and a weedy. Admixture analysis was performed again, considering the three separated cases and the relative contributions of truly wild and cultivated were estimated (Table 3).

Table 3. Admixture analysis in the wild-weedy-crop complex G50879 considering three possible hybrid subpopulations.

| 1. Weedy | | | | |
|---|---|---|---|---|
| *Ms*[1] | SD[2] | *Mc*[1] | SD | *Mc/Ms* |
| 0,4458 | 0,0436 | 0,5518 | 0,043 | 1,2 |
| 2. Wild-hybrid | | | | |
| *Ms* | SD | *Mc* | SD | *Mc/Ms* |
| 0,4669 | 0,055 | 0,5281 | 0,0588 | 1,1 |
| 3. Cultivated-hybrid | | | | |
| *Ms* | SD | *Mc* | SD | *Mc/Ms* |
| 0,3237 | 0,0501 | 0,6766 | 0,0495 | 2,1 |

[1]1000 resampling events

[2]Standard deviation

In the weedy and wild-hybrid subpopulations, the contribution of truly-cultivated was slightly higher than that of truly-wild meaning that both gene flow directions are possible and almost symmetric. For the cultivated-hybrid, the contribution of truly-cultivated was twice that of the truly-wild, suggesting more pollen flow from the cultivated types. Since the sample size of this pilot study is quite low, the analysis of more Colombian wild-weedy-crop complexes through SNP haplotypes is required.

The use of biallelic SNPs for genetic diversity studies has been controversial since they are less informative than multiallelic SSRs (Brumfield *et al*, 2003; Morin *et al*, 2004). Although we have looked only at one wild-weedy-crop complex from Colombia our results show that SNP haplotypes are informative enough to provide evidence about gene flow dynamics.

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### 1.1.2 Phaseolin: variability and standards in wild and cultivated common beans.

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**Introduction**

Phaseolin – the most important seed storage protein in common bean – has been used as an easy-to-detect marker in evolutionary studies and assessment of genetic diversity. We report on phaseolin types not published previously and we establish standards for phaseolin morphotypes which are available internationally as genetic stocks.

**Materials and Methods**

The accessions which are reported here were obtained from the world collection held in CIAT (Table 5). The samples were analyzed in one dimension-SDS-PAGE (Brown et al. 1981) and confirmed later in two dimension-IEF-SDS-PAGE (O'Farrel 1975).

**Results and Discussion**

Variability in banding patterns of phaseolin has been found in the Mesoamerican and the Andean centers of diversity, among wild, weedy and cultivated accessions. Even though this globulin has a narrow range of molecular weights (45-52 kD) and isoelectric points, a total of 63 banding patterns has been found so far, 31 being present in Mesoamerican materials, 21 in the Andean region, 8 in Colombia, 2 in Mesoamerica and Colombia, and 1 in the Mesoamerican and Andean regions (Table 5). More phaseolin types have been found in wild accessions as compared to the cultivated bean, suggesting a founder effect upon domestication. However, the founder effect may not be as severe as previously thought. The accessions reported here as the source of each phaseolin morphotype is maintained at CIAT, and small quantities of seed are available for research.

Table 5. Diversity of phaseolins and reference materials in wild, weedy and cultivated common bean.

| No. | Phaseolin Types | Number G | Standards (Phs morphotypes) | Biological Status[1] | Genetic Pool[2] | Country of origin[3] |
|---|---|---|---|---|---|---|
| 1 | S | G12853 | FI-2380 | WILD | M | GTM |
| 2 | Sb | G12952 | FI-5416 | WILD | M | MEX |
| 3 | Sd | S33761 | FI-2881 | CULT | M | COL |
| 4 | M1 | G23418 | FI-5824 | WILD | M | CRI |
| 5 | M2 | G23652 | FI-1930 | WILD | M | MEX |
| 6 | M3 | G12865 | FI-1389 | WILD | M | MEX |
| 7 | M4 | G23678 | FI-1697 | WILD | M | MEX |
| 8 | M5 | G12851 | FI-4068 | WILD | M | GTM |
| 9 | M6 | G24365 | FI-1712 | WILD | M | MEX |
| 10 | M7 | G12869 | FI-1415 | WILD | M | MEX |
| 11 | M8 | G12879 | FI-4414 | WILD | M | MEX |
| 12 | M9 | G12878 | FI-1457 | WILD | M | MEX |
| 13 | M10 | G11034 | FI-1363 | WILD | M | MEX |
| 14 | M11 | G50869 | FI-3657 | WDY | M | COL |
| 15 | M12 | G10002 | FI-1304 | WILD | M | MEX |
| 16 | M13 | G23439 | FI-3144 | WILD | M | GTM |
| 17 | M14 | G12853 |  | WILD | M | GTM |
| 18A | M15 | G24365 | FI-1714 | WILD | M | MEX |
| 18B | M15 | G11027 A | XX-61 | WEEDY | M | MEX |
| 19 | M16 | G50726 | FI-3976 | WILD | M | HND |
| 20 | M17 | G12882 A | FI-1504 | WILD | M | MEX |
| 21 | M18 | G12855 A | FI-2390 | WILD | M | GTM |
| 22A | M19 | G12854 | FI-3349 | WILD | M | GTM |
| 22B | M19 | G19907 | FI-1273 | WILD | M | GTM |
| 23 | M20 | G24584 | FI-5419 | WILD | M | MEX |
| 24 | M21 | G2721 | FI-4045 | CULT | M | PER |
| 25 | M22 | G23511 A | FI-1629 | WILD | M | MEX |
| 26 | M23 | G12890 | FI-4089 | WILD | M | MEX |
| 27 | M24 | G12949 | FI-1923 | WILD | M | MEX |
| 28 | M25 | G12851 | FI-4070 | WILD | M | GTM |
| 29 | M26 | G23434 A | FI-28 | WDY | M | GTM |
| 30 | T | G50015B | FI-2838 | WDY | A | ARG |
| 31 | To1 | G24776 | FI-4454 | CULT | A | COL |
| 32 | To2 | G23786B | FI-4456 | CULT | A | PER |
| 33 | Ta | G23445 | FI-1029 | WILD | A | BOL |
| 34 | Tcaj | G23600 | XX-72 | CULT | A | PER |
| 35 | K | G23422 | FI-4105 | CULT | A | PER |
| 36 | Ko | G23814 | FI-4655 | CULT | A | PER |
| 37 | H1 | G51049 | FI-2753 | CULT | A | COL |

| | | | | | | |
|---|---|---|---|---|---|---|
| 38 | H2 | G50401 | FI-2514 | CULT | A | COL |
| 39 | C | G21194 | FI-4188 | WILD | A | ARG |
| 40 | Ca | G12857 | FI-1747 | WILD | A | PER |
| 41 | Ca1 | G50850 | FI-3847 | CULT | A | COL |
| 42 | B | G24717 | FI-2038 F2 | CULT | M/COL | COL |
| 43 | J1 | G19895 | FI-998 | WILD | A | ARG |
| 44 | J2 | G23592 | FI-934 | WILD | A | ARG |
| 45 | J3 | G19902 | FI-976 | WILD | A | ARG |
| 46 | J4 | G21194 | FI-4190 | WILD | A | ARG |
| 47 | CH | G50886 | FI-3716 $F_2$ | WDY | A/M | COL |
| 48 | P1 | G23423 | FI-1805 | WILD | A | PER |
| 49 | Pa | G23455 | FI-1831 | WILD | A | PER |
| 50 | L | G24408 | FI-2121 | WILD | COL | COL |
| 51 | LI | G51019 | FI-2493 | CULT | COL | COL |
| 52 | CAR | G50843 | FI-2432 | CULT | COL | COL |
| 53 | HE | G51006 | FI-2490 | CULT | COL | COL |
| 54 | TI1 | G51048 | FI-2849 | CULT | COL | COL |
| 55 | TI2 | G51036 | FI-2896 | CULT | COL | COL |
| 56 | I | G21244 | FI-1 | WILD | A | PER |
| 57 | A | G12857 | FI-1748 | WILD | A | PER |
| 58 | A1 | G12078 | FI-4842 | CULT | A | PER |
| 59 | Qui | G24674 | FI-4421 | CULT | COL | COL |
| 60 | Mu | Ent 12, retrocruza | FI-7118 | CULT | COL | COL |
| 61 | Dur | G11027 | MEXDU-01 | WILD | M | MEX |
| 62 | Tel | G18970 | FI-5791 | CULT | M/COL | CRI |
| 63 | Nvo1? | DGD3195 | FI-12661 | HYBRID | M | CRI[4] |

[1]Biological Status: WILD (Wild), WDY (Weedy), CULT (Cultivated)
[2]The common bean genetic pools: M (Mesoamerican); A (Andean); COL (Colombia).
[3]Country of origin: MEX (Mexico), GTM (Guatemala), HND (Honduras), CRI (Costa Rica), COL (Colombia), PER (Peru), BOL (Bolivia), ARG (Argentina).
[4]The germplasm analyzed to find this new phaseolin is part of the project "Gene flow in *Phaseolus*".

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### 1.1.3 Determination of gene flow among bean species (*Phaseolus* ssp.) from Colombia and Costa Rica with help of molecular markers

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GRU Project

**Introduction**

In the course of our project on gene flow on the bean model, because of past field work, we were wondering whether any other closely related bean species would take part in the natural introgression. We present hereafter evidence on interspecific gene flow events among bean species (*Phaseolus* ssp.) in natural conditions of Colombia and Costa Rica using molecular markers along our previous work (González-Torres et al. 2006). We selected a total of 150 individuals possibly resulting from interspecific hybridizations and 21 individuals as controls of the possible species involved (Table 1 and 2, respectively). The individuals were analyzed as reported by González-Torres et al. (2006) including for the first time the analysis of chloroplast DNA.

Table 1. Identification of *Phaseolus* ssp. materials and number of individuals analyzed in the interspecific gene flow evaluation.

| Country | Department | Accesion | Species | |
|---|---|---|---|---|
| COL | Nariño | G 12709 | *P.* x *vulgaris* | N= 1 |
| COL | Cundinamarca | G24628 | *P.* x *vulgaris* | N= 3 |
| COL | Cundinamarca | G24628B | *P.* x *vulgaris* | N= 1 |
| COL | Cundinamarca | G24628E | *P.* x *vulgaris* | N= 3 |
| COL | Cundinamarca | G24661 | *P.* x *vulgaris* | N= 3 |
| COL | Cundinamarca | G24661A | *P.* x *vulgaris* | N= 1 |
| COL | Cundinamarca | G24666 | *P.* x *vulgaris* | N= 1 |
| COL | Cundinamarca | G24666A | *P.* x *vulgaris* | N= 5 |
| COL | Cundinamarca | G24666D | *P.* x *vulgaris* | N= 2 |
| COL | Boyacá | G24764 | *P.* x *vulgaris* | N= 7 |
| COL | Boyacá | G24765 | *P.* x *vulgaris* | N=15 |
| COL | Boyacá | G24765A | *P.* x *vulgaris* | N= 8 |
| COL | Boyacá | G24765B | *P.* x *vulgaris* | N=11 |
| COL | Boyacá | G24765C | *P.* x *vulgaris* | N=12 |
| COL | Boyacá | G24765D | *P.* x *vulgaris* | N= 9 |
| COL | Boyacá | G24765E | *P.* x *vulgaris* | N=14 |
| COL | Boyacá | G24765F | *P.* x *vulgaris* | N=10 |
| COL | Boyacá | G24765G | *P.* x *vulgaris* | N= 9 |
| COL | Boyacá | G24765H | *P.* x *vulgaris* | N= 2 |
| COL | Boyacá | G24765I | *P.* x *vulgaris* | N= 3 |
| COL | Boyacá | G24765J | *P.* x *vulgaris* | N= 5 |
| COL | Boyacá | G24765K | *P.* x *vulgaris* | N= 4 |
| COL | Boyacá | G24766 | *P.* x *vulgaris* | N= 2 |
| COL | Boyacá | G24767 | *P.* x *vulgaris* | N=14 |
| COL | Boyacá | G24767A | *P.* x *vulgaris* | N= 1 |
| COL | Boyacá | G24767B | *P.* x *vulgaris* | N= 1 |
| COL | Boyacá | G24767C | *P.* x *vulgaris* | N= 1 |
| COL | Boyacá | G24767D | *P.* x *vulgaris* | N= 2 |

Table 2. Identification of *Phaseolus* species used as control for comparisons with the possible hybrids.

| Identification | Specie |
|---|---|
| COC1634. | *P. dumosus* |
| COC 1440 | *P. dumosus* |
| G35758 | *P. dumosus* |
| G35877 | *P. dumosus* |
| COC 1396 | *P. dumosus* |
| 11280 | *P. vulgaris* |
| 11429 | *P. vulgaris* |
| 9590 | *P. vulgaris* |
| 11390 | *P. vulgaris* |
| 6744 | *P. vulgaris* |
| COC 1653 | *P. coccineus* |
| COC 1531 | *P. coccineus* |
| COC 1533 | *P. coccineus* |
| COC 1280 | *P. coccineus* |
| COC 1718 | *P. coccineus* |
| DGD 2095 | *P. costarricensis* |
| DGD 2102 | *P. costarricensis* |
| DGD 2116 | *P. costarricensis* |
| DGD 3120 | *P. costarricensis* |
| S 29699 | *P. costarricensis* |
| PL 3592 | *P. albescens* |

**Results**

In our previous work (González Torres et al. 2006) we found that the evaluated microsatellites were of high discriminatory power (PIC), in spite of the fact that many *loci* belong to the ancestral evolutive *phylum*, and are thus shared. For that reason, we proposed to evaluate another 39 SSR *loci* (with high PIC) using fluorescent techniques that increase the resolution of alleles. Some technical aspects of PCR conditions were improved as shown in Figure 1.

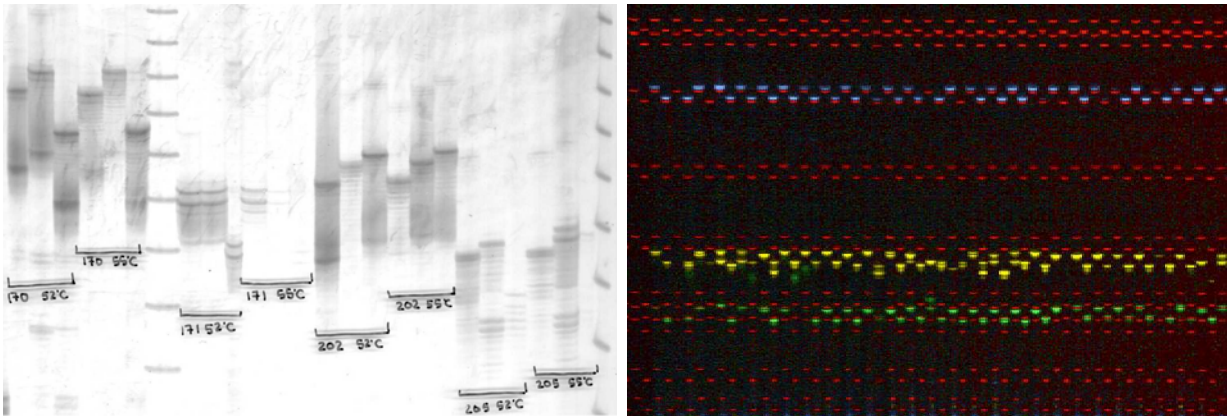

Figure 1. Left: polymorphisms of microsatellite using silver staining; Right: polymorphisms of microsatellite using primer fluorescents.

At the moment, we have run the electrophoresis of all SSR *loci* of the selected set of individuals and the analyses of the gels using GeneScan and Genotyper softwares have been realized. The objective is to analyze parameters of genetic diversity using POPGENE and TFPGA softwares and the contribution of each species to the hybrid population using ADMIX software and MCA with SAS.

The determination of chloroplast haplotypes has been conducted using polymorphism of ten non-coding regions of chloroplast DNA. The PCR restriction fragments of length polymorphism (RFLP) were analyzed using the chloroplast regions proposed by Chacón (2001). The restriction site patterns found in the interspecific hybrids and alien species were significantly different from the ones disclosed in *P. vulgaris* materials (Table 3) by Chacón et al. (2005).

Table 3. Description of ten chloroplast haplotypes found into *Phaseolus* species and interspecific hybrids.

| | | *AccD-psaI* | *NdhA intron* | *Rps14-psaB* | | *TrnL intron* | | *TrnL-trnF* | | | *TrnT-trnL* | *RpL16 intron* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Haplotype* | *Species and frequency* | *DdeI* | *DraI* | *Tsp509I* | *AluI* | *HphI* | *MseI* | *Tsp509I* | *VspI* | *SspI* | *RsaI* | *DraI* | *PacI* |
| H1 | *Weedy (1/148)* | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 |
| H2 | *Weedy (1/148)* | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 |
| H3 | *P.vulgaris (1/5) P.dumo (5/5) P.costar. (4/5) P.coccin. (5/5) P.albesc (1/1)* | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 |
| H4 | *Weedy* | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 0 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *(8/148)* | | | | | | | | | | | | |
| H5 | *P.vulgaris (1/5) Weedy (100/148)* | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| H6 | *P.costar. (1/5)* | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| H7 | *Weedy (5/148)* | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 0 |
| H8 | *P.vulgaris (3/5)* | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 0 | 1 |
| H9 | *Weedy (1/148)* | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 |
| H10 | *Weedy (1/148)* | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 |

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González-Torres, RI, M. Carvajal, O. Toro, M.C. Duque, R. Araya & D.G. Debouck. 2006. Annual Report of the Bean Improvement Cooperative 49: 135-136.

### 1.1.4 Determination of gene flow in common bean in the Central Valley of Costa Rica

Rosa González, Walter Barrantes, Harold Suárez & D.G. Debouck.
GRU Project

**Introduction**

The gene flow event was evidenced in materials of a newly collected year (2006) in Quircot (Central Valley- Costa Rica) using morphological, biochemical and molecular markers as mentioned in our previous works (González –Torres et al. 2003, 2004). In this case, a total of 153 individuals of common bean were evaluated belonging to the Costa Rican complex (43 weedy, 47 cultivated and 63 wild materials) from different locations of Quircot as shown in Table 4.

Table 4. Identification of evaluated populations

| No. Population | Biological status | Geographical localization |
|---|---|---|
| 1 | Weedy | Sector 2 |
| 2 | Weedy | Sector 3 |
| 3 | Weedy | Sector 4 |
| 4 | Weedy | Sur quebrada |
| 5 | Weedy | *P.costaricensis* |
| 6 | Cultivated | Parcela Tali |
| 7 | Cultivated | Parcela Tali |
| 8 | Wild | Sector 2 |
| 9 | Wild | Sector 3 |
| 10 | Wild | Sur quebrada |
| 11 | Wild | Sector 5 |
| 12 | Wild | Sector 4 |
| 13 | Wild | Sector 2 |
| 14 | Wild | Sector 1 |
| 15 | Cultivated | Higuerilla type, Sector 1 |
| 16 | Cultivated | Yellow bean- Farmer: Mr. Tali |
| 17 | Cultivated | Vainica de Palo |
| 18 | Cultivated | Higuerilla type, Farmer: Mr. Tali. Tapado system |

The tendency of the population structure and the dispersion of the individuals, were made by an analysis of multiple correspondence (ACM), by the CORRESP procedure of SAS (SAS Institute, 1989), from a data matrix with different combinations of all the used markers: morpho-agronomic evaluation, phaseolin, nine microsatellite *loci*, and cpDNA polymorphisms by PCR- RFLPs. The contribution of each one of the variables (markers) to the main tendency of the wild-weedy-cultivated complex was considered. The graphical representation allowed to observe spatial localization in a multidimensional plane, of the different biological forms evaluated by their genetic similarity. In the context of the analysis of data for genetic interpretation the MCA is quite sensitive, this fact permits to detect subtle patterns of similarity based on rare alleles that held in common among genotypes.

**Results**

The clusters obtained from the multiple correspondence analyses, using all the markers, allowed to establish five groups that explain 91.5% of the total variation of the evaluated individuals (Figure 3). Figure 3 shows two defined groups of cultivated materials (1 and 5); cluster 5 involves landraces collected from a farm. The majority of wild forms were grouped into two clusters from three sectors. Cluster 3 is conformed by wild and weedy types; these materials were collected in different sectors, with sector 5 being occupied only by weedy types. The resulted descriptors for each cluster are shown in Table 4. All *P. costaricensis* materials, possibly weedy forms, exhibited haplotype of chloroplast "A", as well as 14 weedy forms of *P. vulgaris* from Quircot sector 2 and sur de la quebrada (Table 4).

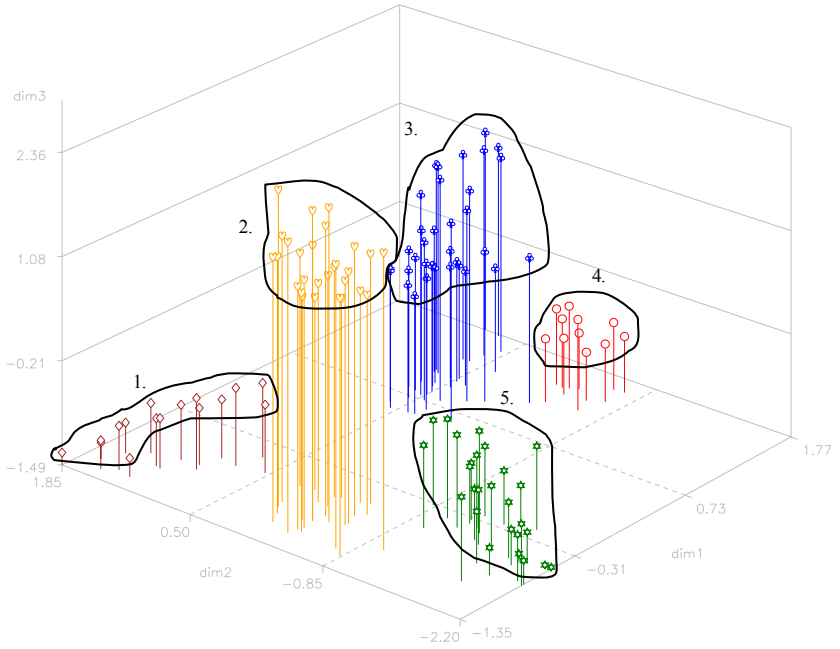


Figure 3. Spatial distribution of evaluated materials from Costa Rica using MCA.

Table 5. Description of cluster obtained by MCA analysis of SSR

| *Cluster* | *Biological status* | *Localization or material type* | *Cp haplotype* | *Seed size* |
|---|---|---|---|---|
| 1 | Cultivated | Higuerilla | "J" | |
| | | Vainica de palo | | Large |
| | | Frijol Amarillo | | Medium |
| | | Higuerilla sistema tapado | | |
| 2 | Weedy | Quircot sector 2 | "L" | |
| | | Quircot sector 3 | "A" | Very Large |
| | | Quircot sector 4 | | Medium |
| | | Quircot sector sur quebrada | | Small |
| | | Quircot sector- *P. costaricensis* weedy | | |
| 3 | Wild | Quircot weedy | "J" | Small |
| | Weedy | Quircot sector sur quebrada | | Medium |
| | | Quircot sector 5 | | Large |
| | | Quircot sector 4 | | |
| | | Quircot sector 2 | | |
| | | Quircot sector 1 | | |
| 4 | Wild | Quircot sector 2 | "L" | Small |
| | | Quircot sector 3 | "H" | Medium |
| | | Quircot sector sur de la quebrada | | |
| 5 | Cultivated | Quircot Farm of Mr. Tali | "A", "L", "J" | Medium |

Figure 4 shows the same 153 individuals according to their biological status in red, blue, and green as cultivated, weedy, and wild, respectively. It exhibits the differentiation of two complexes, in which weedy forms are closer to cultivated forms, possibly because of the higher weight given by the MCA to descriptors such as cpDNA (none of the weedy was of 'H' haplotype) and 100-seed weight. The haplotypes of chloroplast DNA in the weedy populations indicated the following gene flow direction: pollen of wild materials towards cultivated forms.

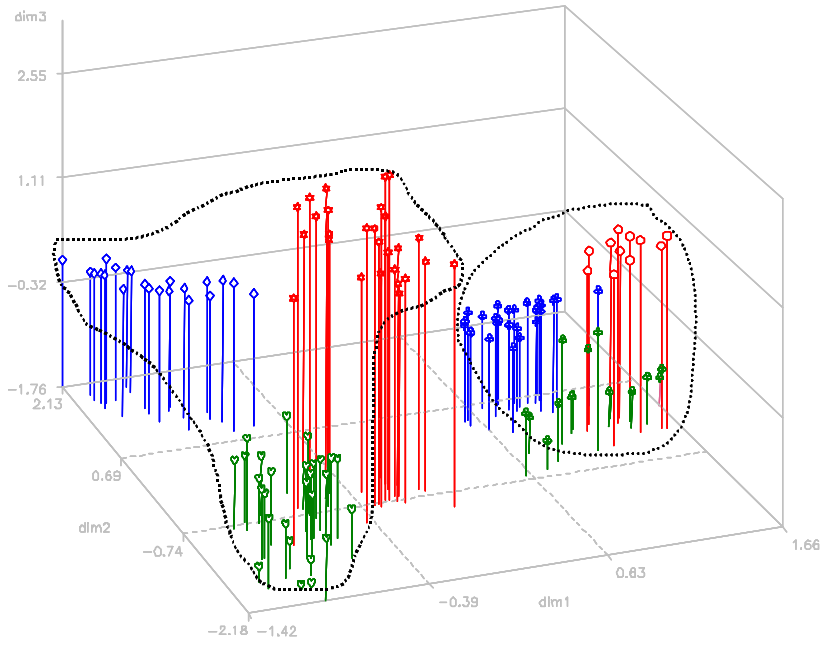


Figure 4. Spatial distribution of evaluated materials from Costa Rica using MCA by biological status. In red: cultivated types, blue: wild forms, and green for weedy population.

Table 6 summarized results for each evaluated population. Wild population shows an average seed weight of 6.85 g and displays mainly haplotypes "L" and "J"; however the haplotype "H" was found at lower frequency. The cultivated population presents an average of 22.1 g, and we principally find haplotype "J". In addition, weedy forms exhibit 21.73 g as mean weight, and shared haplotypes as "L" and "J". However, they also show haplotype "A" in four hybrids, this haplotype was found in 5/5 individuals selected out of *P. costaricensis*. This data suggests a gene flow event between *P. vulgaris* and *P. costaricensis*, where common bean was the pollen receptor.

Table 6. Description of evaluated markers on wild, cultivated and weedy individuals.

| Population N=136 | Biological Status | Mean weight (g/100 seeds) | Phaseolin patterns | | | | | Haplotype of chloroplast | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | S | TEL | C H | M1 | Novel Pattern | H | L | J | A |
| Sector 1 N=2 | Wild N=45 X=6.85g | 5.8 | | | | | | | | 2/2 | |
| Sector 2 N=25 | | 7.5 | | | | | | 7/25 | 8/25 | 10/25 | |
| Sector 3 N=13 | | 6.2 | | | | | | 3/13 | 10/13 | | |
| Sector 5 N=5 | | 7.9 | | | | | | | | 5/5 | |
| Sector 2 N=9 | Weedy N=31 X=21.73 g | 17.9 | | | | | 9/9 | | | | 9/9 |
| Sector 3 N=5 | | 19.2 | | | | 5/5 | | | 5/5 | | |
| Sector 4 N=17 | | 21.6 | | | 1/2 | | | | 2/2 | 15/15 | |
| Sur quebrada N=13 | | 28.25 | | | | 4/10 | 4/10 | | 3/3 | 4/10 | 6/10 |
| Sector 1 N=5 | Cultivated N=47 X=22.1g | 21.2 | 5/5 | | | | | | | 5/5 | |
| Parcela Tali N=42 | | 23 | 27/42 | 14/42 | | | | | 8/42 | 32/42 | |

According to proposed model by Bertorelle & Excoffier (1998), and used by Papa and Gepts (2003), the relative contribution to the weedy population of the wild and cultivated populations were estimated using ADMIX 1_0 software (California University, Berkeley, URL: http://web.unife.it/progetti/genetica/Giorgio/giorgio_soft.html). The contribution is expressed as coefficient of mixing mY (Table 7). We found the SSR alleles frequency for each population and locus, using POWERMAKER software. These results provide data to build a molecular distance matrix to analyze the coefficient of mixing mY (Tables 7, 8, 9). The main difference between mY and the other two estimators is that mY also takes into account the molecular distance between the different alleles. The analysis of contribution was conducted with 1,000 randomly resampling.

The admixture coefficient $M_Y$ was calculated, based on SSR alleles frequencies and the molecular distances between them. The term *Mw* is the relative contribution of wild forms and *Mc* that of cultivated forms. The admixture coefficient for wild forms was slightly higher than that cultivated which means *a priori* that the contribution of wild forms to the weedy types is 8.107 times greater (Table 7). These results suggest that the direction of the gene flow was pollen of wild populations towards cultivated types inferred in weedy forms.

Table 7. Estimation of admix distribution among wild and cultivated forms using SSR alleles.

| *Weedy* | | | | |
|---|---|---|---|---|
| Mwild | | Mcultivated | | Mc/Mw |
| Estimated | SD | Estimated | SD | |
| 0,8902 | 0,0595 | 0,1098 | 0,0593 | 0.123 |

On the other hand, the selection of individuals for evaluation involve some individuals with interspecific characteristics such as in crosses with *P. costaricensis*, therefore an analysis of admix was realized using

these individuals (Tables 8, 9). The estimate of the contribution of the wild population to the cultivated population was significantly higher than the estimate of the contribution of cultivated to the wild in the interspecific individuals.

Table 8. Estimation of admix distribution among wild and cultivated forms with possible interspecific hybrids using SSR alleles.

| *P X vulgaris* | | | | |
|---|---|---|---|---|
| Mwild | | Mcultivated | | Mc/Mw |
| Estimated | SD | Estimated | SD | |
| 0,9504 | 0,0473 | 0,0496 | 0,0525 | 0.052 |

Table 9. Estimation of admix distribution among wild and cultivated forms with possible *P. costaricensis* individuals using SSR alleles.

| *P. costaricensis* | | | | |
|---|---|---|---|---|
| Mwild | | Mcultivated | | Mc/Mw |
| Estimated | SD | Estimated | SD | |
| 0,7966 | 0,0876 | 0,2034 | 0,0502 | 0.255 |

### 1.1.5 Estimation of gene flow of "wild-weedy-cultivated" complexes of common bean along its range of distribution

Rosa González, Carlos Martinez, Harold Suarez, Orlando Toro & D. G. Debouck.
GRU Project

**Introduction**

Although common bean has long been considered as an autogamous plant, it can outcross naturally with its wild relative or even with sister species leading to the formation of complexes "wild-weedy-cultivated" (Gonzalez-Torres et al. 2006). These complexes have been phenotypically observed in the states/ departments of Oaxaca (México), El Progreso (Guatemala), San José (Costa Rica), Boyacá (Colombia), Azuay (Ecuador), Apurimac (Perú) and Tarija (Bolivia). We applied molecular markers using cpDNA (SNPs) and nuclear DNA (SSRs) to individuals putatively resulting from gene flow events under natural conditions (González-Torres et al. 2003, 2004) from this geographic range.

**Results**

The results shown in Table 10 display the diversity of chloroplast haplotypes in elements of the complexes and the direction of the flow. The wild and cultivated populations were characterized with such markers first allowing then to infer about the weedy individuals. The main direction was that of wild pollen towards cultivated materials, although the other direction was also evidenced at significant frequency in many places.

Table 10. Chloroplast haplotypes found in the complex "wild-weedy-cultivated" and their frequencies.

The data for Colombia and Peru seem to indicate cpDNA haplotypes shared between wild, weedy and

| | *Chloroplast haplotypes found* | | | |
|---|---|---|---|---|
| ***Country*** | ***Wild*** | ***Weedy*** | ***Cultivated*** | ***Pollen flow direction and frequency*** |
| Costa Rica | H (540/540) | G (3/481)<br>H (179/481)<br>J (98/481)<br>K (1/481)<br>L (199/481)<br>F (1/481) | J (18/56)<br>K (23/56)<br>L (15/56) | Wild to Cultivated<br>(98+1+199/481) |
| Guatemala | I (6/16)<br>J (10/16) | C (1/32)<br>J (31/32) | K (16/16) | Cultivated to Wild<br>(31/32) |
| Colombia | J (50/68)<br>L (18/68) | L (64/96)<br>J (28/96)<br>A (2/96)<br>C (2/96) | L (14/158)<br>C (3/158)<br>J (140/158) | Wild to Cultivated<br>Or<br>Cultivated to Wild |
| Ecuador | F (28/28) | F (47/51)<br>A (3/51)<br>J (1/51) | C (15/15) | Cultivated to Wild<br>(47/49) |
| Perú | C (51/51) | C (126/181)<br>I (1/181)<br>J (38/181)<br>L (11/181)<br>F (4/181)<br>A (1/181) | C (70/105)<br>J (18/105)<br>L (17/105) | Wild to Cultivated<br>Or<br>Cultivated to Wild |
| Bolivia | A (6/6) | A (1/20)<br>C (2/20)<br>I (1/20)<br>J (16/20) | C (6/12)<br>J (6/12) | Wild to Cultivated<br>(16+2/20) |
| Argentina | D (6/6) | C (40/40) | C (51/51) | Wild to Cultivated<br>(40/40) |

cultivated forms, in the same places of domestication (Chacón 2001), preventing thus to infer about direction of flow. SSR analysis with admixture estimation will be used to solve out this limitation.

The results obtained for the evaluated populations from Ecuador (Azuay) and Guatemala (El Progreso) in Table 10 suggest that the direction of gene flow was from pollen of cultivated materials towards wild forms, since according to Chacón (2001) the haplotypes dominant in the wild forms are 'F' and 'J', respectively.
For Ecuadorian population these data are interesting accordingly both biological statuses mainly have haplotype "F". This haplotype is currently distributed in Central-southern Ecuador and observed in a high frequency in wild population suggesting that in this case the direction of gene flow was from pollen of "cultivated" materials towards wild forms.

The Colombian population involves a set of 26 individuals selected as "escape", therefore an analysis of genetic similarity of Nei 1978 of escape population was required. This study was using POPGEN software, to group this set with their putative genetic counterparts (wild, cultivated or weedy individuals) (Figure 5). The tree displays a genetic similarity of 80.6% between cultivated forms and escaped individuals. For that reason, in the following analysis of genetic contribution (ADMIX) these populations were joined.

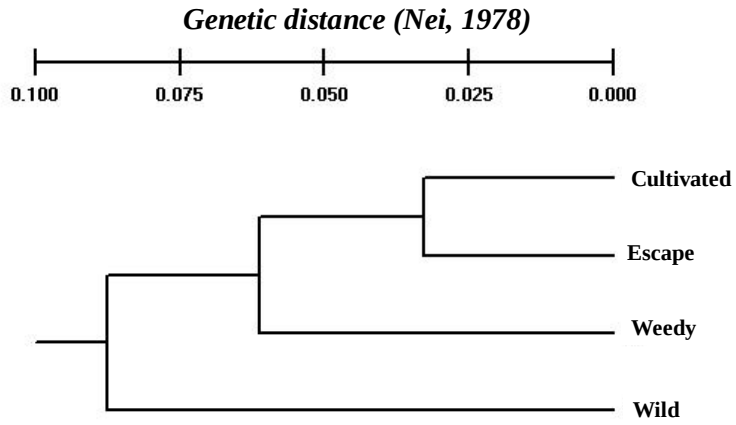


Figure 5. Cluster of genetic distance of evaluated biological forms, according to Nei (1978).

The admixture coefficient for wild forms was similar than cultivated population, indicating that the gene flow has occurred in both directions (wild pollen towards cultivated types and vice versa) almost symmetrically in Colombian populations (Table 11).

Table 11. Estimation of admix distribution among wild and cultivated forms on weedy population using SSR alleles.

| *Weedy* | | | | |
|---|---|---|---|---|
| Mwild | | Mcultivated | | Mc/Mw |
| Estimated | SD | Estimated | SD | |
| 0,4206 | 0,0551 | 0,5794 | 0,0549 | 0.143 |

We were also interested in examining gene flow events over time by sampling materials at the same localities in different years (1987, 1998, 2003, 2004, and 2006), in San José and Cartago, Costa Rica. We evaluated 9 nuclear SSR *loci* in a total of 520 weedy individuals to evidence the gene transfer and its quantification. We determined characteristic alleles for each population belonging to the complex and these were detected in the weedy populations suggesting that the evaluated hybrids are real cases of gene flow. The weedy individuals were confirmed by morphoagronomic and biochemical markers found in wild or cultivated populations (Table 12).

Table 12. Number of real hybrids revealed for each year screened in Quircot and Jerico populations in Costa Rica.

| Year / Population | 1987 | 1998 | 2003 | 2004 |
|---|---|---|---|---|
| **Cartago (Quircot)** | 29 | 40 | 123 | 50 |
| **San José (Jerico)** | -- | 9 | 50 | 40 |

The chloroplast analysis provided evidence on the effects of the evolutionary forces of domestication and gene flow on the levels of genetic diversity in this crop. On the one side, the few domestication events induced a strong founder effect, thus reducing crop genetic diversity. On the other side, the gene flow events have worked against this consequence, over millennia and across the range of the wild relative, contributing to the richness of the common bean gene pools (nuclear and chloroplast genomes). These two forces, among others, have taken part in the formation of domesticated races (Singh et al. 1991; Beebe et al. 2000), an unexpected result in a reported autogamous (!) crop.

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### 1.1.6 Determination of gene flow events in the Andean and Mesoamerican genepools of *Phaseolus lunatus*

Carlos Martínez, Harold Suárez, Rosa González & D.G. Debouck

**Introduction**

After using molecular and biochemical markers to successfully establish gene flow events in weedy forms of *Phaseolus vulgaris* and to indicate pollen direction from the wild into the cultivated and vice versa (González-Torres et al. 2004), we were interested in testing the hypothesis of gene flow in populations of *Phaseolus lunatus*. Lima bean has an interesting reproductive system with a facultative allogamy up to 48% (Baudoin et al. 1998). Wild Lima beans have a high level of HCN glucoside, that can be easily evaluated, allowing thus to assess consequences of gene flow in a food and crop domestication perspective. In this study we propose to evaluate nuclear genome with lectin data, microsatellites, and the analysis of seed cyanide acid. The contribution of the chloroplast genome is evaluated using RFLPs of non-coding regions in order to set the direction of gene flow.

Initial evaluations

The preliminary evaluations of a set of individuals belonging to both gene pools (Andean and Mesoamerican) with all markers were conducted to determine the analysis conditions (Table 18).

Table 18. Populations of *Phaseolus lunatus* evaluated in the preliminary studies.

| Accession<br>Biological status | Average seed weight (g) (n=5) | Color testa of seed | Country |
|---|---|---|---|
| G25704 | 11,5 | Wild type | Mexico |

| | | | | |
|---|---|---|---|---|
| | G25737 | 8,3 | Wild type | |
| | G25759 | 11,5 | Wild type | |
| | G25789 | 10,3 | Wild type | |
| | G25913 | 11,7 | Wild type | |
| | G25914 | 11 | Wild type | Perú |
| | G25916 | 18 | Wild type | |
| CULTIVATED | G25705 | 47,1 | Colored | |
| | G25733 | 31,1 | Colored | |
| | G25760 | 39,7 | Colored | |
| | G25778 | 22,2 | Colored | Mexico |
| | G25786 | 25 | Colored | |
| | G25787 | 37,8 | Colored | |
| | G25826 | 47,5 | Colored | Perú |
| | G25831 | 175,3 | Colored | |
| | G25919 | 99,7 | Colored | |
| | G25930 | 50 | Colored | |
| | G25933 | 83,4 | Colored | |
| | G25943 | 34,8 | Colored | |
| WEEDY | G25955 | 106,4 | Colored | |
| | G25706 | 18,6 | Wild type | Mexico |
| | G25736 | 22 | Colored | |
| | G25778 | 22,2 | Colored | |
| | G25915 | 28 | Wild type | Perú |
| | G25944 | 44,5 | Colored | |
| | G25947 | 53,1 | Wild type | |
| | G25948 | 55,3 | Colored | |
| | G25952 | 36,3 | Colored | |
| | G25952A | 31,4 | Colored | |

*Biochemical markers*

Quantification of cyanide acid
The amount of linamarin compound (as the anti-nutritional marker) was determined using the colorimetric method reported by Essers et al (1993), using a lower amount of seed tissue than previously reported. For that reason, one and two grams were evaluated with this method to compare results.

**Results**

Table 19 shows cyanide concentration (ppm) obtained for each biological status and gene pool. These results fall in line with levels reported for *P. lunatus* by Baudoin et al. (1997) who found very high HCN contents in the wild populations, markedly greater than those found in the cultivated types, as a possible result of domestication. In addition, we found no correlation between seed coat color and cyanide content. Weedy forms exhibited a content of the cyanogenic glycoside intermediate between wild and cultivated types, as a possible result of gene flow events.

Table 19. Cyanide concentration (ppm) and its average for each biological status of *P. lunatus.*

| | *Cyanide concentration (ppm)* | | |
|---|---|---|---|
| | *Wild* | *Cultivated* | *Weedy* |
| *Mesoamerican* | 2345<br>2296<br>1547<br>1421<br>X=1902,3 | 69<br>72<br>38<br>107<br>X=71,5 | <br>1881<br>356<br><br>X=1118,5 |
| *Andean* | <br><br><br>2747<br>3210<br>3684<br><br><br>X=3213,7 | 232<br>94<br>485<br>335<br>114<br>34<br>379<br>X=239 | <br>1625<br>643<br>1386<br>582<br>2077<br>2451<br>X=1460,7 |

Lectins or reserve proteins

Seed storage proteins or lectins are localized between 31 y 45 kDa, and were analyzed for individuals listed previously using the electrophoretic method one di-SDS-PAGE described by Gutiérrez-Salgado et al. (1995). We used accessions G25916 (Andean genepool) and G25704 (Mesoamerican genepool) as electrophoretic controls of protein patterns reported by Gutiérrez-Salgado et al. (1995).

**Results**

The obtained patterns of lectins for each gene pool were similar to those of used controls. The Mesoamerican population shows two different patterns in the cultivated form, while the wild and weedy individuals have the same M1 lectin like the control (Figure 7 white arrows).

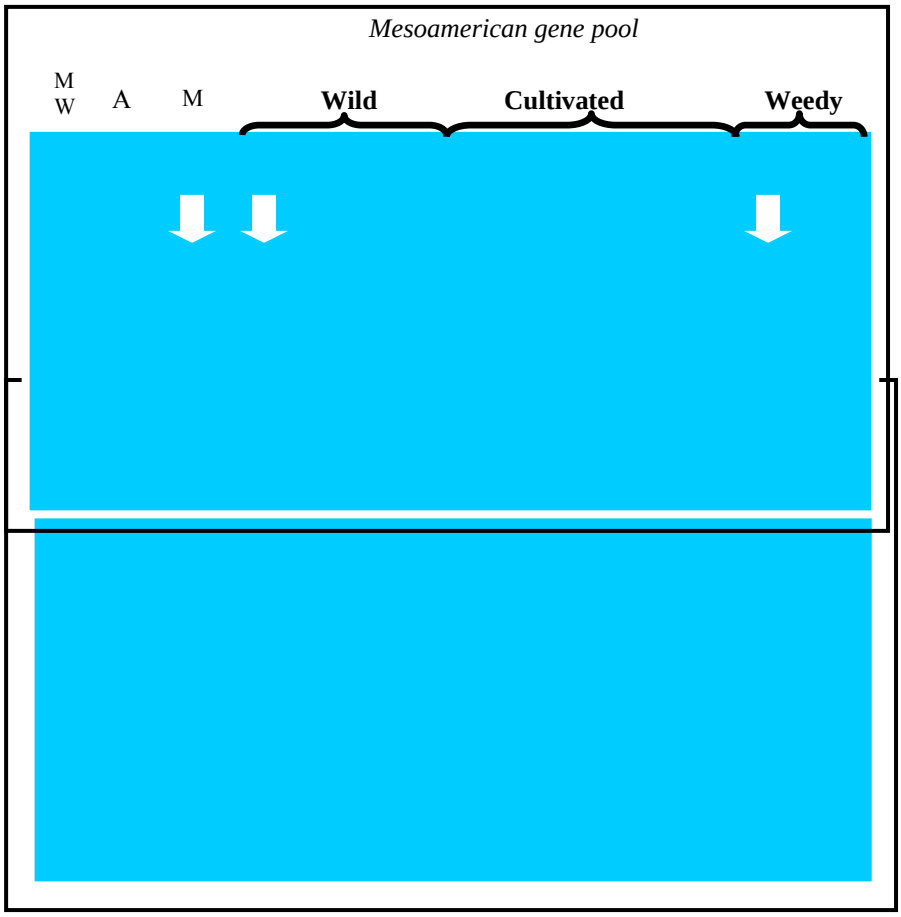


Figure 7. Lectins patterns obtained using SDS-PAGE. A: Andean control, M: Mesoamerican control, MW: molecular weight marker (Kda).

Andean individuals displayed four different patterns of lectins, two of them are shown in Figure 7 as white arrows and stars. One wild individual has the same lectin as the control.

*Molecular markers*

Microsatellites

A total of 68 SSR loci reported by Gaitán et al (2002) for *P. vulgaris* were evaluated on the selected population of *P. lunatus* to evidence polymorphism and improve amplification conditions. The microsatellites were evaluated with silver staining (Figure 8).

**Results**

Polymorphism information content for microsatellite was calculated using POWERMARKER software V3.0, and 20 of the microsatellites were found to have values higher than 0.374 (Table 20), similar to the SSRs used by González (2004), studying gene flow in common bean of Costa Rica. These *loci* will be evaluated to evidence gene flow events in the complex "wild-weedy-cultivated" of *P. lunatus*.

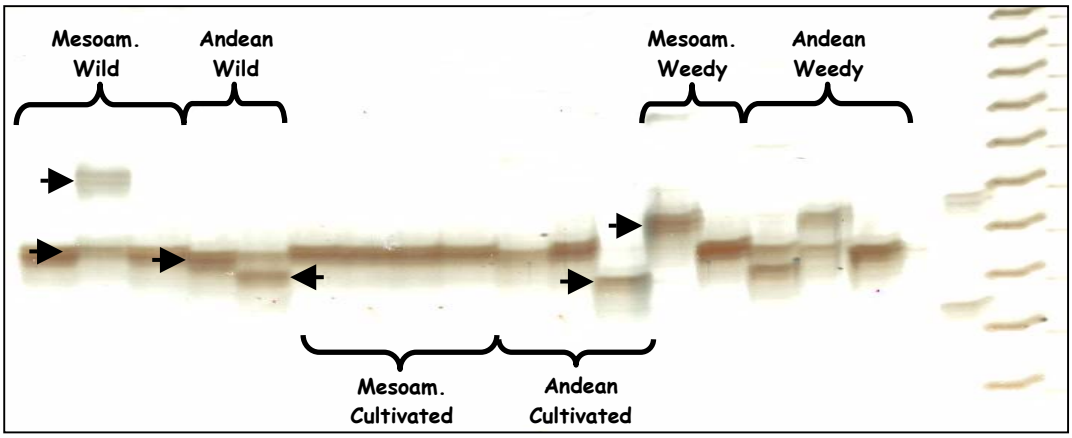


Figure 8. Microsatellies alleles obtained for locus BM 187 in the evaluated population

Table 20. Description of polymorphic SSR with a high PIC.

| *SSR* | *Polymorphic alleles* | *PIC* |
|---|---|---|
| BM209 | 9 | 0,776 |
| GATS91 | 7 | 0,768 |
| BM211 | 6 | 0,746 |
| BM143 | 6 | 0,669 |
| BM140 | 5 | 0,641 |
| BM170 | 6 | 0,611 |
| BM181 | 5 | 0,564 |
| BM154 | 5 | 0,563 |
| BM156 | 3 | 0,551 |
| BM202 | 3 | 0,546 |
| BM183 | 6 | 0,526 |
| BM148 | 5 | 0,515 |
| BM171 | 4 | 0,508 |
| BM212 | 4 | 0,508 |
| BM201 | 4 | 0,481 |
| BM141 | 3 | 0,397 |
| BM155 | 3 | 0,383 |
| BM153 | 2 | 0,374 |
| AG1 | 2 | 0,374 |
| BM146 | 3 | 0,374 |

RFLPs of chloroplast DNA

We are interested in finding specific haplotypes of chloroplast DNA in the *P. lunatus* populations with the objective of determining gene flow direction, as it is maternally inherited. Hence, twelve intergenic regions of cpDNA reported by Fofana et al. (1999) and Demesure et al. (1995) were evaluated to determine the amplification conditions (Table 21).

Table 21. Regions of cpDNA evaluated in this study.

| CpDNA Region | PRIMER SEQUENCE (5`-3) | SIZE (pb) | REFERENCE |
|---|---|---|---|
| atpB-rbcL spacer | GTGTCAATCACTTCCATTCC<br>GTAAAATCAAGTCCACCGCG | 1700 | 1 |
| rps14-psaB spacer | CATTTCACGAAGTATGTGTCCG<br>TGGCGTGGATATTGGCAGGA | 700 | 1 |
| petA-psbE region | GCATCTGTTATTTTGGCACA<br>TACCTTCCCTATTCATTGCG | 1200 | 1 |
| psbC-tRNAser spacer | GGTCGTGACCAAGAAACCAC<br>GGTTCGAATCCCTCTCTCTC | 1700 | 1 |
| tRNAser-tRNAfmet spacer | GAGAGAGAGGGATTCGAACC<br>CATAACCTTGAGGTCACGGG | 1600 | 2 |
| trnT-trnL spacer | CATTACAAATGCGATGCTCT<br>TCTACCGATTTCGCCATATC | 800 | 2 |
| trnL Intron | CGAAATCGGTAGACGCTACG<br>GGGGATAGAGGGACTTGAAC | 630 | 2 |
| trnL-trnF spacer | GGTTCAAGTCCCTCTATCCC<br>ATTTGAACTGGTGACACGAG | 530 | 2 |
| rpl16 intron | GCTATGCTTAGTGTGTGACTCCGTT<br>CGTACCCATATTTTTCCACCACGAC | 1210 | 2 |
| ndhA intron | GGWCTTCTYATGKCRGGTATRGMTC<br>CTGYGCTTCMACTATATCAACTGTAC | 1500 | 2 |
| accD-psaI spacer | GGAAGTTTGAGCTTTATGCAAATGG<br>AGAAGCCATTGCAATTGCCGGAAA | 700 | 2 |
| rpoC1-rpoC2 spacer | GAAGTTCACTATGAATCTTTNGGTACC<br>TAGACATCGGTACTCCAGTGC | 1700 | 2 |

Fofana et al. 1997
Demesure et al. 1995

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### 1.1.7 Race structure within the Mesoamerican gene pool of common bean (*Phaseolus vulgaris* L.) as determined by microsatellite markers

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#### Introduction

Cultivated common beans (*Phaseolus vulgaris* L.) originated in two centers of diversity giving rise to two genepools: Mesoamerican from Central America and Mexico and Andean from the Andes mountains of South America (Singh et al., 1991a, b). Some authors also refer to the Mesoamerican gene pool as the Middle American gene pool but both terms refer to the same set of characteristics. The differences between Mesoamerican and Andean gene pools of common bean include seed size, phaseolin (seed storage protein) patterns, plant morphology, isozymes and RFLP, RAPD or AFLP markers. Cultivated bean gene pools have further been divided into races according to morphological criteria and agroecological adaptation where the term 'race' is used to denote a group of related landraces (Singh et al., 1991c). Members of each race have distinctive and specific physiological, agronomic, biochemical and molecular characteristics and differ from other races in the allelic frequencies at specific loci. Race structure has been analyzed by RAPD markers (Beebe et al., 2000) but less molecular evidence has been accumulated for within gene pool differences as compared to between gene pool differences. The objective of this study was to describe the race structure of the Mesoamerican gene pool using microsatellite markers.

#### Materials and Methods

A total of 60 genotypes were used of which 35 were from Mexico, 8 from Guatemala, 7 from Brazil, 3 from El Salvador, 2 from Colombia and 1 each from Costa Rica, Ecuador and the United States, all of these representing the Mesoamerican genepool; with 2 additional genotypes, 'Calima' from Colombia and 'G19833' from Peru, used as an Andean outgroup. Genotypes were selected based on previous race designations (principally Beebe et al., 2000 and Singh et al., 1991a, b, c) and the phaseolin pattern of each genotype was known to be typical of the Mesoamerican gene pool (S, Sb, Sd and M). The Mesoamerican

genotypes 'ICA Pijao' and 'DOR364' from Colombia and El Salvador/CIAT, respectively, were considered control genotypes for the genepool since they had been evaluated previously (Blair et al., 2006). Genotypes were provided by the Genetic Resources Unit of CIAT and most represented landrace collections rather than bred varieties. DNA was extracted from 10 germinated seeds selected at random and diluted to 10 ng/ml for further experiments. The DNA was used to amplify a total of 52 microsatellites of which 22 were cDNA based and 30 were genomic. Microsatellites were selected based on their high polymorphism information content from Blair et al. (2006) and their even distribution around the genome. PCR conditions were as per this earlier article. Gels were stained with silver nitrate and allele sizes were evaluated relative to a 10 bp molecular weight size standard (Invitrogen, Carlsbad, CA). The allele information coded for band presence or absence was used to determine population structure and other common parameters of genetic diversity (percentage polymorphic loci, allele frequencies, observed heterozygosity (Ho), indices of genetic differentiation (Gst), and gene flow (Nm) for each of the races.

**Results and Discussion**

A total of 267 bands were generated with an average of 5.1 alleles per marker and 0.297 heterozygosity across all microsatellites. Correspondence analysis identified two major groups equivalent to the Mesoamerica race and a group containing both Durango and Jalisco race genotypes. Two outlying individuals were classified as potentially of the Guatemala race although this race does not have a defined structure and previously classified members of this race were classified with other races. Population structure analysis with K=1 to 4 agreed with this classification (Figure 1). The genetic diversity based on Nei's index for the entire set of genotypes was 0.468 (Table 1) while this was highest for the Durango-Jalisco group (0.414), intermediate for race Mesoamerica (0.340) and low for race Guatemala (0.262). Genetic differentiation (GST) between the Mesoamerican races was 0.27 while genetic distance and identity showed race Durango and Jalisco individuals to be closely related with high gene flow (Nm) both between these two races (1.67) and between races Durango and Mesoamerica (1.58). Observed heterozygosity was low in all the races as would be expected for an inbreeding species. The analysis with microsatellite markers identified subgroups, which agreed well with commercial class divisions, and seed size was the main distinguishing factor between the two major groups identified. These results have implications in terms of multiple domestications within the Mesoamerican gene pool and introgression between wild and cultivated genotypes.

**Table 1.** Genetic diversity parameters for Mesoamerican race and Andean control genotypes.

| Groups | N | na | ne | Obs_Het | Nei's | P | % |
|---|---|---|---|---|---|---|---|
| Race Durango | 16 | 2.769 | 1.720 | 0.046 | 0.339 | 45 | 86.54 |
| Race Jalisco | 14 | 3.288 | 2.221 | 0.045 | 0.425 | 48 | 92.31 |
| Total D-J | 30 | 3.808 | 2.081 | 0.046 | 0.414 | 48 | 92.31 |
| Race Guatemala | 2 | 1.577 | 1.526 | 0.067 | 0.262 | 28 | 53.85 |
| Race Mesoamerica | 26 | 3.212 | 1.939 | 0.028 | 0.340 | 42 | 80.77 |
| Total Mesoamerican | 58 | 4.789 | 2.352 | 0.039 | 0.444 | 49 | 94.23 |
| Andean checks | 2 | 1.346 | 1.339 | 0.010 | 0.171 | 18 | 34.62 |
| Grand Total | 60 | 5.077 | 2.447 | 0.038 | 0.468 | 52 | 100.0 |

Abbreviations: Number of genotypes (N), observed number of alleles (na), effective number of alleles (ne), observed heterozygosity (Obs_Het.), genetic diversity according to Nei (1973), number of polymorphic loci (P), percentage polymorphic loci (%).

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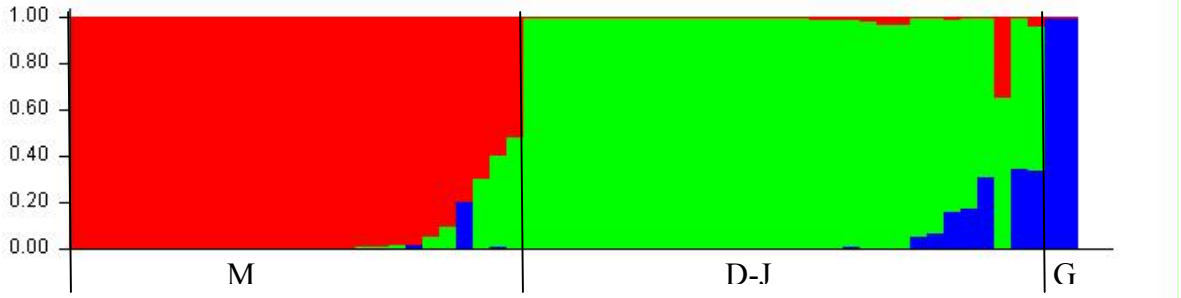


Figure 1. Graph of the population structure (K=3) for 58 Mesoamerican genotypes (x-axis) sorted by membership coefficients (y-axis) within clusters based on highest estimated probability among 50,000 runs using the software program Structure (Pritchard et al., 2000). Cluster / group names indicated below figure (M= race Mesoamerica; D-J = races Durango and Jalisco together and G = race Guatemala).

### 1.1.8 Seed iron and zinc levels in a collection of colombian released varieties

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**Introduction**

Bean breeding in Colombia has produced a wide range of large-seeded Andean varieties for many different agroecological zones. The country is home to a wide range of traditional farmer varieties and landraces. To obtain a baseline of information on nutritional quality in Colombian germplasm we assembled a nursery for the major bush bean varieties released in the country. The objectives of this research, therefore, were to test a representative number of improved and traditional Colombian varieties for seed iron and zinc levels. These genotypes represent all the bush bean varieties released by ICA and CORPOICA over the past thirty years along with standard check varieties and a few standard landraces. The majority of the varieties are red mottled 'Calima' or 'Nima' classes followed by large reds 'Radicales' or 'Duva' types.

## Materials and Methods

Seed was analyzed from the study of 40 bush bean genotypes that were grown in a replicated yield trial in Darien in semester 2004A under two fertilization treatments: low phosphorus, 50kg/ha (7.5 kg. P205) and high phosphorus, 350 kg/ha (45kg P205). Native soil P is 2 to 10 ppm. Experimental design consisted in three replications in randomized complete block with plots separated by DOR390 as check rows. During 2004A, Darien was a favorable site for all the genotypes given its location at 1500 masl and average temperature of 19°C and annual rainfall of 1.200 mm (500 during the season). The genotypes included 22 large-seeded red mottled (Calima) or red (Radical) varieties released by ICA or CORPOICA over the past 30 years (DIACOL and ICA series), 11 landraces from Berruecos, Darien, Sevilla and Tenerife (Valle) as well as Ocaña and Zaragosa (No. Santander) and seven CIAT lines as controls (A36, AFR188, AFR612, AFR619, AFR735, AND279 and CAL96). Seed mineral content was evaluated as described above in the Analytical Services laboratory of CIAT for both iron and zinc concentration measured in parts per million (ppm). Data analysis was conducted with the software program Statistix v. 8.0

## Results and Discussion

The analysis of variance for the seed iron and zinc showed significant differences for both genotype and phosphorus level. The genotype x phosphorus fertilization interaction was significant for iron (P=0.0211), while it was not significant for zinc (Table 1). Among all varieties, iron levels were higher on average under the high phosphorus treatment (56.7 ppm) than under the low phosphorus treatment (52.5 ppm). In contrast zinc levels were higher under low phosphorus (26.3 ppm) than under high phosphorus (23.1 ppm). The range in iron concentration was from 35.1 to 77.0 ppm in the low phosphorus treatment and from 32 to 79 ppm in the high phosphorus treatment. The range in zinc concentration was from 20.1 to 30.7 ppm in low phosphorus and 16.9 to 30.4 ppm in high phosphorus (Figure 1). Coefficients of variation for seed iron and zinc ranged from 9.9 to 18.0 % and were lower for zinc estimates than for iron estimates. Varieites with the highest seed iron levels were ICA Tundama (79.44 ppm), T AFR188 (76 ppm), AFR735 (75.14 ppm) and ICAL-59 ARS-59 (75.12 ppm) in high phosphorus and Radical de Restrepo (69.98 ppm), AFR612 (77.04 ppm) and AFR188 (67.36 ppm) in low phosphorus. AFR188 and AFR612 were also high in seed zinc concentration, along with Cargabello, especially under low phosphorus (Table 2). The correlations between iron and zinc concentrations in low and high phosphorus treatments were high (r=0.79 and r=0.57, respectively (P=0.000)). The correlations of seed mineral content between phosphorus treatments for both iron and zinc was also high (r=0.55 and r=0.56, respectively (P=0.000)). Correlations were not significant between mineral content and the agronomic characteristics evaluated for the experiment (including days to flowering, days to maturity and yield, both in g/plant and kg/ha) (Table 3).

The range in yields for the varieties went from a low of 309 kg/ha to a high of 1781 kg/ha in the low phosphorus treatment and from a low of 580 kg/ha to a high of 3164 kg/ha in the high phosphorus treatment (Table 1). The same variety was the lowest yielding in these two environments, namely the type I genotype DIACOL Nutibara, a variety poorly adapted given its origin in colder climates. The highest yielding variety in the high phosphorus treatment was the type II breeding line AFR619, while the highest yielding variety in the low phosphorus treatment was the type III landrace Palisero (Berruecos) which was also the second highest yielding in high phosphorus. As a result Palisero had a relatively high low P / high P yield index (59.9%) which was similar to several of the landrace genotypes, including Blanquillo (73.8%), Morado Moteado (62.1%), Sangretoro (64.3% and 72.0%). The average index for the landraces (55.7%) was higher than for the improved released varieties (50.8%) showing that landraces tend to be better for low fertility conditions and pointing out a deficiency of some of the released varieties, except for ICA Tundama a cold climate variety with a high yield index (72.0%) and reasonable yields under low P (1032 kg/ha) and high P (1433 kg/ha).

In terms of yield, the more recently-developed breeding lines tended to do better than the released varieties. Two notable breeding lines in this respect were AFR188 and AND279. In addition, AFR619 was an interesting genotype due to its high responsiveness to P fertilization in the high P treatment. Overall, the average yield of the breeding lines (2129.5 kg/ha) exceeded that of the landraces (1776.0 kg/ha) and the improved varieties (1446.3 kg/ha) under high P treatment. The same pattern was seen for breeding lines (1114.1 kg/ha), landraces (989.5 kg/ha) and released varieties (712.7 kg/ha) under low P treatment. The differences between groups may be due to the fact that the landraces included long season, small seeded higher yielding indeterminate genotypes while the released varieties included some with more specific agro-ecological adaptation, especially those from colder climates. This was evident when comparing the days to flowering and maturity differences between the two groups. Meanwhile the breeding lines included genotypes that were locally selected at the experimental site which might explain their greater adaptation. All together, the results are interesting in pointing out the yield stability of traditional landraces, the site-specificity of some of the released varieties and the improvements made in low P adaptation in breeding lines when compared to released varieties. Darien was a good environment to test the genotypes because most genotypes were well adapted there and the native low P soils were uniform and easy to manage for both the low P and high P treatments. Many of the landraces adapted quite well even though they were from very different environments originally. This might reflect a wider stability of some landraces over some of the improved germplasm. Apart from yield, the effect of low P treatment was notable in delaying flowering and days to maturity by an average of 1.8 and 2.2 days respectively overall and in some cases by as much as 3.7 days for flowering date and 7.7 days for maturity date. Interestingly this occurred most notably in some but not all of the varieties with better performance under low P stress (eg. AFR188, AFR619 and Sangretoro) suggesting that delayed phenology may be a mechanism for low P stress tolerance in Andean beans, although significant correlations were observed between many of the traits.

**Table 1.** Analysis of variance for seed iron and zinc in 40 Colombian varieties grown under high and low phosphorus levels in Darien 2004[a].

**a) Iron**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| **P level** | 1 | 3982.4 | 3982.42 | 90.41 | 0.0000 |
| **Genotype** | 38 | 12966.8 | 341.23 | 7.75 | 0.0000 |
| **P level x Genotype** | 38 | 2711.6 | 71.36 | 1.62 | 0.0211 |
| **Error** | 162 | 7136.2 | 44.05 | | |
| **Total** | 239 | | | | |

**b) Zinc**

| | | | | | |
|---|---|---|---|---|---|
| **P level** | 1 | 766.40 | 766.403 | 156.14 | 0.0000 |
| **Genotype** | 38 | 1352.72 | 35.598 | 7.25 | 0.0000 |
| **P level x Genotype** | 38 | 179.50 | 4.724 | 0.96 | 0.5385 |
| **Error** | 162 | 795.17 | 4.908 | | |
| **Total** | 239 | | | | |

iron CV 11.55 // zinc CV 8.82

**Table 2.** Average seed iron and zinc concentration, days to flowering (DF) and maturity (DM) and yield (in g/plant and kg/ha) for 40 Colombian varieties grown under high and low phosphorus (P) levels in Darien 2004[a].

| | Low P | | | | | | High P | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Genotype** | **Iron** | **Zinc** | **YIELD** | **DF** | **DM** | **G/pl** | **Iron** | **Zinc** | **YIELD** | **DF** | **DM** | **G/pl** |
| ICA GUALI | 62.22 | 29.00 | 774.78 | 36.00 | 81.33 | 3.64 | 53.04 | 20.43 | 1377.61 | 36.33 | 79.67 | 6.11 |
| DIACOL CALIMA | 45.87 | 24.41 | 515.89 | 38.33 | 78.67 | 2.23 | 48.00 | 23.72 | 1204.57 | 37.00 | 81.33 | 5.15 |
| L 17. ICA PALMAR. INIA 17 | 47.11 | 24.53 | 666.21 | 42.00 | 83.67 | 3.02 | 55.05 | 21.96 | 1502.34 | 43.33 | 86.67 | 7.35 |
| DIACOL NUTIBARA | 53.43 | 29.63 | 309.33 | 39.00 | 75.67 | 1.42 | 49.76 | 25.17 | 580.11 | 39.67 | 71.67 | 2.38 |
| ICA DUVA | 53.12 | 26.97 | 802.54 | 37.33 | 84.33 | 3.49 | 45.72 | 19.86 | 1913.32 | 37.33 | 82.00 | 8.54 |
| SANGRETORO | 38.28 | 21.30 | 885.23 | 42.33 | 84.67 | 3.66 | 53.97 | 22.92 | 1377.51 | 39.67 | 79.00 | 6.56 |
| DIACOL NIMA | 50.58 | 24.29 | 759.00 | 41.67 | 86.67 | 3.62 | 61.90 | 20.54 | 1694.32 | 40.33 | 85.00 | 8.28 |
| ICA TONE | 46.69 | 26.09 | 450.00 | 38.33 | 80.33 | 2.35 | 48.31 | 21.74 | 943.82 | 38.67 | 77.33 | 4.39 |
| ICA CUNA | 41.67 | 20.86 | 585.33 | 39.67 | 82.00 | 2.43 | 36.48 | 17.78 | 1201.26 | 38.33 | 82.33 | 5.48 |
| ICA TUNDAMA | 49.75 | 25.68 | 1032.35 | 48.00 | 90.00 | 5.03 | 79.44 | 30.18 | 1433.19 | 48.00 | 90.00 | 9.12 |
| DIACOL CATIO | 57.45 | 25.69 | 592.91 | 42.67 | 85.67 | 2.63 | 54.19 | 19.57 | 1480.36 | 40.33 | 85.33 | 7.90 |
| ICA L 24 FRIJOLICA P-1.1 | 47.41 | 24.99 | 849.01 | 43.00 | 87.00 | 3.49 | 60.18 | 24.81 | 852.40 | 41.33 | 79.33 | 3.88 |
| AFR 619 | 35.06 | 21.76 | 1315.11 | 45.67 | 92.33 | 5.53 | 32.00 | 19.58 | 3164.44 | 42.00 | 85.33 | 15.96 |
| ICA L-59 ARS-59 | 60.86 | 28.75 | 966.11 | 42.67 | 87.33 | 4.46 | 75.12 | 26.02 | 1821.52 | 40.00 | 88.67 | 8.46 |
| L 34 ICA BACHUE | 55.57 | 28.15 | 675.43 | 35.67 | 82.00 | 3.06 | 62.37 | 21.83 | 1220.28 | 38.00 | 80.67 | 5.61 |
| L 33341 ICA CERINZA | 53.75 | 27.56 | 560.56 | 42.67 | 84.67 | 2.19 | 68.57 | 24.04 | 1244.86 | 39.33 | 78.00 | 6.17 |
| RADICAL CERINZA | 52.83 | 28.71 | 816.21 | 36.67 | 83.67 | 3.61 | 59.33 | 27.18 | 1805.39 | 37.33 | 84.00 | 8.21 |
| ICA CITARA | 45.62 | 23.10 | 798.11 | 35.67 | 79.33 | 3.31 | 46.68 | 16.86 | 1524.75 | 36.33 | 82.67 | 6.78 |
| AND 279 | 53.89 | 21.61 | 1080.62 | 40.33 | 86.00 | 5.67 | 59.98 | 19.23 | 1741.93 | 39.67 | 84.00 | 10.23 |
| ICA L-66 | 50.91 | 27.56 | 421.00 | 37.00 | 77.33 | 2.06 | 54.81 | 21.97 | 1529.12 | 37.00 | 81.33 | 6.84 |
| ICA CAUCAYA | 59.13 | 28.39 | 988.30 | 44.00 | 87.33 | 4.59 | 46.73 | 24.40 | 1720.50 | 41.33 | 82.00 | 8.55 |
| ICA CAFETERO | 48.48 | 26.67 | 525.00 | 41.67 | 84.00 | 2.37 | 55.83 | 23.02 | 1365.71 | 40.33 | 83.00 | 5.97 |
| ICA QUIMBAYA | 64.36 | 30.23 | 577.67 | 38.00 | 84.00 | 2.52 | 63.85 | 23.60 | 1395.18 | 37.00 | 83.33 | 5.94 |
| CHOCHO (DE TENERIFE) | 41.13 | 24.55 | 572.11 | 39.67 | 78.67 | 2.73 | 59.07 | 22.13 | 1211.56 | 38.00 | 74.33 | 5.39 |
| ICA GUANENTA | 48.22 | 27.88 | 706.56 | 37.33 | 76.67 | 2.98 | 57.37 | 25.46 | 1280.27 | 37.33 | 80.00 | 5.72 |
| RADICAL FROYLAN | 53.02 | 26.46 | 633.67 | 39.67 | 85.00 | 2.71 | 53.90 | 24.23 | 1901.10 | 38.50 | 88.00 | 8.64 |
| CARGABELLO (DAR) | 62.35 | 30.17 | 663.55 | 41.00 | 80.00 | 2.74 | 52.75 | 25.55 | 1405.10 | 40.00 | 80.67 | 6.44 |
| LP 110.ROSADO O ZARAGOZA | 40.78 | 25.53 | 558.45 | 35.67 | 75.00 | 2.66 | 47.32 | 21.85 | 923.06 | 36.00 | 74.33 | 3.89 |
| ROSADO OCA&A | 55.40 | 26.91 | 370.89 | 35.33 | 75.00 | 1.53 | 47.74 | 24.97 | 867.25 | 35.33 | 76.00 | 3.76 |
| RADICAL (DE RESTREPO) | 69.98 | 28.22 | 1072.83 | 40.33 | 85.33 | 4.97 | 60.67 | 24.04 | 1926.96 | 39.67 | 83.67 | 8.08 |
| MORADO MOT (RESTREPO) | 49.57 | 26.07 | 1082.28 | 38.33 | 82.00 | 4.48 | 62.98 | 24.08 | 1741.76 | 37.33 | 82.33 | 7.23 |
| SANGRETORO (BERRUECOS) | 48.55 | 24.22 | 1658.38 | 45.67 | 92.67 | 7.43 | 55.76 | 23.38 | 2303.10 | 43.00 | 90.33 | 11.17 |
| PALISERO (BERRUECOS) | 53.93 | 26.03 | 1781.55 | 45.33 | 93.00 | 7.69 | 57.56 | 24.14 | 2976.88 | 44.67 | 92.33 | 13.84 |
| BLANQUILLO (BERRUECOS) | 54.64 | 27.65 | 1466.15 | 49.00 | 90.00 | 6.03 | 58.63 | 26.30 | 1986.12 | 48.33 | 90.00 | 10.79 |
| MINA (BERRUECOS) | 54.25 | 25.49 | 773.51 | 38.33 | 83.33 | 3.42 | 63.98 | 21.98 | 2816.64 | 37.50 | 80.00 | 12.28 |
| CAL 96 | 46.27 | 23.72 | 706.45 | 39.33 | 80.67 | 3.17 | 45.04 | 18.90 | 1490.14 | 37.33 | 80.67 | 7.23 |
| AFR 188 | 67.36 | 30.73 | 1421.98 | 40.33 | 91.33 | 6.12 | 76.20 | 30.37 | 1660.40 | 38.33 | 85.00 | 7.85 |
| AFR 735 | 62.33 | 27.71 | 1277.79 | 37.67 | 82.67 | 5.32 | 75.14 | 22.00 | 2302.51 | 37.00 | 86.67 | 12.55 |
| A 36 | 41.56 | 24.28 | 850.03 | 47.00 | 95.00 | 4.12 | 51.01 | 24.07 | 2001.13 | 46.00 | 90.00 | 9.43 |
| AFR 612 | 77.04 | 30.72 | 1147.03 | 43.67 | 89.33 | 4.66 | 69.46 | 23.02 | 2545.88 | 41.33 | 90.00 | 13.80 |

**Table 3.** Correlations between seed mineral concentration and agronomic characteristics at the two levels of phosphorus for Colombian varieties grown in Darien 2004a.

| | IronLP | IronHP | ZincLP | ZincHP | DAFLP | DAFHP | DAMFLP | DAMFHP | PExPLALP | PExPLAHP | Yield |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Iron HP** | 0.5537* | | | | | | | | | | |
| **Zinc LP** | 0.7908* | 0.4699* | | | | | | | | | |
| **Zinc HP** | 0.3179* | 0.5716* | 0.5608* | | | | | | | | |
| **DF HP** | -0.0634ns | 0.1721ns | -0.1111ns | 0.3458* | 0.9341* | | | | | | |
| **DM LP** | 0.1320ns | 0.2668ns | -0.0841ns | 0.2340ns | 0.8094* | 0.7374* | | | | | |
| **DM HP** | 0.2439ns | 0.3240* | -0.0107ns | 0.1790ns | 0.6275* | 0.6361* | 0.7996* | | | | |
| **PExPLALP** | 0.1943ns | 0.3073ns | -0.0567ns | 0.2120ns | 0.5642* | 0.5363* | 0.7654* | 0.7035* | | | |
| **PExPLAHP** | 0.1831ns | 0.1737ns | -0.0972ns | -0.0446ns | 0.5159* | 0.4455* | 0.7072* | 0.7403* | 0.752* | | |
| **Yield LP** | 0.2066ns | 0.2968ns | -0.0246ns | 0.2230ns | 0.5591* | 0.5151* | 0.7536* | 0.7001* | 0.9848* | 0.763* | |
| **Yield HP** | 0.1677ns | 0.1079ns | -0.0715ns | -0.0668ns | 0.4114* | 0.3346* | 0.6571* | 0.6862* | 0.7093* | 0.9652* | 0.7295* |

(*) P <0.05

**Figure 1.** Population distribution for seed iron and zinc content in Colombian genotypes grown under high and low phosphorus in Darien 2004A

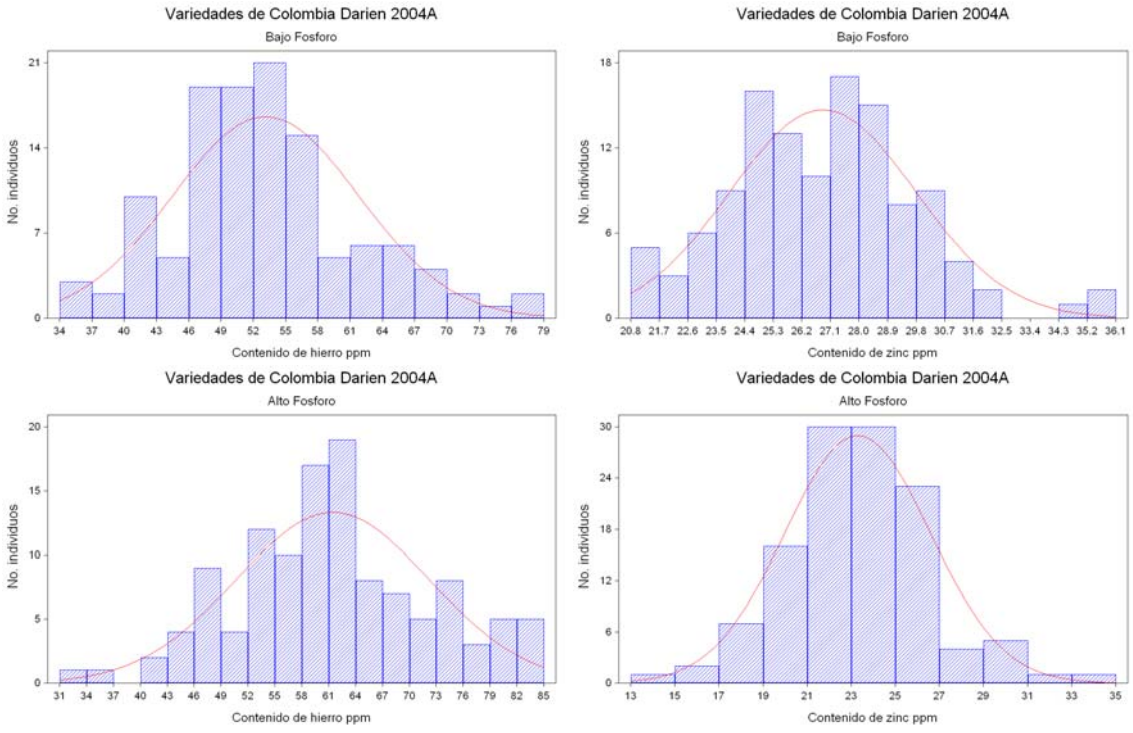
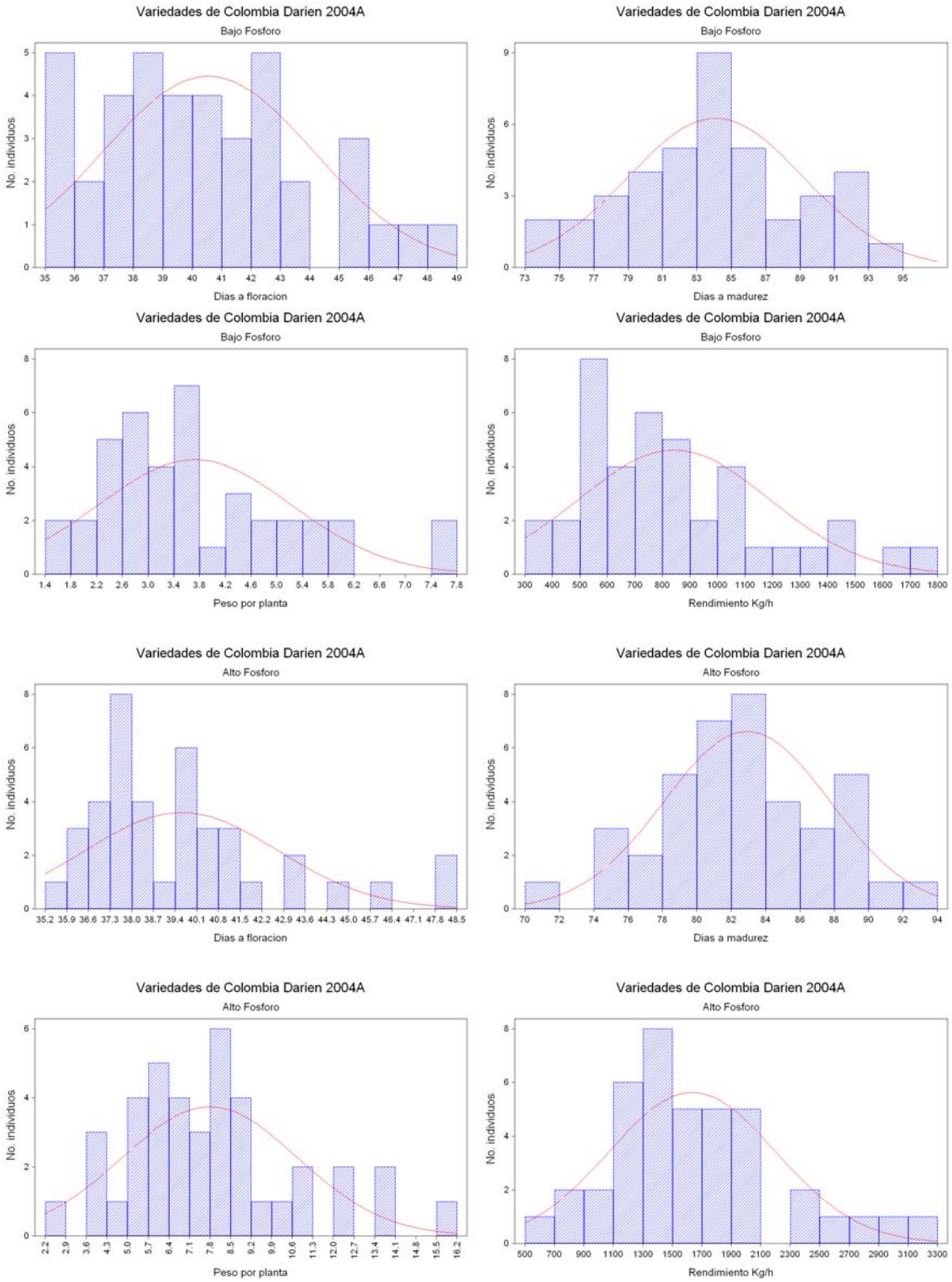


**Figure 2.** Population distribution for days to flowering, days to maturity and yield (in g/plant and kg/ha) in Colombian genotypes grown under high and low phosphorus in Darien 2004A



### 1.1.9 Analysis of seed iron and zinc concentration in a collection of Bolivian landraces and potential varieties

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#### Introduction

Within Latin America, Bolivia is a primary center of diversity for the Andean gene pool of common beans. We have been collaborating with the national germplasm collection and bean programs of Bolivia to characterize their collection of cultivated and wild common beans (*Phaseolus vulgaris)* and relatives *P. coccineus*, *P. lunatus*, *P. augusti*, for seed iron and zinc concentration. In this section we will describe the analysis of over 200 genotypes from Bolivia.

#### Materials and Methods

Two groups of genotypes were analyzed: one consisted of 20 breeding lines that have been or are close to release in Bolivia by the national bean program at Univ. Autonoma Gabriel Rene Moreno and the other consisted of a 250 genotypes from a germplasm collection of landraces and wild accessions held at the Center for Plant Genetic Resources of the Pairumani Foundation. The germplasm collection included a total of 224 accessions of common bean, *Phaseolus vulgaris* (214 cultivated/10 wild); 14 accessions of lima bean, *P. lunatus*; 2 accessions of scarlet runner bean, *P. coccineus*, and 8 accessions of wild lima bean relative, *P. augustii*. Seed was grown at Vallecito, the experiment station of the University, or at the farm of the Foundation (Figure 1) for each of the germplasm groups, respectively over the period of late 2004 to early 2006. Seed from the germplasm bank was multiplied from an initial stock mostly taken from collection sites. The full set of genotypes was roughly milled into a grits and 5 g each were sent to Colombia. Seed mineral content was evaluated at CIAT by grinding 3 g of grain into a fine powder using a modified Retsch mill with teflon chamber and zirconium grinding balls. Powder was transferred to 25 ml plastic tubes and analyzed for both iron and zinc concentration measured in parts per million (ppm) with a wet digestion method and Atomic Absorption spectrophotometry in the Analytical Services laboratory of CIAT. A total of 30 soil samples were taken at the production sites and mixed into 2 bulks before being analyzed for iron, zinc and other common elements.

#### Results and Discussion

Data was obtained for a total of 248 of the germplasm entries and all of the breeding lines. In the germplasm collection, iron and zinc concentration varied by species with higher averages for iron in *P. lunatus* than for the other species (Table 1). Meanwhile, for zinc concentration the highest average was for the accessions of *P. coccineus* compared to both cultivated common bean and *P. augustii* but not to wild common bean. Within *P. vulgaris*, wild accessions were notable in that they showed the highest averages for iron and zinc levels that were consistently higher than for cultivated accessions of common bean. The range of iron values was broader in the cultivated beans (39.7 to 100.7 ppm) than in the wild beans (72.8 to 91.0). The same was true of zinc values which were 20.2 to 51.2 ppm in the cultivated beans and 29.7 to 36.5 in wild beans. The overall range in the collection is shown in Figure 2. Soils from the Pairumani site were analyzed and found to be high in iron and sufficient in zinc. They were mineral soils with low organic material (2.0 to 2.3%), near neutral pH (5.9 to 6.1) and with high levels of phosphorus (P) and potassium (K) as shown in Table 2. The breeding lines from Univ. AGRM were found to be intermediate in iron and zinc levels. Highest concentration for iron were found in FOT 49; ICTA JU 95-13; AND 1090, AND 1088 and Blanco Laran Mejorado with values of 84.1; 83.91; 83.6;

82.8 and 81.3 ppm, respectively. The zinc levels high for some of the same genotypes, namely ICTA JU 95 13; BRB 229; DFA 58; AND 1088 and FOT 49 with values of 48.3; 47.6; 46.5; 44.6 y 43.9 ppm, respectively. Tables 3 and 4 show the low iron and zinc lines as well as the descriptive statistics for the experiment.

**Table 1.** Iron and zinc concentration by species within the Bolivian germplasm collection from the Pairumani Foundation.

| Species | **No** | **Iron** | | | | **Zinc** | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **Avg.** | **SD** | **Max (ppm)** | **Min (ppm)** | **Avg.** | **SD** | **Max (ppm)** | **Min (ppm)** |
| *P. vulgaris* cult. | 214 | 66.3 | 0.695 | 100.7 | 39.69 | 29.38 | 0.345 | 51.16 | 20.18 |
| *P. vulgaris* wild | 10 | 80.2 | 3.206 | 90.98 | 72.81 | 33.33 | 1.595 | 36.52 | 29.72 |
| *P augustii* | 8 | 64.9 | 3.584 | 53.18 | 84.91 | 26.25 | 1.784 | 22.34 | 29.99 |
| *P. lunatus* | 14 | 74.3 | 2.709 | 98.53 | 61.36 | 30.18 | 1.348 | 38.25 | 21.64 |
| *P. coccineus* | 2 | 60.4 | 7.168 | 73.6 | 47.15 | 32.23 | 3.568 | 33.64 | 30.82 |
| **Total** | **248** | **69.2** | | **100.7** | **39.69** | **30.27** | | **51.16** | **20.18** |

**Table 2.** Soil analysis for the Pairumani Foundation farm.

| Sample | Org. Matter. % | Total N % | P mg/kg | K cmol/kg | B ppm | Fe ppm | Zn ppm | pH |
|---|---|---|---|---|---|---|---|---|
| 1 | **2.3** | **0.12** | **53** | **0.33** | **0.54** | **56** | **4.2** | **6.1** |
| 2 | **2.0** | **0.12** | **44** | **0.34** | **0.48** | **62** | **3.6** | **5.9** |

**Table 3.** Iron and zinc concentration in promising breeding lines grown under 2005 fall season in Santa Cruz, Bolivia.

| N° | **Genotypes** | **Fe** | **Zn** | N° | **Genotypes** | **Fe** | **Zn** |
|---|---|---|---|---|---|---|---|
| **1** | FOT 49 | 84.13 | 43.90 | **11** | SEQ 1041 | 72.66 | 36.64 |
| **2** | ICTA JU 95-13 | 83.91 | 48.27 | **12** | LR 93201723 | 71.03 | 39.16 |
| **3** | AND 1090 | 83.58 | 39.03 | **13** | RAA 34 | 70.28 | 41.40 |
| **4** | AND 1088 | 82.76 | 44.65 | **14** | PEROLA | 69.66 | 31.39 |
| **5** | Blanco Laran Mejorado | 81.27 | 35.52 | **15** | BRB 211 | 69.41 | 32.21 |
| **6** | RAA 15 | 78.81 | 40.62 | **16** | TB 94-01 | 67.94 | 35.84 |
| **7** | LP 90-88 | 78.01 | 42.42 | **17** | DFA 58 | 67.40 | 46.48 |
| **8** | POA 13 | 76.57 | 39.70 | **18** | DOR 751 | 65.58 | 35.25 |
| **9** | BRB 229 | 76.18 | 47.57 | **19** | LP 91-90 | 64.28 | 39.21 |
| **10** | POA 11 | 74.65 | 40.45 | **20** | AND 1092 | 61.58 | 35.50 |

**Table 3.** Descriptive statistics for the promising breeding lines grown under the 2005 fall season in Santa Cruz, Bolivia.

| | **Fe** | **Zn** |
|---|---|---|
| **No. of samples** | 20 | 20 |
| Average | 73.984 | 39.761 |
| Std. Dev. | 7.0242 | 4.8146 |
| CV | 9.4942 | 12.109 |
| Minimum | 61.58 | 31.39 |
| Maximum | 84.13 | 48.27 |

**Figure 1.** Multiplication of common bean samples from Pairumani

### 1.1.10 Analysis of seed iron and zinc concentration in a collection of Rwandan landraces

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#### Introduction

As part of the Harvest Plus challenge program the ECABREN and SABRN teams have been assembling germplasm collections for the countries of Eastern and Southern Africa to evaluate for seed iron and zinc concentration. In this section we will describe the initial analysis of the collection from Rwanda which consists in over 1000 genotypes with very variable seed size from small, medium to large and seed colors ranging from white, cream, cream mottled, yellow, yellow mottled, pink, red, red mottled to purple seed classes. Rwanda has often been cited for its bean diversity and this may be explained by the importance of beans in the Rwandese diet and their widespread distribution across almost all the prefectures of the country.

## Materials and Methods

Genotypes were provided by ISAR to the Univ. of Nairobi where they were multiplied and sent for destructive sampling at CIAT in Colombia. The full set of genotypes sent to Colombia was 1021 accessions. Of these sufficient seed was available for 984 genotypes which were used for iron and zinc measurements. Seed mineral content was evaluated at CIAT by grinding 3 g of grain into a fine powder using a modified Retsch mill with teflon chamber and zirconium grinding balls. Powder was transferred to 25 ml plastic tubes and analyzed for both iron and zinc concentration measured in parts per million (ppm) with a wet digestion method and Atomic Absorption spectrophotometry in the Analytical Services laboratory of CIAT.

## Results and Discussion

Iron and zinc concentration ranged from 45.2 to 115.1 and 22 to 49.2 ppm, respectively, in the large set of genotypes from Rwanda evaluated in the present study. Average concentrations were 69 and 34 ppm for iron and zinc, respectively (Table 1, Figure 1). The genotypes with the highest mineral content are shown in Table 2 and include RW688, RW986 and RW1046 for iron and RW820, RW580 and RW1326 for zinc. The genotypes with the lowest concentrations were RW32, RW305 and RW77. correlations between the two minerals was significant (r=0.53; P=0.0000).

## Future Work

In a related project, a total of 600 genotypes have been used for DNA extraction and 376 of these are being genotyped with microsatellite fingerprinting. The best genotypes will be used in crosses. Quarantine grow out of the genotypes at CIAT is pending greenhouse facilities in Bogota.

**Table 1.** Descriptive statistics for seed iron and zinc concentration in genotypes from Rwanda.

| | Iron | Zinc |
|---|---|---|
| N | 984 | 984 |
| Mean | 69.320 | 34.842 |
| SD | 8.8016 | 4.3393 |
| Variance | 77.468 | 18.830 |
| SE Mean | 0.2806 | 0.1383 |
| C.V. | 12.697 | 12.454 |
| Minimum | 45.190 | 22.020 |
| Median | 68.890 | 34.670 |
| Maximum | 115.11 | 49.220 |
| Skew | 0.4256 | 0.2046 |
| Kurtosis | 1.1947 | 0.3968 |

**Table 2**. Genotypes presenting the highest and lowest iron and zinc concentration among 984 Rwandese genotypes analyzed at CIAT.

| Genotype | **Iron** | **Zinc** | Genotype | **Iron** | **Zinc** | Genotype | **Iron** | **Zinc** |
|---|---|---|---|---|---|---|---|---|
| RW 688 | 115.1 | 42.9 | RW 683 | 86.4 | 42.4 | RW 600 | 82.9 | 44.3 |
| RW 986 | 113.2 | 49.2 | RW 298 | 86.4 | 33.3 | RW 439 | 82.9 | 39.9 |
| RW 1046 | 102.9 | 37.9 | RW 1180 | 85.9 | 41.6 | RW 801 | 82.6 | 41.9 |
| RW 820 | 98.0 | 48.9 | RW 1087 | 85.6 | 34.4 | RW 767 | 82.6 | 37.9 |
| RW 582 | 97.6 | 49.0 | RW 839 | 85.6 | 47.3 | RW 880 | 82.6 | 47.0 |
| RW 580 | 96.4 | 42.1 | RW 248 | 85.1 | 36.6 | RW 257 | 82.5 | 43.6 |
| RW 1326 | 95.7 | 23.3 | RW 593 | 84.8 | 35.3 | RW 267 | 82.5 | 37.8 |
| RW 648 | 93.3 | 38.9 | RW 846 | 84.7 | 43.2 | RW 1244 | 82.4 | 42.2 |
| RW 850 | 90.6 | 41.4 | RW 942 | 84.7 | 41.7 | RW 774 | 82.3 | 44.0 |
| RW 806 | 90.0 | 46.2 | RW 731 | 84.7 | 35.6 | RW 601 | 82.2 | 38.4 |
| RW 324 | 90.0 | 33.6 | RW 849 | 84.5 | 46.3 | RW 745 | 82.2 | 42.4 |
| RW 833 | 89.6 | 42.8 | RW 1245 | 84.4 | 40.9 | RW 837 | 82.1 | 45.0 |
| RW 375 | 89.4 | 40.4 | RW 184 | 84.4 | 41.5 | RW 1261 | 82.0 | 45.8 |
| RW 721 | 88.9 | 42.9 | RW 613 | 84.4 | 38.0 | RW 218 | 82.0 | 34.0 |
| RW 615 | 88.9 | 39.1 | RW 297 | 84.4 | 35.7 | RW 805 | 81.8 | 43.0 |
| RW 834 | 88.9 | 46.2 | RW 447 | 84.3 | 40.7 | RW 547 | 81.7 | 43.4 |
| RW 825 | 88.8 | 40.3 | RW 611 | 84.2 | 39.0 | RW 700 | 81.7 | 36.0 |
| RW 835 | 88.2 | 46.0 | RW 971 | 84.2 | 25.8 | RW 667 | 81.7 | 37.4 |
| RW 583 | 87.5 | 39.5 | RW 655 | 84.1 | 39.4 | RW 96 | 51.1 | 31.1 |
| RW 221 | 87.5 | 37.3 | RW 588 | 83.7 | 39.8 | RW 76 | 50.5 | 35.2 |
| RW 581 | 87.1 | 39.5 | RW 684 | 83.6 | 48.0 | RW 1096 | 50.3 | 32.7 |
| RW 697 | 87.1 | 38.9 | RW 438 | 83.6 | 40.1 | RW 519 | 49.2 | 25.9 |
| RW 1234 | 86.9 | 32.6 | RW 1179 | 83.4 | 41.0 | RW 95 | 48.9 | 30.1 |
| RW 500 | 86.7 | 41.9 | RW 651 | 83.4 | 41.5 | RW 40 | 48.4 | 31.4 |
| RW 1172 | 86.6 | 44.3 | RW 896 | 83.3 | 38.8 | RW 1097 | 46.0 | 30.2 |
| RW 216 | 86.5 | 39.1 | RW 474 | 83.2 | 36.2 | RW 32 | 45.8 | 30.0 |
| RW 693 | 86.5 | 36.8 | RW 937 | 83.1 | 40.5 | RW 305 | 45.6 | 31.7 |
| RW 28 | 86.5 | 34.8 | RW 716 | 83.0 | 39.3 | RW 77 | 45.4 | 33.2 |

**Figure 1**. Population distribution for seed iron and zinc content in genotypes from Rwanda.

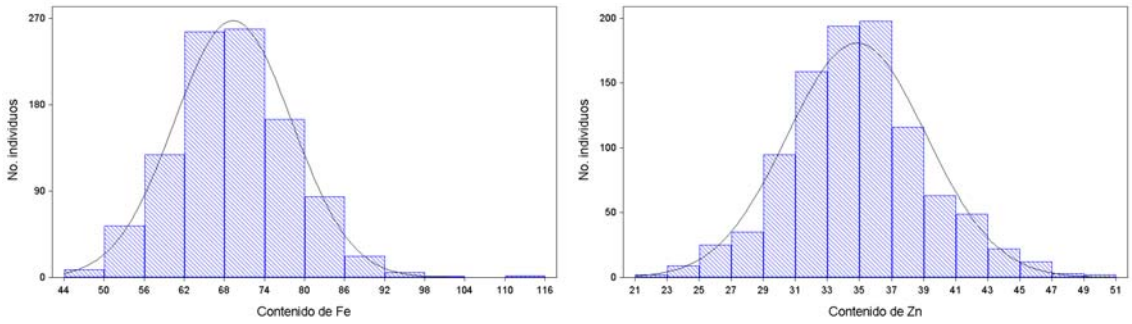


**Figure 2**. High iron genotypes from Rwanda.

**RW 688**
Fe 115.1 ppm
Zn 42.9 ppm

**RW 986**
Fe 113.2 ppm
Zn 49.2 ppm

**RW 1046**
Fe 102.9 ppm
Zn 37.9 ppm

**RW 820**
Fe 98.0 ppm
Zn 48.9 ppm

**RW 580**
Fe 96.4 ppm
Zn 42.1 ppm

**RW 1326**
Fe 95.7 ppm
Zn 23.3 ppm

## 1.1.11 Single sequence repeat marker diversity in cassava: study of the level of genetic redundancy and identification of genotypes

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### Introduction

In an asexually propagated germplasm collection, duplicate accessions may be common. In the case of the cassava collection at CIAT, 20 % to 25 % is estimated to be as internal duplicated. The presence of genetic duplicates in a germplasm collection has serious implication for germplasm conservation, as well as for a breeding program. Such redundancy makes the existing collection more expensive to maintain and manage, and slows down the introduction of new germplasm (Hershey et al. 1991). For cassava, a large number of these possible genetic duplicates were identified using passport, morphological, and isozyme characterization (Ocampo et al. 1993; Jiménez 1994; Sumarani et al. 2004). The combination of molecular markers with morphology/passport/isozymes can give a high degree of confidence into identifying duplicates (Ocampo et al. 1995). Now we propose to confirm these groups of possible genetic duplicates, using the technology of DNA fingerprinting (single sequence repeat markers or SSR); that is, to detect genotypic differences among these groups that otherwise appear identical in their morphology and isozyme-banding patterns (Chavarriaga et al. 1999). The additional objectives are: (1) to develop a description of each accession based on its molecular pattern (fingerprinting) as a criterion to avoid genetic duplicates when new germplasm is introduced in the cassava collection; (2) once known the level of redundancy, to study the distribution of the resulting genetic diversity in the different agroecological zones of Colombia.

### Materials and Methods

*Plant material*. This work has been initiated on the designate cassava germplasm collection of Colombia, consisting of 1,986 accessions (the largest collection by country). The *in vitro* Cassava Laboratory (GRU) provided the accessions to characterize according to their morphological and isozymatic similarities.
*Molecular Markers*. One type of molecular markers that may be suitable for cassava germplasm characterization is the microsatellite (SSR). Microsatellites are considered more sensitive in detecting genotypic differences as compared to morphological and isoenzyme descriptors. Microsatellites, like RFLPs, are considered codominant markers. Their high polymorphism makes microsatellites suitable markers in order to identify redundancies in cassava (Chavarriaga et al. 1998). A set of seven SSR markers, carefully chosen to represent coverage of the cassava genome with moderate to high polymorphism information content (PIC) and robust amplification, were used in this study (Table 1).

Table 1. Different primer combinations evaluated in this study. Parameters of number of alleles per locus and polymorphism information content (PIC) are included (Marin et al. 2003).

| Primer | | Alleles per locus | Polymorphism information content (PIC) |
|---|---|---|---|
| SSRY100 | 498 | 17 | 0.828 |
| SSRY82 | 381 | 11 | 0.813 |
| SSRY106 | 507 | 14 | 0.784 |
| SSRY69 | 313 | 16 | 0.729 |
| SSRY59 | 249 | 17 | 0.672 |
| SSRY105 | 506 | 13 | 0.538 |
| SSRY109 | 521 | ---------- | ---------- |

**Results and Discussion**

The molecular variation was assessed at seven unlinked SSR markers (Fregene et al. 2003) in 188 accessions of cassava landraces grouped into 78 groups of possible genetic duplicates according to their biochemical and morphological similarities. The number per group varied between 2, 3, 4, 5 and 6 accessions, showing 110 redundant accessions among the 188 duplicated accessions (Tables 26 and 27). The molecular grouping obtained on these morphobiochemical groups shows that the SSR polymorphisms obtained were displayed principally among different groups and in a moderate proportion within these groups. This level of polymorphism is moderate due to high similarity among accessions, which are closely related as previously determined by morphological and biochemical markers (Ocampo et al. 1993; Jimenez 1994). The molecular fingerprinting analysis confirms 91 % of the morphobiochemical groups, showing 71 different molecular groups (between 2 and 5 accessions within of each group). Additionally 27 accessions that are uniquely separated from other accessions, represent unique genotypes. Therefore the molecular grouping forms 98 groups, including 27 unique genotypes (Table 2). There is an important reduction of 18 % in the level of genetic redundancy: 90 redundant accessions versus the 110 original redundant accessions, which imply changing 188 duplicated accessions to 161 accessions with the molecular grouping (Table 3). In conclusion, if these accessions are indeed genetically identical, they could be pooled together with no loss in the overall amount of genetic variation. Furthermore, the fact that most of these groups of possible genetic duplicates were confirmed by seven SSR markers suggests that the model developed at CIAT to detect these duplicates is reliable. However it might be desirable to test more SSR markers to confirm with high reliability these possible genetic duplicates.

Table 2. Accessions involved as morphobiochemical duplicates versus molecular duplicates of the Colombian cassava collection held at CIAT as a FAO Designate Collection.

| Group No. | Morphobiochemical duplicates (Accessions) | Group No. | Molecular duplicates (Accessions) |
|---|---|---|---|
| 1 | COL 25, 896 | 1 | COL 25, 896 |
| 2 | COL 45, COL 948C, COL 1008, COL 1431 | 2 | COL 45, COL 948C, |
| | | 3 | COL 1008, COL 1431 |
| 3 | COL 61, COL 1978 | 4 | COL 61, COL 978 |
| 4 | COL 70, COL 78B | 5 | COL 70, COL 78B |
| 5 | COL 76B, COL 912A, COL 927, COL 1962 | 6 | COL 76B, COL 912A, COL 927, COL 1962 |
| 6 | COL 81, COL 647, COL 1067, COL 106, COL 1538 | 7 | COL 81, COL 647, COL 1067, COL 106, COL 1538 |
| 7 | COL 93, COL 1044 | 8 | COL 93, COL 1044 |
| 8 | COL 134, COL 138 | 9 | COL 134, COL 138 |
| 9 | COL 137, COL 140, COL 145 | 10 | COL 137, COL 140, COL 145 |
| 10 | COL 207, COL 1485 | 11 | COL 207, COL 1485 |
| 11 | COL 240, COL 281 | 12 | COL 240, |
| | | 13 | COL 281 |
| 12 | COL 261, COL 547 | 14 | COL 261, |
| | | 15 | COL 547 |
| 13 | COL 376, COL 380, COL 588A, COL 727 | 16 | COL 376, COL 380 |
| 14 | COL 436, COL 2617 | 17 | COL 436, COL 2617 |
| 15 | COL 437A, COL 1934 | 18 | COL 437[a], COL 1934 |
| 16 | COL 467, COL 1720 | 19 | COL 467, COL 1720 |
| 17 | COL 942, COL 958, COL 1955 | 20 | COL 942, |
| | | 21 | COL 958, COL 1955 |
| 18 | COL 1043, COL 1057, COL 1065 | 22 | COL 1043, COL 1057, COL 1065 |
| 19 | COL 1092, COL 1602, COL 1616, COL 1821 | 23 | COL 1092, COL 1602, |
| | | 24 | COL 1616, COL 1821 |
| 20 | COL 2239, COL 1830, COL 1828A, COL 1518, COL 1516, COL 151 | 25 | COL 2239, COL 1830, COL 1828A, COL 1518, |
| | | 26 | COL 1516, COL 151 |
| 21 | COL 1601, COL 1990, | 27 | COL 1601, COL 1990, |
| 22 | COL 2282, COL 2297, COL 2375, COL 2390 | 28 | COL 2282, |
| | | 29 | COL 2297, COL 2375, COL 2390 |
| 23 | COL 2286, COL 2292, COL 2298, COL 2300, COL 2313 | 30 | COL 2286, COL 2292, COL 2298, COL 2300, COL 2313 |
| 24 | COL 1672, COL 1673, COL 1678 | 31 | COL 1672, COL 1673, COL 1678 |
| 25 | COL 1711, COL 1764A, COL 1764B | 32 | COL 1711 |
| | | 33 | COL 1764A, COL 1764B |
| 26 | COL 1772, COL 1777, COL 1781, COL 1895 | 34 | COL 1777, |
| | | 35 | COL 1781, COL 1895, COL 1772, |
| 27 | COL 1786, COL 1879, COL 2023 | 36 | COL 1879, |
| | | 37 | COL 1786, COL 2023 |
| 28 | COL 1889, COL 1893, COL 1894 | 38 | COL 1889, |
| | | 39 | COL 1893, COL 1894 |

| | | | |
|---|---|---|---|
| 29 | COL1896, COL 1900, COL 2062 | 40 | COL1896, |
| | | 41 | COL 1900, COL 2062 |
| 30 | COL 1901, COL 1902, COL 1903 | 42 | COL 1901, COL 1902, COL 1903 |
| 31 | COL 2358, COL 2362, COL 2407 | 43 | COL 2358, COL 2362, COL 2407 |
| 32 | COL 275, COL 290 | 44 | COL 275, COL 290 |
| | | 45 | COL 280, |
| 33 | COL 280, COL 2542 | 46 | COL 2542 |
| 34 | COL 286, COL 328 | 47 | COL 286, COL 328 |
| 35 | COL 303, COL 306 | 48 | COL 303, COL 306 |
| 36 | COL 344, COL 386 | 49 | COL 344, COL 386 |
| 37 | COL 475, COL 1452 | 50 | COL 475, COL 1452 |
| 38 | COL 476, COL 494 | 51 | COL 476, COL 494 |
| 39 | COL 487, COL 509 | 52 | COL 487, COL 509 |
| 40 | COL 488, COL 490 | 53 | COL 488, COL 490 |
| | | 54 | COL 654, |
| 41 | COL 654, COL 667A | 55 | COL 667A |
| 42 | COL 661, COL 663 | 56 | COL 661, COL 663 |
| 43 | COL 671, COL 673A | 57 | COL 671, COL 673A |
| | | 58 | COL 683, |
| 44 | COL 683, COL 1442 | 59 | COL 1442 |
| 45 | COL 777, COL 778 | 60 | COL 777, COL 778 |
| 46 | COL 796, COL 1486 | 61 | COL 796, COL 1486 |
| 47 | COL 800, COL 803 | 62 | COL 800, COL 803 |
| 48 | COL 902A, COL 902B | 63 | COL 902A, COL 902B |
| 49 | COL 844, COL 845A | 64 | COL 844, COL 845A |
| 50 | COL 948A, COL 1967 | 65 | COL 948A, COL 1967 |
| | | 66 | COL 957B, |
| 51 | COL 957B, COL 957C | 67 | COL 957C |
| | | 68 | COL 978, |
| 52 | COL 978, COL 974A | 69 | COL 974A |
| 53 | COL 1019, COL 1023 | 70 | COL 1019, COL 1023 |
| 54 | COL 1231, COL 1347 | 71 | COL 1231, COL 1347 |
| 55 | COL 1409, COL 1413 | 72 | COL 1409, COL 1413 |
| 56 | COL 1440, COL 1917 | 73 | COL 1440, COL 1917 |
| 57 | COL 1463, COL 2189 | 74 | COL 1463, COL 2189 |
| 58 | COL 1471, COL 1472 | 75 | COL 1471, COL 1472 |
| 59 | COL 1478, COL 2305 | 76 | COL 1478, COL 2305 |
| 60 | COL 1504, COL 1632 | 77 | COL 1504, COL 1632 |
| 61 | COL 1505, COL 2054 | 78 | COL 1505, COL 2054 |
| 62 | COL 1513, COL 1514 | 79 | COL 1513, COL 1514 |
| 63 | COL 1552, COL 1553 | 80 | COL 1552, COL 1553 |
| 64 | COL 1563, COL 1564 | 81 | COL 1563, COL 1564 |
| 65 | COL 1566, COL 1717 | 82 | COL 1566, COL 1717 |
| | | 83 | COL 1607, |
| 66 | COL 1607, COL 10 | 84 | COL 10 |
| 67 | COL 1613, COL 1614 | 85 | COL 1613, COL 1614 |
| | | 86 | COL 1630, |
| 68 | COL 1630, COL 1745 | 87 | COL 1745 |
| 69 | COL 1667, COL 1671 | 88 | COL 1667, COL 1671 |

| | | | |
|---|---|---|---|
| 70 | COL 1823, COL 2229 | 89 | COL 1823, COL 2229 |
| 71 | COL 1868A, COL 1868B | 90 | COL 1868A, COL 1868B |
| 72 | COL 1884, COL 1898 | 91 | COL 1884, COL 1898 |
| 73 | COL 1912, COL 1915 | 92 | COL 1912, COL 1915 |
| 74 | COL 2004, COL 2007 | 93 | COL 2004, COL 2007 |
| 75 | COL 2025, COL 2033 | 94 | COL 2025, |
| | | 95 | COL 2033 |
| 76 | COL 2082, COL 2203 | 96 | COL 2082, COL 2203 |
| 77 | COL 2107, COL 2114 | 97 | COL 2107, COL 2114 |
| 78 | COL 2143, COL 2147 | 98 | COL 2143, COL 2147 |

Table 3. Description of the morphobiochemical duplicates versus molecular duplicates of the colombian cassava germplasm collection held at CIAT as a FAO Designate Collection.

| Type of Group | Morphobiochemical duplicates | | | Molecular duplicates | | | |
|---|---|---|---|---|---|---|---|
| | No. of each group | Duplicated accessions | Redundant accessions | No. of each group | Duplicated accessions | Redundant accessions | "Single accessions" |
| Groups containing 2 similar accessions | 59 | 118 | 59 | 59 | 118 | 59 | 27 |
| Groups containing 3 similar accessions | 10 | 30 | 20 | 7 | 21 | 14 | |
| Groups containing 4 similar accessions | 6 | 24 | 18 | 3 | 12 | 9 | |
| Groups containing 5 similar accessions | 2 | 10 | 8 | 2 | 10 | 8 | |
| Groups containing 6 similar accessions | 1 | 6 | 5 | 0 | 0 | 0 | |
| Total | 78 | 188 | 110 | 71 | 161 | 90 | 27 |

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### 1.1.12 Development of a reference SSR marker kit to Analyze Diversity in Cassava (*Manihot esculenta* Crantz)

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CIAT

Funding: Generation Challenge Program (GCP)

**Introduction**

The cassava genetics program has developed over 800 SSR markers. These marker were developed in previous studies (Cano, 2004, Fregene, 1997, Zarate, 2002 and Garcia, 2002) and all of them have been used to evaluate mapping populations looking for association with genes controlling traits of agronomic interest. Up to 67 of those markers have been used in previous diversity studies to characterize germplasm from several countries (MOLCAS, www.ciat.cgiar.org/molcas/index.jsp). The last diversity study included more than 2500 accessions from 3 germplasm collections and a group of 36 SSR markers.

Since the characterization of genetic diversity in cassava helps to identify useful variability for breeding and to eliminate duplicates, it is important to have an established set of markers that can be used to define genetic diversity in any group of cassava accessions and also a reference set of samples showing the expected range of alleles.

This GCP-commissioned project aims to develop a SSR kit for estimation of genetic diversity and also to propose a reference sample set that could be accessible to the entire cassava research community. The genetic information generated in the GCP diversity pilot study (Annual Report, CIAT, 2004) and the GCP global diversity study (Annual Report, CIAT, 2005) allowed the selection of a set of cassava accessions with a complete range of alleles for cassava diversity and a subset, representing the different cassava clusters. It also provides information to evaluate the candidate SSR markers to compose an SSR kit.

Based on the genetic information that we have generated in the last 6 years, a data base compiling all the SSR markers description was generated. The data base includes: molecular pattern, number of alleles, definition of alleles in polyacryilamide gels, and molecular weight differentiation for all the available SSR markers in cassava. Only the SSR markers with a polymorphic pattern, more than 2 alleles, high definition of alleles in polyacrilamide gels and high PIC values were selected as candidates to compose the SSR kit. Some of the candidate markers have been only used in genetic mapping studies so the PIC values were calculated for all of them and the markers showing the highest values were finally selected. Markers used in previous diversity studies showing the highest average PIC value were selected.

The location of the SSR markers in the cassava genetics map is the final selection criteria with the objective of looking for a high representation of the cassava genome. To validate this group of markers in diversity studies, a subset of samples selected in this project were evaluated with the candidate SSR markers and based on the results and their location on the cassava genetic map, 36 SSR markers were selected to compose the cassava SSR kit.

**Methodology**

*Selection of cassava accessions representing the complete range of alleles in cassava diversity.*
36 SSR markers have been used by the cassava genetics program to assess global genetic diversity in Cassava (Annual Report, CIAT, 2004, 2005). Another 11 diversity studies of cassava accessions from different sources previously conducted also employed some of the 36 SSR markers and in some cases up to 67 (Fregene et al. 2003).

Using the results from the GCP diversity pilot study (Annual Report, CIAT, 2004), 45 samples with the complete range of alleles for cassava diversity were selected from the structure defined by the PCoA (Figure 1A and 1B).

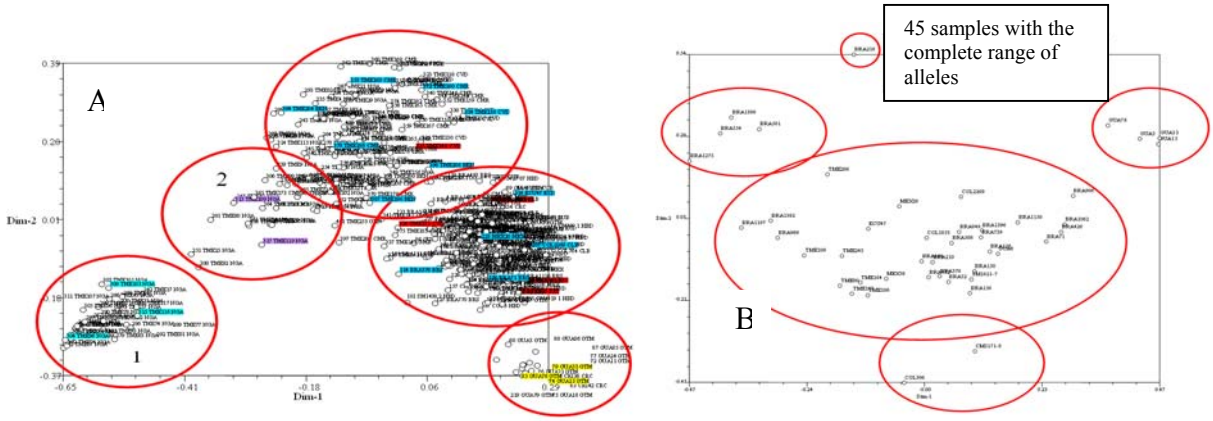


Figure 1. A. Genetic structure of Cassava accessions included in the GCP diversity pilot study. B. Genetic structure and distribution of 45 cassava accessions with the complete range of alleles for cassava diversity.

*Selection of cassava accessions (subssample) to evaluate the SSR markers composing the SSR kit.*
In order to define a group of cassava accessions that can be used to evaluate the candidate SSR markers composing the kit, at least 2 samples were selected from each group defined by the PCoA from the GCP pilot study (Annual Report, CIAT, 2004). To prove the trustworthiness of the 8 selected samples, the polymorphic Information Content (PIC) was calculated for each of the 36 SSR markers used to estimate genetic diversity in the GCP diversity pilot study. The values were compared with the ones obtained for the same markers using genotypic information from 432 samples included in the pilot study.

*SSR markers used in previous studies for genetic mapping*

Approximately 1,200 images were evaluated from 817 SSR markers that have been developed for cassava (Cano, 2004, Fregene, 1997, Zarate, 2002 and Garcia, 2002). From this evaluation a database was developed to compile genetic information for the whole group of SSR markers using the categories presented in Table 1. The database will be accessible to the entire cassava research community through the GCP web site and MOLCAS (www.ciat.cgiar.org/molcas/index.jsp)

Table 1. Different categories used to evaluate the 817 SSR markers.

| 1.Polymorphic pattern |
|---|
| yes = Y |
| no = N |
| 2. Number of alleles |
| No. of alleles in the gel. |
| 3. Definition of alleles (clear and defined bands) |
| Clear band = B |
| Moderate band = M |
| Undefined band = L |
| 4. Mol. Weigth differentiation (distance between bands) |
| high = H |
| moderate = M |
| Low = L |
| 5. Rationale (primer dimmer) |
| high = H |
| moderate = M |
| low = L |
| 6. Source |
| BSA = bulk segregant analysis |
| P = evaluation of mapping population |
| D = diversity study |

Once the database was composed, a selection of candidate SSR markers was done, based on the criteria shown in Table 2

Table 2 List of criteria to select SSR markers

| Criteria | Polymorphic pattern | No. of allleles | Definition of alleles | Mol. Weight differentiation | Rationale |
|---|---|---|---|---|---|
| 1 | Y | More than 2 | H | H | L |
| 2 | Y | More than 2 | M and H | M and H | M and L |
| 3 | N | More than 2 | M and H | M and H | M and L |
| 4 | N | 1 | H | H | L |

*SSR markers used in previous diversity studies*

Looking at previous cassava diversity studies, 67 SSR markers have been used to assess diversity in the crop. Each diversity study has employed at least 30 markers out of the group of 67. PIC values were calculated for the whole group using genotypic information previously generated.

*Selection of SSR markers composing the SSR kit*
Candidate SSR markers from genetic mapping and previous diversity studies were used to select the ones composing the SSR kit following several criteria: high PIC values, high polymorphism, high definition of alleles and distribution genome wide (at least one SSR marker representing each linkage group).

Results
*Selection of cassava accessions representing the complete range of alleles in cassava diversity.*
The structure of the selected accessions and their distribution was analyzed and the list of genotypes that represent the complete range of alleles of cassava diversty is presented in Table 3.

Table 3. 45 accessions with the complete range of alleles of genetic diversity in cassava.

| No. | GENOTIPO | No. | GENOTIPO | No. | GENOTIPO |
|---|---|---|---|---|---|
| 1 | BRA1062 | 16 | BRA370 | 31 | GUA15 |
| 2 | BRA1150 | 17 | BRA426 | 32 | GUA3 |
| 3 | BRA1197 | 18 | BRA432 | 33 | GUA33 |
| 4 | BRA126 | 19 | BRA501 | 34 | GUA6 |
| 5 | BRA1275 | 20 | BRA508 | 35 | GUA76 |
| 6 | BRA1392 | 21 | BRA71 | 36 | MEX20 |
| 7 | BRA1396 | 22 | BRA739 | 37 | MEX58 |
| 8 | BRA1399 | 23 | BRA849 | 38 | SM1821-7 |
| 9 | BRA150 | 24 | BRA906 | 39 | TME103 |
| 10 | BRA156 | 25 | BRA969 | 40 | TME109 |
| 11 | BRA164 | 26 | CM3171-8 | 41 | TME164 |
| 12 | BRA210 | 27 | COL1853 | 42 | TME186 |
| 13 | BRA216 | 28 | COL2269 | 43 | TME206 |
| 14 | BRA32 | 29 | COL306 | 44 | TME243 |
| 15 | BRA354 | 30 | ECU47 | 45 | TME80 |

*Selection of cassava accessions (sub sample) to evaluate SSR markers from the SSR kit.*
A set of 8 accessions representing the genetic structure of cassava was composed (TMS30572, GUA76, BRA376, BRA71, ECU47, TME116, TME119, TME160). Their genetic structure and distribution were accessed with a principal coordinate analysis (Figure 2). TMS3057-2 (NGA 2) was added to the group since this accession has been always used as DNA control for genotyping in cassava.

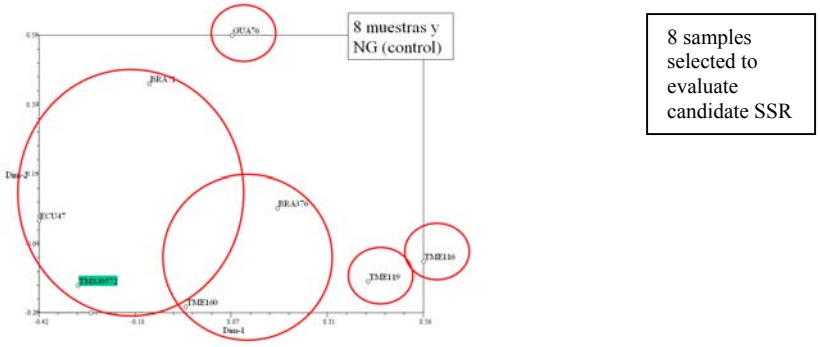


Figure 2 Distribution of 8 samples selected to evaluate the candidate SSR markers composing the SSR kit.

PIC values calculated for the 36 SSR markers based on 432 samples, analyzed in the GCP diversity pilot study, have strong relationship with PIC values calculated for the same makers using the 8 selected samples (Figure 3). The correlation between PIC from 432 samples and PIC from 8 samples is 0.87 and the average of the differences between them is 0.02. Based on this analysis, it can be concluded that the 8 samples selected from the GCP diversity study can be used to evaluate candidate SSR markers to compose the SSR kit as well as to calculate their PIC for selection of markers with the highest values.

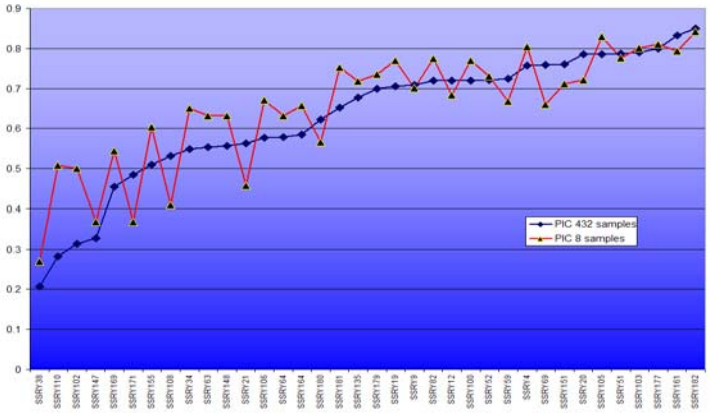


Figure 3. PIC values distribution for 36 SSR markers used in the GCP diversity pilot study. Blue line: 432 samples evaluated. Red line: 8 selected samples.

*SSR markers used in previous studies for genetic mapping*

174 SSR markers were selected from this database following the criteria 1 presented in the Table 1. Those markers, with polymorphic pattern, more than 2 alleles/ locus, high resolution and high molecular weight differentiation, represent the first group of candidate markers to compose the SSR kit. The 8 accessions composing the sub sample were evaluated with the 174 SSR candidate markers and their PIC values were calculated. Only 19 markers with the highest values (up to 0.5) were selected as candidate to compose the SSR kit.

*SSR markers used in previous diversity studies*

PIC average was also calculated for the 67 SSR markers used in cassava diversity studies and their distribution is shown in the Figure 4. Twenty two out of the group of 67 SSR markers have PIC values lower than 0.5. 45 SSR markers used in previous diversity studies, with PIC average higher than 0.5, were selected as candidate markers to compose the SSR kit.

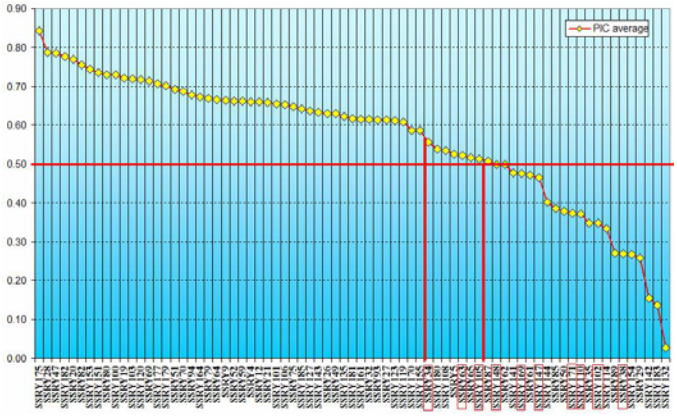


Figure 4. PIC average distribution of 67 SSR markers used in cassava diversity studies. Markers in a red box were used in the GCP diversity pilot study.

*Selection of SSR markers composing the SSR kit*

10 SSR markers were selected from previous studies on genetic mapping and 26 were selected from previous diversity studies, to give a SSR kit with a set of 36 SSR markers (Table 4, Figure 5).

Eight out of 26 and 2 out of 10 SSR markers selected from previous diversity studies and genetic mapping, respectively, are not on the cassava genetic map (SSRY 151, SSRY 69, SSRY 64, SSRY 9, SSRY 127, SSRY 155, SSRY 34, SSRY 180 and SSRY 47, SSRY 5). However, these markers were selected due to their high PIC values (higher than 0.7), high polymorphism, and high resolution of their alleles in the 11 diversity studies where they have been used.

**Conclusion and perspectives**

45 samples with the complete range of alleles for cassava diversity were selected from the GCP cassava diversity study. Eight out of 20 samples corresponding to groups in the genetic structure of that study were also selected to provide a set of genotypes that can be used to evaluate candidate markers to compose the SSR kit. This set can be also be used to test new SSR markers and calculate their PIC in future cassava studies.

A data base with genotypic information of 817 SSR markers was created using the images generated from several genetic mapping studies. Genetic data from 11 diversity studies was used to calculate the PIC values for 67 SSR markers that have been previously used to assess diversity in the crop. Once information from genetic mapping and diversity studies was complete, 36 SSR markers with high PIC values (higher than 0.5), high polymorphism (more than two alleles), high definition of alleles and distribution genome wide (at least one per linkage group) were selected to compose the SSR kit. 10 out of 174 candidate markers selected from genetic mapping were chosen and 26 out of 45 candidate markers used in previous diversity works were added to the kit.

Eight out of 26 SSR markers selected from previous diversity studies are not on the cassava genetic map. Even though, those markers were selected because of their high PIC values (higher than 0.7), high polymorphism and high resolution of their alleles in the 11 diversity studies that employed them. These markers need to placed on the cassava genetic map in further mapping studies.

Table 4. 36 SSR markers selected to compose the SSR kit

| NO | NAME | PIC (Average) | LEFT PRIMER | RIGHT PRIMER | ANNEALING TEMPERATURE |
|---|---|---|---|---|---|
| 1 | SSRY47 | 0.78 | GGAGCACCTTTTGCTGAGTT | TTGGAACAAAGCAGCATCAC | 55 |
| 2 | NS189 | 0.78 | TGGGCTGTTCGTGATCCTTA | CATGAGTTTAAAAATTATCACATCCG | 55 |
| 3 | SSRY182 | 0.77 | GGAATTCTTTGCTTATGATGCC | TTCCTTTACAATTCTGGACGC | 55 |
| 4 | SSRY20 | 0.77 | CATTGGACTTCCTACAAATATGAAT | TGATGGAAAGTGGTTATGTCCTT | 55 |
| 5 | SSRY82 | 0.76 | TGTGACAATTTTCAGATAGCTTCA | CACCATCGGCATTAAACTTTG | 55 |
| 6 | SSRY175 | 0.75 | TGACTAGCAGACACGGTTTCA | GCTAACAGTCCAATAACGATAAGG | 55 |
| 7 | NS376 | 0.75 | TCAAGACCCTTGCTTTGGTT | GGACTATCAAGGCGCAAAAG | 55 |
| 8 | SSRY151 | 0.73 | AGTGGAAATAAGCCATGTGATG | CCCATAATTGATGCCAGGTT | 45 |
| 9 | SSRY100 | 0.73 | ATCCTTGCCTGACATTTTGC | TTCGCAGAGTCCAATTGTTG | 55 |
| 10 | NS911 | 0.72 | TGTTGTTCAGACGATGTCCAA | TTGAAGCAGTTATGAACCGT | 50 |
| 11 | SSRY19 | 0.72 | TGTAAGGCATTCCAAGAATTATCA | TCTCCTGTGAAAAGTGCATGA | 55 |
| 12 | SSRY103 | 0.72 | TGAGAAGGAAACTGCTTGCAC | CAGCAAGACCATCACCAGTTT | 55 |
| 13 | SSRY120 | 0.72 | TCACCGTTAATTGTAGTCTGCG | GCGAGGTTCAAATATGCGAT | 55 |
| 14 | SSRY69 | 0.71 | CGATCTCAGTCGATACCCAAG | CACTCCGTTGCAGGCATTA | 55 |
| 15 | SSRY177 | 0.71 | ACCACAAACATAGGCACGAG | CACCCAATTCACCAATTACCA | 45 |
| 16 | SSRY179 | 0.70 | CAGGCTCAGGTGAAGTAAAGG | GCGAAAGTAAGTCTACAACTTTTCTAA | 55 |
| 17 | SSRY51 | 0.69 | AGGTTGGATGCTTGAAGGAA | GGATGCAGGAGTGCTCAACT | 55 |
| 18 | SSRY164 | 0.67 | TCAAACAAGAATTAGCAGAACTGG | TGAGATTTCGTAATATTCATTTCACTT | 45 |
| 19 | SSRY64 | 0.66 | CGACAAGTCGTATATGTAGTATTCACG | GCAGAGGTGGCTAACGAGAC | 55 |

| | | | | | |
|---|---|---|---|---|---|
| 20 | SSRY 52 | 0.66 | GCCAGCAAGGTTTGCTAC AT | AACTGTCAAACCATTCTA CTTGC | 55 |
| 21 | SSRY 59 | 0.66 | GCAATGCAGTGAACCATC TTT | CGTTTGTCCTTTCTGATGT TC | 55 |
| 22 | SSRY 4 | 0.66 | ATAGAGCAGAAGTGCAG GCG | CTAACGCACACGACTACG GA | 55 |
| 23 | SSRY 12 | 0.66 | AACTGTCAAACCATTCTA CTTGC | GCCAGCAAGGTTTGCTAC AT | 55 |
| 24 | SSRY 21 | 0.66 | CCTGCCACAATATTGAAA TGG | CAACAATTGGACTAAGCA GCA | 55 |
| 25 | SSRY 9 | 0.66 | ACAATTCATCATGAGTCA TCAACT | CCGTTATTGTTCCTGGTC CT | 55 |
| 26 | SSRY 101 | 0.66 | GGAGAATACCACCGACA GGA | ACAGCAGCAATCACCATT TC | 55 |
| 27 | SSRY 127 | 0.64 | CTTCGGCCTCTACAAAAG GA | GCTGAACTGCTTTGCCAA CT | 45 |
| 28 | NS57 | 0.62 | ACCAAAATCTCCACACCC TG | CAACAATTGGACTAAGCA GCA | 55 |
| 29 | SSRY 161 | 0.62 | AAGGAACACCTCTCCTAG AATCA | CCAGCTGTATGTTGAGTG AGC | 55 |
| 30 | SSRY 181 | 0.61 | GGTAGATCTGGATCGAGG AGG | CAATCGAAACCGACGAT ACA | 55 |
| 31 | SSRY 155 | 0.59 | CGTTGATAAAGTGGAAA GAGCA | ACTCCACTCCCGATGCTC GC | 55 |
| 32 | SSRY 34 | 0.56 | TTCCAGACCTGTTCCACC AT | ATTGCAGGGATTATTGCT CG | 55 |
| 33 | SSRY 180 | 0.54 | CCTTGGCAGAGATGAATT AGAG | GGGGCATTCTACATGATC AATAA | 55 |
| 34 | SSRY 108 | 0.54 | ACGCTATGATGTCCAAAG GC | CATGCCACATAGTTCGTG CT | 55 |
| 35 | SSRY 5 | 0.53 | TGATGAAATTCAAAGCAC CA | CGCCTACCACTGCCATAA AC | 55 |
| 36 | SSRY 63 | 0.52 | TCAGAATCATCTACCTTG GCA | AAGACAATCATTTTGTGC TCCA | 55 |

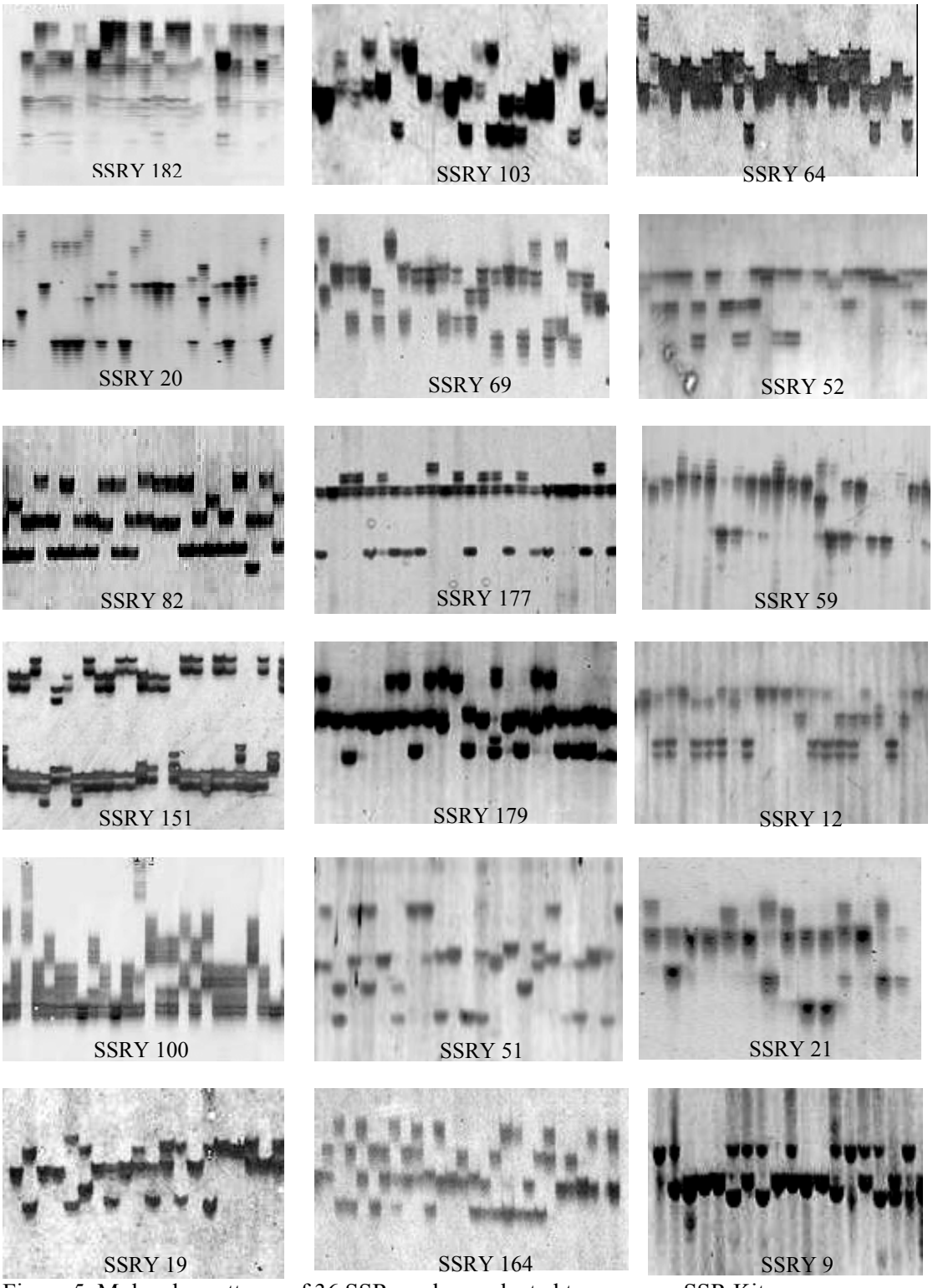

Figure 5. Molecular patterns of 36 SSR markers selected to compose SSR Kit

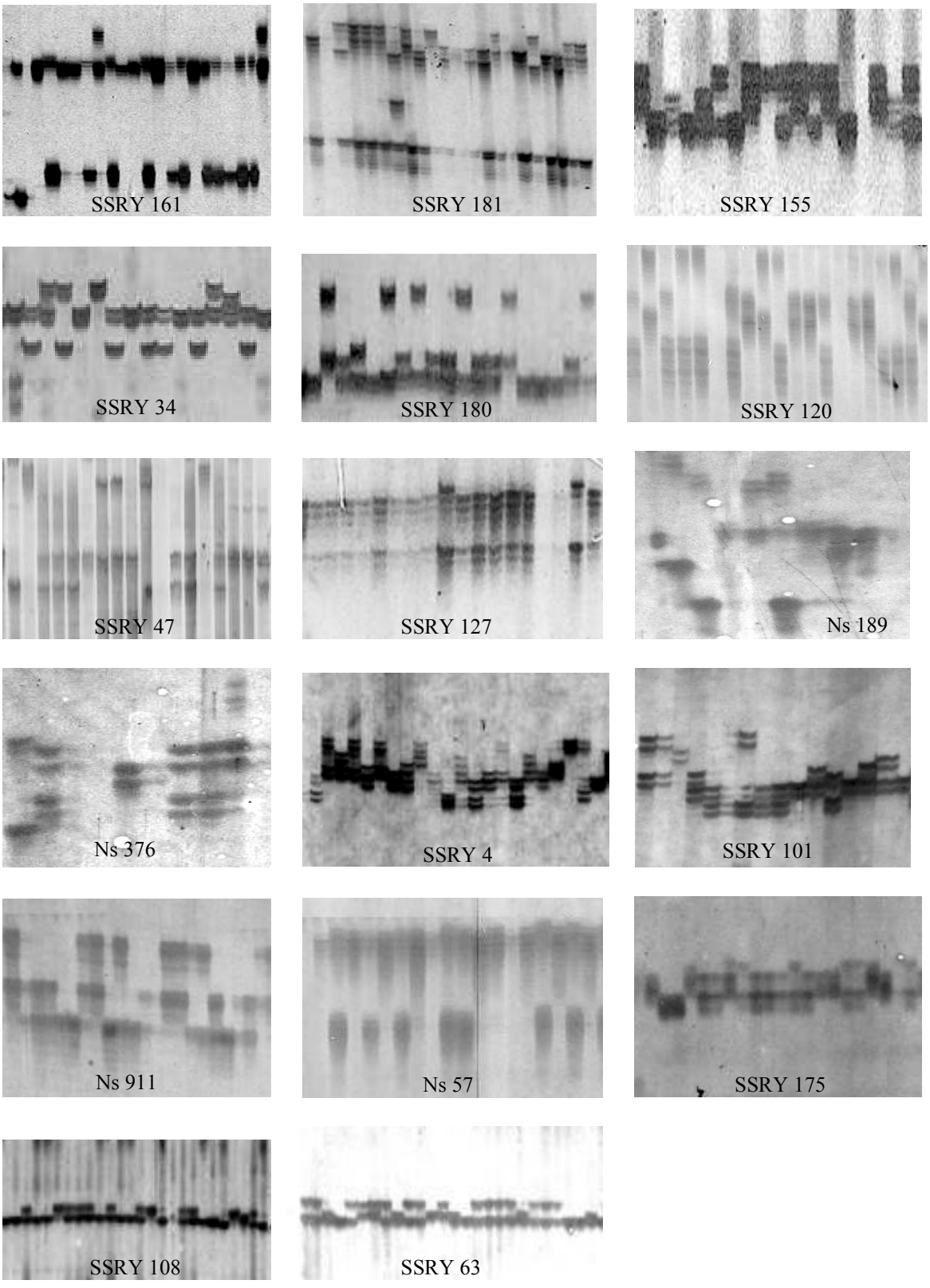


Figure 5 (contd). Molecular patterns of 36 SSR markers selected to SSR Kit

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### 1.1.13 A dataset on allele diversity at orthologous candidate genes in GCP crops (ADOC)

**Introduction**

The (ADOC) project was accepted in February 2006 as a commissioned project from the GCP Management Team and Program Steering Committee. The main objective of this project is to provide an initial dataset of sequence diversity for 10 to 15 orthologous candidate genes for drought tolerance, in a reference sample of around 300 accessions for seven target crops of the GCP program (barley, rice, sorghum, common bean, chickpea, cassava and potato). This project is being conducted in collaboration with advanced research groups who are interested in seeing their favourite candidates documented in an optimized collection of germplasm of the major world food crops. Cassava genetics program was selected as collaborator in this project.

**Methodology**

The crop species under consideration were selected based on the following advantages: Importance, gene complexity, genetic map and segregating progenies in relation to genome colinearity and QTLs for drought tolerance, a large collection of ESTs and a reference sample of some 300 accessions is being defined in perspective of association studies for drought tolerance.

A list of drought-tolerance candidate genes was drawn up based on an updated bibliographic survey and analysis of the results obtained in different laboratories involved in the ADOC project. This list involves different steps in plant response to drought stress (signal perception and transduction, transcription control, stress response), with a special focus on sugar metabolism and water use efficiency. Each crop collaborator has identified a group of potential tolerant varieties for drought tolerance using characteristics of each crop.

**Results**

Cassava accessions tolerant to drought or having high yield in semi-arid agro-ecology were selected as follows:

A set of 97 cassava varieties made up of a group of elite CIAT accessions selected based on high yield level (> 20 ton/h), dry matter content (> 30%) and resistance to pests and diseases in a sub-humid tropic Colombian region ("Magdalena Medio") during the period 1983-2000. Some of these genotypes have been used as drought tolerant clones in previous projects at GCP.

A group of 27 Brazilian accessions selected by Dr Alves using different physiological parameters including leaf conductance, transpiration, photosynthesis, solutes accumulation, accumulation of specific low MW proteins, ABA accumulation, nonstructural carbohydrate reserves in leaves, leaf area, leaf retention, root depth, number of roots, dry matter, starch, productivity and tolerance to cassava green mites (CGM) and other insects

159 Colombian accessions from the CIAT Cassava Germplasm Bank selected based upon their adaptation to semiarid agro-ecologies with an altitude range of between 0-500 masl, rainfall below 800 mm/year and a minimum temperature of 35°C.

DNA from each genotype selected were extracted using a mini-prep protocol of Dellaporta et al. (1983), quality was evaluated in 1% agarose gels (Figure 1) and samples were shipped for diversity and sequencing high throughput analysis to the following Ortholabs:
OL1: Cereals: Brigitte Courtois, Claire Billot, Dominique This, Agropolis (France)
OL2: Roots and tubers: Merideth Bonierbale, Reinhart Simon, CIP (Peru)
OL3: Legumes: Dave Hoisington, Rajeev Varshney, ICRISAT (India)
OL4: Cross species and sequencing : Dominique Brunel, INRA-CNG (France)

Figure 1 DNA of cassava genotypes. Quality of the DNA samples were evaluated using 1% agarose gels.

**Conclusion and perspectives**

This initial period of the project has been used to define a consensus among partners of the ADOC project in the initial choice of candidate genes and the methodology to be used. We expect first results on sequencing and orthologous relationships to come very soon and the next step will be to reveal genetic structures for selected genes in each crop.

**Referentes**

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### 1.1.14 Genetic Diversity Study in Cassava Varieties Cultivated by Small Farmers in The Colombian Region of The Atlantic Coast

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Funding: Gines Mera Fellowship

**Introduction**

In Colombia the main cassava production zone is the Atlantic cost with 42% of the national cassava yield (Alarcón, 2002). In this project we are interested in quantitative estimate of what proportion of cassava acreage in the Colombian North Coast is grown to improved CIAT varieties and genetic diversity of cassava in the North Coast of Colombia compared to samples from several countries and germplasm banks.

Small farmers on the Colombian Atlantic cost use several cassava varieties that have not been clearly identificatied, they could be local varieties or elite varieties from Colombian breeding programs. Since 1970, CIAT has developed elite cassava-varieties, which have been released and disseminated by the national Colombian Institute- ICA y CORPOICA (Ceballos et. al, 2002). However, up date the adoption of those materials by the small farmers has not been monitored in a quantitative fashion and in the entire region.

**Methodology**

The target area of this study and where information was collected is shown in Table 12.18. At a confidence level of 90% of sampling we have visited and interviewed 400 farms (Table 1.) We also

collected stakes from cassava genotypes identified as rare varieties by farmers and planted them in CIAT greenhouses. DNA was extracted from young leaves collected using the Dellaporta's protocol (1983).

Table 1 Location and number of farms visited to collect information in each zone of study.

| COLOMBIAN DEPARTMENT | NUMBER OF TOWNS TO BE VISITED | No. OF INTERVIEWS AND/OR FARMS VISITED |
|---|---|---|
| ATLÁNTICO | 10 | 100 |
| MAGDALENA | 9 | 90 |
| CÓRDOBA | 11 | 110 |
| SUCRE | 10 | 100 |
| TOTAL | 39 | 400 |

A group of 30 accessions collected in the study were randomly chosen and evaluated with aset of 36 molecular markers used routinely for cassava genetic diversity studies in the Cassava Genetics Program (CIAT), After this analysis, nine SSRY molecular markers were selected based polymorphism information content (PIC) using the CERVUS program. These markers were used to evaluated all the materials in this study, a total of 1048 genotypes.

Results

A total of 1048 genotypes were collected in Córdoba (243), Sucre (192), Atlántico (330), and Magdalena (283) genotypes.

The nine SSR markers selected based on highest Polymorphic Information Content (PIC) value and the location of those markers in the cassava genetic map, to assure a large coverage in the cassava genome, are: SSRY12, SSRY51, SSRY63, SSRY82, SSRY100, SSRY135, SSRY151, SSRY155, SSRY179 (annex 1).

Currently the 9 molecular markers selected have been evaluated with the following number of samples (Fig1):

SSRY 51 677 genotypes
SSRY 82 552 genotypes
SSRY 100 460 genotypes
SSRY 151 368 genotypes
SSRY 135 368 genotypes
SSRY 63 276 genotypes
SSRY 179 184 genotypes
SSRY 12 96 genotypes
SSRY 155 0 genotypes

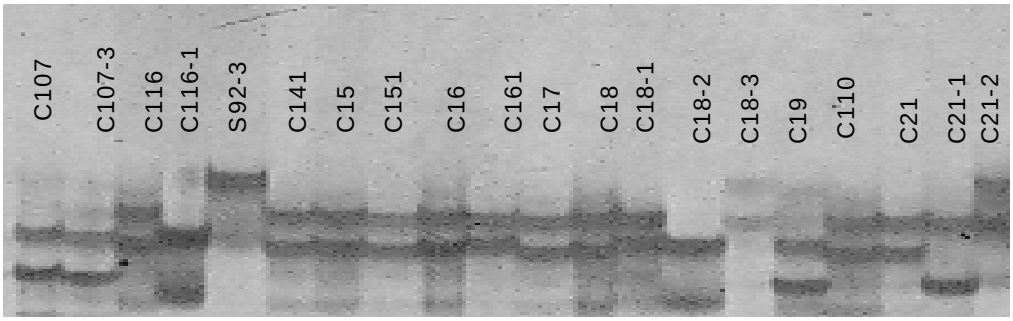


Figure 1: Evaluation of different cassava genotypes cultivated by small farmers in the Colombian Atlantic cost with the SSRY 82 marker.

**Conclusions and Perspectives**

The evaluation with the nine SSRY markers of cassava accessions collected on the North Coast of Colombia is ongoing. Alleles for each marker will be recorded with the Quantity One program (Bio-Rad.Inc) and genetic distance calculated based on the proportion of shared alleles (PSA) using the Microsat program. Additional cluster analysis will be performed by main components analysis using the JMP (versión 3.2.6) and EXCEL spread sheet will be used to graphically present the 1st and 2nd principal component axes. Results should enable an identification of all accessions and the extent of diversity in these materials.

The information collected by interviews will also be organized and correlated with the molecular data analysis to establish the level of adoption of elite varieties released by CIAT and ICA-CORPOICA in the zone of study.

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## 1.1.15 Identification of cassava clones with high commercial value for different industries

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Joe Tohme [4] ; Dominique Dufour[5]
[1]Cassava Germplasm Collection, CIAT; [2]Cassava Breeding Project, CIAT; [3]IP3/SB2 Project – CIAT
[4S]B2 Project – CIAT; [5]CIRAD/CIAT

Financial Support: The Rockefeller Foundation. COLCIENCIAS. IAEA

**Introduction**

Cassava roots are valued for their starchy properties. They are used for fresh consumption, fermentation and drying, rasping and drying, chipping, pelleting, starch extraction and alcohol production. More recently high value-added products, such as precooked, frozen croquettes and fried chips, have been developed and have an increasing presence in urban markets. In addition to its important food security role, cassava is acquiring an increasing role in rural development as raw material for many processing pathways. The two most important industrial uses of cassava are as source of energy in animal diets in the feed industry and for the starch industry. Nowadays there is a growing interest in bio-ethanol production from cassava roots as well. The main strategy used until now to promote cassava as source of raw material for industrial processes has been to increase its productivity and/or reduce production costs, allowing a

competitive price of the roots. However, very little effort has been made to increase the value of cassava roots.

One additional disadvantage for cassava to play a more important role in tropical agriculture is the relatively low genetic variability for root starch traits. Compared with the many economically advantageous mutations found and exploited, for example, in the maize kernel (sweet corn, pop corn, waxy maize starch, opaque 2, etc.), very little variability has been reported for cassava. It is valid to assume that such variability exists in the crop, and at least two main reasons could explain why it has not been readily found and reported: a) Starch mutations in the roots are more difficult to detect than in grain kernels (where they can be easily identified by visual inspection without the need of any sophisticated tests). To detect a mutation in the cassava root starch, the breeder will have to cut the roots and most likely will have to conduct a particular test (i.e. iodine test) or biochemical analysis to be able to pick potentially useful variants. It is possible, therefore, that clones with valuable traits have already been grown in breeding nurseries but could not be detected and, not showing an outstanding agronomic performance, they were unfortunately discarded; b) The known starch mutants are usually recessive. The fact that cassava seldom undergoes inbreeding drastically reduces the chance of (expectedly) low-frequency recessive alleles, to express in the phenotype.

**Materials and Methods**

Different strategies have been implemented simultaneously in search of cassava clones with increased commercial value and are briefly described below:
*1. Massive and systematic screening of germplasm:* Both landraces from the germplasm collection (see report of this kind of activity in this Annual Report). Most germplasm that is evaluated and produces roots is routinely screened for nutritional and starch quality traits (Chávez et al., 2005).

*2. Divergent recurrent selection:* As a result of the routine screening of germplasm a group of 29 clones selected for their low-amylose content in the starch of their roots was selected (Average amylase content 11.2%). Similarly a group of 35 clones selected for their high-amylose content was also selected (Average amylase content 22.7%). Normal range of amylase for cassava typically oscillates around 16-18%. Low-amylose clones have been grown and crosses made between them to generate a segregating population and those clones with low-amylose content will be selected for further crosses (Ceballos et al., 2006). Similarly, high-amylose clones have been grown and crosses made between them to generate a segregating population and those clones with high-amylose values will be selected for further crosses. About 250 genotypes among the two populations have been produced and will be harvested in March 2007.

*3. Genetic transformation:* A technique that has already proved to be useful (Raemakers et al. 2001; Taylor et al. 2004) was implemented in cassava. The transformed plants are currently in the field for phenotyping.

*4. Introduction of inbreeding to expose recessive traits:* This is a fundamentally new approach in cassava genetic enhancement (Ceballos et al., 2004). Most starch mutation of commercial value are recessive (Neuffer et al., 1977). Therefore, after massive screening of landraces from the germplasm bank not surprisingly, little useful variation was found. With resources from the Rockefeller Foundation systematic self-pollinations of landraces and elite germplasm were made and the resulting partially inbred genotypes screened in search of useful variants.

*5. Induction of mutagenesis and TILLING.* Molecular approaches such as TILLING (McCallum, et al., 2000) can improve significantly the efficiency of mutagenesis for traits where little natural variation has been found. Botanical seed from five cassava populations were irradiated with gamma rays or fast

neutrons. The plants grown from this seed ($M_1$ generation) were grown in the field and self-pollinated to overcome the effect of chimeras. The resulting $M_2$ generation was grown in the field and carefully screened for useful variations, particularly in relation to starch characteristics.

*6. Crosses with wild* Manihot *species.*This is the work conducted by the cassava molecular geneticists and will probably be mentioned elsewhere in this report. It is mentioned here just to highlight the integral approach that has been taken.

In addition to all these activities aiming at producing cassava genotypes with increased probabilities to express useful genetic variation an additional step needed to be taken in order to develop the capacity to identify those genotypes. Therefore, with resources from COLCIENCIAS the root quality laboratory was upgraded. It now possesses three rapid viscoanlayzers to screen for differential pasting properties. In addition a DSC (differential scanning calorimeter) was purchased. All samples produced by the different strategies described above are screened in this laboratory that now has the capacity of 15,000 samples per year.

**Results**

March 2006 may turn out to be a turning point for cassava research. Few important mutations were found as a result of the activities described above and this has created considerable interest on the industry.

*a) Amylose – free starch.* This is a long standing request from the cassava starch industry. Starch is made up to two molecules amylose and amylopectine. These are chains of hundred of glucose molecules and they distinguish themselves by the degree of ramification. Cassava typically has between 16-18% amylose. A maize mutation that results in an amylose-free starch is well and commonly known as waxy (*wy*)starch (Neuffer et al., 1997). Similar mutations have been found or developed in other cereals (rice) and root tubers (potato). Amylose-free starch can be identified by the iodine solution test. Normal starch stains blue, whereas waxy starch stains red-brown. Roots from a self-pollinated cassava genotype (AM 206-5) stained reddish when the iodine solution was applied to it. Further tests (amylose quantification by spectrophotometer and amylose quantification by the DSC,) confirmed that the starch from the roots of AM 206-5 lack (or have drastically reduced levels of) amylose. SDS-Page test confirmed that the GBSS enzyme responsible for amylose synthesis is missing in the starch from this genotype. Pasting properties and other starch functional properties tests further support the hypothesis that AM 206-5 is indeed a natural waxy-starch mutation of cassava. Viscosity peak, for instance is twice as high in the starch from AM 206-5 compared with "normal" cassava starch (Figure 1). AM 206-5 starch granule size and shape, on the other hand, were completely normal. This is the first report of a waxy mutation in cassava, which has prompted the starch industry and NARs to show interest in financing CIAT for the development of commercial waxy varieties that began in 2007.

*b) Small starch granule.* In collaboration with colleagues from the John Innes Center in the UK (Allison Smith and Kay Trafford) $M_2$ a large sample of genotypes from the mutagenized populations were screened for abnormal starch phenotypes. As a result, two sister $M_2$ plants (derived from self-pollinations of the same $M_1$ mother plant) showed starch granules distinctively smaller in size. When processed through the rapid viscoanalyzer the starches from these plants showed no viscosity peaks (Figure 1). The proximal analysis of these starches indicated that they have about twice the normal levels of amylose (up to 36%). It is thought that this may be a mutation equivalent to the $ae_1$ (amylose expander) mutation in maize (Neuffer et al., 1977). As in the case of the waxy starch mutant, this small granule mutant can lead to commercial advantages. The small size of the starch granule may require less enzymes in the process to degrade starch before fermentation in the production of bio-ethanol. The mutation in maize has lead to the production of "resistant starches" that offer advantages in human nutrition, particularly for people

suffering from diabetes. In addition, the combination of the *wy* and $ae_1$ mutations in maize leads to third starch phenotype characterized as a sugary type, similar to the sweet corn mutation.

*c) Tolerance to post-harvest physiological deterioration.* In a second batch of $M_2$ plants that was harvested during the second semester of 2006 a plant having adequate amount of starch did not show symptoms of post-harvest physiological deterioration (PPD) two weeks after harvest. Normal cassava roots spoil within 1-2 days as a result of this PPD process that has been identified as one of the most important bottlenecks for cassava production and commercialization (Beeching, et al. 1998; Ceballos et al., 2004). Only few roots from a single genotype had been harvested and evaluated and, therefore, further confirmation of this finding is required. However, it is significant that crosses with the wild relative *Manihot walkerae* has also provided excellent results and hopes that the PPD problem will finally be overcome.

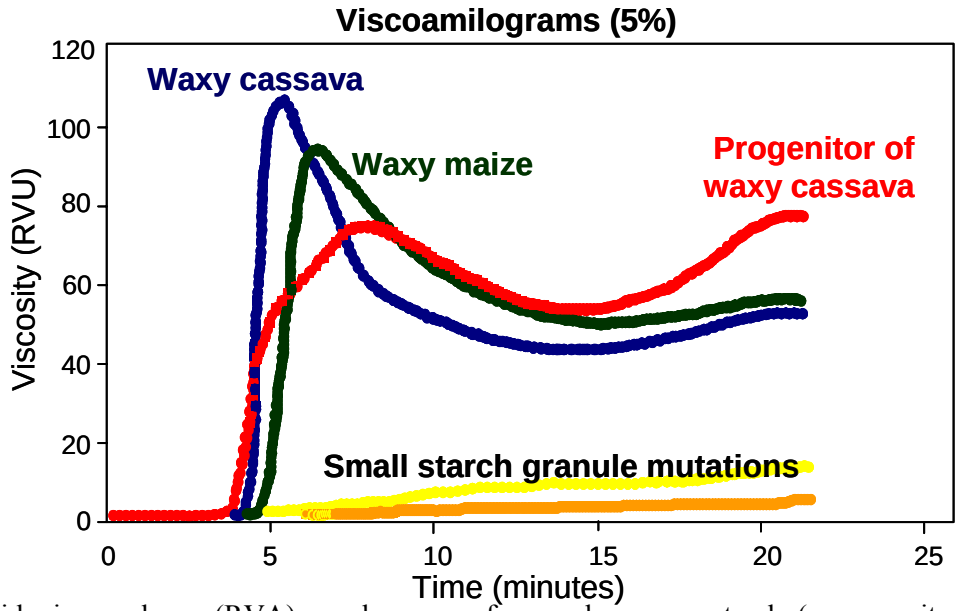


Figure 1. Rapid viscoanalyzer (RVA) amylograms of normal cassava starch (= progenitor of waxy cassava), maize and cassava waxy starch and two 'small granule' mutations.

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## 1.1.16 Characterization of Genetic Diversity: Relationships and potential origin of the weedy rice complex in Colombia

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**Abstract**

The Colombian weedy rice has been characterized in preliminary works conducted by our research group (Gonzalez *et al*., 2003. SB2 Annual Report 2003) using 148 Colombian weedy rice accessions collected in farmer fields evaluated with 19 microsatellites markers. In this report we add up 7 SSR and 13 *Oryza rufipogon* accessions with the objective to evaluate the genetic relationships between weedy rice and *O. sativa* japonica and indica type varieties, landraces, wild species and hybrids between red and cultivated rice to have a better knowledge of the origin of weedy rice and correlate diversity analysis to find strategies to control weedy rice.

**Key Words:** weedy rice, genetic diversity, molecular markers, SSR

**Introduction**

Weedy rice is a common weed in most irrigated rice production areas in the Americas: Bolivia (Llanos et al. 1993), Brazil (Noldin 1988), Chile (Pedreiros and Alvarado 1990), Colombia (Montealegre and Vargas 1989), Guyana (Rai 1973), The United States (Dunand 1988), and Venezuela (Ortiz et al 1997). The only areas where weedy rice is not considered a major problem are California (USA) and Uruguay, but it may still be found infesting some fields (Noldin 2000). This weed is a superior competitor for crop cultivars due to early vigor, greater tillering, and greater height of plants, furthermore has colored pericarps that results in lowered grain quality in most rice markets, and early shattering that reduces harvestable yield (Mortimer et al. 2000). This weed shows a high similarity with cultivated rice varieties in the early growth stages. At maturity it has wide-ranging characteristics, including competitive ability, tillering capacity, flowering date, seed shattering and dormancy, pigmentation of several plant parts in particular of the pericarp (Diarra *et al*., 1985). Different reports including our work (Lentini and Espinoza, 2005) show that some weedy rice can also have intermediate characteristics between wild *O. rufipogon* and cultivated *indica* or *japonica* varieties of *Oryza sativa* (Bres-Patry *et al*., 2001) or could be divided into *O. sativa* ssp. japonica and *O. sativa* ssp. indica-like groups, with some intermediate accessions having characteristics from both groups (Cho *et al*., 1995; Lentini and Espinoza, 2005). In addition, the high rate of fertility in crosses supports the traditional argument that red rice and cultivated rice are both *O. sativa*. However not everyone agrees with this traditional classification. It has been argued that in the tropics,

weedy rice complex may also include other annual *Oryza* species such as *Oryza barthii*, *Oryza longistaminata*, *Oryza rufipogon*, *Oryza perennis*, or *O. punctata* (Kwon *et al*. 1991). These arguments are based on morphological characteristics, but the high degree of variation and lack of a clear classification system make it difficult to definitively categorize red rice. Another hypothesis is that weedy rice may have endo-ferally evolved through the dedomestication of cultivated rice to weedy types, where wild rice is not present (Vaughan *et al*., 2003). In despite of the different hypotesis to solve the origin of weedy rice, its knowledge must begin understanding the variation in the several populations considering that very different very different processes are involved both within and between countries and regions (Mortimer et al. 2000). The Colombian weedy rice has been characterized in works conducted by our research group (Gonzalez *et al*., 2002, Ruiz et al., 2002; Vasquez, 2002; Ruiz, 2003) using 148 Colombian weedy rice accessions collected in farmer fields evaluated with 19 microsatellites markers. This analysis generated five groups three of which grouped all weedy rice accessions. One of these groups clustered closely to *O. rufipogon*. The wild species *O. barthii* and *O. glaberrima* clustered together in the fourth group, and *O. glumaepatula* in the fifth group. Subsequently, a following study reported last year evaluated the genetic variation among weedy rice types, hybrids between weedy rice and cultivated rice, some rice *O. sativa* cultivars japonica and indica types, Colombian landraces and wild species using the same 19 microsatellites markers used before to have a better understanding of the relationships and the potential origin of the weedy rice complex in Colombia. In this report we add up 7 SSR and 13 *Oryza rufipogon* accessions in addition of the materials and markers reported earlier.

**Materials and Methods**

*Plant Material and Genetic Analysis using Microsatellites Markers:* Materials used in this study consisted of 148 weedy rice accessions collected in Colombia; 19 *O. sativa* indica rice commercial varieties and 12 *japonica* type; 16 hand-made manual crosses between the RHBV-resistant transgenic Cica 8 line and non-transgenic variety purple or selected weedy rice accessions; 20 accessions of wild *Oryza* species AA genome, and 15 Colombian landraces. Twenty-six SSR primers derived from rice were amplified in all samples. The PCR products were resolved on silver-stained polyacrylamide gels and microsatellites alleles were sized by comparison to 10 bp molecular weight standard (Promega).

*Statistical Anaysis:* Allelic frequencies were calculated for all materials analyzed. Pearson chi square test was used to evaluate the association between the microsatellites alleles with seed morphological traits. Two multiple correspondence analyses (MCA) were conducted. The first analysis only included the molecular markers data (MCA-M), and the second analysis included both the molecular and seed morphological data (MCA-MSM). The Pearson chi-square and MCA are tests applied to establish the significance of association between categorical variables. The Pearson chi-square test is based on expected frequencies in a two-entry data set, whereas MCA is a modeling technique to analyze associations in multi-entry data set. All analyses were conducted using SAS software (SAS, 1989).

**Results and Discussion**

A total of 372 alleles were scored from the 230 accessions using 26 polymorphic microsatellite markers. The allelic size ranged from 89 to 276 bp and the number of alleles per locus oscillates from 10 to 24 (average of 14.3 alleles per locus). A total of 194 specific alleles were identified from the total number of 372 alleles, thus 52% of alleles are specific and only 48% are shared among different rice types. At least one specific allele is found per each SSR tested in the population analyzed. The large number and origin diversity of wild *Oryza* accessions used, in particular of *O. rufipogon*, account for the largest number of specific alleles (123 specific alleles) found in this study, followed by the weedy rice accessions with 19 specific alleles, indica rice with 16 specific alleles, rice landraces with 10 specific alleles and japonica rice with the lowest number of 6 specific alleles. On the other hand, despite the weedy rice group was

represented by the largest number of accessions (148), this population displayed no more than 19 specific alleles of the total 162 alleles detected in the population.

Multiple correspondence analyses using 26 SSRs (MCA-M) indicated that 93.6% of the variability is represented by 12 groups (Figure 1). The largest group (Group 1) included all accessions of weedy rice, *O. sativa* indica type varieties and manual crosses between the rice and selected weedy rice accessions, 40% of landraces and one accession of *O.nivara* (China), one cross *O.nivara/O.rufipogon* (China), *O.rufipogon* (Taiwan), *O.rufipogon* (Myanmar) and *O rufipogon* (Malaysia). Group 2 is composed by 60% of landraces and all accessions of *O. sativa* japonica type varieties. Group 3 contained six accessions of wild species: *O. nivara* (China), five accessions of *O.rufipogon* from China (2), India(1), Cambodia(1), New Guinea(1). The other nine groups are composed by individual accessions (one per each group) of *O. glumaepatula* (Costa Rica), *O.rufipogon* (two different accessions from China, one from India and one from Bangaldesh), one cross *O. nivara/O. rufipogon* (China), O. *rufipogon* (China), *O. rufipogon* (India), *O. barthii* (Chad), *O. glaberrima* (Africa). The variability obtained in the MCA-M analyses, could be explained by 12 SSRs alleles, all of them which are specific to wild species. It is important to highlight that the weedy rice accessions clustered with all the indica rice varieties and with accessions of *O. nivara*, *O. rufipogon* and one cross *O. nivara/ O. rufipogon*, but with none of the japonica rice accessions. A better resolution of the composition of these groups within the weedy rice and landraces population is obtained when the analysis is expanded to include 99% of the variability (generating 28 groups, Figure 2). The manual crosses between rice and weedy are separated from the main weedy rice group probably due to the high presence of heterozygote individual patterns. Thus, Colombian weedy rice appears to be genetically closer to *O. sativa* indica type varieties. Vaughan *et al.* (2001) also reported that all of the weedy rice accessions were genetically related with indica type varieties, except the accession MS5 which was closer to japonica varieties. Additionally, the analysis showed clear similarity between some accessions (60%) of the Colombian landraces and japonica varieties suggesting a possible origin from *O. sativa* japonica type for these Colombian landraces. The addition of morphological traits to the multiple correspondence analyses did not add more discrimination among the groups analyzed.

**Conclusions**

Multiple correspondence analyses using the selected polymorphic 26 SSRs markers suggest that the Colombian weedy rice are more genetically related to indica type varieties. Additionally, Colombian landraces appear to have a japonica rice type origin.

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Figure 1.- Multiple correspondence analysis (MAC) using 26 SSRs markers. Group 1 ( weedy rice, manual crosses between weedy rice and rice, indica varieties, landraces (40%), *O.nivara* (China), *O. nivara/O.rufipogon* (China), *O.rufipogon* (Taiwan), *O. rufipogon* (Myanmar), *O. rufipogon* (Malasia)). Group 2 (landraces (60%), japonica varieties). Group 3 (*O. nivara* (China), five accesions of *O.rufipogon* (China(2), India(1), Camboia(1), (Nueva Guinea(1)). Group 4 (*O. glumaepatula* (Costa Rica)). Group 5 (*O. rufipogon* (China). Group 6 (*O. nivara/O. rufipogon* (China)). Group 7 (*O. rufipogon* (China)). Group 8 (*O. barthii* (Chad)). Group 9 (*O. glaberrima* (Africa)). Group 10 (*O. rufipogon* (India)). Group 11 (*O. rufipogon* (China)). Group 12 (*O. rufipogon* (Bangladesh)).

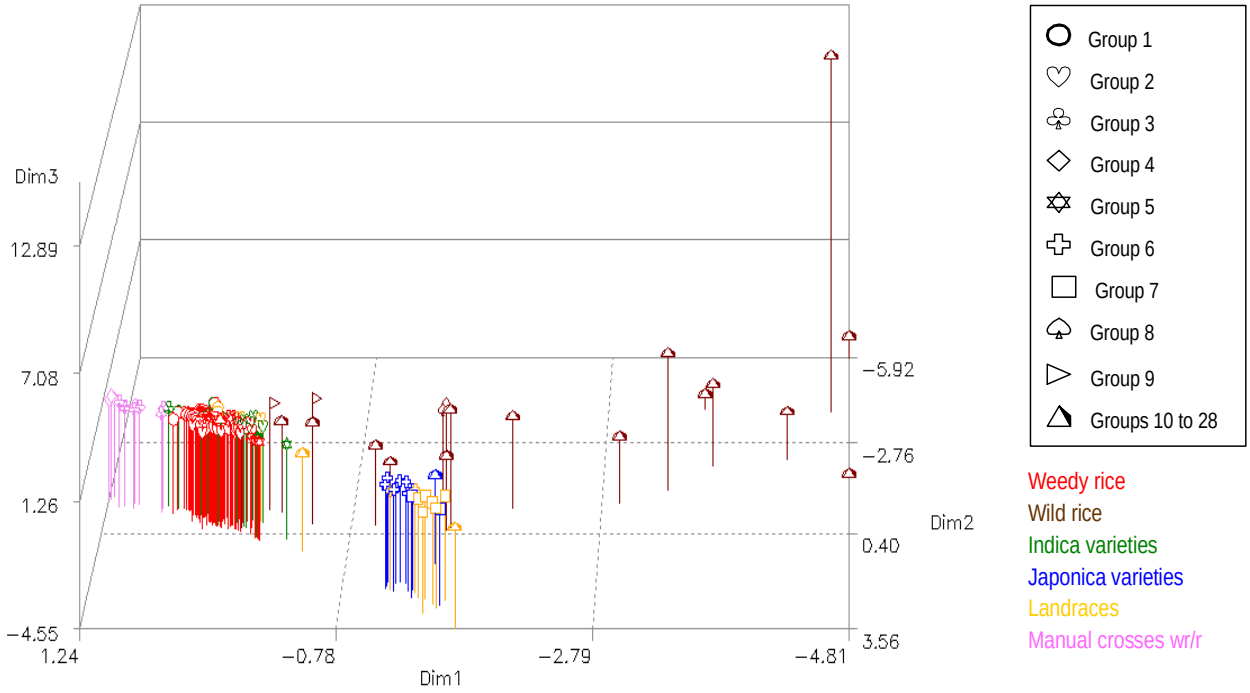


Figure 2.- Multiple correspondence analysis (MAC) using 26 SSRs markers with 230 individuals in 28 groups. Group 1 ( weedy rice (48%), one accesion of manual crosses between weedy rice and rice, indica varieties (28%), landraces (13%)). Group 2 (weedy rice (47%), indica varieties (50%), landraces (20%), *O. nivara/O.rufipogon* (China). Group 3 (manual crosses between weedy rice and rice (63%) and Cica 8. Group 4 (manual crosses between weedy rice and rice (31%). Group 5 ( weedy rice (4%), indica varieties (19%). Group 6 (japonica varieties (82%), landraces (13%)). Group 7 (landraces (40%), (japonica varieties (18%)). Group 8 (two accesions of *O.rufipogon* (China and Camboia). Group 9 (two accesions of *O.rufipogon* (Taiwan and Myanmar). Group 10 to group 28 are composed by one accesion: one accesion of landraces, *O.rufipogon* (Nueva Guinea), O.nivara (China), *O. rufipogon* (Malasia), *O.nivara* (China), *O. rufipogon* (India), *O. rufipogon* (China), one accesion of weedy rice, one accesion of japonica variety, one accesion of landraces, *O. glumaepatula* (Costa Rica), (*O. rufipogon* (China), *O. nivara/O.rufipogon* (China), *O. rufipogon* (China), *O. barthii* (Chad), *O. glaberrima* (Africa), *O. rufipogon* (India), *O. rufipogon* (China), *O. rufipogon* (Bangladesh).

## 1.1.17 Scaling up the analysis of gene flow from rice into weedy rice at landscape under farmers' commercial conditions. Part I. SSR, an alternative for the gene flow evaluation from Clearfield CF205 ® to weedy rice.

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[1]SB2, [2]IP4, [3]FLAR. Funding from GTZ, Germany and CIAT

**Abstract**

This report shows the SSRs molecular characterization of 114 accessions of weedy rice collected from commercial rice fields after planting 2-3 cycles with the variety Clearfield CF205 ® tolerant to the herbicide Imazapic (IMI) in Tolima (Colombia), and the progress of the corresponding characterization of 187 accessions of weedy rice collected from farmers fields prior and after planting 1 cycle with the same herbicide tolerant variety in Jamundi (Valle del Cauca). This information attempts to standardize a methodology for large scale trace of gene flow from rice into red rice and anticipate the emergence of herbicide tolerant weedy rice.

**Key Words:** weedy rice, herbicide tolerance, IMI, CF205 ®, SSR, molecular analysis

**Introduction**

This report is a part of a series of total of four documents presented in this Annual Report associated with the 2nd phase of a project entitled "Gene Flow Analysis for Environmental Safety In the Tropics", which main goal is to generate baseline genetic information for the development of guidelines on the safe introduction and use of novel agriculture traits (biotechnology derived or not native from the place of introduction), while reducing potential environmental impact on native biodiversity in the Neotropics, using two staple crops, bean and rice, as models. The objective of this second phase is to assess the impact of specific non-transgenic traits on biodiversity (genetic structure of recipient population) due to gene flow over time at landscape in countries that harbor land races, weedy/wild species of these two crops. In the case of rice, herbicide is among the current methods preferred by farmers to control weedy rice, a major bottleneck for rice production in this region. Herbicide resistant rice varieties had been released in several productions sympatric to natural environments harboring native wild relatives of rice. Herbicide resistance in rice here derived from mutagenesis (imidazolinone resistance, Clearfield®) had been bred into elite local materials and released as improved varieties in Central America and Colombia. Because of its easy tracing, herbicide resistance provides an excellent model to evaluate the unintended transfer of traits deployed in the crop by cross-pollination to the sexually compatible weedy rice complex for which the herbicide is used as a form of chemical control (positive selection), and in the wild *Oryza* compatible relatives that are found in natural environments in the crop contact zones (neutral selection).In addition, the use of non-transgenic herbicide resistance source is an ideal case study for the comparison of the same trait in transgenic vs. non-transgenic allowing to elucidate the effects due to the trait itself independently from the gene source. This model will give information on impact of introgress non-transgenic resistance genes that may affect fitness of derived hybrids, invasiveness, population dynamics and genetic structure of the corresponding wild/weedy. It will also serve for anticipating a potential impact from a transgenic situation. In this report, the genetic profile of the herbicide resistance donor (CF205 ® variety) and the potential herbicide resistance recipient is presented, as well as progress attained to trace the mutated ALS (Acetolactate synthase) gene sequence involved in the resistance to IMI. These mutations can be determined by means of targeting induced local lesion in genomes (Tilling), a methodology utilized to determine mutants in other species as *Arabidopis*, and or identifying SNPs. It is also necessary to

standardize the methodologies used in field and laboratory for the weedy rice selection resistant to the herbicide Imazapic, which belongs to the imidazolinone group.

**Materials and Methods**

*Plant Material.* The materials used in this study consisted of: 290 accessions of weedy rice and 188 accessions Clearfield CF205 ® variety collected in Tolima and Valle del Cauca. Six biotype (1-3-4, 5-48-2, 5-38-5, 4-12-2, 1-21-3 and 5-36-4) collected in Tolima in 2001, four commercial *indica* type rice varieties (Fedearroz-50, Oryzica I, Cimarrón and Coprosem II), four accessions of wild Oryza species (*O.rufipogon* IRGC-105491 Malasia, *O.rufipogon* IRGC-100916 China, *O. barthii* IRGC-104119 Chad, *O.glumaepatula* Costa Rica) and the cultivated rice *O. glaberrima* IRGC-103544 were used as reference.

*Characterization of the weedy rice accessions from Jamundi-Alsacea.* In order to identify the alleles of weedy rice, we collected weedy rice samples prior planting for the first time in the field the CF205 variety in the *Jamundi-Alsacea*. The plants were labeled and transplanted under greenhouse conditions. The molecular and morphological characterization of the collected seeds are in progress following similar methodologies previously used to characterized weedy rice accessions collected from the Tolima region in year 2001 prior the commercial introduction of CF205 in Tolima in 2003.

Plot selection, localization of plants and seed harvest. *Four plots planted with CF 205 were identified in Tolima and Valle del Cauca departments. The parameters used for sampling the weedy rice population included: 1) plots with known agronomic history and with high weedy rice infestation up to 60 %; 2) synchronization of flowering between CF205 variety and weedy rice; 3) sampling area larger than 6 ha; 4) in the Tolima, the two plots selected (7 and 20 ha) had two consecutive cropping cycles of CF205. In Tolima (Saldaña and Espinal), weedy samples were collected at random through each plot from weedy rice spread in patches and intermingled and in contact with CF205. In Jamundi, samples were collected at random from an area between 200 and 250 $m^{2\,from}$ each of the two plots. Each plot was sub-divided into squares of 52 $m^2$ (Figure 1). The samples were collected and located by GPS, and the topographic map constructed in each case. In all cases (Tolima and Jamundi) weedy samples collected flowering panicles were in physical contact with flowering panicles from CF205. Each mother plant was labeled; progeny seeds harvested, leaf tissue samples collected for molecular analyses, and the original plant transplanted from the field to greenhouse conditions.*

*DNA extraction and bulk analysis by SSRs* The genomic DNA of parental material was extracted from rice leaves according to McCouch (1988). Six or eight polymorphic microsatellite markers clearly distinguishing weedy rice and Clearfield CF 205 were used to determine the profile of the materials collected.
*Statistical analysis.* A multiple correspondence analysis (MCA) was conducted. This analysis was conducted using SAS software (SAS, 1989).

**Results and Discussion**

Multiple correspondence analysis (MAC) using SSRs markers generated eight groups and a clear separation between the weedy rice and the wild species, *O. barthii*, *O. glumaepatula* and *O. rufipogon,* and the African cultivated species *O. glaberrima* (Figure 1). These results are in accordance to the previous report presented herein by González et al. 2006 suggesting that Colombian weedy rice accessions are genetically close related to *O. sativa* species. Weedy rice formed four distinguishable groups (Figure 1). Group 1 included 59 weedy rice (73 %) accessions from Saldaña of which 77 % (62) have awns. Group 2 enclosed all Clearfield CF205 ® individual plants collected from the Saldaña and Espinal rice field; 30 % (14) of the weedy rice accessions from Espinal and three (3) from Saldaña. This group included 98 % of the awn-less weedy rice accessions. Group 3 was composed by 95% accessions from

Espinal and awn-less (80 %). Group 4, included accessions No. 10-32 and 11-56 with brown-black hull and awns clustering together with *O. rufipogon* IRGC-105491 (Malaysia) and weedy rice 5-48-2 (clustering with *O.rufipogon* in earlier reports as well) used herein as control representing a weedy rice *O. rufipogon* like. These results support the hypothesis that the weedy rice is a complex of several species of the *Oryza* genus. In the case of population from Jamundí, 149 Clearfield CF205 ® accessions and 180 accessions weedy rice are being evaluated using eight SSRs. The analysis is in progress.

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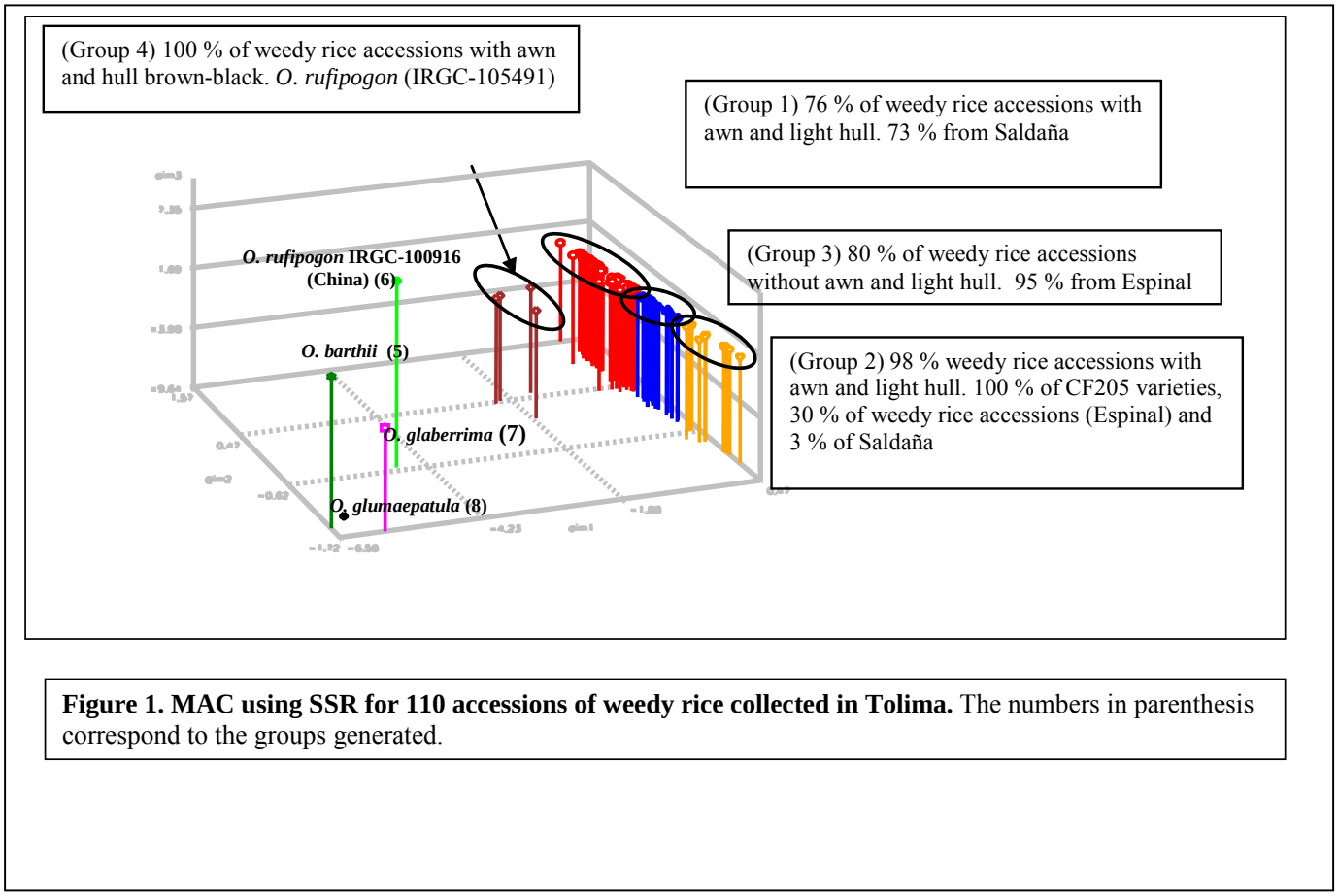


**Figure 1. MAC using SSR for 110 accessions of weedy rice collected in Tolima.** The numbers in parenthesis correspond to the groups generated.

## 1.1.18 Scaling up the analysis of gene flow from rice into weedy rice at landscape under farmers' commercial conditions. Part II. Molecular detection of IMI herbicide resistance gene in the Clearfield CF205 ® variety.

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**Abstract**

Main objectivity of this work is to trace gene flow from the imazapic herbicide resistant Clearfield CF 205 variety into weedy rice population in farmer's fields in main rice crop areas of Colombia, as a first step to standardize methodologies applicable to environmental biosafety in the tropics. DNA primer sequences were tested and used to detect the herbicide resistant gene. Herein we report the partial sequence of the ALS gene found in CF205 variety, since there is no clear information on the mutant used as progenitor donor of the herbicide resistance.

**Key Words:** weedy rice, herbicide tolerance, IMI, CF205 ®, ALS sequence analysis

**Introduction**

Acetohydroxy acid synthase, also known as acetolactate synthase (ALS), is a key enzyme in the biosynthesis of the branched chain amino acids valine, leucine and isoleucine which are fundamental for the normal development of the plants. The ALS is the target of several classes of herbicides such as sulfonylureas, imidazolinones, triazolopyrimidines, pyrimidyloxybenzoates and sulfonylaminocarbonyl-triazolinones. ALS herbicide resistance is conferred by a single mutation dominant gene. The nuclear gene is inherited via seed and pollen (Tan et al., 2005). The resistance occurs as a result of reduced sensitivity of the target ALS enzyme to inhibition by the herbicide or the resistance can be the result in rapid detoxification of the herbicide. In plants, five highly conserved amino acids (Ala 122, Pro197, Ala205, Trp574, and Ser653) have been identified that when mutated, confer resistance or cross resistance to one or more ALS herbicides (Tranel and Wright, 2002). Two SNPs mutations in the rice ALS gene conferring resistance to the imazethapyr herbicide were also reported in Clearfield 161 variety (CL 161). Of these two mutations, the substitution for amino acids serine to asparagine (codon 653), prevents binding of imidazolinone herbicides to its catalytic site and confers resistance to herbicide (Tang et al., 2005; Rajguru et al., 2005). Up to these investigations, a DNA –based method that involves design and application of allele specific primer using PCR assay to distinguish herbicides susceptible and resistant alleles in homozygous or heterozygous genotypes produced between CL161 and weedy rice have been reported (Kadaru et al., 2006).

**Materials and Methods**

*DNA sequencing and alignment.* In order to sequence the plants resistant to the imazapic herbicide, CF205 seeds were placed in a solution of herbicide (25 ppm) during 8 days. Following the methodology described in this annual report (Fory et al., 2006. SB-2 Annual Report 2006), the plants that survived the application of herbicide were used for the ALS gene sequence analysis. DNA was extracted from young leaves according to McCouch et al. (1998). A polymerase chain reaction (PCR) based approach was used to identify the gene coding ALS sequence in rice. Five primers reported by Rajguru et al. (2005) were used. These primers amplify and locate 2047 pb rice ALS according to Figure 1. The primers were designed on the basis of ALS sequence of rice in the GenBank database (AB049822) (http://www.ncbinlm.nih.gov/). PCR was performed in 25 µL reactions consisting of 1X PCR buffer, 0.2 µM of each dNTPS, 1.5 mM, dNTP's 0.2 mM, primer forward y reverse and 0.3 µL de Taq polymerase. The cycling conditions of 95°C for 2 min, 40 cycles of (94°C for 30s; 55°C or 63°C for 1 min, depending

annealing temperature, 72°C for 30 s) and 72°C for 5 min. The amplicons were separated on 1.5 % agarose and all fragments of correct length were purified with a wizard® PCR Clean up system (Promega) The PCR fragments were cloned into the PCR®2.1 (TA Cloning. Invitrogen Life Technologies. USA) following the instructions of the manufacturer. TOPO10 Coli cells were plated on selective LB Medium [1 % (w/v) Tryptone, 0.5 % (w/v) Yeast Extract, 1 % (w/v) NaCl, 1.5 % (w/v) Agar] that contain 50 µg/mL ampicillin, X-Gal and IPTG and grown overnight at 37 °C. One to five recombinant clones were selected and grown over night a 37 °C in liquid LB medium [1 % (w/v) Tryptone, 0.5 % (w/v) Yeast Extract, 1 % (w/v) NaCl]. Plasmid DNA was extracted from each of the clones with a Wizard® minipred DNA purification system. The insertion of the PCR fragment into the plasmid vector was confirmed with Eco RI digestion. This enzyme restriction released the fragment cloned. DNA fragment was sequenced using T7 and M13 reverse flanking the multiple cloning sites. The ALS sequences were compared with sequences in the GenBank using the BLASTX algorithm to confirm similarity. The sequences were aligned using NT-Vector 10.

*Allele specific polymerase chain reaction (AS-PCR).* Seven weedy rice biotypes collected in Tolima (11-41, 11-73, 11-99, 11-113, 11-121, 11-123 and 10-123) and two Clearfield CF205 ® accessions were used. DNA was extracted from young leaves according to McCouch et al. (1998). The cycling conditions were 95°C for 2 min, 27 cycles of (95°C for 15 s, 60°C for 15 s, 72°C for 15 s) and 72°C for 5 min (Kadaru et al., 2006). For the standardization, we changed the temperature and number of cycles. The products were observed by electrophoresis on a 2 % agarose gel and non-denaturing 6.0 % acrylamide bis acrylamide (19:1) polyacrilamide gel stained with silver.

**Results and Discussion**

Three of five combinations, with specific homology at ALS gene were cloned in vector TA cloning. The clones between 320 to 530 pb corresponding to PCR product size obtained from several plants were sequenced (Figure 2). The sequences were edited and cleaned of the vector and an assemblage by the Secuencher v 3.0 programs was carried out. A partial sequence of the gene (1164 pb) was obtained after joining all individual sequences. The similarity of the sequence of the Clearfield CF205 ® ALS gene was verified through BLAST-N and BLAST-X using the information displayed on the NCBI web site. The sequences from CF205 were aligned and compared to japonica rice type resistant to herbicide (AB049823) and to japonica rice type susceptible to the herbicide (AB049822). The ALS partial sequence of Clearfield CF205® showed 97% similarity, Score of 2116 and E-value 0.0 with the gene resistance japonica (AB049823) and 98 % similarity and score of 2139 and E-value 0.0 with susceptible japonica (AB049822). This indicates that the ALS sequence CF205 is highly conserved among cultivated rice. However, change in the sequence may occur as result of rearrangements, insertions and deletions, which may result in intra-specific variations.

Results showed that when comparing the ALS gene terminal region at the aminoacid level the two sequences reported by the GenBank, japonica rice type resistant to imazathepyr (AB049823) and to japonica rice type susceptible to imazathepyr (AB049822) a change of serine for asparagine (codon 653 *A. thaliana* reference) reported by Tranel and Wright (2002) and Tang Rajguru et al. (2005) was observed. But when comparing the CF205 sequence with accession resistant japonica (AB049823), three changes at the aminoacid level are registered on the sequence but the mutation in the codon (654) is the most important where glycine was substituted by glutamic acid. These two changes in the codon 653 and 654 have precisely been associated specifically with the resistant to imidazolinones. The sequences of the progenitor 93AS3510 and PW16, PWC23, CMC29, WDC33 and WDC38 haven been reported. The encoded ALS protein sequence showed that the position of the mutation for 93AS3510 in codon 654 where glycine was substituted by glutamic acid and target site mutation for the PW16, PWC23, CMC29, WDC33 and WDC38 are at codon 653 where serine is substituted by aspargine. The mutation in the (codon 653) position of ALS gene has been reported for X112 maize mutant and the PM1 oilseed rape mutant (Tan et al., 2005). It is important to take into account that the development of rice tolererant to

imidazolinones was accomplished by chemically induce mutagenesis of the seed with EMS using rice line 93-AS-3510 and Cypress. Since then, several rice cultivated tolerant to imidazolinones have been developed through breeding programs using 93-AS-3510 and Cypress as the male parent line. CL121 and CL141 were developed from 93-AS 3510 and these lines were the first commercially released in 2001 (Crouhgan 1994). CL 161 and XL8 are mutants directly developed up to the seven lines (PWC16, PWC23, CMC29, CMC31, WDC33, WDC37 and WDC38) with tolerance to IMI. These lines were developed by using a mutated cypress source (Tan et al., 2005; Wenefrida et al., 2004 cited by Leavy, 2004).

There was no discrimination between the susceptible and resistant accessions to the imazapic herbicide (Figure 3). Although a 134 pb fragment was visualized in agarose gel, the amplification was not specific when using the two sets of primers reported by Kadaru et al. (2006). These results were confirmed when visualizing the fragment in polyacrilamide gel and at a higher level of resolution. Some unspecific bands were observed in the polyacrilamide gel. The temperature of aniling and the number of cycles in the PCR was modified. The ALS gene is variable when comparing japonica and indica varieties. Two hundred SNPs between these two types of genome for the ALS gene have been reported. These results indicate that the sequence at the nucleotide level Cl 161 (japonica type) is different from the CF205 variety (indica type), affecting the design of primers. The mutation in CF205 is in the codon 654 whereas the designed SNPs for CL161 japonica variety is in codon 653.

**Future Activities**

Test the primers designed to detect the herbicide resistance gene in Clearfield CF205 ® , in its progenitors (Yacu 9, herbicide susceptible; and Cypress, herbicide tolerant) in order to confirm its utility for tracing the gene in potential hybrids with weedy rice.

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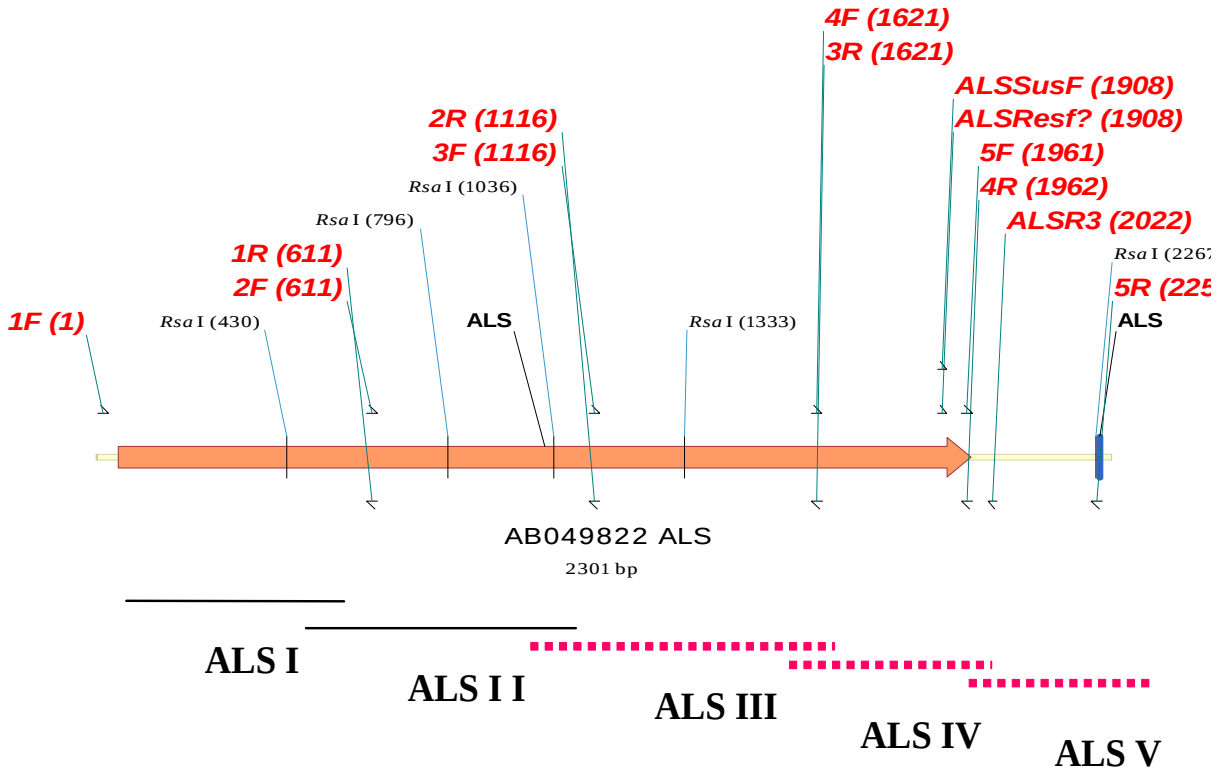


Figure 1. Schematic representation of the ALS gene (AB049822) indicating region amplified by different primers. Red lines indicate the sequenced region. Red letter indicate the primers.

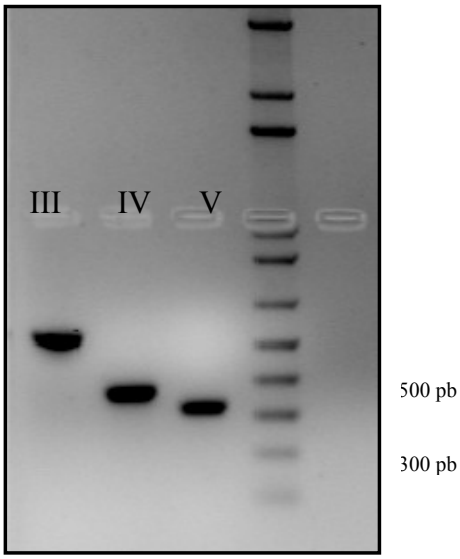


Figure 2. PCR of the *ALS* gene for the regions III, IV

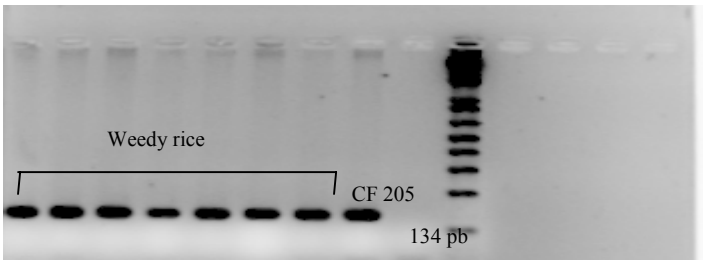


Figure 3. AS-PCR results for *ALS* gene for seven representative weedy rice and Clearfied CF205 ® varietv on the 2%

## 1.1.19 Scaling up the analysis of gene flow from rice into weedy rice at landscape under farmers' commercial conditions. Part III. Tilling as an alternative method to evaluate gene flow.

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[1]SB2, [2]IP4.Funding from GTZ, Germany and CIAT

**Abstract**

Herein it is reported the use of tilling as an alternative method to trace gene flow from rice into weedy rice by large scale analysis applicable to farmer fields, in this case using IMI herbicide resistance as a model

**Key Words:** weedy rice, herbicide tolerance, IMI, CF205 ®, SSR, tilling analysis

**Introduction**

Targeting induced local lesion in genomes (TILLING) is a reverse genetic method that combines random chemical mutagenesis with Polymerase Chain Reaction (PCR) –based screening of gene regions of interest associated with the hetero-duplex analysis or mismatch cleavage to detect mutations in specified target region. Chemical mutagens generate an allelic series at any target locus, resulting in a change of function, reduced activity or specificity or a knockout mutation (McCallum et al., 2000a; McCallum et al., 200b). Tilling protocols were first implemented by Seattle TILLING Project (STP) for screening ethyl mehanesulfonate (EMS) mutagenized *Arabidopis* populations (Till et al., 2004, McCallum et al., 2000a). TILLING have also been used in *Drosophila melanogaster* (Bentley et al., 2000 cited by Colbert et al., 2001), and maize (Purdue University, Lafayette, IN, USA; http:// genome.purdue.edu/maisetilling/). TILLING is currently being used at CIAT in crops such as rice where gene mutations associated with iron transport are identified. In beans, mutant populations (BAT 93) is being generated with EMS, and in the case of the cassava, it is being used to identify natural mutants involved in starch synthesis. TILLING is a simple methodology and the advantage is that it is not necessary to sequence each individual, DNA from various individual can be pooled together (bulk analysis) to increase throughput. The PCR product is heated and re-annealed allowing forming hetero-duplex between mutant and non-mutant DNA. Hetero-duplex are identified through cleavage of mismatched sites by the Cel I endonuleases (Slade et al., 2005). This plant enzyme has been purified from celery (*Apium graveolens*), and the raw extract is used to cleavage the full length PCR products that then can be visualized by size on agarose or polyacrilamide gel (Raghawan et al., 2006). Since the IMI herbicide resistance gene contained in Clearfield CF205 ® is a variety generated by direct mutagenesis, it is possible to use TILLING for mass analysis of weedy rice populations collected from rice fields and determine if the weed had acquired the imazapic resistant gene from Clearfield CF205 ® through outcrossing.

**Materials and Methods**

*DNA extraction and PCR*. DNA was extracted from 100 mg leaf tissue (McCouch et al., 1988). DNA was quantified and diluted to a concentration of 20 ng/µL. To test the optimum pool size for TILLING, tissues of Clearfield CF205 and the variety Cica 8 (herbicide susceptible) were combined at ratios of 1:1, 1:3, 1:5, 1:7, 1:10, 1:20. Alternatively for comparison, Clearfield CF205 DNA was diluted with DNA from the variety IR64, also herbicide susceptible, using the same ration as for Cica 8. Six highly polymorphic SSRs markers distinguishing weedy rice from Clearfield CF 205 were used to determine the optimal DNA pool size for bulk analysis. The ALS gene located in chromosome 2 was the target in this study. The primers used to detect the ALS gene were those designed by Rajguru et al. (2005). The PCR was performed as follows: one cycle at 95°C for 2 min, 40 cycles following the sequence of 94°C for 30s; 55°C or 63°C for 1 min (depending annealing temperature) ending with 72°C for 30 s; and then 72°C for

5 min. The PCR reaction was carried out at a final volume of 25 µL (DNA 50 ng, PCR buffer $MgCl_2$ 1.5 mM, dNTP's 0.2 mM, concentration of primers forward and reverse of 0.2 µM, and 0.3 µL Taq polymerase.

*Heteroduplex.* The PCR products were denatured at 95°C for 10 minutes and re-natured initially at 85°C for 20 seconds followed by 69 cycles to decrease the temperature up to 25°C step-wise by intervals of 0.1°C per cycle.

*Digestion.* The celery extract juice produced by CIAT Bean laboratory was used as enzyme source: 10 µl PCR amplified DNA was mixed with 0.4 µL of celery enzyme, 2 µl CEJ buffer (10 mM de HEPES pH 7.5, 10 mM $MgSO_4$, 0.002% Triton X-100, and BSA 20 ng/mL) and 7,6 µL of water per reaction. The digestion was carried out at 45°C for 35 minutes. Enzyme activity was stopped by adding 5 µL EDTA, and incubated at 45 °C for 30 minutes. Digestion products were resolved in 1.5 % (p/v) agarose gel by staining with ethidium bromide.

**Results and Discussion**

ALS gene sequence of Clearfield CF205 ® variety (reported in this Annual Report by Fory et al. 2006) was compared with those of reference genotypes, IR64 indica rice and Nipponbare japonica rice, genotypes for which complete genome sequences are available (International Rice Genome Sequencing Project 2005). These sequences showed the high level of variation present in the ALS gene between different genotypes.

Six and seven mutations are identified when comparing the ALS gene partial sequence of Clearfield CF205® with those of IR-64 and Nipponbare respectively. The mutations are not located in the same DNA bp supporting the finding of high ALS gene variability between indica and japonica genomes. Although a mutation is associated with the imazapic herbicide resistance in Clearfield CF205®, it may also be possible that other specific mutations could had been generated by the radiation and be present in the variety, thus those Clearfield CF205® specific mutations could also be useful for identifying potential hybrids between Clearfield CF205® and weedy rice as product of outcross in the field..

Once the ALS gene is sequenced, it is possible to determine and confirm the expected fragments by digestion with the Cel I enzyme. SNP detection analysis of Clearfield CF205 ®, IR 64 variety and two weedy rice accessions is shown in Figure 1. Un-digested DNA is shown in the first four rows of each panel (ALS-V and ALS-III PCR amplified regions) indicating the absence of small size fragments as expected corroborating the specificity of Cel I enzyme for cutting hetero-duplex. When the ALS-V PCR amplified region of the ALS gene [region reported by Rajguru et al. (2005)] was digested with Cel I enzyme, two hetero-duplex fragments of 320 pb and 149 pb are observed (Figure 1, tracks 5 and 7) which are not present in the undigested DNA samples of Clearfield CF205® variety and the IR64 variety (Figure 1, left panel tracks 1 and 2), and are specific to Clearfield CF205®. Similarly when digesting the ALS-III PCR amplified region of the ALS gene, in addition to the reference 530 pb fragment, two additional fragments product from the Cel I digestion are resolved corresponding to the hetero-duplex fragments 360 bp and 170 pb, which correspond to the ALS specific mutation present in Clearfiled CF205 ® (Figure 1, right panel tracks 7 and 8). In both cases, the hetero-duplex fragments detected in ALS-V and ALS-III regions are specific Clearfield CF205® and those fragments are present in neither IR64 nor the weedy rice 1-21-3 and 4-12-2. By combining the analysis of ALS-III and ALS-V, three possible specific markers for CF205® are identified, which may or not be associated with the herbicide resistance trait, association that needs to be determine. The CF205® specificity of these hetero-duplex fragments needs to be corroborated in larger populations including increased number of weedy rice accessions.

The CF205® hetero-duplex specific fragments were resolved up to dilution of 1:20 ratio when mixing hetero-duplex DNA with Cica8 DNA. The reproducibility of this result is being tested. Other reports showed that SNP detection in agarose gel can be an efficient method to map gene from parental lines that show low levels of polymorphisms and it is possible to detect the SNPs in pools of 15 samples (1:15 ratio) [Raghawan et al., 2006]. At CIAT, assays in rice to genes associated with iron metabolism indicate that the specific iron SNPs may be efficiently detected in ratios of 1: 16 (Sanabria et al., 2006 SB-02 annual report). In addition to TILLING, the analysis can be complemented with bulk analysis using specific polymorphic SSRs, which already had been used by our research group and demonstrated an efficiency detection at 1: 20 ratio.

Future Activities

To design and evaluate other primers that may detect ALS other gene mutation located in the ALS-IV region and its association with the imazapic herbicide resistant. TILLING protocol needs to be standardized and its reproducibility needs to be confirmed using older leaf tissues from filed samples.

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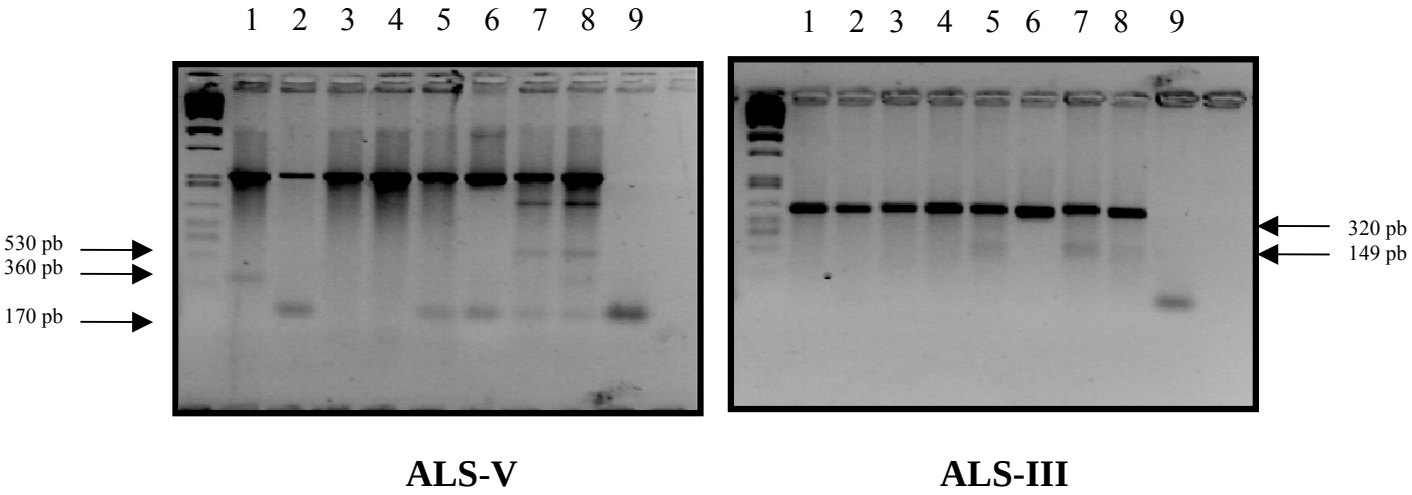


Figure 1.- Match of SNP detection between Clearfield CF205 (CF205) and IR 64 at ALS-V and ALS-III PCR amplified regions of ALS gene. Row 1: CF 205; row 2: IR 64 ; row 3: weedy rice 1-21-3; row 4: weedy rice 4-12-2; from row 1 to 4 undigested DNA. From row 5 to 9, Cel I digestion. Row 5: IR64 + CF205; row 6: IR64 + CF205 not digestion with Cel I; row 7: weedy rice 1-21-3 + CF205; row 8: weedy rice 4-12-2 + CF205; row 9: Control, PCR without ADN. The arrows are indicating the CEL 1 cleaved products SNPs between CF205 and IR64.

## 1.1.20 Scaling up the analysis of gene flow from rice into weedy rice at landscape under farmers' commercial conditions. Part IV. Standardization of herbicide resistance evaluation under field and laboratory conditions using Masterkey DG.

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**Abstract**

Following it is reported the standardization of IMI herbicide tolerance evaluation in rice under field and laboratory conditions using the Masterkey DG® system recommended by BASF. The methodology has been adapted to assess at large scale the detection of possible hybrids between the herbicide resistant Clearfield CF205® variety and weedy rice.

**Key Words:** weedy rice, herbicide tolerance, IMI, CF205 ®, weedy rice, Masterkey DG®, field, laboratory

**Introduction**

Three systems of rice herbicide tolerance had been developed in the past decade. Transgenic rice tolerant to glyphosate or glufosinate, and non-transgenic rice derived from mutagenesis with tolerance to imidazolinones (IMI) herbicide had been generated, which IMI rice is the only one commercially available since 2002. Current evidence suggests that out-crossing has led to the formation of IMI resistant weedy rice hybrids in the USA (Gealy, 2005). The IMI herbicide is absorbed by the leaves and roots, and has residual activity in the ground. This herbicide controls different types of weeds, including weedy rice, by inhibition of the acetohydroxyacid synthase enzyme (AHAS, also called ALS) (Tan *et al.,* 2005). In Colombia, the Clearfield System created by BASF and the Louisiana University was used by Fedearroz to develop Clearfield CF205® variety tolerant to the Masterkey DG® herbicide system. Clearfield CF205 ® is derived from crosses made between USA IMI rice variety (herbicide tolerant donor) and Colombia (herbicide tolerance recipient). Clearfield CF205 ® has been grown commercially in Colombia since 2002 in the Tolima and Huila region, and first sown in Jamundi (Valle del Cauca) in 2005. Herbicide tolerance provides an excellent model to evaluate the unintended transfer of crop traits from gene flow by cross-pollination to the sexually compatible weedy rice complex for which the herbicide is used as a form of chemical control (positive selection), and in the wild *Oryza* compatible relatives that are found in natural environments in the crop contact zones (neutral selection). In addition, the use of non-transgenic herbicide tolerance is an ideal case study for the comparison of the same trait in transgenic vs. non-transgenic allowing to elucidate the effects due to the trait itself independently from the gene source. In order to assess the impact of specific non-transgenic traits on genetic structure of recipient population due to gene flow over time at landscape between CF205 variety and weedy rice, a total of 1083 accessions of weedy rice were collected from Clearfield fields in Tolima and the Valle del Cauca in 2005 (Fory et al, 2005. SB2 Annual Report). Here it is reported the evaluation and standardization of IMI herbicide tolerance for rice under field and laboratory conditions using the Masterkey DG® system recommended by BASF, in order to assess gene flow in the F1 generation of self-progeny seeds derived from the original weedy rice collected in the field. Herbicide tolerance evaluation in the field requires more labor than in the laboratory, reason why in addition of the proposed field evaluation attempts were also conducted to establish a laboratory protocol.

## Materials and Methods

*Standardization of the evaluation system of the Masterkey DG® herbicide under field conditions.* The experimental materials used in this preliminary study consisted of 28 weedy rice accessions that were collected in Tolima and Huila (15 accessions) in 2001, and in Jamundí (13 accessions) in 2005, in both cases prior the release and cultivation of Clearfield CF205 ® put under the action of the herbicide. These materials were chosen according to their genetic and morphologic diversity (Lentini and Espinoza, 2005; Fory et al. 2005; Hernandez, 2006). Six commercial rice varieties (Cica 8, Fedearroz 50, Oryzica 1, Cimarron and Coprosem II) and the variety Clearfield 205 were used as controls.

*Experimental design and treatments.* Replicated randomized split-plot design was used with a total of four treatments and three repetitions by treatment. Each repetition was subdivided in four seed beds (6 m long by 1,2 ms wide, row size). Fifty (50) seeds per each accession were sown per rows and 10 cm between rows. Treatment consisted of various number of herbicide applications at different times of crop cycle: 1) One application of imazapic at pre-emergence (PRE) followed by one at post-emergence (POST) 15 days after seed sown (DAS). 2) One POST at DAS. 3) Two POST applications at 15 and 25 DAS. 4) No herbicide application, control. Imazapic herbicide was applied using a concentration of 115g/ha according to the manufacturer recommendation (BASF, 2003). Herbicide was diluted in water, and a nonionic surfactant was added to solution at 0.5 %. The herbicide was applied by aspersion with a backpack sprayer A-Z at a height of 1,20 m. Clearfield CF205 ® and the susceptible rice commercial varieties were included three timed in each repletion to assess the efficiency of the herbicide application. The number of alive and dead plants was recorded at 15 days, 25 days and 35 days after the herbicide final application (DAA). Plant toxicity was evaluated at 15 and 25 DAA, following the ordinal herbicide susceptibility scale established by Finol et al. (1999). Score 1 refers to zero damage, plants similar to non-herbicide application control. Score 3, refers to moderate damage, characterized by general plant chlorosis; and score 5 refer to severe damage indicated stunted plants or death.

*Evaluation of herbicide tolerance under laboratory conditions.* The variety Cica 8 (susceptible control) and Clearfield CF205® (tolerant control) were used. Surface sterilized seeds were sown on either germination paper or standard paper towel. Four doses of herbicide (0, 5, 15 and 25 ppm) were evaluated in two treatments: 1) Seeds were imbedded in the herbicide solution (50 mL) by 1 hour followed by two washes with distilled water, then incubated for germination in the dark on either germination paper or standard paper towel damped with water. 2) Seeds were sown on either germination paper or standard paper towel and then damped with the herbicide solution by 16 hours or 8 days, after which were transferred and incubated for germination in the dark on either germination paper or standard paper towel damped with water. Treatments were arranged using a completely randomized block design with three repetitions of 20 seeds per treatment. The percentage of plant emergence and survival were recorded at 7 and 15 DAS. Plants that survived the treatment were transplanted to pots in the greenhouse and their survival (%) was recorded at 21 DAS. The percentage of absolute reduction in survival was calculated and referred as $\%R = [(T - X) \times 100]/T$, where X refers to the survival of the control (without herbicide) and T the survival of the treatment.

## Results and Discussion

Clear cut visible damage caused by the herbicide in susceptible plants (including susceptible controls) was observed 10 to 15 DAS. Symptoms included chlorosis, withering and some cases plant death. As expected, the resistant CF205 variety did not show any damage symptoms, although seed germination without herbicide treatment was lowered than the other commercial verities. No significant differences were observed between the different treatments in the field. In all the cases, 80% mortality was noted (Figure 1). Similar damaged were observed at 15, 25 and 35 DAA, which indicates that the herbicide is highly effective on weedy rice and susceptible varieties Cica 8, Fedearroz 50, Oryzica 1, Cimarron and

Coprosem. Other reports had indicated that weedy rice (97 %) is controlled with another IMI herbicide (imazathapyr) and no differences are observed between the imazethapyr PRE y POST applications (Leavy, 2004). It is important to highlight that in the control treatment (without application) the natural mortality of the weedy rice and commercial varieties including the resistant variety did not surpass the 16%, which indicates that the test was not affected by external factors that may influence the normal development and growth of in the field. It is also important to highlight that one weedy rice accession (No 9-11 ) collected from Jamundi showed some tolerance to the herbicide about 40 % survival indicated by the recovery of plants after treatments. This tolerance should be subject of future studies, since in this case a cross-herbicide resistance may be present since this accession was collected prior IMI herbicide had been used for the first time in that location and other reports suggest the possibility that other herbicides may have the same mode of action.

In the case of the laboratory treatment, higher germination was obtained when using paper towel than germination paper without herbicide. It appears that the germination paper has some toxic inhibitory substance that is released after watering the paper. Herbicide treatment did not affect the percentage of germinating seeds in neither susceptible variety Cica 8 nor the tolerant Clearfield CF205 ® . From 90% to 100% reduction in plant survival of the susceptible control was obtained by sowing the seeds on standard paper towel, damped it with the herbicide (5, 15 and 25 ppm) solution for 8 days, and then transferred the seeds onto fresh paper damped with water and incubated in the dark. Similarly studies made in Brazil using transgenic seeds of resistant rice to ammonium gluphosinate have demonstrated that both methods when dampening the paper with the herbicide in a concentration 0,004% and submerging the seeds in the solution of herbicide 0,4% showed a good control for the selection of resistant seeds to the herbicide (Gilneililge et al., 2003). It has been possible to implement the fast test in the selection of sorghum seeds resistant to imidazolinones, exposing the seeds to a 4.6 p.p.m dose of imazathapyr during five or six days (Beadle, 1998 cited by Gilneililge et al., 2003). The simple, efficient and not expensive methodology described in this report for assessment of tolerance of imazapic offers an alternative when field evaluations cannot be done, and may be more practical since IMI herbicide has long residual effect in the soil impeding the cultivation of rice in the same plot for about 1 semester.

**Future Activities**

The herbicide tolerance of F1 generation of self-progeny seeds (41.947 plants) derived from the original weedy rice accessions collected in Tolima and Huila from farmer fields planted with Clearfield CF205 ® will be tested on December 2006 , and the tolerant plants will be assayed molecularly to determine weather or not these tolerant plants contain the IMI resistance gene from Clearfield CF205 ® .

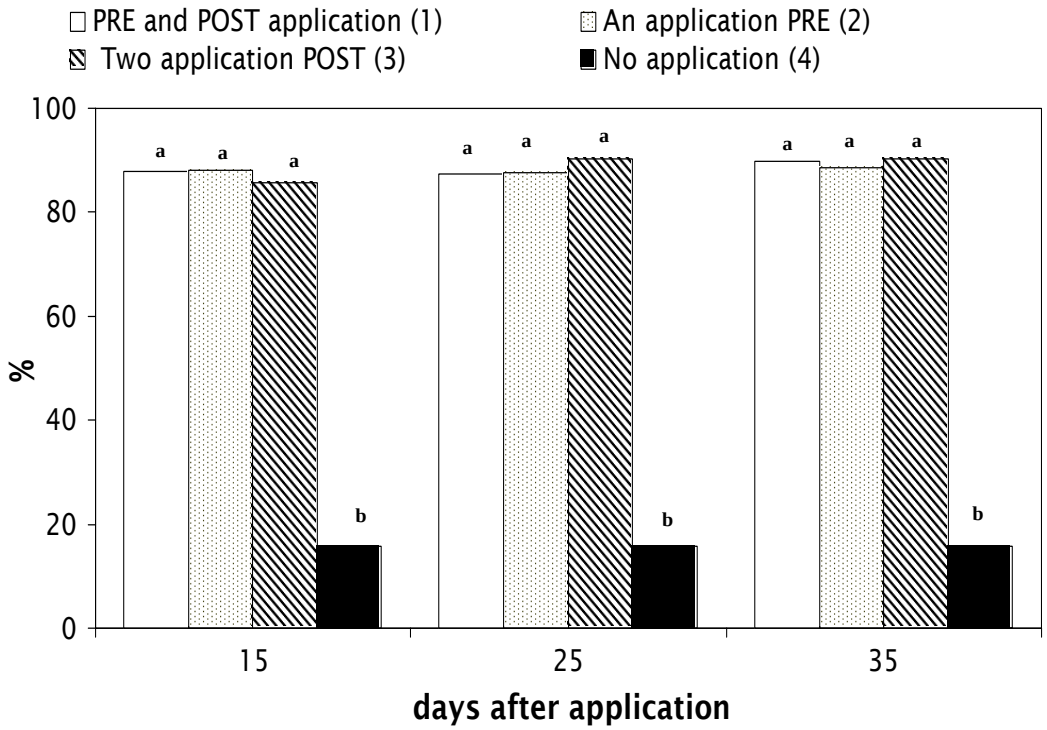


**Figure 1. Mortality percentage of plants 15, 25 and 35days after application of the herbicide Masterkey ® under field conditions.** Values followed by the same letter are not significantly different (*p=0.05*) Ryan-Einot-Gabriel-Welsch multiple range test.

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### 1.1.21 Use of chloroplast DNA polymorphisms for gene flow analysis in rice. Part I. characterization of wild rice species collected in Colombia and Venezuela.

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[1]SB2, [2]IP4. Funding from GTZ, Germany and CIAT.

**Asbstract**

This work is part of the project **"**Gene Flow Analysis for Assessing the Safety of GMOs in the Neo-Tropics" directed to analyze the gene flow from non-transgenic or transgenic rice into wild/weedy relatives in the Neotropics, and its effect(s) on the population genetic structure of the recipient species. The objective of this part of the work is to evaluate cp and nuclear DNA regions to quantify gene flow and direction from rice into weedy rice and wild *Oryza* species. This report describes preliminary work of the use of this markers in the characterization of samples collected in Colombia and Venezuela.

**Key Words:** cpDNA, nuclear molecular markers, gene flow, weedy rice, *Oryza* species

**Introduction**

The genus *Oryza* is composed of two cultivated and 21 wild species with 10 recognized genome types (A, B, C, BC, CD, E, F, G, HJ and HJ) (Vaughan et al., 2003). The cultivated species are *Oryza sativa* and *Oryza glaberrima* of Asian and African origin, respectively. Four wild *Oryza* species have been reported in Latin America: four tetraploids (CCDD genome) *O. alta*, *O. grandiglumis* and *O. latifolia*, (Vaughan, 1994), and one dploid (AA genome) *O. glumaepatula*, sometimes described as *O. rufipogon* americana (Vaughan, 1994; Lentini and Espinoza, 2005). All the described diploids species for the American continent have the genome AA likewise *O. glumaepatula*; whereas the *O alta*, *O. latifolia* and *O. grandiglumis* species have the CD genome. *O alta*, *O. latifolia* and *O. grandiglumis,* are tetraploids species very closely related and the key characteristics that distinguish them are not clear. In the Americas, only Costa Rica and Brazil have conducted complete collection and characterization of the wild *Oryza* present thoughout each country. Different molecular methods are available and reported as tools for identifying rice genomes or species. Maternal inherited genome analysis using either mitochondrial DNA (mtDNA) or chloroplast DNA (cpDNA) can be accomplished and complemented by RFLP analysis of PCR amplified nuclear DNA regions (Ge et al., 1999; Buso et al., 2001). One of these markers is the restriction site-polymorphism of PCR-amplified alcohol dehydroenase (Adh) genes and internal transcribed spaces (ITS) (Ge et al., 2001; Ying and Ge, 2003). These molecular tools can be used to facilitate the identification of collected wild rice germplasm, and and also to clarify phylogenetic relationships and provide a rationale for choosing strategies for breeding, and used of genetic resources (Ge *et a*l., 2001). This report describes the use of cpDNA polymorphisms (maternal inheritance) to give a comprehensive understanding of the hybridization and dynamic introgression under field conditions.

**Material and methods**

The materials used in this study consisted of weedy rice accessions (3) selected as indicators of genetic diversity collected in Colombia based on previous work, the commercial rice varietie CF205, 4 accessions of *O. rufipogon*, *one accession each of O. glaberrima* and *O. barthii* (1), 4 accessions of *O. glumaepatula* (4), accessions of *O. alta*, (1), *O. grandiglumis* and (3) *O. latifolia* (7). Total DNA was isolated from young leaves according to the method described by McCouch et al. (1988). The PCR was carried out in 50 µL total volume containing the following components: 80 ng of genomic DNA, 0.2 mM dNTPs, 2.5 mM

$MgCl_2$, 2.0 U Taq polymerase, 1X PCR buffer 0.5 μM of each primers was used. Some primers were redesigned on the chloroplast sequence NC001320 (www.ncbi.nlm.nih.gov/entrez). These primers had been designed to amplify non-coding regions of chloroplast sequences of *Oryza sativa* and *Nicotiana tabaccum* (Demessure et al., 1995; Chacon, 2001). Thirty one endo-nucleases were tested and some enzymes have been reported by Busso et al. (2001) due to their ability to restrict and identify polymorphic fragments. The amplification was carried out using 1 cycle of 2 min at 94°C, 40 cycles of 30 s at 94°C, 30 s at 58°C, at 72°C for 5 min and one cycle of 10 min at 72°C. The PCR products were separated on agarose gel (1.4 %), stained with ethidium bromide. The regions including the ITS were amplified using universal forward and reverse primers (ITS1 and ITS4). The PCRs included the following cycles 1 cycle of 2 min at 94°C, 30-40 cycles of 30 s at 94°C, 1 min at 52°C or 62°C, depending on the annealing temperature and one cycle of 10 min at 72°C. These PCR products were then digested by incubation with 10μL of PCR product with different enzymes at 37°C, for 3 h. The digested amplifications were then separated on 1.5% agarose gel.

**Results and Discusion**

Fragments between 300 and 2800 bp were amplified using 13 primer combinations. As expected, more polymorphism was found between the AA and CCDD genomes, followed by different species with the same genome. A 400 bp fragment of TrnL-Trnf region sequence was identified in the cpDNA of all seven accessions of CCDD genome species represented by 6 acessions of *O. latifolia,* (including one from Venezuela and two from Colombia), one accession *O. alta* and three accessions *O. grandiglumis*. This fragment was not noted in AA genome diploids in which a fragment of 344 pb was observed (Figure 1). This result indicates that non-restriction PCR amplified fragment of TrnL-Trnf sequence can be used to easily distinguish tetraploid (CD) from diploid (AA). These results were confirmed by means of the amplification and restriction of three regions of chloroplast PsbC [pstI 44 kd protein] and trnS [tRNA-Ser-(UGA)], trnS [tRNA-Ser- (UGA)] and trnfM [tRNA-fMet (CAU)], TrnC [RNA-Cys (GCA)] and trnD [tRNA-Thr-(GGC)] with seven restriction enzymes. These combinations also allowed differentiating the species CD from the AA (data not showed). Three of the four accessions of the *O. glumaepatula* revealed cpDNA polymorphisms in the region CP8 corresponding to the non-coding regions between the amino acid trnS [TRNA-Ser- (GGA)] and trnT [tRNA-Thr (UGU)]. Two polymorphic fragments of 298 bp and 98 pb were observed in *O. glumaepatula* accessions from Costa Rica, Colombia and Venezuela, after digestion with DraI (Figure 2). These fragments also were observed in the sample of *Oryza sp* collected from the "Estero de Camaguan" in Venezuela, while the other species of the genome CD and AA also including *O. glumaepatula* (Brazil) showed a 396 pb fragment. It is important to include more species of *O. glumaepatula* since three or three ecotypes of this species has been described using genotypic, biochemical and molecular analyses recognizing ecotypes groups for Central America and the Caribbean, the Amazon and the Pentanal region in the Southeastern of Brazil (Akimoto et al., 1998, Vaughan et al., 2003). Only one polymorphic combination in region CP3/ RSA for *O. rufipogon* and the weedy rice was found. This combination allows separating the weedy rice accession 5-48-2 from the other two weedy rice used.

The analysis of similarity using the cpDNA regions allowed a clear separation between the tetraploids and diploids genomes (Figure 3). Nevertheless, although the wild species of *Oryza* and rice are closed related (92 % similarity), with these sequences is possible to identify four groups: the first group contains CD species, *O. alta*, *O. grandiglumis* and *O. latifolia*. Two *O. latifolia* samples collected in Colombia (Meta and Pacifico) and one sample from Venezuela (Portuguesa) are found in this group. The cpDNA sequences analyzed do not allow differentiation among the three CCDD genome species. Nevertheless, studies at molecular level using nuclear and chloroplasts sequences indicate that *O. grandiglumis* and *O. alta* are more close related and this complex is different from *O. latifolia*. Comparison analysis of the ITS region allow separation of *O. alta* and *O. grandiglumis* from *O. latifolia* (Ying and Ge, 2003) when using digestion with restriction enzymes Dra III and Fok I. The second group contains all accessions *O.*

*glumaeputula* from IRRI , Costa Rica, Colombia and Venezuela and the *Oryza* sp sample collected in the "Estero de Camaguán", Venezuela. These species are clearly different from *O. rufipogon*. An interesting results is that one accession of *O. glumaepatula* from Brazil clustered in the same group as *O. glaberrima*. The fourth group contains the species of *O. rufipogon*, *O. sativa* and *O. barthii* and weedy rice accessions. The weedy rice accession 5-48-2 clustered with *O. rufipogon* and *O. barthii.* The accession 5-48-2 weedy rice has been also been associated at molecular and morphological level with the accession *O. rufipogon* (IRGC 105491) in previous studies (Gonzales *et al*., 2003, SB2 Annual Report 2003).

**Future Activities**

The evaluation of cpDNA, amplified product using other additional restriction enzymes will be tested in order to identify specific polymorphic patterns between varieties, weedy rice and wild *Oryza* species, and the amplified DNA fragment will be sequenced. Other markers allowing differentiating the weedy rice complex, *O. sativa* and *O. rufipogon* will be tested including V-ATPase B-subunit (p-VATPase) region, and a nuclear pseudogene with low pressure to selection. This region has been used in previous works and shown its utility indicating that the two subspecies of rice (indica and japonica) were domesticated from geographically different wild rice gene pool. The identification of specific haplotypes is in progress.

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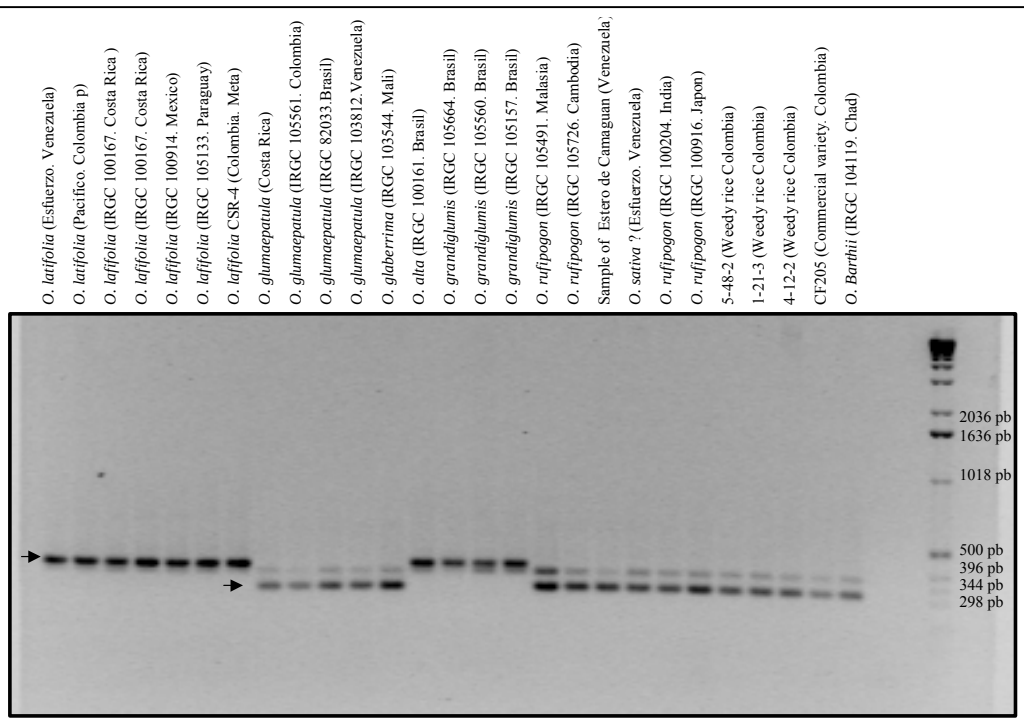


**Figure 1. Amplification of cpDNA TrnL-Trnf sequence of several *O. sativa* wild species, weedy rice and rice.**

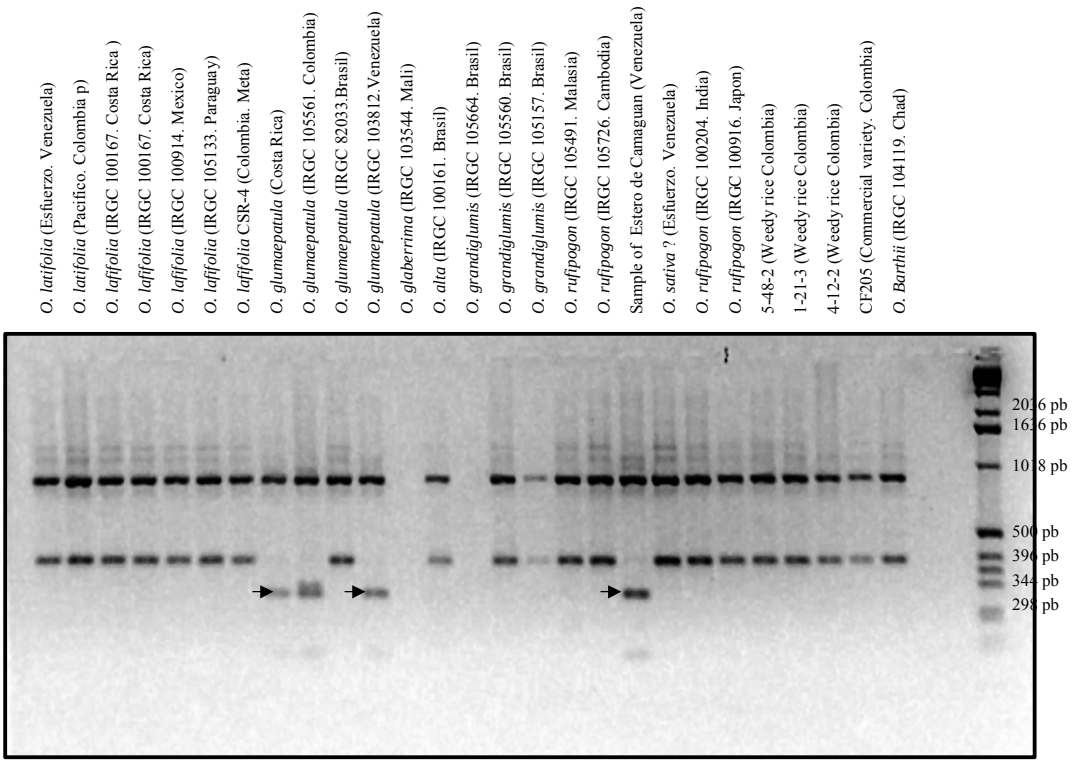


**Figure 2. Amplification of trnS [TRNA-Ser- (GGA)] and trnT [tRNA-Thr (UGU)] sequence of several *O. sativa* wild species, weedy rice and rice.**

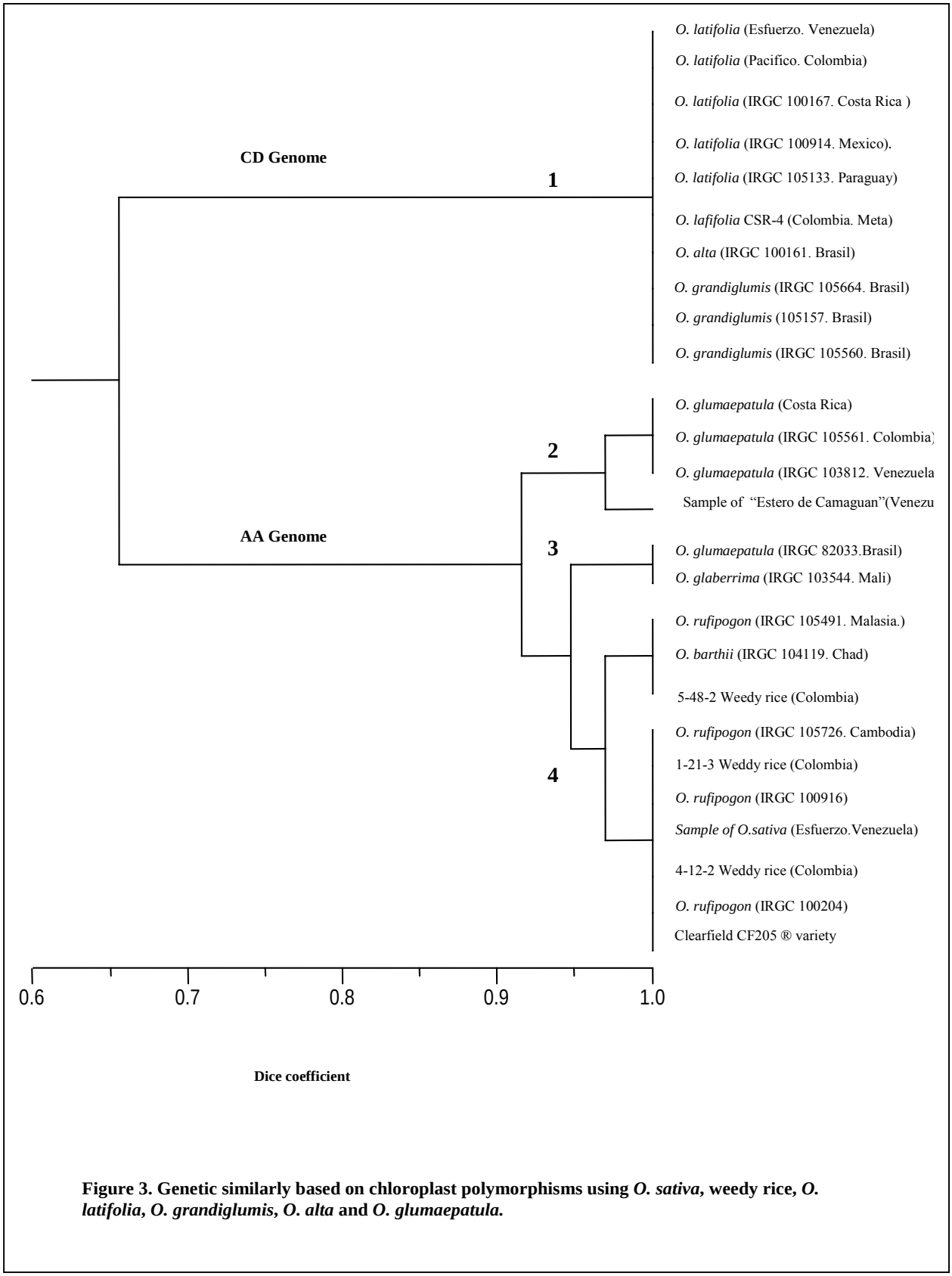


**Figure 3. Genetic similarly based on chloroplast polymorphisms using *O. sativa*, weedy rice, *O. latifolia, O. grandiglumis, O. alta* and *O. glumaepatula.***

## 1.1.22 Use of chloroplast DNA and nuclear sequences for characterization of weedy rice. Part II. Characterization of weedy rice species collected in Colombia.

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[1]SB2, [2]IP4. Funding from GTZ, Germany and CIAT.

**Abstract**
The objective of this work is to standardize methodology for bulk analysis of large number of field samples allowing discerning weedy rice and wild *Oryza* species, and direction of gene flow with cultivated rice, as pre-requisite for completing environmental safety dossier of potential release of transgenic rice in the Neo-tropics..

**Key Words:** cpDNA, nuclear molecular markers, gene flow, weedy rice, *Oryza* species

### Introduction

Several hypotheses exist about the origin of the weedy rice complex. One of them suggests it has arisen from hybridations between the subspecies *O. sativa* japonica and indica types, since weedy rice showed intermediate characteristic to both groups. Another theory suggests that weedy rice evolved from wild *Oryza* species or by degeneration of the cultivated rice form present in marginal no cultivated sites (Suh et al., 1997 Bres-Patry et al., 2001, Lentini and Espinoza, 2005). Some markers that had been used by other groups for taxonomy and phylogeny analysis of rice were selected in this study for the characterization and identification of the sample populations. The amplification of the chloroplast sequence ORF100 using the combinations of primers reported by Sun et al (2002) and Garris et al. (2005) was standardized. This sequence has been used to differentiate between indica and japonica type varieties, and also between tropical and temperate japonica cultivars at the cytoplasmic level (Chen et al. 1993). The current report showed the characterization of 251 Colombian weedy rice accessions using cytoplasmic markers that correspond to the sequences of the ORF100 region. The RFLPs of cpDNA and specific nuclear sequences (Ge et al., 2001 and Ying and Ge, 2003) were used to identify the genome type and ploidy level, and discern if either any of the American Oryza species (*O. latifolia, O. grandiglumis, O. alta and O. glumaepatula*) maybe part of the weedy rice complex. Additionally 5 cp-SSRs that show intra- e inter-specific variation in AA genome were used in the characterization of the weedy and wild rice accessions. The combined data of cytoplasm and nuclear genome profiles would help elucidate the potential origin of this weedy rice population.

### Material and methods

*Plant materials*. Accessions of weedy rice (251) collected in Huila and Tolima in 2001 and 2005, Clearfield CF205 ® accession (40) collected in Clearfield field commercial rice also include indica (22) and japonica (12) rice varieties types, *O. rufipogon* (8), *O. glaberrima* (1), *O. barthii* (1), *O. glumaepatula* (1), *O. alta*, (1), *O. grandiglumis* (1), *O. latifolia* (3), two cross of O. nivara /O. rufipogon (2) and O. nivara (2). Total DNA was isolated from young leaves according to the method described by McCouch et al. (1988).

*cpSSR.* The molecular analysis was conducted with five cp-SSRs chloroplast markers (RCt1, RCt3, RCt5, RCt8 and RCt9) reported by Ishii et al. (2001). PCR was performed in 20 µL containing: 40 ng of genomic DNA, 0.75 mM dNTPs, 1.8 mM $MgCl_2$, 1.U Taq polymerase, 1X PCR buffer 0.5 µM of each primers. The amplification was carried out using 1 cycle of 3 min at 94°C, 34 cycles of 15 s at 94°C, 15 s at 55°C, at 72°C for 15 s and one cycle of 5 min at 72°C. The PCR products were resolved on silver-

stained polyacrylamide gels and microsatellites alleles were sized by comparison to the 10 and 25 bp molecular weight standards (Promega). Five cp-SSRs and one nuclear-SSR (RM234) were evaluated using accessions of weedy rice (46) and all wild rice accessions and also commercial rice (15) and wild species (18).

*ORF100 region and RFLPs- chloroplast.* The plastid sequence, which captures the ORF100 region, was amplified as described in Fory et al., 2005 (SB2 Annual Report 2005). The region CP8 corresponding to the non-coding regions between the amino acid trnS [tRNA-Ser- (GGA)] and trnT [tRNA-Thr (UGU)] and TrnL-Trnf F, were amplified following the protocol describe here in Fory et al., 2006. SB2 Annual Report 2006.

*Adh-2.* The regions including the Adh-2 gene were amplified using universal forward and reverse primers (Adh F1 and adh RR) reported by Ge et. (2001). PCR was performed in 20 µL volume containing the following components: 200 ng of genomic DNA, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1.U Taq polymerase, 1X PCR buffer 0.1 µM of each primers The reaction was carried out using the following program; cycles 1 cycle of 70°C 4 min, 94°C 1 min, 52°C 30 s 72°C 1 min 30 s, 35 cycle of 94°C 20 s, 55°C 20s, 72°C 1 min 30 s one cycle of 10 min at 72°C. These PCR products were then digested by incubating 10µL of PCR product with EcoNI at 37°C, for 3 h. The digested amplifications were then separated on 1.4% agarose gel.

**Results and Discusion**

Multiple correspondence analysis (MAC) using SSRs markers generated seven groups. All wild *Oryza* species were distributed in four different groups (Figure 1A). The weedy rice accessions are separated in two groups (1 y 2). Group 1: included 38 accessions, which 31 are weedy rice. Eighty one percent of the weedy rice showed dark hull. This group also included six commercial varieties (Bonanza, Cica 8, Fedearroz 50, IR64, Taducan and Fedearroz 200). One accession of *O. rufipogon* (IRGC 105726) was included in this group. It is important to remark that the accessions that belong to this group had indica type chloroplast. Group 2: include 11 weedy rice accessions seven of which are black. This group enclosed four japonica varieties Moroberkan, Azucena, Bluebelle and Cypress and one variety indica, Cuba 65. All varieties classified as japonica showed japonica type chloroplast. Cuba 65 was the only indica variety included in this group that showed chloroplast japonica type. Group 3: it included the tetraploids *Oryza latifolia* (Pacifico), *Oryza latifolia* IRGC 100167 (Costa Rica) *Oryza alta* IRGC100161 (Brazil) y *Oryza grandiglumis* IRGC 105664 (Brazil). Group 4: contains five (5) *Oryza rufipogon* (IRGC 100204, IRGC 105349, IRGC 100923, IRGC 103823, IRGC 100916), two *Oryza nivara* (IRGC 103821, IRGC 103824), two (2) crosses *Oryza nivara /Oryza rufipogon* (IRGC 103813 y IRGC 103814). The varieties CO25 and Agostano are included in this group. Group 5. *Oryza glaberrima* and one weedy rice 4-19-2. Group 7: *O. barthii* (Chad) Group 6: *O. glumaepatula* (accession from Costa Rica).

Multiple correspondence analyses (MAC) were conducted using cp-SSR (MAC-cpSSR) and were complemented with one nuclear SSR markers, and ORF100 chloroplast region and seed morphological traits (cpSSR-SSR-ORF100-MT). This total analysis generated eight groups. Wild *Oryza* species were distributed in six different groups (Figure 1B). Group 1: contained 83 % weedy rice accessions, six indica rice type commercial varieties (Cica 8, Fedearroz 50, Fedearroz 2000, Bonanza Taducan and IR64) and one accession of *O. rufipogon* (IRGC 105726). The 100 % of the accessions showed the indica type chloroplast, and 83% and 68% of the accessions had showed straw hull and straw apiculus, respectively. Group 2: it contains seven (7) weedy rice accessions, which six accessions (85 %) had brown hulls and awns and 100 % of the accessions had japonica type chloroplast. This group enclosed four (4) commercial varieties. They were considered a morphologic level as japonica type cultivated rice (Azucena, Bluebelle and Cypress), and one indica type cultivated rice (Cuba 65). Group 3: *O. latifolia* (Colombia) and *O. alta* IRGC 100161 (Brazil) and *O. grandiglumis* IRGC 105664 (Brazil). Group 6: *O. glumaepatula* (Costa

Rica). Group 7: one crosses between *O. nivara* and *O. rufipogon* from China (103813). Group 8: *O. barthii* (Chad). Some wild species were clustered together with weedy rice and commercial varieties. For example, the Group 4: contains four *O. rufipogon* accessions (100204 India, 105349 India; 103823 China; 100923 Myanmar) and two *O. nivara* 5 IRGC 103821 (China), and *O. nivara* 7 IRGC 103824 (China), one cross between *O. nivara* and *O. rufipogon* from China (IRGC 103814). This group also included three (3) commercial varieties Agostano, CO25, Moroberekan (BCF 363), and one weedy rice accession 7-10-2. Group 5: *O. glaberrima* IRGC 103544 (Mali), *O. rufipogon* IRGC 100916 and two weedy rice (4-19-2 and 7-3-2). The weedy rice accessions were principally clustered in two groups.

When comparing the analyses of MAC-cpSSR and MAC-cpSSR-ORF100-MT, it is important to note that both analyses separate the majority of wild Oryza species, O. *glumaepatula, O. barthii* and *O. latifolia, Oryza alta* and *Oryza grandiglumis.* Some *O. rufipogon* accessions were clustered with other *O. rufipogon / O.* nivara accessions or with weedy rice, and *O. sativa.* In both analyses some weedy rice accessions were associated with the japonica type varieties, and the rest of weedy rice accessions were indica type. It was also found that 14% of individuals present japonica type chloroplast with ORF100 region. When the ACM analysis was carried out using nuclear SSR (González et al., 2003-2005), the weedy rice was associated mainly with the indica varieties. However, Vaughan et al. (2001) report that one weedy rice accession (MS5) was similar to the japonica type varieties. These results indicate that in addition to indica type, a small proportion of weedy rice may have a different origin in this case of japonica type including 6 chloroplast regions (RCt1 RCt3 RCt5 RCt8 RCt9, and OR100 region). Similar results were obtained analyzing the ORF100 region. The OR100 region allows a clear separation of *O. sativa* types since $X^2$ Test (17.01 $p<0.0001$) showed an association chloroplast type with the morphological classification given for the japonica or indica type cultivated *O. sativa*.

So far of the weedy rice accessions analyzed (79) none of them are similar to *O. glumaepatula* by analyzing neither the CP8 [tRNA-Ser- (GGA)] nor the trnT [tRNA-Thr (UGU)]/Dra I specific sequences. Two polymorphic fragments of 298 and 98 were observed in the *O. glumaepatula* accessions from Costa Rica (Figure 2A), whereas the weedy rice accessions showed 1000 pb band which is common in all species. No tetraploid individuals had been detected in population of 146 weedy rice individuals analyzed using the TrnL-Trnf F spacer sequence. This marker allows having a clear distinction between AA diploids and the CD tetraploid using as reference *O. alta*, *O. grandiglumis* and *O. latifolia* (Figure 2B). Similar results were obtained in the analysis of the nuclear gene Adh-2 and amplified sequence digested with EcoNI. This combination gene/enzyme allows a clear differentiation *Oryza sativa* (AA) from the complex from *Oryza officinalis* (CD), where the CD accession as in the case of *O. grandiglumis* and *O. latifolia* show 3 additional bands which correspond to 0.6, 1.0 kb y 1.6kb whereas the A genome shows an only 1.6 kb band (Figure 2C). Ge et al. (2001) were able to discern 10 genome types conducting restriction-enzyme Adh-2 and Adh-1 gene sequences. Results suggest that the weedy population analyzed is composed by AA genome individuals, including those accessions characterized by tall plants with broad leaves resembling tetraploid *Oryza* species. So far it cannot rule-out the presence of wild species in the weedy rice complex since some accessions are closely related to *O. rufipogon* by SSR analysis.

**Future Activities**

Detail analysis of weedy rice population collected from Jamundi (Valle del Cauca) and *Oryza* sps from Venezuela is in progress. These population will be analyze using the ORF100 and the CP3 TrnC [tRNA-Cys- (GCA)] and trnD [tRNA-Asp (GUC)] sequences among others. The specific alleles and identification of haplotypes are in progress

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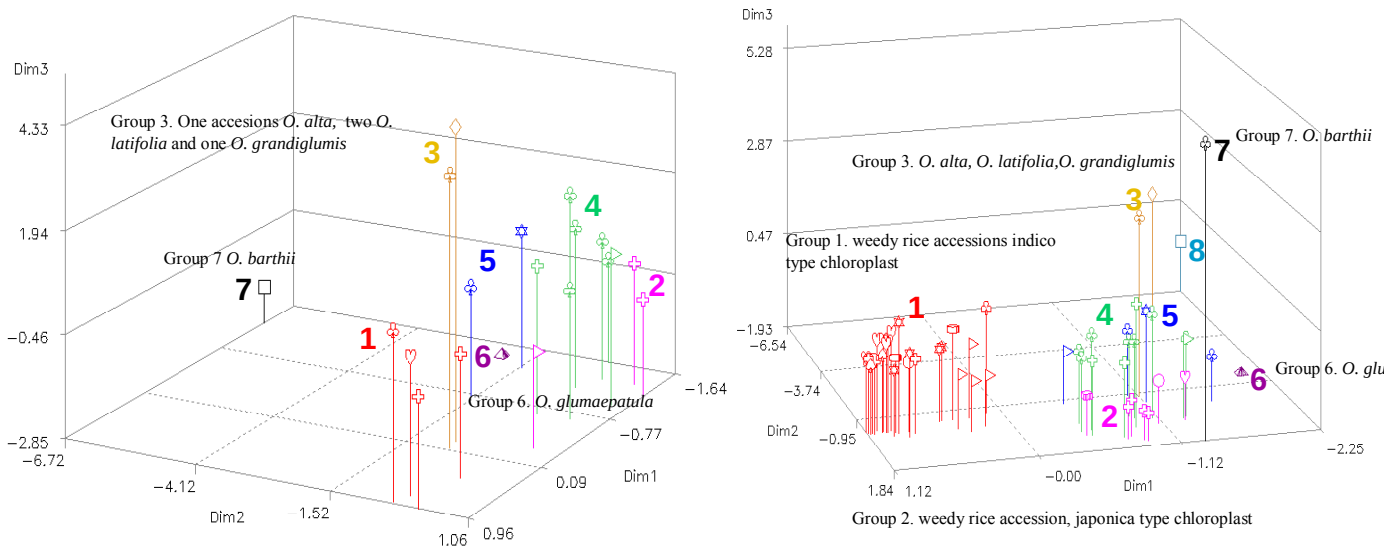


**A. MAC-cpSSR** **B. MAC-CpSSR-ORF100-MT**

Figure 1. A) MAC-cpSSR and B) MAC-CpSSR-ORF100-MT. 1A). In the Figure 1 A) The group 4 contain: five *Oryza rufipogon*, two *Oryza nivara*, two crosse of *Oryza nivara /Oryza rufipogon* and two varieties. The Group 5: *Oryza glaberrima* and one weedy rice 4-19-2. In the Figure 1 B. The group 4: contain four *O. rufipogon* accessions and two *O. nivara*, one crosses between *O. nivara* and *O. rufipogon*, three commercial varieties and one weedy rice accession 7-10-2. Group 5 contain *O. glaberrima*, *O. rufipogon* and two weedy rice (4-19-2 and 7-3-2).

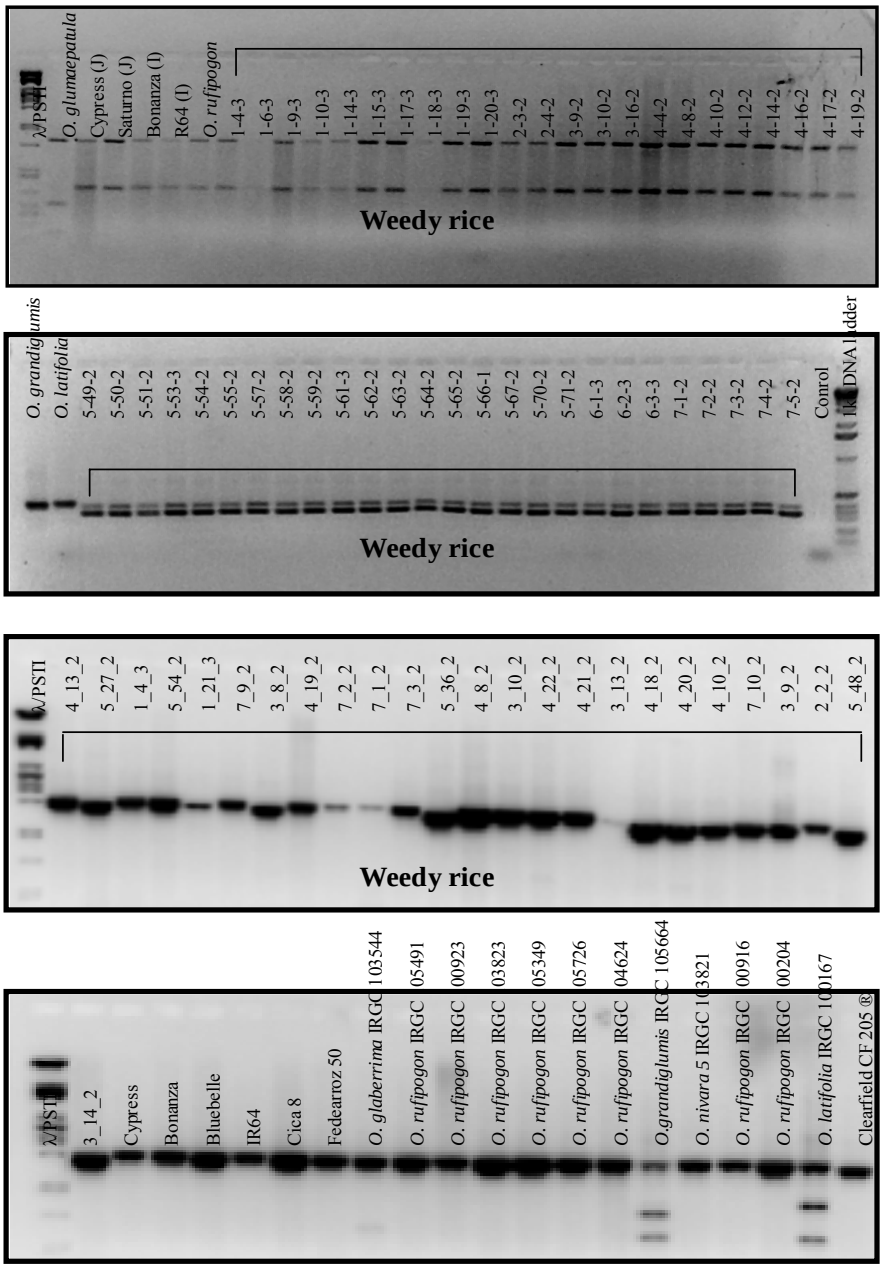


**Figure 2. A Amplification of trnS [TRNA-Ser- (GGA)] and trnT [tRNA-Thr (UGU)] sequence and cut with Dra I. B, PCR of the cpDNA TrnL-Trnf sequence. D, Restriction profiles of the PCR amplification of the Adh –2 and cut with EcoNI**

### 1.1.23 Exploring wild introgressions in rice

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*These authors contributed equally to the work

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**Introduction**

The future of crop improvement depends on the availability of genetic variation. Most modern crop varieties have undergone a genetic bottleneck associated with the process of domestication resulting in a restriction of the genetic options that are available to plant breeders. There is a larger pool of genetic variation available in landraces and wild relatives of crops. These resources are known to contain many interesting traits for breeding, including good to strong tolerance to abiotic and biotic stresses and various nutritional traits of interest (Sun et al 2001). However, it is often difficult to utilize these natural sources of genetic diversity because of fertility barriers, linkage drag, the time and resources required to recover useful recombinants.

To take advantage of the unexploited reservoir that exists in the wild relatives of cultivated rice (*Oryza sativa* L.), we started to develop interspecific introgression lines that will be of immediate use to breeders and will simultaneously serve to enhance our understanding of the "wild alleles" that contribute favorably to plant performance under drought stress. These lines are called Chromosome Segment Substitution Lines (CSSLs).

CSSLs are particularly valuable when complex, quantitatively inherited phenotypes are the breeding target. Because they represent permanent (inbred) genetic resources that can be easily replicated by seed and distributed to collaborators working in different environments. Each set of CSSLs consists of a relatively small number of lines that can be evaluated in replicated trials. They are constructed to provide maximum power of statistical analysis because each line can be compared to all others or may simply be compared to the recurrent parent, making it possible to extract a great deal of valuable information from a relatively small number of lines crops. For phenotypes that are difficult to measure, or require repeated evaluation over years and environments, the ability to focus quickly on a small number of lines is a critical component of success (Ghesquière et al, 1997).

In addition to the targeted introgression of traits that can be identified phenotypically in the wild material, such as biotic or abiotic stress tolerance, it has been demonstrated that alleles hidden in low yielding, agronomically undesirable ancestors can enhance the productivity of many of the world's most important crop varieties. These yield-enhancing alleles are the basis of 'transgressive variation' and may confer an advantage in both favorable (irrigated) and unfavorable conditions (drought and weed competition) (Moncada et al., 2000; Gur and Zamir, 2004). Thus, the use of wild and exotic germplasm for CSSLs construction carries with it the possibility that favorable transgressive segregants will be identified, providing the basis for studies aimed at understanding the genetic basis of transgressive variation associated with the trait of interest.

Wide spread utilization of *O. sativa* relatives remains limited due to the fact that: (1) no extensive study has been carried out to explore the range of allelic diversity in any of the *Oryza* AA genome relatives, (2) the genetic basis of heterosis or transgressive variation in interspecific crosses remains largely unknown, (3) interspecific crossing barriers have hampered full utilization of rice relatives for breeding and genetic studies, (4) very few genomic resources have yet been developed to facilitate breeding efforts using *O. sativa* relatives. In particular, the lack of a cost effective, high throughput marker system that targets gene-based polymorphisms impedes efforts to efficiently and systematically select the best introgression lines and to evaluate the gene content of those lines in the context of comparative cereal genomics.

**Methodology and Results**

We are currently developing introgression lines from four cultivated x wild crosses, where the wild species are *O. meridionalis, O. rufipogon, O. glumaepatula* and *O. barthii*.

*O. sativa* x *O. meridionalis*
Laura T Moreno - CIAT
Interspecific cross: *O. sativa* ssp. tropical *japonica* cv. Curinga x *O. meridionalis* acc. OR44
We have worked at the construction of a genetic map based on unique-site microsatellite markers (SSRs) screened on a backcross population of 87 BC1F1 lines obtained from an interspecific backcross between the cultivated rice *O. sativa* BRSMG Curinga and its wild relative *O. meridionalis* accession OR44 (or W2112) from Queensland, Australia. The donor parent was the wild accession. A total of 387 SSRs were screened and 123 markers showed polymorphism between the two parents. These 123 SSRs were evaluated on the BC1F1 population. The genotyping data of this population was used for the construction of a genetic map using the MapDisto beta version 1.7 (121) software (Lorieux 2007).

From the development of the F1 and the BC1F1 population, we can conclude that it exists a strong hybrid inviability as a consequence of sterility barriers between the species *O. sativa* and *O. meridionalis*, though these barriers are hoped to be broken during the next backcross generations. From the screening of the SSRs, we could observed a high degree of polymorphism between these two species with 66% (81 SSR) out of 123 evaluated with an allelic variation of 10 or more base pairs. The construction of the linkage map allowed the identification of 12 linkage groups with a distance coverage of 2178 cM (Kosambi function). The colinearity of the map was good with only a few inversions that seem to be related to segregation distortion. These regions are being verified to fix possible scoring errors and data manipulation.

The 87 BC1F1 lines were backcrossed to the recurrent parent Curinga during the second semester of 2006 to generate a BC2F1 population. We are currently working on the selection of the BC2F1 lines using the CSSL Finder program (Lorieux 2005). The genotyping of this population will be worked at the University of Cornell in Ithaca, NY during the months of April and May of 2007. These lines will be used to generate the BC3F1 families and the crossing work is expected to start at the end of march 2007.

*O. sativa* x *O. rufipogon*
Juan David Arbelaez - Fedearroz
Interspecific cross: *O. sativa* ssp. tropical *japonica* cv. Curinga x *O. rufipogon* acc. IRGC105491

A preliminary linkage map has been generated using 80 plants from a $BC_1F_1$ population derived form the cross [Curinga x IRGC105491] x Curinga. A set of 130 SSR markers distributed homogeneously along the whole rice genome was chosen from the Core Map. These markers were chosen from the Core Map for being polymorphic between the parents and being distributed homogeneously across the rice genome in an average distance of 3 Mpb.

The resulted map has total genetic distance of 1930 cM, very similar to those reported for others maps between interspecific crosses populations (Lorieux et al 2000), and shows high colinearity with the physical map, confirming the use of these markers for the analysis of the next generations lines.
Also, this genotyping work allowed us to identify the introgressed fragments from the donor parent in each line.

With this information and the use of the CSSL Finder program, around 50 of these 80 plants were chosen to be backcrossed to the recurrent parent Curinga to obtain 50 BC2F1 families (representing in 600 plants). This 50 BC1F1 plants were selected to represent the entire donor genome of IRGC 105491 in small desirable overlapping chromosomal segments.
These BC2F1 plants will be analyzed with the SSR markers according to the introgressed fragment of interest, tracking the introgressions and optimizing the recovery of desirable genetic background in each line in order to later on develop the Chromosome Segments Substitution Lines (CSSLs) set.

*O. sativa* x *O. barthii*
Gustave Djedatin - WARDA
Interspecific cross: *O. sativa* ssp. tropical *japonica* cv. Curinga x *O. barthii* acc. IRGC101937
The cultivar Curinga, used as female parent, was crossed at CIAT with the *O. barthii* IRGC101937 accession. The $F_1$ plants were backcrossed to Curinga to produce the population of $BC_1F_1$ lines. A total of 80 $BC_1F_1$ seeds have been obtained and sown at two times. Among them, 64 have germinated.

A total of 233 SSR markers from the Core Map have been used to survey the polymorphism between the two parents on each anchor in order to cover the whole genome through the 12 chromosomes. A set of 131 polymorphic markers well distributed on all 12 chromosomes were used to evaluate the population. They have been set up in 17 multiplex. For each multiplex, the markers have been amplified and PCR products checked on 4 % polyacrylamide or 4 % Metaphor® agarose gels. The separation of PCR fragments of the BC1F1 DNAs analysis was done on an Applied Biosystems 3730x1 capillary DNA Analyzer. The ABI results have been analyzed by GenMapper® version 3.0 program.

The linkage map was computed and drawn using the MapDisto program. The total length of the first draft of the map was of 2646 cM, which is significantly longer than the expected size. Most of the markers were regularly distributed along the chromosomes. The genetic distance between 2 markers varied from 14.81 cM on chromosome 7 to 28.0 cM on chromosome 4 with a mean interval length of 22.0 cM.
A significant part of the map expansion observed in this population is supposed to originate from genotyping errors. We are currently double-checking the genotyping data using the “Show error candidates” option of the MapDisto program. This will allow us to correct the data in order to

attain a correct map size, and to make sure that we are actually targeting the right chromosomal segments.

Using the program CSSL Finder, several *O. barthii* introgressions could be detected in each line, while the proportion of introgressed regions varied across chromosomes and individuals. The proportion of introgressed *O. barthii* genome varied among the 12 chromosomes. Introgressions at several loci were observed at high frequency.

A BC2F2 population was produced at CIAT and the seeds have been sent to WARDA for sowing and genotyping. The BC3F1 population will be produced at WARDA.

*O. sativa* x *O. glumaepatula*
Priscila Rangel – Embrapa-CNPAF
Interspecific cross: *O. sativa* ssp. tropical *japonica* cv. Curinga x *O. glumaepatula* acc. GEN1233
A genetic map was obtained for the $BC_1F_1$ population derived from the interspecific cross *O. sativa* (Curinga) x *O. glumaepatula* (GEN1233). The map was constructed with 127 SSR markers using the software MapDisto v. 1.6. The CIAT Core Map was used as a reference to establish markers orders.

The genetic distances and genotyping data were used to detect wild chromosomal segments introgressed to the $BC_1F_1$ plants using the software CSSL Finder v. 0.8. A total of 60 $BC_1F_1$ plants were genotyped and one plant per targeted wild fragment was selected.
All $BC_1F_1$ plants were backcrossed to Curinga and 10 seeds from each plant were sown in pots in the greenhouse. Ten $BC_2F_1$ plants, representative of each selected $BC_1F_1$ plant, were genotyped with three to four markers that covered the wild fragments. The detection of flanking markers was done using the software QGene version 2.3 for Macintosh (Nelson 1997). The $BC_2F_1$ plant that showed the wild allele for all three or four markers on each fragment was selected for the third backcross. A total of 60 $BC_2F_1$ plants were selected and are being backcrossed to Curinga to generate the $BC_3$ population. The $BC_3$ crosses will be ready by the end of March 2007 and the $BC_3F_1$ plants must be available for genotyping by the end of April 2007 at Cornell University.

**Conclusion**

CSSLs were proven as very a powerful tool for gene discovery in different crops. They are of particular value for studies involving wild progenitors as they 1) often permit to overcome interspecific sterility barriers as a large part of the cultivated species is recovered in advanced generations, 2) allow a direct comparison of the introgressed lines to the cultivated parent, permitting to display the effect of the wild progenitor on the phenotype.
We hope that the development of full-genome coverage CSSL populations will contribute significantly to the set of genetic and genomic tools available for breeding and gene discovery in rice.
To date, the project allowed us to obtain many important results. Among them, we may want to mention in particular the following:
- Four interspecific genetic maps were developed,
- Four cultivated x wild BC1F1 populations were genotyped,
- Four cultivated x wild BC2F1 populations were derived,

- A software (CSSL Finder) was designed for the specific purpose of helping at developing CSSL lines,
- Several students and research assistants were trained,
- Four students do shuttle research between their respective centers and Cornell University,
- The international collaboration between several ARIs, CG centers and NARS was strengthened,
- Several publications are in preparation.

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### 1.1.24 Soil microbial population analysis by Real-Time PCR to study Biological nitrification Inhibition (BNI) activity of crops

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[1]SB-2 and [2] IP-5 CIAT projects

**Introduction**

In soil, nitrogen (N) available to plants in the form of nitrates ($NO_3^-$) does not bind to soil particles due to its negative charge. Therefore, this form of nitrogen is highly susceptible to leaching and thereby is lost when water runs off. Nearly 70% of the nitrogen fertilizer applied to agricultural soils is lost due to the nitrification process (oxidation of the relatively immobile ammonium - $NH_4^+$ - into the highly mobile nitrate - $NO_3^-$ -). In the same way, the loss of N from soil to the atmosphere in the form of other compounds, cause a negative impact on the environment by contributing to worldwide global warming and the greenhouse effect. Moreover, the soil N that is lost by leaching and/or denitrification can be substantial, which promotes pollution of ground water.

For these reasons, it is imperative to identify natural compounds that inhibit the nitrification process, which is carried out by soil nitrifying microorganisms including Archaea, as suggested by recent studies (Leininger et al, 2006). In collaboration with JIRCAS of Japan, CIAT found that *Brachiaria humidicola* has the ability to suppress the activity of specific nitrifying microorganisms (bacteria and Archaea) by releasing inhibitory compounds from its roots to the soil. The inhibitory effect was mainly demonstrated by a bioluminescence assay using root exudates which will give indirect evidence for the phenomenon. To demonstrate direct evidence of the BNI phenomenon in soil, we report here the implementation and standardization of a Real-Time PCR technique to monitor microbial populations in soil, which allows us to study direct effects of roots exudates of several crops on the soil microorganisms involved in the nitrification process.

**Materials and Methods**

Soil samples used in this report were harvested from the CIAT HQ field. The field has been used for BNI field work for three years, since 2004, and the work was partially supported by JIRCAS. The crops used in the field study were: Soybean, *Panicum maximum,* Hybrid Mulato*, Brachiaria humidicola* 679, *Brachiaria humidicola* 16888 and bare soil (used as a control). The soil sampling was done at the end of the grasses $4^{th}$ growing cycle and before and one day after nitrogen fertilization of the $5^{th}$ growing cycle. The soil samples were taken from a depth of 10 cm from a 1m x 1m sub-plot for every treatment using a metal tube with one side open. The two soil samples were mixed and the soil DNA extraction was performed using a FastDNA SPIN kit for Soil (Q-BIOgene, USA) according to the manufacturer's directions. Afterwards, the extracted DNA was quantified using the PicoGreen reagent and then was electrophoresed onto a 1% agarose gel to check its quality.

Four target genes (Bacteria and Archea *amoA* genes and 16S rRNA genes) were amplified using specific primer sets: *amoA*-1F/*amoA*-2R, *amoA*19F/*amoA*643R, BACT1369F/PROK1541R, and Arch 20F/Arch 958R, respectively. These primers were demonstrated to be useful for this work elsewhere (Suzuki et al, 2000, Leininger et al, 2006). The *amoA* primers were designed to amplify t*amoA* genes, which encode a subunit of the ammonia-oxigenase enzyme that is presumably involved in the nitrification process in ammonia-oxidizing bacteria and Archaea. The other primers were used to amplify 16S rRNA gene, which was used as an internal control to track the population dynamics of the ammonia oxidizing bacteria and Archaea populations in soil.

**Results and Discussion**

To establish the PCR techniques for quantifing soil bacterial populations, we first generated standard curves to quantify the copy number of the target genes using gDNA from *Nitrosomonas europaea* for *amoA* gene and *E. coli* for 16S rRNA gene, and plasmid DNA with the specific DNA inserts for Archaea amoA and 16S rRNA genes (Figures 1 and 2). There were strong linear (R2 = 0.99) inverse relationships between Ct and the $\log_{10}$ number of amoA and 16s rRNA gene copies. This set a detection limit of gene copy numbers per DNA sample of interest in the reaction mixture.
In addition, the TaqMan technique was also carried out as a preliminary experiment to evaluate the viability of the technique to amplify the bacteria 16S rRNA gene using a specific probe and the primers mentioned earlier (data not shown). A universal Tm was used for all the primers to reduce variability among samples as much as possible, but the primer concentration was specific for each primer set. The TaqMan technique also proved to quantify accurately the bacteria 16S rRNA (data not shown) and therefore can be use for further studies.

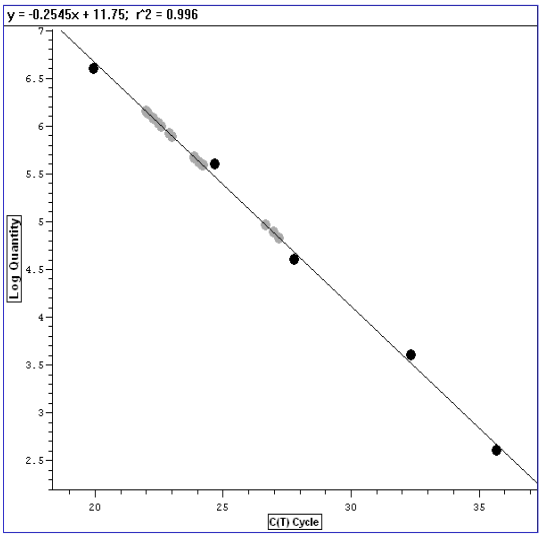


**Fig 1.** Standard curve generated from *E. coli* gDNA to quantify the copy number of the bacteria *amoA* gene.

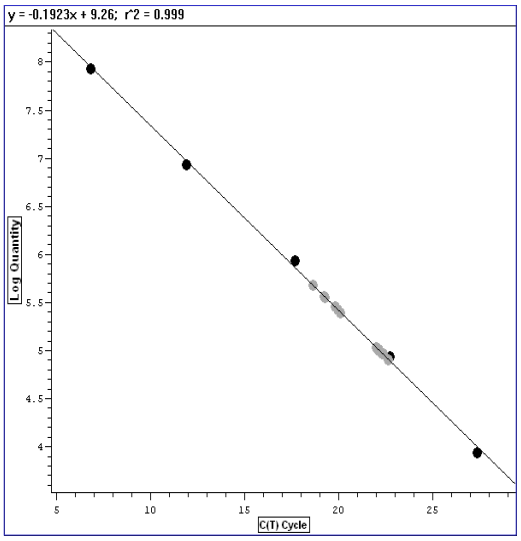


**Fig 2.** Standard curve generated from plasmid DNA to quantify the copy number of the Archaea 16S rRNA gene.

Likewise, as we observed in Figure 3, the sharp fluorescence plot of the standard curve obtained from Real-Time PCR experiments will ensure an accurate quantification of the target genes. In addition, the fluorescence plot of the melting curve showed that specific PCR products are being obtained with the primers used, which gives more reliability to the experiment (Figure 4).

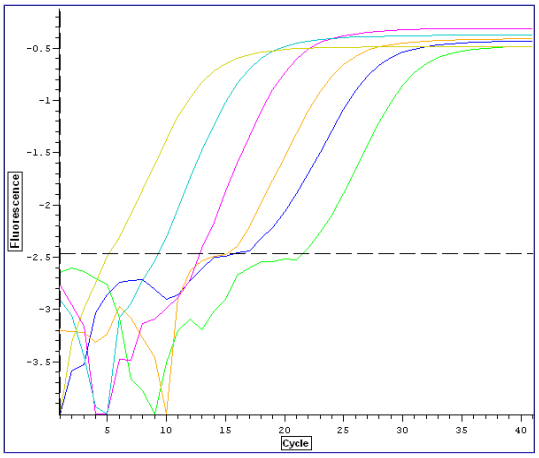


**Fig 3.** Fluorescence plot of PCR products of bacteria *amoA* gene using diluted *Nitrosomonas* genomic DNA

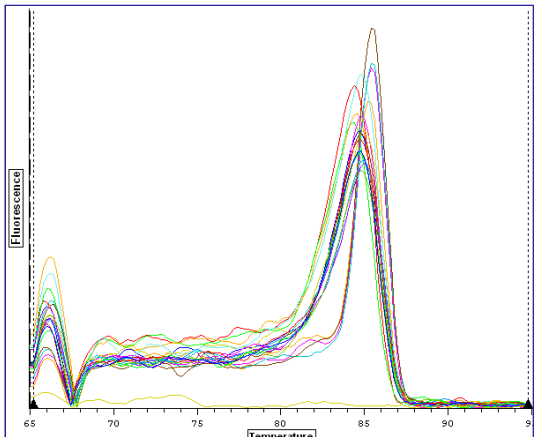


**Fig 4.** Melting curve of PCR products using a primer set for Archaea 16S rRNA gene The DNA template used for this experiment was a diluted plasmid DNA containing Archaea 16S rRNA.

Our results suggested that the PCR technique developed in this project is precise and reproducible for monitoring soil bacterial populations. For the soil samples, we have isolated good quality DNA from the soil samples that were described above. Currently, we are implementing this technique along with a detailed statistical analysis to monitor effects of BNI in the field conditions on bacterial populations of ammonium oxidizing bacteria and Archaea.

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## 1.1.25 Patterns of genetic diversity in the Colombian collection of avocado (*Persea americana* Mill.) using molecular markers

Collaborative project with CORPOICA, initially funded by Ministerio de Agricultura y Desarrollo Rural, Colombia.

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**Introduction**

The Colombian collection of avocado (*Persea americana* Mill.) with 60 accessions, maintained *ex situ* by CORPOICA in Palmira, is the major collection of this fruit in Colombia. *P. americana* is a subtropical diploid (2n=24) tree and has been commonly divided into three distinguishable ecotypes or horticultural races, known as Mexican, Guatemalan and West-Indian (Bergh & Ellstrand 1986; Storey et al. 1986). Being the avocado with open pollination, it contains a large genetic variability in its germplasm. This diversity has had a great impact on the development of the crop and the avocado industry worldwide (Bergh 1992). To make an efficient use and a suitable management of the Colombian collection of avocado we proposed to know its patterns of diversity and the levels of genetic redundancy present in this collection, using DNA molecular PCR based markers.

**Materials and Methods**

*Plant material*. 60 accessions of *Persea americana* Mill. maintained by CORPOICA in Palmira, of which we report here the analysis for 56 accessions. In addition, two wild species: *Persea caerulea* and *P. rigens* sampled in Colombia were included as outgroup. In Table 4 these accessions are reported with their geographical origin and horticultural race.

*Methodology*. The DNA molecular markers used were AFLPs. This technology was selected because of the magnitude of genome coverage and high reproducibility. The AFLP fingerprinting was performed basically as described in the manual protocol provided by Vos et al. 1995, as well as additions and changes made in our lab for avocado. Due to the moderate size of the avocado genome (8,83 x $10^8$ bp) (Bergh 1992), primer combinations of type 2/3 (EcoR1/Mse1) were used for the AFLPs. For the amplification a total of sixteen primer combinations were selectively tested to identify at least four that can show polymorphism and quality of the amplified fragments, which are in descending order: E-AC/M-CAC, E-AC/M-CTC, E-AG/M-CTA, E-AG/M-CAT.

Table 4. Avocado accessions and wild species of *Persea* from Colombia included in this study.

| Accession | Race[1] | Origin[2] | Accession | Race[1] | Origin[2] |
|---|---|---|---|---|---|
| *P. caerulea* | W | Valle, COL | Nativo 2011 | WI | Valle, COL |
| *P. rigens* | W | Quindio, COL | Booth 5 | GxWI | Florida, USA |
| Trapica | WI | Valle, COL | Hulumana | WI | Canal Zone, PAN |
| Lorena | WI | Valle, COL | 1607 | M | California, USA |
| Oriente 1 | WI | Valle, COL | Bacon | GxM | California, USA |
| Hass | G | California, USA | 135–27 | GxM | California, USA |
| Jim | GxM | California, USA | Collinred | GxWI | Florida, USA |
| HX 48 | GxM | California, USA | Booth 7 | GxWI | Florida, USA |
| 135–15 | GxM | California, USA | Waldin | WI | Florida, USA |
| Papelillo | WI | Valle, COL | Semil 44 | GxWI | Rio Piedras, PRI |
| Simmonds | WI | Florida, USA | Monroe | GxWI | Florida, USA |
| Peterson | WI | Florida, USA | Trapp | WI | Florida, USA |
| Itzamna | G | Santa MJ, GTM | 135-21 | GxM | California, USA |
| Nabilico | G | Desconocida | Pollock | WI | Florida, USA |
| Kanola | G | Antigua, GTM | Booth 1 | GxWI | Florida, USA |
| Tumaco | WI | Nariño, COL | Lula | GxWI | Florida, USA |
| Winslowson | GxWI | Florida, USA | G755 | G | Coban, GTM |
| Hayes | GxM | California, USA | Puebla | M | Puebla, MEX |
| Fuerte | GxM | Atlixco, MEX | Gottfried | M | Florida, USA |
| 135–20 | GxM | California, USA | La Selva | GxM | Antioquia, COL |
| Costa Rica | GxM | Antioquia, COL | Booth 8 | GxWI | Florida, USA |
| 143–77 | GxM | California, USA | Dr. Sardi | WI | Valle, COL |
| Trinidad | GxWI | Canal Zone,PAN | Zutano | GxM | California, USA |
| Choquette | GxWI | Florida, USA | Ibague | WI | Tolima, COL |
| Gripiña | GxWI | Rio Piedras, PRI | Los Silos | WI | Antioquia, COL |
| Mayapan | G | Purula, GTM | Gwen | GxM | California, USA |
| Duke 7 | M | California, USA | Marcus | WI | Florida, USA |
| Marzala | WI | Canal Zone,PAN | Ruehle | WI | Florida, USA |
| Oculta 1 | WI | Antioquia, COL | Fairchild | GxWI | Florida, USA |

[1] Designation of the avocado botanical races: W (wild species); WI (West Indian); G (Guatemalan); M (Mexican); GxA (Hybrid between the Guatemalan and West Indian races) y GxM (Hybrid between the Guatemalan and Mexican races).

[2] The origin corresponds first to the province and second to the country (according to FAO).

To report the botanical race and the origin, the following sources were consulted:

CORPOICA, Colombia (Bernal y Diaz, 2005; CORPOICA, 2004); PROFRUTALES LTDA, Colombia (Rios-Cataño y col, 2005); "Instituto de Investigación de Recursos Biológicos Alexander von Humboldt", Colombia (Vargas and Palacios, Pers. Com.).
Also the following WEB pages were consulted:
Virtual library on avocado: http://avocadosource.com
Florida avocados (USA): http://edis.ifas.ufl.edu/HS284
California avocados (USA): http://www.avocado.org/about/varieties.php

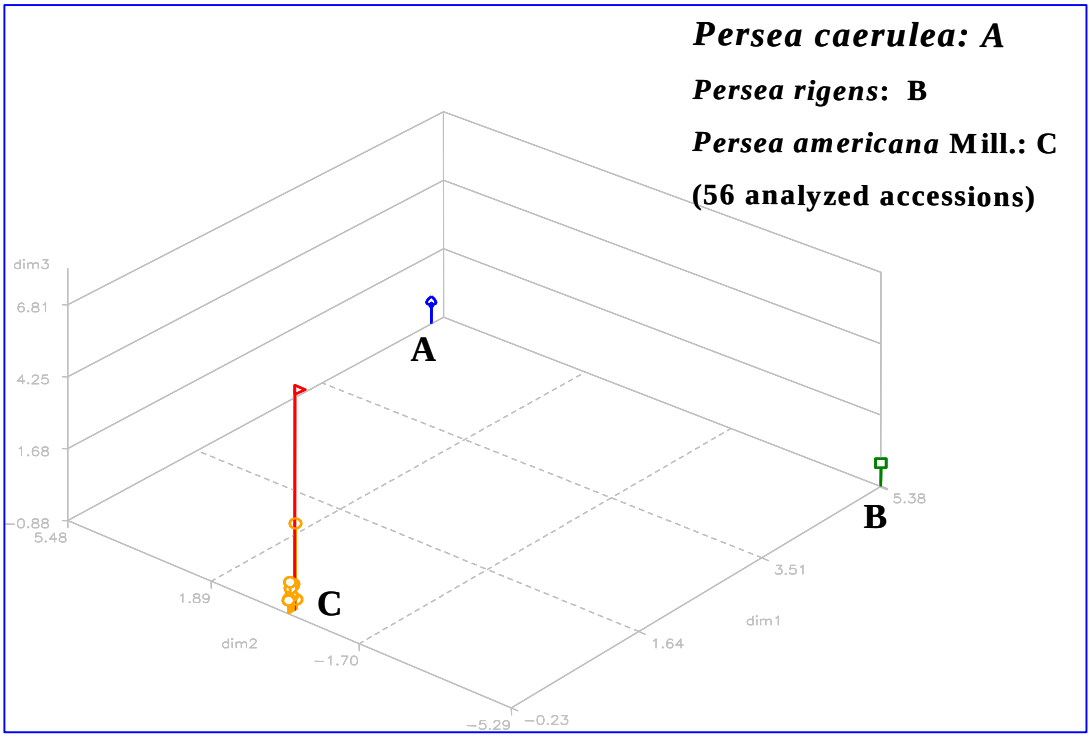
**Results and Discussion**

*Assessment of the genetic diversity*. The dendrogram derived from a UPGMA cluster analysis (using the four selected primers combinations), shows only a 1.8 % of genetic redundancy (two accessions as a possible genetic duplicate) in 56 analyzed accessions (Fig. 1). The possible genetic duplicate reported here corresponds to the accessions "Lorena" and "Trapica", which are similar in fruit, and plant habit, are of West Indian race and were originated by mass selection in the Valle. However, there are some morphoagronomic differences among them (Rios-Castaño, pers. com.; Rios-Castaño et al. 2005). The level of genetic redundancy in the collection is minimal, which facilitates the management and utilization. This analysis also shows that all the accessions are different with a similarity level of 95 % with exception of Lorena and Trapica. Therefore most of the accessions (98.2 %) could be characterized by specific molecular prints and then certainly identified (Fig. 1). Consequently, these results indicate the presence of genetic variability with a high similarity among the 56 analyzed accessions, suggesting that the genetic diversity should be increased, especially, with alleles of interest for avocado improvement.

*Genetic diversity patterns*. The pattern of genetic variability shows clearly the separation, without any relation, of the two *Persea* wild species sampled in Colombia from the rest of analyzed germplasm (Fig. 2). The West Indian race is most adapted to the climatic conditions of Colombia, to the extent that some authors suggest that the West Indian race originated in South America, with the North coast of Colombia, as the most probable place (Morton 1987; Patiño 2002). The UPGMA cluster analysis (Fig. 1) as well as the Analysis of Multiple Correspondence (Fig. 3) show the compact grouping of most of the West Indian avocados. From 12 original accessions of Colombia, 10 are West Indian and of these, 8 form the compact West Indian group. In order to investigate the relationship among the different avocado accessions, the racial distribution of the analyzed material shows the domain of the interracial hybrids with a 48 % (GxM = 25 % and GxWI = 23 %) and of the West Indian race with a 34 %. On the other hand, the Guatemalan and Mexican have respectively 11 % and 7 % of the analyzed accessions (Table 4). Relating these racial/ ecological designations with the genetic variability of the collection, we find a pattern of constant distribution without a clear differentiation among the races, with the presence of the interracial hybrids in between (Fig. 1). These patterns possibly result from gene flow caused as much by the management of the accessions as by their improvement. These results question the racial/ ecological designation for most of the analyzed accessions; nevertheless it was not possible to have this designation confirmed *a priori.* On the base of these results, it is advisable to preserve most of the accessions of the Colombian avocado collection as a new gene pool of *Persea americana* Mill germplasm.

Figure 1. Dendrogram (derived from a UPGMA cluster analysis) showing the 56 analyzed accessions of *P. American*, one of *P. caerulea* and other of *P. rigens*.

Figure 2. Three-dimensional graph based on multiple correspondence analysis (MCA).



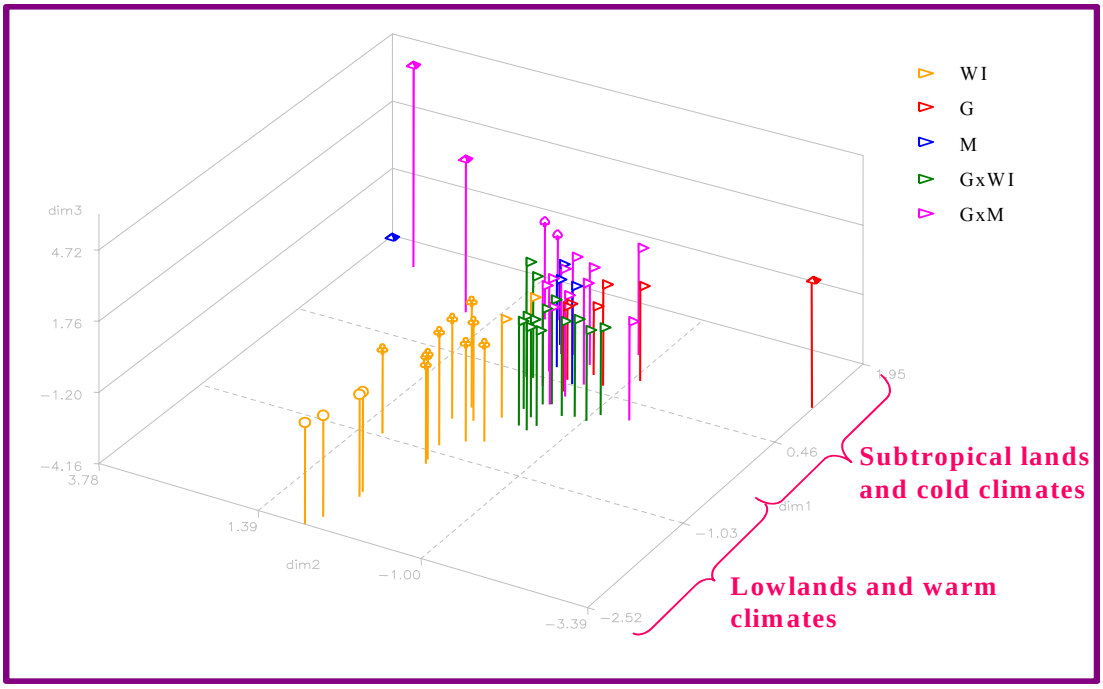




Figure 3. Three-dimensional graph based on MCA. Grouping done according to the horticultural race. The Tumaco and Marzala accessions were not included (see conventions in Table 25).

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# Activity 1.2 Development of molecular techniques for assessing genetic diversity and mapping useful genes

## 1.2.1 SNP markers validation and their integration to the common bean linkage map.

Contributors: C. Quintero[1], E. Gaitán-Solís[2], C. Quigley[3], P. Cregan[3], M.C. Duque[1] and J. Tohme[1]
[1]SB-2 Project; [2]Donald Danforth Center; [3]Soybean Genomics and Breeding Lab-USDA.

### Introduction

Single nucleotide polymorphisms are becoming the marker of choice for a wide variety of organisms, due to their high abundance in genomes (Gupta *et. al.*, 2001), their evolutionary stability and the availability of high throughput technologies for their detection (Landegren *et. al.*, 1998; Syvanen *et al.,* 1999). Genotyping of the CIAT's principal bean mapping population with SNP markers started in 2004 (Gaitán *et al.,* 2004) and some of them were added to the linkage map (Quintero *et al.*, 2005). Since then, efforts were made to enhance multiple SNP detection in the flow cytometer platform Luminex[100].

### Materials and Methods

Ten *Phaseolus vulgaris* genotypes from Andean and Mesoamerican genepools (wild and cultivated) were used for the validation of SNP markers derived from soybean by Cregan and Quigley (USDA). *Phaseolus* SNPs were discovered and validated by Gaitán as previously reported (Gaitán & Tohme, 2002; Gaitán *et al.,* 2004).

A population of $F_9$ plants from 87 RILs developed at CIAT from the cross between G19833 (Andean genepool) and DOR364 (Mesoamerican genepool) was genotyped with the validated SNPs, which were added to the existing linkage map (Beebe *et al.*, 1998) using MAPMAKER/EXP (version 3.0) (Lander *et al.*, 1987). The framework map had 345 markers including 237 RAPDs, 51 RFLPs, 34 AFLPs, 8 SCARs, 7 SSRs and 8 RGAs, distributed across the eleven linkage groups.

Once the position of the SNPs was known by linkage mapping, haplotype block partition was done using the HAP algorithm (Halperin & Eskin 2004). Association between individual SNPs and haplotypes with disease response was evaluated with a $\chi^2$ independence test in an RxC table ($P<0.01$) through the FREQ procedure (SAS v.8.2; SAS Institute, 1989). The following hypothesis was tested: There is no association between SNPs and the response to isolates of angular leaf spot and anthracnose.

## Results

SNP Validation

Microsphere fluorescence was measured using a Luminex$^{100}$ flow cytometer equipped with a Luminex XY Platform plate reader. Allele calling was done using Masterplex GT software (MiraiBio, Inc).

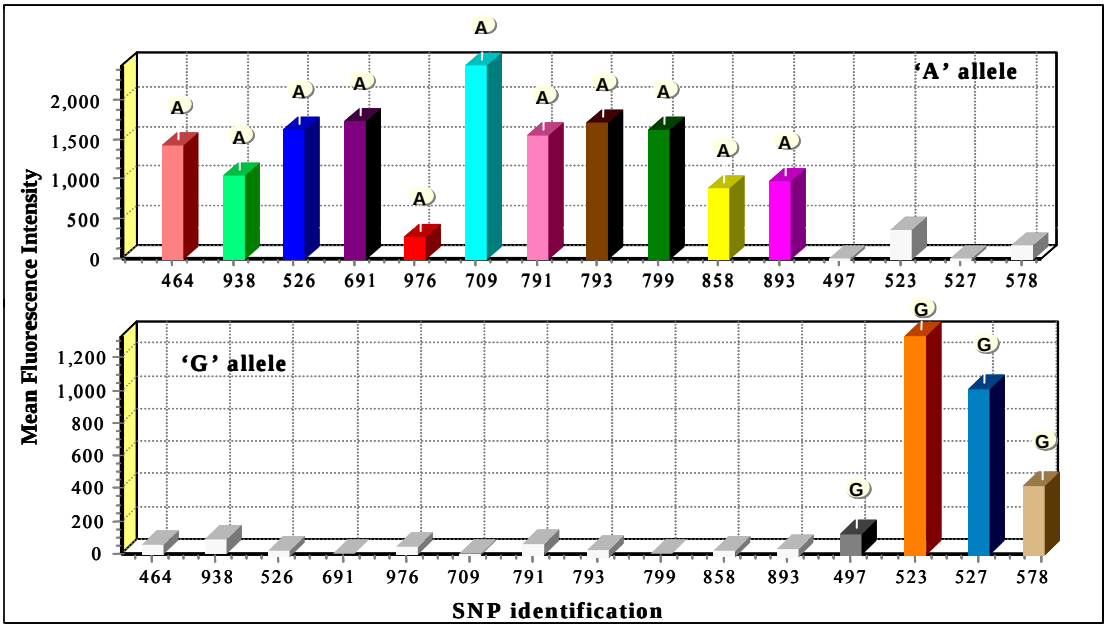


Figure 1. Simultaneous genotyping of 15 SNP alleles in the bean variety DOR 364.

Improvement of multiple detection of SNPs, compared to last year results (Quintero *et al.*, 2005) was observed and then simultaneous genotyping up to 15 alleles in one single SBE reaction was achieved (Figure 1).

Only one of the SNP markers was monomorphic and heterozygote individuals were easily and accurately identified. Polymorphism information content (Botstein *et al.*, 1980) was calculated for the 166 validated SNPs using PowerMarker software (Liu, 2005). 84% of the validated SNPs had PIC > 0.3 which is considered to be high for these biallelic markers since the highest achievable value is 0.5 (Table 1).

**Table 1.** Polymorphism information content (PIC) calculated from 10 *P. vulgaris* genotypes.

| PIC value | **Number of SNPs[1]** |
|---|---|
| 0.16 | 5 |
| 0.18 | 1 |
| 0.19 | 1 |
| 0.22 | 1 |
| 0.27 | 17 |
| 0.29 | 2 |
| 0.30 | 2 |
| 0.33 | 26 |
| 0.34 | 1 |
| 0.35 | 8 |
| 0.36 | 37 |
| 0.37 | 19 |
| 0.38 | 46 |

[1]Number of SNPs that reached this PIC value.

**SNP mapping**

Segregation data for 155 SNPs in 87 RILs of the DOR364/G19833 mapping population were obtained. 136 SNPs were added to the existing linkage map at a minimum LOD score of 4.0.

The resulting map has 470 markers and a total cumulative length of 1980cM. Each of the eleven *P. vulgaris* linkage groups had at least six SNP markers attached and B02 and B03 had the highest number of SNPs placed on them (n=23). The order of the markers inside linkage groups was consistent with Blair *et al*. (2003) and Beebe *et al*. (2006) who worked with the same population.

In almost all linkage groups, SNPs covered regions (Figure 2) that were not previously covered by SSRs, after comparison of our map with the one published by Blair *et al*. (2003). The number of these regions might be underestimated but this remains unknown since there is still no access to the segregation data of the mapped SSRs.

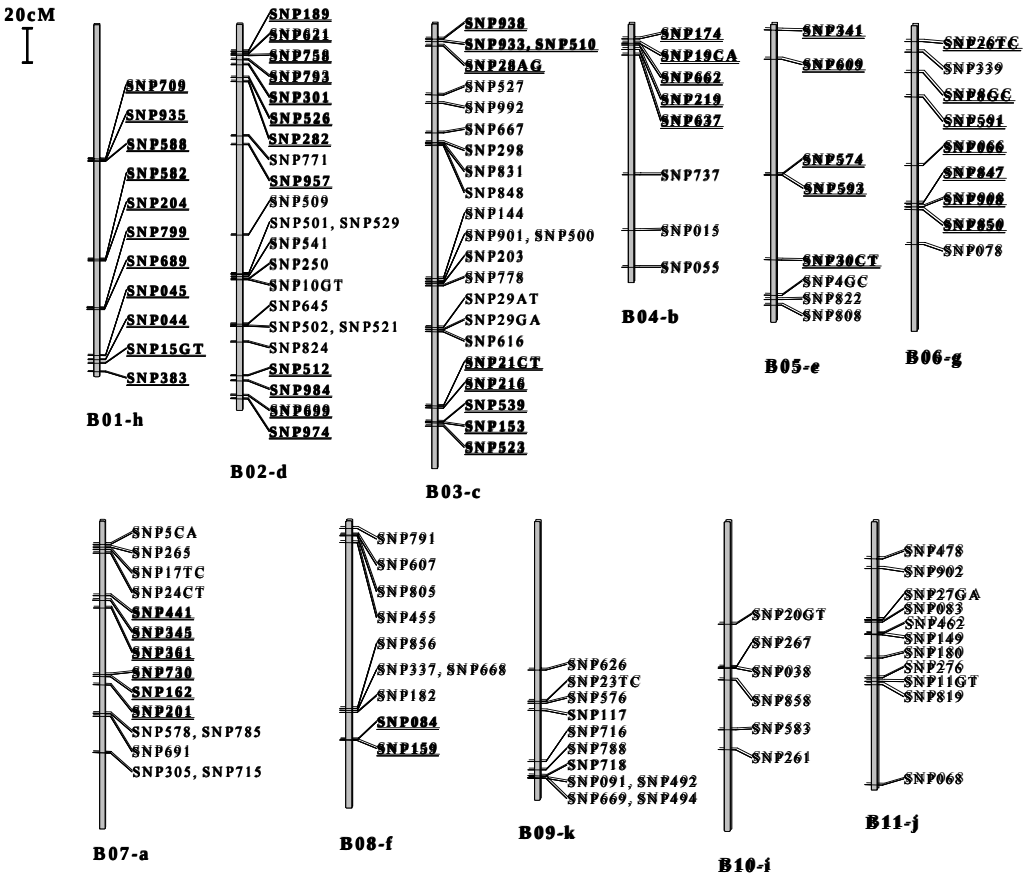


**Figure 2.** Integration of SNP markers to the linkage map of the cross between DOR364 and G19833. Bolded and underlined SNPs are covering regions still not reached by SSRs.

SNP block partition and haplotype prediction inside blocks was done for each linkage group with the HAP software (Halperin & Eskin, 2004). Blocks contained from two to seven SNPs and several haplotype variants were observed (between 4 and 14).

Chi square tests in a RxC table was conducted for individual SNPs and haplotypes to test their association with resistance to isolates of angular leaf spot (3COL, 260COL, 30CRI, 12MEX) and of anthracnose (5DOM, 20COL, 43COL, 77CRI), the same data used by López *et al.* (2003). Unfortunately, no significant association between phenotypes and SNPs was encountered.

**On-going activities**

To design PCR and SBE primers for 100 SNP-containing sequences found by Ramirez *et al*, (2005).

Validate the SNPs mentioned above and map those that are polymorphic for the parents of the population.

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### 1.2.2 Mutagenesis and generation of a TILLING population for common beans

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**Introduction**

This project has initiated the development of TILLING (Targeting Induced Local Lesions in Genomes) mutant resources for common bean given that fewer mutant populations have been created for grain legumes compared to those for Arabidopsis, forage legumes (Lotus Principally) and the cereals. TILLING mutant collection are both forward and reverse genetics tools that can be used to either find new phenotypic mutants or to screen for mutations in candidate genes through a PCR approach based on pools of total DNA from mutated individuals. A comprehensive mutant population is a basis for exploring gene function. For this reason, an important task is handling the phenotypic information. TILLING databases for mutant populations have been developed in Soybean and Lotus. Our objective is to develop a similar resource for common beans. So far, the mutant population of common bean has been advanced in the field to the M3 generation. Traits recorded show the differences between the mutants and wild-type plants of BAT93 and form the basis for gene discovery through reverse and forward genetics approaches. On the other hand, we have evaluated chemical mutagenesis with EMS (ethyl metanesulfanate) to find the ideal concentration to increase our mutant population under CIAT greenhouse conditions.

**Materials and Methods**

*Population Advance:* We have continued the advance of the populations in the field keeping the same methodology as in the greenhouse (six or eight plants per M1 family in greenhouse and six or eight rows per M2 and M3 family in the field). To create a large seed source for further experimentation, bulk seeds were collected from M2 and M3 families.

*TILLING database:* Data was collected in the greenhouse and in the field. The traits were grouped in phenotypic categories describing plant morphology at various developmental stages from germination to harvest (seed, plant size, leaf morphology, leaf color, grown habit, flower morphology, harvest and others). This information has been entered into the database using Microsoft® Visual Basic for Excel.

*EMS experiment:* We have evaluated five concentrations of EMS (0, 0.2, 0.5, 0.8 y 1.1%). Each treatment consisted of 200 seeds following a protocol used in barley (Caldwell et al; 2004). The seeds mutated were sown in the greenhouse and germination was evaluated.

**Result and discussions**

*Field testing and generation advance:* The population advance was made following the schema that we established for TILLING in common bean (Figure 1). The M2 and M3 generations were sown in semester 2006A. In the field trial for each of these generations, we have seen that traits like leaf color, dwarfism, semi-drawfism and late maturity are stable mutations in the M2 and M3 generations while leaf morphology mutations observed in the M1 generation are generally not stable (Figure 2). Similar results were obtained in tomato where in some M3 generations the M2 phenotype was not preserved (Menda et al; 2004). We have also found that in M2 and M3 generations some segregation for the mutated trait occurs both in between rows (families) and within rows (Figure 2a). This suggests that we need to

advance the M2 and M3 families to fix the targeted traits. In summary, we have evaluated 514 families, 3152 M1 plants, 1807 M2 rows and 473 M3 rows so far. Furthermore, we have extracted DNA for genotypic screening from at least 2 individuals per family.

*TILLING database:* To allow a simple searchable interface for our database, a catalog with categories from germination to harvest was developed. The categories were selected to allow characterization of a mutant phenotype for observable traits that could be rapidly evaluated in the field on a large scale over a relative short period of time, following the methodology of Menda et al. (2004). The input and outputs of the database are shown in the Figure 3. The input matrix allowed us to save genotypic and phenotypic information along with photographs and data along with the possibility of adding additional information (Figure 3a). The current family option shows the information for each of the families in each generation with the corresponding photos and observations (Figure 3c).

*EMS experiment:* In general, germination of the EMS treated seeds occurred later than seeds not treated with EMS (Figure 4a, 4b). In addition, germination was reduced with increases in EMS concentration (Table 1) indicating that common bean is susceptible to high concentration of EMS such as 0.5, 0.8 and 1.1%. Similar results were obtained in beans previously where it was the best concentrations were between 0.05 and 0.15 % (Yanhulov et al; 1980). Other research in the crop showed that concentrations of 0.25% produced germination of 75 % and high mutation frequency (Barbosa et al; 1988). Treated plants showed morphological differences in comparison to control (Figure 4c) that suggest that $M_0$ plants can be chimeric (Till et al; 2003)

**Conclusions and future work**

Mutant stocks in common bean will allow researchers to conduct both forward (systematic phenotypic screening) and reverse genetics (TILLING) experiments aimed at understanding the genes involved in abiotic and biotic stress tolerance, as well as those genes involved in biological nitrogen fixation. Mutations will be sought in common bean genes that have been isolated at CIAT and shown to be associated with drought tolerance (eg. DREB transcprition factors). The phenotypic effect of these mutations will be analyzed as a proof of concept for the value of the mutant stocks generated by this project. The relevance of TILLING is high given the difficulty of genetic transformation of common bean and the impossibility of establishing T-DNA or heterologous transposon-based mutagenesis.

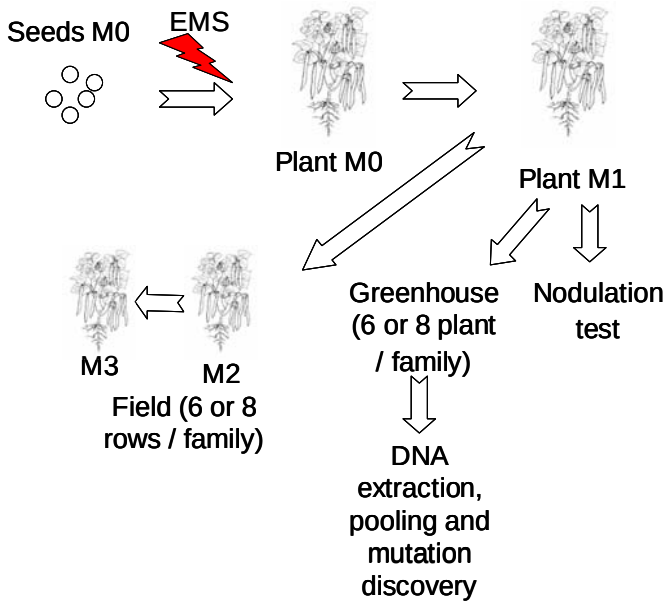


Figure 1. TILLING population scheme. BAT93 seeds are mutagenized with EMS. the $M_0$ generations is self-pollinated, and M1 progeny are used for DNA screening. M2 and M3 generations are grown in the field.


Figure 2. Examples of M2 generation plants under field conditions: a. mutant leaf coloration; b. dwarf plants; c. stem etiolation.

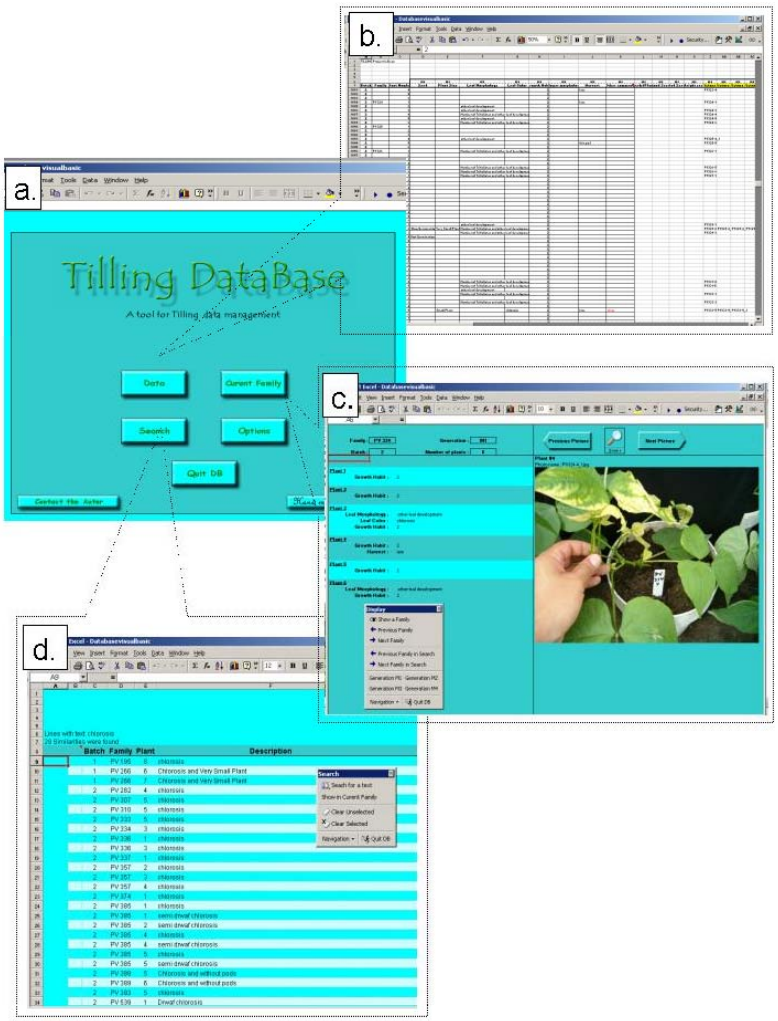


Figure 3. Database developed for TILLING in bean. a) main menu; b) input matrix for phenotypic data; c) current family information; d) search tool.

Table 1. Effect of EMS concentration on germination frequency in common bean.

Table 1. Germinatior

| Treatment | Concetration EMS % | Germination % | % of control |
|---|---|---|---|
| 1 | 0 | 94 | 100.00 |
| 2 | 0.2 | 65 | 69.15 |
| 3 | 0.5 | 1.5 | 1.60 |
| 4 | 0.8 | 0.4 | 0.43 |
| 5 | 1.1 | 2.5 | 2.66 |

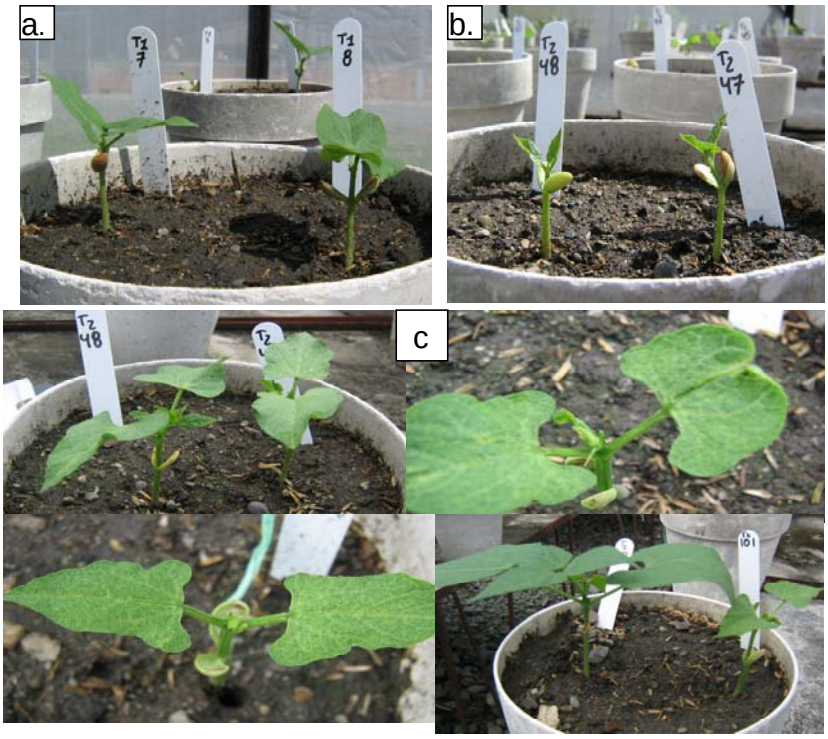


Figure 4. Plants shown seven days after EMS treatment of germinating seedlings. a) control plants with 0% EMS; b) 0.2% de EMS; c) examples of mutant genotypes from 0.2 % EMS.

### 1.2.3 Marker assisted selection of anthracnose resistance in Andean red seeded beans

MW Blair, HF Buendia (IP-1/SB-2 Project, CIAT), G. Ligareto (Univ. Nacional – Bogotá)

**Introduction**

Anthracnose, caused by the fungal pathogen *Colletotrichum lindemuthianum*, is a serious biotic constraint on common bean (*Phaseolus vulgaris*) in many areas of East Africa, Central America and South America. A set of differentials and the mapping population BAT93 x Jalo EEP558 have been used to identify over a dozen genes and QTLs affecting resistance/ susceptibility reactions of different races of the pathogen to different common bean genotypes. Co–evolution is known to have occurred between fungal pathotypes and the two gene pools found in common bean so that Andean races attack Andean genotypes and Mesoamerican races attack Mesoamerican genotypes, while Andean genotypes resist Mesoamerican races and Mesoamerican genotypes resist Andean races. Therefore the best sources of resistance for breeding programs is often found in the complementary gene pool and genes for resistance must be introgressed through wide crosses and recurrent selection or backcrossing between Andean and Mesoamerican genotypes. Our objective in this study was to introgress resistance from the Mesoamerican genotypes G2333 into red-seeded Andean climbing beans.

**Materials and Methods**

DNA was extracted from leaf tissue taken from field grown plants in the breeding nurseries in Darien and Popayan during 2006A. Bulk harvests were made per row consisting of four leaf disks collected together in the same cell of an ELISA plate. Each leaf disk was harvested from a separate plant within the row to evaluate whether the family was segregating or not for the resistance genes. The tissue samples were harvested in the morning and kept on ice during and after sampling. Once back in the laboratory, the tissue samples were stored at – 20 °C until proceeding with Alkaline Extraction (a high-throughput "microprep", 96-well format method based on alkaline lysis of fresh leaf tissue disks). The extract was diluted 1:10 and stored frozen until PCR amplification with the SAS13 and SAB3 markers where 5 µl of the dilution was used in 15 µl reaction volumes. The SAS13 marker is diagnostic for *Co-4*$^2$ (Young et al., 1998) while the SAB3 markers is diagnostic for *Co-5* (Vallejo and Kelly, 2000) and both have been used in our laboratory for the testing of G2333 derived genotypes. After PCR amplification 5 µl of blue juice was added per well and the samples were electrophoresed in 1.5 % agarose gels in 0.5X TBE buffer using 30 well combs and multiple loading for a total of three separate "tracks" spaced at 5 minute intervals and then run for a total of 45 minutes. A total of 470 genotypes were evaluated of which 188 were F5 derived families grown in Popayán and 282 were F2 derived families grown in Darién. The first group was derived from pedigree selection of simple crosses made with BRC lines which are derived from G2333 and which contain the SAS13 and SAB3 markers while the second group was derived from gamete selection of triple and double crosses made with either the BRC lines or G2333 which was the original source for the markers when they were developed.

**Results and Discussion**

Although we used both the SAB3 marker and the SAS13 marker on the collected samples, only the SAB3 marker amplified reliably for the alkaline extraction DNA from adult field grown plants. This differs from previous results we had where SAS13 showed some amplification at the 1:10 dilution and even better amplification at 1:20 dilution. The different results may have been due to the older stage at which the plants were sampled in this semester compared to the previous semester or to the fact that bulk tissues were harvested per row. In addition since we were trying to amplify both markers with the same dilution

we did not try the 1:20 or higher dilutions that may have worked with SAS13, but rather proceeded with the SAB3 marker directly on all samples. The results of the SAB3 marker amplification are summarized in Tables 1 and 2. For pedigree selection in Popayán the total number of positive selections was 81 and the total number of negative selections was 107. For gamete selection in Darien there were 137 positive and 145 negatives. In both groups of genotypes the observed ratios roughly fit the expected ratios given the generation and types of crosses employed.

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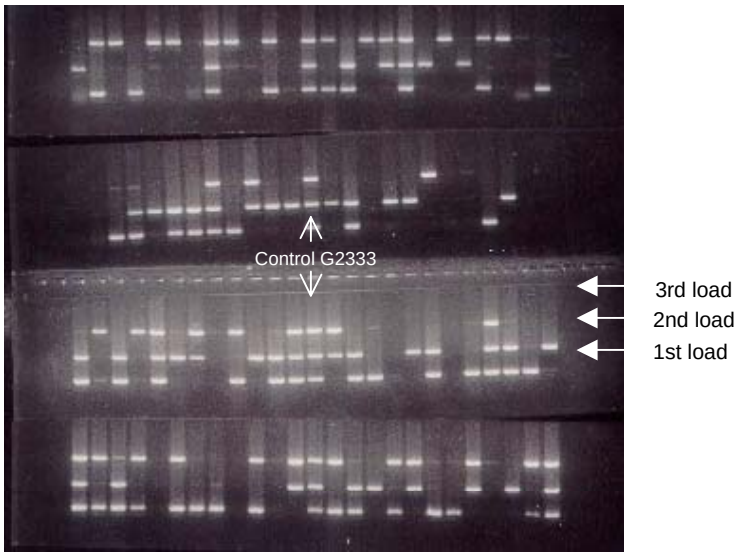
Table 1. SAB3 evaluation of F5 derived families from pedigree selection grown in Popayán in 2006A.

| | No. of families | | |
|---|---|---|---|
| Pedigree | Negative | Positive | Total |
| BOLIVAR x BRC 12 | 13 | 3 | 16 |
| BOLIVAR x BRC 14 | 11 | 1 | 12 |
| BOLIVAR x BRC 31 | 18 | 0 | 18 |
| BRC 3 x DAR 5068 | 0 | 5 | 5 |
| BRC 3 x G 16881 | 1 | 0 | 1 |
| BRC 3 x G 22041 | 0 | 21 | 21 |
| BRC 3 x MAC 56 | 0 | 2 | 2 |
| BRC 3 x MICHOACAN | 1 | 14 | 15 |
| BRC 3 x Q'OSQOPOROTO | 0 | 1 | 1 |
| BRC 32 x BOLIVAR | 23 | 0 | 23 |
| BRC 32 x G 2333 | 5 | 3 | 8 |
| G 2333 x BRB 153 | 7 | 5 | 12 |
| G 2333 x BRB 181 | 2 | 4 | 6 |
| G 2333 x BRB 189 | 0 | 10 | 10 |
| G 2333 x BRB 211 | 4 | 11 | 15 |
| LAS 399 X ( LAS 399 X BRC 12 ) | 11 | 1 | 12 |
| LAS 399 X ( LAS 399 X BRC 14 ) | 1 | 0 | 1 |
| Grand Total | 107 | 81 | 188 |

Table 2. SAB3 evaluation of F2 derived families from gamete selection grown in Darién in 2006A.

| Pedigree | Negative | Positive | Total |
|---|---|---|---|
| (G 2333 X AGRARIO ) X MBC 34 | | 1 | 1 |
| (G 2333 X AGRARIO )X MBC 28 | 1 | 2 | 3 |
| (G 2333 X AGRARIO )X MBC 33 | | 5 | 5 |
| (G 2333 X BOLIVAR ) X G 23823E | 3 | 7 | 10 |
| (G 2333 X MAC 47) X G 23823E | 12 | 9 | 21 |
| (G 21242 X MBC 26) X (G 2333 X BOLIVAR ) | 9 | 5 | 14 |
| (G 21242 X MBC 26) X (G 2333 X MAC 47) | 3 | 10 | 13 |
| (G 21242 X MBC 28) X (G 2333 X BOLIVAR ) | 10 | 14 | 24 |
| (G 21242 X MBC 28) X (G 2333 X MAC 47) | 4 | 3 | 7 |
| (G 23823E X MBC 28) X (G 2333 X BOLIVAR ) | | 4 | 4 |
| (G 23823E X MBC 28) X (G 2333 X MAC 47) | 2 | 3 | 5 |
| (G 23823E X MBC 34) X (G 2333 X BOLIVAR ) | 6 | 8 | 14 |
| (G 23823E X MBC 34) X (G 2333 X MAC 47) | 4 | 4 | 8 |
| G 14519 X (G 2333 X BOLIVAR ) | 4 | 3 | 7 |
| G 14519 X (G 2333 X MAC 47) | 5 | 1 | 6 |
| G 21242 X (G 2333 X BOLIVAR ) | 4 | 3 | 7 |
| G 21242 X (G 2333 X MAC 47) | 2 | 3 | 5 |
| MBC 26 X (G 2333 X AGRARIO ) | 10 | 3 | 13 |
| MBC 26 X (G 2333 X CARGAMANTO ) | 7 | 2 | 9 |
| MBC 28 X (G 2333 X AGRARIO ) | 14 | 6 | 20 |
| MBC 28 X (G 2333 X D.MORENO ) | 8 | 3 | 11 |
| MBC 33 X (G 2333 X CARGAMANTO ) | 6 | 3 | 9 |
| MBC 33 X (G 2333 X D.MORENO ) | 6 | 7 | 13 |
| MBC 33 X (G 2333 X AGRARIO ) | 11 | 6 | 17 |
| MBC 34 X (G 2333 X AGRARIO ) | 4 | 9 | 13 |
| MBC 34 X (G 2333 X CARGAMANTO ) | 6 | 8 | 14 |
| MBC 34 X (G 2333 X D.MORENO ) | 4 | 5 | 9 |
| Grand Total | 145 | 137 | 282 |

Figure 1. Multiple loading on agarose gels of SCAR marker SAB3 for anthracnose resistance.



## 1.2.4 Genetic mapping of the bean golden yellow mosaic geminivirus resistance gene *bgm*-1 and synteny with potyvirus resistance in common bean

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### Introduction

Bean golden yellow mosaic virus (BGYMV) is a whitefly-transmitted geminivirus of the Begomovirus family that causes important yield losses to common beans grown in tropical and sub-tropical countries of Latin America and the Caribbean. The disease is devastating in bean production areas below 1000 masl in many countries especially Dominican Republic, Guatemala, El Salvador, Haiti, Honduras and Nicaragua. BGYMV is also prevalent in parts of Costa Rica and southern Mexico and has been reported in Florida. Yield losses can be from 45 to 100% during epidemics and the disease continues to expand its range into new seasons and environments at higher altitudes and latitudes. A major resistance gene that has been widely deployed in this region is the recessive locus *bgm*-1 that prevents the development of severe yellowing typical of the disease. In this study, our goal was to genetically map a sequence characterized amplified region (SCAR) marker that is tightly linked to the *bgm*-1 resistance gene through comparative mapping using two genetic maps for the species. In addition we sought to map the SR2 marker relative to *bgm*-1 in a segregating population of recombinant inbred lines developed from the resistant x susceptible cross of DOR476 x SEL1309.

### Materials and Methods

*Population Development:* A recombinant inbred line (RIL) population was developed from the cross of SEL1309 x DOR476 through single seed descent from the F2 generation until the F5 generation. Subsequently the harvests for each of the 100 RILs were bulked until the F7 generation. The source of BGYMV resistance in the cross came from the paternal parent, DOR476 that contains the *bgm*-1 resistance gene and the associated marker alleles derived from the A429 source, while the maternal parent SEL1309 is susceptible to BGYMV. DOR476 has small red seed while SEL1309 has reddish-brown colored seed but both are of the Mesoamerican genepool and have type II growth habits. The population was tested in the greenhouse at CIAT to identify the most BGYMV resistant and susceptible progeny for combining into DNA bulks. The greenhouse experiment was mechanically inoculated with a BGYMV–Guatemala strain maintained in a susceptible genotype ('Topcrop') grown in whitefly–proof cages in the Virology Unit of CIAT. Ten plants were evaluated per treatment and the RILs were evaluated for overall symptom score and four separate characteristics (chlorosis, dwarfing, flower abortion and pod deformation). In both cases a 1 to 9 scale was used where 1 was equivalent to resistant and 9 to susceptible. DNA was extracted from the DOR476 x SEL1309 RILs by a standard method and quantified with a Hoefer DyNA Quant 2000 fluorometer for dilution to a standard concentration of 10 ng/ul for marker amplification. DNAs from two other RIL mapping populations, DOR364 x G19833 and BAT93 x Jalo EEP558 as described in Blair et al. (2003) were used for marker locus placement.

*SCAR marker development:* SCAR development was carried out based on the RAPD band (R2) identified by Urrea et al. (1996). Briefly, amplification of the RAPD fragment was carried out in 25 volume reactions with the same reaction components as in this previous study. The polymorphic bands associated with resistance and susceptibility from this previous study were cut out of 1.5% low melting point agarose gels and cleaned individually with the Wizard PCR prep purification system (Promega, Madison, WI). The purified insert DNAs were cloned into the pPCR-Script Amp SK(+) plasmid vector (Stratagene, La Jolla, CA) using T4 DNA ligase (Promega) in a reaction at 20ºC for 1 hr followed by 16 hr incubation at 4 ºC. The ligation reaction was transformed by heat shock into XL1-Blue MRF´ host cells and the bacteria were plated onto selective media. Several recombinant clones were picked per ligation reaction and their DNA extracted with a standard miniprep protocol. These minipreps were checked for insert size with PCR

amplification and *Sac*I and *Eco*RI digestions. Clones having the expected fragments were selected and end sequenced with standard techniques, T7 and T3 primers using the Sequenase™ Version 2.0 DNA Sequencing Kit. Sequences were compared and searched for inverted repeats and SNP polymorphisms using the software Sequencher v.4.1 or DNAman and analyzed for sequence homology to each other and to NCBI database using BLAST. Specific primers were designed from the fragment ends and at an internal location using Primer 3.0 software and were tested for ability to amplify single-copy SCAR products as described below.

*SCAR testing:* PCR reactions for the SCAR markers were carried out in 25 µl reaction volumes containing 50 ng of genomic DNA, 0.2 uM each of forward and reverse primers, 20 mM of total dNTP and 1 unit of Taq polymerase in 1X PCR buffer. Final MgCl2 concentrations of 1.5 to 2.5 mM were tested by adding this component separately to the PCR reaction mix. After amplification, 12 µl of the PCR products were combined with 5 µl of loading buffer (30% glycerol; 0.25% bromphenol blue dye) and run on 1.5% agarose gels in 0.5X TBE buffer and visualized on a ultraviolet transiluminator. The new SCAR was tested for amplification on a panel of genotypes representing parents of the test population described above and mapping populations used for marker placement: DOR476, SEL1309, DOR364, G19833, BAT93, Jalo EEP558. Segregation for the SCAR was evaluated on the entire population of 100 individual RILs from the DOR476 x SEL1309 population where the marker was co-dominant. For the other two populations polymorphism was uncovered by CAPS marker analysis as described below.

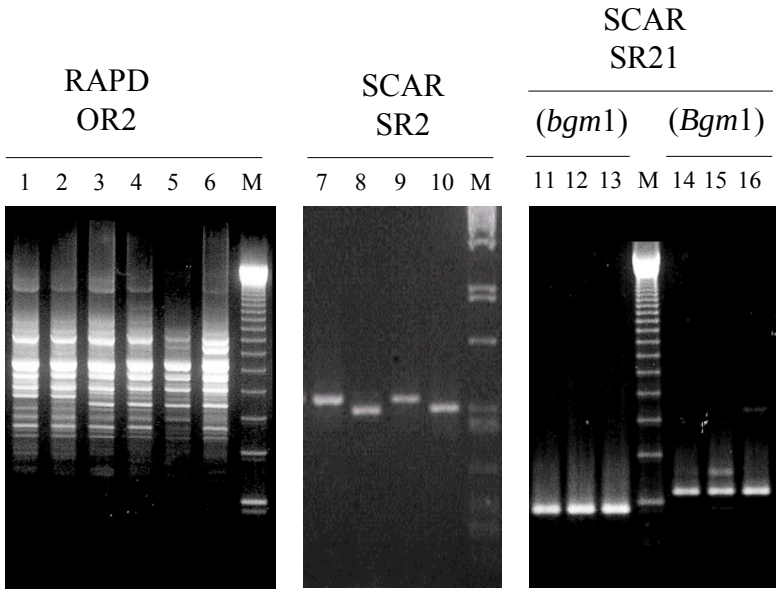
*CAPS marker development and testing:* A restriction analysis was carried out on the sequenced fragment from above using the software DNAman (Lynnon Biosoft, Inc.) and nine restriction enzymes (AluI, BamHI, DpnI, EcoRI, HaeIII, HinfI, MseI, RsaI and TaqI). These enzymes were then used to digest 12.5 µl of the SCAR product with 5 units of each enzyme in separate 20 µl reaction volumes for periods of 4 hours at the recommended temperatures for each enzyme. The restriction fragment patterns were analyzed on 2% agarose gels run in 0.25X TBE using the undigested PCR product as a control reaction as described above. Polymorphisms were scored between the parents described above and segregation of the CAPS marker was tested on 87 individuals of the DOR364 x G19833 and 78 individuals of the BAT93 x JaloEEP558 mapping populations from Blair et al. (2003) and Freyre et al. (1998), respectively.

*Data Analysis:* Genotypic data for the SCAR and CAPS markers were used for linkage analysis in the DOR476 x SEL1309 population and in two additional populations for which genetic maps have been constructed, DOR364 x G19833 (Blair et al. 2003) and BAT93 x JaloEEP558 (Freyre et al. 1998). All genetic mapping was conducted using the software Mapmaker 2.0 for Macintosh and a minimum LOD of 3 under the 'assign' command. Segregation distortion was evaluated with chi-square tests and marker-trait associations in the DOR476 x SEL1309 population were determined with analysis of variance and F tests with the software program qGENE. Based on this last analysis, F-test values are reported for the significant marker trait associations with a probability threshold of $P>0.001$.

**Results and Discussion**

The RAPD band was successfully converted into two SCAR markers named SR2 and SR21 (Figure 1). Polymorphism of the SR2 marker was shown to be based on a 37 bp insertion event in the allele associated with susceptibility compared to the allele associated with resistance. The SR2 marker was significantly associated with overall disease symptoms and with three of the four symptoms associated with the disease (yellowing or chlorosis, flower abortion, pod deformation) in a greenhouse trial in Colombia with the mechanically transmissible BGYMV–Guatemala strain and the mapped at a distance of 7.8 cM from the resistance gene *bgm*-1 based on the chlorosis score. The SR2 marker was successfully converted into a polymorphic CAPS based marker for mapping in both the DOR364 x G19833 and BAT93 x Jalo EEP558 mapping populations (Figure 2). In this mapping excercise, SR2 was located near the end of linkage group b03 (chromosome 5) suggesting a sub-telomeric position. Interestingly, the

position of the *bgm*-1 resistance gene was syntenic with that of *bc*-1, a strain-specific resistance gene for bean common mosaic virus (BCMV), based on linkage of SR2 with the SCAR marker SBD5 in the DOR364 x G19833 mapping population. It is fascinating to us that there is synteny between these two




recessive resistance genes as this may suggest that there is an association between resistance genes for both begomovirus and potyvirus pathogens.

Figure 1. Conversion of RAPD marker OR2 (left panel) to SCAR markers SR2 (middle panel) and SR21 (right panel). Lane genotypes are: Susceptible RIL bulk (1, 9 and 15), SEL1309 (2, 7 and 14), DOR476 (3, 8 and 13), A429 (breeding line source of bgm-1) (4 and 12), G24404 (Garrapato – landrace source of bgm-1) (5 and 11), Resistant RIL bulk (6 and 10) and DOR364 (negative control) (16). Lanes labeled as M are molecular weight standards (100 bp ladder for OR2 and SR21; lambda PstI for SR2).

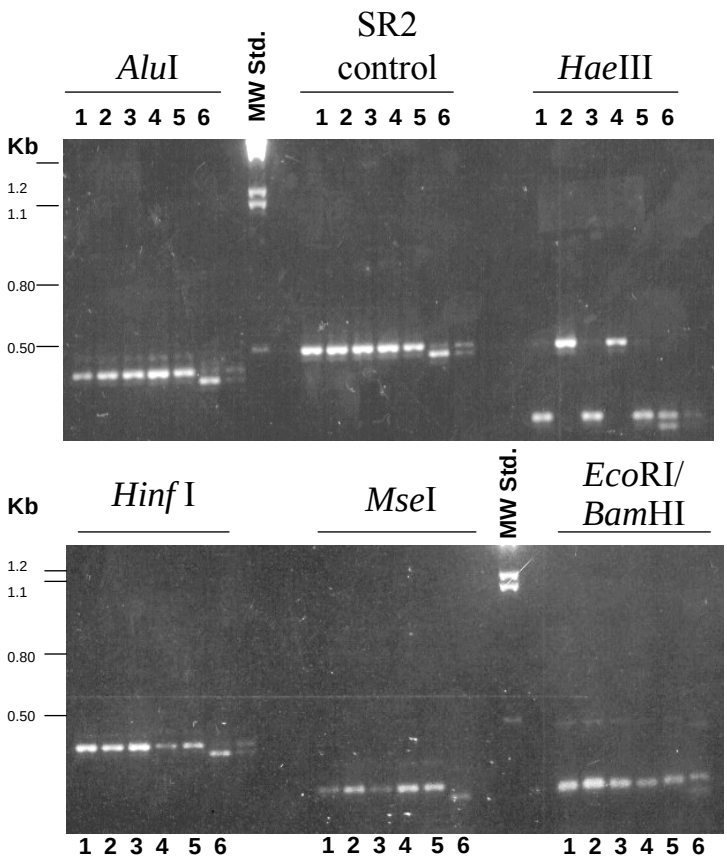



Figure 2. Development of CAPS marker for SR2 using five restriction enzyme based on 4 or 5 bp recognition sites (*Alu*I, *Dpn*I, *Hae*III, *Hinf*I and *Mse*I) and a double digestion with two restriction enzymes with 6pb recognition sites (*Eco*RI and *Bam*HI). Parental survey represented by genotypes DOR 364 (lane 1), G19833 (lane 2), BAT93 (lane 3), JaloEEP558 (lane 4), SEL 1309 (lane 5), DOR 476 (lane 6).

## 1.2.5 Generation means analysis of climbing ability in common bean (*Phaseolus vulgaris* L.)

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### Introduction

Climbing common bean (*Phaseolus vulgaris* L.) genotypes have among the highest yield potential of all accessions found in the species. Climbing bean varieties are morphologically distinct from bush bean varieties of common beans characterized by tall growth, long internodes and climbing ability. They are an important component of traditional agriculture in several parts of Latin America, especially Mexico, Guatemala, Colombia, Ecuador, and Peru and have spread to the Great Lakes region of Africa. Climbing bean cropping systems are classified as monoculture or inter-cropping, whereby farmers produce two or more species in the same area. Climbing beans are often grown in association with maize, either in relay or simultaneous plantings, and maize provides the support required for the climbing beans to grow upwards. In monoculture, climbing beans are planted with the support of wood or bamboo stakes or trellis systems. Trellising, a widespread system in the Andean region, is an alternative that reduces the need for stakes, but requires an investment in wires, string and labor for tying up bean vines. Trellising of climbing beans is economically justified because yield may surpass 4500 kg ha-1. Therefore, climbing beans are particularly useful for small landholdings in situations where labor is not limiting and where demand for beans is high. Genetic improvement of climbing beans would benefit from an understanding of the inheritance of climbing capacity (made up of plant height and internode length traits). Therefore, the objective of this study was to determine the inheritance of climbing capacity traits in three crosses made within and between gene pools using generation means analysis.

### Materials and Methods

*Plant Material:* Three populations were developed, both within and between gene pools i.e. Andean × Andean (BRB32 × MAC47), Mesoamerican × Mesoamerican (Tío Canela × G2333), and Mesoamerican × Andean (G2333 × G19839). Each cross combination contrasted for growth habit but not for indeterminacy with either a type II (BRB32, Tio Canela) or type III (G19839) parent crossed with a type IV (MAC47 or G2333) parent. A total of 50 pollinations were made per cross to generated F1s and 40 pollinations per backcross of the F1 hybrids with their respective parents to create the BC1P1 and BC1P2 generations. Crosses were confirmed to be hybrids based on flower and seed color and F1 plants were used to obtain seed for the F2 generation.

*Experimental Design:* For each population, we used six generations (P1, P2, F1, F2, BC1P1 and BC1P2) in a randomized complete block design experiment with three replications each planted in Darién, Colombia (elevation 1450 masl, yearly rainfall 1288 mm, average yearly temperature 20ºC). The experiments were planted as bean monocultures using trellis systems in which each individual plant was tied with a string made of polypropylene to a heavy weight wire that was suspended horizontally and in parallel above the row at a height of 2 m above soil level on sturdy wooden bamboo posts that were placed every 5 m and at every sixth row. Seed was planted two to a planting hole at distances of 0.2 m between planting holes within rows and 1.0 m between rows. Plantlets were thinned to one plant per planting hole when the seedlings reached a height of 10 cm.

*Traits Evaluated:* Each plant was evaluated for plant height (PH), internode length (IL), and number of branches (BN). Plant height was measured in meters from the insertion of the plant stem at the ground level to the last trifoliate leaf axis along the main stem. Internode length was evaluated by selecting an internode from the mid point along the main stem of the plant and recording its length in centimeters.

Similarly, the number of branches was evaluated by counting the number of stems that climbed around the guide string. PH, IL and GS were evaluated twice during the growing season on an individual plant basis, first at 40 and then at 70 days after planting (DAP). In the evaluations, those plants at the end of each row were not considered to avoid border effects.

*Data Analysis:* Analysis of variance for each of the three populations was conducted separately. Branch number was transformed for normalization with square-root of x. In those variables for which the analysis of variance showed significant differences between generations, separation of means was carried out with Tukey's w procedure for multiple comparisons ($P \leq 0.05$). Those variables that showed significant differences by orthogonal contrasts between parents P1 and P2, were submitted to generation means analysis using the methodology proposed by Mather and Jinks (1971): $gk = m + (\alpha k)a + (\delta k)d + (\alpha k2)aa + (\alpha k \delta k)ad + (\delta k)2dd$; where, gk = mean of generation k; m = mean of the parental homozygotes; αk, δk = coefficients determined by the degree of relationship of generation k; a = additive genetic effects; d = dominant genetic effects; aa = epistatic effects of additive × additive type; ad = epistatic effects of additive × dominant type; dd = epistatic effects of dominant × dominant type. To estimate the additive and dominant parameters a stepwise linear regression analysis was carried out using the statistics package SAS. Regression analysis was weighted based on the inverse of the variance of means and the matrix of parameters or coefficient of genetic effects. R2 and the "goodness of fit" (F test), were used to determine which parameters were acceptable within the model. In addition to generation means analysis, we estimated the broad and narrow sense heritabilities for each population x trait combination.

**Results and Discussion**

Table 1 shows Tukey's multiple means comparison tests for the six treatments across the three populations. For the G2333 × G19839 population dominance was important in controlling both PH and IL and that this trend is more evident early in the season at 40 DAP rather than late in the season at 70 DAP. Means of the backcross generations were observed to be similar to the means of their respective recurrent parents which themselves were contrasting for both PH and IL. In the other two populations, the F1 and F2 treatments had means intermediate between the parents. Generation means analysis showed the importance of additive compared to the dominant-additive portion of the genetic model for all three population (Table 2). Broad sense heritabilities for the traits varied from 62.3 to 85.6% for plant height and from 66.5 to 83.7% for internode length (Table 3). Narrow-sense heritabilities calculated from additive and environmental variances were similar to broad sense heritabilities, with the highest values for G2333 × G19839 and BRB32 × MAC47 (66.9 and 65.4%, respectively) and the lowest for Tío Canela × G2333 (52.5%). The generation means analysis and estimates of heritability suggested that the inheritance of plant height and internode length in climbing beans is relatively simple and mostly additive although a dominant-additive model was also significant in the inter gene pool cross but not the intra gene pool crosses. It is interesting that dominance effects in the first population were less evident at flowering and became more notable as the plants developed into latter growth stages.

Table 1. Tukey's multiple means comparison for plant height and internode length evaluated at 40 and 70 DAP [a] in three populations.

| Treatment | Plant Height – 40 DAP | Plant Height – 70 DAP | Internode length – 40 DAP | Internode length – 70 DAP |
|---|---|---|---|---|
| G2333 x G19839 | | | | |
| P1 (G2333) | 1.893 A | 2.626 A | 21.38 A | 21.81 A |
| $BC_1P_1$ | 1.630 A | 2.806 A | 18.74 A | 19.89 A |
| $F_1$ | 1.710 A | 2.626 A | 18.42 A | 20.98 AB |
| $F_2$ | 1.173 B | 2.136 B | 14.25 B | 16.60 BC |
| $BC_1P_2$ | 0.74 C | 1.523 C | 9.75 C | 13.45 C |
| P2 (G19839) | 0.396 D | 0.803 D | 6.20 C | 8.07 D |
| Tio Canela x G2333 | | | | |
| P2 (G2333) | 1.743 A | 2.573 A | 19.95 A | 18.16 A |
| $BC_1P_2$ | 1.443 B | 2.503 A | 17.73 B | 17.93 A |
| $F_1$ | 1.150 C | 2.176 B | 16.50 B | 15.35 B |
| $F_2$ | 0.860 D | 1.693 C | 12.05 C | 13.52 B |
| $BC_1P_1$ | 0.506 E | 1.016 D | 7.68 D | 9.76 C |
| P1 (Tio Canela) | 0.173 F | 0.396 E | 3.03 E | 4.44 C |
| BRB32 x MAC47 | | | | |
| P2 (MAC47) | 1.650 A | 2.636 A | 22.01 A | 23.10 A |
| $BC_1P_2$ | 1.536 A | 2.360 AB | 19.20 AB | 20.01 A |
| $F_1$ | 1.233 B | 2.170 BC | 15.00 BC | 16.10 B |
| $F_2$ | 1.183 B | 1.936 C | 13.95 C | 14.72 BC |
| $BC_1P_1$ | 0.90 C | 1.576 D | 11.53 C | 12.77 C |
| P1 (BRB32) | 0.476 D | 0.83 E | 7.04 D | 7.49 D |

[a] Means followed by the same letter within each column not significantly different at P = 0.05.

**Table 2.** Sum of squares for the generation means analysis of additive (m [a]) and dominant-additive (m [a][d]) inheritance models of plant height and internode length evaluated at 40 and 70 DAP in three populations derived from crosses G2333 x G19839, Tio Canela x G2333 and BRB32 x MAC47.

| Model | Plant Height – 40 DAP | | | Plant Height – 70 DAP | | | Internode Length – 40 DAP | | | Internode Length – 70 DAP | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | SSq[2] Model | SSq Corr. Model | $R^2$ | SSq Model | SSq Corr. Model | $R^2$ | SSq Model | SSq Corr. Model | $R^2$ | SSq Model | SSq Corr. Model | $R^2$ |
| G2333 x G19839 | | | | | | | | | | | | |
| M | 6315.7 | | | 9995.4 | | | 8119.7 | | | 13927.0 | | |
| m [ a ] | 9376.3 | 3060.6 | 95.83 | 12796.7 | 2801.3 | 88.71 | 10010.6 | 1890.9 | 96.1 | 15420.3 | 1493.3 | 86.90 |
| m [ a ] [ d ] | 9433.7 | 3118.0 | 99.26 | 13136.2 | 3140.8 | 99.46 | 10071.7 | 1927.7 | 99.2 | 15624.6 | 1697.6 | 98.80 |
| **SSq Total** | 9509.5 | 3193.8 | | 13153.2 | 3157.8 | | 10087.4 | 1967.6 | | 15645.1 | 1718.1 | |
| Goodness of fit [d][a] | | | $2.27^{ns}$ | | | 59.98** | | | $7.02^{ns}$ | | | 29.91** |
| **Tio Canela X G2333** | | | | | | | | | | | | |
| M | 3355.9 | | | 7453.0 | | | 8359.8 | | | 8834.1 | | |
| m [ a ] | 6742.2 | 3386.4 | 97.75 | 11148.3 | 3695.3 | 93.67 | 11937.2 | 3577.4 | 91.86 | 11028.4 | 2194.3 | 93.44 |
| m [ a ] [ d ] | 6753.3 | 3397.5 | 99.74 | 11362.2 | 3909.2 | 99.35 | 12173.7 | 3813.9 | 97.94 | 11170.3 | 2336.3 | 99.49 |
| **SSq Total** | 6820.1 | 3464.2 | | 11397.9 | 3944.9 | | 12254.1 | 3894.2 | | 11182.3 | 2348.3 | |
| Goodness of fit [d] | | | $0.50^{ns}$ | | | 17.95* | | | $8.33^{ns}$ | | | 35.57** |
| **BRB32 X MAC47** | | | | | | | | | | | | |
| M | 6330.1 | | | 7766.8 | | | 7014.9 | | | 10274.0 | | |
| m [ a ] | 7735.7 | 1405.6 | 97.70 | 8842.2 | 1075.4 | 94.72 | 7990.0 | 975.1 | 99.29 | 11627.4 | 1353.4 | 98.50 |
| m [ a ] [ d ] | 7764.1 | 1433.9 | 99.67 | 8900.7 | 1133.9 | 99.87 | 7990.9 | 976.0 | 99.38 | 11631.4 | 1357.2 | 98.78 |
| **SSq Total** | 7768.7 | 1438.6 | | 8902.2 | 1135.4 | | 7997.0 | 982.1 | | 11647.9 | 1373.9 | |
| Goodness of fit [d] | | | 18.18* | | | 121.95** | | | $0.46^{ns}$ | | | $0.70^{ns}$ |

[a] Goodness of fit (calculated F statistic) with probability of P >0.05 (ns), <0.05 (**), <0.01 (**) 2/ SSq = Sum of squares, SSq = Sum of squares for corrected model, $R^2$ = determination coefficient

**Table 3.** Genetic variance and heritability of plant height and internode length evaluated at 40 and 70 DAP in three populations derived from crosses G2333 x G19839, Tio Canela x G2333 and BRB32 x MAC47.

| Parameter | Population | Plant Height 40 DAP | Plant Height 70 DAP | Internode Length 40 DAP | Internode Length 70 DAP |
|---|---|---|---|---|---|
| Genotypic Variance | G2333 x 19839 | 0.174 | 0.386 | 16.98 | 15.60 |
| | Tio Canela x G2333 | 0.085 | 0.334 | 10.83 | 13.00 |
| | BRB32x MAC47 | 0.170 | 0.519 | 36.58 | 13.73 |
| Additive Variance | G2333 x 19839 | 0.143 | 0.252 | 13.65 | 14.71 |
| | Tio Canela x G2333 | 0.071 | 0.330 | 9.33 | 12.70 |
| | BRB32x MAC47 | 0.142 | 0.480 | 36.17 | 10.87 |
| Environmental Variance | G2333 x 19839 | 0.0397 | 0.064 | 5.13 | 3.94 |
| | Tio Canela x G2333 | 0.051 | 0.080 | 4.93 | 5.24 |
| | BRB32x MAC47 | 0.047 | 0.119 | 7.19 | 6.90 |
| Broad Sense Heritability | G2333 x 19839 | 81.45 | 85.60 | 76.80 | 79.80 |
| | Tio Canela x G2333 | 62.32 | 80.64 | 68.72 | 71.24 |
| | BRB32x MAC47 | 78.30 | 81.30 | 83.56 | 66.54 |
| Narrow Sense Heritability | G2333 x 19839 | 66.95 | 56.03 | 61.71 | 75.26 |
| | Tio Canela x G2333 | 52.53 | 79.63 | 59.19 | 69.62 |
| | BRB32x MAC47 | 65.42 | 75.27 | 82.63 | 52.65 |

### 1.2.6 Analysis of condensed tannins through HPLC in genotypes from the DOR364 x G19833 population

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**Introduction**

Seed coat color in *P. vulgaris* is determined by the amount and presence of flavonol glycosides, tannins and anthocyanins (Beninger 1999, Takeoka 1997). These compounds are synthesized by the flavonoid pathway and although the pathway is well characterized in some species, in common bean the genes and the pathway are not well understood although extensive genetic analyses have identified specific Mendelian genes that control seed coat pattern and color. Unfortunately until now, it has not been possible to identify the genes responsible for producing specific flavonoid compounds which would clarify the relationship between genes which control the enzymes in the pathway and the Mendelian genes for seed coat color. With this in mind and because of our previous studies on QTL mapping for tannin content, we decided to begin analyses of tannin composition and anthocyanin content in some genotypes from the DOR364 x G19833 recombinant inbred line common bean population.

## Materials and Methods

*Plant Material:* We chose 17 genotypes from the DOR364 x G19833 population that contrasted in seed coat color and tannin content. Samples of 10 seeds were analyzed for each genotype. The seeds were peeled and the seed coats were ground for analysis. Condensed tannins were extracted using 70% acetone and converted to anthocyanidins by the butanol-HCl method as we reported previously in past annual reports.

*HPLC analysis:* For the HPLC analysis we dried 1 mL of each sample after the reaction with butanol-HCl in a sample concentrator. The dried samples were re-dissolved in a methanol-HCl 1% solution and then 0.5 mL of each sample was placed in a vial for the injection. Separation of anthocyanidins (resulting from the depolimerization of the tannins via butanol-HCl method) was achieved using a 8 x 100 mm Nova-Pack C18 column (4 um, Waters). The solvents were A, 100% Methanol and B, 5% acetic acid. The gradient consisted of: 40% B for 1 min, 30% B for 1.3 min, 35% B for 20 sec, 40% B for 1.3 min, 60% B for 2 min. Detection was carried out in a UV detector (Shimadzu CL-10A) using 520 nm as wavelength.

*Standards used:* The standards that we used for the identification were cyanidin chloride, delphinidin chloride and pelargonidin chloride (Supplied by Apin Chem Ltd, Abington, UK). Because we are standardizing the methodology in common bean we also evaluated purified tannins extracted previously from several bean genotypes.

## Results and Discussion

The chromatographic analysis of the samples treated with butanol-HCl, showed the existence of 3 principal anthocyanidins. The retention times for delphinidin, cyanidin and pelargonidin were 4.1, 4.9 and 6.5 minutes respectively and the results for the 17 genotypes are shown in Table 1. The main anthocyanidin was delphinidin, which means that the tannins from these genotypes were constructed mainly from the monomer gallocatechin (see Figure 1). The second most important anthocyanidin was cyanidin, derived from the monomer catechin. Pelargonidin, in some cases, was also significant. We also found a fourth anthocyanidin with 3.2 as retention time that could not be identified. The differences between genotypes show the existence of segregation in the population for tannin composition; therefore we think the next step is the evaluation of the entire population with the improved methodology and the QTL analysis. This would give use more specific information about the genomic regions that control condensed tannin production and along with the anthocyanin analysis we could establish the genes that are specific for steps in the pathway and at the same time evaluate associations between these and the Mendelian factors for seed coat color.

**Future Work**

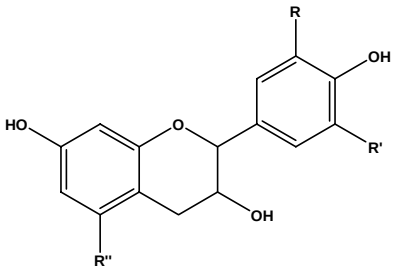
We have developed a basic method to identify three tannin constituents in common beans. However we suspect that improvements to the methods would require sample purification which would be tedious and costly to perform on the whole population. Therefore, we plan to improve the conditions for the HPLC analysis using the butanol-HCl method with the hope that it will be possible to identify a fourth anthocyanidin based on the retention time, by comparing with these present patterns. We also plan to implement a methodology for quantification of anthocyanins that we can also use with the DOR364 x G19833 population. We plan to realize QTL analysis for tannin (composition) and anthocyanin content once we have collected data for the entire population.

**Table 1.** Percentage of three types of anthocyanidins derivate from condensed tannins in bean genotypes from the DOR364 x G19833 recombinant inbred line population.

| Genotype | Color | % Anthocyanidin from solubles tannins | | | % Anthocyanidin from insolubles tannins | | |
|---|---|---|---|---|---|---|---|
| | | Delphinidin | Cyanidin | Pelargonidin | Delphinidin | Cyanidin | Pelargonidin |
| 5 | Yellow-red | 66.42 | 33.58 | 0 | | | |
| 14 | Red | 57.37 | 17.96 | 24.67 | 84.87 | 15.13 | 0 |
| 16 | Cream-purple | 84.86 | 15.14 | 0 | 77.83 | 6.59 | 15.58 |
| 24 | Cream-red | 64.4 | 20.09 | 15.51 | 68.16 | 18.36 | 13.48 |
| 30 | Yellow | 90.31 | 9.69 | 0 | 79.61 | 5.64 | 14.75 |
| 32 | Red | 13.32 | 0 | 0 | 91.27 | 8.73 | 0 |
| 35 | Red | 69.89 | 30.11 | 0 | 74.47 | 25.53 | 0 |
| 38 | Cream-yellow | 91.44 | 8.56 | 0 | 77.02 | 10.59 | 12.4 |
| 45 | Cream-brown | 100 | 0 | 0 | 74.7 | 8.85 | 16.45 |
| 47 | Brown | 43.18 | 17.71 | 39.11 | 82.5 | 17.5 | 0 |
| 53 | Red | 74.33 | 25.67 | 0 | 69.81 | 19.23 | 10.95 |
| 54 | Yellow-brown | 100 | 0 | 0 | 76.14 | 10.7 | 13.16 |
| 60 | Yellow-red | 100 | 0 | 0 | 100 | 0 | 0 |
| 69 | Brown | 87.34 | 12.66 | 0 | 76.71 | 10.75 | 12.54 |
| 84 | Yellow | 65.04 | 20.82 | 14.15 | 75.06 | 24.94 | 0 |
| 86 | Red | 76.82 | 23.18 | 0 | 82.18 | 17.82 | 0 |
| 87 | Yellow | 100 | 0 | 0 | 79.05 | 9.38 | 11.58 |

**Figure 1.** Basic structure of different types of flavan-3-ol monomeric units and the name of their respective anthocyanidin (produced under treatment with butanol acid and heating). Adapted from Hagerman (1998).

| | *Monomer (Flavan-3-ol)* | *Oligomer (proanthocyanidin)* | *Anthocyanidin (depolymerizing product)* |
|---|---|---|---|
| **R'' = OH** | | | |
| **R = R'= H** | Afzelechin, | Propelargonidin, | Pelargonidin |
| **R = OH, R'= H** | Catechin, | Procyanidin, | Cyanidin |
| **R = R'= OH** | Galocatechin, | Prodelphinidin, | Delphinidin |
| **R'' = H** | | | |
| **R = R'= H** | Guibourtinididol, | Proguibourtinidin, | Guibourtinidin |
| **R = OH, R'= H** | Fisetinidol, | Profisetinidin, | Fisetinidin |
| **R = R'= OH** | Robinetinidol, | Prorobinetinidin, | Robinetinidin |

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## 1.2.7 Phytate analysis through HPLC in common bean RILs from the cross AND 696 x G19833


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## Introduction

Phytic acid, InsP6 (or abbreviated as IP6), is a naturally occurring seed component and the principal storage form of phosphorus in cereals, legumes and nuts. However, phytic acid is considered an anti-nutrient, mainly due to its ability to bind essential dietary minerals including calcium, iron, and zinc, as well as proteins and starch, and to consequently reduce their bioavailability in human diets (Chen 2004). Phytates can reach concentrations of 2% of total seed weight but vary greatly between genotypes, growing seasons and species and therefore sensitive quantification techniques are needed to determine the amount of phytates present in seeds. For that reason, we decided to evaluate a bean population grown in both high and low phosphorus environments using an HPLC technique for better quantification of phytic acid content.

## Materials and Methods

*Plant Material:* the population evaluated in this study consisted of 78 RILS derived from the cross AND696 x G19833. The population was grown in a split plot design with 3 replications each under low and high phosphorus treatment in Darien. Seeds of each genotype were prepared within two months of harvest for the phytic analysis, by lyophilizing a sample of 3 g of seed in a Modulyo Liofilizer and grinding the freeze dried seed in a Retsch mill as described previously for mineral analysis.

*Extraction and purification*: An aliquot of ground seed powder weighing 0.5g was mixed with 20 mL of 0.65M HCl that was then mixed mechanically for 2 hours. The samples were centrifuged at 4000 rpm for 15 minutes and supernatants were transferred through a filter to a glass vial where the liquid was stored at 20ºC until further use. After this step, 3 mL of the samples were diluted with distilled water to a volume of 15 mL which was loaded onto SAX solid phase columns (500 mg Varian) for purification. Phytates were eluted with 3 mL of 2M HCl in glass vials and then lyophilized. The dry samples were dissolved in 1 mL of mobile phase for HPLC analysis.

*HPLC analysis*: The HPLC analysis was done according to the method of Graf and Dintzis (1982). Analysis of phytic acid was achieved using a 3.9 x 150 mm symmetry column (5 um, C18 Waters). Optimum elution conditions were 30-min isocratic system from 50 mM sodium acetate at a flow-rate of 1 mL/min. A standard curve for the quantification of IP-6 was obtained by injecting various quantities of sodium phytate solution (10 mg/mL dissolved in mobile phase). The detection of phytic acid was realized with a refractometer.

## Results and Discussion

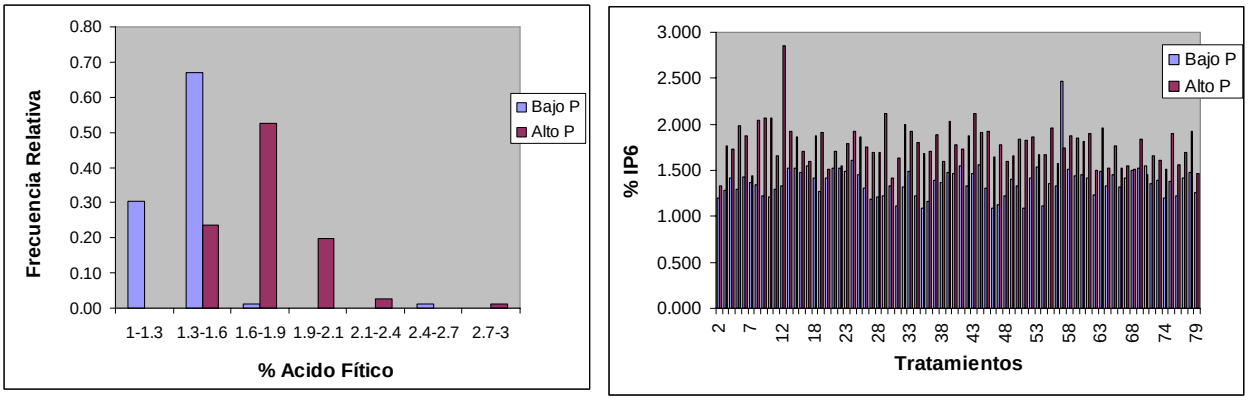
In HPLC analysis, the retention time for IP6 was 1 minute. The correlation coefficient for the standard curve was $R^2 = 0.9998$ and the detection/quantification limit was 0.396 mg/mL in terms of concentration and 0.52% as percentage of phytic acid in whole seed. The coefficient of variation for the methodology was 4.6%. The analysis of variance for phytic acid content is shown in Table 1 where analysis was as a split plot design. Sources of variance were significant for genotype and P level but not for genotype x P level. In this case the term of error used was repetition x genotype x environment. Individual ANOVAs were done to verify the results, using as an error term the P level x repetition and genotype x repetition interactions and the significant differences between high and low phosphorus conditions was confirmed however due to the interaction effect genotype differences were not stable across low and high P levels. G19833 was a good genotype for low phosphorus environments because of its mechanism of phosphorus acquisition from the soil, and many progeny lines from the population had the same behavior in low phosphorus. It also may explain the interaction environment x genotype no significant that we found. In this case, would be necessary establish a correlation between phytic acid content and total phosphorus and determine the mechanism of the AND696 genotype for phosphorus acquisition.

**Table 1.** Analysis of variance for percentage of phytic acid content in bean whole seed from AND696 x G19833 population.

**a) error term = P x Genotype x REP**

| Source | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| P level | 1 | 15.2126 | 15.2126 | 191.66 | 0.0000 |
| Genotype | 75 | 8.7687 | 0.1169 | 1.47 | 0.0144 |
| P level x Rep | 4 | 6.1952 | 1.5488 | 19.51 | 0.0000 |
| P level x Genotype | 75 | 7.5951 | 0.1013 | 1.28 | 0.0850 |
| Error | 256 | 20.3193 | 0.0794 | | |
| Total | 411 | | | | |

**Figure 1**. Distribution of the phytic acid percent in recombinant inbred lines of the AND696 x G19833 population.




## 1.2.8 Characterization of the ferritin gene family and intron sequences.

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### Introduction

An understanding of the basis for iron uptake, transport, and accumulation in the sedes of common bean will require a careful dissection of the entire pathway of genes involved in these processes. This knowledge will assist us as plant breeders to increase bean iron content through the careful selection of alleles and potential parents to use in the breeding program. The objective of this study was to analyze the genes for the major iron storage protein, ferritin. This protein is one of the major non-heme iron storage proteins in many organisms and is conserved in animals, plants and microorganisms (Thiel, 1990). Ferritin consists of a mineral core of hydrated ferric oxide and a multi-subunit protein shell that encloses the core and assures its solubility in an aqueous environment (Thiel et al., 1987). A total of 24 subunits are used to make this protein shell. Ferritin is involved in iron homeostasis because of its ability to sequester thousands of Fe atoms intra-cellularly in a safe form until needed for metabolic functions such as respiration and photosynthesis. Ferritin is encoded by gene families in all plants analyzed so far, with four genes identified in the fully sequences *A. thaliana* genome, one to four genes in the legumes, peas

and soybeans, and two genes in maize. Sequence comparisons between plant ferritin genes has shown them to be highly conserved with variability at the nucleotide level limited to the 3'UTR region and intronic sequences. Expression level differences have also been well documented. We were interested in pursuing the intron sequences to identify polymorphisms that could be applied to the development of molecular markers as part of the current research. The introns of ferritin genes are conserved in position and their total number (7) but vary in length and individual sequences between paralogous genes. Our starting point was the full length mRNA/cDNA sequence of one seed expressed ferritin from common bean and the high homology of this gene with a soybean ferritin gene for which intron/exon boundaries and gene structure have been elucidated.

**Materials and Methods**

Exon sequences were identified from an alignment of the common bean seed ferritin cDNA with the highest homology ferritin gene sequence from soybean using the BLAST subprogram *bl2seq*. Primers were designed using the software Primer 3 within the exon sequences at the exon/intron boundaries flanking specific introns that were identified in this manner. For example, the primer pair for intron 1 was designed at the 3' end of exon 1 and the 5' end of exon 2. For the region between introns 4 and 7, a primer pair was designed at the 3' end of exon 4 and the 5 'end of exon 8. The gene sequences were then PCR amplified using DNA from the maping parents G21242, G21078, G14519, G4825, DOR364, G19833, BAT93 and Jalo EEP558 to determine the allele sizes and sequences for each of these genotypes. PCR products were purified using Promega Wizard PCR prep kits (DNA Purification Systems) and sequenced from both ends on an automated sequencer (ABI3700). Sequence analysis was conducted with the software program Phred v. 4.25 to determine sequence quality and Sequencher v.4.1. (DNAstar) and Vector NTI 10.1.1 (Invitrogen) to make alignments.

**Results and Discussion**

*PCR amplification and sequencing:* The amplification of the eight mapping parents with the two intron based primer pairs is shown in Figure 1. The marker Fer_int1, produced a single band that was approximately 800 bp long while the Fer_int4-7 marker produced a single band that was approximately 850 bp long. These were both larger than the expected size based on the intron lengths in soybean which are 436 bp and 563 bp for the two markers, respectively. No size polymorphism was identified in the PCR products for the different mapping parents with each of the newly-developed markers. Sequencing fo the PCR fragments produced good quality data for the mapping parent alleles that were evaluated for both markers.

*Sequence analysis:* After alignment of the mapping parent alleles, intron 1 presented a high A-T content which was characteristic of intronic regions compared to exons in the ferritin gene and the full length of the consensus sequence was 767 pb long. The region between Exon 3 and Exon 8 containing introns 4, 5, 6 and 7 as well as exons 5, 6 and 7 was 802 bp long and aligned well with the soybean sequences both at the genomic and cDNA level (Figure 2). An early observation was that the intron sizes for common bean were different than for soybean. A blastN search with Intron 1 sequences did not show homology of the common bean sequences with those of soybean nor with the ferritin gene sequences reported in Genbank, therefore this sequence information is unique. A blastX search for the Fer_Int4-7 marker using the nr database at Genbank showed that the sequenced PCR products were indeed for a ferritin gene family member since the exons aligned well and had high homology with soybean ferritins from the expected exon 5, 6 and 7 region (Figure 3). The introns in the same region (ie. introns 4, 5, 6 and 7) showed no sequence similarity to the soybean ferritin gene sequences or any other sequences for that matter except adjacent to some of the splice junction as defined previously by Proudhon et al. (1996) for the ferritin gene at the conserved 5'GT and 3'AG sites flanking each intron. The intron/exon boundaries of the common bean sequences and the reference soybean gene is show in Table 1. The alignment of the translated

common bean gene sequence and other ferritin gene sequences from the legumes, garden pea and soybean, as well as that of *A. thaliana* is shown in Figure 4 where the highly conserved exon sequences are easily observed. Single nucleotide polymorphism and small insertion/deletions were observed among the alleles for the different mapping parents in the case of the sequences for both new markers.

**Future Work**

Protein domain recognition for the translated common bean ferritin sequence is pending using PROSITE as is the genetic mapping of the two markers developed in this study based on the polymorphisms that show most promise based on our sequence analysis of the mapping parent alleles. In addition we plan to analyze possible paralogs of the common bean seed ferritin in the rest of the genome and orthologous relationships with other legume ferritin genes. We hope to achieve a full understanding of the entire gene family. Screening will be conducted in cDNA and BAC libraries of common bean to identify related sequences. Our final goal is to test whether any of the ferritin sequences are associated with QTL for iron accumulation which we have been identifying in common bean mapping populations. Once we complete the ferritin gene analysis we have several other candidate genes for which we will conduct a similar analysis.

**References**

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Proudhon D., Wei, J., Briat J. F., Theil E. C. 1996. Ferritin gene organization: differences between plants and animals suggest possible kingdom – specific selective constraints. J. Mol. Evol. 42:325-336.

**Table 1**. Comparison of the 5' and 3' splice sites at intron/exon boundaries within the common bean ferritin gene and a soybean ferritin gene for the intron sequences between exon 3 and exon 8.

| Intron | | 5' splice site | 3' splice site |
|---|---|---|---|
| **4** | **Common Bean** | | TTC AG- |
| | **Soybean** | | TTC AG- |
| **5** | **Common Bean** | AGT GTA ATG | CTA ACA GG- |
| | **Soybean** | AGT GTA TG | CTA ACA GG- |
| **6** | **Common Bean** | CAG GTT AAA | TTT GTA CAG- |
| | **Soybean** | CAG GTT AAA | TTT GTG CAG- |
| **7** | **Common Bean** | CAC GGT ATG T | |
| | **Soybean** | CAC GGT ATG T | |

**Figure 1.** PCR amplification of two markers for the common bean ferritin gene as described in the text.

a. Fer_Int1 b. Fer_int4-7

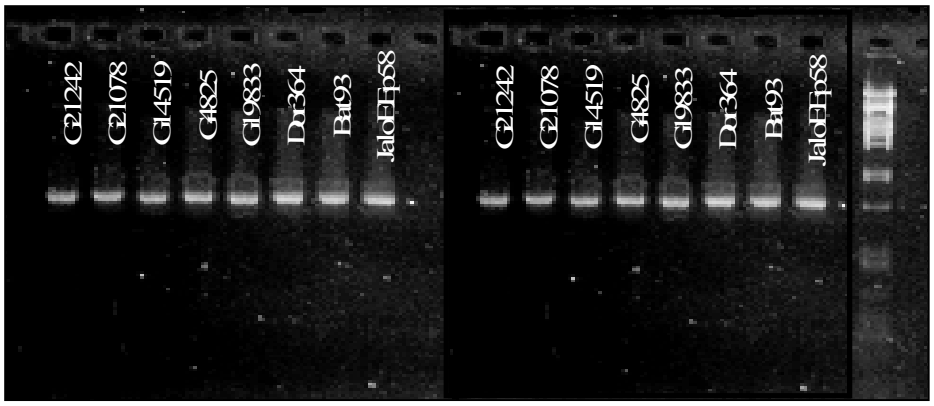



**Figure 2**. Analysis of the exons and introns in the sequence amplified by the marker Fer_int4-7.

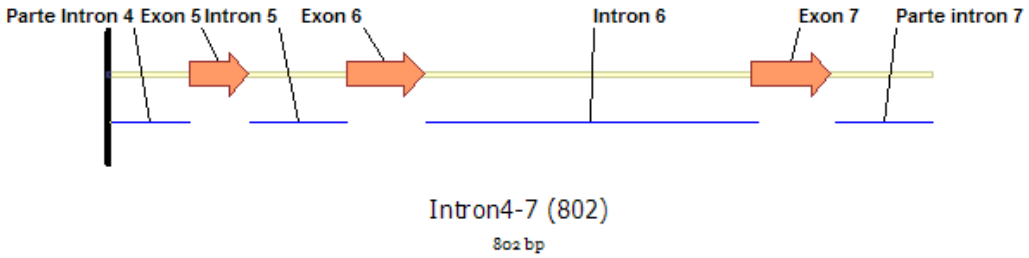



**Figure 3**. Similarity and position of common bean ferritin exons 4, 5 and 6 compared to a soybean ferritin cDNA.

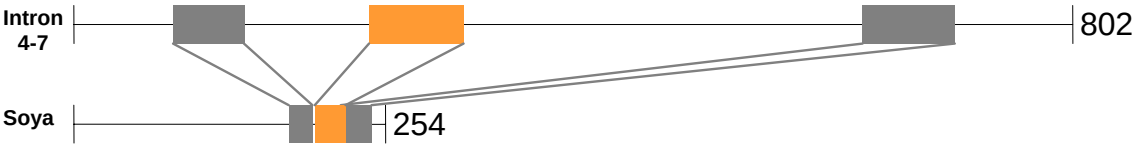
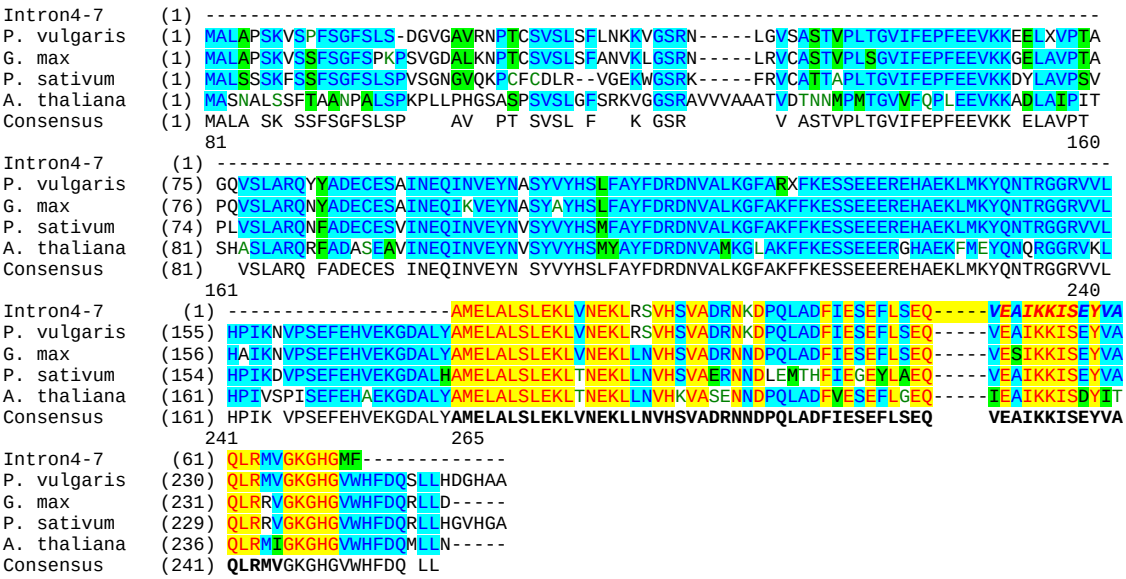


**Figure 4**. Translated sequence alignment of the common bean ferritin exons 4, 5 and 6 with ferritin genes of *Pisum sativum*, *Glycine max* and *A. thaliana*.




### 1.2.9 QTL analysis of adventitious root formation in common bean

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#### Introduction

Eighty-two percent of the soils in Latin America are deficient in phosphorus. Phosphorus deficiency is widespread in East Africa as well, possibly affecting over 50% of soils there. Studies at CIAT have identified several sources of tolerance to low phosphorus conditions including G19833, G19839 and G2333. This last genotype is thought to take up and use phosphorus more efficiently through ample production of adventitious roots. In the breeding programs at CIAT, one of our long-term objectives is to increase the capacity of new breeding lines to grow under low P conditions. The objectives of this study were to identify quantitative trait loci (QTL) for P accumulation and associated adventitious rooting traits in a population derived from two P efficient genotypes G2333 and G19839.

#### Materials and Methods

*Plant Material:* A total of 84 F5:8 recombinant inbred lines (RIL) were developed at CIAT by single seed descent from the cross G2333 x G19839. In this cross, G2333 ('Colorado de Teopisca') is a small red-seeded Mexican climbing bean landrace belonging to race Mesoamerica with type IV growth habit; and G19839 is a large, yellow and red-mottled seeded Peruvian landrace with type III growth habit that belongs to race Nueva Granada of the Andean gene pool.

*Experimental Design:* The RILs and parents were planted in a field trial in Darien, Colombia, during the rainy season from September to December 2001. Several years of fertility management to generate high and low phosphorus plots in this location resulted in plots having markedly different phosphorus availability with an average phosphorus concentration of 64.3 ppm and 1.68 7 ppm for high and low phosphorus plots respectively. Prior to planting, the high phosphorus plot received 45 kg/ha phosphorus and the low phosphorus plot received 7.5 Kg/ha phosphorus as triple super phosphate. To prevent zinc and boron deficiency typical of these soils, 600 g ha-1 of ZnSO4 and 600 g ha-1 of borax (Na2B4O2(H2O)10) were applied as a foliarly feed every week after emergence for three consecutive weeks. Phosphorus-free fungicides and pesticides were applied several times as needed. Seeds were sown at a depth of 7-8 cm and at 15 days after emergence plants were manually hilled with soil to slightly below the cotyledons. The experimental design consisted in a randomized complete block design (RCBD) with a split plot arrangement of treatments where phosphorus levels were the main plots and RIL genotypes were the subplots. The experiment consisted of 3 replications over time to allow for efficient sampling of adventitious roots. Each experimental unit was a single 1m-row plot sown with 15 seeds. One meter spacing between rows was used to accommodate both climbing and semi-climbing genotypes. Total plant density was ca. 150,000 plants ha-1.

*Root traits evaluated:* Root systems were collected for two plants per row at 42 days after sowing, corresponding to the early flowering growth stage R6 by carefully excavating the plants to reveal as many adventitious and basal roots as possible, and counting the number of adventitious roots emerging from the hypocotyls. Sub-samples of both adventitious and basal roots were preserved in 25% ethanol and kept at 4° C until analyzed for root length. Shoots and remaining adventitious roots were oven dried at 60º C for 2-3 days for dry weight determination. Adventitious and basal root sub-samples were stained with 0.1g L-1 neutral red dye for 24 hours and mounted on a clear acrylic tray in shallow water for scanning and quantification of root length as well as other root parameters using a flat bed scanner. Scanned images were analyzed with WinRhizo Pro. Specific root length (SRL) was calculated as length per unit of root dry weight (meters per gram). SRL for adventitious root samples was used to estimate total adventitious root length.

*QTL analysis:* A genetic map was constructed with microsatellites and PCR-based markers (RAPDs and SCARs) as described in Ochoa et al. (2006) covering all eleven linkage groups. QTL were detected using the computer software program QTL Cartographer version 2.0 for Windows. QTL for a given adventitious root traits and their combined effects were identified with both Composite Interval Mapping (CIM) and Multiple Interval Mapping (MIM) features. Parameters for CIM and MIM analysis included a forward/backward regression with a window size of 10 cM, a walk speed of 1 cM and probability thresholds of 0.05 each for the partial F-test for both marker inclusion and exclusion. The empirical thresholds for QTL detection with the CIM method were estimated using 1000 permutation tests. Additivity estimates for each adventitious root trait were computed by the QTL Cartographer program at the peak of the LOD profile.

**Results and Discussion**

A total of 13 QTLs were identified for adventitious root-related traits (Table 1) by means of composite interval mapping (CIM) and multiple interval mapping (MIM) analyses performed for

each phosphorus treatment level (high and low phosphorus). Significant QTLs were detected in linkage groups B2, B4, B6, B7, B8, B9, B10, and B11, and the phenotypic variation explained by the individual QTL for adventitious root traits ranged from 11% to 36%. Among the QTLs, 10 were observed under high phosphorus conditions, while 3 were detected under low phosphorus conditions. The number of QTL identified for each trait are given below.

*Adventitious root number:* Three QTLs for the number of adventitious roots in the field were identified on linkage groups B2 (under low phosphorus), B7 (high phosphorus), and B9 (high and low phosphorus). The QTLs on linkage groups B7 and B9 together accounted for 33% of the total phenotypic variation for this trait in high phosphorus; while QTLs on linkage groups B2 and B9 explained up to and for 61% of the observed variation for the same trait in low phosphorus.

*Adventitious root biomass:* Three QTLs for adventitious root biomass under high phosphorus conditions were detected in the same regions of linkage groups B2 and B9 as well as on linkage group B6. Together the three QTL explained 59% of the variation for adventitious root biomass and individually from 18 to 24%.

*Adventitious root length:* For adventitious root length, three QTLs were identified in the field environment and these were located on linkage groups B2, B9, and B11, together explaining 58% of the variation for the trait. Of the three QTLs mapped for adventitious root length in high phosphorus the ones on linkage groups B2 and B9 coincided in location with the QTLs mentioned above for adventitious root biomass in high phosphorus as well as for the number of adventitious roots under high and low phosphorus.

*Specific root length:* For specific root length (SRL) measured on adventitious roots grown under high phosphorus conditions, QTLs were identified for the field experiment on linkage groups B2 and B7. These QTL accounted for 19% to 39% of the total variation for the traits. For adventitious SRL under low phosphorus conditions in the field only one QTL was detected in linkage group B2 and this explained 25% of the observed variation. The biochemical marker phaseolin (*Phs*), on linkage group B7 was significantly associated with a QTL for SRL of adventitious roots under high phosphorus conditions and this QTL explained 18% of the variation for this trait.

It was notable that the positive alleles for all QTL detected on linkage groups B9 and B11 were inherited from the maternal genotype line G2333, while the positive alleles for the QTLs located in linkage group B2 were inherited from the paternal genotype line G19839 with the exception of the QTL for SRL of adventitious roots under high phosphorus conditions. The QTLs for adventitious rooting under high and low phosphorus conditions detected on linkage group B9 near markers P070.45 and M130.34 had positive alleles that were inherited from G2333, while the other QTLs identified on linkage groups B6 and B7 had positive alleles inherited from both parents.

**Table 1**. QTLs for adventitious root traits under high and low phosphorus availability.

| Adventitious Traits | QTL name† | Linkage group | Nearest Marker locus | LOD‡ | §$R^2_{CIM}$ | ¶$R^2_{MIM}$ | Additivity# |
|---|---|---|---|---|---|---|---|
| <u>High P</u> | | | | | | | |
| Number | HPAdvNoF.1 | B7 | V100.29 | 3.68 | 0.14 | 0.12 | -3.53 |
| | HPAdvNoF.2 | B9 | P070.45 | 4.21 | 0.20 | 0.21 | 3.67 |
| | | | Total†† | 2.74 | | 0.33 | |
| Biomass | HPAdvDWF.1 | B2 | P101.6 | 3.95 | 0.18 | 0.10 | -2.66 |
| | HPAdvDWF.2 | B6 | P010.6 | 4.55 | 0.24 | 0.16 | -2.99 |
| | HPAdvDWF.3 | B9 | M130.34 | 4.47 | 0.23 | 0.30 | 3.14 |
| | | | Total | 5.40 | | 0.55 | |
| Length | HPAdv_LF.1 | B2 | P101.6 | 3.66 | 0.18 | 0.14 | -2.88 |
| | HPAdv_LF.2 | B9 | M130.34 | 5.55 | 0.25 | 0.33 | 3.63 |
| | HPAdv_LF.3 | B11 | Bmy002 | 3.74 | 0.15 | 0.10 | 2.96 |
| | | | Total | 4.83 | | 0.58 | |
| SRL | HPSRL_AdvF.1 | B2 | Q171.7 | 3.59 | 0.15 | 0.07 | 9.45 |
| | HPSRL_AdvF.2 | B7 | Phs | 3.30 | 0.18 | 0.04 | 9.44 |
| | | | Total | 1.27 | | 0.19 | |
| <u>Low P</u> | | | | | | | |
| Number | LPAdvNoF.1 | B2 | P101.6 | 3.37 | 0.13 | 0.10 | -2.86 |
| | LPAdvNoF.2 | B9 | P070.45 | 6.63 | 0.36 | 0.49 | 4.84 |
| | | | Total | 4.41 | | 0.61 | |
| SRL | LPSRL_AdvF.1 | B2 | Bm142 | 5.14 | 0.25 | | -8.48 |

† HP and LP in front of the abbreviation QTL name means high phosphorus and low phosphorus respectively. For abbreviation descriptions refer to Table 2.

‡ Maximum $Log_{10}$ likelihood peak

§ Proportion of the phenotypic variance explained by QTL at test site using CIM

¶ Proportion of the phenotypic variance explained by all significant QTLs using MIM

# Effects of substituting a single allele from one parent to another. Positive values indicate that allelic contribution is from G2333 and negative from G19839

†† Maximum LOD and the amount of phenotype variation simultaneously explained by all markers identified for a trait and determined by multiple QTL model

## 1.2.10 Molecular marker-assisted selection (MAS) for the improvement of local cassava germplasm in Tanzania for pest and disease resistance

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[1]ARI-Mikocheni); [2]SRI-Kibaha [3]ARI-Ukiriguru; [4]IITA-Nairobi; [5]CIAT

Funding: Rockefeller Foundation, CIAT

### Introduction

Centralized breeding cassava program in International Agricultural Research Centers (IARCs) and National Programs is a multi-stage evaluation lasting 8-10years with farmers being introduced at the very end of the scheme. The low adoption of improved cassava genotypes coming from centralized breeding programs in many African counties have led to the proposal of a decentralized breeding scheme involving molecular marker-assisted selection (MAS), to quickly reduce the size of breeding populations derived from crossing local varieties to improved introductions, followed by participatory plant breeding (PPB). A project to test this idea was initiated in Tanzania in October 2003 with support from the Rockefeller Foundation and is in its fourth year. Improved varieties with good resistance to the cassava mosaic disease (CMD) and the cassava green mites (CGM) were introduced from CIAT and evaluated for resistance to pest/diseases and other traits of agronomic interest. Selected introductions were then crossed to local farmer preferred genotypes collected in the Eastern, Southern, and Lake regions of the country. We describe here generation of crosses, establishment of the seedling nursery, evaluation for pest and disease resistance, and molecular marker-assisted selection for resistance to CMD. The project is expected to produce improved cassava varieties acceptable to farmers within the next 3 years but more importantly it is expected to provide a proof of concept for the MAS-PPB paradigm in cassava breeding capable of accelerating the production of highly productive and stable farmer-preferred varieties.

### Methodology

*Genetic crosses and establishment of seedling nursery*
A total of 80 introductions from CIAT were selected based on resistance to CMD and CGM and crossed to 51 local varieties selected based on CBSD resistance and farmer-preferred traits.

Three crossing blocks were established in February 2005 at Chambezi Experimental Station, as follows:

Controlled pollinations comprising 58 CIAT genotypes, 25 local varieties from the Eastern zone, 18 from the Southern zone and 3 improved varieties from the breeding program.

Polycross design of 20 CIAT genotypes, 22 local varieties from the Eastern zone and one improved variety Kibaha.

Polycross design of 20 CIAT genotypes, 16 local varieties from the Southern zone and three improved varieties from the southern zone.

During the multiplication of introduced varieties they became infected with CBSD and therefore could not be moved to Maruku in the Lake zone. Pollen was flown from Chambezi to Maruku for crossing, although poor flowering in Maruku meant that only 1,612 crosses were made at that location.

Over 60% of the seeds produced were planted in a seedling nursery in March 2006. The CMD-resistant progenies were also crossed with genotypes with high contents of carotene and protein. About 60% of seeds harvested from the crosses were germinated plastic seed boxes in two batches, floating and non-floating seeds during thee test of viability, in March 2006. After 4-8 weeks the seedlings were transplanted to the field at the Chambezi experimental station of Mikocheni Agricultural Research Institute (ARI) situated about 60km North-west of Dar es Salaam. The first batch of non-floating seeds was replicated by families and planted in 3 blocks in April 2006, while the second batch, of floating seeds was planted in 3 replications of floating seeds planted in June 2006. All genotypes were planted at 1.m x 0.7m spacing in 25-plant rows.

*Field evaluation of resistance to CMD, CBSD and CGM and collection of samples*
Evaluation of the first and second batches for resistance to CMD, CBSD and CGM were carried out at 3, 6 and 9 months respectively after planting. Absence or present of symptoms was reported at three months and the steam was painted with a different color identifying plants with symptoms for each disease. At 6 and 9 months evaluation, absence or present of symptoms was recorded as well as recovery for each plant. Healthy plants without CMD symptoms at 6 months in each batch were labelled and the youngest leaves collected for MAS using available markers linked to *CMD2*.

*MAS for CMD2*

Young leaves from 3016 labelled plants from batch 1 and 75 parents were collected in eppendorf tubes for DNA extraction. Young leaves from 1225 labelled plants from batch 1 and batch 2 were collected in paper envelops and put in oven at 48°C for two days. Dried leaf samples were then shipped to CIAT.
A scar marker, RME-1 and two SSR, NS158 and SSRY28 were used for molecular evaluation of the parents and the plants without symptoms in the field. Evaluation of the parents was used to identify markers polymorphic in each family and subsequent molecular characterization of the progenies. The scoring of these markers is based on presence or absence of the allele associated with *CMD2*. Positive controls was the source of CMD2, the genotype TME-3.

DNA extraction was following the modified Dellaporta (1983) protocol. Between 0.5 to 1 g of young leaves was harvested from all plants and oven dried at 48oC for 24 h. The dried leaves were ground, using a power drill (or manually) fitted with a metal pestle and some acid-washed sand. DNA was extracted from 200 mg of dried leaf tissue, using a mini-prep version of the Dellaporta et al. (1983) protocol. DNA quality and quantity was checked for all the samples before molecular marker analysis. A single 10X dilution with TE (10 mM Tris, 1 mM EDTA, pH = 8.0) was employed for all DNA samples. Amplification by polymerase chain reaction (PCR), polyacrylamide gel electrophoresis, and silver staining were carried out as described by Mba et al. (2000) for the SSR marker. PCR products of SSR markers were also scored on 4% Synergel. In contrast, the PCR product of the SCAR marker was run on 1% agarose gels at 60 V for 2 h and stained with ethidium bromide. The gel image was captured by scanning and transferring to a Microsoft Excel file containing field resistance data.

**Results**

*Genetic crosses and establishment of seedling nursery*
At Chambezi, 57,463 crosses were made. From the controlled crossing block 27,107 seeds were collected. From the polycross block consisting of materials from the Eastern zone 7,376 seeds were collected, while 4,933 seeds was obtained from the southern zone polycross block, and 24, 566 seeds from open pollinated crosses in the controlled crossing block. About 60% of these seeds were planted in seed trays in the screen house at ARI-Kibaha in February 2006. They were transferred to the field at Chambezi in March 2006. The remaining seeds were stored at Kibaha at 4°centigrade. Germination was over 90% for non-floating seeds and 70% for floating seeds. A total of 26,592 seedlings were transferred to the field at Chambezi in

March 2006. The relatively lower number of seeds planted in the field compared to those obtained was due to high fungal infection in the seedlings after 5 weeks of sowing.

During Phase I an attempt was made to produce crosses between local varieties from the Lake zone and the CIAT introduction. However, seed set was low due to poor flowering and probably low pollen viability. Due to importance of transferring CMD tolerance to local varieties in the Lake region, a second year of crossing will be undertaken by shipping pollen from CIAT introductions to the lake region during the first year of the project's second Phase. Pollen will be collected from CIAT genotypes at Chambezi and flown to ARI Maruku for crossing with about 20 local varieties from the Lake zone. During the second year of Phase II, botanical seeds from the crosses will be germinated, and planted in a seedling nursery for multiplication and MAS.

*Field evaluation of resistance to CMD, CBSD and CGM and harvest of the seedling nursery*
Summary of field evaluation for resistance CMD, CBSD, and CGM at 3, 6, and 9 months respectively in the first block of seedlings from batch 1 are presented by family in Table 1. Evaluation and data entry for the remainder of block 1 and batch 2 is in progress.

*MAS for CMD2 : Selection of families*
Seventy five parental lines used to generate breeding populations were evaluated by PCR using the three CMD2 markers (RME-1, SSRY28 and NS158) and PCR amplification bands scored on 1% Agarose gels for the SCAR or Synergel 4% for the SSR markers (Figure 1).

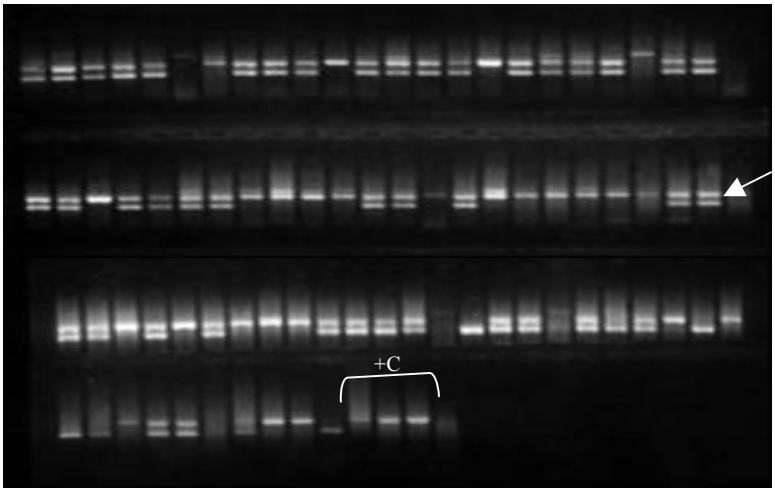


Figure 1. Tanzanian parents evaluated with the scar marker (RME-1). Positive controls (+C) represents the resistant controls TME-3.

Based on the evaluation of the parents, families with 1 or 2 of the 3 markers being polymorphic in the parents were selected for MAS (Table 2). Additional markers are being developed from BAC contigs to evaluate families for which polymorphic markers in the parent are yet to be identified.

Table 1 CMD severity score in families of controlled and open crosses between local varieties and CIAT introductions

| Family | Number of plants | Average CMD severity at 3 months after planting | Average CMD severity at 6 months after planting |
|---|---|---|---|
| KHO16 | 454 | 1.66 | 1.81 |
| KHC87 | 224 | 2.29 | 2.49 |
| KHC154 | 193 | 2.09 | 2.27 |
| KHC4 | 178 | 2.21 | 2.31 |
| KHC110 | 172 | 2.03 | 2.36 |
| KHO12 | 135 | 2.00 | 2.18 |
| KHC80 | 130 | 1.82 | 2.11 |
| KHC37 | 122 | 2.80 | 2.87 |
| KHC22 | 120 | 2.55 | 2.60 |
| KHC111 | 119 | 1.43 | 1.71 |
| KHC9 | 119 | 2.61 | 2.71 |
| KHO13 | 118 | 1.57 | 1.81 |
| KHC98 | 92 | 1.74 | 2.11 |
| KHO27 | 89 | 2.14 | 2.35 |
| KHC78 | 81 | 2.32 | 2.44 |
| KHC21 | 79 | 2.26 | 2.33 |
| KHO8 | 75 | 1.98 | 2.24 |
| KHC11 | 74 | 2.73 | 2.66 |
| KHO14 | 72 | 2.43 | 2.54 |
| KHC23 | 71 | 1.98 | 2.18 |
| KCH11 | 68 | 2.59 | 2.54 |
| KHC36 | 68 | 2.07 | 2.41 |
| KOH27 | 67 | 1.66 | 1.90 |
| KHC20 | 64 | 2.14 | 2.27 |
| KHC81 | 62 | 2.22 | 2.45 |
| KHC83 | 60 | 2.24 | 2.45 |
| KHC1 | 54 | 2.29 | 2.39 |
| KHC17 | 52 | 2.63 | 2.67 |
| KHC7 | 51 | 2.44 | 2.41 |
| KHC82 | 51 | 1.82 | 2.12 |
| KHC2 | 49 | 1.95 | 2.41 |
| KHC67 | 48 | 2.30 | 2.31 |
| KHC79 | 45 | 1.83 | 2.00 |
| KHO17 | 45 | 2.38 | 2.53 |
| KHO35 | 45 | 1.87 | 2.13 |
| KHO15 | 44 | 2.85 | 2.98 |
| KHC124 | 43 | 2.00 | 2.33 |
| KHC156 | 42 | 2.50 | 2.57 |
| KHC14 | 40 | 2.86 | 3.18 |
| KHO20 | 40 | 2.08 | 2.28 |
| KOH17 | 35 | 2.20 | 2.20 |
| KHO25 | 32 | 1.68 | 1.75 |
| KHC8 | 31 | 2.56 | 2.84 |
| KHO18 | 29 | 1.60 | 2.24 |
| Average | | 2.15 | 2.33 |

Table 2. Number and percentage of families selected for MAS based on polymorphism of markers associated with *CMD2* in the parents.

| | RME-1 | | SSRY28 | | NS158 | |
|---|---|---|---|---|---|---|
| | No of families | Percentage | No of families | Percentage | No of families | Percentage |
| Controlled crosses | 73 | 36.1 | 44 | 21.8 | 10 | 4.9 |
| Open pollinated crosses from Polycross block | 26 | 61.9 | 19 | 45.3 | 26 | 61.9 |
| Open pollinated crosses from controlled crossing block | 45 | 71.4 | 25 | 39.7 | 45 | 71.4 |

The selected families are being evaluated with the three markers for selection of resistant genotypes (Figure 2).

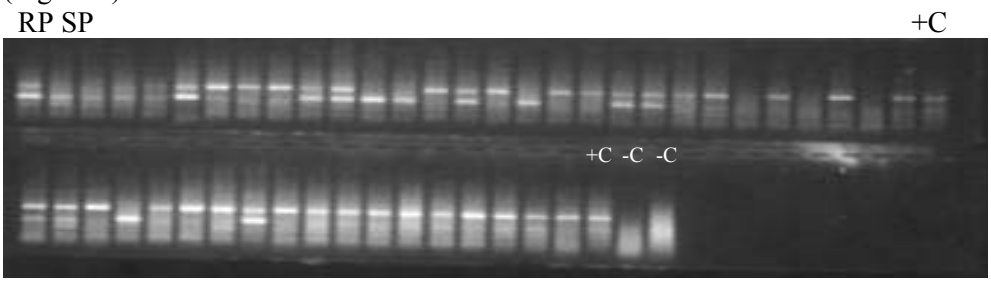


Figure 2 Agarose gel stained image of PCR product of the SCAR marker RME-1 evaluated in 49 progeny of the family KHC16 (controlled cross). First well shows the resistant parent (RP) CR27-24 (CIAT accession), second well shows the susceptible parent (SP) Nachinyaya (Tanzanian local variety), wells labeled as +C represent the positive control *CMD2* donor parent TME-3, wells labeled as –C represent the negative controls, the remaining wells correspond to progeny of KHC16.

Harvest of the first batch is currently ongoing and will be completed early May 2007. Harvest of batch 2 will begin immediately after and will end in June 2007. Criteria for selections will be field resistance to CMD, including molecular data from MAS, CBSD, CGM, and the ability to produce 8 stakes. About 8000 clones are expected to be established in the CET. Selections will be immediately established in a clonal evaluation trial (CET) station at Chambezi, a location with strong CBSD and CMD pressure. The CET will be evaluated for field resistance to CMD, CBSD, and CGM as well as yield components such as harvest index, number of roots, and total plant biomass, and farmer-preferred characteristics. Selections from the CET will be re-evaluated on-station in a preliminary yield trial (PYT) and on-farm in farmer participatory trials according to the original PPB and MAS scheme (Figure 3). About 5% of the 8,000 clones will be selected for on-farm trials in 2008 and 2009.

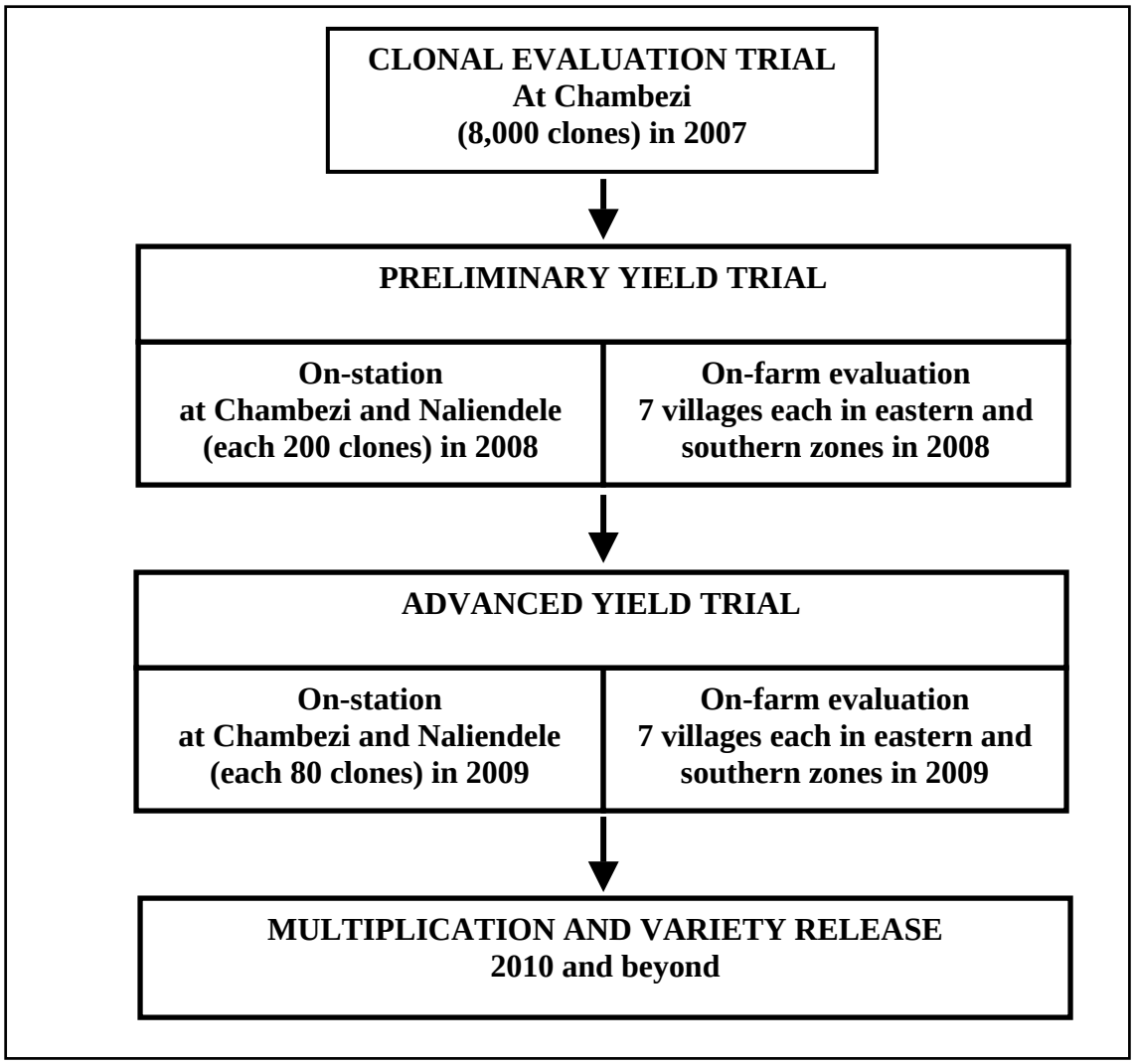


Figure 3. PPB Scheme for selections from the seedling nursery.

Table 3 shows villages that have been earmarked as sites for on-farm trials establishment.

Table 3. Villages selected in the target regions for end-user participatory evaluation

| Southern zone | Eastern zone |
|---|---|
| Mtwara | Zongowahale |
| Newala | Chanika |
| Nachingwea | Tongwe |
| Masasi | Bungu |
| Ziwani | Magoda |

**Conclusion and perspectives**

The Tanzanian MAS-PPB project seeks to improve local varieties for disease and pest resistance and provide a proof of concept for the MAS-PPB paradigm in cassava breeding but more importantly it is expected transfer useful variability from the crop's center of diversity of cassava to Africa. The concept has already been extended to three other NARs in Africa, namely Nigeria, Ghana, and Uganda, under the

auspices of a Generation Challenge Program (GCP) competitive grant project 'Development of Low-cost Marker Technologies for Pyramiding Useful Genes from Wild Relatives of Cassava into Elite Progenitors'.

The second and most important phase of the project is the farmer participatory evaluation of cassava genotypes that have resistance to CMD, CGM, and CBSD. Detailed records of why farmer select certain genotypes, including perceptions of disease response, yield, dry matter content, cooking quality and taste will be made and used to guide future germplasm transfer to NARS cassava breeding program.

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### 1.2.11 Deployment of Cassava Mosaic Disease (CMD) resistant Latin American Germplasm in Nigeria

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Funding: NRCRI, GCP, CIAT

**Introduction**

Cassava germplasm held in CIAT's gene bank represents millenniums of farmer selection and thirty years of breeding and it constitutes a very valuable resource for cassava world-wide. Direct introductions of Latin American (LA) germplasm to Africa suffers from susceptibility to CMD (Okogbenin et al. 1998), the most widespread cassava disease of economic importance in Africa. CMD does not occur in the Neotropics and its begomoviruses are unknown in the Americas. A MAS program to incorporate CMD resistance genes into LA germplasm began in 2001 and led to the shipment of CMD resistant germplasm from CIAT to Tanzania, Nigeria, Ghana, Uganda, and India.

We present results of direct evaluation of CIAT introductions in the field in Nigeria. We also describe the different stages of evaluation of selections from the initial introductions, including national pre-release trials in 8 locations spread across the major cassava growing agro-ecological zones.

## Methodology

*Introduction and evaluation of CIAT germplasm*
Germplasm was introduced as *in vitro* cultures with inspection by the Nigerian plant Quarantine service (PQS). Plants certified by PQS were hardened and planted in the filed. A total of 156 genotypes CMD resistant germplasm were introduced in 2004 and evaluated at the Nigerian Starch Mills (NSM) over 2 seasons for response to diseases and yield. First year evaluation was 8 plants per genotype and second year was between 30 to 100 plants per genotype in replicated trials. Another set of 138 genotypes was received from CIAT in 2006 and has also been planted in the field at NRCRI in preparation and is currently being evaluated.

Selections from two years of evaluation at NSM were introduced into the regional trials also known as the Nationally Coordinated Research Projects (NCRP) in Nigeria. It involves distribution of varieties to be tested to partners in Universities, National Agricultural programs that have breeding, agronomy or crop science programs and IITA Ibadan. The coordinating agency in Nigeria is the National Root Crops Research Institute (NRCRI), Umudike. Between June – July 2006, a total of 13 varieties (Table 2) were established in replicated trials in a randomized complete block design in 8 locations (Table 1) mostly in the south and central zones spanning the agro-ecological zones of the major producing areas in Nigeria. Data to be collected include response to pests and diseases especially CMD and cassava green mite, yield, dry matter and other yield components at various stages of plant growth. Each partner manages their own data collection and field maintenance. At the end of the cropping season the trials would be harvested.

Table 1: List of locations, agro-ecological zones and partners in the cassava pre-release trials in Nigeria

| S/No. | Location | Agro-ecological zone | Managing Institute |
|---|---|---|---|
| 1 | Abakaliki | Forest-savanna transition | Ebonyi State University |
| 2 | Ibadan | Forest-savanna transition | International Institute of Tropical Agriculture |
| 3 | Ikenne | Humid forest | Institute of Agricultural Research & Training, Obafemi Awolowo University |
| 4 | Minna | Southern Guinea savanna | Federal University of Technology |
| 5 | Otobi | Southern Guinea savanna | National Root Crops Research Institute |
| 6 | Umudike | Humid forest | National Root Crops Research Institute |
| 7 | Uyo | Humid forest | University of Uyo |
| 8 | Zaria | Northern Guinea savanna | Institute of Agricultural Research |

## Results

*Response to CMD in CIAT introductions*
Results of field evaluation of the CIAT introductions in 2005/2006 indicate that about 68% of the 2005 introduced germplasm selected with markers for CMD showed resistance to the disease (Table 2). The results indicate the efficiency of markers for CMD selection at CIAT. Seedling nursery results for crosses of pure Latin American parents (high beta carotene lines) were 100% susceptible to CMD.

*(b) Yield of CIAT introductions*
Table 3 shows yield of CIAT introductions at 8MAP. The results suggest that these materials are early yielding, an important trait desired by farmers. Three genotypes (CR 14A-1, CR 41-10, and CR 38-3) of the introduced germplasm from CIAT with good yields have been included in the uniform yield trial (UYT) at NRCRI and in national coordinated regional trails (NCRP). Ten other clones are also being evaluated in the uniform yield trial stage. The clones are CR 36-5, CR36-2, CR42-4, CR 12-45, CR 52A-41, AR 15-5, AR1-82, AR 37-108, CR 52A-25 and CR26-1. Both trials represent last stages in the evaluation scheme prior to varietal release.

Table 2. Disease rating of 156 genotypes introduced as cultures in 2006

| CMD index severity | # genotypes | % |
|---|---|---|
| 1 | 84 | 53.8 |
| 2 | 21 | 13.5 |
| 3 | 31 | 19.8 |
| 4 | 19 | 12.2 |
| 5 | 1 | 0.6 |

Note: Disease rating in classes and 2 are considered as resistant

Table 3. Yield of some cassava genotypes with the gene *CMD2* harvested at 8 months after planting at the Nigerian Starch Mills farm in Opuoma and advanced to UYT in 2006

| Genotypes | CMD Severity index | Yield (8MAP) |
|---|---|---|
| AR 38-3 | 1 | 33.2 |
| CR14A-1 | 1 | 37 |
| AR37-108 | 1 | 14 |
| CR36-2 | 1 | 20 |
| CR36-5 | 1 | 31.8 |
| CR52A-25 | 1 | 35.4 |
| CR41-10 | 1 | 46 |
| CR42-4 | 1 | 33.3 |
| CR26-1 | 1 | 34.3 |
| AR12-45 | 1 | 40.3 |
| AR1-82 | 1 | 33 |
| AR15-5 | 1 | 26.7 |
| CR52A-41 | 1 | 32 |
| CR52A-22 | 1 | 41.3 |

*Nationally Coordinated Research Projects (NCRP) trials*

Evaluation of the CIAT introductions to pests and diseases in the NRCRP trials at 8 locations is ongoing. Preliminary data collected in single location trials in Nigeria in 2005 indicated the promising performance of the selected genotypes (Table 4). The trials will be harvested 12 months after planting at the various locations. There would be a repeat trial in the different locations as well as on-farm adaptive trials for assessment by selected farmers in different parts of of Nigeria. Varieties selected after a meeting with all stakeholders will be submitted for release to Nigeria's Plant Varietal Release Committee most probably in late 2008.

Table 4: List of genotypes in the NRCRP trial and response to CMD and potential yield estimates.

| S/No. | Genotype | Source | CMD* response | Yield (t/ha) | Harvest index |
|---|---|---|---|---|---|
| 1 | CR 14A-1 | CIAT | 1.00 | 37.0 | 0.76 |
| 2 | CR 41-10 | CIAT | 1.00 | 46.0 | 0.77 |
| 3 | AR 38-3 | CIAT | 1.00 | 33.2 | 0.70 |
| 4 | NR 01/0004 | NRCRI | 1.33 | 26.3 | 0.68 |
| 5 | NR 01/0161 | NRCRI | 2.66 | 23.5 | 0.66 |
| 6 | NR 01/0071 | NRCRI | 2.50 | 26.1 | 0.67 |
| 7 | TMS 01/1086 | IITA | - | - | - |
| 8 | TMS 00/0210 | IITA | - | - | - |
| 9 | TMS 00/0203 | IITA | - | - | - |
| 10 | TMS 00/0214 | IITA | - | - | - |
| 11 | TMS 01/0040 | IITA | - | - | - |
| 12 | TMS 30572 | National Check | 3.40 | 21.5 | 0.68 |
| 13 | Local best | Depends on locality | - | - | - |

*CMD severity is measured on a scale of 1 – 5, where 1 = no apparent symptoms and 5 = very severe infection. - data not available

**Conclusion and perspectives**

MAS for breeding CMD resistance in Latin American cassava provide an opportunity to utilize directly Latin American cassava germplasm in Africa. This is the first time in Africa that LA cassava germplasm are being successfully deployed in Africa without previous breeding in Africa. It serves as a first step and a model for adoption in other African countries where cassava is important for food security and poverty alleviation. The *CMD2* gene was introgressed into LA germplasm with very high yields and dry matter content as well as tolerance to acid soils which are common in sub Saharan Africa. Successful release of these germplasm and those that may follow presents a great opportunity for African farmers to exploit the vast cassava genetic resources abundant in LA. Similar efforts are underway in Ghana and Uganda using the same set of varieties. It also provides evidence on the potential of breeding for CMD resistance using markers alone should the disease finds its way into LA.

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## 1.2.12 Controlling Delayed post harvest physiological deterioration in cassava

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Funding: GCP; Bio Cassava Plus, CIAT

### Introduction

A new source of genes for dramatically delayed post-harvest physiological deterioration was identified in an interspecific hybrid between cassava and a wild relative *Manihot walkerae* at the International Center for Tropical Agriculture (CIAT) in Colombia. This inter-specific hybrid (CW 429-1) has been used to develop first backcross generation families as the first step in an advanced backcross scheme (Tanksley and Nelson, 1996) to transfer the delayed PPD into cassava gene pools. We report here the progress made in the evaluation of the $BC_1$ families and subsequent crosses to cassava.

### Methodology

The PPD status of the inter-specific hybrid CW 429-1 and 8 elite cultivars with the widest variation for delayed PPD was evaluated in replicated trials in two environments, namely CORPOICA, Palmira and CIAT station Santander de Quilichao, in order to compare delayed PPD in the inter-specific hybrid with known levels in the cultivated gene pool. PPD quantification was done using the method of Wheatley et al. (1985) with a slight modification. Three evaluations were made on the 5th, 10th and 15th day after harvest (DAH) with five roots each. At each evaluation, seven 2-cm thick transversal cuts were made all along the root from the proximal end. A score, corresponding to the percentage of cut surface showing discoloration, of 0 – 10 (0=0%, 1=10%, 2=20%, etc.), was assigned to each slice. The mean PPD score for each root was calculated by averaging the score from 7 slices.

A half-sib backcross population ($BC_1$) was developed using CW 429-1 as the donor parent and two elite genotypes (MTAI 8 and SM 909-25) as recurrent parent. The $BC_1$ families will be used to identify molecular markers associated with delayed PPD and also to begin the process of transfer of the genes for delayed PPD into the cultivate gene pool. Another $BC_1$ family made up of 250 sexual seeds from open-pollination of CW 429-1 was also developed. The $BC_1$ population numbering 405 genotypes was established *in vitro* from embryo axes, micro-propagated and 4-10 plants per genotype hardened in the screen house and established in the field. Harvesting and PPD evaluation of the $BC_1$ population has commenced. Due to insufficient number of roots, the protocol for PPD evaluation was slightly modified to two evaluations on the $7^{th}$ and $14^{th}$ day after harvest.

The $BC_1$ population will also be genotyped with SSR markers to map genes for delayed PPD as well as to select progenies with minimum M. walkerae genome during for further back crossing (reduction of linkage drag). For molecular marker analysis genomic DNA was isolated from young leaves of the parental genotypes and 405 $BC_1$ genotypes according to Dellaporta et al. (1983). The DNA samples from the two parents were surveyed for polymorphism using 530 SSR markers, selected on a genome-wide basis, as described by Mba et al. 2000. PCR amplification products were mixed with 10 µl of loading dye, denatured for 5 min at 95°C and chilled on ice, loaded onto 4% polyacrylamide gel. Electrophoresis was in 1 X TBE at 80 V for 1hr 30 min. Amplified DNA fragments were visualized by silver staining.

**Results**

Evaluation of the inter-specific hybrid CW 429-1 revealed that novel delayed PPD in the *M. walkerae* has been transferred to the hybrid. Results of mean PPD values at 5 days after harvest (DAH) ranged from 0% in CW 429-1 and MBRA 337 to 44.85% in CM 523-7 (Table 1). At 10 DAH, mean values ranged from 0% in CW429-1 to 58% in CM 523-7, respectively. The same trend was observed 15 DAH with CW 429-1 still displaying no visible sign of deterioration (Figure 1). Previous evaluation of delayed PPD showed nothing better than reduction at the 7th DAH (Sanchez et al. 2005).

Of the 450 SSR markers surveyed, 243 (54%) of them detected polymorphism between the parents of the $BC_1$ family obtained from controlled crosses or detected 2 alleles in CW429-1. These markers are currently being used to genotype the 405 $BC_1$ progenies for identification of genes associated with delayed PPD (Figure 2).

Even though phenotypic evaluation of the $BC_1$ family is still ongoing, we have identified a good number of $BC_1$ genotypes with resistance at the $14^{th}$ DAH. These include BIPD 280-18, BIPD 280-19, BIPD 280-14, BIPD 280-21, and etc. Evaluation is still in progress since harvesting is being done in small fractions in order to be able to effectively manage the large population size. We have also started crossing $BC_1$ genotypes with delayed PPD to elite cassava lines to generate $BC_2$ families

Table 1: Mean scores for post-harvest physiological deterioration (PPD) of the inter-specific CW 429-1 and 8 other elite varieties.

| Genotype | Previous PPD status | Root colour | Dry matter (%) | PPD 5 DAH (%) | PPD 10 DAH (%) | PPD 15 DAH (%) | Mean PPD scores (%) |
|---|---|---|---|---|---|---|---|
| CW 429-1 | Inter-specific | white | 28.15 | 0.00 | 0.00 | 0.00 | 0.00 |
| MTAI 8 | Susceptible | cream | 41.49 | 33.70 | 42.30 | 22.85 | 32.95 |
| CM 523-7 | Susceptible | white | 39.37 | 44.85 | 57.99 | 52.86 | 51.90 |
| HMC-1 | Susceptible | white | 36.34 | 37.43 | 35.40 | 30.86 | 34.56 |
| MCOL1505 | Susceptible | white | 35.07 | 26.57 | 29.70 | 27.14 | 27.81 |
| MBRA 337 | Resistant | yellow | 21.33 | 0.00 | 22.00 | 6.43 | 7.76 |
| MPER 183 | Susceptible | white | 32.12 | 39.71 | 19.60 | 45.71 | 36.19 |
| CM 2772-3 | Resistant | yellow | 34.31 | 24.85 | 18.90 | -* | 22.43 |
| MCOL 2279 | Resistant | cream | 24.39 | 12.86 | 30.00 | 7.14 | 11.27 |
| LSD (P=0.05) | | | 2.12 | 26.84 | 31.68 | 25.27 | 16.99 |

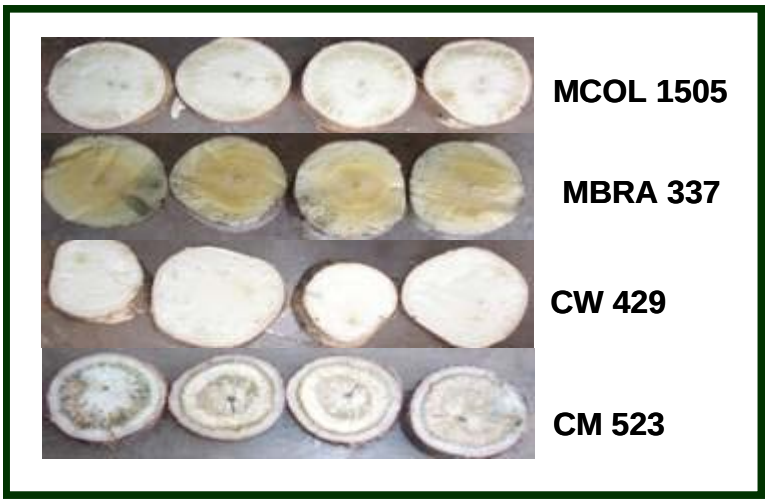


Figure 1: Delayed post-harvest physiological deterioration (PPD) in the inter-specific hybrid CW 429-1, 14 days after harvest.

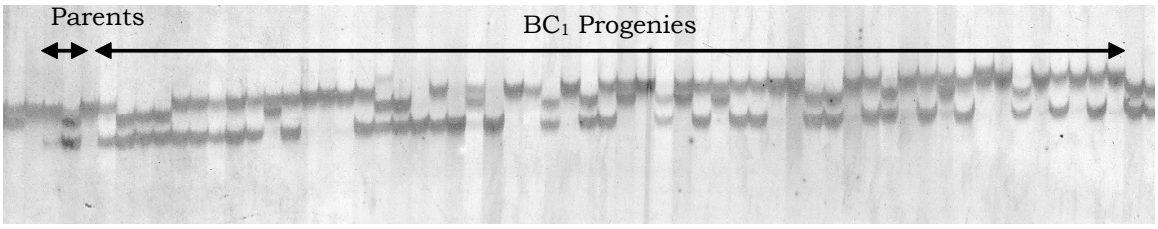


Figure 2: Silver stained PAGE gel of PCR amplification product parents and part of the progeny of a $BC_1$ derivative of CW429-1.

**Conclusion and perspectives**

This study confirms introgression of delayed PPD genes into cassava as expressed in an inter-specific hybrid, CW 429-1. The stage is set for use of this new resource in cassava breeding. The discovery of delayed PPD in cassava from crosses with a wild relative further underlies the usefulness of genes from wild species for use in cassava.

Currently, $BC_1$ mapping populations of 405 individual genotypes derived from the inter-specific hybrid are being evaluated for PPD and with data being derived from genotyping will be used in linkage and QTL analyses for the identification of markers associated with genes controlling the trait. Successful identification of molecular markers linked to this trait will facilitate its breeding through marker-assisted selection (MAS).

$BC_1$ genotypes with delayed PPD further crossed to elite parental lines will be widely distributed to national programs in Africa, Asia and Latin America for introgression into local adapted germplasm. Other future plans also include the mapping of candidate genes for delayed PPD, based on previous molecular studies of the trait, to increase the power of genetic mapping of delayed PPD.

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## 1.2.13 Genetic Mapping of Quantitative Trait Loci (QTL) Controlling High Protein Content in the Primary Gene Pool of Cassava (*Manihot esculenta* Crantz) and its Utilization in Cassava Breeding

Olalekan Akinbo, Javier Lopez, Theresa Sanchez, and Martin Fregene (CIAT)

Funding: Rockefeller Foundation and CIAT

### Introduction

A modified advanced back crossing QTL (ABC-QTL) introgression scheme is being used transfer high root parenchyma protein content from accessions of *M.esculenta* sub *spp flabelifolia*, *M.esculenta* sub spp *peruviana* and *M.tristis*, with between 10-18% (dry weight basis) protein in the storage roots, to cassava. Three $BC_1$ mapping populations for high protein content were established in 2004 from embryo axes, micro-propagated, hardened, and transferred to a single row trial in the field at ICA-Corpoica, Palmira in August 2005. In 2006 the mapping populations were harvested and planted in replicated trials at CIAT Head quarters in preparation for evaluation of the high protein content trait. We describe here progress in the advanced back crossing QTL (ABC-QTL) introgression scheme for high protein content.

### Methodology

Field evaluations in 2000 of 579 accessions of *M. esculenta* sub *spp*. *flabelifolia, M.trisitis, M. esculenta* sub *spp*. *peruviana* identified over 100 genotypes with high root parenchyma protein content (CIAT 2001). Inter specific $F_1$ hybrids were obtained by crossing selected wild accessions to elite cassava lines and hybrids evaluated for protein content. Three high protein content 3 $F_1$ genotypes with good root size were crossed to a cassava variety, MTAI8 (Table 1) to obtain first backcross ($BC_1$) populations, between 200-250 genotypes per family was obtained. The $BC_1$ were established in vitro from embryo axes, micro-propagated to obtain 8-10 plants per genotype in 2004. Sexual seeds ($BC_1$) derived from embryo axes, were hardened in the screen house and later planted in the field in 2005 in single rows (SRT) to generate

sufficient planting materials for replicated trials in the second year. In July 2006 the 3 $BC_1$ mapping populations were planted in a randomized complete block design of 8 plants per genotype replicated three times at CIAT, Colombia. Planting space was 0.8 m x 1.6 m.

Molecular marker analysis of parental lines of the $BC_1$ population was using over 530 SSR markers available at CIAT. The parents and 10 selected genotypes from the $BC_1$ were screened for polymorphism (Figure 1). Markers found to be polymorphic in the parental genotypes will be used to genotype the entire mapping population.

**Results**

An earlier evaluation of root protein content in 579 wild accessions revealed that wild genotypes OW 230 -3, OW 231-3, OW 280-2, OW 132-2, and OW 284-1 had high root protein content of between 10 and 18% (fresh weight basis) and good root size. Some $F_1$ hybrids from these accessions had protein content of over 10% (fresh weight basis) and were crossed to the variety MTAI 8 to give a total of 657 genotypes organized into 3 families. The $BC_1$ families will be evaluated later this year for high root protein content in a randomized complete block design experiment in three replications with 8 plants per row per genotype.

Three hundred and seventy three out of 530 SSR markers were found to be polymorphic in the parental screening. These markers are being used for the genotyping of the mapping population. (Figure 2).

**Conclusion and perspectives**

Future perspectives include QTL mapping of root protein content in the $BC_1$ population and selection of BC1 individuals for crosses to elite cassava lines with high beta carotene and CMD resistance to generate $BC_2$ families. Other activities include establishment from embryo axes and distribution $BC_2$ genotypes selected by MAS for high root protein content, high beta carotene content, and CMD resistance for distribution to African countries where the crop is an important food staple.

Table 1. List of $F_1$ inter-specific hybrids with high protein content from which the $BC_1$ parents were selected

| Family name | Wild parent | F1 inter-specific hybrid | BC1 rec. parent | SEED NO | % Protein content of F1 hybrid parent |
|---|---|---|---|---|---|
| B1P2 | OW 230 -3 | CW 198-11 | MTAI 8 | 227 | 11.28 |
| B1P5 | OW 231-3 | CW 205-2 | MTAI 8 | 209 | 10.54 |
| B1P6 | OW 230-3 | CW 201-2 | MTAI 8 | 221 | 10.2 |
| Total | | | | 657 | |

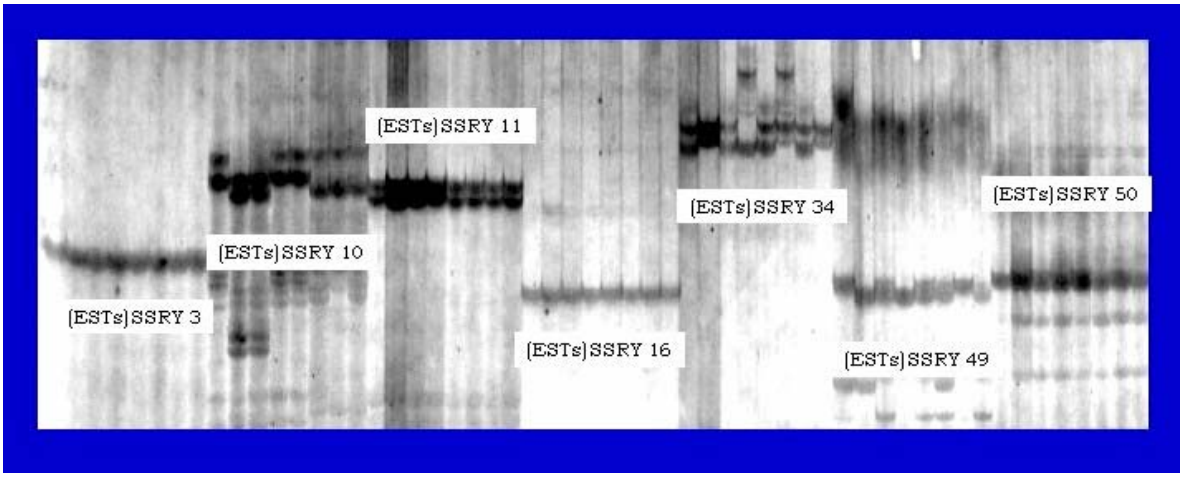


Figure 1: Silver stained PAGE gel of PCR amplification product of parents 10 $BC_1$ progeny of SSR markers being used in a parental screen for polymorphic markers

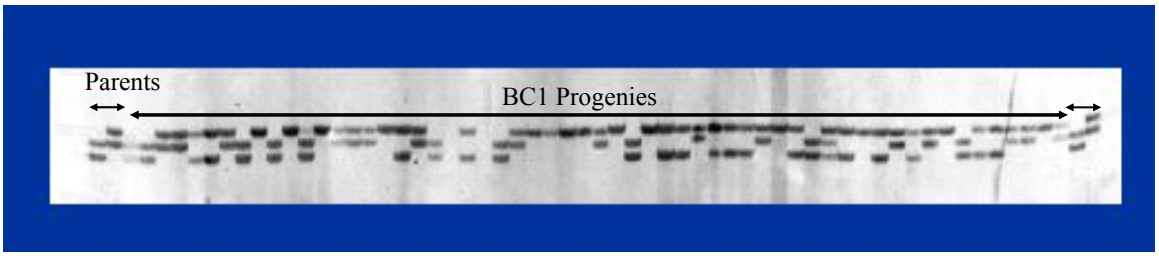


Figure 2: Silver stained PAGE gel of PCR amplification product of parents and part of the $BC_1$ population for QTL mapping of high root protein content

## 1.2 14 Activities in the GCP Project 'Development of Low Cost Technologies for Pyramiding Useful Genes from Wild Relatives of Cassava into Elite Progenitors' at NRCRI, Umudike, Nigeria

C. Egesi, E. Okogbenin, and M. Fregene

Funding: GCP and IAEA

### Introduction

Nigeria is the world's largest cassava producer and its cassava transformation is the most advanced in Africa. The scope for increasing the use of cassava in industries is, to a large extent, determined by the development of improved cassava varieties with high quality traits required by domestic industries and export market. Identification and creation of useful germplasm for the genetic improvement of novel quality traits is important in developing improved cassava varieties of high quality.

Improving the root quality traits of cassava is a key step in the development of value-added cassava. This will enhance its competitive ability with other crops that readily offer alternative sources of carbohydrates in the industrial sector. The major constraint of cassava production is its high susceptibility to pests and diseases amongst which the cassava mosaic disease and cassava green mite are the most important. This crop is mainly grown for its starchy roots which unfortunately are low in protein and beta carotene. In addition the roots on harvest don't store very well since post harvest physiological deterioration sets in immediately leading to severe root losses to farmers.

Genetic variation for these traits in germplasm collections of many breeding is highly limited. Breeding to address these problems have been slow due to limited genetic variation in existing gene pools of breeding programs and partly to its highly heterozygous nature This project, seeks to explore useful genetic variation in wild relatives to breed for these traits. This project therefore seeks to develop new improved cassava varieties for improved quality traits, resistance to disease and pests, and high yield through improved low cost technologies for efficient molecular breeding via MAS.

**Methodology**

In 2005, cassava seeds generated at CIAT from beta carotene rich varieties were used introduced to NRCRI and established in a seedling nursery for the evaluation of pulp color. Yellow pulp color has been found to be associated with beta carotene. The seedling nursery was evaluated in the 2005/06 planting cropping season.

Plant materials received from CIAT which on evaluation were found to have high beta carotene were selected crosses to adapted African germplasm as part of activities to introgress useful genes into Africa. Crosses for this purpose commenced in September 2006. A total of 122 genotypes of CIAT germplasm are being used for this exercise. These genotypes are being used in crosses with 52 varieties comprising elite clones developed at NRCRI and IITA as well as farmer preferred varieties. The LA materials are being used as female parents. Open pollinated seeds will also be collected and used in the NRCRI breeding program.

*Introduction and evaluation of IAEA irradiated germplasm*
Germplasm were introduced for this project from IAEA, Austria. Irradiated germplasm of *in vitro* plantlets of two varieties (SM 909-25 and Col 2215) for different dosages 10Gy, 15Gy, 20 Gy and 30 Gy) of gamma radiation were introduced into Nigeria. A set of 108 plants of the IAEA introduction were hardened and were planted on the field for evaluation for cassava mosaic disease and quality traits. The rating for reaction to cassava mosaic disease (CMD) was based on the standard five class scoring method: class 1 = no symptoms to class 5 = severe mosaic distortion of in the entire leaf. The materials were planted at 1m x1m. The germplasm was harvested at 12 MAP in November 2006. Several quality traits were analyzed in the IAEA germplasm. They include % peel loss, cortex/peel ratio, ease of peeling fresh root, color of parenchyma, boiled color, taste, carotenoid, mealiness, dry matter content and % starch. The germplasm was also evaluated for yield

*Diversity study*
To improve parent selection in breeding program there is a need to assess genetic diversity, using molecular markers, elite cassava genotypes used as parents in breeding programs. To this end, cassava germplasm, local and improved accessions, at NRCRI and other groups of clones from the International Institute of Tropical Agriculture (IITA) and the International center for Tropical Agriculture (CIAT) were used in this study. The local accessions represent the diverse Nigerian agro-ecologies from where they were collected, viz, rain forest, Guinea savanna, Sudan savanna and derived savanna. Eighty cassava genotypes (32 local varieties, 10 improved from NRCRI, 12 from IITA and 26 from CIAT) were used for

DNA isolation. A set of 36 SSR primers with wide genome coverage was selected for diversity analysis. PCR amplified products were separated on PAGE gels as described by Mba et al 2001.

*MAS for early bulking*
Early bulking is an important trait in cassava given the crop's long growth cycle. Combining early bulking with root quality traits and disease resistance is one of the breeding program objectives in Africa. Early bulking mapping populations for identification of markers were developed from six parents - three early bulking (TMS 30572, TMS 97/2205 and TMS 98/0505) and late bulking (TMS 30555, NR 8212 and NR 8083) parents. A seedling nursery of the mapping populations was established, harvested and replanted for evaluation of early bulking.

**Results**

*(a) Qualitative assessment of beta carotene*
The seedling nursery of beta carotene rich genotypes was evaluated for root parenchyma color and results indicate that 87% of the germplasm had cream pulp color and 10% were yellow and 3% were white. The high percentage of cream and relatively low percentage of yellow and white pulp color in the nursery indicate the recessive gene action of high beta carotene content

*(b) Introgression of CIAT germplasm with African elite cultivars*
Crosses between CIAT and African cassava germplasm are crucial to the development of new varieties combining attributes of both germplasm. So far about 7,000 seeds have been generated from controlled crosses. Several thousand of seeds have also been collected from open pollination. Seeds are still been collected from the field. The newly developed materials will be grown and via MAS promising materials will be selected for evaluation in the breeding programs in Africa

2. IAEA *germplasm*
The introductions from IAEA were evaluated primarily for CMD. Results indicate that the IAEA materials which were of LA origin were highly susceptible to CMD. To address this constraint local materials are now been used for irradiation. These materials are well-adapted to the humid, sub-humid and savanna ecologies of Nigeria; and are highly desired for their good yield and resistance to pest and disease. The use of these local materials for irradiation is expected to rapidly result in the development useful mutants as varieties by farmers including the prospect of improved chance of adoption by farmers.

Yield data from the IAEA germplasm was based on data collected on one plant per genotype and it ranged from 0.1-5.0 kg per plant. The IAEA plant materials will be further assessed in subsequent yield trials. The materials have been replanted in a replicated trial (RCBD of two replications).

Results from quality traits analyzed showed interesting data for carotenoid analysis where five genotypes showed high carotenoid content above 100ug/100g fresh weight of roots (Figure 1). The highest carotene content observed was 237.27 ug/100g. Some 12 genotypes also showed high dry matter percentage above 40%. These candidate genotypes will be explored further in subsequent analysis as likely useful mutants for our breeding program. Variation in data for other quality traits were observed, but we have not detected significant values for those traits in the IAEA germplasm. The IAEA germplasm will be selfed to identify recessive traits.

*Diversity study of Nigerian cultivars*
Twenty five primers were evaluated in 80 genotypes selected for the diversity study. Except for SSRY 110 which was monomorphic, all the markers assessed were polymorphic. Markers revealed alleles varying from 2 to 12 (Figure 2) which reflects the heterozygosity found in cassava. Preliminary analysis

indicates high genetic variation in the Latin American accessions included in the study. This study will be concluded in March 2007 with the genotyping of accessions with additional 11 markers.

*Early bulking*

Evaluation of the parents used in early bulking population showed that there was significant difference in yield between the early bulking and late bulking parents. Early yielding parents had a mean yield of 15-17 t/ha while the late yielding had mean yield of 5-9 t/ha. All the populations showed segregation for yield and would be used for bulk segregant analysis to identify markers linked to this trait after field evaluations in the second year.

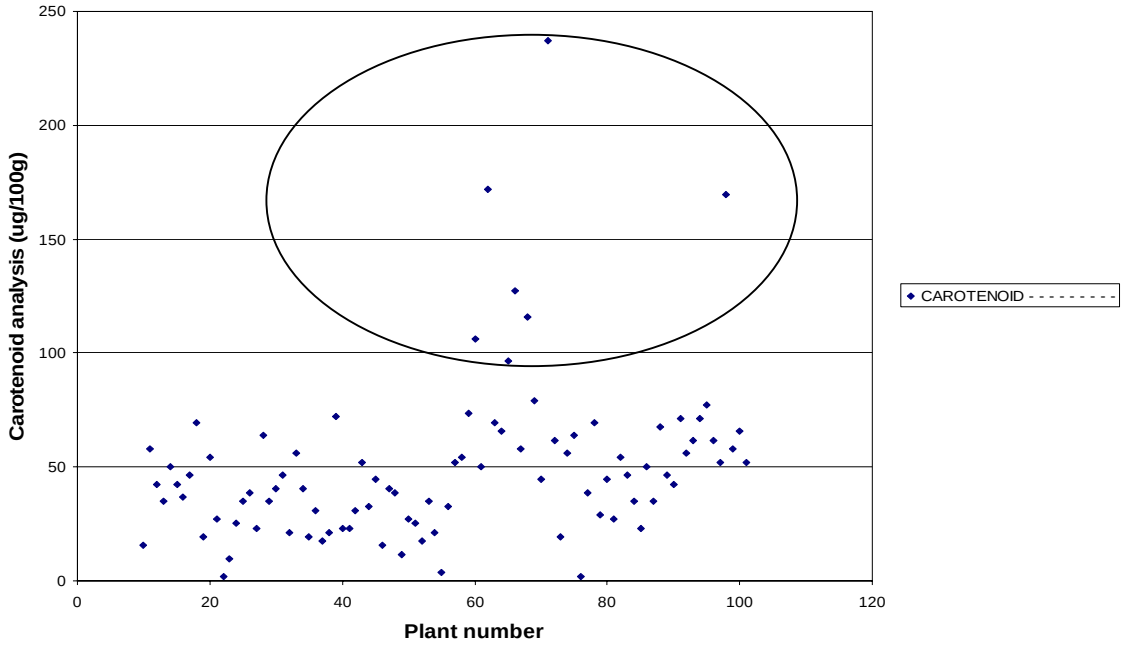


Figure 1. Carotenoid content distribution in the irradiated germplasm received from IAEA with six clones (in the circle) showing high dry matter content above 40%

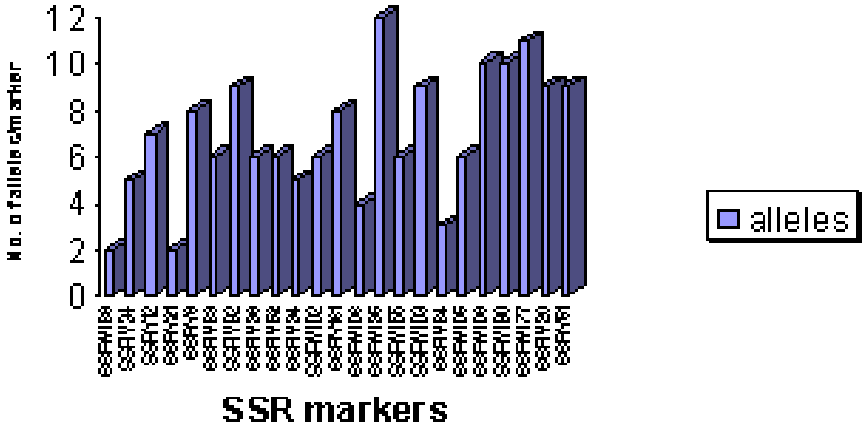


Figure 2 Distribution of alleles per SSR marker in a set of 80 African local and improved cassava accessions

### 1.2.15 Genetic mapping of multiple sources of resistance to the cassava mosaic disease (CMD)

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Funding: Rockefeller foundation

**Introduction**

The cassava mosaic disease (CMD) has proven to be a shifty, changing, constantly evolving enemy of cassava production in Africa capable of wreaking havoc on livelihoods of rural communities that rely on cassava in Africa. However, certain land races of cassava from several African countries have been shown to possess very high levels of resistance to CMD, a total of seven sources of natural resistance have been described (Dixon pers comm. 2004; Akano et al. 2002). These sources of natural resistance represent a very important resource in the struggle to contain cassava mosaic begomo- viruses that have shown a remarkable ability to recombine into more virulent forms. A project, funded by the Rockefeller Foundation at IITA and CIAT seeks to tag and pyramid multiple sources of CMD resistance via molecular marker-assisted selection (MAS) into elite cassava varieties to enable cassava farmers with durable resistance remain one step ahead of the CMD scourge. Markers developed by the project will be indispensable in combining different sources of genes that are undistinguishable by their phenotypes alone. This project will also initiate a molecular marker-assisted pyramiding of resistance genes into elite parental lines for use in different MAS projects working on the development of durable CMD resistance ongoing in several African countries.

**Methodology**

For CMD resistance, there are seven putative sources of which two have already been mapped (Akano et al 20002; Fregene et al. 2006). Populations for the putative 5 additional sources are $S_1$ and $F_1$ crosses from TME1, TME419, TME225, and half-sib crosses of TME279 and 96/1089A. These populations range from 150 to 500 individuals in size and are currently in a seedling trial (one plant per genotype) at

IITA Ibadan. They will be planted in a single row trial in Ibadan beginning June 2007 for evaluation of resistance to CMD.

Given the large number of populations to be mapped for the identification of multiple sources of genes, there is a need to develop high throughput genotyping in this project. We have therefore initiated in collaboration with 2 advanced research institutes (ARIs) the development of SNP marker resources for high throughput gene tagging and molecular marker-assisted selection (MAS) for pyramiding multiple sources of CMD resistance genes. Activities include construction of a physical map of the cassava genome by fingerprinting 70,000 BAC clones, build a minimal tiling path (MTP) of the cassava genome and sequence the ends of 9,000 clones distributed throughout the genome. This is part of a new GCP project.

Selected sequences will be re-sequenced in 10 cassava genotypes that represent diversity in global cassava genetic resources, namely : TMS30572 and CM2177-1, which are the parental genotypes of the segregating F1 population used to construct the first genetic map of cassava, TME3, the source of the BAC library, MTAI1, MNG19, MCOL22, MGUA76, MBRA12, MVEN309, and MMEX17.
Primer pairs will be designed from the EST and BAC-end sequences using the program Primer3 (http://frodo.wi.mit.edu/primer3/primer3_code.html). Primers, already arrayed in 96-well plates, will be purchased (from Invitrogen, CA) and PCR reactions carried out in 96-well plates. High fidelity sequences will be obtained from the 10 cassava genotypes and compared to the reference BAC-end sequences using sequence alignment software such as MUMmer (http://mummer.sourceforge.net) or BLAST (Altschul et al. 1997).

We anticipate being able to identify and validate approximately 2000 SNP sites suitable for genetic mapping. For each SNP, an additional primer will be designed immediately adjacent (5' or 3') to the polymorphic nucleotide. This primer will be used for single base extension into the SNP site, which is the molecular basis of the FP-TDI assay (see below). The FP-TDI assay is a single-base extension assay for genotyping SNP markers. This assay involves the use of Taq polymerase to extend by one base an oligonucleotide primer that ends immediately upstream of the SNP position, using one of two fluorescent dye-labeled terminators. The identity of the base added is then determined by the increased fluorescence polarization of its linked dye.

The high specificity and sensitivity of FP-TDI allows high-throughput SNP genotyping in 384-well plate format. In addition we will emphasize transfer of this technology by training of partners in the use of high throughput SNP marker systems.

**Results**

Several $F_1$ and $S_1$ populations have been produced and established in the field directly from sexual seeds at IITA Ibadan between May and July 2006. They will be cloned in the 2007 growing season and planted at Ibadan and Onne, a high CMD pressure site.

Genotyping of the mapping populations: We expect to phenotype and genotype one $F_1$ and $S_1$ population for the 5 unmapped sources of resistance to CMD, this is a total of 10 populations. Assuming the minimum size of 200 genotypes per population and 150 markers to make a map of each population, this is a total of 300,000 molecular marker data points. CIAT, IITA, and ACGT, at the University of Pretoria will be responsible for generating this data.

Polymorphic markers and CMD resistance will be evaluated in individuals of the mapping populations and strength of association between markers and traits measured by simple regression or the interval QTL mapping approach (Lincoln et al. 1992; Liu 1998).

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## 1.2.16 Progress in the Genetic Mapping of dry matter content in cassava

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CIAT

Funding: Rockefeller Foundation, CIAT

**Introduction**

In 2002 a project was initiated to develop molecular markers for dry matter content (DMC) based upon initial quantitative genetic analysis of the trait. SCA and GCA estimates from an earlier genetic analysis diallel experiment (CIAT, 2003) were used to select families for bulked segregant analysis (BSA) of dry matter content (DMC). A marker was found to be associated with DMC in the selected families having SM 1741-1 as parent. Based upon the above results larger populations were generated for QTL analysis. More than 1500 $F_1$ seedlings from 8 families (CIAT, 2004) were established in the field and evaluated for DMC. Cuttings from the seedlings were used to establish a clonal trial. DMC data from the seedling evaluation was used to initiate bulked segregant analysis (BSA) in the larger population. We describe here further evaluation of the $F_1$ families and selections to develop additional crosses to test the utility of markers identified earlier for dry matter content.

**Methodology**

A seedling nursery consisting of 8 crosses derived from parents selected from the three major cassava growing areas- mid-altitude, low land semi-arid and acid savannah for dry matter and yield related agronomic traits was evaluated for dry matter content in May, 2005. Dry matter content (DMC) of the tubers was estimated using the standard CIAT procedure. The stems from individual plants were stored separately to provide the planting material for the $F_1$ clonal evaluation. In July 2005, the $F_1$ clonal evaluation trial was established from the stored stems. Of the 8 families available, three families (GM 252, GM 901 and CM 9953) with genotypes greater than 200 were replicated, the rest were planted as single row plots. A total of 220, 147 and 91 genotypes from families GM 252, GM 901 and CM 9953 were established as a replicated trial (4reps, 4 plant plots) and 41, 64 and 49 as a 3 rep, 4 plant plots trials respectively. Overall 261, 211 and 150 genotypes of GM 252, GM 901 and CM 9953 respectively were established as replicated trials. Plants were planted on ridges on spacing of 1.6 by 0.7 m, the spacing being mainly to reduce inter-genotype and increase intra-genotype competition.

Plants were harvested in early March 2006 and a selection was done based on evaluations conducted in the 2005/2006 season. Because of a the long delay before planting in the 2006/2007, a period of 3 and a half

months, a considerable amount of selections were lost. However, in late July 2006 a new trial was established with the selected $F_1$ clones.

Results

In the 2004/2005 season, 1236 genotypes from 8 families were harvested. These genotypes were re-planted in replicated trials in 2005/2006. Harvest data was averaged per genotype and family to give an indication of a family's performance (Table 1). Selection based on dry matter content, as a percentage of materials in the field in 2006/2007 with respect to the total number of harvested materials in 2005/2006 are also shown in Table 1.

Table 1 Means and Standard Deviations of root quality characteristics of 7 families evaluated at harvest in CIAT, Palmira in March, 2006 and percentage of selected materials planted in 2006-2007 season

| FAMILY | PARENTAGE | V A R I A B L E | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | HI | Yield | DM | Dyield | TbWt | selected % |
| CM 9953- | SM1219-9 x SM1741-1 | Average | 54.38 | 22.43 | 34.64 | 7.98 | 0.36 | 16.1 |
| CM 9953- | | StdDev | 10.84 | 11.83 | 3.54 | 4.42 | 0.12 | |
| CM 9958- | SM1411-5 x MTAI-8 | Average | 45.16 | 16.05 | 38.84 | 6.32 | 0.21 | |
| CM 9958- | | StdDev | 9.03 | 8.88 | 5.15 | 3.55 | 0.07 | |
| GM 252- | SM1665-2 x SM805-15 | Average | 54.78 | 15.55 | 33.40 | 5.29 | 0.37 | 7.7 |
| GM 252- | | StdDev | 12.33 | 9.49 | 2.71 | 3.38 | 0.16 | |
| GM 256- | SM1219-9 x SM1565-15 | Average | 51.38 | 22.98 | 35.90 | 8.32 | 0.35 | |
| GM 256- | | StdDev | 9.87 | 10.35 | 2.81 | 3.77 | 0.17 | |
| GM 536- | SM1565-15 x CM4574-7 | Average | 43.01 | 17.26 | 35.80 | 6.24 | 0.27 | 6.0 |
| GM 536- | | StdDev | 9.11 | 8.09 | 2.87 | 3.06 | 0.10 | |
| GM 853- | CM8027-3 x CM6754-8 | Average | 42.49 | 10.05 | 35.37 | 3.58 | 0.23 | |
| GM 853- | | StdDev | 11.01 | 5.86 | 3.06 | 2.25 | 0.10 | |
| GM 901- | SM1741-1 x MPER183 | Average | 56.31 | 23.32 | 33.08 | 7.89 | 0.44 | 24.8 |
| GM 901- | | StdDev | 10.90 | 12.90 | 2.89 | 4.60 | 0.18 | |

The dry matter content (DMC) in the families ranged between 16.3% in the family CM9953 and 64.22% in the family CM9958. The family CM9953 (with SM1741-1 as parent) showed the highest DMC average (34.64), higher than the average of the best parent (MPER183) in the cycle 2004-2005 (30.08%). The family showing the best segregation for DMC in the mapping populations was CM9953 (41.25% - 16.3%, followed by GM252 and GM901. Families with the highest number of selected genotypes were GM9953 and GM901 both having SM1741-1 as parent. Molecular markers for DMC had been identified earlier in the family GM9953 and GM901 (CIAT2005). The highest root yield was observed in the genotype CM9953-088 (67.18ton/ha) and the average for the family was 22.42ton/ha.

Selections, based on performance and sufficient stems, of 114 genotypes were made and planted in the cycle 2006-2007 with 3 replications per genotype. Plot size was 5 plants with a distance of 0.75m between plants and 0.8m between rows. They will be harvested in April 2007 re-established in a crossing block for
crosses to elite clones for an evaluation of markers associated to DMC in different genetic backgrounds.

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## 1.2.17 Genetic changes as a result of cassava domestication: a study of genes controlling selected traits important for cassava improvement

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Funding: SIDA-FORMAS; Swedish Institute (SI); Department of Plant Biology and Forest Genetics, Swedish University of Agricultural Science (SLU); CIAT

**Introduction**

QTL mapping has arisen as a genetic tool to understand the genetic basis of adaptation and the genetic changes as a result of domestication (Zeng et al, 2005; Westerbergh and Doebley, 2002). We are combining QTL mapping, functional markers, genes controlling adaptive traits, to study traits important for the domestication of cassava in a $S_1$ population derived from a cross between the cassava cultivar MCOL1734 and its wild ancestor *M. esculenta* spp *flabellifolia,* accession OW181-2. Key traits being looked at include starch, protein, β-carotene, fibrous roots and morphological stem traits. Three different environments, Colombia, Sweden, and Vietnam have been selected for phenotypic evaluation of the mapping population. The main goal of this project is to study the genetic and phenotypic changes that have occurred during cassava domestication, especially to identify the genes/QTLs, and linked molecular markers, that control differences in selected traits between cassava and its wild ancestor and to compare the genetic variation in cassava and its wild ancestor and other *Manihot* species. This knowledge is of great importance for the use of wild relatives as genetic sources in breeding programs based on marker-assisted selection as well as a preliminary knowledge about the evolution of the crop.

**Methodology**

A $S_1$ population obtained from a cross between a cassava cultivar and its wild ancestor *M. e.* spp. *flabellifolia* with approximately 200 individuals is used as mapping population for QTL mapping. Polymorphic SSR and EST-SSR markers in the $F_1$ inter-specific parent are being analyzed in the mapping

population using standard PCR and PAGE protocols (Mba et al 2001). Candidate genes for target traits (adaptive traits) will be selected and mapped onto the linkage map using SNP variation in the sequences of the corresponding genes (Castelblanco and Fregene, 2006). Candidate genes include SBE being used to study the genetic variation within the genera *Manihot* through direct sequence analysis. New statistical approaches are also being adapted in order to obtain the best possible QTL analysis (Shizhong Xun, statistical genomics course, 2006, Umeå, Sweden).

**Results**

The mapping population has now been established at CIAT and SLU. Field and screen house evaluation will be conducted in 2007. The scoring of the polymorphic markers in the $S_1$ population is already ongoing. Plant materials for several species of the genera *Manihot* are being collected and a preliminary analysis of the gene SBE is in progress.

**Conclusion and perspectives**

The mapping population is being analyzed both at the genetic as well as the phenotypic level in different environments. We expect to obtain a linkage map at the end of 2007 together with initial phenotypic data. An analysis of the starch synthesis as a trait under strong selection pressure during domestication is also being studied.

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## 1.2.18 Genetic mapping of beta-carotene content from multiple sources in cassava

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Funding: Harvest Plus.

**Introduction**

Deficiency of Vitamin A in Developing countries is the leading cause of child blindness in the developing world. Beta-carotene is the precursor of vitamin A, an important micronutrient in human diet. A project to fortify cassava varieties grown by rural communities with higher levels of beta-carotene has been initiated as a way of combating deficiency of this micronutrient in areas where cassava is major staple. The discovery of a wide segregation pattern of root color in two family ($S_1$) from Colombian races and the Thai variety MTAI8 led to the commencement of molecular genetic analysis of the inheritance of beta-carotene in cassava.

The objectives of this project is to analyze with molecular markers and phenotypic evaluation 846 genotypes from 6 families representing multiple sources of genes for Beta-carotene content in cassava roots to identify regions of the genome and multiple alleles associated with B-carotene production. Another objective is to study the genetics of beta-carotene accumulation in cassava roots. This information can be used for functional diversity analysis of natural variation of beta-carotene content for a more rational exploitation of naturally occurring variability. We present here results of biochemical characterization and Bulks Segregant analysis (BSA) for identification of regions of the cassava genome associated with beta-carotene content.

**Methodology**

The $F_1$ progeny (about 846 individuals) were established in a non-replicated trial at CIAT headquarters in Palmira, Colombia, and harvested after 10 months. Root parenchyma was visually measured using a scale of 1 (white parenchyma) to 9 (pinkish parenchyma) based on a color chart. Root parenchyma color is known to be highly correlated to beta-carotene content ($r=0.85$ at $P<0.05$). The quantification of carotenes total was done using a spectrophotometer with the purpose of selecting the best families and initiating the molecular analysis.

For DNA isolation, 2g of young leaves was harvested from selected families. The leaves were dried for 24h in an oven at 48 C and then ground into a fine powder using a power drill and washed sand. DNA was isolated using a mini prep. version of the Dellaporta (1983) protocol. The bulks of white and orange/ pink were then created per family to give a total of 12 bulks. DNA from the bulks and the parents were then genotyped with about 700 available cassava SSR markers according to methods described by Mba et al (2001) We then selected markers polymorphic in the bulks and parents for analysis of the individuals within the bulks. Markers that remained polymorphic were then are been analyzed in the entire family.

A simple linear regression of phenotypic data on marker genotype marker class means (single point analysis) using Microsoft Excel was used to determine association between orange/pink color and molecular makers. The amount of phenotypic variant explained by each marker will obtained from the $R^2$ value.

**Results**

Families segregating for high content carotene were GM 708,GM 705, GM 734, GM 893, GM 734, GM 9816 (with sizes ranging from 10 to 65 individuals). Firstly A total of 7 markers, namely SSRY313, NS980, SSRY92, SSRY251, SSRY9, and SSRY66, and NS717 earlier found to be associated with root parenchyma color were also used. In the cross GM708 all 7 SSR markers were polymorphic in the bulks. Other crosses had between 2 and 5 polymorphic markers each. A simple regression of root parenchyma color in individuals of the GM708 cross on marker classes of the 7 markers revealed markers SSRY313 and SSRY92 each explained between 27 and 36% of phenotypic variance . A similar result was found for crosses GM705 and GM734, where markers SSRY313 and SSRY9 explained between 25 and 30% of phenotypic variance (Figure 1). Analysis of marker association in the other crosses is yet to be completed.

The above results suggest the presence of multiple alleles with different effects on beta-carotene content. The cost-effectiveness of breeding for high beta-carotene content in cassava can be considerably enhanced if the contribution of each of these alleles to beta-carotene content and the inheritance are known.

Preliminary observations showed that the marker SSRY 86 and ESTY76 are polymorphic in bulks from the families GM 893, GM 705 and GM 9816 and may represent novel loci. However association is not so strong with these markers. These families are characterized by most individuals having medium

to high beta carotene content.

**Conclusion and Perspectives**

A search for markers more tightly associated with beta-carotene in the genetic map of cassava is ongoing. A modified BSA method using recombinants and multiples sources of cassava will allow the identification of SSR markers and associated with beta carotene content. This result, including and other earlier efforts, suggests multiple alleles for yellow root parenchyma color in cassava.

We intend to carry out a test of allelism to estimate the effect of different alleles as a first step towards increasing beta-carotene content via combination of favorable alleles. However, the inheritance mechanism that governs the content of beta carotene has not still been totally elucidated.

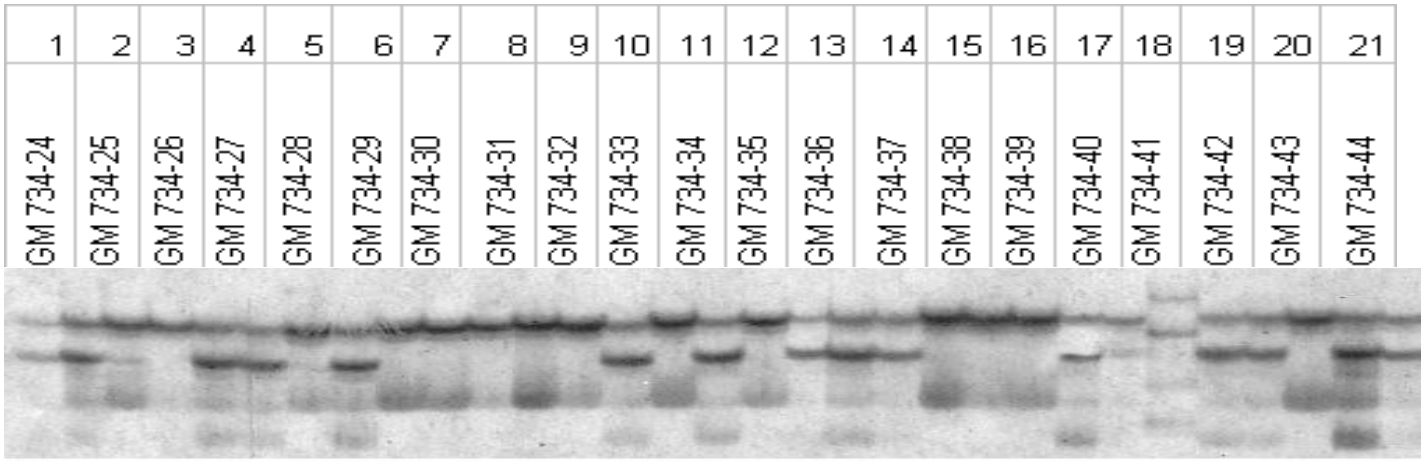


Figure 1. Silver stained polyacrylamide gel of PCR amplification product of marker SSRY313 in individuals of the F1 family GM734, the figures under the gel are root parenchyma color scores.

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## 1.2.19 Identification of SSR Markers Associated With Genes Controlling Leaf Retention in Cassava

Ovalle, T., Gutierrez, J.P., Ospina, C., Barrera, E., Ceballos, H. and Fregene, M.
Funding: CIAT

### Introduction

Studies have established that leaf retention plays an important role in cassava productivity (El-Sharkawy et al. 2003). Leaf retention is also thought to be associated with drought tolerance and high productivity under water stress, (Lenis et. al 2006). The objective of this study was to develop a SSR marker identification associated with genes that control leaf retention in cassava as a means understand the genetics of this trait and to provide an easily assayable and highly heritable marker for enhancing leaf retention in cassava breeding programs.

### Methodology

Five families were used in this study, of these two families are open pollinated crosses from female parents with good leaf retention (Table 1). Selection of the families was based on the phenotypical evaluation in clonal trials field in 2002 /2003 on the North Coast of Colombia (Santo Tomas – Atlántico). In 2006 we carried out evaluations of additional individuals of the two open pollinated crossed (SM2783 y SM2615) in replicated trials at CIAT.

A set of 500 SSR markers were evaluated in bulks of high and low leaf retention genotypes of the 5 families otherwise known as Bulk Segregant Analysis (BSA). Markers polymorphic in the bulks were evaluated in individuals of the bulks and eventually in the entire cross.

Table 1. Parents of the families evaluated and number of individuals.

| Family | Crosses | Progeny (N. Individuals) Evaluation 2002-2003 | Progeny (N. Individuals) Evaluation 2006 |
|---|---|---|---|
| SM2615 | CM4365-3 * | 29 | 35 |
| SM2783 | SM1511-6* | 22 | 80 |
| CM9775 | CM7514-7 x MNGA 19 | 9 | - |
| CM9791 | SM1433-4 x MNGA 19 | 17 | - |
| CM9794 | SM1438-2 x MNGA 19 | 15 | - |

* = This cross was generated from open pollinations.

Association between molecular markers and leaf retention in the selected families was done using the Chi-square test and the SAS statistical program additionally.

### Results

The three smaller families from controlled crosses were discarded due to their small sizes. We concentrated on the two larger sized families that also showed the highest segregation, as demonstrated by the presence of individuals with low, medium and high leaf retention scores.

BSA of bulks and female parents of the 2 families identified 5 candidate SSRs markers in SM2615 and 18 in SM2783. These 23 markers were evaluated in all progeny of these two families. Using Chi square

analysis of molecular data and phenotypic data from the 2002/2003 evaluation, the molecular marker EST-SSRY 295 was identified as being associated with leaf retention in the SM2783 family (Fig. 1).
To minimize the statistical error related to population number we increased the number of individuals to 80 in the SM2783 family and re- evaluated leaf retention in the family in 2005/2005 growing season. The new genotypes were also evaluated with the polymorphic candidate marker. Chi-square test also showed the association between the EST-SSRY 295 marker and leaf retention in the family SM2783.

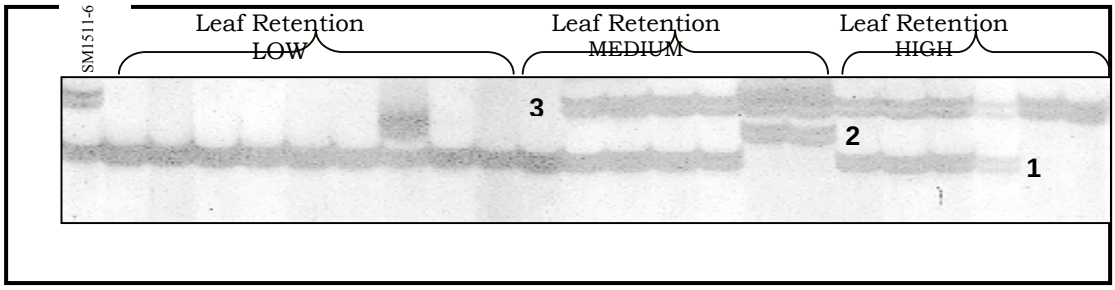


Figure 1. Silver stained polyacrylamide gel of PCR amplification product of marker EST-SSRY 295 in female parent and individual SM2783 family

The reliability for this marker has to be proven further as the majority of individuals in this population have only one field evaluation of the trait. Although, the leaf retention is known to be highly heritable phenotypic expression has an environmental effect. To avoid bias due to the environment it is necessary have more field evaluations in different years and environment to obtain a more accurate score of leaf retention in individual genotype.

**Conclusions**

We have identified the SSR marker EST-SSRY 295 as a putative marker associated with high leaf retention in the family SM2783 based on a chi square test of between molecular marker data and phenotypic data analysis from evaluations at two sites in Colombia (Atlantico and Valle del Cauca). Future perspectives include a second year evaluation of leaf retention aims to understand the genetic basis of the leaf retention in cassava, an important contribution to plant breeding programs looking for optimize this crop.

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## 1.2.20 Identification of SSR molecular markers associated with green mites (*Mononychellus tanajoa)* resistance in cassava (*Manihot esculenta* Crantz).

Eliana Macea, Cesar Ospina, Martin Fregene, and Anthony Bellotti

### Introduction

The cassava's green mites (CGM), *M. tanajoa* is a native specie of the Neotropics, it was first found in the North East region of Brazil in 1938 and was first report in Uganda, East Africa in 1971. This pest colonizes the apical zone of the plant (leaf buds, young leaves, and green parts of the stem), the populations of *M. tanajoa* prefer meristems and the under side of the young leaves. When the attack is severe, the foliar area is reduced and meristems lose all leaves and the apical stems become dry. The CGM is a serious problem during the dry season because it causes defoliation and destruction of the shoot tips in the plant. In Colombia this pest is found largely in regions having a long dry period such as Atlantic cost. We present efforts to use SSR molecular markers to identify and map genome regions that confer resistance, derived from wild progenitors of cassava, to the green mites.

### Methodology

Phenotypic evaluation of green mites under field conditions was conducted for four families inter-specific $F_1$ between cassava varieties and a single accession of *M.esculenta* sub spp *flabelifolia* (CW 65, 66, 67, 68). Selected inter-specific F1 hybrids were back crossed to the elite cassava parents to obtain $BC_1$ families. The BC1 families were evaluated over three seasons beginning 2002/2003 season. In order to identify markers associated with resistance to CGM, bulks were created from CGM resistant and susceptible genotypes from the $BC_1$ families that have been evaluated over three cycles. Genotypes with symptom damage of less than 3 made up the resistant bulk, and those with 4-6 constituted the susceptible bulks, bulks were made on a family basis.

Young leaves were collected and the DNA was extracted using the Dellaporta protocol (1983). SSR analysis of parental genotypes, resistant and susceptible bulks was as earlier described (Mba et al. 2001). The bulks were then evaluated with 500 SSR markers available for cassava. Markers polymorphic in the bulks were evaluated in each individual of the bulk and then in the entire population. Association between marker and phenotypic data was by a chi squared test and regression analysis.

### Results

After the first evaluation eleven candidate molecular markers were identified. In the opened open bulks, three markers continued to be polymorphic and showed clear differences between resistant and susceptible individuals: SSRY11, NS1099 and NS346 (Fig. 1)

The regression analysis showed that two of the three molecular markers selected show a significant relationship between mechanical damage caused by the green mites and the SSR marker alleles from NS1099 and NS346 in individuals derived from the parents CW 66 and CW 67 (Table 2)

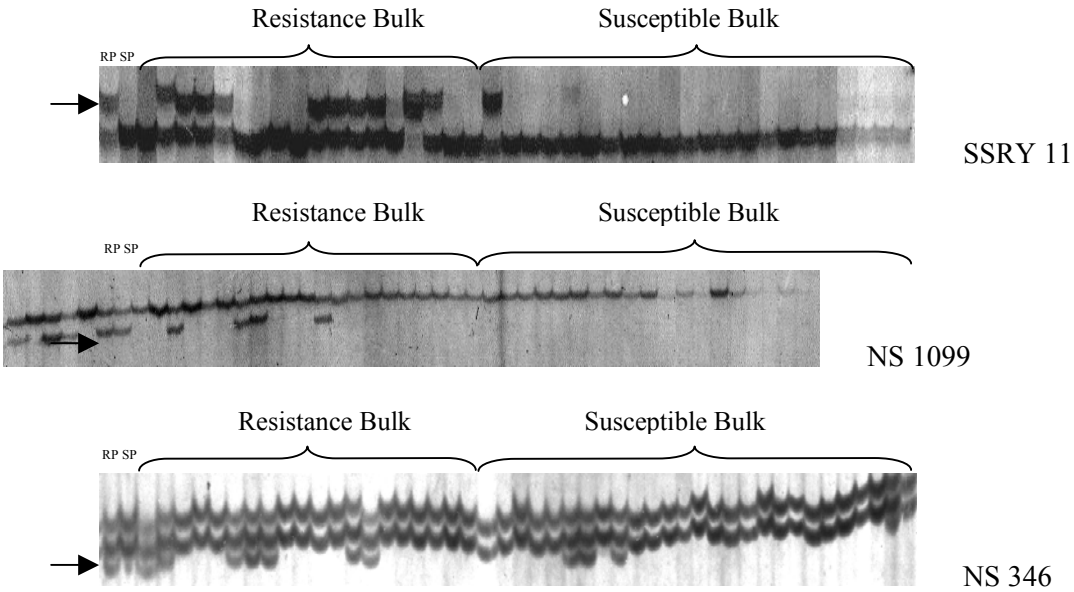


Figure 1. Silver stained PAGE gels of SSR marker amplification product of the evaluation of the $BC_1$ population with markers SSRY11, NS1099 and NS346. The black arrow indicates the the polymorphic band in the Resistant parent (RP) and Susceptible Parental (SP).

Table 2. Statistical analysis results (Correlation and Lineal Regression) of the CW families with the two polymorphic markers.

| Marker | Family | Correlation Coefficient | Regression $R^2$ |
|---|---|---|---|
| NS 1099 | CW 66 | - 0.56* | 0.32 |
| | CW 67 | - 0.61* | 0.37 |
| NS 346 | CW 67 | - 0.61* | 0.37 |

We have also found significant association with the same markers NS 1099 y NS 346 using a chi square test (Table 3). The resultsof these tests suggests that the associations are not random events and there is association between these markers and resistance to green mites.

Table 3. Independence probe results of the CW families with the two polymorphic markers

| Marker | Family | Valor $X^2$ | G.L | α | Significant |
|---|---|---|---|---|---|
| NS 1099 | CW 66 | 6.59 | 1 | 0.5 | 0.01 * |
| | CW 67 | 3.83 | 1 | 0.5 | 0.05 * |
| NS 346 | CW 67 | 3.83 | 1 | 0.5 | 0.05 * |

**Conclusion and Perspectives**

In $BC_1$ families derived from CW 66 y CW 67 association between NS 1099 y NS 346 markers and resistance to green mites have been found. Individuals carrying the resistant allele are being crossed with elite cassava varieties to test the value of this marker in selecting for resistance to mites. The larger populations derived will also provide greater statistical power to confirm associations

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## 1.2.21 Validation of SNP markers located in rice iron homeostasis candidate genes

C. Quintero[1], F. Rodríguez[1], G. Plata[1], O.X. Giraldo[1], C.P. Martínez,[2] and J. Tohme[1].
[1]SB-2 Project; [2]IP-4 Project.

**Abstract**

This activity was conducted as a proof of concept; 32 SNP-containing sequences (100bp length) were selected to test the multiplex capacity of PCR and single base extension reactions. Results across the different assays were consistent and no ambiguous detection was observed. Percentage validated SNPs ranged between 50% and 72%. The most desirable assay, that assemble high multiplex capacity and large amount of results was the one that involved two multiplex PCR reactions of 16 amplicons and one single SBE reaction of 32 SNP alleles. To date, 56 SNPs are ready to be used for the screening of rice genotypes having contrasting levels of iron content in the polished grains.

**Introduction**

New rice varieties with high iron content in the polished grain, would have a great positive impact in human nutrition. The development of these lines could be achieved by breeding, using the available variability, or by genetic engineering. Both approaches would benefit from a better understanding of the physiological processes involved in iron uptake by the plant, translocation and proper allocation into the grain. In its way towards the rice grain, iron flux may be limited in several steps; in previous studies scientists tried to identify the “bottle neck” and the genes able to overcome their limitations in “high iron in the grain” genotypes. Several proteins are involved with iron mobilization, transport and storage in

plants. Gross *et al.* (2003) identified 43 genes potentially encoding such proteins, comprising five distinct gene families: eighteen YSL (Yellow Stripe Like), two FRO ($Fe^{3+}$ -chelate reductase oxidase), thirteen ZIP (Zinc Regulated Transporter/Iron Regulated Transporter Protein), eight NRAMP (Natural Resistance – Associated Macrophage Protein) and two Ferritin genes. Some of the objectives of this project are the identification and validation of single nucleotide polymorphisms located in these candidate genes. Then, rice lines with high and low iron content in the polished grain will be genotyped and tagged SNPs associated with high iron content could be used in breeding programs for marker-assisted selection purposes.

## Materials and methods

### Plant material

Genomic DNA from eight rice genotypes was extracted following Dellaporta (1983) protocol with some modifications. Three belonged to *Oryza sativa* subsp. *japonica* (Koshihikari, Nipponbare and Caiapo), three to *indica* subsp. (93-11, BG90 and Oryzica Llanos 5) and the remaining to the African rice species *Oryza glaberrima* and *Oryza barthii*.

*SNP identification and primer design*

Plata, Rodríguez and Tohme (this issue), designed a database to identify putative SNPs (*indica/japonica*) in 43 genes related to iron metabolism (Gross. *et al.*, 2003). SNP genotyping was carried out with the single base extension method using the flow cytometer Luminex$^{100}$ as platform (Quintero *et al*, 2005) following the these steps:

PCR amplification of SNP-containing DNA fragments.
Enzymatic removal of excess dNTPs and primers.
Single base extension (SBE ) or minisequencing.
Hybridization of extended SBE primers with the beads.
SNP detection as Mean Fluorescent Intensity with the Luminex$^{100}$
Allele calling with Masterplex GT (Miraibio, Inc).

As a proof of concept, 32 SNP-containing sequences (100bp length) were selected to test the multiplex capacity of PCR and single base extension reactions. PCR primer pairs were designed under multiplex conditions using the FastPCR software (Kalendar, 2007). Single base extension primers were designed through SBEprimer software (Kaderali *et al.*, 2003).

Once the accuracy of the methodology was demonstrated, either for primer design and SNP detection, Plata and Rodríguez designed a set of scripts for the construction of multiplex PCR sets in an automated way, since the FastPCR design was found to be time-consuming. Then two sets of 27 multiplex PCR based SNPs were assayed in this second stage of the validation process.

## Results

*SNP Validation proof of concept*

Among the three primer combinations (forward and reverse) reported by the FastPCR software for each SNP-containing region, the least interfering with each other were selected for the assay. Individual fragment amplification was carried out using conventional *Taq* polymerase to confirm the presence of single and bright bands. The AccuPrime™ *Taq* Polymerase System (Invitrogen™) was used to perform multiplex PCR reactions. Single base extensions were carried out in multiplex of 8, 16 and 32 SBE

primers. No fluorescent signal was observed for 7 SNPs in all three assays, which meant that they were probably arising from sequencing errors.

Results across the different assays were consistent and no ambiguous detection was observed. Percentage validated SNPs ranged between 50% and 72% (Table 1). The most desirable assay, that assemble high multiplex capacity and large amount of results was the one that involved two multiplex PCR reactions of 16 amplicons and one single SBE reaction of 32 SNP alleles.

Table 1. Comparison between the assays performed for the validation of the first set of SNPs located on iron metabolism genes.

| **PCR amplification** | **Number of SNP alleles per SBE reaction** | **Validated SNPs** | |
|---|---|---|---|
| | | **Number/total** | **%** |
| Individual | 8 | 23/32 | 72 |
| | 32 | 21/32 | 66 |
| Multiplex (16 amplicons) | 16 | 22/32 | 69 |
| Multiplex (32 amplicons) | 32 | 16/32 | 50 |

Every time a scorable signal was detected, the predicted polymorphisms of Nipponbare and 93-11 were reproduced unequivocally. For 20 markers, SNPs alleles could be detected in the *O. sativa* relatives, *O. barthii* and *O. glaberrima*.

*Enhancing multiplex capacity*

After automated primer design performed by Plata, 54 SNPs were assayed. Multiplex PCR was done in sets of 13, 14 and 27 primer pairs.

Eighteen did not yield any fluorescence, three were monomorphic for all the rice genotypes tested, two were monomorphic between Nipponbare and 93-11, and unexpectedly, three A/T SNPs showed opposite alleles when compared with the predicted ones. Finally, 28 SNPs genotyped correctly the two sequenced rice varieties, with high mean fluorescent intensity values observed. No differences were found between the results obtained from PCR multiplex of 13, 14 and 27 primer pairs.

To date, 56 SNPs are ready to be used for the screening of rice genotypes having contrasting levels of iron content in the polished grains.

**On-going activities**

Continue the validation of the remaining 111 SNPs from which PCR and SBE primers have been already designed.

Initiate the genotyping of 536 rice lines with high iron content and checks.

Scale single base extensions up to 50 SNP alleles per reaction.

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## 1.2.22 Design of multiplex PCR panels for high-density SNP haplotyping of rice candidate genes

Germán Plata[1], Fausto Rodriguez-Zapata[1], Constanza Quintero[1], Joe Tohme[1], Mathias Lorieux[1].
[1] SB2-Project

### Introduction

Tagging SNPs are useful for the discovery of gene variants associated with complex phenotypes (Weale, et al., 2003). Designing SNP markers within gene sequences involved in stress response or other interesting phenotypes and further screening of this markers on a wide variety of germplasms, would allow the identification and selection of promising alleles for the improvement of rice through a candidate gene approach. Given the huge amount of sequence data available for rice, care must be taken to choose genes, SNPs and experimental procedures. Computational techniques are required for experiment design so that the number of screened SNPs is maximized while the number of reactions and reactants is reduced through multiplex PCR.

### Methods

*SNP detection and primer design*

Two lists of genes were used for the detection of SNPs; one set consisted of 40 genes related to iron metabolism while the other included 374 genes related to stress response. The Gene Ontology annotation of these genes shows that they code for enzymes with various molecular activities (transporter, hydrolase, isomerase, oxidoreductase) and that most of them are related to signal transduction pathways and response to stimulus.

For each of the genes selected, mRNA sequences where downloaded from GenBank and aligned to a database of rice transcripts; the best hit of each gene in this database was then aligned to the Oriza sativa ssp. japonica and ssp. indica genomes using BLAST (Altschul, et al., 1997). Genomic sequences for these genes where retrieved including 3 Kb upstream and downstream of the aligned region. The fragments were masked for repeats and aligned to each other in order to detect the SNPs, these were selected when a

conserved region of 20 base pairs upstream and downstream of the polymorphic site was present (Figure 1).

```
gagtattgttagagaaaaatcacccaaggagcctctaaagtggagtaaactgg
||||||||||||||||||||||| ||||||||||||||||||||||| |||
gagtattgttagagaaaaatcacctaaggagcctctaaagtggagtaaagtgg
```

Figure1. Condition for SNP detection: 20 conserved base pairs flanking the polymorphic site.

A relational database was created in order to store information about the genes, SNPs and primers designed. For the 414 genes, 7664 SNPs were detected. An attempt was made for each of the SNPs to design PCR primers in order to amplify a region of 80 - 120 base pairs around the polymorphic site, and to design primers for single base extension (SBE) adjacent to the SNP position. Using very stringent conditions for the design of primers, SBE primers for 5831 SNPs could be designed, along with 2885 primer pairs for PCR; together these primers allow the amplification of 1928 SNPs of 341 genes: 28 belonging the iron list and 313 from the stress list. The ePCR software (Schuler, 1997) was used to discard primer pairs that would amplify more than one locus.

PCR Primers were designed with Primer3 (Rozen and Skaletsky, 2000) using the following restrictions: Length: 19-30, GC%: 40-60, Max_Diff_Tm: 5.0, Tm: 50-60.5. SBE Primers were designed with SBE Primer with these restrictions: Length: 18-20, Tm: 50-75

*Design of multiplex PCR panels*

Building multiplex PCR sets in order to reduce the time and costs of genotyping large quantities of SNPs is a process in which several factors must be taken into account: A) PCR primers should not form stable dimers at the annealing temperature used in the multiplex reaction. B) PCR primers included in a multiplex reaction should have similar melting temperatures. C) SBE primers should not form dimers with each other at the annealing temperature used for the mini-sequencing reaction. D) SBE primers must not anneal to a different template than that from which it was designed.

The process of finding pairs of SNPs that are compatible is computationally more intense and time consuming as the number of SNPs increases, therefore our set of 1928 SNPs was divided into several groups as a workaround to this problem. However, there is a drawback for this solution: as the number of SNPs from which multiplex sets are to be designed is reduced, it gets harder to find an optimal solution, especially if the desired level of multiplexing is high (Rachlin, et al., 2005).

The formation of primer dimers was tested using the AutoDimer software (Vallone and Butler, 2004); false priming of the SBE primers designed was tested using SBE Primer (Kaderali, et al., 2003).

After various attempts to find the best solution, an algorithm was designed to create multiplex sets using a strategy in which those SNPs that are harder to multiplex are the first to be included in the tubes. This strategy seems to work better than the fixed-assignment best-fit algorithm tested by Rachlin et al (Rachlin, et al., 2005).

**Results and Discussions**

Due to the difficulties inherent to the primer design process there is a severe reduction in the amount of SNPs per gene that can be genotyped compared to the total number of SNPs detected per gene. Starting with a mean of 17.8 SNPs per gene we ended with 6.5 SNPs per gene as the mean value, which is almost a threefold reduction.

Previous work has shown that as many as 40% of the SNPs detected could be useless, either because they are not polymorphic or because they fail to amplify (Quintero et al. this issue). This would reduce even more the amount of SNPs per candidate gene in our assay. As an alternative, it was proposed to design longer PCR fragments that include more than one SNP. It is easier to design primers for these longer fragments, moreover, they would reduce the amount of primers needed to obtain information for more SNPs. PCR primers were designed for 921 out of 1657 such fragments, these would allow the amplification of 2200 SNPs; these, together with 956 SNPs not included in the long PCR set would give a total of 3156 SNPs, or an average of 9.89 SNPs per gene for 360 genes. Below we present the results of the multiplexing algorithm used for the creation of PCR panels.

| | Iron | Stress | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| No. Genes | 30 | 325 | | | | | | | |
| Partition | ALL | RJA | RIA | YJC | YIC | K | M | S | W |
| No. SNPs | 192 | 335 | 341 | 335 | 326 | 210 | 195 | 150 | 236 |
| Multiplex reactions (SNP number) | | | | | | | | | |
| Tube1 | 32 | 33 | 37 | 33 | 32 | 31 | 33 | 36 | 31 |
| Tube2 | 32 | 33 | 37 | 33 | 32 | 31 | 33 | 36 | 31 |
| Tube3 | 32 | 33 | 37 | 33 | 32 | 31 | 33 | 36 | 31 |
| Tube4 | 32 | 33 | 37 | 33 | 32 | 31 | 33 | 18 | 31 |
| Tube5 | 32 | 33 | 37 | 33 | 32 | 31 | 33 | 11 | 31 |
| Tube6 | 17 | 33 | 37 | 33 | 32 | 31 | 23 | 7 | 31 |
| Tube7 | 14 | 33 | 37 | 33 | 32 | 17 | 7 | 5 | 31 |
| Tube8 | 4 | 33 | 37 | 33 | 32 | 7 | | 1 | 15 |
| Tube9 | 1 | 33 | 37 | 33 | 26 | | | | 3 |
| Tube10 | | 33 | 8 | 33 | 26 | | | | 1 |
| Tube11 | | 5 | | 5 | 11 | | | | |
| Tube12 | | | | | 4 | | | | |
| Tube13 | | | | | 3 | | | | |

Table 1. There would be 87 reactions to amplify 2324 SNPs using 6972 primers.
R = A/G, Y = C/T, K = G/T, M = A/C, S = G/C W = A/T;

**Conclusions**

We have implemented two Perl modules, that along with a MySQL relational database allow the detection and handling of a large number of SNPs for rice genomic sequences, we have also designed a set of scripts for the design of primers and the construction of multiplex PCR sets; these steps, as necessary as they are for SNP haplotyping, currently rely on different pieces of software running on different platforms. This problem should have to be solved in order to obtain a completely automatic system.

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# Activity 1.3 Identification and Mapping of useful genes and gene combinations

## 1.3.1 Sequence characterization and genetic mapping of the RGC7 family of TIR-NBS-LRR genes linked to Angular Leaf Spot (ALS) resistance in common bean (IV).

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[1] SB2-Project.

### Introduction

We have previously sequenced a BAC clone (57M14) containing the RFLP marker RGC7 (Acosta *et al*., 2003) that is tightly linked to a major QTL for resistance against ALS in common bean (López *et al.*, 2002). The 90kb sequence of BAC 57M14 includes 5 members of a single family of disease resistance like genes from the TIR-NBS-LRR class (Acosta *et al.*, 2003). Even though BAC 57M14 comes from the susceptible cv. 'Sprite', it served to characterize the RGC7 family at sequence level. Subsequently BAC 57M14 sequences were used for isolating the corresponding candidate genes in the resistance source G19833 using a *genome walker* method. As the resistance phenotype was screened in the DOR364 X G19833 mapping population (Acosta *et al*., 2003), we used the same approach to obtain the RGC7 sequences from the susceptible genotype DOR364. The sequencing of the RGC7 members of both contrasting parentals was intended to help in discerning resistance cluster members from unlinked duplicates that are not involved in the resistance. With this aim genetic markers designed on the candidates were added to the existing DOR364 X G19833 genetic map. We expect that the polymorphisms between resistant and susceptible alleles could account for the difference in response to ALS. Here we report the isolation of disease resistance candidates from the RGC7 family in G19833 and DOR364, as well as the corresponding sequence annotation and genetic mapping.

### Methodology

*Candidate Gene Sequencing*.
From the multiple sequence alignment of the RGC7 paralogs in BAC 57M14 (Acosta *et al.*, 2003) we designed consensus primers on the conserved regions of the TIR and NBS domains. Since these primer sequences are shared by most or all RGC7 paralogs in BAC 57M14, we expected them to maximize of number amplified RGC7 members in G19833 and DOR364 through the Vectorette-PCR method (Hagiwara & Harris 1996), also known as *genome walker*. Total genomic G19833 and DOR364 DNA was extracted (Tohme *et al.*, 1996) and digested with *EcoRV*, *DraI*, *HincII, ScaI* (New-England Biolabs, Gibco), and then each digestion was ligated to the Vectorette adaptors using T4 ligase (Gibco). The digestions-ligations were used as Vectorette-PCR templates for amplification with the adaptor 224M13 and the RGC7 consensus primers (Rodríguez-Zapata 2005; Plata, 2005) or gene specific primers when considered pertinent. In this way the adaptor primer allowed amplification of adjacent unknown sequence downstream the known primer sequence. In this method, the combination of restriction enzyme and consensus or gene specific primer produced the adequate DNA fragment overlap for *contig* assembly (Acosta *et al.*, 2003). We ligated the Vectorette-PCR amplicons to pGEMT-easy vector (Promega) and constructed a library in *E.coli* DH10B for each bean genotype. Finally the clone inserts were reamplifed and sequenced with T7 and SP6 primers.

*Sequence Annotation*.
The sequence reads were edited by hand, trimmed of vector, adaptor and primers, and assembled in Sequencher 3.5 (Gene Codes). The final *contig* sequences were aligned with BAC57 M14 paralogs using ClustalW and HMMER and the resulting alignment was corrected by hand in MacClade. This multiple sequence alignment allowed the automated annotation, through the use of Perl scripts, of all the RGC7 members. The features annotated on RGC7 candidates were gene structure with GeneScan, NetPlantGene, GeneSeqer and GenMark; protein domain and motif architecture with EMBL REPSEARCH and MAST search on the *Arabidopsis thaliana* NBS-LRR; and ORF integrity with *in-home* Perl scripts.

*Determination of allelic relationships between RGC7 members*.
Two sequences were taken as orthologous, and therefore alleles, if they 1) were the best BLAST bidirectional hit and 2) conformed a supported monophyletic group in a parsimony phylogenetic reconstruction (100 *bootstrap* replicates tree using phylip *dnapars*). This criterion takes into account both the fragmentary nature of the isolated sequences and the phylogenetic inference where comparable data could be obtained. The groups of 2 (G19833, DOR364) or three (Sprite, G19833, DOR364) alleles found this way were named as putative RGC7 sub-loci corresponding to different paralog copies in each bean genotype.

*Polymorphism detection and Genetic Mapping*.
G19833 and DOR364 orthologous sequences were compared with the *diffseq* program from EMBOSS suite. Results from *diffseq* were parsed with a Perl script for single nucleotide polymorphisms in order to design single base extension (SBE) primers and CAPS markers. Locus specific PCR primers were designed by eye based on the BAC 57M13 multiple sequence alignment of RGC7 members and checked for optimal PCR parameters in NetPrimer (http://www.premierbiosoft.com/netprimer/). CAPS markers were checked for polymorphism in 2% agarose gel electrophoresis and genotyped in the 87 RILs F9 G19833 X DOR 364 population. From these, two new polymorphic markers were appended to the data matrix used by López *et al.* (2002) where the original RGC7 RFLP was reported and the BAC57M14 microsatellite SSR07 was added in 2003 by Acosta *et al*. For genetic mapping the markers were first tested for linkage in Mapmaker 3.0 with the command `group` ( LOD 15, 30 cM) and once the likage to the B10I group was confirmed the RGC7 derived makers were finally located with the command `try`. QTL mapping was carried out in Qgene by single marker and interval analysis.

**Results**

We obtained partial sequences for different RGC7 paralogs in both G19833 and DOR364, however no disease resistance gene was sequenced in full length. In spite of the fragmentary nature of the isolated sequences, the RGC7 members in G19833 and DOR364 fit to the basic gene structure and domain architecture found in the locus RGC7B from BAC 57M14 (Figure 1C). This structure, from 5' to 3', consists of two exons that contain respectively the TIR and the NBS domains separated by a well defined intron 1; a less supported intron 2 that is found between the NBS and a short exon coding for a NBS-LRR (NL) transition peptide; and finally, a few LRRs likely interrupted by one or more introns. This basic organization has been dramatically modified by deletions and/or intergenic recombination in two RGC7 members that show either the attachment of LRRs to the TIR domain in G-TIR01, or the fusion of TIR to NBS motifs in the case of G-CHITIN01 and D-QUIMERA.

In G19833 the 13 segments sequenced (average size 1295bp) correspond to 6 TIR, 10 NBS, and 3 NL/LRR protein domains (Fig 1b). From these we identified just 3 disease resistance genes candidates (G-NBS01, G-NBS01B and G-NBS07) with integral open reading frames (ORFs). The remaining 10 G19833 fragments are expected to be pseudogenes since they are non-functional chimeras of different proteic domains, or have disruptions in their ORFs. In a similar way, the DOR364 sequences obtained constitute 14 different fragments (average size 1000bp) representing partial sequences of 10 TIR, and 9 NBS

resistance protein domains. As in G19833, most of the isolated candidates from DOR364 are likely to be pseudogenes, only D-TIR-NBS01 and D-TIR04 have integral ORFs.

Ortholog detection allowed us to propose 6 allelic relationships (Table 1), from which just RGC7D and RGC7H loci could be mapped. Although we found in DOR364 a clear ortholog a G19833 candidate with integral ORF, G-NBS01, its corresponding locus RGC7F could not be mapped due to its lack of polymorphism. This conservation of sequence suggests that RGC7F has retained its function through most of the divergence time between Mesoamerican (DOR364), and Andean (G19833) gene pools. In contrast to RGC7F, we infer from the chimerical structure of RGC7H that it has remained non-functional since its origin, most likely by a recombination or deletion event, which in turn must precede the Andean-Mesoamerican split. Thereby RGC7H has been accumulating mutations ever since and, consequently, of all the locus considered it shows the greatest divergence between its allelic variants.

Table 1. Orthologous (i.e. allelic) groups and polymorphisms found in RGC7 members.

| DOR364 | G19833 | Locus | SNPs | Indels | Alignment Length | Distance between SNPs (bp) |
|---|---|---|---|---|---|---|
| D-NBS04 | G-NBS02 | RGC7 B | 2 | 0 | 1495 | 747.5 |
| D-TIR04; D-NBS03 | G-TIR09-NBS05 | RGC7 D | 4 | 2 | 1198 | 299.5 |
| D-TIRNBS01 | G-NBS01 | RGC7 F | 0 | 0 | 962 | >962 |
| D-QUIMERA | G-CHITIN01 | RGC7 H | 10 | 2 | 622 | 62 |
| D-TIRNBS03 | G-TIR05-NBS06 | RGC7 I | 8 | 1 | 1113 | 139.1 |

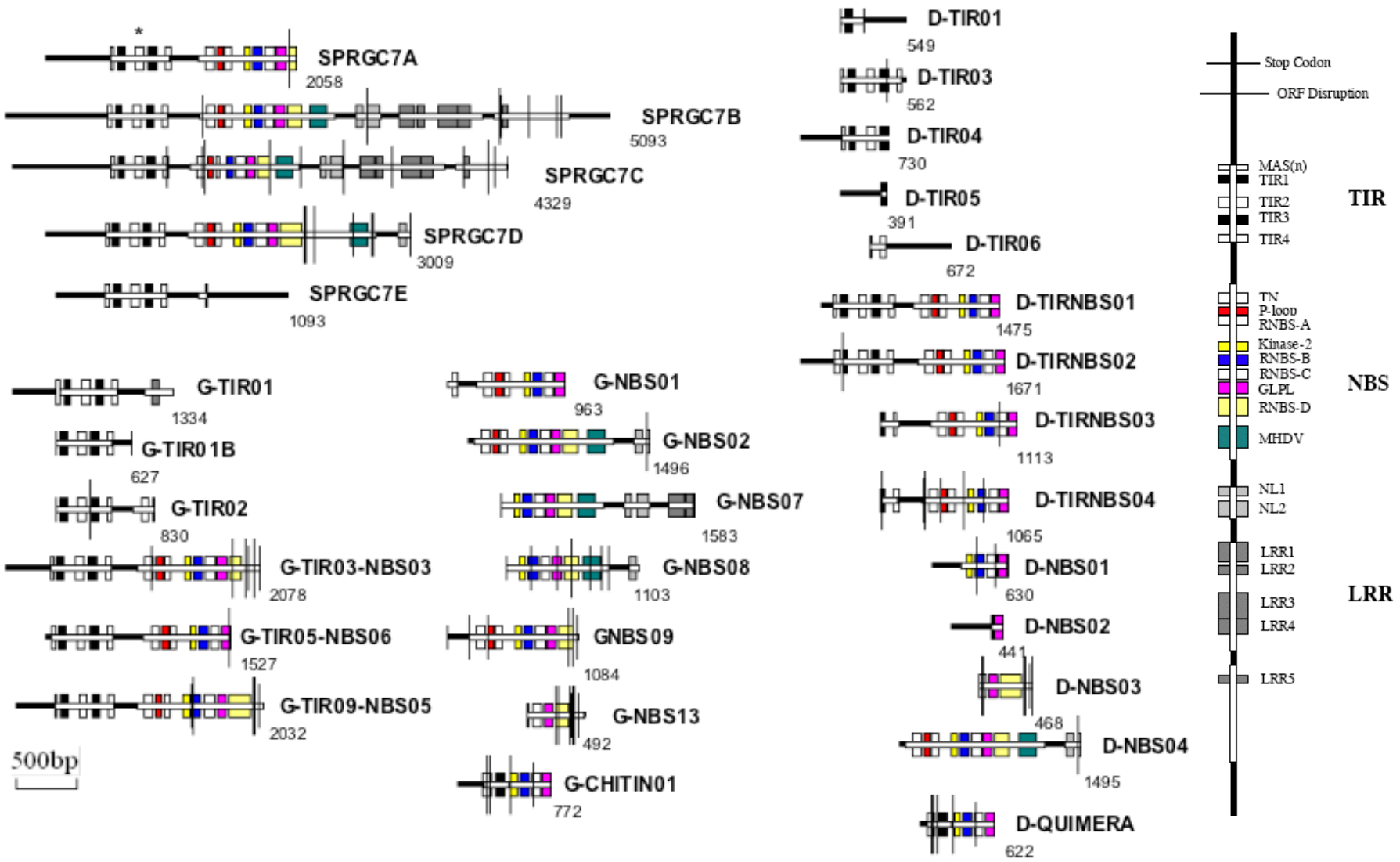


Figure 1. RGC7 gene family fragments isolated from *Sprite*, G19833 and DOR364. Exons shown as white bars. * SPRGC7A is interrupted by a .4 kb retroelement in the TIR2 motif.

The genetic mapping shows that RGC7H and RGC7D loci are tightly linked with the RGC7 RFLP and SSR07.1, two previously mapped markers of the RGC7 cluster (Figure 2). The interval spanned by all the RGC7 is about 7 centimorgans, a far greater figure than expected according to the gene density in BAC 57M14. This suggests that the RGC7, locus has a low R-gene density or that it could be a recombinogenic cluster. The QTL analysis (Figure 2) demonstrates, nonetheless, that the interval delimited by the RGC7 markers is bounding a major quantitative locus of resistance to ALS. As expected the presence of the G19833 allele in the four RGC7 markers is strongly correlated with the ALS resistance. These markers show the highest percentages of explanation of the phenotypic variance in the resistance to 12 MEX ( 50-70 %) and 30 CRI ( 30-49 %) isolates.

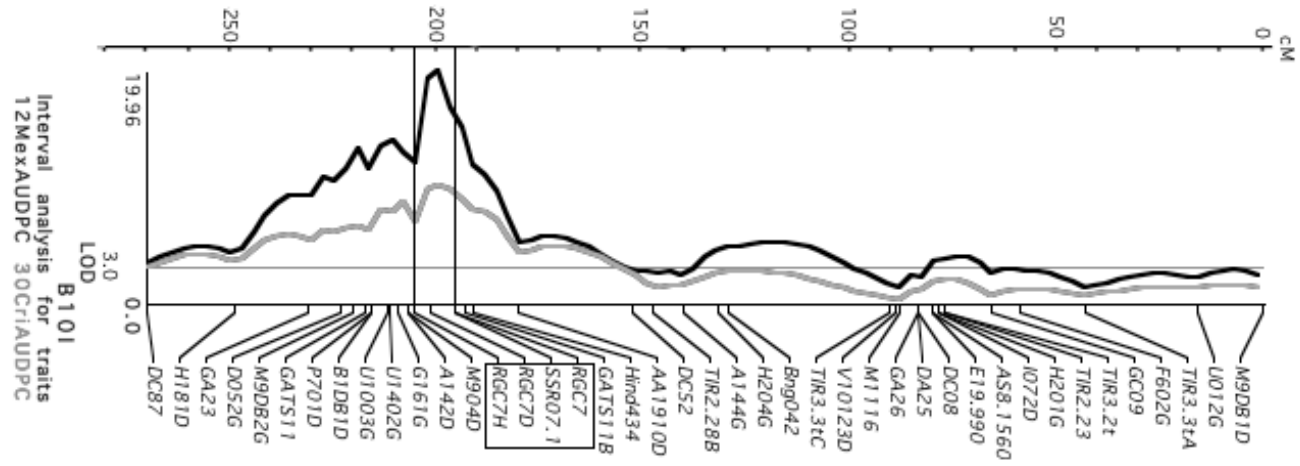


Figure 2. QTL analysis for resistance to ALS isolates12MEX and 30CRI.

**Conclusions**

A major quantitative resistance locus to ALS in bean is bounded by members of the RGC7 family of NBS-LRR disease resistance like genes. At least 3 different members of the RGC7 family map to the same genetic interval containing the resistance QTL. However, there are as much as 10 other RGC7 members whose actual genetic location is unknown, or whose relationship to the resistance is unconfirmed.

Most of the RGC7 members, inlcuiding two mapped in the present work, should be pseudogenes whose translated protein could not be involved in the response to pathogens.

The RGC7 family diversified before the Andean-Mesoamerican split of the common bean gene pools.

**Ongoing Work**

To screen the available G19833 BAC library for clones containing the RGC7 probe in order to get a physical map and to select a minimum tilling path for shotgun sequencing. This complete picture will allow us to tag the functional RGC7 genes in the RIL population.

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### 1.3.2 Validation of Diversity Arrays Technology (DArT) as a Platform for whole Genome Profiling in Orphan Crops

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[1]CIAT; [2]Biodiversity

Funding: Generation Challenge Program (GCP)

**Introduction**

There are many constraints to the widespread use of molecular markers for diversity analysis of germplasm and the subsequent identification of associations between traits and genes. Limitations include the following: theoretical and practical lack of knowledge of the tools, cost of development, low reproducibility, low data yield (limited throughput) of the experiments, restriction of access to proprietary technologies and insufficient resources (laboratory facilities, equipment, chemicals, etc).

The project proposed to test the usefulness of Diversity Array Technology (DArT) as an alternative for detecting DNA variation in ways that will be more effective and resource-efficient. DArT offers the highest throughput available up to date and allows for whole genome scanning in a speedy manner. In addition, the types of polymorphism detected by DArT (single nucleotide polymorphisms, insertion-deletions and methylation changes) may expand the potential of traditionally used markers, increasing the power to ascertain the structure of germplasm collections. Last, the experimental procedures to obtain DArT take into account the complexity of genomes and its effect on the extent of diversity shown by a collection of germplasm. Therefore, a set of different cases addressed, using the biological diversity of the crops and the issues, focusing the development of new resources on orphan crops that are not likely to gain much attention elsewhere.

To validate the effectiveness of DArT for a range of applications, a group of 6 crops species (Musa, Sorghum, Coconut, Cassava, Wheat and Rice) was used as model to span the range of crops in the Generation CP. In order to do the validation, a number of accessions of each crop were evaluated by using SSR and DArT and the results compared between the two marker systems.

**Methodology**

*Compilation of SSR and DArT data for 6 crops*

SSR and DArT data sets were sent to CIAT by the researchers responsible for the molecular characterization of each crop (Table 1). Different numbers of accessions were included in SSR and DArT data sets for each crop and therefore individual names and numbers in the DArT and SSR marker data sets were verified before data analysis,. Only individuals analyzed with the two marker systems in each crop were included in the analysis (Table 2).

Table 1. Description of crop data sets

| Crop | SSR data | File description | Submmission date Submitted by.. | DArT data | File description | Submmission date Submitted by.. |
|---|---|---|---|---|---|---|
| Wheat | X | 94 individuals x 73 SSR markers | June 13 2006[1] François Balfourier | X | 94 individuals x 714 DArT markers | June 13 2006 François Balfourier |
| Rice | X | 91 individuals x 39 SSR markers | September 20 2006 Kenneth McNally | X | 90 individuals x 519 DArT markers | February 15 2006 BrigitteCourtois |
| Sorghum | X | 90 individuals x 34 SSR markers | May 5 2006 Sophie Bouchet | X | 92 individuals x 520 DArT markers | May 3 2006 Sophie Bouchet |
| Coconut | X | 192 individuals x 21 SSR markers | June 8 2006[2] Patricia Lebrun | X | 223 individuals x 337 DArT markers | April 25 2006 Chandrika Perera |
| Cassava | X | 134 individuals x 36 SSR markers | June 15 2005 Paula Hurtado | X | 124 landraces x 424 DArT markers 19 wild accessions x 1710 DArT markers | May 30 2006 Andrzej Killian / Prapit Wongtiem |
| Musa | X | 184 individuals x 12 SSR markers | June 9 2006 Isabelle Hyppolyte | X | 187 individuals x 463 Pst1/BstNI DArT markers 187 individuals x 373 Pst1/TaqI DArT markers | June 9 2006 Ange-Marie Risterucci |

1. The first SSR data set corresponding to Wheat was sent by Francois Balfourier on November 16 2005. This file was corrected and the updated version was sent on June 13 2006.
2. The first SSR data set corresponding to Coconut was sent by Patricia Lebrun on April 11 2006. This file was corrected and the updated version was sent on June 8 2006.

Table 2. Summary of individuals and markers to be compared in the diversity analysis.

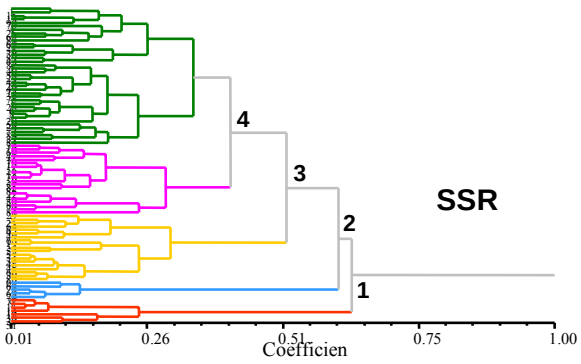
| | | SSR data | | DArT data |
|---|---|---|---|---|
| Crop | Number of Individuals | Number of SSR Markers | Number of alleles | Number of DArT Markers |
| Wheat | 94 | 73 | 686 | 714 |
| Sorghum | 90 | 34 | 360 | 520 |
| Coconut | 191 | 21 | 243 | 337 |
| Rice | 86 | 39 | 419 | 519 |
| Cassava | 132 | 22 | 212 | 239 |
| Musa | 169 | 12 | 182 | 836 |

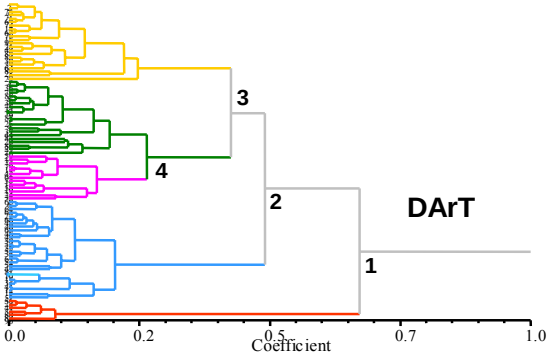
*Comparison of SSR and DArT markers*
Comparison of SSR and DArT markers was performed at two levels:
A. Separate analysis of SSR and DArT data with NTSYSpc v 2.10:
Similarity matrix calculation based on Jaccard or Nei's coefficient with SSR or DArT data respectively
Principal coordinate analysis (PCoA) for each similarity matrix
Cluster analysis (UPGMA) based on the Euclidian distances resulting of the PCoA of each data set
Group content comparison defined at the first four similarity subdivision levels
B. Comparison of the two marker systems:
Comparison of clusters (trees) by means of frequency tables for each subdivision in the populations (SAS version 9.1)
Coincidence (%) of genotypes in the clusters defined by SSR and DArT at each subdivision

## Results

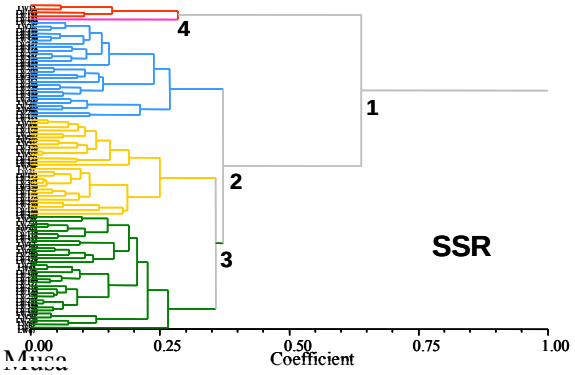
For the first level of comparison, a Cluster analysis (UPGMA) is shown for each crop SSR and DArT data sets (Figure 1). The first four similarity subdivision levels are indicated by numbers and the clusters defined by the different subdivisions are represented by colors (red: group 1, blue: group 2, yellow: group 3, pink: group 4, green: group 5).

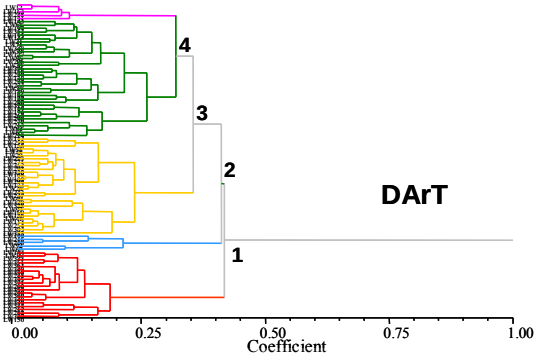
Sorghum



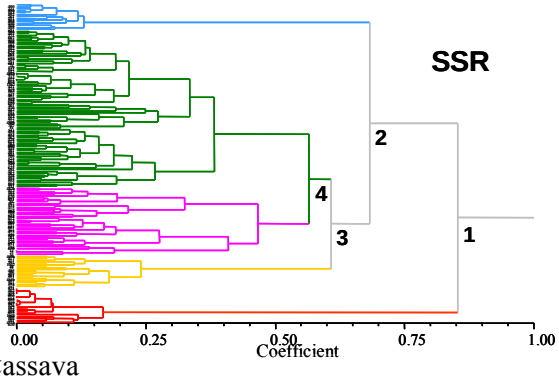



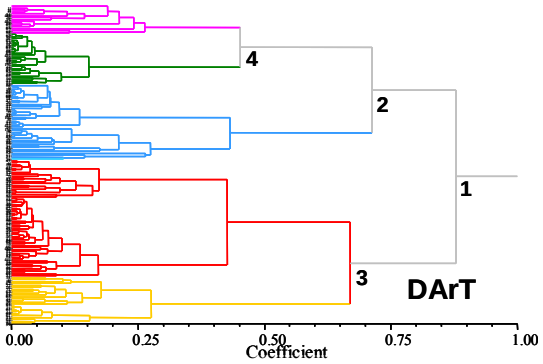

Wheat



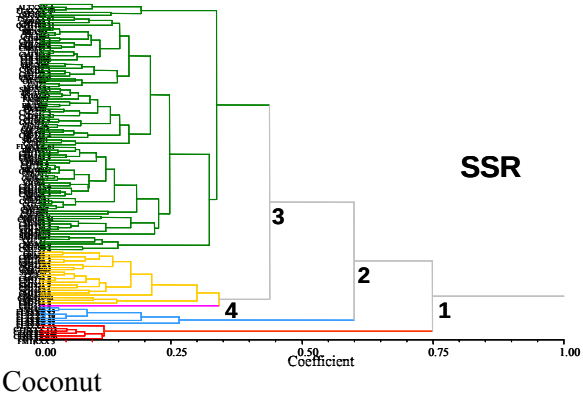



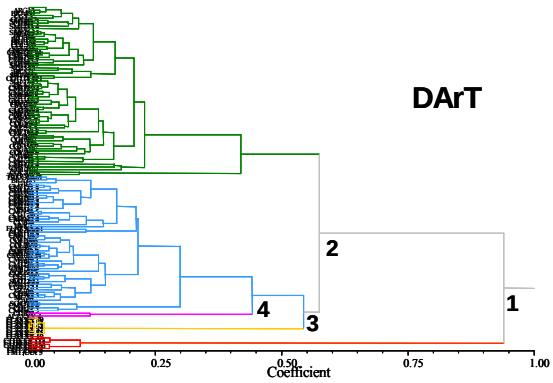

Musa




Cassava




Coconut

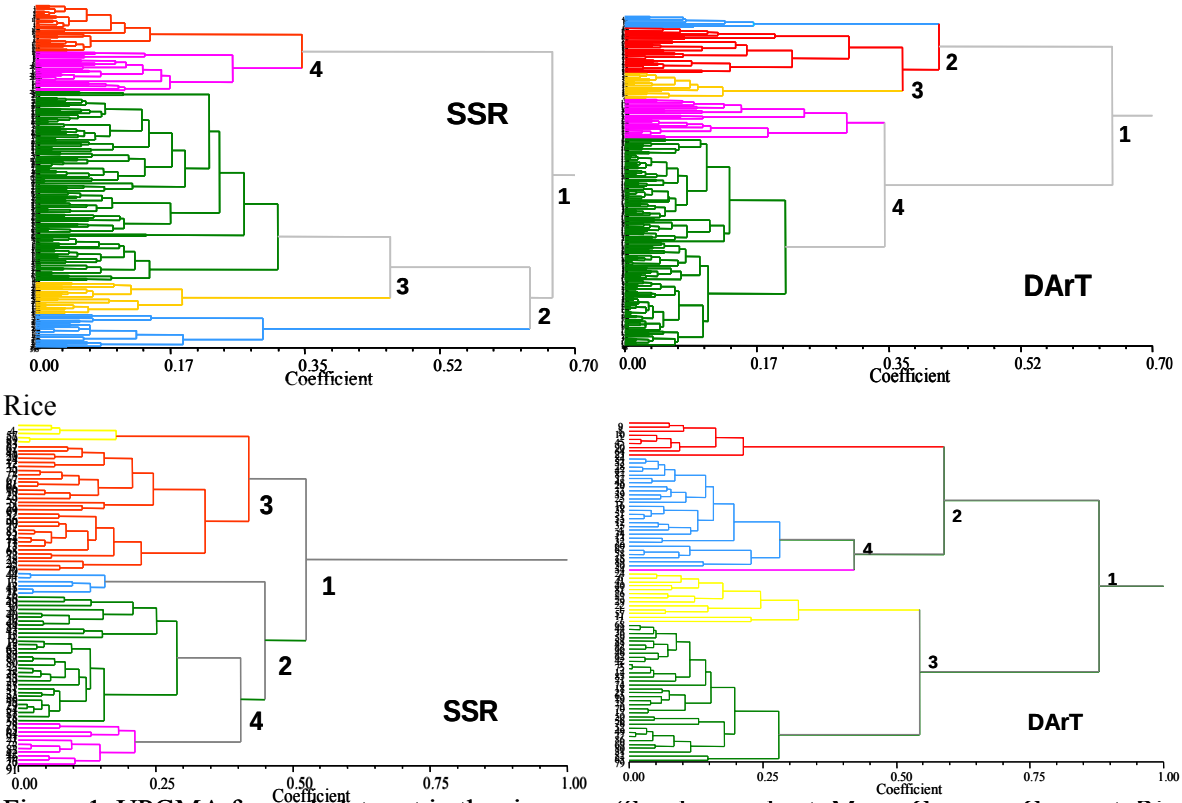



Figure 1 UPGMA for each data set in the six crops (Sorghum, wheat, Musa, Cassava, Coconut, Rice)

For the second level of comparison, coincidence of accessions composing (%) the clusters defined by each molecular marker was calculated (Table 3).

Table 3 Coincidence between accessions composing the clusters defined by SSR and DArT.

| Subdivision | Number of clusters | Sorghum | Wheat | Musa | Cassava | Coconut | Rice | Average |
|---|---|---|---|---|---|---|---|---|
| 1st | 2 | 85,6 | 73,4 | 62,7 | 100,0 | 100,0 | 54,6 | 79,4 |
| 2nd | 3 | 65,6 | 80,9 | 54,4 | 59,1 | 87,8 | 45,3 | 65,5 |
| 3th | 4 | 61,1 | 73,4 | 50,3 | 57,6 | 70,4 | 41,8 | 59,1 |
| 4th | 5 | 56,7 | 76,6 | 52,1 | 57,6 | 82,5 | 34,8 | 60,0 |
| Average | | 67,2 | 76,1 | 54,9 | 68,6 | 85,2 | 44,1 | 66,0 |

The average coincidence for the accessions found in the equivalent clusters with SSR and DArT was 66%. Passport (geographical) or phenotypic information is needed to determine which marker system better explains the resulting classification. In the six crops, differences were found between and among clusters as defined by SSR or DArT. At the first subdivision, coconut and cassava showed 100% coincidence in the accessions encompassed by groups 1 and 2. Coconut showed the highest coincidence between accessions at different subdivision levels (85.2%) while Rice showed the lowest (44.1%). The marker system that better explain the diversity of the crop must be defined by rice specialists.

The average coincidence between both marker systems was 66%, so more than half of the accessions evaluated in each crop were classified in the same clusters by SSR and DArT.

Cassava was taken as a model crop for doing the comparisons since passport data and geographical origin of the samples were available. Cassava study included some of the wild progenitors (*M. flabellifolia*), two wild *Manihot* species (*M. filamentosa* and *M. carthagenensis*), some cassava landraces from South America and breeding lines. Figure 2 shows the comparison between the cassava accessions classified in the different clusters by SSR and DArT. A frequency table (Table 4) was used to calculate the percentage of coincidence between accessions composing clusters with both systems.

At the first subdivision level SSR and DArT are showing the same cassava accessions composing the clusters. The two wild *Manihot* are clearly separated (red cluster) from the others with both marker systems. At the second level, the accessions representing the wild progenitors of cassava are composing a different cluster defined by SSR (blue cluster). At the same level, DArT markers are showing a cluster composed by the wild progenitors and some breeding lines. The wild progenitors were separated from the rest of accessions in the third subdivision level by DArT (yellow cluster). Cluster number 5 (Green) is mainly composed by breeding lines with the two marker systems.

Even the significant difference detected for the coincidence in the first subdivision and the other three subdivisions, the average coincidence for the accessions found in the equivalent clusters between both systems was 68,6%. The group of SSR markers used in this study has been used in previous diversity studies showing a high resolution of the clusters and separating accessions according to the origin. DArT was used as an alternative system to resolve diversity in cassava and to get genetic information from more than 200 loci in short time. The utility of DArT in cassava association studies will be also assessed in another GCP commissioned project.

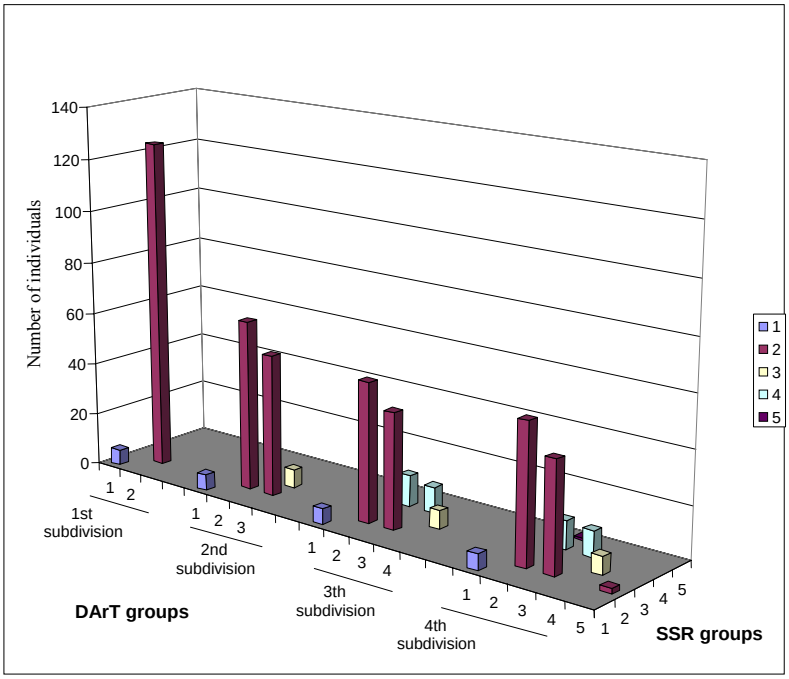


Figure 2. Comparison between accessions composing the clusters at each subdivision level in cassava.

Table 4. Frequency table used to estimate the coincidence (%) for the accessions found in the equivalent clusters with SSR and DArT in Cassava.

| | | | SSR | | | | |
|---|---|---|---|---|---|---|---|
| | Subdivision | Cluster number | 1 | 2 | 3 | 4 | 5 |
| DArT | 1st | 1 | 6 | 0 | | | |
| | | 2 | 0 | 126 | | | |
| | 2nd | 1 | 6 | 0 | 0 | | |
| | | 2 | 0 | 65 | 0 | | |
| | | 3 | 0 | 54 | 7 | | |
| | 3th | 1 | 6 | 0 | 0 | 0 | |
| | | 2 | 0 | 53 | 0 | 12 | |
| | | 3 | 0 | 44 | 0 | 10 | |
| | | 4 | 0 | 0 | 7 | 0 | |
| | 4th | 1 | 6 | 0 | 0 | 0 | 0 |
| | | 2 | 0 | 53 | 0 | 11 | 1 |
| | | 3 | 0 | 42 | 0 | 10 | 0 |
| | | 4 | 0 | | 7 | 0 | 0 |
| | | 5 | 0 | 2 | 0 | 0 | 0 |

**Conclusions and perspectives**

The average coincidence for the accessions found in the equivalent clusters with SSR and DArT was 66%. Passport (geographical) or phenotypic information is needed to determine which marker system better explains the resulting classification.

In six crops, differences were found between and among clusters as defined by SSR or DArT. At the first subdivision, coconut and cassava showed 100% coincidence in the accessions encompassed by groups 1 and 2.

The study of cassava benefited from phenotypic and geographic information – a significant difference was detected for the coincidence in the first subdivision and the other three subdivisions. The first subdivision clearly separates wild *Manihot* species (***M. filamentosa, M. Carthagenensis***) from the wild progenitor and other cultivated species.

*Rice* showed the lowest coincidence between accessions (44.1%) in the clusters. Crop specialists should discuss which marker clustering better explains the diversity of the crop.

### 1.3.3 Use of Molecular Markers to Estimate Level of Heterozygosity in Selfed Lines of Cassava (*Manihot Esculenta* Crantz)

Castro, AM. Perez, JC. Ceballos, H. and Fregene, M.
Funding: CIAT

#### Introduction

The use of molecular markers permits a measurement of the level of heterozygosity of selfed progenies with to respect to the progenitors and by so doing accelerate the process of attaining homozygosity. The objective of this study is to estimate the level of heterozygosity in four $S_1$ familes using simple sequence repeat (SSR) markers and to determinate the best genotypes for additional crosses. This will serve to eliminate the confounding effect of vigor on level of homozygosity and establish a corelationship between molecular analysis and phenotypic data and to select the best progenitors in order to continue to the second generation of selfing.

#### Methodology

A total of 300 $S_1$ lines were chosen for this study, they were produced from 4 elite clones: SM1219-9 (AM331 family 72 genotypes) SM1511-6 (AM 335 family 70 genotypes), SM1665-2 (AM337 family 88 genotypes), SM1669-5 (AM338 family 70 genotypes). The four $S_1$ families were planted at CORPOICA, Palmira in August 2006 and phenotypic data obtained[1].

Young leaf tissue was collected and dried at $45^0$C for 48h. DNA was isolated using the mini Dellaporta (1983) protocol. Preliminary screening of 500 SSR markers in the parent of each family was conducted to identify markers that were heterozygous in the progenitors. These polymorphic markers were evaluated in the entire families. SSR markers were analyzed on 4% polyacrylamide gels as described earlier (Mba et. al. 2001). Gel image was captured by scanning and transferred to a Microsoft Excel file for the inclusion of heterozygosity data and interpretation.

#### Results

A set of 73 SSR markers (at least 4 markers per each chromosome) were selected. Heterozygosity of the parents was determined based on the number of SSR alleles possessed. Parent of AM 331 family (SM 1219-9) showed an average of heterozygosity of 1.57, this means that 57% of the evaluated loci are heterozygote, presence of two bands in the gel (Figure 1) Parent of AM 335 family (SM 1511-6) showed an average of 1.51 of heterozygosity, 51% of the evaluated loci are heterozygosis. Parent of AM 337 family (SM 1511-6) showed an average of 1.48, 48% of the evaluated loci are heterozygosis. And parent of AM 338 family (SM 1669-5) showed an average of 1.62, 62% of heterozygosity. Heterozygosity of the $S_1$ families is currently being evaluated.

[1] Provided data by Perez, J.

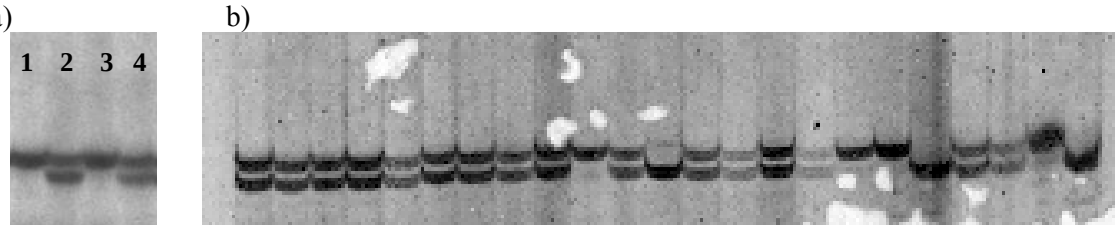



Figure 1 Evaluation of parents, SM 1219-9 (AM331), SM 1511-6 (AM335), SM 1665-2 (AM337) and SM 1669-5 (AM338) and part of the AM335 family with SSR marker SSRY90. Individuals with two bands are heterozygote.

**Conclusions and Perspectives**

The heterozygosity status of the parental lines of the $S_1$ families has been determined it ranges from 50% to 60%. Future activities include SSR marker evaluation of the $S_1$ families, assessment of heterozygosity, and statistical correlation with vigor from field phenotypic evaluation in field. Finally, we will recommend the best genotypes to be advanced to the $S_2$ generation based on results of the molecular marker analysis.

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### 1.3.4 Association analysis of Candidate Genes Associated with Dry Matter Content in Cuban Cassava Germplasm

Yoel Beovides García, Paula X. Hurtado, Wilson Castelblanco, Martin Fregene, Janneth P. Gutiérrez, Charles Buitrago, Edgar Barrera, Marilys Milián y Sergio Rodríguez.

Funding: Casava Biotechnology Nerwork (CBN)

**Introduction**

The characterization, evaluation, conservation and sustainable use of the genetic resources, is one of the priorities cassava research world-wide. The development of SSR markers (SSR-simple sequence repeats), and others like the SSCP-SNP (single-strand conformational polymorphism) associated with traits of agronomic interest, offer powerful tools to transfer traits from exotic sources into adapted varieties.

A study to assess the association of starch biosynthesis genes and dry matter content (DMC) in 50 Cuban cassava accessions was conducted as a first step to developing markers for MAS of DMC. Association analysis in allogamos organisms is based on the knowledge of genetic structure of germplasm accessions, accurate phenotypic evaluation of the target trait, and markers physically close to the gene of interest. An SSR study of structural diversity in 92 Cuban cassava accessions has been conducted previously

(Beovides, 2005). DMC had also been measured for 50 of these accessions in multi-locational trials in Cuba. A list of starch biosynthetic genes of starch biosynthesis that are candidate genes for DMC was also drawn up from previous studied. We present here an association analysis of DMC in the Cuban germplasm and the starch biosynthesis genes.

**Methodology**

*Selection of germplasm*
50 accessions were selected based genetic structure previously defined in a diversity study including 92 Cuban accessions evaluated with 36 SSR markers (Beovides, 2005). Those accessions were collected in Cuba and are held at the germplasm bank of the Instituto de Investigaciones en Viandas Tropicales (INIVIT), Cuba.

*Dry matter content (DMC) evaluation*
DMC was measured at 10 months after planting in the 50 accessions. The procedure included three replications of grinded fresh roots (200gr) placed in the oven at 85°C. DMC measurements were taken as percentages of fresh rot weight and the evaluations were done during three consecutive years.

*DNA extraction and PCRs*
DNA was extracted following the modified Dellaporta protocol (Dellaporta, 1983). The quality and quantity were checked and dilutions were done to be used as PCR templates.

*Search for homologue sequences to 12 starch genes and primer design*
Selected starch biosynthesis genes include:

| | |
|---|---|
| SBE I, SBE II | Starch branching enzyme I y II |
| SS I, SS II, SS III | *Starch synthase* |
| aINV | *Acid invertase* |
| apoINV | *Apoplastic invertase* |
| SPS | *Sucrose phosphate synthase* |
| SUT | *Sucrose transporter* |
| HET | *Hexose transporter* |
| SUSY | *Sucrose synthase* |
| MEX 1 (RCP1) | *Maltose transporter* |

Sequences of the above starch genes were obtained from Genbank and tBlastx using a Cassava EST data base conducted to identified cassava sequences ones with a significant *E*-value. Primes could be designed for Invertase (INV), SUSY, HET, SPS, MEX1, SSSIII and SBE genes but sequences SS I, SS II, apoINV and alINV were not specific enough for primer design. *Primer-3* program was used for primers design. In this study, 16 primers were used based on afore-mentioned genes codifying for starch synthesis proteins.

*PCR conditions and visualization of products*
Concentration of PCR template was 10ng/ul of each genotype. A total of 8 PCR reactions using 16 primer pairs were carried out for molecular characterization. PCR conditions were adjusted for each primer and the amplification was checked on Agarose gels 1.5%. The PCR products were denatured and separated according to their structural conformation on a MDE gel (Mutation Detection Enhancement) (Martins-Lopes et al, 2001). Fragments were separated by electrophoresis at 8W running for 16-18 hours at room temperature and the conventional silver staining protocol was used to visualize PCR amplification (Bassan

et al, 1991). PCR products were scored based on presence (1) or absence (0), missing data was recorded as 9.

*Linkage disequilibrium analysis*
Linkage disequilibrium data was calculated using the software POPGENE version 1.32 based on the genotypic data previously generated in the diversity study of Cuban accessions (Beovides, 2005).

*Association analysis*
Association was detected between genotypic and phenotypic data using Mann-Whitney-U non-parametric independence test by means of the software SPSS/PC.

**Results**

*Phenotypic data analysis*
The mean of three measurements of DMC recorded over3 years for each of the 50 genotypes was calculated. The data was divided into 5 categories and the frequency of genotypes according to the established ranks identified (Table 1).

Table 1. Frequency of genotypes included in each DMC rank

| Category | Rank (percentage of CMD) | First evaluation (Year 1) | Second evaluation (Year 2) | Third evaluation (Year 3) | Average |
|---|---|---|---|---|---|
| 1 | 30.8 - 33.8 | 5 | 5 | 4 | 4 |
| 2 | 33.9 - 36.8 | 9 | 11 | 9 | 10 |
| 3 | 36.9 - 39.8 | 11 | 11 | 10 | 11 |
| 4 | 39.9 – 42.8 | 21 | 20 | 24 | 24 |
| 5 | 42.9 – 45.8 | 4 | 3 | 3 | 3 |

Correlation was calculated between the 3 evaluations and between each evaluation and the average to define the group of phenotypic data used in the association analysis. Results from correlation analysis showed that there is a strong correlation (0.97-0.99) between and within the three evaluations and the average. It represents a high confidence in the evaluation; so the average of DMC percentage for each genotype during the three years of evaluation was used for the association analysis.

Genetic structure from a previous diversity study (Beovides, 2005) including 34 SSR loci showed a clear separation between the 50 accessions included in this study. Two groups, A and B were detected.

Genotypic data was correlated to the average DMC in the 50 accessions distributed in groups A and B. Seventeen accessions or 82% of group A have a DMC percentage lower than 40%. On the other hand 32 accessions or 69% of group B, have a DMC higher than 40%. These results suggest that one of the causes of the genetic structure found in the 50 Cuban accessions is dry matter content. It means that the 50 samples selected in this study are ideal for association studies using molecular markers highly linked with this trait.

*Linkage disequilibrium (LD)*
LD was calculated using the information from 50 genotypes included in this project. The genotypes were divided in two groups (A and B) according to the observed genetic structure. 34 SSR markers were used in the previous diversity study and a matrix composed of 198 alleles in the 50 genotypes was generated

and employed to calculate LD. In the group A there was a significant LD between 9 allele pairs (Table 2) and only one of them belongs to the same linkage group, LG, according to their location in the cassava genetic map (Fregene et al., 1997; Mba et al., 2001). In the group B there was a significant LD between 40 allele pairs (Data no shown) and only two of them belong to the same LG.

Table 2. Allele pairs with significant LD in genotypes from group A. In blue is indicated the allele pair belonging to the same LG.

| Locus | Allele | - | Locus | Allele | Burrows | Correlation | Chisq* | Probab. |
|---|---|---|---|---|---|---|---|---|
| SSRY19 | H | - | SSRY21 | C | 0,0138 | 0,5 | 4,25 | 0,0393 |
| SSRY19 | F | - | SSRY105 | D | 0,026 | 0,5 | 4,25 | 0,0393 |
| SSRY20 | E | - | SSRY108 | D | 0,0519 | 0,5 | 4,25 | 0,0393 |
| SSRY20 | B | - | SSRY110 | A | 0,0138 | 0,5 | 4,25 | 0,0393 |
| SSRY20 | F | - | SSRY151 | D | 0,026 | 0,5 | 4,25 | 0,0393 |
| SSRY34 | C | - | SSRY69 | E | -0,04 | -0,5195 | 4,05 | 0,0442 |
| SSRY34 | A | - | SSRY100 | H | 0,0138 | 0,5 | 4,25 | 0,0393 |
| SSRY63 | F | - | SSRY155 | D | 0,0138 | 0,5 | 4,25 | 0,0393 |
|  | A | - | SSRY171 | F | 0,0311 | 0,5165 | 4 | 0,0454 |

Although significant LD was detected in low number of allele pairs from the same LG, it is important to consider the low number of markers used for the estimation and the small sample size. To detect an accurate LD in a crop, a high number of markers covering the genome must be evaluated or a specific genome region with high density of markers must be study. The present study was focused on a small genome region highly associated with DMC using evaluation of molecular markers related with starch biosynthesis.

*Primers evaluation and association analysis*

From 16 primers designed for genes related with starch biosynthesis, amplification conditions were standardize for 12 of them (1-SUSY, 2-SUSY, 1-HET, 2-HET, 1-SPS, 2-SPS, SSIII, 1-SBE I, 2-SBE I, SUT, 1-SBE II, INVERT). One of them was monomorphic (1-SBE) and between 3 and 10 alleles were detected in the remaining 11 polymorphic primers generating a matrix of 65 alleles. The analysis was conducted separately for genotypes from each group (A or B). Association was detected between the genotypic classes (1/0) and the phenotypic evaluation (DMC percentage) in group A the alleles 2 (marker: 2-SPS), 5 and 7 (marker 2-HET), 8 (marker: 1-SUSY), 2 and 7 (marker: 2-SBE I) using the independence test Mann-Whitney-U when $P<0.1$. Group A is mainly composed by genotypes with DMC lower than 40%, indicating association between these alleles and low DMC. In group B there were no alleles associated with DMC higher than 40%. The probabilities for the genotypic classes in all the markers were higher than 0.1.

**Conclusion and perspectives**

6 alleles were associated with low DMC in the evaluated germplasm.

Only some genes related with the starch process of synthesis and degradation were included in this study. New studies including more genes have to be done in order to identify alleles or markers related with DMC.

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### 1.3.5 Population structure, phenotypic information and association studies in long-generation crops

Paula Hurtado, Charles Buitrago, Myriam C. Duque, Amparo Rosero, Martin Fregene
Funding: Generation Challenge Program (GCP)

**Introduction**

Cassava improvement is mostly field-based and the process can take over 8 years due to the growth cycle of the crop and the multiple evaluations that must be done to identify superior genotypes. A more cost-effective and reliable way of determining the genotype, is the use of molecular marker assisted selection (MAS). In the same way, the search for genetic markers can be accelerated using approaches that does not require development of mapping populations, for example association mapping. Thirty years of cassava breeding has accumulated phenotypic data for 800 improved lines. These materials have been evaluated over several years in different locations and in replicated trials for traits of agronomic importance (harvest index, dry matter content, and yield). A subset of 175 varieties out of the 800 was randomly selected for the determination of cassava linkage disequilibrium (LD) in the cassava genome. Genetic relationship between the accessions was assessed using CIAT germplasm collection databank and breeding program database for further pedigree analysis to guide association mapping analysis.

**Methodology**

*Compilation of phenotypic information in a complete data base*
Phenotypic data have been compiled for the 175 lines by the CIAT breeding program. These materials have been evaluated over several years in different locations (59 places) and replicated trials for traits of agronomic importance such as fresh and dry root yield, root dry matter content, starch content, number of roots and resistance to pests and diseases. Additional information corresponding to the breeding program, the ecozone, the type of trial and the harvest time were also included for each genotype.

*Genotyping of cassava accessions*
A sub-set of 175 varieties (breeding lines and landraces/elite accessions) were randomly selected out of 800 improved lines for association analysis and linkage disequilibrium determination. For genotyping of

the sub-set, 100 SSR markers were previously selected (CIAT, 2005) and up today, 63 of them have been used for molecular characterization. Genotypic data has been stored in a data base composed also by phenotypic information for each accession.

*Data quality assessment*
A workshop on association analysis was organized by GCP for the crops involved in the project to evaluate data quality and identify a list of things to do for completion of the association study. Outstanding tasks for cassava include:
Completion of the genotyping work: Evaluations of the remaining 37 SSR markers to reach the proposed 100 markers and filling in the gaps for the genotypic data, some accessions have more than 20% of missing data.

Confirm the population structure in the sub-set using the statistical software package Structure; this has to be included in the association analysis as a variable.
Review the position of the selected SSR markers in the genetic map to define accurate linkage disequilibrium decay in each chromosome.
Complete the pedigree information for each genotype and calculate the coancestry and fraternity coefficients between genotypes. It must be included as an additional variable in the association analysis.

To assess the quality of the available phenotypic data, looking at month/year of planting, type of trial, location and number of harvested plants. Since the data sets are unbalanced (not all the varieties have been evaluated in the same places and years), variance and co-variance analysis must be done to provide descriptive statistics of the data.
Based on the type of genotypic and phenotypic data we have, the association analysis must be done using a mixed model and numerous variables will have to be included to detect reliable associations between molecular and phenotypic information.

**Results**

Compilation of phenotypic data has been done, including all the variables affecting the data in the data base. Descriptive statistics is being performed to assess the data quality.

Genotypic information has been generated from 175 accessions evaluated with 63 SSR markers (Figure 1) and a preliminary population structure obtained by principal Coordinate analysis, PCoA (Figure 2).

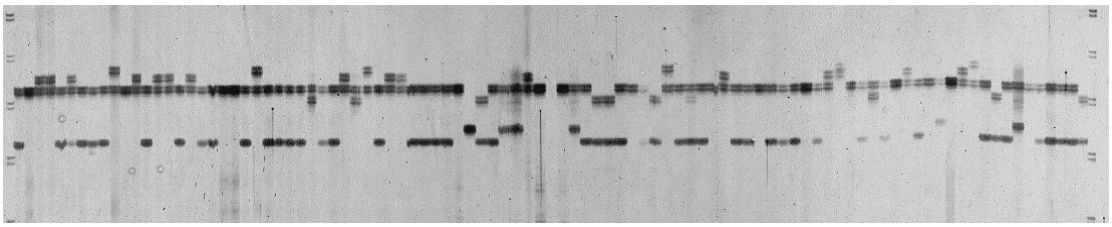

Figure1 Silver stained PAGE gel of PCR amplification of 96 cassava accessions out of 175 evaluated with SSRY 177.

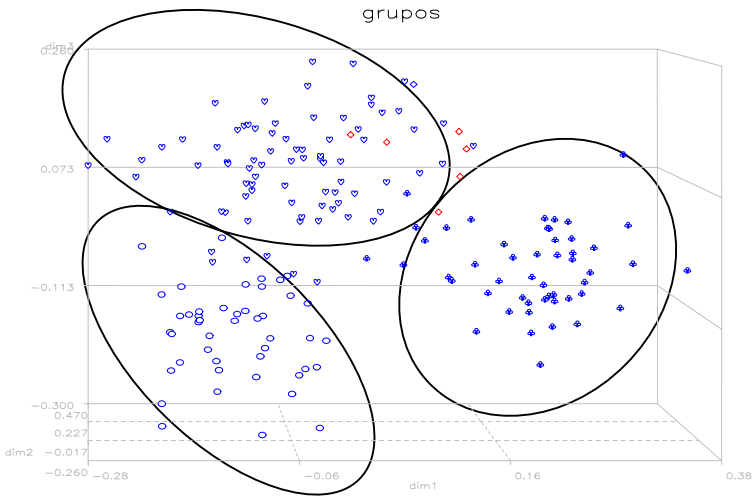


Figure 2 PCoA generated from 175 accessions evaluated with 63 SSR markers.

**Conclusions and perspectives**

Once genotyping is completed marker haplotypes will be defined in the 175 genotypes using the computer software Tassel, developed by GCP-sub-programme 4 (Bioinformatics). The haplotype data and the position of each SSR marker in the genetic map will be used to calculate linkage and structural disequilibrium between alleles of the SSR loci in the selected genotypes.

Population structure will be defined using the complete data set and the Structure software; if any clusters are found, it will be included as a new variable in the association analysis.
Pedigree information will be completed for each genotype and the coancestry and fraternity coefficients will be calculated for inclusion in the analysis.

Descriptive statistics will be completed to assess the quality of the data.
According to the numerous variables affecting the data, a mixed model will be used to detect reliable associations between molecular and phenotypic information.

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### 1.3.6 Progress in Positional Cloning of the *CMD2* Gene Based on Comparative Genomics with Castor Bean (*Ricinus Communis*)

I. Moreno (CIAT); J. Tomkins (CUGI); E. Okogbenin (CIAT-NRCRI), P. Rabinowicz, and M. Fregene (CIAT)

**Introduction**

Comparative genomics is a powerful tool to obtain useful genomic information from well studied organism that can be applied in unknown related genomes. In this study we are using already available information from Castor Bean, a member of the *Euphorbiacea l*ike cassava to advance the chromosome

walking process to the *CMD2* gene. The basic concept is find new molecular markers closer to the gene. However, a set of contiguous low copy sequences are required to identify homologous sections of the castor genome.

Previously we had identified BAC33 is contig constructed around the SCAR marker RME-1 2cM away from *CMD2* and BAC 36 from the SSR marker NS158 contig as candidates for BAC clone sequencing (CIAT 2005). Low copy sequences can then be used for primer design for new molecular marker for evaluation in parentals and bulks as well as to identify a homologous contig in castor bean.

**Methodology**

Sub libraries from each BAC clone was constructed by digestion with Hind III, and cloning into pBluescript vector using standard protocols. Low copy sequences were analyzed using public genome database, the cassava database at CIAT, and the Blastx sequence comparison algorithm (Altschul et al 1997). Primers were designed for each sequence selected to produce an amplification product of between 300-350bp using the Primer 3.0 program, the PCR conditions for a final volume of 25 ul were 1X of Buffer; 2mM of MgCl2, 0.2mM of dNTPs and 0.2 uM of each primer and 1 U of taq polymerase, the amplification program consisted on a initial denaturation step at 95ºC 2min and 30 cycles of 94ºC 30 sec, 55ºC 1 min, 72ºC 1min and a final extension step of 72ºC for 5min.

The PCR amplification products for each pair of primers corresponding to parentals and bulks (resistant and susceptible parental, resistant bulk and susceptible bulk) were resolved on agarose gels and monomorphic profiles denatured and separated by single-strand conformational polymorphism (SSCP) gels using a mutation detection enhancement (MDE) gel solution following conditions recommended by the author (Bertin et al 2005). The candidate marker was evaluated in each individual of each bulk and the recombinant susceptible individuals.

**Results**

A low copy sequence was obtained from each BAC clone. The low copy sequences from BAC 36 (BA36A35) matched a glutamate receptor and from BAC 33 sequnce (BA33C53TV) hits a gene in the GenBank, which is an unknown wound-induced protein, found in different plant species.

After amplification and SSCP gel analysis, we obtained a clear difference between CMD resistant and susceptible parents and bulks with the new S-BAC33c SSCP-SNP marker that was consistent in individuals of the bulks (Fig1)

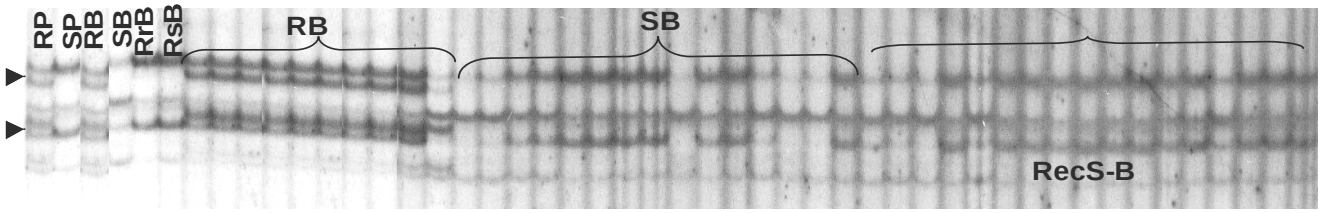


Fig 1 Segregant Bulk Analysis with the S-BAC33c SSCP-SNP primer. The sample order is: RP (Resistant parental), SP (Susceptible parental), RB (Resistant bulk), SB (Susceptible bulk) and RecS-B (Recombinant susceptible-Bulk).

**Conclusion and perspectives**

We have advanced in the chromosome walking process toward to the CMD2 gene by the identification of a new molecular marker associated to this characteristic. The entire BAC 33 clone is currently being sequenced at TIGR using a different strategy (ASAP library) to obtain a higher number of low copy sequences. Sequence homology with castor bean genome will be sough and a castor bean contig selected as template for design of a new group of primers for BAC pool screening and BAC end sequencing in cassava.

Additionally, Two SCAR markers have been developing from BAC33b and S-BAC33c polymorphic band to be used in new rounds of BAC library screening and chromosome walking. Those markers also represent potential tools for MAS.

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## 1.3.7 Development of COS Markers for Positional Mapping in Cassava based on homologus Castor Bean Sequences

Isabel Moreno1, Pablo Rabinowicz2, and Martin Fregene1
[1]CIAT; [2]TIGR

**Introduction**

*Ricinus communis*, also known as castor bean, is native to Africa and warmer parts of Asia and it is a member of the family Euphorbiaceae. Castor oil is extracted from R. communis seeds and is used for industrial, medicinal and cosmetic purposes. The United States is one of the world's largest importers of castor oil and its derivatives. Considering that a deeper understanding of the castor bean genome will allow for improvements in castor oil production a sequencing project was initiated at TIGR (The Institute for Genome Research). So far TIGR has sequenced and assembled a 4X draft of the ~400 Mbp castor bean genome using a whole genome shotgun strategy. In addition, ~50,000 ESTs from different tissues have been produced to help gene discovery and annotation (Rubanowicz et al 2006, personal communication).
The purpose of this study was to identify single-copy castor bean sequences that have a single best match to one region of the cassava ESTs bank and hence would qualify as potential orthologs that can be useful applied as molecular markers in cassava genetic research.

**Methodology**

Sequences of the molecular markers associated with the *CMD2* resistance gene (SSRY28, NS158, RME-1, GY159, GY1) and sequences obtained from BAC ends were compared with the castor Bean genome by Blastn and tBlastx analysis at TIGR.

Castor bean contig sequences obtained were analyzed with Genescan to identify putative genes and proteins. Proteins were analyzed by BLASTbatch against cassava ESTs bank (CIAT) and results were evaluated under following parameters to identify conserved ortholog set or COS markers:

A unique castor bean sequence was selected as a conserved ortholog if it met the following criteria: it matched a single cassava EST at an expect value of E-15, and the next best cassava match was of lower significance (i.e., there was a difference of 10 between expect scores (Fulton et al 2002). Cassava ESTs sequences were analyzed by BLAST2 against castor bean contig sequences to define exon and intron regions. Primers were designed from exonic regions containing introns as general rule using as template both cassava ESTs (COS primers) and castor bean sequences (2CAST primers).

The PCR conditions for COS primers in a final volume of 25 ul were 1X of Buffer; 2mM of $MgCl_2$, 0.2mM of dNTPs and 0.2 uM of each primer and 1 U of taq polymerase, the amplification program consisted on a initial denaturation step at 95ºC 2min and 30 cycles of 94ºC 30 sec, 55ºC 1 min, 72ºC 1min and a final extension step of 72ºC for 5min.

PCR conditions for 2CAST primers in a final volume of 25 ul were 1X of Buffer; 2mM of $MgCl_2$, 0.2mM of dNTPs and 0.2 uM of each primer and 1 U of taq polymerase, the amplification program consisted on a initial denaturation step at 95ºC 2min and 30 cycles of 94ºC 30 sec, 55ºC 1 min, 72ºC 1min and a final extension step of 72ºC for 5min.

Primers were evaluated to CMD resistance using parentals and bulk (resistance and susceptible) in a Bulk segregant analysis. Amplification products were resolved in agarose gels and monomorphic profiles were resolved in SSCP-SNP gels (Bertin et al 2005).

**Results**

We have obtained a set of 17 COS primers designed from sequences selected of cassava ESTs and a set of 16 2CAST primers designed from the corresponding castor bean sequences.

The amplification products obtained didn't show polymorphisms that permit the differentiation between resistant and susceptible individuals. Some Mono morphic profiles showed polymorphisms between parentals and bulks in SSCP-SNP gels (Fig 1) but we have not found consistent polymorphic bands in the individuals of the bulks as shown in Figure 2

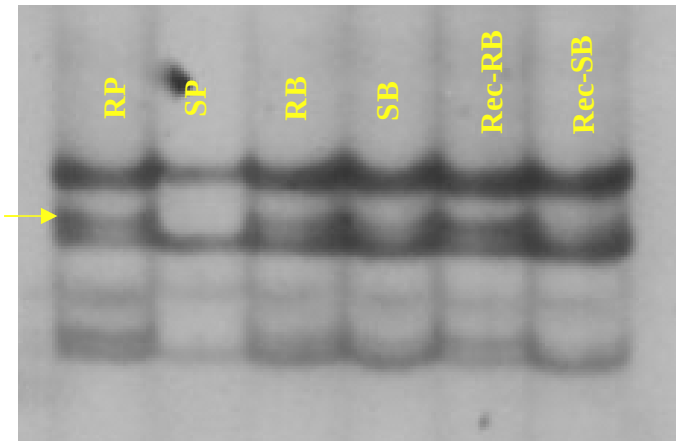

Figure 1 Segregant Bulk Analysis with COS 8A primer. The sample order is: RP (Resistant parental), SP (Susceptible parental), RB (Resistant bulk) SB (Susceptible bulk), RecS-B (Recombinant susceptible-Bulk) and RecS-B (Recombinant resistant-Bulk).

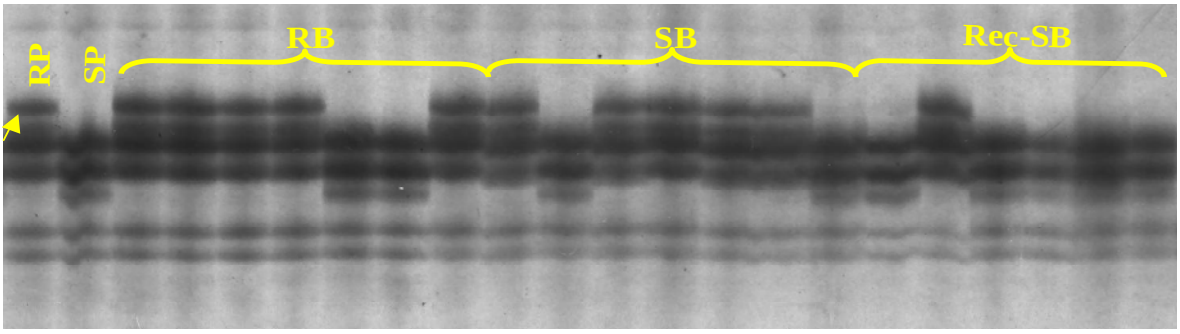


Figure 2 Open Bulk Analysis with 2CASTA11primer. The sample order is: RP (Resistant parental), SP (Susceptible parental), RB (Resistant bulk individuals) SB (Susceptible bulk individuals) and RecS-B (Recombinant susceptible-Bulk individuals).

**Conclusion and perspectives**

We have conducted a comparative analysis between two related genomes to develop a new set of molecular markers, this strategy open possibilities for future studies to define syntenic relationships among those genomes.

A variety of primers were evaluated for resistance to CMD that implied standardization of amplification conditions that permit its use in different agronomic trait studies looking for molecular markers association and fingerprinting analysis.

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## 1.3.8 Isolation of Differential Sequences Expressed during the Defense Response to whitefly attack (*Aleurotrachelus socialis* Bondar) in Cassava *Manihot esculenta* Crantz) using Functional Genomics

A. Bohorquez, A. Bellotti, B. Arias and J. Tohme

**Introduction**

White fly is one of the most serious pest and disease vector that affects agriculture around the world. In cassava (*Manihot esculenta* Crantz), white fly causes between 70 to 80 percent of economical losses (Bellotti, 2002). The Tropical Whitefly IPM (Integrated Pest Management) Project (CIAT), identified resistance to the white fly *Aleurotrachelus socialis* (Bondar) in several cassava clones from Ecuador, like

genotype Mecu 72. Due to the importance of white flies as pests and disease vectors, it is very useful to understand the nature of genes that confer resistance in Mecu 72. Understanding the gene expression profile of cassava in response to attacks of white flies implies characterizing genes involved in the downstream signaling cascade, produced during the plant defense response. One tool that may permit unraveling the complex pattern of gene expression is the analysis of a cDNA library. Such library was developed by Subtractive Hybridization (Luo et al, 1999). Using this approach, two mRNA populations, extracted from the resistant and susceptible genotypes, will be examined to elucidate differential gene expression. Functional genomic tools such as Microarrays (Bouchez & Hofte, 1998) may give a first comprehensive overview of the molecular basis of the cassava defense response to whitefly attacks. Microarray-expression profiling will be used to identify putative, early response, regulatory, and/or signaling genes.

**General Objective**

Have a better understanding of the molecular mechanisms of defense, at the gene expression level, displayed by cassava (*Manihot esculenta* Crantz) when attacked by white flies (*Aleurotrachelus socialis* Bondar).

*Specific Objectives*

1-Identify sequences differentially expressed in cassava during the attack of white flies (*A. socialis*) using substractive hibridization.

2-Assign putative functions to sequences identified in 1, using BLAST (Altschul, *et al.,* 1990) as an algorithm to search for homologous sequences.

3-Use microarrays to identify genes that are timely regulated, turned on or off, in cassava during the attack of *A. socialis*.

**Methodology**

To obtain differentially expressed sequences we developed the bioassay described below, in growth chambers at 28 ± 1ºC, 70 ± 10% RH and 12 hours of light, located in CIAT, Palmira, Colombia.

*A. Infesting cassava with white flies in the Growth Chambers*

It is known that resistance to white flies of genotype Mecu72, comes as a combination of antibiosis and antixenosis (Gomez 2004). Mecu72 and CMC40 have been previously evaluated in field tests in CORPOICA-Nataima (Department of Tolima, Colombia). The former has shown high levels of resistance in different environments and throughout time, while the latter is considered susceptible. Evaluation of susceptibility/resistance to white flies is usually scored using a scale (from 1 to 6; Arias, 1995) that quantifies the population of white flies and the damage suffered by the plant. This year a second trial to obtain sequences expressed during the attack of *A. socialis*, was run between June and July of 2006. Fifty four plants of 45 days of age were seeded in plastic pots with sterile soil and maintained in wooden, nylon mesh lined cages (1m x 1m x 1m), and placed in the growth chamber. Of the 54 plants, 36 were Mecu 72 while the other 18 were CMC40. This last clone is commonly used for rearing *A. socialis* (Figure 1). Plant infestation was accomplished by introducing 20 whitefly adults into small leaf cages, supported by plastic straws (Figure 1). Each leaf cage has a small lateral opening and with the aid of a pasteur aspirator, *A. socialis* adults are encouraged to enter the leaf cages (Figure 1). Three leaf lobes were infested on each plant. Three treatments were set up as follows:

1) Mecu 72 infested
2) CMC40 infested.
3) Mecu 72 without infestation.

There were three repetitions (18 plants) per treatment (Figure 2).

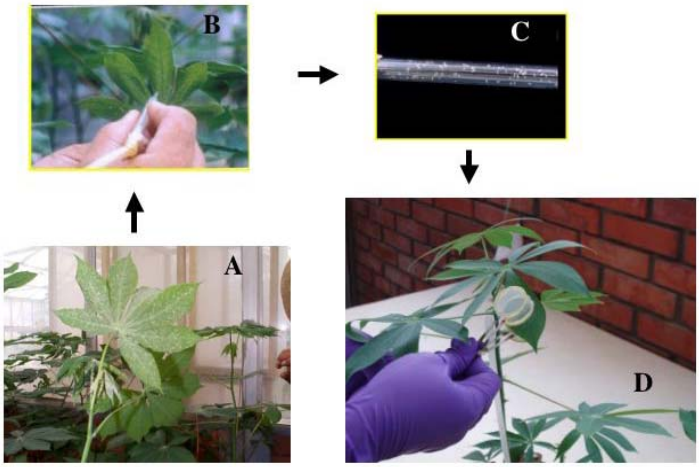


Figure 1: A: Colony of *A. socialis* growing on cassava clon CMC40, maintained at the IPM program of CIAT. B: Adults of *A. socialis* on the axial side of a leaf, being harvested with the aid of a pasteur aspirator. C: Adults harvested with pasteur aspirator. D: A 45 day old cassava clone MEcu72, infested with *A. socialis* adults using clip-cages. Source: Cassava Entomology and A. Bohórquez, CIAT 2006.

*A. socialis* adults were allowed to oviposit for 72 hours, thereby assuring a uniform population. Leaf cages and adults were then removed and egg infested plants were distributed in a complete randomized design due to the existing microclimate inside the growth room (Castrillón & Perlaza, 2000). Infested and non-infested leaves were collected according to Table 1. Collection times matched the life cycle of *A. socialis*. Collected plants were marked not to use them again.

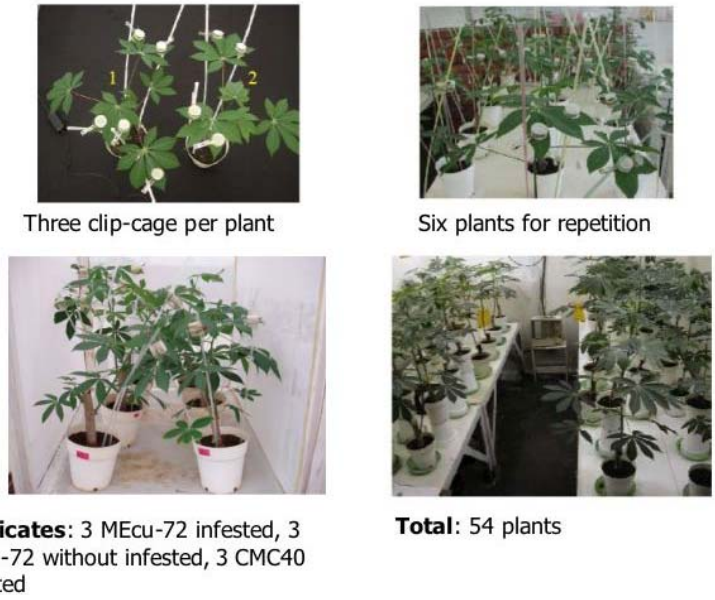


Figure 2. Fourty five day old cassava clones infested with *A. socialis* adults using clip cages,. (source: A. Bohórquez, CIAT 2006).

Table 1: Collection times of infested and not infested leaves. The times match different stages of development of *A socialis.*

| *A. socialis* Stage | Time of collection after infestation |
|---|---|
| Adult | (1) 5 hours, |
| Egg | (2) 7 days |
| Nymph I | (3) 14 days |
| Nymph II | (4) 18 days |
| Nymph III | (5) 20 days |
| Nymph IV | (6) 27 days |

*B. Functional Genomics*

Strategy for Differential Subtraction

For the isolation of differentially expressed sequences we are using two strategies. In the first one the genotype Mecu-72, infested with *A. socialis,* was used as tester, while infested CMC40 was used as driver (Figure 3). This will allow obtaining constitutive resistant genes. In the second strategy infested Mecu-72 was used as tester, while the driver was the same genotype with no infestation. The objective is to obtain genes implicated in the downstream signaling cascade of the plant defense response. The representational difference analysis of cDNA comprises the generation of a PCR amplicon, which is representative of the original mRNA from Mecu-72 and CMC40. The subtractive hybridization of these amplicons, during which amplified portions of differentially expressed genes are enriched and common sequences are depleted, is performed using *Differential Subtraction Chain* (DSC) (Luo et al, 1999) between MEcu-72 (Tester) and CMC40 (Driver). The same substractive hybridization is carried out between the amplicons of Mecu-72 infested (Tester) and Mecu-72 not infested (Driver). Ultimately the DSC technology leads to the cloning and screening of sequences differentially expressed.

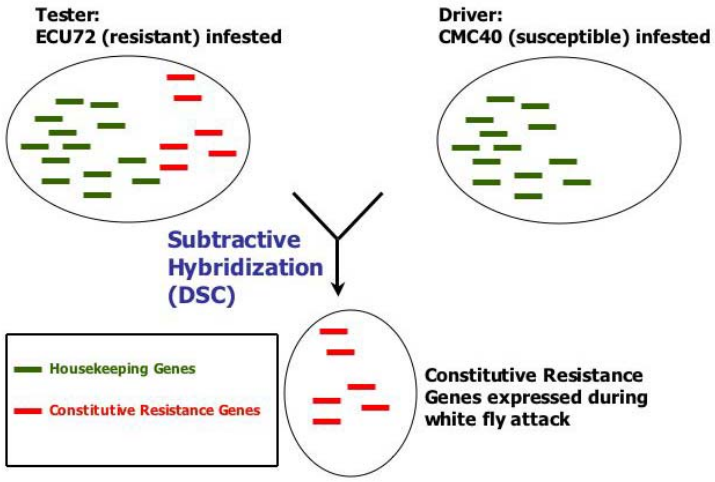


Figure 3: Strategy 1 for the isolation of resistance gene sequences expressed during white fly attack.

Isolation of total RNA, mRNA, cDNA synthesis and substractive library construction
Total RNA was isolated from young leaves using the *SV™ Total Isolation System™ (Promega)*. To isolate mRNA, six pools of total RNA were created within each treatment. Each pool grouped the three repetitions, and corresponded to one single time of collection (i.e., at 5 hours and 7, 14, 18, 20 and 27 days). Then, mRNA was isolated from the 18 pools (3 treatments x 6 collection times) using the *Oligotex™ mRNA isolation kit (Qiagen)*. The first-strand cDNA synthesis and the cDNA amplification were made using SMART™ PCR cDNA Synthesis kit (Clontech). PCR products were purified using

*Wizard® SV Clean-Up System. (Promega).* A total of 12 subtractive hybridizations will be performed, which will generate 12 different subtractive libraries.

**Results**

*Total RNA isolation*
We isolated high quality, total RNA of Mecu-72 and CMC40 (Fig. 6). The RNA concentration was measured using GENios™ specterfluorometer (TECAN).

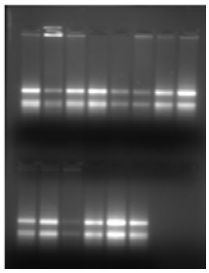

Figure 6: Total RNA isolated from leaves of of Mecu-72 infested with *A. socialis*.

*Poly A+ mRNA isolation, cDNA amplification and purification*.
Poly A+ mRNA was isolated from 100 µg of total RNA from each pool to synthesize cDNA.
*Ongoing activities*
-Digestion-Ligation of cDNAs amplified and purified and amplicons generation.
-Subtractive hybridization of amplicons
-Cloning, sequencing and library construction of resulting products (twelve subtractive libraries).

**Future activities**

-Microarray expression profiling will be used to identify putative early-response, regulatory and/or signaling genes, and to test the function of selected candidate genes using real time PCR technology.

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### 1.3.9 Gene Sequence Analysis of a Natural Waxy Cassava Variety

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**Introduction**

Starch from cassava contains about 20% amylose and 80% amylopeptin. A natural waxy mutant was identified by selfing a cassava genotype from the gene bank (Ceballos et al. 2006, in preparation). The GBSSI gene in the natural waxy mutant and a wild type genotype was sequenced to identify the mutation that led to the waxy phenotype.

**Methodology**

A set of six overlapping primers were designed from the sequence of the gene GBSSI reported in the GenBank using Primer 3.0 software with a expected size between 350-450 bp. The PCR conditions for a final volume of 25 ul were 1X of Buffer; 2mM of $MgCl_2$, 0.2mM of dNTPs and 0.2 uM of each primer and 1 U of taq polymerase, the amplification program consisted on a initial denaturation step at 95ºC 2min and 30 cycles of 94ºC 30 sec, 55ºC-60ºC 1 min, 72ºC 1min and a final extension step of 72ºC for 5min.

Amplification products from the waxy mutant was run on agarose gels 1% and specific bands were eluted and cloned into the pGemT-easy vector (Promega) The quality of cloned bands was evaluated by PCR with universal primers T7 and Sp6 and miniprep of five clones for each primer were sent to sequencing at Iowa State University facility sequencing center (http://www.dna.iastate.edu). Sequences obtained with each primer were edited from vector contamination and analyzed in the genome database with the Blastn algorithm (Altschul et al 1997).

After homology with the cassava GBSSI gene sequence was confirmed a contig of the six sequences was constructed using the Vector NTI program ( Invitrogen corp). Sequence consensuses were aligned using ClustalW (Thompson et al 1994) to find differences between them (Fig 1) and a wide-type cassava GBSS gene found in the public database (Genbank).

```
                    51                                  100
          CLONA     (1) ------------------------------------------------
          CLONB     (1) ----------------------------------GAATTCACTAGTGATTT
          CLONC     (1) ----------------------------------GAATTCACTAGTGATTT
gi_437041_emb_X74160.1_MEGBSS                                              (51)
AGCTTCTACACCGGAGAGAGCACCATGGCAACTGTAATAGCT-GCACATT
          CLOND     (1) ----------------------------------GAATTCACTAGTGATTT
          CLONE     (1) -----------------------------------CGAATTCACTAGTGATTT
        Consensus   (51)                        GAATTCACTAGTGATTT
                    101                                 150
          CLONA     (1) ------------------------------------NNGGCTAAAGGT
          CLONB                                                            (18)
TCGTTTCCAGGAGCTCACACTTGAGCATCCATGCATTAGAGACTAA-GGC
          CLONC                                                            (18)
TCGTTTCCAGGAGCTCACACTTGAGCATCCATGCATTAGAGACTAA-GGC
gi_437041_emb_X74160.1_MEGBSS                                              (100)
TCGTTTCCAGGAGCTCACACTTGAGCATCCATGCATTAGAGACTAA-GGC
          CLOND                                                            (18)
TCGTTTCCAGGAGCTCACACTTGAGCATCCATGCATTAGAGACTAA-GGC
          CLONE                                                            (19)
TCGTTNCCAGGAGCTCACACTTGAGCATCCATGCATTAGAGACTAA-GGC
        Consensus                                                          (101)
TCGTTTCCAGGAGCTCACACTTGAGCATCCATGCATTAGAGACTAA GGC
                    151                                 200
          CLONA                                  (13)          TNATAAATTNNT---
CNCNNNNACNNGGACCCAAACTATCACTCCCAATG
          CLONB                                                            (67)
TAATAATTTGTCTCACACTGGACCCTGGACCCAAACTATCACTCCCAATG
          CLONC                                                            (67)
TAATAATTTGTCTCACACTGGACCCTGGACCCAAACTATCACTCCCAATG
gi_437041_emb_X74160.1_MEGBSS                                              (149)
TAATAATTTGTCTCACACTGGACCCTGGACCCAAACTATCACTCCCAATG
          CLOND                                                            (67)
TAATAATTTGTCTCACACTGGACCCTGGACCCAAACTATCACTCCCAATG
          CLONE                                                            (68)
TAATAATTTGTCTCACACTGGACCCTGGACCCAAACTATCACTCCCAATG
        Consensus                                                          (151)
TAATAATTTGTCTCACACTGGACCCTGGACCCAAACTATCACTCCCAATG
                    201                                 250
          CLONA                                                            (60)
GTTTAAGGTCCCTCAACACTATGGATAAACTCCAAATGAAGACACAATCA
          CLONB                                                            (117)
GTTTAAGGTCCCTCAACACTATGGATAAACTCCAAATGAAGACACAATCA
          CLONC                                                            (117)
GTTTAAGGTCCCTCAACACTATGGATAAACTCCAAATGAAGACACAATCA
gi_437041_emb_X74160.1_MEGBSS                                              (199)
GTTTAAGGTCCCTCAACACTATGGATAAACTCCAAATGAAGACACAATCA
          CLOND                                                            (117)
GTTTAAGGTCCCTCAACACTATGGATAAACTCCAAATGAAGACACAATCA
```

CLONE (118)
GTTTAAGGTCCCTCAACACTATGGATAAACTCCAAATGAAGACACAATCA
Consensus (201)
GTTTAAGGTCCCTCAACACTATGGATAAACTCCAAATGAAGACACAATCA
251 300
CLONA (110)
AAAGCTGTGAAAAAGGTCTCTGCCACCGGCAATGGTAGGCCTGCTGCCAA
CLONB (167)
AAAGCTGTGAAAAAGGTCTCTGCCACCGGCAATGGTAGGCCTGCTGCCAA
CLONC (167)
AAAGCTGTGAAAAAGGTCTCTGCCACCGGCAATGGTAGGCCTGCTGCCAA
gi_437041_emb_X74160.1_MEGBSS (249)
AAAGCTGTGAAAAAGGTCTCTGCCACCGGCAATGGTAGGCCTGCTGCCAA
CLOND (167)
AAAGCTGTGAAAAAGGTCTCTGCCACCGGCAATGGTAGGCCTGCTGCCAA
CLONE (168)
AAAGCTGTGAAAAAGGTCTCTGCCACCGGCAATGGTAGGCCTGCTGCCAA
Consensus (251)
AAAGCTGTGAAAAAGGTCTCTGCCACCGGCAATGGTAGGCCTGCTGCCAA
301 350
CLONA (160)
AATTATTTGTGGTCATGGAATGAATTTAATCTTTGTTGGAGCTGAAGTTG
CLONB (217)
AATTATTTGTGGTCATGGAATGAATTTAATCTTTGTTGGAGCTGAAGTTG
CLONC (217)
AATTATTTGTGGTCATGGAATGAATTTAATCTTTGTTGGAGCTGAAGTTG
gi_437041_emb_X74160.1_MEGBSS (299)
AATTATTTGTGGTCATGGAATGAATTTAATCTTTGTTGGAGCTGAAGTTG
CLOND (217)
AATTATTTGTGGTCATGGAATGAATTTAATCTTTGTTGGAGCTGAAGTTG
CLONE (218)
AATTATTTGTGGTCATGGAATGAATTTAATCTTTGTTGGAGCTGAAGTTG
Consensus (301)
AATTATTTGTGGTCATGGAATGAATTTAATCTTTGTTGGAGCTGAAGTTG
351 400
CLONA (210)
GTCCCTGGAGCAAAACTGGTGGACTTGGTGATGTTCTTGGAGGACTCCCC
CLONB (267)
GTCCCTGGAGCAAAACTGGTGGACTTGGTGATGTTCTTGGAGGACTCCCC
CLONC (267)
GTCCCTGGAGCAAAACTGGTGGACTTGGTGATGTTCTTGGAGGACTCCCC
gi_437041_emb_X74160.1_MEGBSS (349)
GTCCCTGGAGCAAAACTGGTGGACTTGGTGATGTTCTTGGAGGACTCCCC
CLOND (267)
GTCCCTGGAGCAAAACTGGTGGACTTGGTGATGTTCTTGGAGGACTCCCC
CLONE (268)
GTCCCTGGAGCAAAACTGGTGGACTTGGTGATGTTCTTGGAGGACTCCCC
Consensus (351)
GTCCCTGGAGCAAAACTGGTGGACTTGGTGATGTTCTTGGAGGACTCCCC
401 450

```
                CLONA                                                   (260)
CTTGCCATGGCCGTAAGTAGAGGACCCTTCTTTTGTTCTTTCTGCCTCGC
                CLONB                                                   (317)
CCTGCCATGGCCGTAAGTAGAGGACCCTTCTTTTGTTCTTTCTGCCTCGC
                CLONC                                                   (317)
CCTGCCATGGCCGTAAGTAGAGGACCCTTCTTTTGTTCTTTCTGCCTCGC
```

Fig1. Clustal analyisis between GBSSI sequence reported at GenBank and consensus sequences

**Results**

Sequences consensus obtained from amplification of genomic DNA of waxy variety were aligned against GBSSI cDNA sequences reported in GenBank. Putative introns are shown in blue color and exons in yellow color (Figure 1). A single base deletion (763) in exon 6 leads to a frame shift that trunates the GBSS I protein (Figure 2).

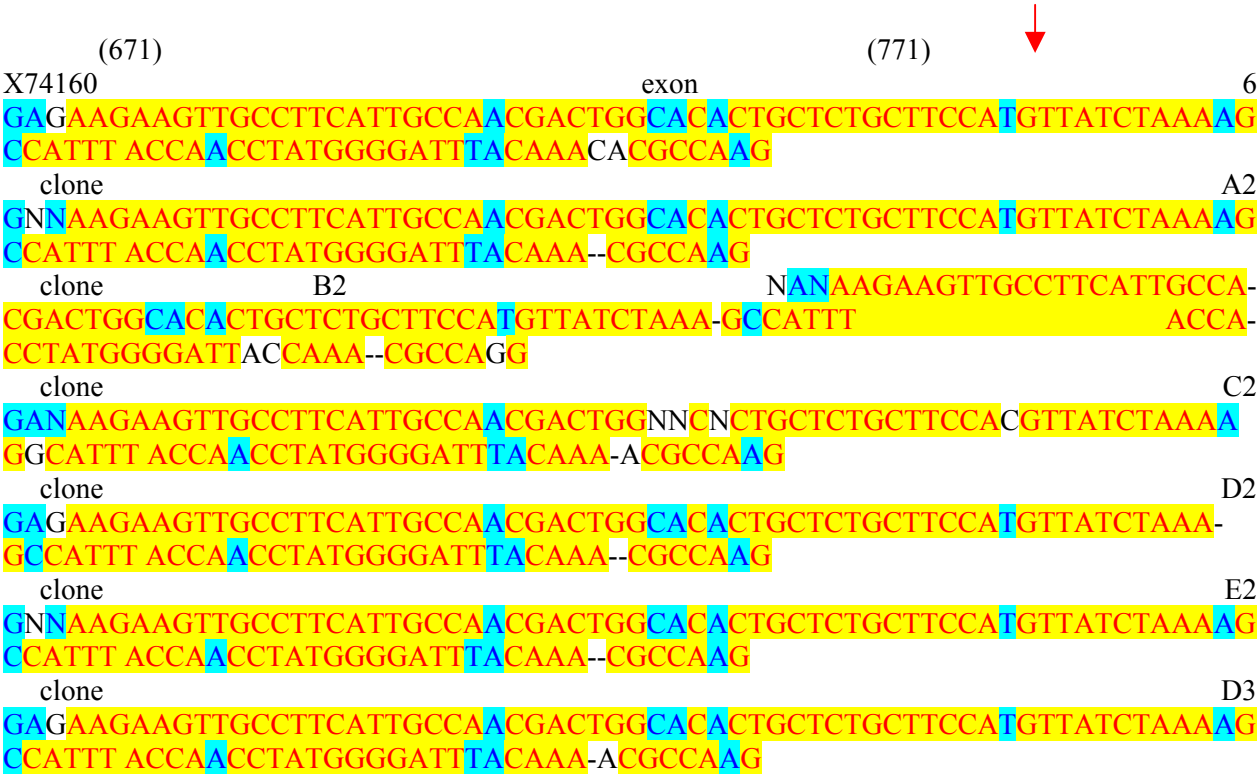


Figure 2 Analysis of GBSSI sequences. The red arrow indicates a single base deletion in exon 6.

**Conclusion and perspectives**

A single base deletion in exon 6 inactivates the GBSS I gene by a frame shift. This is the only consistent difference between waxy mutant and wild-type sequence detected so far.

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## 1.3.10 Development of a TILLING (Targeting Induced Local Lesions in Genomes) Protocol for Cassava

Isabel Moreno, Jaime Marin, and Martin Fregene
CIAT

### Introduction

TILLING (Targeting Induced Local Lesions in Genomes) is a new technique that can identify polymorphisms in a target gene by heteroduplex analysis. We are using a variation of this technique (ECOTILLING) as a means to determine the extent of natural variation in selected genes (Comai et al 2004).

ECOTILLING is an additional use of TILLING methodology to discover and genotype natural variation in populations. ECOTILLING can provide a low cost alternative to full DNA sequencing when searching for rare polymorphisms. We are in the process of assaying natural variation in gene regions associated to starch synthesis pathway in a panel of cassava accessions establishing differences between wild and cultivar accessions using a homemade CELI enzyme.

### Methodology

Homemade CELI was extracted from celery following the instructions reported by Till et al 2004, the activity of this enzyme was evaluated using the commercial enzyme surveyor (Transgenomics) as positive control following the indications of the Manufacturer (Figure 12.28. For standardization process we used two wild and two cultivar cassava genotypes CRA-013, CW14-11, MCOL-1508 and MCOL-2269 respectively. We selected two primers 2-HET (Hexose transporter) and 1-SPS (Sucrose Phosphate Synthase) from a group of primers designed from cassava ESTs sequences showing homology with reported genes associated to starch synthesis pathway in other species (Castelblanco & Beovides personal communication) as they give clear amplification products.

The PCR conditions for those primers in a final volume of 25 ul were 1X of Buffer; 2mM of $MgCl_2$, 0.2mM of dNTPs and 0.2 uM of each primer and 1 U of taq polymerase, the amplification program consisted on a initial denaturation step at 95ºC 2min and 30 cycles of 94ºC 30 sec, 55ºC 1 min, 72ºC 1min and a final extension step of 72ºC for 5min. Amplification products were pooled and cleaned using polyethylene glycol PEG to avoid primer-dimer presence.

Amplification products cleaned from each genotype were hybridized for heteroduplex formation in a thermocycler using the following program: 95°C for 10 min; 95°–85°C at –2°C/s; and 85°–25°C at –

0.1°C/s. Annealed DNA was digested in a 20 ul reaction mixture containing, 0.1M KCl, 0.1M MgSO4, 100mM HEPES 7.5, 0.02% Triton-x100, 0.002 mg/ml BSA and 1ul of CELI. The digestion reaction was incubated at 42 °C for 40 min and was stopped by addition of 5 uL of 0.15M EDTA. The entire reaction mixture (20 uL) was subjected to 2% agarose gel electrophoresis in the presence of EtBr.

**Results**

We have probed the activity of the homemade CELI using controls and kit provided for transgenomics as DNA endonuclease that cleaves mismatches in heteroduplex formations containing any single-base substitution and insertion/deletions up to at least 12 nucleotides (Fig 1). In our assays we have observed differences between wild and cultivar genotypes with the 2-HET primers at level of single nucleotide polymorphisms (Fig 2).

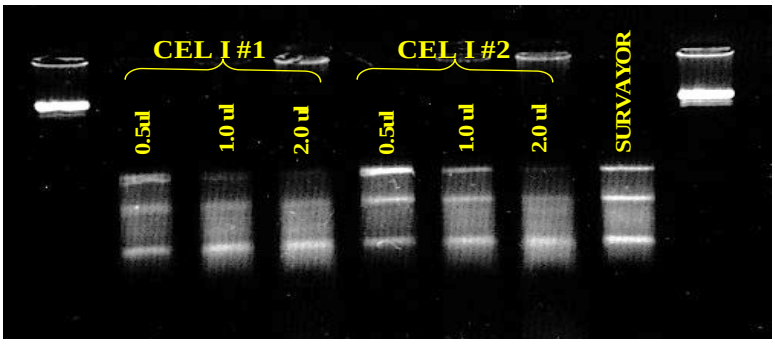


Fig 1. Ethidium bromide stained gel of a CELI digestion assay. Homemade CELI detected the controls mismatched provided in the transgenomics Kit and the control commercial enzyme (surveyor).

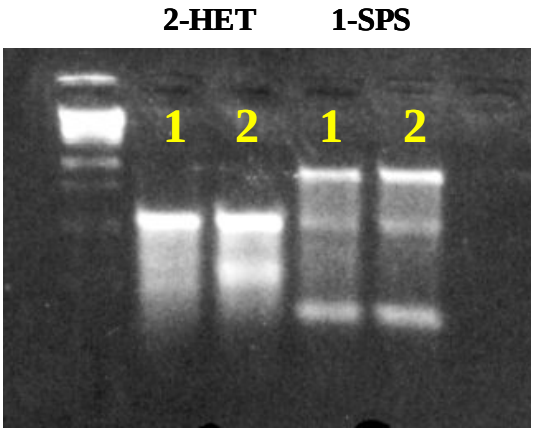


Fig 2. Ethidium bromide stained gel of cassava genotypes amplified with 2-HET and 1-SPS primer and digested with CELI. 1=wild accession, 2= cultivar accession.

**Conclusion and perspectives**

We report a simple method of enzyme mutation detection using CELI already tested in different crops for efficient identification of mutations and polymorphisms can be applied in cassava. Evaluation of different populations of cassava with primers designed from starch biosynthesis genes and confirmation of the mutations by sequencing consists on the second part of this project.

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## 1.3.11 Evaluation of $S_1$ Cassava Lines with 100 SSRY Markers

Collaborators: Jaime Marín, Nelson Morante, Hernan Ceballos, Martin Fregene (CIAT).

Funding: CIAT

**Introduction**

The sequencing of the cassava genome is important to establish regions of the genome that could be of great interest related to economic traits. In order to prepare materials for the sequencing of cassava, ten genotypes belonging to the series $S_1$ - $S_5$ lines were evaluated for their level of heterozygosity in order to establish that there was no conservation between them.

**Methodology**

Five $S_3$ genotypes from the variety MCol1505 produced at CIAT and one $S_4$ and one $S_5$ genotype from improved variety TMS30572 were plant materials from the study. Also included was TME117 a local variety tolerant to drought. Molecular markers were 100 SSR markers selected from across the cassava genetic map. The selected materials are also shown below:
AM560-1
AM560-2
AM560-3
AM560-4
AM560-5
MCOL1505
NIG-2
S-4 30572
S-5 0110903
10. TME-117

**Results**

DNA extraction from all ten genotypes was following Dellaporta's 1983 protocol and it gave good quality DNA (Figure 1). Two individuals were selected and screened with 100 SSR markers following methodology described by Mba et to (2001) to optimize the PCR conditions and gel electrophoresis was on PAGE gels (Figure 2). The complete set of samples was evaluated with 100 SSR markers using the modifications based on the initial assay (Figure 3). The number of allele per SSR loc was scored in an excel sheet and averaged for each genotype to estimate level of heterozygosity. A total of 74 markers gave complete data set and was the basis of estimation of heterozygosity (Table 1).

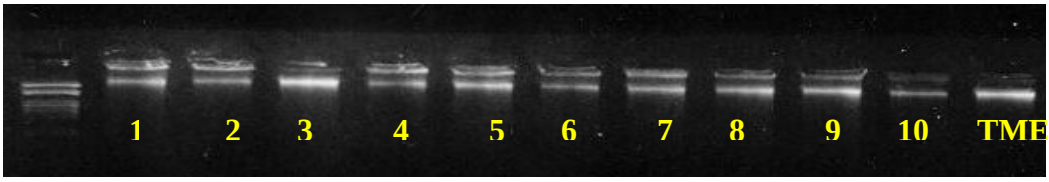


Figure 1. Ethidium stained agarose gel of total genomic DNA samples from 10 $S_3$ $S_4$, and $S_5$ genotypes and their parents.

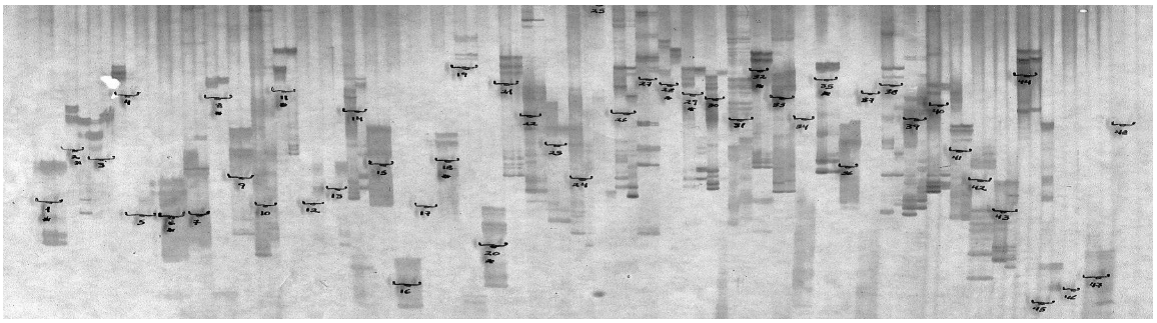

Figure 2 Silver stained PAGE of PCR amplification of two individuals of $S_3$ lines with 100 SSR markers.

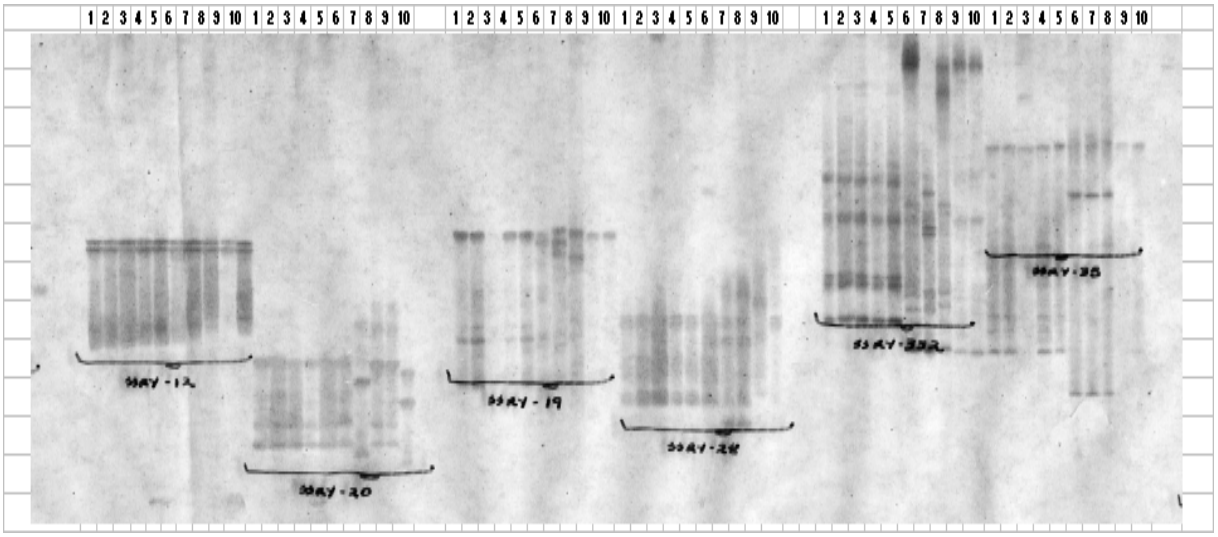

Figure 3 Silver stained PAGE of PCR amplification of the ten genotypes with 6 SSR markers after optimization

Table 1. Score of SSR alleles in S3, S4, S5 genotypes and their parental genotypes

| | | AM560-1 (S-3) | AM560-2 (S-3) | AM560-3 (S-3) | AM560-4 (S-3) | AM560-5 (S-3) | MCOL-1505 | 30572 | 30572 (S-4) | 01/0903 (S-5) | TME-117 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | SSRY296 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 2 | SSRY309 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 |
| 3 | SSRY172 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 1 |
| 4 | SSRY178 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 |
| 5 | SSRY331 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 |
| 6 | SSRY103 | 2 | 1 | 2 | 2 | 1 | 2 | 1 | 2 | 2 | 2 |
| 7 | SSRY106 | 2 | 1 | 2 | 2 | 2 | 2 | 1 | 2 | 2 | 2 |
| 8 | SSRY251 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 |
| 9 | NS9 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 2 |
| 10 | NS33 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 2 | 1 |
| 11 | NS72 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 1 | 2 | 1 |
| 12 | NS149 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 |
| 13 | NS905 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 |
| 14 | SSRY70 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 |
| 15 | SSRY229 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 |
| 16 | SSRY236 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 1 |
| 17 | SSRY239 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 2 |
| 18 | SSRY240 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 1 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 19 | SSRY250 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | |
| 20 | SSRY282 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 1 | |
| 21 | SSRY298 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 1 | 1 | |
| 22 | SSRY319 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 1 | |
| 23 | NS384 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | |
| 24 | NS584 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 1 | 1 | |
| 25 | NS49 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 1 | 2 | 2 | |
| 26 | NS193 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 2 | 2 | |
| 27 | NS235 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 1 | 2 | 1 | |
| 28 | NS1054 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 1 | |
| 29 | SSRY20 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | |
| 30 | SSRY19 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 1 | 1 | |
| 31 | SSRY28 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 1 | 1 | |
| 32 | SSRY35 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 1 | 1 | |
| 33 | SSRY299 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 2 | 1 | |
| 34 | SSRY301 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 1 | |
| 35 | SSRY331 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 1 | |
| 36 | SSRY339 | 2 | 1 | 2 | 2 | 2 | 2 | 1 | 2 | 2 | 2 | |
| 37 | NS40 | 2 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 1 | 2 | |
| 38 | NS169 | 2 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 1 | 2 | |
| 39 | NS271 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 1 | |
| 40 | NS341 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | |
| 41 | NS664 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 1 | |
| 42 | SSRY302 | 1 | 1 | 2 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | |
| 43 | SSRY313 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 2 | |
| 44 | SSRY314 | 2 | 2 | 1 | 2 | 2 | 2 | 1 | 1 | 1 | 1 | |
| 45 | SSRY328 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 2 | |
| 46 | SSRY233 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 1 | |
| 47 | SSRY284 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 2 | |
| 48 | NS53 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 2 | 1 | |
| 49 | NS74 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 1 | |
| 50 | NS92 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | |
| 51 | SSRY191 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 2 | |
| 52 | SSRY192 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 2 | |
| 53 | SSRY203 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | |
| 54 | SSRY100 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 2 | |
| 55 | SSRY110 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | |
| 56 | SSRY144 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 2 | |
| 57 | SSRY145 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 2 | 1 | 2 | |
| 58 | SSRY170 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 1 | |
| 59 | SSRY63 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 1 | |
| 60 | SSRY68 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | |
| 61 | SSRY75 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | | 1 |
| 62 | SSRY98 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 1 | 1 | |
| 63 | SSRY101 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | |
| 64 | SSRY105 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 1 | 2 | |
| 65 | SSRY50 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 | 1 | |
| 66 | SSRY52 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 2 | 2 | |
| 67 | NS565 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 2 | 1 | 2 | |
| 68 | NS890 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 69 | NS911 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 |
| 70 | NS368 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 2 | 1 |
| 71 | SSRY4 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 1 | 2 |
| 72 | SSRY23 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 |
| 73 | SSRY122 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 |
| 74 | NS1014 | 1 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 2 | 2 |
| | AVERAGE | 1.09 | 1.03 | 1.09 | 1.08 | 1.12 | 1.65 | 1.64 | 1.49 | 1.49 | 1.51 |

Based on assessment of the selfed genotypes with 74 SSR markers the level of heterozygosity in the $S_3$ lines was between 3 and 12 percent compared to 65% in the MCol 1505 parent. Heterozygosity in the $S_4$ and $S_5$ genotypes was 49% for both, a rather surprising result. The $S_3$ genotype with the lowest AM560-2 has been selected for genome sequencing at the DOE-JGI sequencing facility in the USA.

Initially, nuclear DNA was extracted from about 5g of leaf tissue and shipped to the DOE-JGI facility. Later, AM560-2 was introduced *in vitro* and 18 plants shipped to the Donald Danforth Center, St Louis for further multiplication, green house hardening and shipment of large quantities of lead tissue to the DOE-JGI sequencing facility

**Conclusion and perspectives**

We have selected a $S_3$ genotype, AM560-2, which presents the lowest level of heterozygosity. The cassava sequencing project is being conducted using this genotype at DOE-JGI sequencing facility Institute.

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## 1.3.12 Sequencing analysis of 20.000 full-length cDNA clones from cassava reveals recent duplications of stress-related genes.

Germán Plata[1], Fausto Rodríguez-Zapata[1], Tetsuya Sakurai[2], Motoaki Seki[2], Andrés Salcedo[1], Joe Tohme[1], Yoshiyuki Sakaki[3], Atsushi Toyoda[3], Atsushi Ishiwata[2], Kazuo Shinosaki[2], and Manabu Ishitani[1].

[1] SB-2 Project ; [2] RIKEN Plant Science Center; [3] RIKEN Genomic Sciences Center

**Introduction**

Cassava, an allotetraploid known for its remarkable tolerance to abiotic stresses is an important source of food carbohydrates for over 500 million people in the tropics. It can be grown in marginal, low fertility acidic soils showing increased nutrient use efficiency and remaining photosynthetically active during

prolonged water stress (El-Sharkawy 2004). In order to understand the regulatory networks involved in stress response and to detect candidate genes suited for the improvement of cassava cultivars, a library of 20.000 full-length cDNA clones from cassava plants under various abiotic stresses was sequenced and assembled.

**Methods**

*Library construction and sequence assembly*

Full-length cDNA libraries from MTAI16 plants exposed to the conditions depicted in table 1 were built by the biotynilated CAP trapper method (Seki et al. 2002). 20.000 clones were sequenced from both ends and assembled using CAP3, the resulting contigs were used to build scaffolds based on clone names. Different transcripts with sequence reads assembled in a single contig, as well as those showing a percent identity higher than 98% to other transcripts were regarded as alternative splicing variants.

Table 1. Conditions used for mRNA extraction.

| Treatment | Age | Tissue | Duration of treatment |
|---|---|---|---|
| No treatment | 9, 11, 12 weeks | leaf | |
| No treatment | 9 month | root | |
| Drought shock | 7-weeks | leaf | 3,6,24,72 hours |
| Heat | 9-weeks | leaf | 3,6, 24, 72 hours |
| PPD | 9 month | root | 24,48,120 hours |
| Acid soil (high Al, low pH) | 9 weeks | leaf | 3,6,24,72 hours |
| Acid soil (high Al, low pH) | 9 month | root | 6,24,48 hours |

*Functional and structural annotation*

Sequences were annotated with Gene Ontology (GO) terms and KEGG Orthology accessions on the basis of sequence similarity running BLASTX against the GO lite and Arabidopsis KEGG GENES databases (e-value 1e-5) (Rodriguez-Zapata et al. 2007) The BLAST report was also parsed to find start and stop codons in the reading frame defined by known proteins, this allowed the annotation of UTRs and coding sequences (CDS). The Vienna RNA package was used to find the folding energies of 5' UTRs, 3'UTRs and CDS.

*Comparative genomics*

Reciprocal runs of TBLASTX were performed between the cassava sequences and the transcripts of *Ricinus communis*, *Populus trichocarpa* and *Arabidopsis thaliana*. Orthologs were initially defined as reciprocal best hits (RBHs), the WEGO server (http://wego.genomics.org.cn/) was used to identify GO terms enriched amongst the sequences with assigned orthologs and those without (Figure 1). Recent gene duplications were detected defining groups of cassava sequences with the same best hit in two or more species (Figure 2) after filtering alternative splicing variants.

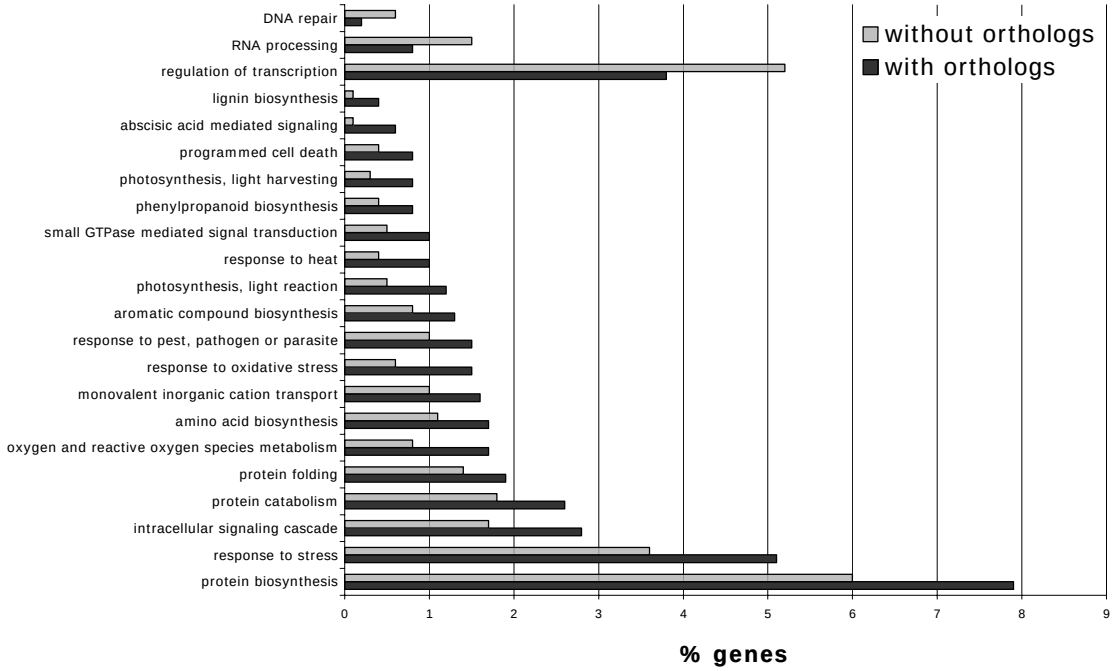


Figure 1. Overrepresentation of Gene Ontology terms related to stress between sequences with putative orthologs and sequences without

## Results

*Sequencing and assembly of the fl-cDNA library*

We obtained 6355 contigs and 9026 singletons that were grouped into 10577 scaffolds, of these, 6967 were not present in public databases. 26% of the transcripts correspond to alternative splicing variants of 2096 distinct genes, we thus captured 7796 genes in the library (Table 2). A Compound Poisson process model (Wang et al. 2005) revealed that there should be about 50698 distinct transcripts in cassava.

Table 2. Summary of library properties and assembly results.

| Library feature | Sequence count |
|---|---|
| Clones | 19968 |
| Sequence reads (trimmed) | 35400 |
| Contigs | 6355 |
| Singletons | 9026 |
| Scaffolds | 10577 |
| Full-length sequences | 2005 |
| Genes | 7796 |
| Novel cassava transcripts | 6967 |
| Novel plant transcripts | 1521 |

*Functional annotation*

A function was assigned to 8227 (78%) sequences, we were able to map the captured sequences to pathways of agronomical interest such as 'starch and sucrose metabolism' or 'carotene biosyntheis'. Comparison of the annotation of sequences with RBHs and sequences without revealed an enrichment in the library of transcripts related to stress response (Figure 1). We also found that 5'UTRs of our sequences had on average lower folding free energies, were longer and had higher GC% than those of Populus and Arabidopsis; altogether this suggest a higher post-transcriptional regulation of stress genes.

*Gene duplications*

230 gene duplications were detected, the annotation of these sequences showed that many of them were related to oxidative stress, which is a common theme in plant response to the treatments used to build our library. Important enzymes in $H_2O_2$ scavenging pathways were found duplicated as well as enzymes involved in signal transduction and stress response.

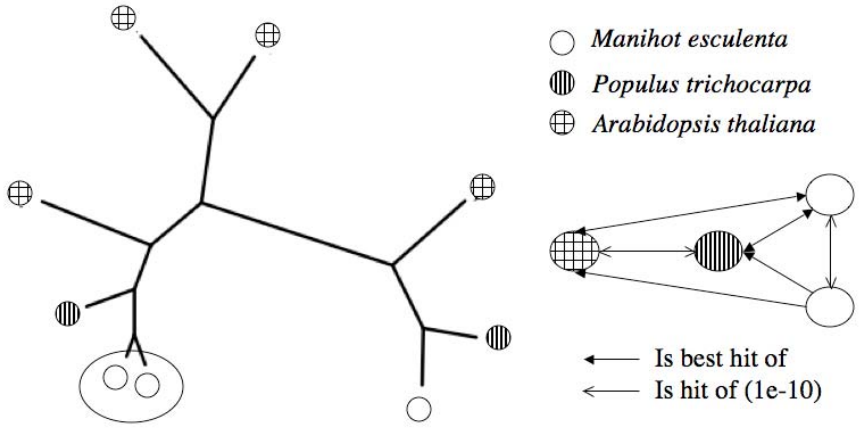


Figure 2. Definition used to find duplicate genes: Groups of sequences (white) with the same best hit in other genomes that are similar to each other

**Conclusions and Perspective**

For the cassava research community, a full-length cDNA library is an important resource for gene identification, annotation and cloning; our analysis has lead to the identification of non-redundant sequences as well as recent gene duplications related to stress response. A detailed analysis of the expression of these sequences can be conducted building oligonucleotide arrays from the library. This should prove useful for understanding the evolution of stress tolerance in cassava. Also, the full length library will be an invaluable resource for the annotation of the upcoming cassava genome.

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## 1.3.13 Further characterization of candidate genes for Al resistance in *Brachiaria*

Rodriguez M.E.[1], Salcedo, A.F.[1], Recio M.E. [2], Chavez, A. L.[1], Rao I. [2], Thome J.[1], and Ishitani, M [1].
[1]SB-2 and [2] IP-5 CIAT project funded by BMZ

**Introduction**

Previous studies have demonstrated that there is pronounced difference in aluminum (Al) resistance between *B. decumbens* (resistant) and *B. ruzisiensis* (susceptible). The objective of this work is to identify candidate genes responsible for high levels of Al resistance in *B. decumbens* using PCR-based technology.

**Materials and Methods**

Plant material: Seeds of *B. decumbens* and *B. ruziziensis* were germinated in 200 μM $CaCl_2$ (pH 4.2) for 4- 5 days in the greenhouse. Homogeneous seedlings with root length between 4 and 5 cm were subjected to aluminum treatment with continuously aerated solutions consisting of 200 μM $CaCl_2$ (pH 4.2) with and without 200 μM $AlCl_3$. Root tips (1cm length) of *B. decumbens* and *B. ruzisiensis* were collected at 0, 3, 6, 24 and 72 hours of the treatment.

Candidate genes for Al resistance: 18 candidate genes have been isolated from a cDNA subtraction library between *B. decumbens* and *B. ruziziensis* as previously reported (Salcedo et al. 2005) and were used for this study.

cDNA synthesis: Total RNA was isolated from the root tips using Trizol® (Invitrogen, USA) according with manufacture's protocol. Total RNA was treated with DNaseI (Invitrogen, USA) to remove genomic DNA. cDNA for PCR experiments was synthesized using SuperScript III reverse transcriptase

(Invitrogen, USA). We used a co-amplification reverse transcription (Co-RT) strategy for priming cDNA, which combines oligo-(dT) with an 18S-RNA-specific primer in the initial reverse transcription reaction (Zhu and Altmann, 2005).

Gene expression analysis: Comparative expression analysis of each 18 genes at 0, 6 and 24 hours in *B. decumbens* and *B. ruziziensis* root tips was conducted by real time PCR using a gene-specific primer. Real-time PCR was carried out in MJ research Opticon II as follows: 20µl reaction volume containing: 10µl of Master Mix (2X) SYBR Green I kit (Stratagene, USA), 175 nM of each primer, and 5µl of 1:10 diluted cDNA template. PCR conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, 50-60°C for 20 seconds and 72°C for 30 seconds. The fluorescence reading was done at 72°C and 83°C and specificity of amplified products were confirmed by a melting curve from 65°C to 95°C.

Data analysis: Sequence analysis and homologous searchs were performed with Vector NTI (Invitrogen, USA) and the BLAST algorithm of NCBI. We used qBase software v 1.3.5 (http://medgen.ugent.be/qbase) to analyze real time PCR data. The software employs a delta-Ct relative quantification model with PCR efficiency correction and multiple reference gene normalization. To estimate efficiency of PCR amplification of each gene, a standard curve was prepared using a serial dilution of cDNA or plasmid DNA carrying candidate genes. As reference gene for normalization we used 18S-rRNA gene.

Results and Discussion

Among the 18 genes we examined, six genes named *AlBdec3*, *AlBdec5*, *AlBdec8*, *AlBdec10*, *AlBdec15* and *AlBdec16* showed significant differential expressions in *B. decumbens* in response to aluminium toxicity compared with *B. ruziziensis*. *AlBdec3, 8, 10 and 16* showed a similar pattern of gene expression. At 0 hour, the constitutive expression of each gene was observed. Upon exposure to Al toxicity, transcript level of these genes increased at 6 hours. Interestingly, *B. decumbens* maintained the same expression level for these genes at 24 hours as found at 6 hours. On the other hand, we observed significant reduction of the transcript level of these genes in *B. ruziziensis* at 24 hours. Other two genes, *AlBdec5* and *15* had a similar type of expression pattern which indicated higher expression in *B. decumbens* at the all time points we tested compared with *B. ruziziensis*. Figure 1 shows gene expression patterns of *AlBdec3* and *AlBdec15* as examples of the two expression patterns described above.

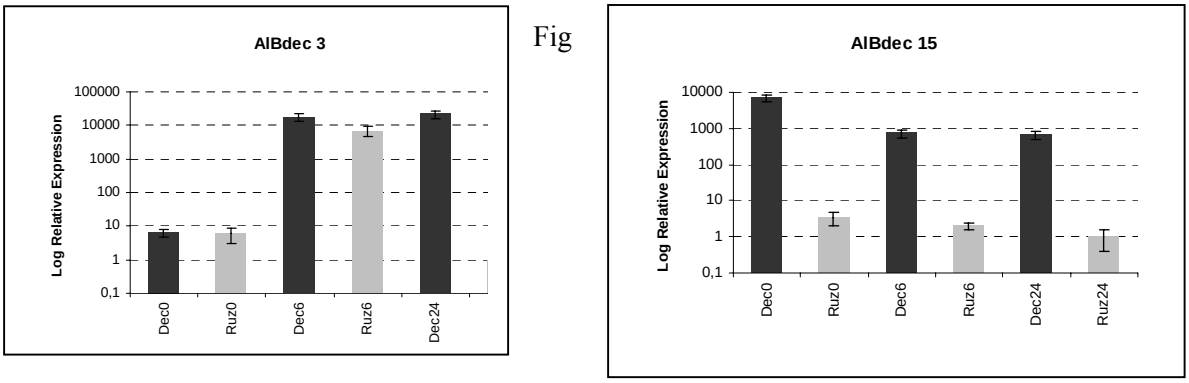


Fig 1. Transcript analysis of *AlBdec3* and *AlBdec15* at 0, 6 and 24 hours of Al treatment Black bar: *B. decumbes*; Gray bar: *B. ruziziensis*. The relative expression level was shown in a logarithmic scale.

In a 2005 CIAT Annual Report, we described 3 candidate genes that are presumably regulatory genes such as transcription factors and kinases based on BLAST analysis. However, these genes did not show any difference in transcript levels at the time points that we tested. Among the six genes confirmed by this study as differently expressed genes in *B. decumbens, AlBdec3*, *AlBdec5*, *AlBdec15* and *AlBdec16* did not

show any significant homology or showed no homology to any genes in the NCBI database. This could be in part due to the short cDNA sequence of the gene with less than 300 bp. The other two genes, *AlBdec8* and *AlBdec10* showed a high homology to a putative cysteine proteases and a metal-dependent protein hydrolase family protein found in *O. sativa*, respectively.

Table 1: Summary of Blastx search for six candidate genes in the NCBI database

| Name | Organism | E-value | Score (Bits) | Description |
|---|---|---|---|---|
| AlBdec03 (262bp) | No significant HIT | | | |
| AlBdec05 (356bp) | *Solanum tuberosum* | 6.3 | 36.8 | Low E-value, probably unknown gene: acidic ribosomal protein P1a-like |
| AlBdec08 (247bp) | *O. sativa* | 3e-46 | 191 | Transglutaminase-like enzymes, putative cysteine proteases |
| AlBdec10 (548bp) | *O. sativa* | 3e-35 | 151 | Metal-dependent protein hydrolase family protein |
| AlBdec15 (242bp) | No significant hit | | | |
| AlBdec16 (271bp) | No significant HIT | | | |

**Ongoing Activities**

1. Isolation of full length cDNAs for selected candidate genes for POC work

Once comparative analysis of the genes is completed we will obtain full length cDNA of selected genes by Rapid Amplification of cDNA Ends (RACE) technology to conduct functional analysis of genes by transgenic approach. Dr. Koyama at University of Gifu in Japan is collaborating with us to conduct the POC work.

3 Diversity analysis of aluminum resistance

An aluminum treatment experiment will be performed during the upcoming weeks for different genotypes of *Brachiaria* which show different degrees of aluminum tolerance, including *B. decumbes (Al tolerant), B. brizantha* cv. Marandú,, a hybrid Mulato II (inermediate), and *B. ruziziensis* (sensitive) (I. Rao com. pers.). Total RNA will be extracted from root tips and levels of expression for each candidate gene will be evaluated.

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## 1.3.14 Development of introgression lines of *Oryza sativa* with chromosome segments substitutions from the African cultivated species

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*Project funded by:* The Generation Challenge Program; IRD; CIAT; The French Ministry of Foreign Affairs

### Introduction

Although the methodologies for mapping of genes underlying quantitative traits have progressed considerably, development of new population structures have been poorly documented so far (Eshed and Zamir, 1995). The most used populations for QTL mapping in self pollinated crops have been recombinant inbred lines (RILs), doubled haploids (DH), backcross (BC) or $F_2/F_3$, which have several limitations such as low resolution power for QTLs with minor effects (Eshed and Zamir, 1995; Koumproglou *et al.*, 2002). In the past twelve years, researchers have developed novel mapping populations such as introgression lines (ILs) in tomato (Eshed and Zamir, 1995), *Brassica napus* (Howell *et al.,* 1996) and *Brassica oleracea* (Ramsay *et al.*, 1996), stepped aligned inbred recombinant strains (STAIRS) in *Arabidopsis* (Koumproglou *et al.*, 2002), recombinant chromosome substitution lines (RCSLs) in barley (Matus *et al.,* 2003) and chromosome segment substitution lines (CSSLs) in rice (Jena *et al.,* 1992; Doi *et al.,* 1997; Sobrizal *et al.,* 1999; Kubo *et al.,* 2002; Yu *et al.,* 2004; Wan *et al.,* 2004; Li *et al.,* 2005; Ebitani *et al.,* 2005).

The CSSLs materials contain one or few contiguous chromosomal segments of the donor genotype in the background of a recurrent genotype, limiting the interactions between donor alleles to those homozygous substituted tracts, reducing the effects of interferences with the genetic background (Howell et *al*., 1996). These materials allow to make detailed analyses, as far as, marker assisted selection and map-based cloning (Li *et al*., 2005; Ebitani *et al*., 2005). Finally, CSSLs represent a small number of lines that can be evaluated in replicated trials and they provide a very good alternative to understand the genetic bases of reproductive barriers between species.

The aim of this work is to describe the development and selection of two populations of Chromosome Segment Substitution Lines (CSSLs) between the two cultivated species of rice, *Oryza sativa* L. and *O. glaberrima* Steud. Furthermore, we present a QTLs detection analysis for yield and yield components and resistance to RSNV (Rice Stripe Necrosis Virus) in order to illustrate the advantages of using this kind of materials in genetic analysis and breeding of rice.

### Methodology and Results

Genotyping of the IR64 x TOG5681 $BC_2F_4$ & $BC_3F_3$ populations
Interspecific cross: *O. sativa* ssp. *indica* cv. IR64 x *O. glaberrima* acc. TOG5681
A population made of $BC_2F_4$ and $BC_3F_3$ lines (Pre-CSSLs) was developed at IRD, Montpellier, France through marker-assisted backcrossing from the cross IR64 (*O. sativa* ssp. *indica*) x TOG5681 (*O. glaberrima*). This population is currently analyzed for its genomic content at CIAT.

Twenty-seven microsatellite markers selected from Rice Universal Core Map data base and corresponding to eight chromosomes were added to the previous data set. Altogether, 128 SSRs markers have been evaluated for the two sub-populations. The search for a set of lines as candidate for CSSLs was done using program CSSL Finder v. 0.8a11 (Lorieux 2005). The following parameters were taken into account: size of introgressed segments, minimum number of segments that cover the genome and treat heterozygotes as donor homozygotes. As a result, 57 lines were selected using 107 of 128 SSRs markers that showed an

even distribution across the twelve rice chromosomes. These lines covered the complete *O. glaberrima* genome with introgressions, except for some small regions on chromosomes 3, 4 and 9. New $BC_1F_1$ lines derived of the same cross have been developed in order to 1) validate the genetic map and 2) to recover the lost segments (see next section).

A preliminary QTL analysis was carried out for various traits scored in the field at CIAT. Using the program CSSL Finder, we could identify several QTLs for tillering, panicle size and plant height.
Each one of these QTLs are being compared to those obtained in the Caiapo x IRGC103544 population (see next section) and with QTLs for yield and yield components found in the literature and in the databases like Gramene (www.gramene.org).
Finally, this population has been sent to WARDA, Cotonou, Benin in order to be evaluated in 2007 for response to drought stress in the field.
Development of a new IR64 x TOG5681 $BC_1F_1$ population
Interspecific cross: *O. sativa* ssp. *indica* cv. IR64 x *O. glaberrima* acc. TOG5681
As some gaps were observed in the genome coverage of the IR64 x TOG5681 $BC_2F_4$ and $BC_3F_3$ populations, we decided to develop a new $BC_1F_1$ population from the same cross. This will let us monitor the derivation of $BC_2$ and $BC_3$ lines that will complete the first set of lines.
A total of 125 $BC_1F_1$ lines were produced and 62 of them could be successfully backcrossed to IR64 to advance to the next generation.

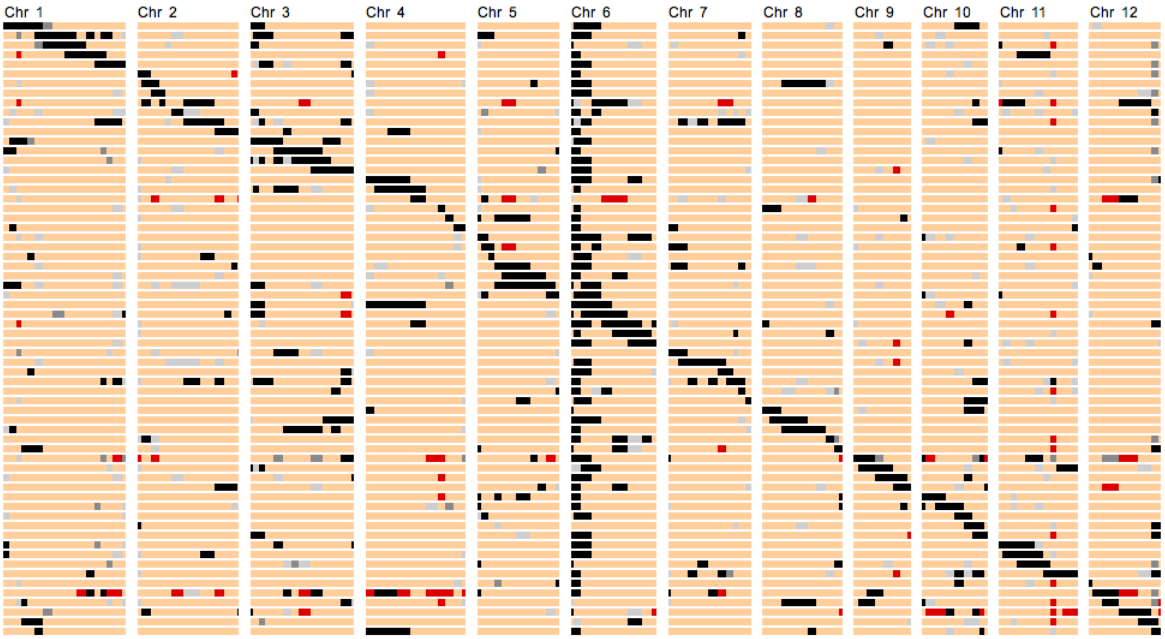
The fertility of the $BC_1F_1$ lines was also evaluated with the scope of tagging the interspecific sterility genes. As a result, two lines showed a high fertility (> 50 %), several showed intermediate fertility while the majority of the lines showed almost complete sterility.

Construction of a new IR64 x TOG5681 genetic map
Interspecific cross: *O. sativa* ssp. *indica* cv. IR64 x *O. glaberrima* acc. TOG5681
The new population derived from the cross (IR64 x TOG5681) x IR64 (125 individuals) has been evaluated for 141 anchors belonging to the Universal Core Genetic Map. This allowed us to compute a new interspecific genetic map. The advantages of obtaining this new map are 1) to confirm and to improve the previous *O. sativa* x *O. glaberrima* interspecific genetic map (Lorieux *et al*., 2000), 2) to derive graphical genotypes of the lines to monitor the derivation of $BC_2$ lines, 3) to validate the SSRs of the Core Map for their genetic location, 4) to provide information about the interspecific sterility loci 5) to allow a direct comparison of the location of the *O. glaberrima* introgressions to the location of wild species introgressions obtained from this project.
The data were analyzed with the MapDisto v. 1.7b126 program (Lorieux 2007). Of the 141 polymorphic SSRs markers that have been evaluated in the $BC_1F_1$, 136[AG□□□□ ▪ ▪ □□1] mapped to the expected position based on the Nipponbare pseudomolecule, leading to a map of about 1950 cM (Kosambi mapping function).
Eight regions showing segregation distortion (SD) have been found on chromosomes 1, 2, 3, 6, 7 and 11. Of these regions, six matched with the twelve *O. sativa/O. glaberrima* sterility loci described so far: S30(t) (Li *et al*., 2005), S29(t) (Hu e*t al.*, 2004), S19 (Taguchi *et al.*, 1999), S1 (Sano 1990), S21 (Doi *et al*., 1999) and S3 (Sano 1983). Two new regions with SD that have not been described for the cross were found in the long arms of chromosomes 3 and 6.
As expected, the strongest SD was found to be located at the short arm of chromosome 6, corresponding to the expected position for the locus S1.
Using the program CSSL Finder, we looked for candidate lines for $BC_2$ generation, in such a way that the introgressed fragments would overlap with the gaps observed in the previous population. We successfully found several lines that will able us to finish the production of a complete set of introgression lines for this cross.
Genotyping of the Caiapo x IRGC103544 $BC_3F_1$DH population
Interspecific cross: *O. sativa* ssp. tropical *japonica* cv. Caiapo x *O. glaberrima* acc. IRGC103544

A $BC_3F_1$ population was obtained at CIAT HQs from the cross between Caiapo (an elite tropical *japonica* from Brazil) and *O. glaberrima* (IRGC103544) (César P. Martinez). From these lines, anthers were collected and a population of 695 lines $BC_3DH$ was obtained through in vitro culture of anthers (Zaida Lentini).
A subset of 312 $BC_3DH$ lines were genotyped using 199 SSRs. Sixty-four lines that cover the *O. glaberrima* genome were selected as candidate for CSSL development by means of the program CSSL Finder using the 125 best markers in terms of their distribution across the twelve rice chromosomes (Figure 1). The overlapping targeted chromosomal segment size was 10 cM on average. New markers from intervals between RM71-RM300 (Chr. 2) and RM185-RM241 (Chr. 4) have been evaluated to fill the gaps in these regions.
A new $BC_1F_1$ population has been generated to validated the genetic map and to recover lost segments. This population will be mapped in 2007.
A preliminary QTL analysis allowed to detect several QTLs for plant height, yield, tillering, 1000-grain weight and sterility located on chromosomes 1, 3, 4, 5, 6, 9 and 11. One highly significant QTL could be located on Chr. 11 for resistance to the Rice Stripe Necrosis Virus (RSNV) and fine mapping of this major QTL can be envisaged using $BC_4F_2/F_3$ lines. All the analyses were performed in considering 1) the ANOVA1 F-test value and 2) the graphical genotypes of the lines that showed extreme phenotypes for the trait.

In order to optimize and to purify the development of the CSSLs, 59 $BC_3DH$ lines of the 64 candidates were backcrossed to Caiapo and selfed to obtain 59 $BC_4F_2$ populations. These materials will be evaluate with microsatellite markers for their genetic background and for the presence of the targeted *O. glaberrima* segment. Also, 36 $BC_3DH$ lines were selected and backcrossed to both parental accessions, Caiapo and IRGC103544, in order to study the genetic basis of sterility in interspecific crosses.

**Figure 1.** Graphical genotypes of the 64 CSSLs lines selected with the help of the program CSSL Finder. The 12 chromosomes of rice are displayed vertically. They are covered by 125 evenly dispersed SSRs marker. The introgression (CSSL) lines are displayed horizontally. The black rectangles indicate homozygous introgressions from *O. glaberrima*. The light pink rectangles indicate homozygous fragment of the recurrent genotype Caiapo, the red rectangles indicate possible alien alleles and the gray rectangles indicate missing data.



## Conclusion

This work allowed us to advance significantly in the construction of CSSLs between the two cultivated species of rice, in both *indica* and *japonica* genetic backgrounds.
Specifically, the results may be summarized as follows:
- A new interspecific genetic maps were developed, that will allow us to complete the IR64 x TOG5681 CSSL population.
- Two *O. sativa* x *O. glaberrima* populations are almost finished and ready for distribution to partners.
- Four cultivated x wild $BC_1F_1$ populations were genotyped.
- Four cultivated x wild $BC_2F_1$ populations were derived.
- The use of Universal Core Genetic Map for rice, combined with the data analysis using the CSSL Finder program, will allow us to easily compare the gene or QTL locations discovered with those two populations, to the results obtain with the new cultivated x wild CSSL populations (see the companion article "Exploring wild introgressions in rice").
- Several students and research assistants were trained.

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### 1.3.15 Utilization of Wild Rice Species at CIAT to Broaden the Genetic Base of Cultivated Rice in Latin America

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Funding: CIAT Core and CIDA-Canada

**Abstract**

Nearly 13,000 breeding lines in different stages of development were evaluated in Santa Rosa and Palmira; percentage of selected material varied depending on the type of cross combinations. About 1224 advanced lines were selected by participants from diverse NARs in a Breeder's Workshop held in Santa Rosa . Wide segregation for diserable traits including grain quality and a good number of plant selections were made for further testing in crosses involving *O.latifolia*. Elite lines derived from crosses with *O.glaberrima*, *O.barthii*, and *O.rufigon* showed good field performance and high yield potential in replicated trials run by Fedearroz in high yield-environments.

**Introduction**

Wild species are valued as a unique source of genetic variation; however, they have rarely being used for the genetic improvement of quantitative traits. Since 1994 the CIAT Rice Project in close partnership with CIAT Biotechnology Unit has been characterizing and utilizing wild rice species to broaden the genetic base of cultivated rice in Latin America. The strategy in place make use of molecular maps in combination with backcrossing to elite breeding lines or commercial varieties to develop populations that are used to identify and transfer QTLs associated with traits of agronomic importance to cultivated rice. Recent progress in this area will be reviewed and presented in this paper.

Data that will be presented suggest that several traits of agronomic importance, including yield and yield components, and tolerance to biotic and abiotic stresses, have been transferred from *O.rufipogon*(IRGC105491), *O.glaberrima* (IRGC103544), and *O.barthii* (IRGC104119) to improved cultivars. Breeding implications will be discussed.

On the other hand, samples of wild rice populations collected in two sites in Colombia were classified as *O.latifolia*, a tetraploid species carrying the CCDD genome. Preliminary evaluations suggest that these accessions carry resistance to all rice blast lineages found in our "hot spot" Santa Rosa, as well as resistance to rice hoja blanca virus and *Tagosodes orysicola*.

**Materials and Methods**

Breeding lines from different crosses (Table 1) were planted and evaluted under different biotic and abiotic stresses in Santa Rosa, Villavicencio and Palmira Experiment Stations. Data on reaction to main diseases and insect pests, and main agronomic traits(including grain quality) were taken and used to identify and select promissing lines for further testing in 2007 or to be distributed to NARs via CIAT-ION nurseries.
On the other hand, selected elite lines were evaluated in a high yielding environment(Aipe, Huila) in collaboration with Fedearroz.

**Results**

Data in Table 1 shows that percentage of selected material was different depending on the type of genetic material. There was a high disease pressure in Santa Rosa , specially in terms of rice blast ; high panicle sterility was present in cross combinations including *O.glaberrima* and *O.barthii* accessions. However, promising interespecific breeding lines with high yield potential, tolerance to main diseases and good grain quality were identified and included in the CIAT-ION nursery made available to NARs in 2006. Out of the 194 lines, 65 were from interspecific crosses.
About 1224 lines from different cross combinations were selected by breeders that attended a Breeder's Workshop held in Santa Rosa in August 2006 with funding from FAO. Breeders from both private and public institutions from Colombia, Cuba, Bolivia, Dominican Republic, Nicaragua, Panama, Guatemala , Argentina, and Ecuador made selections, which are being seed-increased in CIAT-Palmira for distribution in mid-2007.

About 1370 plant selections for seed multiplication and molecular testing in CIAT Palmira were made out of a collaborative breeding activity initiated in 2003 aimed at the introgression into susceptible rice varieties of Pi1, Pi2, and Pi33 genes shown to confer stable resistance to rice blast(Rice Annual Report 2004,2005). These backcrossed lines have undergone several cycles of phenotypic selection for both leaf and neck blast infection in Santa Rosa and there is a need to use molecular markers associated with the Pi1, Pi2, and Pi33 genes in order to identify which lines are carrying these genes. Best lines will be distributed to our partners in 2007.

Table 2 shows the breeding behavior of F5 lines derived from crosses with *O.latifolia*, which has been showed by our Rice Pathology and Virology groups to be resistant to rice blast, rice hoja blanca virus and its vector *Tagosodes oryzicola*. This finding is very relevant to our breeding work since these biotic stresses have been the most important and difficult production constraint afecting rice production in Latin America and the Caribbean. Data shows a wide segregation for diserable traits including grain quality and a good number of plant selections were made for further testing in Palmira.

Finally, Tables 3, 4, and 5 show field performance of interspecific lines derived from different cross combinations in contrasting environments. Lines from the cross Lemont/O.barthii// Improved line are

about 10 days early compared to local checks but with lower yield potential and milling yield. Preliminary data suggest that lines from the cross CT17237 have higher iron content(6-7 mg/kg) than Fedearroz 50(4-5 mg/kg) in milled rice. Some lines from the cross Bg90-2/5980// Fedearroz 50 had better yield potential than local checks; a similar case was observed in lines from the cross Perla2/ *O.rufipogon*. All these lines are the result of the shuttle breeding program carried out between Santa Rosa and Palmira, where diverse climatic, soil, and biotic stresses are found.

## Future activities

1. Use of additional wild rice species( *O.meridionalis*, *O.glumaepatula*, other accessions of *O. latifolia*) in our breeding program .
2. Continue the evaluation of segregating populations in collaboration with our partners .
3. Distribution of CIAT-ION nurseries to NARs and collaborators.
4. Attendance to international conferences and meetings to present our data .

**Table 1**. Summary of breeding lines evaluated and selected in Santa Rosa and CIAT Experiment Stations in 2006-2007.

| | | 2006 | | 2007 | % |
|---|---|---|---|---|---|
| **Material** | **Generation** | **CIAT** | **S. Rosa** | **CIAT** | **Selections** |
| O. *latifolia/O.sativa* | $F_4$-$F_5$ | **3315** | **3315** | **4070** | **61.4** |
| O. *latifolia* /O. *sativa* | $F_2BC_2$ | **16** | | **64*** | **100** |
| *O. rufipogon* / Fedearroz 50 | $F_4BC_2$ - $F_5BC_2$ | | **611** | **651** | **100** |
| O.*glaberrima* / Fedearroz 50 | $F_5BC_2$, $F_5BC_4$, $F_6BC_2$ | **377** | | | To be planted |
| Lemont/ O. *barthii* // WC | $F_7$,$F_8$, $F_9$ | **336** | | | For CIAT-ION |
| Lemont / O.*barthii* | $R_6$ | **15** | | | Germplasm Bank |
| Advanced interspecific lines | $F_{11}$, $F_{12}$ | | **123** | **45** | **36.6** |
| Elite breeding lines | $F_8$,$F_9$ | **691** | | | NARS |
| Introgressions in elite lines | $F_4$ | | **964** | **675** | **70** |
| Purification of breeding lines | $F_4$-$F_7$ | | **126** | **182** | **100** |
| Pyramiding of genes Pi1, Pi2 y Pi33 | $BC_2F_6$ - $F_7BC_1$ | | **722** | **1370** | **100** |
| Biofortification (IRRI) | $F_4$-$F_5$ | **1445*** | | | **-** |
| Biofortification | $F_3$ | **1693*** | | | **-** |
| CIAT-ION-Biofortificación | $F_8$,$F_9$ | | **288** | **71** | **24.7** |
| Biofortificacion elite lines | $F_8$,$F_9$ | | | **50** | **-** |
| Material introduced from NARs | | **100** | **177** | | Fe and zn analysis |
| Multiplication high *Fe lines* | $F_2BC_4$ | **28** | | **28** | **100** |
| Recurrent selection (PCT 8, 19,21,22) | $S_3$ | | **209** | **200** | **95.7** |
| Recurrent selection populations | | | | **4** | **-** |
| Breeder's Workshop FAO | | | **1645** | **1224** | **74.4** |
| **Total** | | **4878** | **8180** | **8542** | **78.4** |

**Table 2. Breeding behavior of F5 lines from *O. latifolia* / *O .sativa crosses***

| | Resistant % | Intermediate % | Susceptible % |
|---|---|---|---|
| **Rice blast(P.oryzae)** | **70.03** | **21.3** | **8.6** |
| **Rice Hoja Blanca Virus** | **12.6** | **27** | **60.3** |
| | (< 0.8) % | | (>0.8) % |
| **White belly/center *** | **88.3** | | **11.7** |
| | Extra Long % | Long % | Medium % |
| **Grain Lenght (LG)*** | **4.69** | **92.93** | **2.07** |
| **% lines combining all desirable traits** | **7.58** | **25.42** | **66.98** |

* Data from Santa Rosa 2006

**Table 3.Performance of** F8 Lines from the **Lemont / O.barthii // WC Cross In AIPE-Huila .Fedearroz and CIAT 2006.**

| | | Piedrapintada-Aipe-Huila | | | | CIAT-Palmira -Valle | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PEDIGREE | Vg | Flower days | Height cm | Exs (cm) | Yield (Kg/ha) | Flower days | Clk | Len | Amy | Hb | Milling % | Yield (Kg/ha) |
| CT17237-1-5-7-2-2-1-M | 2 | 86 | 94.7 | 2.3 | 7737 | 95 | 1.0 | L | 27.7 | 9 | 46.9 | 4813 |
| CT17237-1-5-7-2-2-2-M | 2 | 83 | 99.3 | 4.0 | 7326 | 95 | 1.6 | L | 27 | 5 | 44.7 | 3505 |
| CT17237-1-5-7-2-2-3-M | 3 | 85 | 103.0 | 3.7 | 7173 | 98 | 3.4 | L | 26.4 | 7 | 43.5 | 4581 |
| CT17238-1-1-1-2-1-3-M | 2 | 85 | 107.0 | 2.7 | 6609 | 95 | 0.8 | E.L | 27.2 | 5 | 39.0 | 3918 |
| CT17238-1-1-1-2-1-4-M | 2 | 85 | 110.0 | 2.0 | 7029 | 100 | 1.4 | E.L | 25.2 | 7 | 48.5 | 3590 |
| CT17238-1-1-1-2-1-6-M | 1 | 83 | 105.0 | 2.3 | 5647 | 98 | 1.8 | E.L | 25 | 7 | 49.4 | 3922 |
| CT17238-1-1-1-2-2-5-M | 2 | 84 | 107.0 | 2.7 | 6830 | 100 | 1.6 | E.L | 25.8 | 5 | 50.3 | 4146 |
| CT17238-1-1-1-2-3-1-M | 2 | 83 | 108.3 | 2.7 | 6067 | 103 | 0.4 | E.L | 26.8 | 5 | 55.6 | 3563 |
| CT17238-1-1-1-2-3-4-M | 2 | 84 | 113.0 | 2.7 | 5871 | 100 | 0.6 | E.L | 26.6 | 5 | 51.4 | 3797 |
| CT17238-1-1-1-2-3-6-M | 1 | 83 | 111.3 | 2.7 | 6754 | 100 | 0.6 | E.L | 26.1 | 5 | 53.5 | 3630 |
| CT17238-1-1-1-2-4-4-M | 2 | 85 | 108.3 | 3.0 | 7035 | 100 | 1.0 | E.L | 27.1 | 5 | 44.3 | 3958 |
| CT17238-1-1-1-2-4-5-M | 2 | 84 | 108.7 | 1.3 | 6060 | 100 | 2.0 | E.L | 26.2 | 5 | 51.4 | 4131 |
| FEDEARROZ 50 | 1 | 96 | 104.3 | 2.3 | 8539 | 111 | 0.2 | L | 29.5 | | 59.9 | 3549 |
| Oryzica 1 | 2 | 90 | 94.3 | 3.3 | 7969 | 100 | 0.2 | L | 32 | | 57.4 | 4631 |
| Lemont | 2 | 76 | 81.3 | 1.3 | 5963 | | | | | | | 4508 |
| T.L. FED 473 | 2 | 92 | 93.3 | 3.0 | 8903 | | | | | | | |
| Fedearroz 275 | | | | | | | | | | | | 4799 |

**Table 4. Performance of F8 lines from the cross Bg90-2 / 5980 // Fedearroz 50 in Piedrapintada-Aipe-Huila.Fedearroz 2006**

| PEDIGREE | Vg | Flower days | Height cm | Exs (cm) | Yield (Kg/ha) |
|---|---|---|---|---|---|
| CT17334-2-1-6-3-1-1-M-M | 2 | 92 | 111 | 4 | 8552 |
| CT17334-2-1-8-3-1-1-M-M | 2 | 92 | 122 | 7 | 8489 |
| CT17334-2-1-6-2-5-3-M-M | 2 | 93 | 120 | 6 | 8318 |
| CT17334-13-7-1-5-M-1-M-M | 1 | 93 | 122 | 4 | 7682 |
| CT17334-13-7-2-1-4-6-M-M | 2 | 95 | 112 | 3 | 7541 |
| CT17334-13-7-2-1-2-5-M-M | 2 | 94 | 119 | 3 | 7538 |
| CT17334-13-7-2-1-4-3-M-M | 2 | 94 | 118 | 4 | 7321 |
| CT17334-13-7-2-1-4-5-M-M | 2 | 96 | 116 | 3 | 7179 |
| CT17334-13-7-2-1-4-1-M-M | 2 | 95 | 115 | 3 | 7021 |
| CT17334-13-3-1-2-3-5-M-M | 2 | 94 | 110 | 3 | 6821 |
| CT17334-13-3-1-5-6-2-M-M | 1 | 89 | 110 | 3 | 6751 |
| CT17334-13-3-1-3-1-M-M | 2 | 93 | 107 | 4 | 6625 |
| FEDEARROZ 50(Local ckeck) | 2 | 98 | 110 | 4 | 8171 |
| FEDEARROZ 369(Local ckeck) | 3 | 81 | 108 | 6 | 7275 |
| FEDEARROZ 473(Local ckeck) | 3 | 91 | 103 | 4 | 8156 |

Table 5. **Performance of F9 Lines from the cross Perla 2/ O. rufipogon // WC in Piedrapintada-Aipe-Huila. Fedearroz 2006**

| PEDIGREE | Vg | Flower days | Height cm | Exs (cm) | Yield (Kg/ha) |
|---|---|---|---|---|---|
| CT16658-4-1-1SR-3-2-3-4-1-M-M | 1 | 88 | 118 | 4 | 8535 |
| CT16658-4-1-1SR-3-2-1-1-1-M-M | 1 | 85 | 109 | 6 | 8518 |
| CT16658-4-1-1SR-3-2-3-4-2-M-M | 1 | 91 | 108 | 2 | 8140 |
| CT16658-4-1-1SR-3-2-3-2-1-M-M | 1 | 87 | 112 | 3 | 7706 |
| CT16659-8-2CT-1-3-5-1-2-M-M | 3 | 93 | 109 | 3 | 7391 |
| CT16659-8-2CT-1-3-5-5-2-M-M | 1 | 92 | 113 | 3 | 7297 |
| FEDEARROZ 50(Local check) | 2 | 98 | 110 | 4 | 8171 |
| FEDEARROZ 369(Local check) | 3 | 81 | 108 | 6 | 7275 |
| FEDEARROZ 473(Local check) | 3 | 91 | 103 | 4 | 8156 |

## 1.3.16 Development of high iron and zinc rice lines to Combat Malnutrition in Latin America and the Caribbean.

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Funding: CIDA-Canada and CIAT_Core

### Introduction

A fast-track approach is under way to screen rice lines in our germplasm banks. About 13,000 breeding lines, mainly insterespecific crosses, originated from CIAT rice project and 1445 F4/F5 lines from IRRI were evaluated under biotic and abiotic stresses in CIAT-Palmira and Santa Rosa, Villavicencio. Based on preliminary data 28 cultivars were selected out of 533 screened for fe/zn content, and distributed to NARs for evaluation under local conditions. Fedearroz 50, a successful rice variety grown commercially in several countries in Latin America, showed 2-3 times the amount of iron found in rice bought by consumers(2-3 ppm). Main achievements have to do with the near-completion of a clean lab for handling and preparing rice samples for iron and zinc analysis, establisment of a methodology for running iron and zinc analysis in rice at CIAT, and establisment of base lines for iron and zinc. Five breeding lines with intermediate iron and zinc content were introduced from IRRI(HP+). Recurrent selection and mutation breeding activities were started to increase levels of iron and zinc in our populations.

### Background

Micronutrient malnutrition, the result of diets poor in vitamins and minerals, affects more than half of the world's population. Women and children are especially susceptible to deficiencies in micronutrients, particularly vitamin A, iron and zinc. As a result they are at risk of disease, premature death, lower cognitive capacity, and poor quality of life. The costs of these deficiencies are high. In Latin America and the Caribbean (LAC) economic and health indicators have been deteriorating. To meet this challenge, the CGIAR is implementing a new paradigm that views agriculture as an instrument for improving human health and nutrition, as well as for increasing productivity. Nutritionally improved staple food provides an inexpensive, cost-effective, sustainable, long-term means of delivering micronutrients to the poor. The goal of the Biofortification Challenge Program (BCP) is to improve the health of the poor by breeding staple foods that are rich in iron, zinc and vitamin A, for poor consumers with priority on Africa and Asia. This program gets funding from diverse sources, including among others, The Melinda and Bill Gates Foundation.

A project funded by CIDA-Canada complements the Biofortification Challenge Program and extends its benefits to Latin America and the Caribbean, through the development of and deployment of high iron and zinc rice lines. Rice has become the most important food grain in LAC, supplying consumers with more calories than other staple crops. Rice has become particularly important in the diets of poor people, who make up about 40% of LAC's total population. Food purchases account for more than half of all expenditures by the poor, and rice accounts for about 15% of their food purchases. Among the poorest 20% of the population, rice supplies more protein to the diet than any other food source, including beef and milk. However, people living in several areas where rice consumption is high have been suffering from a number of major nutritional problems. This is the result of vitamins and/or minerals naturally present in the rice grain but otherwise removed during the milling process or that naturally are not present in sufficient amounts. Preliminary data obtained at CIAT from 11 cultivars planted under irrigated conditions indicated that on the average59 and 26% of the total iron and zinc present in brown rice is lost after milling, respectively. There were significant differences at the 5% level among genotypes tested.

Research carried out at IRRI in close collaboration with NARS suggests that there is genetic variability in the rice genome to increase Fe and Zinc in the rice grain. More recently, Haas et al (J.Nutr. 135:2823-

2830,2005) reported that consumption of biofortified rice, without any other changes in diet, is efficacious in improving iron stores in women with iron-poor diets in the developing world.

In this project for LAC we plan to increase iron and zinc content in the rice grain using a breeding strategy in two phases. On a fast track, landraces and breeding lines conserved in the germplasm banks are screened for mineral content to identify products that could have immediate utility, as potential varieties or donors. Meanwhile, a crossing program was also started to combine high-iron and zinc with high yield potential, tolerance to main biotic and abiotic stresses, and good grain quality. This project is carried out in close partnership with research institutions in Colombia, Bolivia, Cuba, Brazil, Dominican Republic, Nicaragua and more recently Panamá. Preliminary results from the screening process and breeding activities will be presented.

**Materials and Methods**

Several breeding strategies are being used for the development and deployment of high iron(6-8 ppm) and zinc(22-25 ppm) rice lines for micro-nutrient deficiency areas in Colombia, Cuba, Bolivia, Brazil, Nicaragua, Panama, and Dominican Republic. Breeding activities include: 1.Test existing rice germplasm and breeding lines for increased Fe and Zinc content in milled rice; 2. Create segregating populations to increase Fe and Zinc in the rice grain in combination with other desirable traits(high yield potential, tolerance to main diseases and insect pests, good grain quality,etc); 3.GxE studies to determine the influence of climatic and soil factors in the expresion of iron and zinc in the rice grain; 4. Initiate some work on marker assisted selection.and 5. Visits and coordination of collaborative activities carried out by participating NARs.

**Results**

1.Test of existing rice germplasm and breeding lines for increased iron and zinc content
Seed of 533 traditional, commercial varieties and elite lines from the CIAT breeding program (including interspecific crosses) were planted in two contrasting environments (Ciat-Palmira and Jamundi) and10-grms sample of paddy rice/entry was sent to IRRI for iron and zinc evaluation on milled rice.There were indications of iron contamination in the samples, but after some statistical analysis and a careful selection for both iron and zinc, a set of 28 genotypes(Table 1) was selected for further evaluation and distribution to our Agrosalud partners. IR68552-100-1-2-2 had the highest value for both iron (8.12mg/kg) and zinc(23.2mg/kg); Tox 1859-102-4M-4 and Norin 22 had 7.3 mg/kg of iron.
An experiment was set up to verify iron and zinc data reported by IRRI. Milled and brown rice samples of 10 genotypes(Table 2), four replications each, were prepared and sent for iron/zinc analysis to both WAS(Waite Analytical Service, Adelaide University) and CIAT (Analytical Service Lab). In order to minimize contamination, two known rice lines (Icta Motagua and P5746-18-11-1-2-2A-1) having around 1 mg/kg of iron and 9 mg/kg of zinc were run as “blanc controls”between samples of selected genotypes during the milling process; 12 grams of paddy rice were used per sample. A Suzuki rice mill(Figure 1) was used to de-husk and milled the rice samples whilst a locally made mill (Figure2) already tested and validated by the bean AgroSalud project, was used to produce rice flour for iron and zinc analysis. Data shown in Table 2 indicate that iron and zinc values obtained at both WAS and CIAT were significantly lower than IRRI, and no indication of contamination was found. Results indicate that higher values reported by IRRI were probably due to contamination. There was high and positive correlations between WAS and CIAT values(Figures 3,4). Data shows that Fedeaaroz 50, a commercially grown variety in several countries, had the highest iron content in milled rice followed by TOX 1859 and FL04052( advanced breeding lines). These findings have the following implications:a) No contamination was found by WAS indicating that we already have in place at CIAT a protocol to handle, prepare and analyze rice samples for iron and zinc; b) Correlation between iron and zinc values obtained by WAS and CIAT are

positive and very high;c) Fedearroz 50, a successful rice variety grown commercially in several countries in Latin America, has about 2-3 times the amount of iron found in rice bought by consumers(2-3 ppm).
We are now in the final process of finishing up the construction of a clean room and milling /polishing facilities at CIAT to assure good quality data and a fast turn over for our breeding program. It is expected that this new facility will be up and running by February/07.
We also got some information to stablish a base line for iron and zinc. A total of 57 rice samples(brown, parboiled, and milled rice) were collected in supermarkets and stores in several places in Colombia(19), Bolivia(16), Nicaragua(5), and Dominican Republic(9). These samples represent the kind of rice bought by rice consumers for consumption and come directly from rice mills that use commercial rice mills in rice processing. It is expected that these samples have some kind of contamination. Samples were milled and sent for iron and zinc analysis by atomic absorption to the CIAT Analytical Service Lab; three rep./sample were used.
There were statistical differences in the level of iron and zinc found in milled and brown rice samples collected in different locations.Brown ricehad 11-13 mg/kg of iron and 20-25 mg/kg of zinc, compared to 2-3 mg/kg of iron and 17-19 mg/kg of zinc in milled rice(Figure 7). No significant diferences were found in iron and zinc content in both brown and milled rice across countries, indicating that these values can be taken as base line for iron and zinc. Some parboiled rice samples that were analyzed showed iron levels similar to milled rice, contrasting with what is reported in the literature. Similarly, some samples of rice sold as fortified rice had less than 2 mg/kg.. Results suggest that the level of iron and zinc in milled rice used by consumers is low and similar to values reported by HP+ in Asia.

2. Development of segregating populations to increase iron and zinc in the rice grain in addition to other desirable traits, and germplasm exchange.
Based on HP data Azucena, Madhukar, Ketan Lumbu, Gundil Kuning, Perurutong, IR685552-100-1-2-2, and IR71703-657-3-1-2 were used as donor parents in crosses with improved lines and commercial varieties from the CIAT-germplasm bank. Out of 112 single crosses, a total of 147 backcrosses (BC1) were made and planted in CIAT-Palmira in May, 2005. High sterility and poor combining ability was observed in most crosses, specially in crosses with Azucena and Perurutong. A total of 366 F2 families were planted and evalauted in 2006 in CIAT-Palmira; based on agronomic traits and yield potential 1693 single plant selections were made for further evaluation in 2007.
Out of the 30 F2-F3 populations introduced from IRRI in 2004, a total of 2672 plant selections were further evaluated as F3-4 progenies in CIAT-Palmira in 2006; based on agronomic traits, fertility and yield potential, a total of 1445 F4-F5 plants were selected for further evaluation in 2007. Best lines will be evaluated for iron and zinc content as well.
It has been shown by HP+ that land races and wild rice species contain more iron and zinc than modern cultivated rice. Based on this aprox. 13,000 breding lines derived mainly fom crosses between elite lines and *O.rufipogon*, *O.glaberrima*, *O.barthii* and *O.latifolia* were evaluated under biotic and abiotic stresses in Santa Rosa, Villavicencio and CIAT-Palmira. Best lines have been identified in terms of agronomic traits, tolerance to main diseases and insect, yield potential and grain quality. These lines will be evaluated for iron and zinc in 2007.
Four populations (PCT-8CG/1/CG/1, PCT-19, PCT-21, and PCT-22) developed by the CIAT Rice project and carrying cytoplasmic male-sterility gene were selected to start a population improvement program through recurrent selection for high iron and zinc.This is based on the successful use of recurrent selection by Dudley et al,1974 to increase protein conten in maize from 10.0% to 26,6%. Male -sterile plants were selected in each population and crossed to the seven high iron/zinc cultivars mentioned early on. This activity is carried out in collaboration with Fedearroz, our partner in Colombia. F1 seed from each cross was planted and evaluated in 2006 and F2 seed was mixed in equal proportion to form new populations named as PCTBF1,PCTBF2,PCTBF3, and PCTBF4;these populations are undergoing the first recombination cycle and single plants selections will be made in 2007 for iron and zinc analysis. Best plants will be used for the next recombination cycle. The breeding squeme used is shown in Figure 8.

On the other hand, five elite lines from the CIAT and FLAR breeding programs were selected and 3 kg. of seed was sent to the International Institute of Atomic Energy for mutagenic treatment to start a mutation breeding program aimed at the identification of rice mutants with high levels of iron and zinc. One kilogram each of seed of lines BG 90, CT11275-4-M-1-M , FL03191 5P-10-1P-3P-M-M-M , FL04577-3P-11-4P-1P-M , and FL03188-7P-5-4P-1P-M was used for mutagenic treatment following two radiation treatments (150 and 250 GY) using Cobalt 60 as the mutagenic source. Aprox 27000 seeds were planted in CIAT as M1 populations. A total of 8000 plants were selected at random and harvested individually. So far 2000 plants were planted in the greenhouse(1 seed per plant)and leaf tissue was harvested for DNA extraction and detection of mutants using the methodology described by Till et al, 2003; these plants were subsequently transplanted to the field for phenotipic evaluation and seed production. Molecular and mutation evaluation in several genes involved in iron homeostasis will be carried out in 2007. For each gene, three or four primer pairs were designed in order to make screening on the whole sequence of the gene with special attention in the expressed sequences reported in Gramene.
An active germplasm exchange between AgroSalud and HP+ took place; seed of 20 rice lines was sent to Dr. Parmender Virk for field evaluation and iron/zinc analysis at IRRI. This germplasm included several advanced interspecific lines having high yield potential, tolerance to main diseases and insect pests, and good grain quality under our local conditions. On the other hand, 10 breeding lines, including five lines with intermediate iron content(6-8 ppm iron) were sent to us by Dr. Virk for evaluation. This material will be distributed to our AgroSalud partners and used as progenitors in crosses.Seed of this material was also sent to Dr. Janette Palma, one of our collaborators in Brazil.

3. Visits and coordination of collaborative activities carried out by NARs

Colombia(Alejandro Vargas): A collaborative recurrent selection program is being carried out with Fedearroz; four populations have been developed and are in the first cycle of recombination in two contrasting places. A nursery of about 2,000 F3-F4 interspecific lines were sent to Fedearroz for evaluation and selection under adverse climatic conditions(high temperature and humidity).
Bolivia(Roger Taboada): Out of 45 segregating lines from interspecific crosses sent in 2005, 19 lines were selected for further testing in 2006-2007; selected lines performed better than local varieties Epagri 109, Amboro and Paititi. Seed of 100 lines including local varieties grown in Bolivia, were sent to CIAT for iron and zinc evaluation in 2007. Some preliminary evaluations done by Ciat/Aspar in collaboration with small-traditional farmers indicate that Azucena is a good option for small-poor farmers. Preliminary cooking and eating tests done in local restaurants in rural areas suggested that Azucena is acceptable to consumers ; some people specially liked Azucena because of its aroma, taste and flavor. More cooking and eating tests will be conducted in 2006-07. A new set of 136 segregating lines were sent to Bolivia for planting and evalaution in 2006-2007.
Brazil(Jose Luis Viana): The methodology for sample preparation and mineral analysis for polished rice and whole rice was optimized and implemented;97 polished and brown rice samples were analyzed and selected among the Embrapa recommended cultivars, lines introduced from CIAT and breeding lines from Embrapa Rice and Beans.Results indicate that cultivars BRA 02598 and BRA 01506 had 4.4 to 4.8 ppm of iron in milled rice whilst cultivars IAC435, IAC120, Cateto Seda and Vens de Abril had 19 to 20,6 ppm of zinc in milled rice. Besides, 150 local varieties (landraces) collected in Maranhao, Pernanbuco and Ceará States were planted in a experimental field at Embrapa Mid-North. These varieties were harvested in December for iron /zinc analysis in 2007.
Nicaragua(Lázaro Narvaez): A total of 25 advanced lines were evaluated under irrigated and favored upland conditions in two locations( Horno-Sébaco and Posoltega) and six lines were selected for further evaluation , seed multiplication and iron/zinc analysis in 2006-2007. Rice producers were invited to evaluate this material and their observations and suggestions were considered for the final selection . On the other hand, a set of 203 lines was evaluated in a un-replicated yield trial in Posoltega and best lines selected for further evaluation in 2006-07. A regional workshop was carried out in Octubre 2005 in Posoltega with the objective of re-inforcing the breeding capacity of NARs in the region( Nicaragua, El

Salvador, Costa Rica) and to promote Agrosalud activities; 22 people from different organizations attended and selected breeding material for testing under local conditions. Finally a new set of 231 breeding lines were sent to INTA for evaluation in 2006-07. We received a set of 100 lines for iron and zinc analysis in 2007.
Dominican Republic(Angel Adames): A set of 100 lines, including traditional varieties, was sent to CIAT for iron/zinc analysis in 2007. Our collaborator (Angel Adames) visited our breeding site in Villavicencio in August-September/07 to evaluate and select breeding material for his program.About 248 lines were selected.
Cuba(Violeta Puldon): There was a considerable delay in signing the research contract with our counterpart(IIA) and activities are just getting under way.

4: GxE studies to understand climatic and soil factors affecting the expression of high iron and zinc in the rice grain.

It has been shown by HP+ that there is a significant GxE interaction in the expression of iron and zinc in the grain ; in wheat the expression of these minerals in the grain depends on soil conditions and fertilization practices. Similar findings have been reported in rice in Asia. Climatic conditions and crop management practices also affect the yield potential of rice. Therefore, there is a need to determine main factors affecting the expression of iron /zinc in rice to be able to better define best agronomic practices for rice in our regions of interest to assure not only high yield potential but also good expression of iron/zinc in the rice grain.
Acording to Angel Adames,ratooning is a very important management practice in Dominican Republic, very profitable and used specially by small-poor farmers. This practice has spread to other countries like USA, Colombia, Venezuela and Central America. No information is available on the effect of ratooning in the content of iron and zinc in the rice grain. Two experiments were planted in two sites using 28 genotypes to assess how much iron/zinc is left in the rice grain affter ratooning compared to iron and zinc in the rice grain harvested from main crop. These experiments are under way and rice samples from each treatment will be sent to CIAT for iron/zinc analysis in 2007.
It has been reported( Caballero et al,2006.UNAM-Mexico, and Roger et al,1992) that the application of N-fixing bacteria such as Azospirillum, increases the grain yield potential in maize and at the same time reduces the negative impact of fertilizers on the ecosystem.It is suggested that these bacteria has the capacity to provide readily available nitrogen to the crop, promotes root growth, and water/ nutrient absorption from the soil. Several experiments were conducted in 2005-2006 by Dr. Luis Armando Castilla (Fedearroz) to study the effect of climatic and soil conditions on the iron/zinc content in the rice grain. In order to determine the effect of the application of iron and zinc, two rice lines( CT11275-4-M-1-M and IR 68552-100-1-2-1) were planted during the rainy and dry season in Tolima ; iron and zinc was either added to the seed or applied as soil treatment or as foliar spray . There was a control treatment with no iron and zinc in a factorial design. Results indicates that under favorable wheather conditions(high solar radiation) lines CT 11275 and IR 68552 yielded 36 and 22% more than under low solar radiation. Seed samples were sent to CIAT for fe/zn analysis in 2007. Two other experiments were carried out during the rainy and dry season to study the effect of three nitrogen dosages(0,1/2, and 2X) and 4 potassium dosis(0,1/2,1 and 2X) on the Fe/Zn content in the rice grain. A factorial design was used with 3 reps. No NxK interaction was observed whislt 250Kg/ha of nitrogen are needed for high rice yield. Seed samples were taken for iron/zinc analysis.
Lastly, 10 genotypes(*O.glaberrima*, *O.rufipogon*, Fedearroz 50, BG90-2, Linea 30, and five interspecific lines) were planted to determine the effect of the application of N-fixing bacteria (*Azotobacter chroococum* and *Azospirillum amazonense*) on the iron/zinc content in the rice grain.Three nitrogen dosis(0,125 and 250 Kg/ha) were used in a split-plot design. There were highly significant differences in grain yield among genotypes and significant differences in the GxN interaction and Nitrogen x inoculation. No significant differences were found due to the inoculation per se. Lines CT13941 and CT13943( Bg90-2/*O.rufipogon*) yielded significantly more when 50% of total nitrogen was applied in

addition to the inoculation with the N-fixing bacteria. Seed samples were taken for fe/zn evaluation. This finding suggest that high yield can be obtain with less nitrogen (50%) when N-fixing bacteria is also applied to the crop. This will lower production cost for the farmer.

5. Marker assisted selection for iron and zinc.

Molecular markers tightly linked to desired genes are being used in the development of varieties having the desired gene combination. Recent developments in SNP technology has made it possible to identify SNP associated with particular genes controlling specific traits (Fe and Zn), which can be used to select desired genotypes in breeding populations
Some collaboration was started with Dr. Janette Palma, in Brazil in a Harvest Plus funded project entitled" Identification and expression of genes important for iron translocation to the rice grain " This activity was started to look into iron transporters to the rice grain. New rice varieties with high iron content in the grain (specially in the polished grain) would have a great positive impact in human nutrition. The development of these lines could be achieved by breeding, using available variability, or by genetic engineering. Both approaches would benefit from a better understanding of the physiological processes involved in iron uptake by the plant, translocation into the shoots and proper allocation into the grain. In its way towards the rice grain, iron flux may be limited in several steps, which we assume to be more efficient in rice genotypes with higher iron levels in the grain. We intend to identify these "bottle neck" steps and the genes able to overcome their limitations in "high iron in the grain" genotypes. The Yellow Stripe gene family has obvious candidates to play such important roles, since different Yellow Stripe Like (YSL) genes are considered responsible for uptake and long-distance transport of iron-chelates in different grass species. We have identified eighteen YSL genes in the rice genome, and we propose to determine which ones play major roles towards higher iron allocation into the grain. We will compare their expression in rice lines with high and low iron in the grain, as well as specifically in the polished grain (with higher allocation of iron into the endosperm as opposed to the aleurone layer). In a complementary approach, we will evaluate rice mutant lines with insertions (of the endogenous retrotransposon Tos17 and/or T-DNA) in different YSL genes, looking for the genes that, when mutated, result in altered flux of iron into the grain. We will also search for other genes, presently unforeseen, but potentially also playing major roles in determining iron content in the rice grain. For that, we will use the RDA (representational difference analysis) technique to identify genes with altered expression in "high iron" rice lines. Promising genes identified in this project (YSL and others) will be available to be used as new tools in HarvestPlus breeding programs and/or in transformation of high yielding rice lines aiming at higher iron content in the grain.
As a complement to this approach a thesis project( Olga Ximena Giraldo) was initiated to design SNPS markers that will be evaluated in rice lines with high and low iron content in milled rice. This SNPS could be associated with genes pertaining to the YSL family and/or other genes and would be very valuable in a MAS program.

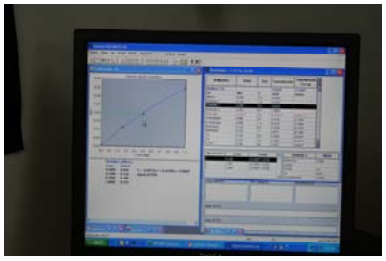
**Future Activities**

Continue the evaluation of cultivars and breeding lines from the germplasm banks and conventional breeding programs for increased iron and zinc content.
Evaluation of segregating populations obtained trough the conventional and recurrent selection methods, as well as trough mutation breeding.
Continue the work on identification of SNIPs for iron and zinc.
Conduct more field experiments to understand GxE factors affecting the expression of iron and zinc in the rice grain.

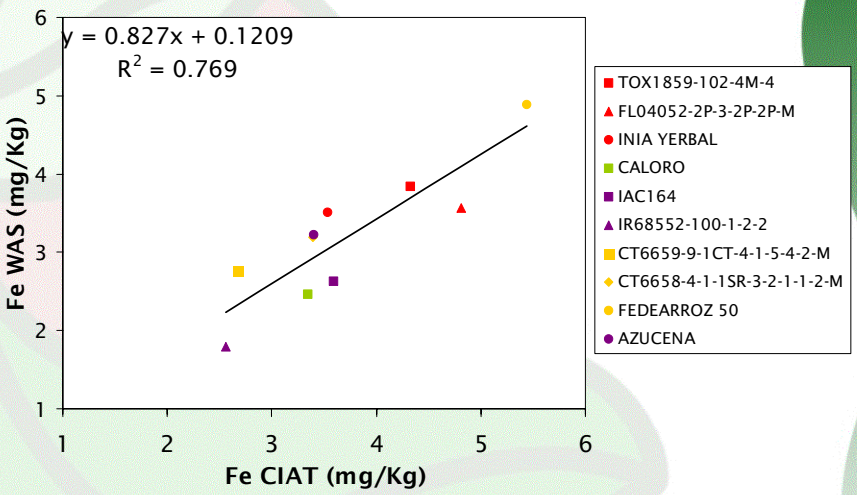
Field visits to Agrosalud partners and assistance to confrences and meetings related to biofortification.
Distribution of data and nurseries to our collaborators.

**Annex.**

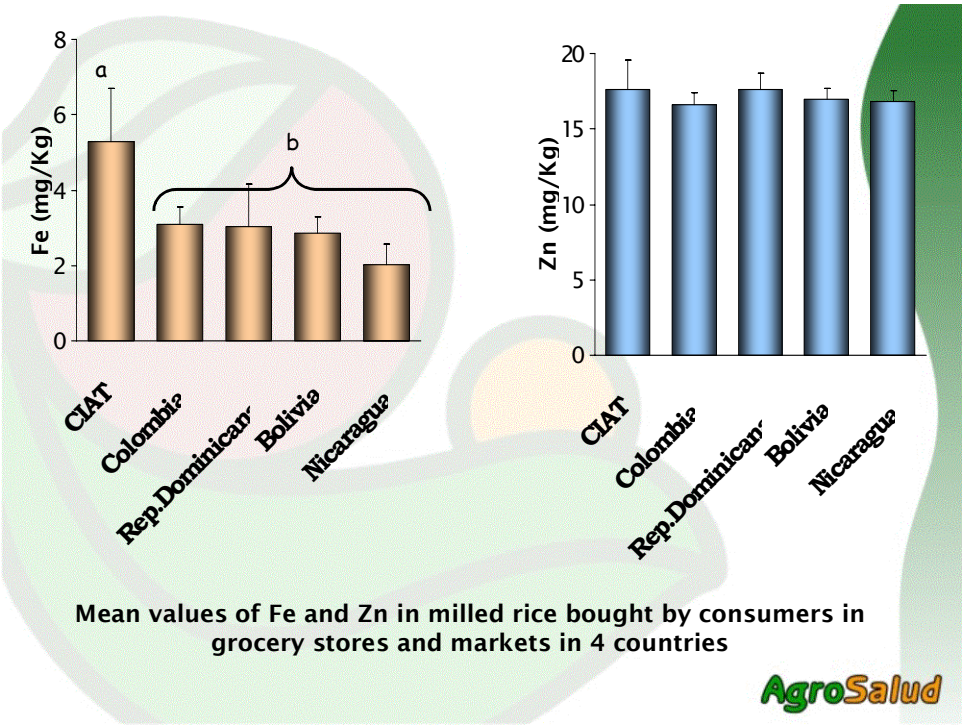
## Analysis of Fe and Zn by Atomic Absorption Method



| Muestra | Descripcion | Fe (mg/kg) | Zn (mg/kg) |
|---|---|---|---|
| 1 | 201 | 2.62 | 13.08 |
| 2 | 202 | 2.05 | 15.28 |
| 3 | 203 | 2.22 | 17.16 |
| 4 | 204 | 2.13 | 17.69 |
| 5 | 205 | 1.78 | 14.42 |
| 6 | 206 | 2.21 | 12.79 |
| 7 | 207 | 2.80 | 16.39 |
| 8 | 208 | 1.90 | 11.20 |
| 9 | 209 | 2.57 | 16.83 |
| 10 | 210 | 2.67 | 10.90 |
| 11 | 211 | 3.88 | 13.49 |
| 12 | 212 | 3.13 | 11.90 |
| 13 | 213 | 1.79 | 14.06 |
| 14 | 214 | 7.83 | 8.37 |
| 15 | 215 | 1.99 | 13.33 |
| 16 | 216 | 2.36 | 13.27 |
| 17 | 217 | 2.82 | 12.71 |




**Correlation between mean values of Fe found in milled rice of 10 rice cultivars determined at CIAT and WAS-Australia.2006**




**Mean values of Fe and Zn in milled rice bought by consumers in grocery stores and markets in 4 countries**

### 1.3.17 Isolation and Expression Analysis of Cysteine Protease Sequences Induced During Brachiaria-Spittlebug Interaction.

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SB-2 Project

## Introduction

Spittlebug is the major pest of brachiariagrasses in the neotropics. Its attack causes important losses in great extensions of unfertile well-drained lands, where the culture of these forages has a greater potential (Hollmann and Peck, 2002). Although Brachiaria lines with a multiespecific antibiotic effect on spittlebugs have been developed (Miles *et al*. 2006), difficulties were found when trying to combine resistance to the insect to tolerance to infertile soils. A characterization of the molecular events that underlie resistance is a fundamental step for the execution of marker assisted selection programs or for the development of transgenic Brachiaria lines combining biotic and abiotic resistance traits. Because the transcriptional effect of the attack of xylem sucking insects has barely been studied in crops, this characterization would also enlarge the knowledge of the mechanisms of resistance of plants to these important plagues.
The first approximation to the molecular bases of the antibiotic effect was the realization and sequencing of a sustractive library comparing the transcriptome of resistant plants infested and uninfested with spittlebug nymphs (Romero, 2003). This leaded to the identification of 19 gene products that are induced by insect attack. The putative roles for these transcripts cover the perception of the invader, signaling and efection of antibiotic responses. One of the most interesting candidates was a 740 bp fragment coding for a cysteine protease. This sequence (named BCP for Brachiaria Cysteine Protease) shares a high similarity with proteases whose function in resistance of plants to biotic stresses has been proved. In this work I report the isolation of the full-length transcribed and genomic sequences of this candidate and his homologs in resistant and susceptible accessions of Brachiaria. BCP expression during plant-insect interaction was also evaluated by means of the Real Time PCR technique.

## Methodology

In this study two Brachiaria accessions where compared:

CIAT 36062, an apomictic tetraploid hybrid resulting from the contrasting cross *B. brizantha* accession CIAT 16829 * *B. ruzizienzis* 44-3. This genotype has a multiespecific antibiotic effect on spittlebug that is manifested in an increased mortality of nymphs and adults, a delay in the time required to attain maturity and reduction of weigth (Cardona *et al*. 1999, Cardona *et al*. 2003, Pabon *et al*. 2005). Spittlebugs reared in this accession feed less and leave the spittle mass more often, wandering over the soil surface and eventually dying of dehydratation (Cardona *et al.* 2003). This effect seems to be under the control of few genes (Miles *et al.* 1995) and has been associated previously to an antifeedant or toxin (Lapointe et al. 1992)
The other genotype studied is BRX4402 (B. ruziziensis), a highly spittlebug susceptible accession (Pabon *et al*. 2005).

Genomic sequences where obtained by the application of the genome walker protocol described by Hagiwara and Harris (1992) to total DNA extracted by the CTAB method and digested with

HincII, EcoRV and XmnI enzymes (New England Biolabs). 5 ug of these digestions where ligated to the *vectorette* adapter. PCR reactions performed with gene specific primers and the 224M13 primer (complementary to the vectorette unit) where carried using these ligations as template. The amplification products where cloned and sequenced.

RNAs where derived from superficial roots of resistant (R) and susceptible (S) genotypes infested (I) and uninfested with 200 *Aenolamia varia* s nymphs. Three plants of each condition (RI, R, SI, S) where collected at five post-infestation times (6 hours, 1day, 2days, 4days and 8days), their RNAs where extracted separately with the *SV Total RNA Isolation System kit* (Promega, Madison, WI, U.S.A.) then pooled and retrotranscribed with Power Script enzyme and SMART primers (CLONTECH.) following constructor instructions.

Translated full length sequences where obtained by the utilization the *BD SMART™ RACE cDNA Amplification Kit* (CLONTECH). The amplification products from PCR reactions conducted in pools of cDNA from resistant and susceptible accessions where cloned and sequenced. Sequences were assembled, annotated and analyzed with bioinformatic tools.

The quantification of gene expression levels was performed by the RT-PCR technique on DNA Engine OPTICON 2 TM System (BioRAD) with SYBR green detection method (DyNAmo kit, Fynnenzymes). qPCR reaction conditions where stringently standarized for the reference genes (18S ribosomal RNA and GAPDH gene) and the cysteine protease primers. A standard curve was built with five tenfold dilutions (10E9-10E5 molecules/ul) from a solution of a plasmid containing the evaluated gene. Reactions where run in duplicate for the 20 different cDNAs. Melting curves were performed and the PCR products were submitted to electrophoresis in a 2% agarose gels to check the specificity of the amplification.
The evaluation of the mean normalized gene expression and the statistical analysis of this data where done by the relative quantification method using the REST® (Pfaffl and Horgan, 2002) and qBase v1.3.5 software.

**Results**

I-Isolation and analysis of genomic and transcribed sequences
Eight very similar cysteine protease sequences were isolated in this study (table I, Figure 1). Four transcripts corresponding to alternative polyadenilations of one gene product are expressed in the resistant genotype. These transcripts code for a 338 amino acid (aa) CP (predicted MW=36.6 KDa) that shares all the characteristics of functional proteases belonging to the papain family (C1A) (Wiederanders, 2003): It is produced as a pre-pro-protein, with a signal sequence ("pre" region) that defines its cellular location and a propeptide that must be hydrolyzed for its activation. The 20 aminoacids located at the N-terminal form a hidrophobic alpha helix that works as a signal directing the CP to the secretory pathway. The propeptidic region is composed of the next 104 aas, it contains ERFNIN (Ex3Rx3Fx2Nx3I/Vx3N) and GNFD (GxNxFxD) conserved motifs involved in the inhibition and processing of the enzyme (Rzychon et al. 2004). The 214 aa long mature protein is formed by two structural domains separated by a furrow containing the active site aas Cys25, Gln19, His 162 and Asn 182 (Figure 1 B and C).

Table 1: Properties of the isolated sequences

| | cDNA | | | Genomic | | | |
|---|---|---|---|---|---|---|---|
| | 36062-CPcDNA 01,02,03,04 | 4402-CPcDNA01 | 4402-CPcDNA 02 | 36062-CP01 | 36062-CP02 | 36062-CP03 | 4402-CP01 |
| Codifying (bp) | 1014 | 1014 | 561 | 1014 | 1014 | 618 | 399 |
| 3´UTR (pb) | 189, 350, 455, 600 | 65 | | | | | |
| 5´UTR* (bp) | 28 | 59 | | 17 | 75 | | |
| Intron (bp) | | | | | 104 | 116 | |
| Similiarity + | | 98% | 99% | 97% | 100% | 100% | 99% |
| Variable sites + | | 6 | 10 | 30 | 0 | 0 | 3 |
| Variable aas + | | N80A I129T A188T T228A P307S | Y105C | N76C, Y105 C, | | | |
| Covering | 18.0X | 7.2X | 3.0X | 3.0X | 6.3X | 1.3X | 2.3X |
| Comments | Reported by Romero (2003). Multiple polyadenila-tion copies | Ortholog to 36062-CPcDNA | Fragment. Pseudogene with a duplication. | Paralog. Intron-less sequence | Gene transcri-bed during spittlebug attack | Fragment Gene transcri-bed during spittlebug attack | Paralog sequence not transcibed during spittlebug attack. |

(*) 5´UTR sequences may be incomplete due to DNA degradation.
(+) In comparison to 36062-CPcDNA.

CP mRNAs present long AU rich 3´UTRs that may be involved in the regulation of their stability and translation rate (Backheet *et al.* 2006).
Two copies of the gene coding for this protease were isolated in the genome of CIAT 36062. Because these copies differ only in the length and sequence of the unique intron it cannot be stated if they are alleles (CIAT 36062 is an allotetraploid hybrid) or paralogs. An intron-less paralog sequence that is not expressed in Brachiarias roots but is very similar to the other two genes was also detected. The promoter region of these genes was not isolated in this work.
In the susceptible accession BRX 4402 two transcripts where sequenced. One codes for a cysteine protease almost identical (one aa difference) to that found in the resistant genotype. The

other is a pseudogene shearing a 3´dupplication that leads to an interruption of the reading frame. One incomplete genomic sequence that is not expressed under the evaluated conditions was detected in this accession.

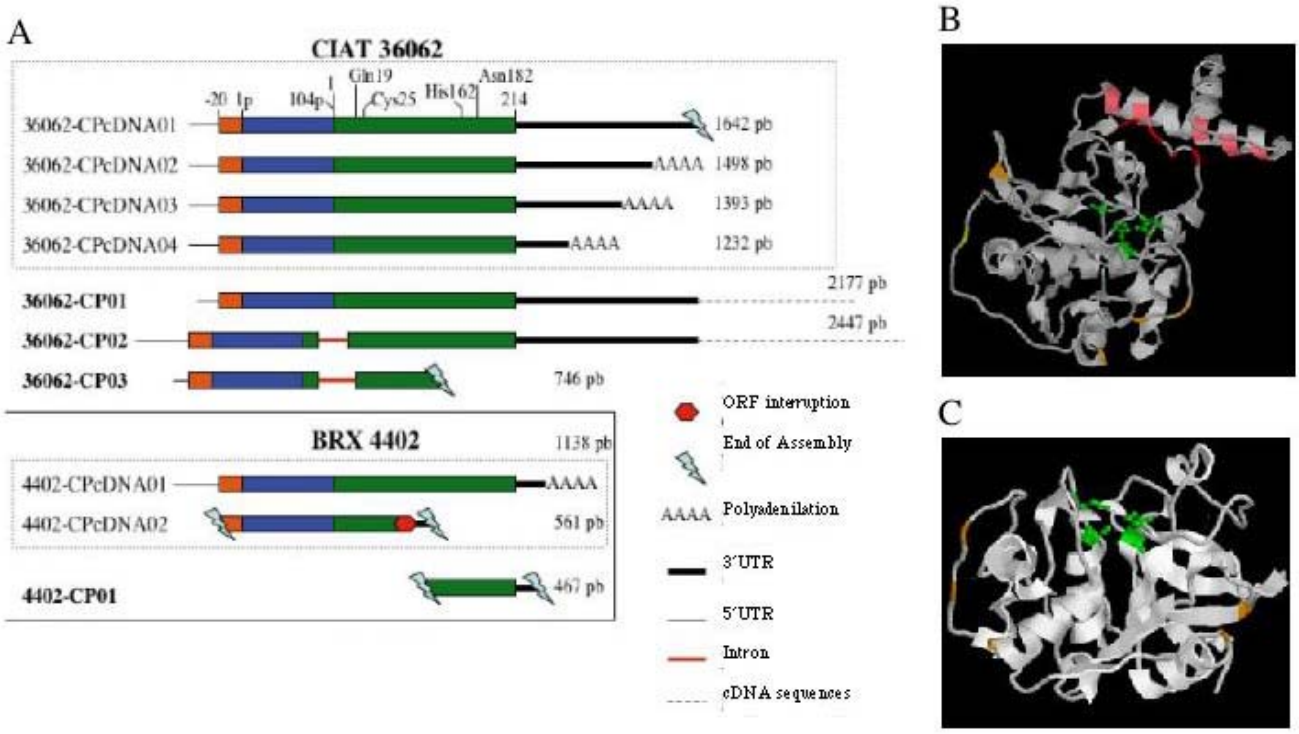


Figure 1: Structure of The BCPs. (A) Characteristics of the main sequences isolated in resistant and susceptible genotypes. Signal peptide is in orange, propeptide in blue and catalytic domain in green. (B and C) BCPs structural prediction. Catalytic aas appear in green and variable aas in yellow. In (B) the preprotein is shown, ERFNIN and GNFD motifs appear in red and pink respectively. (C) Mature enzyme showing the catalytic clift between his two structural domains.

Many papains have been identified in plants, where they participate in diverse metabolic events of physiological importance including the mobilization of nutrients, programmed cell death processes, tissue development and responses to various stress (Wiederanders, 2003, Grudowska and Zavdanska, 2003). The evolution of these proteases is marked by the appearance of some structural domains (Figure 2): Propeptidic regions played an important role as they regulate the activity of the enzymes. For instance the acquisition of ERFNIN-GNFD containing propeptides has occurred once in the evolution of the family and lead to the appearance of the Cathepsin L like subgroup of papains (to which BCPs belong) (Karrer *et al*. 1993). Because the function of a protease is defined by its subcellular location, the nature of the signal peptide is one of the main factors that define C1A functional and evolutionary classification. Signal peptides have coevolved with the machinery responsible for the package and transport of the proteases that contain them (Hara-Nishimura *et al.* 2004).

The BCPs share all the distinctive features of papains but differ markedly from all the other groups of cathepsin L like proteases. This prevents the realization of reliable inferences about their function on uninfested plants. Nevertheless in previous works many extracellular papains were found to mediate developmental and stress response mechanisms in xylematic and other

conductive tissues (Funk *et al.* 2002, Konno *et al.* 2004) This points to the possibility that this protein is secreted to the xylem.

Papains mediate processes at all the levels of incompatible interactions between plants and insects (Van der Horn and Jones 2004): perception of the agent (Kruger *et al*. 2002), signaling (Avrova *et al*. 1999) and execution of an antibiotic effect (Jiang *et al.* 1995, Pechan *et al.*1999, Pechan *et al.* 2000, Pechan *et al.* 2002, Konno et al. 2004, For this reason papains associated to biotic stress resistance are not a monophyletic group (figure 2).

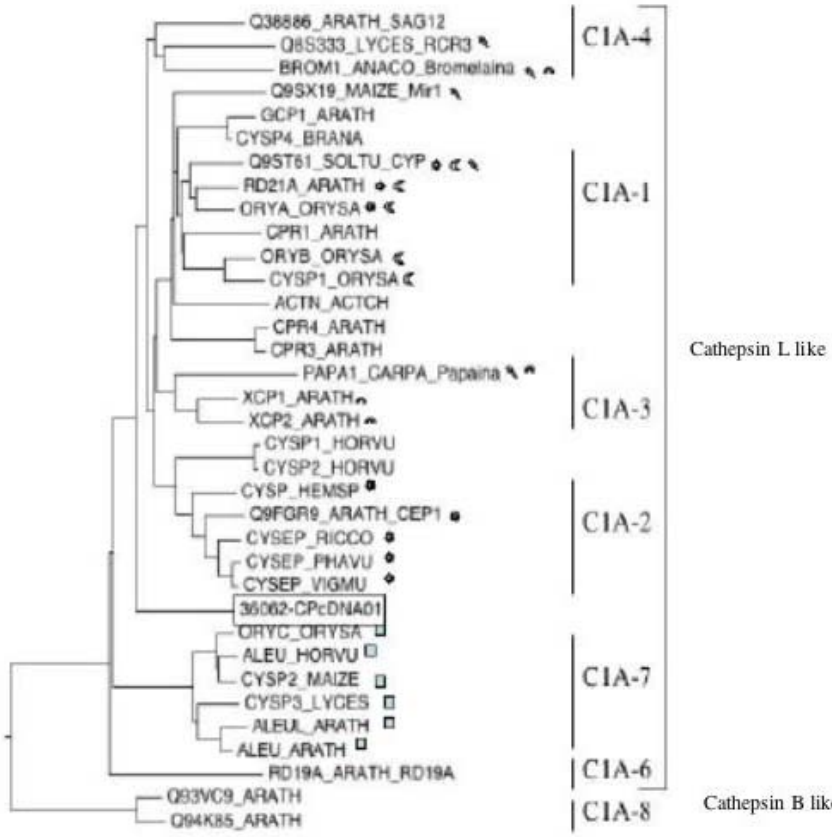


Figure 2: ML Phylogenetic tree of some representative vegetal papains. BCPs appear in a box. Clades are named according to the classification proposed by Beers et al. (2004). (□) Vacuolar and expressed in seeds. (↗) Associated to pathogen and insect resistance. (◠) Expressed in xylem or laticifers (Ξ) KDEL location signal. (□) Granulin domain.

II Gene expression quantification

The BCPs are constitutive genes whose transcripts are produced in infested and control plants of the two studied genotypes. Nevertheless Real Time-PCR results show that their expression is affected by the interaction between the plant and the insect:

Gene transcription is increased under *A. varia* attack in the resistant accession CIAT 36062: According to the randomization test performed by REST software quantity of BCP transcripts is significantly greater ($p<0.001$) in plants attacked by the insect that in controls. A six fold up-

regulation was detectable 6 hours after infestation and maintained during the 8 days evaluation period.
On the other side, insect attack does not have any statistically significant effect ($p>0.05$) on BCP basal expression in susceptible material.

Conclusion

The full-length sequence of the CP gene that was previously reported in sustractive libraries of infested versus control spittlebug-resistant material was isolated. It codes for a secreted functional papain that is constitutively expressed in Brachiaria roots. The proteases generated in resistant and susceptible accessions are very similar but differ in their expression profile: In CIAT 36062 the gene is upregulated in response to insect attack whereas in the susceptible accession its expression is unchanged by the interaction with the plague.
This supports the idea of the protease playing a role in the resistance phenomenon: In fact most of the papains whose antibiotic effect has been proved are also involved in other physiological processes; their utilization in biotic stress responses usually comes from a refinement of a more general function (Funk *et al.* 2002, Van der Horn and Jones, 2004). On the other hand, BCPs share all the properties of the agent that may cause the antibiotic effect of CIAT 36062: It is a an extracellular peptide that could be systemically secreted to the xylem an then be ingested by the insect, generating an antifeedant or toxic effect similar to that reported for papains like maize mir1 and latex proteases. Finally, because the resistance of brachiariagrasses to spittlebugs is an allopatric multiespecific phenomenon, it must result from the activation of a pre-existent general mechanism of defense. From this scope, the inability of BRX 4402 to enhance BCP expression in response to insect attack may cause its susceptibility to spittlebugs. On the other hand the 5 aa differences between 4402-CP01 and the resistant protein could suppress the antibiotic effect. Finally, the existence of transcripts coding for truncated inhibitory propeptides in the susceptible accession could also be involved in this effect.
Nevertheless the participation of BCPs in resistance is not yet proved. A more accurate study of the expression of those genes in a longer period of time, including post-infested samples and other genotypes (with a broaden resistance) could clarify enzymes function. This study can be at done at the transcription level or evaluating enzyme activity. Mapping the genes in the progeny of a contrasting cross may also be an important step in this process. Finally the administration of BCPs in artificial diets would permit the evaluation of the in vitro antibiotic effects of these proteins.

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## 1.3.18 Study of gene expression during pistil development in apomictic and sexual *Brachiaria* spp

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SB-2 Project

### Introduction

Apomixis is a plant reproductive mode through seeds, which contain an embryo produced without genetic recombination by avoiding both meiosis and fertilization. As a result, the progeny of an apomictic plant is genetically identical to it. Thus, hypothetically apomixis could offer tremendous benefits to poor farmers because they would be able to save their hybrid seed for replanting year after year, while indefinitely maintaining advantageous traits. Additionally, apomixis technology could offer faster and less expensive plant breeding strategies to tailor plant cultivars to specific local crop breeding needs.

Facing this situation, a project which seeks to identify candidate genes to be responsible for the apomicitic reproduction in *Brachiaria decumbens* and *B. brizantha* is being carried out at CIAT.

### Strategy

The strategy we are currently following approaches the problem at two levels: the genome and the transcriptome level. At the transcriptome level we are trying to identify differentially expressed genes between the pistils of apomictic and sexual plants during the reproductive development. To do so, we compare the gene expression level of the full set of genes expressed during *Brachiaria* reproductive development using a dedicated cDNA microarray meaning that it contains ESTs from the tissue at the time of interest, pistils found at specific decisive reproductive developmental stages for this case. Afterward the differentially expressed ESTs are sequenced to be able to assign putative biological functions to each one of them, and to look for polymorphisms that will enable us to map them, thus entering the genome level. At this level we look for mapping the reproductive mode in the same population where the differentially ESTs are mapped. The capacity to reproduce apomictically is a qualitative trait that segregates as a simple dominant mendelian trait. We expect to find a complete or at least a very high co-segregation between some of the differentially expressed genes and the apomictic capacity, and such genes are very likely to be fundamental to conferring the capacity to reproduce apomictically to *Brachiaria spp* plants.

### Results

Reproductive developmental stages chosen to evaluate gene expression

Three developmental reproductive stages were chosen to identify differentially expressed ESTs between apomixis and sexuality, given the decisive events that take place at each one of them. The length of the apomictic and sexual pistils (from the base of the ovary to tip of the estigmas, Fig. 1) and the events occurring at each one of these three reproductive stages was determined for *B. decumbens* by Dusi et al. (1999), and for *B. brizantha* by Araujo et al. (2000). Based on this information a narrow range for the pistil length (PL) to obtain pistils at each stage was selected. Following, the chosen PL and the crucial events occurring at each stage are described in table 1.

| Reproductive mode | Events | Pistil length (mm) | Spikelet length (mm) | Stage |
|---|---|---|---|---|
| Apomictic | Differentiation of Aposporic initial cell (AI). Circumvention of meiosis | <0.3 | <3.0 | |
| Sexual | Differentiation of Megaspore Mother cell (MMC). Meiosis | <0.3 | <3.0 | I |
| Apomictic | Embryo sac (ES) development from mature AI. No need of meiosis. | 1.1 - 1.6 | | |
| Sexual | Embryo sac (ES) development from mature megaspore. Only after meiosis. | 1.7 - 2.2 | | II |
| Apomictic | Parthenogenesis | >7.2 | | |
| Sexual | No partenogenesis | >5.88 | | III |

Table 1. Description of crucial events at the three stages chosen to build a cDNA microarray and perform gene expression comparison between pistils of apomictic and sexual plants.

Pistils at stage I are very time-consuming to dissect, therefore inner florets were taken instead of pistils to perform gene expression comparison. Inner florets comprise the pistil, the set of 3 inner anthers, and two leaves surrounding the named structures. Pistils of PL < to 0.3 mm are found in florets with spikelet length (SL) < 3 mm (Dusi et al. 2000). Nevertheless a cDNA library of pistils at stage I from an apomictic (6294) and a sexual (16114) accession will be generated to improve the existing one. Also a cDNA library of pistils at stage II from all the accessions will be generated.

Table 2 shows the *Brachiaria* accessions used to collect pistils to complete the apomictic Vs. sexual gene expression comparison and their reproductive mode according to the analysis of cleared mature ES performed by Naumova et al. (1999). Number of pistils collected at each stage is also shown.

| Accession CIAT ID | Species | Reproductive mode | Number of inner florets at stage I | Number of pistils at stage II | Number of pistils at stage III |
|---|---|---|---|---|---|
| 16114 | B. brizantha | Sxl | 137 [84]* | 200 | 200 |
| 16493 | B. decumbens | Sxl | 200 | 200 | 206 |
| 16494 | B. decumbens | Sxl | 200 | 200 | 206 |
| 44-2 | B. ruziziensis | Sxl | 200 | 141 | 200 |
| 606 | B. decumbens | Apo | 0 | 0 | 200 |
| 6012 | B. decumbens | Apo | 200 | 200 | 209 |
| 6294 | B. brizantha | Apo | 200 [86]* | 200 | 200 |
| MX0356 | Hybrid | Apo | 0 | 0 | 0 |

Table 2. *Brachiaria* accessions used to collect pistils to complete the apomictic Vs. sexual gene expression comparison, their reproductive mode and the number of pistils (or *inner florets) collected.
Most of the used accessions are found only at Popayan CIAT station. A duplication of each of thes accassion has been brought to CIAT Palmira t have tissue more readily available.

cDNA libraries and microarrays
Full-length and subtracted cDNA libraries had been already obtained for stage III pistils from a number of apomictic and sexual hybrids from the forage breeding program at CIAT on 2003 (Bernal et al. 2003).

Full-length cDNA libraries from pistils at stages I (PL <0.2mm) and at late I (0.2 – 0.4 mm), of six apomictic and seven sexual hybrids (Bernal et al. 2005) were obtained following the same methodology reported by Cortes et al. (2002) with the following modifications:

RNA was isolated with PicoPure™ RNA Isolation System from Arcturus and quantified using Ribogreen fluorescence RNA quantification kit, which delivers more sensitive and accurate measurements than absorbance. This data allowed to estimate total RNA quantity obtained per pistil: ~2.95 ng / stage I pistil and ~4.95ng / stage late I pistil (Graph 1). In spite of using around 200 pistils for RNA extraction a small quantity of total RNA was obtained and mRNA was not isolated before synthesizing cDNA.
During full-length cDNA library construction cDNA samples were divided into large, medium and small fractions to perform ligation and electroporation separately to avoid the competition between large and small fragments.

At the end four cDNA libraries were obtained: two for apomictic pistils at I and late I stages, and two for sexual pistils, also at I and late I stages. In the table 3 the titre and number of picked clones of each cDNA library is shown.

| Library | Titre (total cfu) | Number of picked clones |
|---|---|---|
| Apo I | 4.25E+05 | 1536 |
| Apo late I | 4.84E+05 | 1536 |
| Sxl I | 5.83E+05 | 1536 |
| Sxl late I | 4.94E+05 | 1536 |
| Total | | 6114 |

Table 3. Full length apomictic and sexual cDNA libraries showing titre (CFU, colony forming units) and number of picked clones.

Subtracted cDNA libraries from stage I and late I was attempted following the subtractive suppression hybridization as in Bernal et al. (2003). However subsequent sequencing of 100 random clones showed that only 2% of the clones proved to have homology to functional sequences. Almost certainly all the cDNA was spent on the full-length cDNA libraries, and the PCR based SMART cDNA amplification technique resulted in artifact amplification instead of authentic cDNA.

To build the dedicated cDNA microarray, the subtracted cDNA libraries from stage III pistils and the full-length cDNA libraries from stage I and late I pistils have been PCR amplified under standard conditions, reallocated to appropriate 384-well plates with spotting buffer and are ready to spot cDNA microarrays.

Methodology used to perform pistil gene expression comparisons between apomictic and sexual *Brachiaria* plants

Dedicated cDNA microarrays allow researchers to perform transcriptome wide gene expression analysis, obtaining valuable information about the expression profile of almost all the relevant genes involved in a certain condition. These expression profiles are more meaningful the more specific tissues, or cell types they represent. In many cases, such highly specific samples are available only in small quantities, such that not enough total RNA is obtainable to hybridize a cDNA microarray (around 10 ug of total RNA per microarray per sample). To overcome this problem, several protocols where the RNA is amplified by *in vitro* transcription have been validated, and are widely used (Marko et al. 2005). In the *Brachiaria* reproductive mode research, our target tissues are the pistils at different developmental stages. Ideally the expression profile of a single pistil should be acquired to avoid mixin different developmental stages. Yet, after several RNA extractions performed from *Brachiaria* pistils, it is known that enough total RNA is not

obtained even from stage III single pistils. In fact according to graph 1, the RNA that would be obtained from single stage I pistils, would not be enough to hybridize cDNA microarray even after performing *in vitro* transcription amplification of the RNA. A modified RNA amplification method based on Marko et al. paper (2005) was established to allow us to perform gene expression comparisons from stage I to stage III pistils. Briefly, the modification is to synthesize the transcription promoter double strand by performing a cDNA synthesis with E. coli DNA polymerase for 2 hours, instead of a 15 minutes cDNA synthesis with T4 DNA polymerase. The modification improved RNA amplification from 25 fold to 60 fold, therefore we have a modified RNA *in vitro* transcription method that is suitable to use with stage I pistils samples. This method needs at least 500 ng of total RNA, thus according to graph 1 ~160 pistils at stage 1 and ~100 pistils at stage II should be dissected.

Differentially expressed ESTs at stage III pistils between apomictic and sexual plants

After improving gene expression analysis of stage III pistil comparison between apomictic and sexual plants, a total of 121 genes were found to be either up or down regulated.

The distribution of these clones among the subtractive cDNA libraries leads to thinking that the subtraction hybridization didn't work as expected because clones from each of the libraries were found with opposite gene expression patterns as expected depending on which library they came from (Fig. 3).

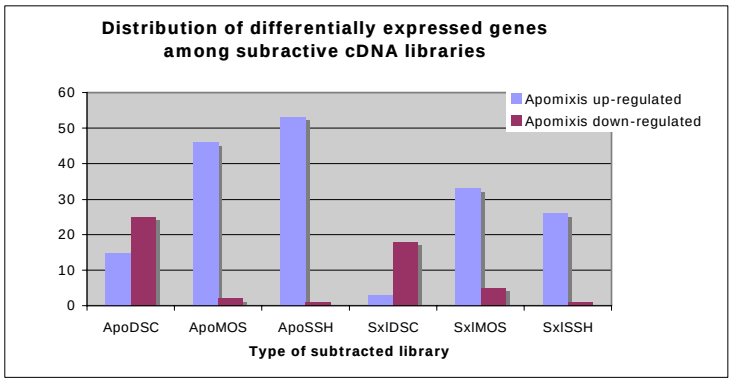


Fig. 3. Distribution of differentially expressed ESTs among subtractive cDNA libraries.

GO terminology was assigned to each EST by automated BLAST search against GO database and retrieval of the associated GO terms. Functional analysis does not yield enough information to identify which of the 121 regulated genes is/are more important during the determination of the kind of reproduction shown by an individual, therefore the mapping approach should help us identify the more important genes, since they should closely co-segregate with the apomictic capacity. Therefore PCR amplification conditions have been established for 38 differentially expressed ESTs. None of them has shown polymorphism among the parentals of a current *Brachiaria* mapping population. Polymorphisms within these sequences should be looked for using CAPs (Cleaved Amplified Polymorphisms) or SNPs (Single Nucleotide Polymorphism) methodologys. Primers for the amplification of the rest of differentially expressed ESTs have been design,

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## 1.3.19 Orthologs Markers Generation For Comparative Mapping in *Brachiaria decumbens*

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SB-2 Project

**Introduction**

The soils that present aluminum-toxic conditions are one of the main restrictive factors of the agricultural productivity in a great variety of crops that are developed in acid soils. These types of soils are distinctive of a main portion of tropical and subtropical regions. Different programs at CIAT have tried to improve the natural adaptation of these crops to these restrictive conditions, using diverse approaches that go from plants traditional improvement to soils management and the use of molecular tools. In relation with this last point a molecular map and a QTLs detection were performed by CIAT agrobiodiversity unit in the highly aluminum resistant grass *Brachiaria decumbens* (Rosero *et al*, 2005). Three QTLs were detected; the first of them located in the linkage group 2, related with root's diameter, explaining the 4% of the phenotypic variation. The second QTL, located in the linkage group 7, related with root's length and root's tips number, explaining the 13.4% of the phenotypic variation and the third one, located in the linkage group 10, related with root's diameter, explaining the 4%. Although three QTLs were detected, they were relatively low explaining just a small fraction of the genetic response of *B. decumbens* to aluminum-toxic conditions.
For this reason is mandatory to try to increment the explained variation by these QTLs or in other way, to identify other QTLs that explain better the phenotypic adaptation of this crop.

Different approaches can be adopted to increase the QTLs values. One is to develop again the phenotypic evaluation of the analyzed population due to is well known that QTLs are really affected by environmental and experimental conditions (Mohan *et al,* 1997) and other way to do it, is detecting new more significant QTLs by means of a molecular map saturation. For the last mentioned approach which was taken in count for the present research a completely new set of orthologs markers were generated by means of comparative analysis within the *Poaceae* family to perform a subsequent comparative mapping in *B. decumbens*. Orthologs markers were generated, because they are informative by themselves due to is well known that in phylogenetically close species orthologs genes of response to a specific treatment are located on homeologs chromosomic regions associated with specific molecular markers, in other words, orthologs markers can identify specific regions associated with a given treatment in phylogenetically close species (Fulton *et al,* 2002).

A set of 43 pair of primers based on orthologs markers were design by means of bioinformatics tools, afterwards, in the next step in the present research they will be used to perform a comparative mapping in the signal grass *Brachiaria decumbens*.

**Materials and Methods.**

Identification of orthologs markers associated with aluminum resistant within the *poaceae* family.
A set of markers associated with aluminum resistant locus in the locus Altph of wheat, locus Alp in Barley, locus Alt+3 in rye, locus Altgb in sorghum and specific resistant regions on rice chromosomes 1,2,3,6,8 and 13 were identified. Specific markers that had been mapped simultaneously in two or more of these species identifying homeologs regions for aluminum resistance were considered orthologs markers, almost all of them were also considered as orthologs by the literature.

Markers sequences obtaining and BLAST-N searching.
Using open source databases as *Gramene*, the sequence of the orthologs markers were obtained, subsequently a BLAST-N searching was performed in order to identify sequences highly similar to the marker sequence reported in other species or in the same specie but different source.

Sequences alignment and conserve regions identifications.
Using *Align X*, different sequences related with a specific marker were aligned and by means of *Vector NTI* software, conserve regions were identified in the same way the variable region within the conserve ones was different enough among aligned sequences to detect those differences as differences in molecular size.

Primer Design
By means of *Vector NTI* software proper primers were design inside the conserve regions to amplify the variable regions. Amplicons were between 200 bp and 400 bp.

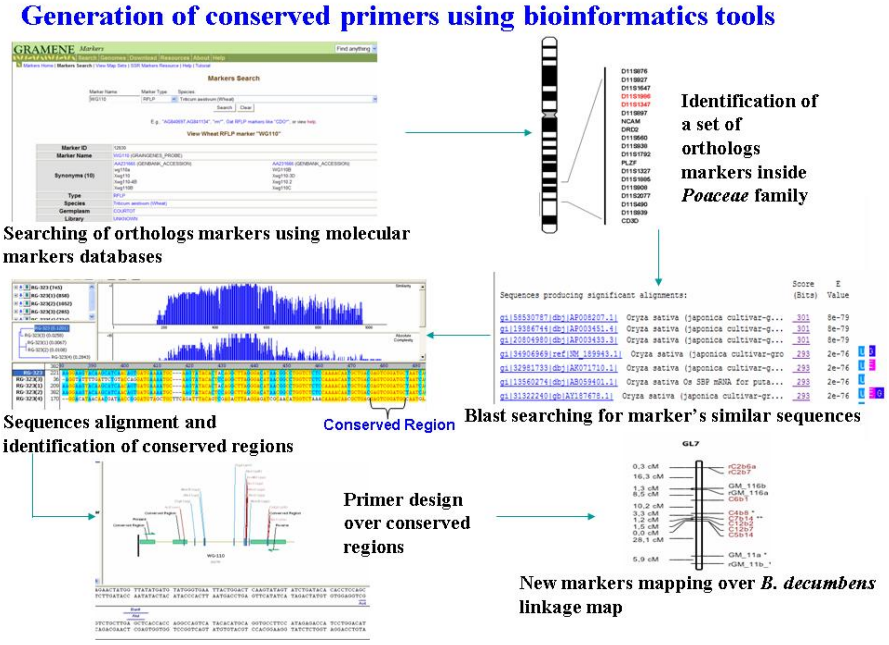


Fig 1. Generation of conserve ortholog markers by means of bioinformatics tools.
3. Results.

A set of 43 pair of primers were designed. Table 1, describes the most important characteristics of these orthologs markers.

Table 1. Designed Orthologs Markers.

| Sequence Name | Crop | Kind of Sequence | Length bp |
|---|---|---|---|
| WG-110 | Rice | EST | 333 |
| RG-109 | Rice | STS | 632 |
| CDO-345 | Oats | EST | 213 |
| RG-323 | Rice | STS | 750 |
| RG-667 | Rice | STS | 583 |
| RZ-142 | Rice | STS | 386 |
| C-86 (Seq 1) | Rice | EST | 448 |
| C-86 (Seq 2) | Rice | EST | 359 |
| R-2625 | Rice | EST | 629 |
| R-2510 (Seq 1) | Rice | EST | 323 |
| R-2510 (Seq 2) | Rice | EST | 372 |
| R-2460 (Seq 1) | Rice | EST | 452 |
| R-2460 (Seq 2) | Rice | EST | 339 |
| S-1520 (Seq 1) | Rice | EST | 309 |
| S-1520 (Seq 2) | Rice | EST | 270 |
| G-200 (Seq 1) | Rice | STS | 322 |
| G-200 (Seq 2) | Rice | STS | 287 |
| CSU-155 | Maize | EST | 201 |
| P-bnlg 1828 | Maize | SSR | - |
| P-bnlg 238 | Maize | SSR | - |
| P-nc 003 | Maize | SSR | - |
| P-bnlg 161 | Maize | SSR | - |
| WG-464 | Sorghum | SSR | - |
| XCDO-1395 (Seq 1) | Oads | EST | 343 |
| XCDO-1395 (Seq 2) | Oads | EST | 378 |
| XBCD-1117 | Barley | EST | 228 |
| RG-650 | Rice | STS | 373 |
| RZ-997 | Rice | STS | 494 |
| R-2549 (Seq 1) | Rice | EST | 441 |
| R-2549 (Seq 2) | Rice | EST | 410 |
| RM-201 | Rice | STS | 685 |
| RG-257 | Rice | STS | 633 |
| RG-1028 | Rice | STS | 637 |
| RZ-543 | Rice | STS | 360 |
| C-1408 (Seq 1) | Rice | STS | 290 |
| C-1408 (Seq 2) | Rice | STS | 342 |
| C-1419 | Rice | STS | 462 |
| CDO-122 (Seq 1) | Oads | EST | 606 |
| CDO-122 (Seq 2) | Oads | EST | 277 |
| RG-190 | Arroz | STS | 644 |
| C-1121 | Rice | EST | 348 |
| RM-215 | Rice | SSR | - |

**Ongoing work**

The designed markers will be used to perform a mapping procedures over the *Brachiaria decumbens* molecular map. With the saturation of the map is possible to increase the value of the already found QTLs or in the same way, the location of new QTLs is very probable.

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## 1.3.20 Comparative Mapping in *Brachiaria decumbens*

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SB-2 Project

**Introduction**

Several species of grass show tolerance to acid grounds among them *Brachiaria decumbens*; this variety has been identified as an extremely resistant specie among the grasses (Wenzl 2000), and as more resistant than most of other genotypes of cultivated gramene, like wheat, maize and rye (Piñeros et al to the 2005). The resistance to Aluminum in *B. decumbens* is one object of investigation in the CIAT by different approaches, within these, the project of comparative genomic tries to find molecular markers associate to QTLs to Aluminum resistance, located in the linkage map of *B. decumbens*.

Clones of orthologous sequences and comparative mapping of genes have been used for the identification of major genes in resistance to Al in several species of the Poaceae family (Jurandir et al 2004, Mitftahudin et al 2004, 2005), which is possible due to the sequences of genes that are conserved from this family. The use of molecular markers associate to resistance to Al in near species (rice, maize, rye and sorghum), help to explain the QTLs resistance to Al in *B. decumbens*, which considers that this variety is important and promissory to find major genes of resistance to Al. (International workshop on advances in improving acid adaptation of tropical crops and forages, and management of acid soils, Brazil 2005). In this phase of the project a set of 43 orthologous markers where used, previously selected from different species of the Poaceae family and clones from a subtractive library; to find possible candidates of resistance to Aluminum. The objective is to establish molecular markers based on PCR of type SSR, CAPs and ESTs, which is useful to saturate the map of QTLs of resistance to Al in *B. decumbens*.

## Materials and methods

2.1 Plant Material and DNA extraction: An F1 hybrid population (182 plants) from the site-specific cross between *B. decumbens (*606, Al resistant) and *B. ruziziensis* (4402, susceptible). Total DNA was extracted from fresh or frozen tissue using approximately 0,03g of leaf tissue. The pulverized plant material was transferred to a microtube and 600 ul of preheated buffer-CTAB (65 $^{o}$C) solution was added. The tube was vortexed for a few seconds and incubated at 65 $^{o}$C for 10 min. Following incubation, 600 ul of chloroform/isoamyl alcohol (24:1) was added and the mixture was shaken vigorously. The extract was centrifuged for 10 minutes at about 12,000 r.p.m. and the supernatant transferred to a new microtube. The chloroform/isoamyl extraction step was repeated two times more and 1 ml of 100% cold ethanol was added to the final supernatant. A DNA pellet became visible upon gentle swirling. It was transferred to a new tube, rinsed two times with 70% ethanol and dissolved in 400 ul of 10 mM TE buffer solution .

2.2 Tests of amplification: A set of 99 sequences (43 selected by genomic comparative, 40 selected from a subtractive library, 4 of sequences UTR and 13 of yeast. They were proven in the parents 606 and 4402, for standardize the amplification conditions and selecting that methodology to evaluate each marker in the hybrid population, like CAPS or PCR marker. The sequences of the markers whose did not display polymorphism in the amplified by PCR are evaluated like CAPS marker. For this sequence it was necessary to elaborate one restriction map by using the Neb cutter 2.0 program.
3. Ongoing work - Molecular Characterization: The sequences identified as polymorphic are going to use to evaluate the hybrid population.

## Results

A total of 57 sequence are amplified in parents that are summarized in table 1 by using information of primers amplified, which where identified as CAPS and PCR markers. The figure 1 show an example of some of the amplified sequences, the presence polymorphism bands between resistant parental and susceptible allow classified the primers in PCR marker, for the sequences without differences between the resistant and susceptible is classificated CAPs marker.

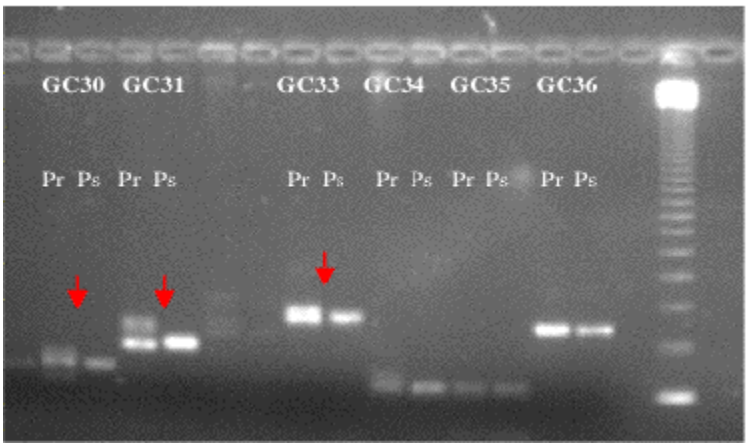


Figure 1. Amplification of sequences in resistant parental (Pr) and susceptible parental (Ps), the arrows indicate primers that display polymorphic bands between the parents, the sequences GC34, GC35 and

Gc36 present polymorphism. This is a 4% agarose-gel stained with ethidium bromide to the right the molecular marker size of 123 bp.

Table 1. Listing of sequences classified like CAPs marker or PCR marker for being evaluated in the hybrid population.

| Name | Kind of Sequence | Characterization |
|---|---|---|
| 9E Bra Al | EST | CAPS marker |
| 4K Bra Al | EST | PCR Marker |
| 12O Bra Al | EST | PCR Marker |
| 22H Bra Al | EST | CAPS marker |
| 22M Bra Al | EST | PCR Marker |
| 1H Bra Al | EST | PCR Marker |
| | EST | CAPS marker |
| 3B Bra Al | EST | CAPS marker |
| 21J Bra Al | EST | CAPS marker |
| 15J Bra Al | EST | PCR Marker |
| 10H Bra Al | EST | CAPS marker |
| 12B Bra Al | EST | CAPS marker |
| Contig 13 Bra Al | EST | PCR Marker |
| Cont11 Bra Al | EST | CAPS marker |
| Cont10 Bra Al | EST | PCR Marker |
| Contig 8 Bra Al | EST | CAPS marker |
| Cont4 Bra A | EST | PCR Marker |
| Cont9 Bra Al | EST | PCR Marker |
| Cont3 Bra Al | EST | CAPS marker |
| 1F | EST | CAPS marker |
| 4B | EST | PCR Marker |
| 4C | EST | CAPS marker |
| 4D | EST | CAPS marker |
| 4G | EST | CAPS marker |
| 5B | EST | PCR Marker |
| 5F | EST | PCR Marker |
| 5G | EST | PCR Marker |
| 6B | EST | PCR Marker |
| 6D | EST | CAPS marker |
| 5A | EST | PCR Marker |
| 7C | EST | PCR Marker |

| | | |
|---|---|---|
| 7G | EST | PCR Marker |
| 7H | EST | CAPS marker |
| 8A | EST | CAPS marker |
| 2E | EST | PCR Marker |
| 3D | EST | PCR Marker |
| 3E | EST | PCR Marker |
| 3G | EST | CAPS marker |
| CD0345 | EST | PCR Marker |
| RZ142 | STS | PCR Marker |
| C -86(2) | EST | PCR Marker |
| C-86 | EST | PCR Marker |
| R2 2625 | EST | PCR Marker |
| R2-2460 | EST | CAPS marker |
| C-1419 | STS | PCR Marker |
| CDO-122 | EST | PCR Marker |
| 31P9L12 | EST | CAPS marker |
| A2 Bra Al | EST | CAPS marker |
| A7 Bra Al | EST | PCR Marker |
| A9 Bra Al | EST | PCR Marker |
| B3 Bra Al | EST | CAPS marker |
| B4 Bra Al | EST | CAPS marker |
| cont10 Bra Al | EST | PCR Marker |
| 15J Bra Al | EST | CAPS marker |
| A2 Bra Al | EST | PCR Marker |

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## 1.3.21 QTL analysis in the population of DOR 364 x BAT 477, and comparison with QTL in the MD 23-24 x SEA 5 population

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### Introduction

Breeding for drought resistance has become an important trait within the Mesoamerican bean breeding program. Evidence suggests that some drought resistance traits may contribute to yield potential in other, non-drought environments. This heightens interest in this trait and in finding QTL associated with drought resistance. BAT 477 was one of the first genotypes recognized in CIAT for a degree of drought resistance, and we have made considerable efforts to elucidate its resistance. We report on QTL analysis of Recombinant Inbred Lines (RILs) of BAT 477 x DOR 364 planted over several environments.

### Materials and methods

In a project looking at the nitrogen fixation potential of RILs of the DOR 364 x BAT 477 population, a subset of the larger population was planted under varied environmental conditions in Mexico, Colombia and Bolivia. Thirty RILs were planted in replicated yield plots, with standard agronomic management for the respective zone, as well as in phosphorus-deficient conditions in some trials in Colombia. Some of the environments were subject to occasional drought.

Validation of putative QTL over sites is a requisite to have confidence in their value, and to eventually implement them in MAS. However, it is not practical for most breeding programs to evaluate large sets of RILs over many sites. Rather, the methodology being tested in this trial was whether a small set of RILs over multiple environments could be employed to validate putative QTL identified in a larger set of RILs in a limited number of experimental sites (as few as one site!). QTL analysis was executed using the joint analysis option of QTL Cartographer, which detects QTL based on a correlation matrix among variables (in this case, yield at several sites). Thus, we wished to test whether the statistical power that was lost due to using a small set of RILs could be regained through multiple sites and the joint analysis.

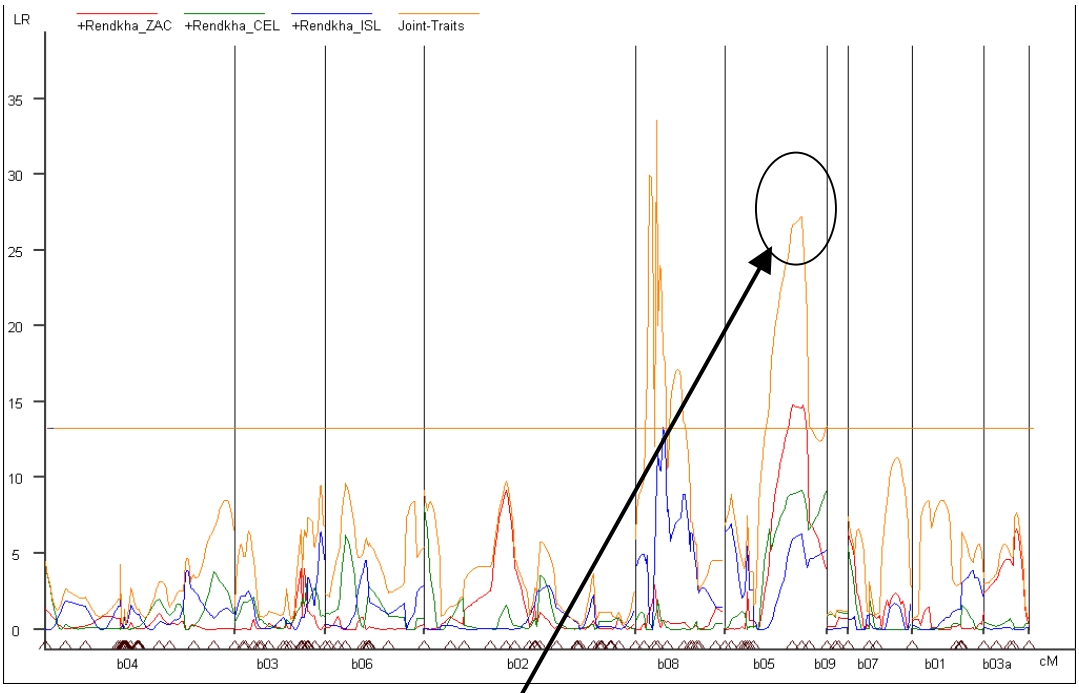
### Results and discussion

One region that emerged as contributing to yield in several sites, as revealed by the joint analysis, was found on linkage group b05. In the Figure 1.a is represented the joint analysis for three environments in Mexico: Zacatecas, Celaya and Islas. Zacatecas is subject to intermittent drought in most growing seasons, Celaya occasionally has drought, and Islas can be subject to terminal drought. The peak for this QTL centered on a microsatellite marker BMd20. Figure 1.b is repeated from last years' report, and illustrates this same peak at BMd20 being expressed in the population of MD 23-24 x SEA 5.

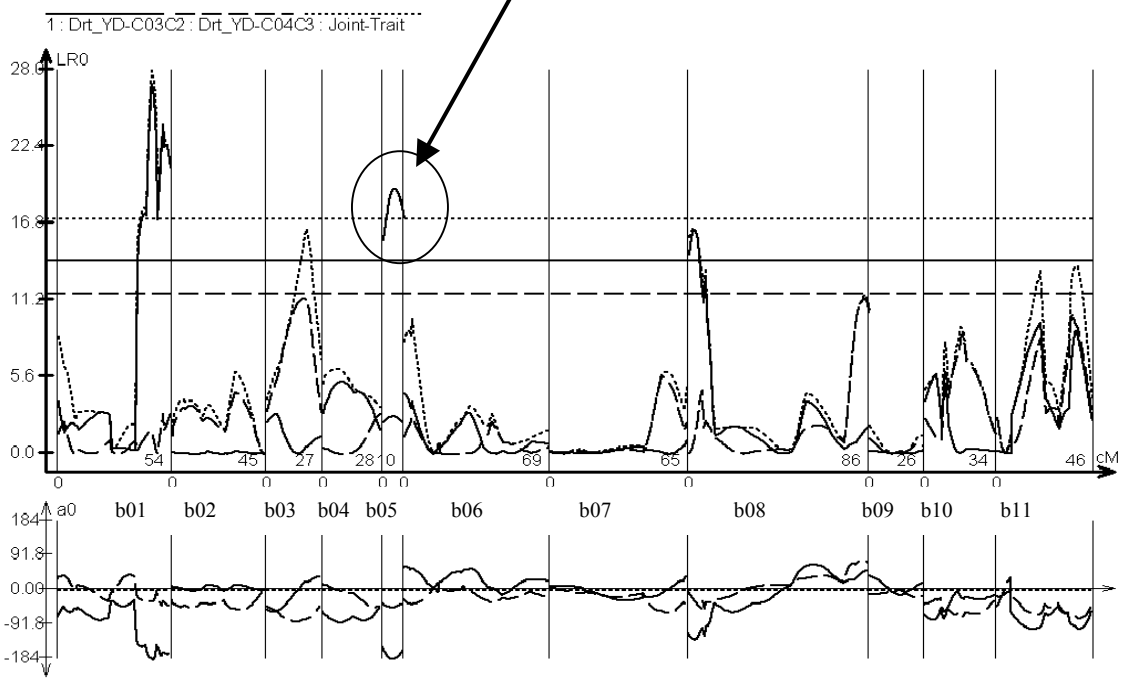
SEA 5 is a progeny of a double cross in which BAT 477 is one of the four parents, and SEA 5 has inherited the BMd20 allele from BAT 477. Thus, a QTL that was first recognized in the population of MD 23-24 x SEA 5 has been validated in a population of DOR 364 x BAT 477.

Figures 1a and 1b: QTL for yield under drought in two populations of RILs. Fig. 1a shows the results of joint analysis for the population BAT 477 x DOR 364. Figure 1b is based on CIM analysis of SEA 5 x MD 23-24.

1.a.




1.b.



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# OUTPUT 2. Genes and Gene Combinations use to Broaden the Genetic Base

# Activity 2.1 Transfer of gene and gene combinations using cellular and molecular techniques

### 2.1.1 Development of tepary x common bean interspecific hybrids with improved competence to *Agrobacterium* mediated transformation

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In the past twelve years different agronomic important transgenes have been introduced to common bean cultivars through particle bombardment (for a review see Svetleva et al 2003) and Agrobacterium tumefaciens (Liu et al. 2005). However, the fact that there is worldwide no transgenic breeding line or marketed common bean cultivar produced through this technology, may indicate that it is not ready for routine application. Low transformation efficiency, failure of transgene expression (Azzam et al. 1996), or transgene elimination (Romano et al. 2005) are among the constraints of the available methodologies for the production of transgenic common bean plants through particle bombardment.

Compared to particle bombardment, Agrobacterium-mediated transformation (Agro-transformation) offers different and unique advantages for transforming plant cells, such as the possibilities to: (i) transfer only one or few copies of DNA fragments carrying the genes of interest (ii) transfer of very large DNA fragments of a size as large as 150kb, (iii) produce transgenic plants with fragments of foreign DNA of the desired size, and (iv) produce more efficiently transgenic plants free of antibiotic marker genes.

Yet, also with Agrobacterium as vector the genetic transformation of common bean has been difficult. In the genus Phaseolus only the tepary bean (P. acutifolius) has been reproducibly Agro-transformed (Dillen et al. 1997; Mejía et al. 2000; De Clerck et al. 2003; Zambre et al. 2005).

Since traits of tepary bean have been introgressed stably into common bean cultivars, and breeding lines through interspecific crosses, the use of tepary bean accessions competent to Agro-transformation as bridge for the production of transgenic common bean cultivars has been proposed (Dillen et al 1997). However, this strategy has not produced common bean transgenic plants so far. The principal constraints of such strategy are two:

1. Most of the tepary and common bean accessions tested so far have been incompatible when crossing directly, producing lethal or self sterile $F_1$ hybrids (Waines et al.1988)

2. Only a relatively small proportion of the genome of tepary bean is transferred to common bean through recurrent or congruity backcrosses (an average of 5.2% in recurrent backcrosses and 8.8% in congruity backcrosses; Muñoz et al. 2004). This presupposes that a transgene should integrate into a tepary bean locus that can be introgressed into common bean.

Instead of using tepary bean accessions as target for the Agro-transformation we propose the development of interspecific common x tepary hybrids that are competent to Agro-transformation and cross-compatible with common bean cultivars, for using them as bridge to efficiently produce transgenic common bean

plants. Once the transgenic hybrid is produced, two or more backcrosses to a desired cultivar of common bean, would be enough for transferring the transgenes.

During 2006 we continued with our effort to develop such hybrid lines. We also made investigations on the optimization of the transformation protocol.

**Methodology**

Hybrid progenies involving the wild tepary bean accession NI576, which have been competent to Agro-transformation, and the common bean cultivar Bayo Madero (among others used as facilitators of the interspecific cross) were generated through double congruity backcrossing (http://gene3.ciat.cgiar.org/blast/docs/DCBCHybrids.pdf).
Whole mature seeds without one cotyledon or cotyledonar nodi were used as explants in the transformation experiments. The cointegrate strain of Agrobacterium LBA4404 pTOK233 (Hiei et al., 1994), and the binary strains C58C1 pTARC B1B (Dillen et al., 1997) and C58C1 pCambia1305.2 (http://www.cambia.org.au) were used for transformation, following the previously described protocol (Mejía-Jiménez et al., Annual Reports 2000 and 2001).

**Results**

Attempts to transfer a transgene from an intraspecific tepary bean hybrid to common bean through sexual crosses

A transgenic plant obtained after Agro-transformation of intraspecific tepary bean hybrids (G40022 x NI576, event 23A; Mejía et al. 2000), which shows a strong expression of the GUS transgene, was used to test the possibility of transferring a transgene from tepary to common bean through sexual crosses.
The transgene could be easily transferred to other accessions of tepary bean or interspecific tepary x common hybrids with the cytoplasm of tepary bean. However all the attempts to transfer the same transgene to common bean cultivars or interspecific hybrids with the cytoplasm of common bean, failed. No fertile or cross-fertile hybrids carrying the transgene could be produced. Even after hundreds of backcrosses to interspecific A-DCBC or V-DCBC hybrids aimed to break possible linkages between the transgene and interspecific incompatibility alleles, the production of cross fertile hybrids with the cytoplasm of common bean was not possible.

Thus this transgene might be linked to an interspecific incompatibility allele that prevents the production of fertile hybrids with common bean.

This example illustrates the difficulties that may arise by trying to introgress transgenes introduced into tepary bean accessions to common bean, as has been proposed (Dillen et al. 1997).

Only transgenes which integrate into chromosomal loci of tepary bean that are readily introgressed to common bean, will be efficiently introgressed to common bean through sexual crosses.
This supports our strategy of using interspecific common x tepary hybrids that are competent to Agro-transformation, instead of using tepary bean accessions themselves as bridge for the introgression of transgenes into common bean. The chance of insertion of a transgene in a locus that can be readily introgressed to common bean will increase proportionally to the amount of chromosomal segments of common bean present in the hybrid.

**Optimization of the transformation protocol**

In the last two years none of the Agro-transformation protocols developed for tepary bean could be successfully applied to common bean cultivars or common x tepary bean hybrids, for the production of transgenic plants. It is possible that the two plant species, which have quite different growth habitats in the wild, require also different growing conditions when cultured in vitro and that the conditions developed for the transformation of tepary bean are not the optimal for common bean.
The most critical step in the genetic transformation of grain legumes through the cotyledonar node approach seems to be wounding and inoculation of the pre-existing meristems in the explants (embryo axes or cotyledonar nodes). In soybean, the most investigated grain legume regarding genetic transformation, the transformation efficiencies could be increased through the use of thiol compounds during inoculation. It is believed that this occurs through the inhibition of enzymes involved in plant pathogen and wound response (Olhoft et al. 2003).

In the second half of 2005 we started a reevaluation of the different important parameters during inoculation and coculture with Agrobacterium. We tested the combined use of thiol compounds, reduced mineral salt concentrations, increased concentration of osmotic active substances, among other parameters during co-culture (Table 2). For measuring the gene transfer, the standard transient expression assay of the GUS-Intron transgene was used, three days after inoculation.

Compared with the methodologies used before, a clear increment of the transient GUS-expression is being achieved with the modified inoculation methodology (Table 2). Levels of 100% transient expression are routinely being obtained with selected interspecific common x tepary hybrid lines (Fig.1). It is still necessary to determine which one of the modifications made to the protocol is responsible for the increase of the transient GUS expression. It is expected that this high transient expression of the GUS-Intron gene results in increased transgenic plant recovery.
These experiments have not concluded and will be continued during 2007.

Production of fertile interspecific common x tepary bean hybrid lines using parental accessions which show competence to Agro-transformation, or good response to methods for in vitro culture and plant regeneration

Different accessions of tepary bean such as the wild genotype NI576 (Dillen et al. 1997; Clercq et al. 2002), the cultivated ones TB-1 and PI440795 (Zambre et al. 2005) and the intraspecific hybrid G40022 x NI576 (Mejia et al. 2000) have been Agro-transformed, while with similar or slightly modified methodologies, the Agro-transformation of wild and cultivated accessions of common bean has not been possible

In other crops such as rice and maize it has been demonstrated that the response to in vitro tissue culture methodologies for plant regeneration or genetic transformation is influenced by genetic traits. It would be therefore possible to improve the response of low responsive accessions of common bean to Agro-transformation through classical breeding.
In order to breed common bean genotypes which are responsive to the Agro-transformation, in the year 2000 we started interspecific crosses in which we included the tepary bean genotype NI576, as a putative source of competence genes for Agro-transformation. We also included tepary bean genotypes, G40022 and G40065, which were selected as the best in forming regenerable, meristematic callus (a trait that can be useful during selection of transformed tissues), and the best-identified genotype of common bean Bayo Madero, which also forms similar callus type. The development of fertile interspecific hybrids involving NI576 and Bayo Madero was possible only through a complex series of backcrosses we called Double Congruity Backcrosses (DCBC; see http://gene3.ciat.cgiar.org/blast/docs/DCBCHybrids.pdf). These

crosses have yielded in the past years hundreds of fertile interspecific hybrid lines with the cytoplasms of common or tepary bean.

For accumulating alleles that may play an important role in competence to Agro-transformation, hybrid-lines, which show a good response to Agro-transformation methodologies, are selected and used again as parentals in the next DCBC.

During 2005, 3 generations of interspecific and 10 of intraspecific DCBC with the common bean cytoplasm, and 3 generations of interspecific and 9 of intraspecific DCBC hybrids with the tepary bean cytoplasm succeeded and more than 500 fertile hybrid lines were produced (See annual report on interspecific crosses). The number of crosses performed and the embryos rescued have not been yet evaluated to calculate and compare hybridization efficiencies among the different DCBC generations.
It is expected that the level of recombination between the genomes of common and tepary beans increases with every new full cycle of DCBCs.

The fertile populations produced during 2006 are being multiplied and will be screened during 2007 with the improved inoculation methodology.

**Conclusions**

Current Agro-transformation methodologies developed for tepary bean have not been effective for the production of transgenic common bean cultivars.
The use of interspecific common x tepary interspecific hybrids competent to Agro-transformation, and cross compatible with common bean cultivars, in combination with improved transformation methodologies may represent an alternative for the production of transgenic common bean cultivars.

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## 2.1.2 Implementation of the cryopreservation technique's using a cassava core collection as a model

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### Introduction

After adjust a basic cryopreservation encapsulation-dehydration technique with a few cassava clones, a core collection (640 clones) has been included in the experiments to test the clone's effect. Partially data has shown 3 groups of response after freezing phase: Lowest response (less than 30% plant recovery), Intermediated (more than 30 and less than 70 % plant recovery) and Highest responding group (more than 70% plant recovery).
Logistical considerations have been considered to make a duplicate of Core collection under frozen conditions.

### Material and methods

The Encapsulation-dehydration technique has been implemented (Annual Report 2000) using *in-vitro* cassava plants supply by GRU. Last year we start to make a duplicate of the Core under liquid nitrogen conditions (called Cryo-CoreII). *In-vitro* cassava material used for makes a second copy coming from clones maintained under BRU lab conditions.

### Results

During 2005-2006 periods we start a Cryo-Copy II (Annual Report 2005).
To make these activities it was necessary make subculture of plants coming from clones maintained at BRU on 4E medium (Roca 1984). Due for growth conditions (temperature of the growth-room and culture media), those materials needs make subculture each 4-8 months. Basically those materials suffer a rapid deterioration at BRU laboratory (fast growth rate and quit aging of the material).

During this year we include only 5 new clones to the Cryo-Core I. Nowadays its collection just wait for 4 clones to be ended the inclusion on the entire Core collection.

More than 99% of the Core's clones are maintained on liquid nitrogen conditions. This collection could be considered unique in the world. Our results show that more than 60% of the clone tested respond up-to 30% as plant recovery (intermediated and highest group) (Table 1)

For the Cryo-Core II we include 182 new clones during 2005-2006 period (Table 1). It represents two-times the number of clones included during the last year (Annual Report 2005). Nevertheless, data obtained by grouping responds by clones are not consistently with the first copy (Table 2). Based on our experiences the management of aged tissue has strong influences on response after freezing. Additionally, in some cases when it initiated a new subculture it appears bacterial contaminations, making difficult the tissue inclusion in liquid nitrogen tank.

**Table 1**: Establishment of Core collection under liquid nitrogen conditions.

| | Cryo-Core I | | Cryo-Core II | |
|---|---|---|---|---|
| | No. Clones | % of the core | No. Clones | % of the core |
| Frozen | 626 | 99.4 | 266 | 41.5 |
| Evaluated | 623 | 98.8 | 242* | 38.4 |

* Clones without evaluation 18. It's on running experiments

For the Cryo-Core II we include 182 new clones during 2005-2006 period. It represents two-times the number of clones included during the last year. Nevertheless, data obtained by grouping responds by clones are not consistently with the first copy. Based on our experiences the management of aged tissue has strong influences on response after freezing. Additionally, in some cases when it initiated a new subculture it appears bacterial contaminations, making difficult the tissue inclusion in liquid nitrogen tank.

Some clones from Intermediated group change its behavior to lowest group. The highest group is more consistently in its responses.

Table 2**: Group of response of copies of the Core collection maintained under liquid nitrogen conditions.**

| | | % Clones based on grouping responses | |
|---|---|---|---|
| | | Cryo-Core I | Cryo-Core II |
| Group of response* | Lowest | 33.6% | 59.6 |
| | Intermediate | 40.5 | 17 |
| | Highest | 25.8 | 23.4 |

CIAT has been using Humboldt's Cryopreservation-tanks to maintain its copies on L.N. Inputs to the collections, from both institutions, are very active. For space's reason, it must be necessary stop our inclusion to their tanks and consider other options for Cryo-Core II.

CIAT made a safety duplication of its in-vitro's collection to CIP-Peru. In the same way, it will consider that, when cryopreservation technique is fine-tuning, CIP (at Peru) or INIBAP (at Belgium) could be act as depositary of the copy of the Cryo collection in a black box option. Transfer of these collection need to be consider as soon as possible to adjust the process, before initiation of the duplication (a certified currier to Peru spend 3 days and to Belgium 5 days).

**Conclusions**

CIAT has an entire copy of the Cassava Core-collection under liquid nitrogen. More than 98% of the clones have been tested after freezing step.
Actually a duplicate of this collection have been build (41.5% of the core)
Tissue aging affect the response after freezing. It is necessary maintain a younger tissue to ensure the best grouping responses after freezing.
It is necessary makes a consensus to design a logic scheme to make a duplicate of the collection. It must include manpower requirement and equipment and input buying

**Future activities**

Determine a procedure for make a duplicated collection under L.N.
Recover plants from different period of conservation and transfer to the field conditions to observe its behavior
Continue with a critical point analysis to monitoring the cryopreservation activities.
Supplement media with AgNO3 to maintain the *in-vitro* material without excessive deteriorations.
Make a consensus work-plan between Technician and Manager that include all items (inputs, manpower, equipment, spaces among others) that allow us cost estimation and duration of inclusion of the entire collection.

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## 2.1.3 Development of an *In vitro* Protocol for the Production of Cassava Doubled-Haploids and its Use in Breeding

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**Abstract**

As part of the Global Cassava Strategy, a meeting funded by the Rockefeller Foundation was held at Bellagio (Italy) on October 2002, involving relevant stakeholders for cassava. In that meeting two important conclusions were drawn in: 1) In every continent there was an increased interest in cassava as a vehicle for rural development through the development of processing facilities; and 2) The introduction of inbreeding in cassava genetic improvement in general and the capacity to produce doubled-haploids in particular, were identified as priorities. Following it is described the progress attained in the development of haploid technology in cassava during 2006.

Key words: **doubled haploids, cassava, microspore culture, androgenesis, microsporogenesis**

**Introduction**

Doubled haploid (DH) plant is of importance for cassava breeding, besides of its potential application to biotechnology and genomics research, especially considering its inbreeding depression and highly heterozygous nature which has impeded the efficiency of traditional breeding strategy. Androgenesis, the process by which microspores develop to form embryiods and subsequently regenerate to DH plants, has been applied in plant breeding since 1964 (Guha and Maheshwari). However, the success of *in vitro* production of DH lines by androgenesis is subject to the constraints from many factors, including the

physiological status of donor plants, special developmental stage of microspore, proper pretreatment on the microspores (stress), and microspore culture condition (the medium species and its composition, the cell density, the temperature regime, the application of plant growth regulators, and the conditioned media). Successfully induced microspores (embryogenic microspores) will experience a rapid growth (2-3 weeks after pretreatment) and can be recognized by their increased size in the cases of barley (Wang *et al*., 2000), rapeseed (Custers *et al*., 1994) and wheat (Liu *et al*., 2002) while the nonembryogenic microspores have a smaller size. The right developmental stage of microspore suit for pretreatment mainly, if not all, ranges from late uni-nucleate to early bi-nucleate microspores in barley, rapeseed, rice, wheat, maize and rye. Another parameter for successful induction of embryogenic microspores is the cell density using for pretreatment and/or culture, commonly with the range from $10^4$-$10^5$ cells/ml medium (Kernan and Ferrie, 2006).Based on the work in 2005, we are able to enrich homogenous microspores (of similar developmental stage) in relatively high concentration. The activities of this year mainly fall into the following aspects: (a) further improvement of tetrads enrichment with short protocol to increase the viability; (b) histological study on cassava *in vivo* microsporogenesis to generate the understanding of reproduction in cassava, which established the guidelines regarding the experiment design to work with right microspores at specific developmental stage; (c) cell culture: different population of cells at certain developmental stage, from early freed spores (EFS) to late-uninucleate or even putative early-binucleate microspores, were cultured on different medium with/without different plant growth regulator combinations after serious of pretreatments and under different conditions, and (d) anther culture as an alternative for DH plant development.

**Materials and methods**

*Plant Material.* Donor plants were planted in the fields of either CIAT campus headquarters or ICA experimental station at Palmira under natural conditions. Clones TAI-8, SM1219-9, TMS60444, HMC-1 and MCol1505 were stagger planted at 2-months interval in order to provide inflorescences year round. Inflorescences were harvested from field and immediately stored in a polystyrene box with refrigerant gel (Glacier Ice Co., USA). Only those plants with healthy growth and profuse flowering and of similar morphology and developmental stage were used. If available, depending on the genotype, 1-3 young leaves were kept attached to the excised inflorescences to prevent deterioration of microspores.

*Histological study on in vivo microsporogenesis.* Identification of the microspore developmental stage is crucial for culturing the microspore in optimal responsive stage for inducing cell division *in vitro*. However the thick and auto-fluorescent exine wall of cassava microspore hinder the use of fluorescence staining for developmental stage examination and subsequent cell division monitoring. Thus, a histological study was conducted on the processes of microsporogenesis and the sporopollenin deposition in the exine using both light and electron microscopy. Buds, anthers and microspore sizes from inflorescences of similar stage of development were measured using a microscopic scale. Anthers were fixed in 2.5% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.2) for 24 h at 4ºC and then dehydrated in a series of ethanol (25%, 50% and 75%). Specimens were immersed in 2% uranyl acetate (in 75% ethanol) for 12 h at room temperature followed by a series of ethanol (90% plus 100% three times), pure acetone rinses for 20 min each and then gradually infiltrated and embedded in Spurr epoxy resin. Embedded anthers were sectioned in slides of 800 to 1000 nm thick using an ultra-microtome with diamond knives. For light microscopy analysis, the slides were stained with fresh Toluidine Blue O (2%) and sodium borate (2%) prepared at the time of being used and examined/imaged under an Olympus Microscope. Standard procedures were used for electron microscopy analysis.

*Microspore isolation.* Clone SM1219-9 was chosen as a model for experiment design because of its high yielding of homogenous microspore suspension. Floral buds were precisely measured and grouped into categories differing in 1mm in diameter: [2.2,2.3) mm, [2.3,2.4)mm, [2.4,2.5)mm, [2.5,2.6)mm, [2.6,2.7)mm, and [2.7,2.9] mm in. Bud selection was conducted on ice. Selected buds in groups of 300-

500 buds were placed in a baby food jar and surface sterilized using 5.25% NaClO solution with 2 drops of Tween 20. The NaClO solution was descanted and the buds were rinsed with sterile water three times of 5 min each. After washing, the buds were transferred into a blender cup containing 40 ml of cold 0.4 M mannitol solution and blended in a blender (Warings, USA) for 20s at low speed. The slurry was filtered through a series of nylon meshes (150, 105/102, 88 and 41 μm pore size in turn). The cells blocked on the filters of 104/102, 88/74 and 41μm were recovered separately by mannitol washing and pelleted by centrifugation at 100g for 1min. The composition of the pellets was determined under light microscope. The pellets were purified by percoll gradient centrifugation (70, 80 and 90% percoll gradient; or only 75% percoll depending on the different purposes) at 150g for 3min. Resultant cell suspension was washed with medium 3 times. The cell concentration was determined using a haemocytometer (Nebauer). The suspension with desirable cell concentration was cultured in 30×15mm Petri dishes at 26 or 28ºC in the dark. All the media were refreshed every 2 or 4 weeks depending on different experiments and cultured for at least two months. Each experiment was repeated between 3 to 10 times.

*Microspore culture*. Various culture media were tested including basal media MS (Murashige and Skoog, 1962), NLN (Lichter, 1982), NN (Nithch and Nitch, 1969), B5 (Gamborg *et al*., 1968), GD (Gresshoff and Doy, 1972), SH (Schenk and Hildebrandt, 1972) and NPB (Liu *et al*., 2002), with or without plant growth regulator (PGR), for microspores culture in various experiments first. Three temperature regimes 4ºC, 10ºC and 30/37ºC were chosen for pretreatments on the whole inflorescences, excised buds or isolated microspores for 3d, 6d, 9d and 12d. High osmolarity and sugar starvation treatments were also tested: isolated microspores were placed in 0.4M mannitol solution at 4ºC or 28ºC for 3d and 6d. Chemical inducer, 2-HNA, was used in two ways: (a) 2-HNA with concentrations of 0, 0.7, 1.4 and 3.6 mM contained in $10^{-6}$ M 2, 4-D and $10^{-6}$ M BAP solutions was used to culture the whole fluorescence at 32ºC for 24 and 72h. Only the basal side of the inflorescences axes was immersed into the solution in a beaker and the beaker was sealed with parafilm (Sigma) to avoid evaporation, and (b) isolated microspores were cultured in media NPB or NLN containing 0.1, 0.2 and 0.3 mM of 2-HNA at 26ºC and 28ºC in the dark. $AgNO_3$ has been wildly used in somatic embryogenesis and androgenesis (Lentini *et al*., 1995) by interfering with the ethylene biosynthesis. The effects of $AgNO_3$ have been tested on enlarged microspore induction. NLN medium containing 0, 2, 5, 10 and 15 μM of $AgNO_3$ was used to culture isolated microspores at 28ºC in the dark. Seven PGRs (BAP, Kinetin, 2,4-D, PAA, picloram, NAA and ABA), wildly used in androgenesis induction and maintenance or cassava tissue culture were tested. For most species, the optimal microspore culture density is about $10^4$ to $10^5$ cells /ml. However because of their large size, cassava microspores overlapped one onto another in suspensions at concentrations of $10^5$ cells/ml. Thus lower densities ranging from $10^2$ to $10^4$ cells/ml were tested.

*Ovary dissection and culture.* Doubled haploids may be induced either from microspores or ovules cultures *in vitro*. Ovary cultures were tested independently or in co-culture with microspores. Different sizes of female flowers prior to opening for pollination were excised from donor plants of HMC-1, SM1219-9 and TMS60444 and placed in a cold box immediately as described above for male flower collection. After washing with running water, these female flowers were disinfected in 5.25% NaClO solutions with 2 drops of Tween 20 for 20 min, followed by sterile water washing for 3 times. The ovaries were excised at about 0.5 mm above the flower base where calyces are attached to, and surface sterilized. The stigma was removed sometimes to test the mechanisms underlying the induction effects on cell division by ovary. Meanwhile, the viability of ovary was tested during storage at 4ºC before dissection.

**Results and discussion**

The microspores at the late uninucleate to early binucleate stage in cassava are characterized by i) an oppressed significantly reduced cytoplasm, harbouring the nucleus/nuclei with one or two nucleoli, ii) one or several large vacuoles, accounting for more than 95% of the cell in volume, and iii) well developed exine wall with strong auto-fluorescence (Figure 1). In HMC-1 and TAI-8 cassava clones, this stage of

microspore is contained in flower buds of 2.5-2.6mm in diameter collected from field grown plants during rainy season.

Different treatments were tested to induce microspore division *in vitro*, the first step for standardization and development of a reproducible haploid technology. Some microspore increased its volume in about 4 fold (enlarged microspore, EM) respect to those "un-induced" microspores. EM it is usually associated with the acquisition of embryogenic potential in model systems. Optimal treatments reproducibly induced 20% of EM in culture. In some cases, multi-cellular structures (MCS), embryo-like structures (ELS) and micro-calli were observed indicating more advanced structures than EM (Figure 2). Among these findings, EM has been considered as the marker for acquisition of embryogenic potential, MCS as a marker for initiation of cell division, and ELS involves in the pattern formation according to the time line during androgenic development in the model species barley (Maraschin et al., 2005). As EM was highly reproducible, extensive factors were tested for EM induction in culture. EM stage, however, is still recalcitrant to DAPI and FDA staining due to its strong auto-fluorescence (Figure 2B). Thus DAPI staining cannot be used to monitor cell division inside the cassava microspore at this stage, as it has been used successfully for other model species to document the androgenic process initiation and progression of subsequent nucleate division.

Of the media tested, preliminary results indicate that NLN is the optimal medium for EM induction, followed by NN and B5. Another four media used in cassava tissue culture successfully were also compared with NLN. The somatic cell derived regeneration system, developed in model clone TMS60444, uses MS based medium, GD and SH media plus 50 mg/l picloram (Schopke et al., 1996). The biggest difference between NLN and other media is that NLN contains high concentration of organic nitrogen (glutamine and glutathion) rather than inorganic nitrogen ($NO_3^-$ and $NH_4^+$) and has only 1/8 to 1/5 amount of potassium of other media. This is consistent with the findings that high concentration of organic nitrogen favors the androgenesis in barley microspore culture using FHG medium (Kasha et al., 2001).

In all the cases, the addition of ovaries and glutathion in the culture medium significantly induced more EM. Similarly a combination of NAA, 2,4-D and kinetin in 2.0 mg/l significantly induced EM formation, suggesting that the cassava exine is permeable in spite if its thickness of about 10 microns. However, NAA and 2,4-D independently showed that could inhibit the EM induction at certain concentrations (data not shown). Of the other growth regulators tested, picloram, PAA, and ABA also induced more EM. In contrast BAP and 2-HNA showed an inhibitory effect, and AgnO3 did not have any effect. Picloram has also been found to be a better cassava-somatic cell division inducer than 2,4-D (Dr. Zhang Peng, ETH, Switzerland, personal communication). PAA is a widely used PGRs species in the medium for androgenesis in barley (Kasha et al., 2001), wheat (Hu et al., 1999) and maize (Zheng et al., 2002). As all the androgenic processes have been triggered by stress, an ABA signal system has been postulated for its role in androgenesis induction via (a) preventing the death of microspores and thus make more microspore survive from the pretreatment stress and (b) repressing the development of microspore into mature pollen (Wang et al., 2000), based on the results of the relationship between the endogenous ABA peak and stress application (Imamur and Harada, 1980; van Bergen et al., 1999) or exogenous application shows positive effects in androgenesis (Hu et al., 1995; Kyo and Harada, 1985), especially ABA enhanced the microspore viability (van Bergen et al., 1999; Wang et al., 1999).

Starvation and osmotic stress effects on EM induction were investigated by culture the isolated microspores at 4°C for 3, 6, 9 and 12d, respectively. The starvation and osmotic stress may be not suitable for cassava androgenesis on isolated microspores in spite of the androgenesis has been triggered by starvation the pollen for 3d in 0.4 M mannitol to exhaust the starch in the cell prior to culture in carbon source-containing medium in tobacco (Kyo and Harada, 1985). The results may be improved by culturing the buds, instead of isolated microspores, under starvation and osmotic stress condition as evidence in

barley by Hoekstra et al. (1992). The optimal temperature pretreatment using excised buds and isolated microspores are to incubate the material at 10°C for no more than 3d prior microspore isolation and culture. High temperature (30°C) can inhibit the RM induction. Accumulated evidence suggests that the heat shock may be not enough to trigger the androgenesis in cassava.

Preliminary results also suggest that rain precipitation may significantly affect plant donor conditions in the field and therefore, microspore response *in vitro.* Caution should be taken when collecting material from different seasons and field plots bearing rain precipitation differences. Similarly, more reproducible results and increased EM induction is attained when cassava plants are maintained with regular watering.

**Conclusions**

Our results show that it is now possible to induce EM in a highly reproducible manner (Figure 2). Multi-cellular structures (MCS), embryo-like structures (ELS) and micro-calli were observed at lower frequency perhaps because of thick and strong cassava microspore exine wall which may hinder further development inside the microspores. In the cases of androgenesis in coffee (Neuenschwander and Baumann, 1995) and lupin (Bayliss et al., 2004), the muticellular microspore were limited to expansion and failed to release globular embryos by the strong exine wall. The occasional appearances of MCS, ELS and even callus formation in our experiments seem to be resulted by unexpected mechanical damage to the exine wall during bending step of isolation protocol. A ruptured microspore that was releasing the protoplast-like cell has been witnessed (Figure 2F-G) and this kind of protoplast has appeared independently in the culture several times (Figure 2H) with a membrane surface of dark yellow color. These observations suggested that the involvement of mechanical forces during the microspore isolation may aid the development of more advanced structures, MCS, ELS and callus. Because the major component of the exine, sporopollenin, is extremely resistant to chemical degradation procedures (Dominguez *et al.,* 1999), the attempt to digest the exine wall of cassava microspores may not be an alternative (Wang et al., Annual Report 2005). Alternatively, the application of physical treatments to weaken the exine or increase its permeability may be more suitable in this case. Currently, different treatments are under investigations.

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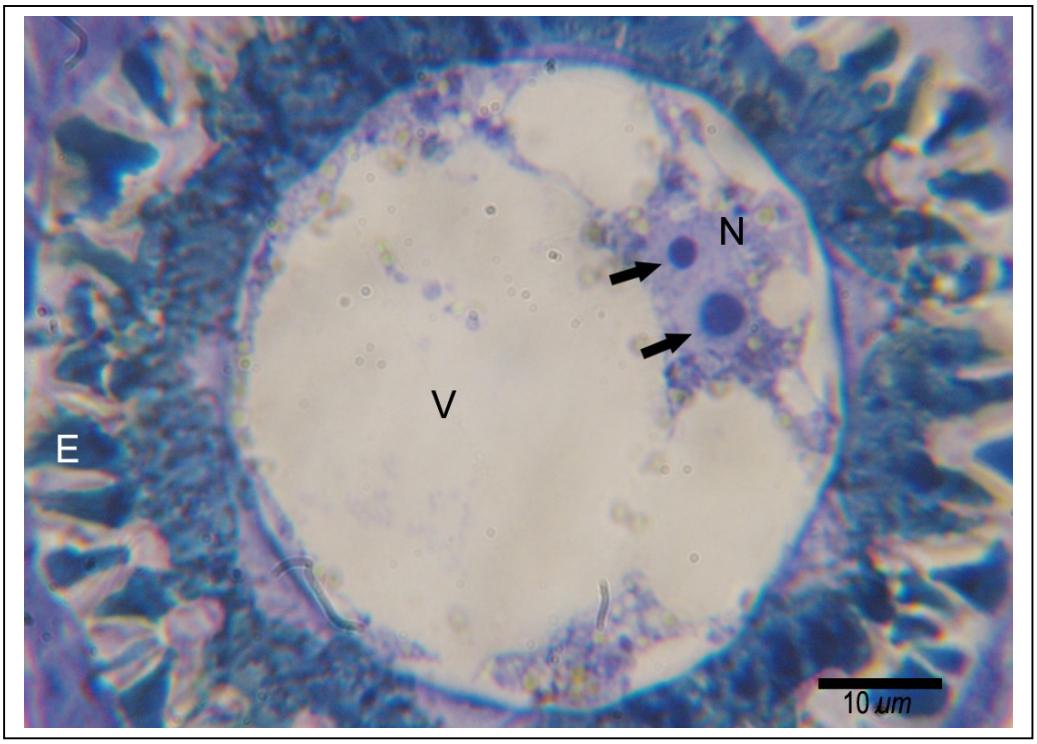


**Fig.1.-** Detail of a microspore at the late uninucleate to early binucleate stage, showing the vacuole (V), nucleus (N), nucleolus (arrow) and exine (E).

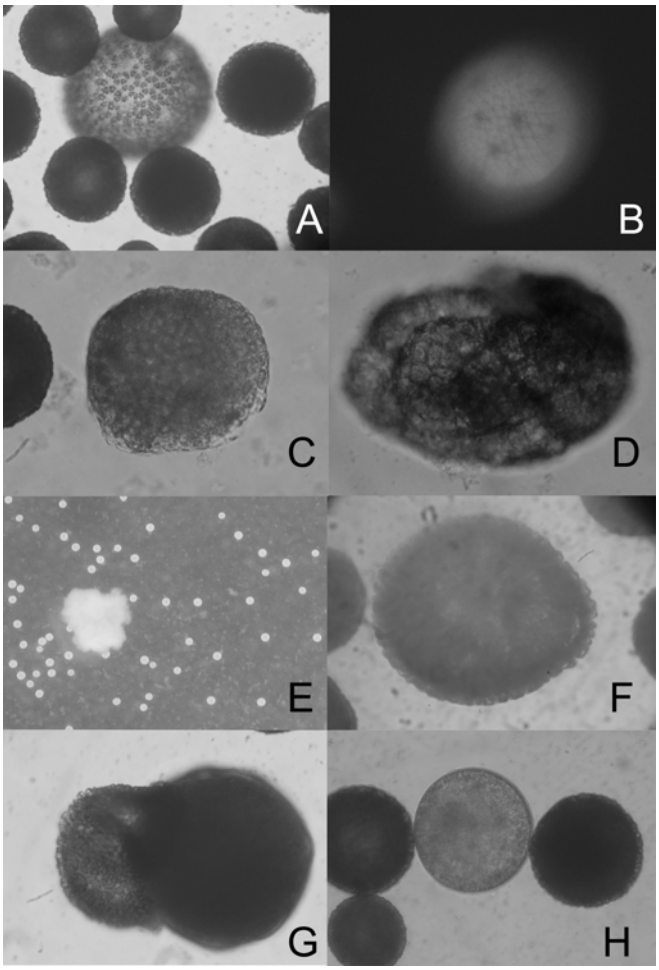


Figure 1. Enlarged microspore (EM), multicellular structure (MCS), Embryoid-like structure (ELS), microcallus and cell rupture to form protoplast derived in microspore culture. (A) an EM with bigger size and different cell surface comparing to its counterparts; (B) an autofluorescence image of the EM which will interfere the DAPI application; (C) Putative MCS; (D) ELS ; (E) microcallus; (F) a microspore with its protruding exine wall, maybe leads to cell rupture as shown in G; (G) a microspore was rupturing to release a protoplast. Note that the membrane of the protoplast and the ornamentation surface of the ruptured microspore; (H) a protoplast formed in the microspore culture.

## 2.1.4 Modification of flowering in cassava by genetic transformation

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Funding: The Rockefeller Foundation

Important Outputs

Forty six and 42 independent transgenic lines of the model transformation cassava genotype 60444 transformed with the constructs Apetala 1 (AP1) and Constans (CO) in an ethanol-inducible promoter vector were transferred to the green house for biochemical and molecular evaluations
A second set of genetic transformation experiments were conducted with flowering locus T (FT) and Suppressor of Constans (SOC1) constructs in the ethanol-inducible vector using the cassava varieties 60444 and CM 3306-4.

**Introduction**

The amount and timing of flowering in cassava is being manipulated through controlled expression of Apetala 1 (AP1), Constans (CO), flowering locus T (FT) and Suppressor of Constans (SOC1) under the control of an ethanol-inducible promoter, of flowering time genes. The AP1 and CO genes represent 2 different mechanisms of flower induction in plants, one that induces flowering from a leaf-derived signal (CONSTANS) and one that generates flowering from the meristem (APETALA). FT and SOC1 are among the most potent activators of flowering, they cause extreme flowering when over expressed (Kardailsky et al.1999). FT and SOC1 are also at the point of convergence of several flowering-time pathways and are often therefore described as floral integrators. (Searle and Coupland 2004). The immediate benefits of this work include making accessible to conventional breeding the many excellent cassava genotypes that are recalcitrant to flowering and easing the difficulties of synchronizing flowering between cassava genotypes which currently flower at different times in the breeding cycle.

**Methodology**

Gateway cloning technique was used to clone APT, CO, FT, and SOC1 from Arabidopsis thaliana into the pNEW vector having the ethanol- inducible promoter AlcR. The chemical inducible expression system used was that of ethanol. The switch is formed by two components: (i) a transcriptional factor AlcR that upon ethanol induction binds to and (ii) activation of the transcription of a target promoter AlcA (Odell et al, 1985). The constitutive expression of the flowering genes CO (constans), API (apetala1), is driven by the cauliflower mosaic virus 35S promoter (CaMV35S) upstream of the alcR transcription factor. This activates the transcription of the target promoter alcA controlling the expression of the flowering gene with the orientation in the antisense direction (Fig 1).

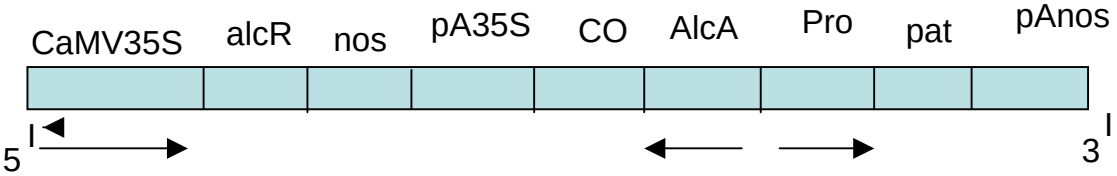


Fig 1: Schematic representation of the orientation of the construct. nos, pA35S and pAnos are transcriptional terminators and pat is the selectable marker coding for phosphinothricin, herbicide resistance.

Friable Embryogenic Callus (FEC) used for this transformation was obtained from the cassava variety TMS 60444 or CM 3306-4. Detailed methodology of genetic transformation has been described elsewhere (Li et al. 1996; CIAT 2005). Regenerated transgenic lines were moved to green house and hardened in preparation for evaluation of herbicide resistance and molecular analysis. Green house transgenic plants 6-8 weeks of age were sprayed with varying concentrations of phosphinithricin (PPT) from 1mg/L, 10mg/L,50mg/L to 100mg/Lidentify stable transformants.

Molecular analysis of the transgenic event was using PCR primers designed from the 3'and 5'ends of the APT, CO, FT, or SOC1 genes respectively. PCR conditions have also been described earlier (CIAT 2005). *A. thaliana* transformed with APT FT, CO, or SOCI constructs were also evaluated for flowering using flowering time induction kinetics. Briefly, T2 non segregating transgenic seeds were germinated for 3 days in agar water and transferred to the green house under short day conditions 8hrs light/16hrs darkness. They were then sprayed with basta thrice at 3 days interval to select the primary transgenics. Selected lines were transplanted and after 3days induced by exogenous chemical inducers: 1% ethanol vapour, 1% ethanol spray, and 1% acetaldehyde. The induction was performed twice a day for 3 days with a gap of 3hours between the first and second induction.

Leaves and apical meristem samples were harvested on the 3rd day after the second induction for RNA extraction and subsequent analysis. RNA was extracted using the RNeasy mini prep extraction protocol, RNA was quantified using eppendorf biophotometer 6131 and RT reactions were conducted using 1µg of RNA samples. Then RT-PCR was conducted using ubiquitin as standard. Analysis was with 6 independent events per construct.

**Results**

A total of 46 independent transformation events were obtained for the CO construct and 23 events for the API construct and transferred to the green house (Figure 2). Herbicide resistance evaluation of the transgenic events revealed little to moderate damage compared to the control (Fig 3 and 4) in the transgenic lines. Only the 100mg/L concentration showed physical damage to the leaf which was often not consistent when sprayed at different times suggesting a variation in strength of different lots of the herbicide.

Fig 2  Independent transgenic events with the CO and API constructs in the green house.

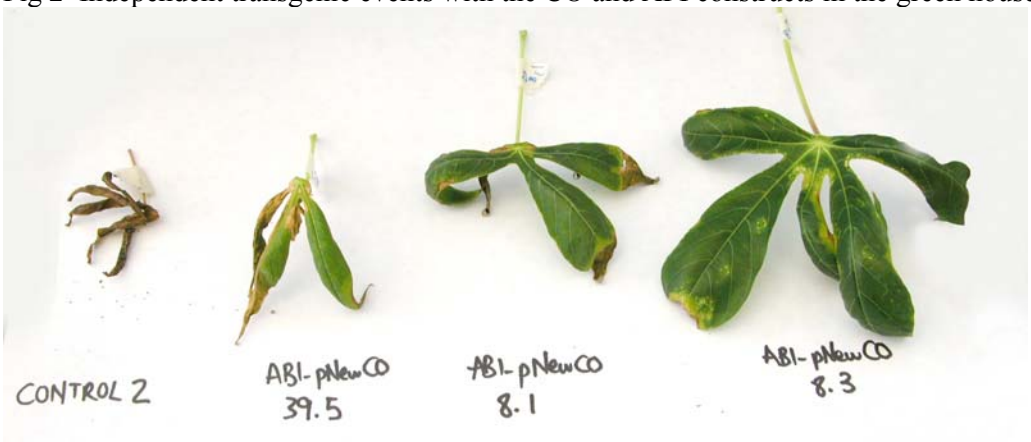


Fig 3. Response in leaves of independent cassava transgenic events transformed with the CO construct and a 60444 control plant to100mg/L of PPT.

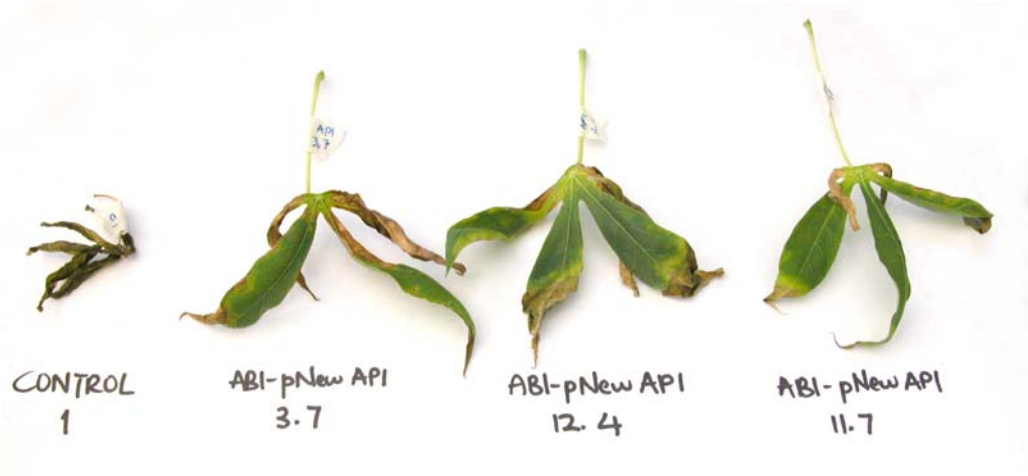


Fig 4. Response in leaves of independent cassava transgenic events transformed with the CO or API constructs and a 60444 control plant to100mg/L of PPT.

Molecular characterization using PCR primers designed from the 3'and 5'ends of the respective genes revealed consistent amplification in 6 CO lines (Fig 5) and none of API lines. PCR amplification with the CO gene primers yielded an expected amplification product size of 441bp fragment in the transgenic plants respectively (Fig 5). The Real time PCR showed slight amplification for lines 6 and 11 carrying the CONSTANS genes.

Molecular tests were conducted for the green house putative transgenic lines.
Southern blot hybridization was used to compare that observed from the Real time PCR using pat PCR product as probe. Results confirmed no transgenics were present because of the signal that was observed from the plasmid positive control which wasn't present in the other samples.

Counting on the transformation expertise of CIAT, it was advised that 1mg/l of ppt is a lethal dosage of selection for transgenics but it seems not to have worked. Different untransformed cassava tissues with increasing concentration of ppt were tested find out which level kills them best.

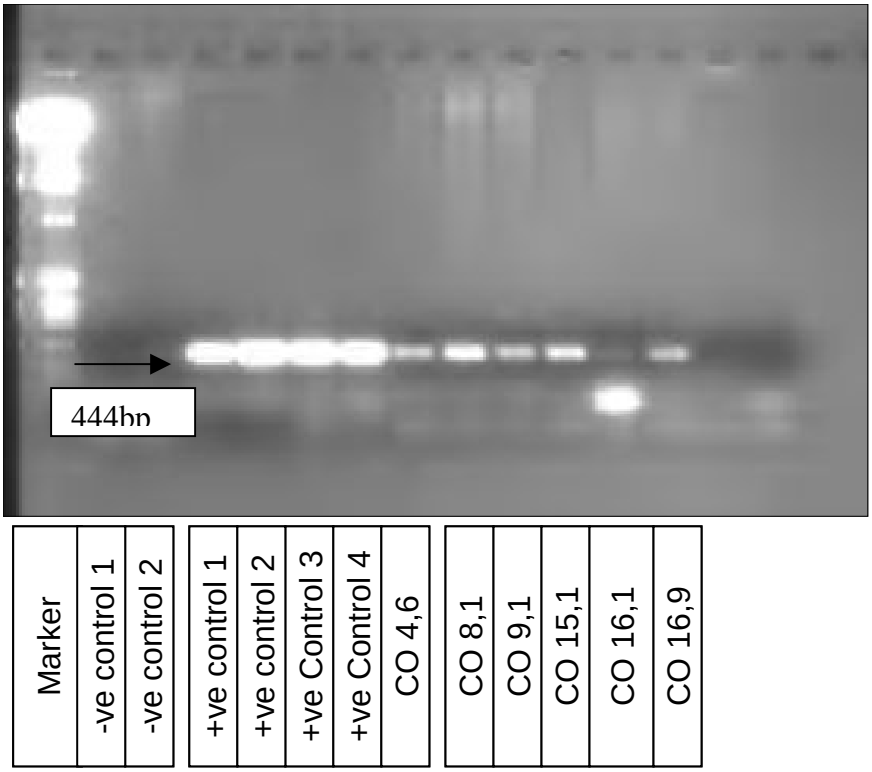


Fig 5. Ethidium bromide stained 1% agarose gel electrophoresis showing PCR amplification product. Of 4 independent

Transformed seeds of Arabidopsis mutant lines were selected using the herbicide basta and viable seeds were bulked. These lines were self-fertilized to find multiple, independent transgenic for each construction where the transgene is not segregating and flowering induction kinetics were conducted on the T2 Arabidopsis lines using the Reverse transcriptase PCR (RT-PCR) to monitor the gene expression of the insert genes after the application of varying ethanol dosage. CO, API and AGL 24 lines showed expression of these genes 3days after spraying with 1% ethanol and 1% acetaldehyde.

◀A second set of transformation experiment is being conducted using FT and SOC1 using friable embyrogenic callus from the cassava varieties 60444 and CM 3306-4. They are at the second selection stage in solid proliferation media, GD2-50Pi with 5mg/L PPT. A major delay in the second set of transformation experiment has been with preparation of FECs to generate a large number of independent transgenic lines. Preparation of FEC remains a laborious and time-consuming task.

With the Friable Embryogenic Callus (FECs) in proliferation medium GD2-50Pi with varying concentrations of PPT, there is 3fold increase in the controls while there is 2fold growth in 1mg/l and there are no significant growth in 10,50,100 and 200mg/l concentration of PPT. FECs in maturation medium into somatic embryo cotyledons, there are no significant difference between the somatic embryos generated by the control and that medium containing 1mg/l ppt while there was no differentiation into embryos in the other concentrations. Based on these experiments, new transformation events have been set to use the concentration of PPT that kills them best.

**Conclusion and perspectivas**

There is great expectation in the new transformation that has been set up. Successful production of transgenic cassava genotypes expressing inducible flowering genes as a result of these new transformation will "open up" conventional breeding of many excellent genotypes that are recalcitrant to flowering and thus synchronize flowering of genotypes that currently flower at different times. This will lead to increased income and improved livelihoods of rural communities who will eventually grow the high yielding disease resistant cassava varieties developed as a result of improved ability to make desired crosses.

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## 2.1.5 Over-expression of yeast-derived invertase in cassava

Yina J. Puentes, Edgar Barrera, Martin Fregene
CIAT

Funding: Rockefeller foundation
Important outputs

Constructs with the Apoplastic invertase gene
Production of lines from Friable Embryogenic Callus (FECs) variety 60444 for transformation.
Successful transformation of the constructs into the model cassava variety 60444 (MNig 11)

**Introduction**

In most plants, sucrose is the main photosynthesis product exported from the source cells in the leaves (active tissue) to photosynthetically less active or inactive tissue such as stems, flowers, and roots (sink tissues) (Heineke et al., 1992). Therefore, when over-expressing the apoplastic invertase gene would more quickly catalyze the conversion of sucrose to glucose + fructose and increase export to the sink tissues and by further processes form sucrose and starch. The objective for this project is therefore to increase the content of starch and therefore dry matter.

**Methodology**

The construct used in this work was kindly provided by Annette Klocke and Babette Regierer from Max Planck Institute in Germany. This construct has Kanamycin selection gene for propagation in bacteria as well as in plants (Figure 1).

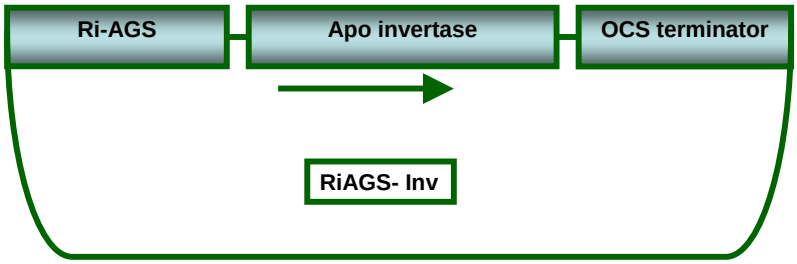



Figure 1. Plasmid RIAGS-Inv

For production of FEC, nodal cuttings from *in vitro* plantlets were cultured in 4E media at a density of about 25 cuttings per Erlenmeyer glass flask. This first step takes about 2 months and the explants were then finely shredded and placed in MS4 media in glass compote, 10 pieces per glass, for 3 to 4 weeks. Somatic embryos formed were excised from the rest of the tissue and placed in GD2-50Pi media solid for the induction of friable embryogenic callus (FECs; maximum of 9 clusters per dish). After 30 days, FECs that have developed in the clusters were sub-cultured in fresh GD2-50Pi media solid to increase its amount. FECs obtained above were cultured again in GD2-50Pi media solid for one month.

To transform the pure FECs, acetosiringone [200μM] was added to each petri-dish and the FECs collected and re-distributed in clusters of 5mm width and about 0.082g weight, about 20 clusters per dish. Following, 10μl of *agrobacterium* already transformed with the construct was added to each cluster and left for 2-3 days at 21°C. The transformed FECs was collected with a sterile spatula and washed with GD2-50Pi liquid media supplemented with Cefotaxima or Claforan [0.5 mg/ml] for one week and an additional week under appropriate selection pressure (Kanamicine 10mg/ml). Individual cell-lines of transformed FECs were allowed to proliferate in the GD2-50Pi solid media for 5 weeks after which they were transferred to MS2-1μMANA media for 2 to 3 weeks to allow the development of cotyledenous embryos and to continue with the respective process of regeneration plants.

**Result**

The construct was transformed into Friable Embryogenic Callus (CEF) of the model transformation genotype MNIG11 via *Agrobacterium tumefaciens*. At the moment it is in phase of obtaining of transgenic CEF lines.

**Conclusion and perspective**

We hope to soon obtain transgenic cassava plants genetically over-expressing the invertase gene with superior in dry matter to the original wild type plant.

**References**

Dieter Heineke, et al. 1992 Apoplastic expresión of Yeast-Derived Invertase in Potato Plant physiol. 100, 301-308

## 2.1.6 Transgenic lines of cassava carrying novel promoter-gene combinations to enhance the expression of bacterial carotenoid genes in roots.

JJ Ladino[1], O Vacca[1], D López[1], JA. Beltran[1], AL Chávez[1], LM Zúñiga [1], P Chavarriaga[1], J Arango[2], S Al-Babili[2], P Beyer[2] and Joe Tohme[1]
[1]Agrobiodiversity and Biotechnology Project, CIAT
[2]University of Freiburg, Germany

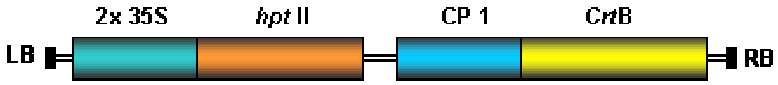
### Introduction

Increasing the nutritional value of cassava roots, by augmenting the amount of β-carotene accumulated in them, requires extensive testing of promoters to pick the best promoter-gene combinations for root-specific expression of bacterial carotenogenic genes. We present preliminary results of introducing three promoter-gene combinations into cassava, in an attempt to direct the expression of the genes crtB, crtI and crtY to the roots.

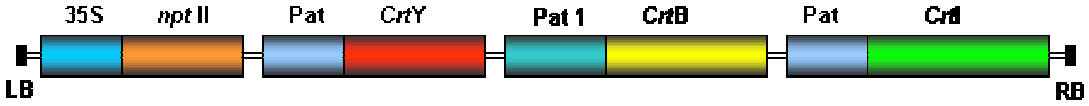
### Mehodology

*Gene constructs*
The following gene constructs were provided by collaborators from the University of Freiburg in Germany:

**pCas-Phyt**: A vascular-specific promoter isolated from cassava that drives the expression of crtB




**pPat-ErwII**: Promoters from Patatin, the major tuber storage protein of potato, have been placed in front of three genes of the carotenogenic pathaway (crtB, crtI and crtY)




**pCa-Ext-Phyt**: A promoter from Extensin, a gene that seems to be over 700 times more expressed in roots than in leaves of cassava (Al-Babili personal communication ), is directing the expression of the gene crtB. The selectable marker gene is the hptII gene as in pCas-Phyt.

Gene constructs number 3 was also designed to have the GUS gene in front of their respective root-specific promoter. It was introduced into cassava as well to have a more expedite indication on the strength of root specific expression. Therefore a total of four constructs were tested.

*Genetic transformation of cassava*
Constructs were introduced into cassava cultivars 60444 (model), MCol2215 (Venezolana) and CM3306-4 (Ica Negrita), by using *Agrobacterium* as vector. The tissues selected to introduce gene constructs and regenerate plants were Friable Embryogenic Callus (FEC) and Cotyledons from Somatic Embryos (SE). Two genes, hpt and nptII, were used as selectable markers to isolate transgenic lines resistant to either

hygromycin or geneticin, respectively. Once plants were regenerated in vitro, samples of leaves were taken for DNA extraction before they were moved to the biosafety greenhouse.

*HPLC analysis of carotenoid content of transgenic tissues*
Samples from calli, in the case of cell lines, or roots of transgenic, in vitro plants were processed to extract carotenoids and run HPLC to detect and/or quantify phytoene and β-carotene.

*Molecular detection of carotenoid genes in cassava putative transgenic lines using conventional and RealTime PCR*
The methodology and results for the molecular characterization of putative transgenic plants is attached as a separate report at the end of this document.

**Results**

*Trangenic cassava lines*
Since February until December of 2006, **sixteen transformation experiments** (Table 1) were performed to introduce the gene constructs depicted in the methodology into cassava embryogenic tissues. Until the writing of this report, over **one thousand independent cell lines** were established for all experiments (Figure 1). Out of the cell lines obtained, **thirty five** putatively **transgenic plants** were regenerated from three cultivars, which gives a **conversion rate of 3%** (actual number of cell lines that produce a plant). Several plants have been currently transferred to the greenhouse. For the latest experiments (Table 1) there are no plant lines established yet since those experiments were recently transferred to fresh selection media where they may eventually regenerate plants.

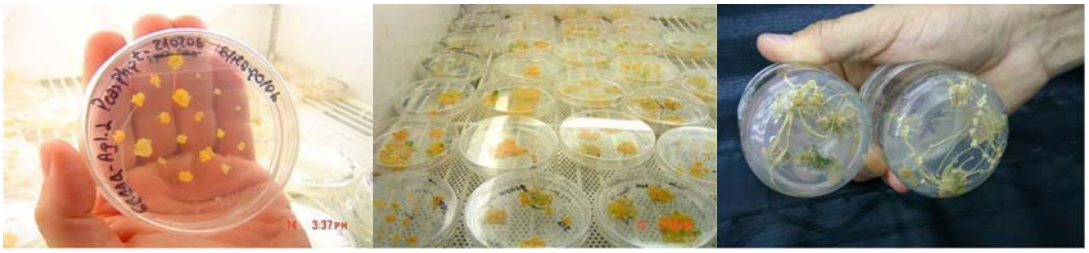


**Figure 1**. Transgenic cell lines of cassava cultivar 60444, transformed with pCP1-Phyt, having an unusual yellow color, most probably due to accumulation of lutein, indicating higher carotenoid content (left and central pictures). On the right panel, fully regenerated somatic embryos, shoots and roots in vitro from which samples were taken for carotenoid content analysis using HPLC.

Table 1. Transformation experiments carried out with four gene constructs described in methods. The last column indicates the ID number, and the total number of plants regenerated from each single experiment. The column before the last shows that, at the moment of writing this report, there were several lines still growing in vitro, including experiments done with constructs pCa-Ext-Phyt and pCa-Ext-Gus.

| **Date of Experiment** (dd-mo-ye) | **Clone** | **Plasmid** | **Explant (FEC or SE)** | **Number of putative transgenic cell lines** | **ID of independent transgenic plants regenerated (total number of plants regenerated)** |
|---|---|---|---|---|---|
| 24-02-06 | 60444 | pCP1-Phyt | FEC (Friable Embryogenic Callus) | 508 (Agl 1) | 22, 60, 84, 88, 122, 139, 143, 146, 196, 220, 487, 509 (**12 lines**) |
| 21-04-06 | Mcol 2215 | pCP1-Phyt | SE (Somatic Embryos) | Discarded | |
| 280406 | CM 3306-4 Mcol 2215 | pPat ERW II | SE | Discarded | |
| 06-05-06 | CM 6740-7 | pPat ERW II | SE | Discarded | |
| 12-05-06 | Mcol 2215 CM 3306-4 60444 | pPat ERW II pCP1-Phyt | FEC | Discarded | |
| 09-06-06 | CM 3306-4 60444 | pPat ERW II | SE | Discarded | |
| 30-06-06 | CM 3306-4 HMC1 60444 Mcol 2215 | pPat ERW II | FEC | 6 lines Discarded Discarded 47 lines | No plants regenerated 1, 5, 8, 10, 14, 17, 21 (**7 lines**) |
| 07-07-06 | Mcol 2215 | pPat ERW II | FEC | Discarded | |
| 21-07-06 | Mcol 2215 | pPat ERW II | FEC | Discarded | |
| 28-07-06 | 60444 | pPat ERW II | SE | Discarded | |
| 13-10-06 | 60444 Mcol 2215 | pPat ERW II | FEC | Growing Discarded | |
| 27-10-06 | 60444 | pPat ERW II | FEC | Growing | |
| 17-11-06 | 60444 Mcol 2215 | pPat ERW II | SE | Growing 200 Lines 377 líneas | No plants regenerated yet |

| | | | | | |
|---|---|---|---|---|---|
| | CM3306-4 | | | | 22, 33, 3, 285, 161, 132, 184, 160, 77, 20, 75, 185, 175, 24, 35, 2 (**16 lines**) |
| 24-11-06 | 60444 | pPat ERW II | SE | Growing | |
| 01-12-06 | Mcol 2215<br>Mcol 2215<br>60444<br>60444 | pCa- Ext Gus<br>pCa-Ext-Phyt<br>pCa- Ext Gus<br>pCa-Ext-Phyt | SE | Growing<br>Growing<br>Discarded<br>Discarded | |
| 07-12-06 | CM 3306-4<br>CM 3306-<br>60444<br>60444 | pCa- Ext Gus<br>pCa-Ext-Phyt<br>pCa- Ext Gus<br>pCa-Ext-Phyt | FEC | Growing<br>Growing<br>Growing<br>Discarded | |

*HPLC analyses of phytoene content in cell lines and roots transformed with pCasPhyt*

The phytoene content in roots varied from 0 to more than 17 μg/g FW, depending upon de line analyzed (Table 2). Similarly, the total carotene content was also variable ranging from 0,29 to 4,55 μg/g FW in roots. One sample of yellow calli (240206-7) had the highest amount of total carotenoids, possibly due to the accumulation of lutein in this tissue (not shown). The non-transgenic roots had quantifiable total carotenoids, although no detectable phytoene was registered.

Quantification of phytoene was straightforward from the HPLC analysis (Table 2 and Figure 2). It was not the case for β-carotene however. The peak of β-carotene was barely detectable, too small to be quantified. We hypothesized that in vitro roots may not have the carotenoid pathway mature, so the enzymes in charge of processing phytoene into β-carotene might have not been expressed yet, or at very low levels. Therefore, no HPLC analyses were performed for in vitro roots of plants transformed with pPatErwII. Instead, they were taken directly to the greenhouse to produce secondary root growth, where larger and thicker tissues, having more plastids, may encourage the formation of β-carotene.

The experiments performed with pCa-Ext-Phyt and pCa-Ext-Gus were too young for plant regeneration and carotenoid analysis.

**Table 2.** Phytoene and total carotenoid content in roots and calli of transgenic cassava plants transformed with pCP1-Phyt.

| Line | Tissue | Phytoene (µg/g FW) | Total carotenoid (µg/g FW) |
|---|---|---|---|
| 240206-122 | Plant roots | 17,40 | 4,29 |
| 240206-487 | Plant roots | 6,21 | 4,55 |
| 240206-143-1 | Plant roots | 1,18 | 2,82 |
| 240206-143-2 | Plant roots | 1,26 | 2,3 |
| 240206-143-3 | Plant roots | 0,68 | 1,74 |
| 240206-143-4 | Plant roots | 0,00 | 0,44 |
| 240206-143-5 | Plant roots | 1,52 | 0,45 |
| 240206-141 | Callus roots | 0,80 | 1,08 |
| 240206-220 | Plant roots | 6,88 | 2,83 |
| 240206-60-1 | Plant roots | 0,00 | 0,29 |
| 240206-60-2 | Plant roots | 2,89 | 1,27 |
| 240206-7 | Yellow calli | 15,34 | 7,85 |
| 240206-60444 non-transgenic | Plant roots | 0,00 | 1,27 |

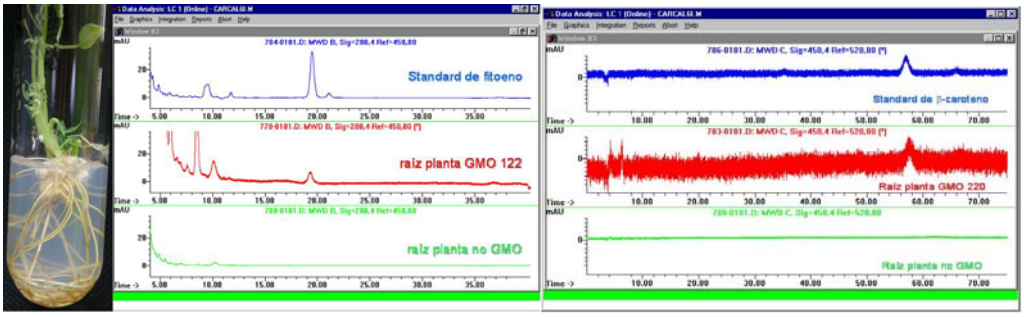



**Figure 2.** Examples of HPLC detection of phytoene (left panel) and β-carotene. In both panels the blue line is the standard, phytoene (left) and β-carotene, the red line corresponds to the transgenic root, and the green is a non transgenic root. The transgenic lines selected for this examples are 240206-122 (for phytoene) and 240206-220, both transformed with pCP1-Phyt. The inset shows a trangenic line in vitro, producing abundant roots.

**Conclusions and Perspectives**

Transgenic cassava plants that carry the construct pCP1-Phyt, with the crtB gene from *E. uredovora*, driven by a cassava vascular-specific promoter, have increased phytoene accumulation in roots taken from in vitro plants. The accumulation of β-carotene was not as evident in the same plants probably due to the low –or lack of- activity of other genes implicated in carotenoid biosynthesis. It is also possible that in vitro roots do not reflect the actual state of carotenoid synthesis in mature, thicker roots of cassava. We expect that the introduction of three genes of the carotenoid pathway, crtB, crtI and crtY, in the construct pPat-Erw-II, improve the conversion of phytoene into β-carotene.

There seems to be now two available methods, FEC and SE, of producing transgenic cassava plants from at least three genotypes: 60444, Mcol2215 and CM3306-4. Although the conversion rate of somatic

embryos into plants is still low (3%) for all genotypes, the efficiency of transformation –scored as the number of regenerated plants that are actually transgenic- tends to increase. Judging by the molecular analyses presented in the following report, the efficiency may be higher than 50%. However, the final proof of transgenicity is given by Southern blots, which have not been performed yet, so we advice caution with interpretation of the data.
For most genotypes, the time of obtaining transgenic plants has shortened to less than six months from the date of transformation until plants are potted in the greenhouse.

### 2.1.7 Molecular detection of carotenoid genes in cassava putative transgenic lines using conventional and RealTime PCR

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Agrobiodiversity and Biotechnology Project, CIAT

**Introduction**

Increasing the nutritional value of cassava roots, by augmenting the amount of β-carotene accumulated in them, requires extensive testing of promoters to pick the best promoter-gene combinations for root-specific expression of bacterial carotenogenic genes. Three genes (crtB, crtI and crtY) coding for enzymes of the carotenoid biosynthetic pathway , driven by root-specific promoters, were introduced into the cassava genome, via *Agrobacterium*, to express enzymes that permit accumulation of carotenes in roots. The evaluation of the putative transgenic plants indicated the presence of the marker genes, and the genes of interest, in most of the lines evaluated.

**Methodology**

The three inserted genes were driven by root- or vascular-specific promoters, according to figure 1. We evaluated two groups of putative cassava transgenic lines that were produced whit the pCasPhyt and pPAT-ERW II plasmids. With the first construct, a *crt*B gene coding for phytoene synthase and the *hpt* II selectable marker gene (fig 1a), four TMS 60444 clones were evaluated. With the second construct, the presence of the genes *crt*B, *crt*Y for lycopene cyclase, *crt*I for carotene desaturese and the *npt* II marker (fig 1B) were tested in six Mcol 2215 and CM 3306-4 cassava clones. We used conventional PCR, and melting curve analysis by Real Time PCR, to determine the molecular status of the putative transgenic cassava lines. The results showed evidence that all the lines analyzed contained all the introduced genes.

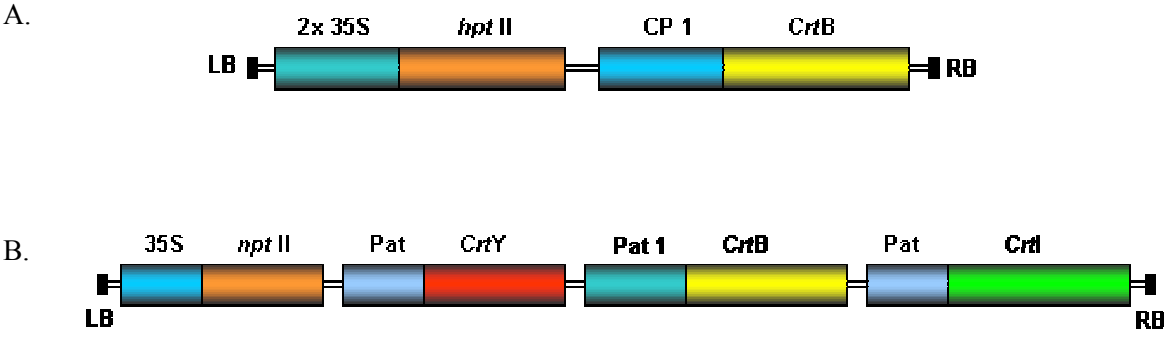



Fig 1. Schematic representation of the pCas-Phyt (A) and pPAT-ERW II (B) T-DNAs.
CP 1: glutamic rich protein promoter from cassava roots (Vascular-specific); Pat: Patain promoter (tuber specific); Pat 1: Patatin I promoter; *crt*B, *crt*Y and *crt*I are, respectively, phytoene synthase, lycopene cyclase and carotene desaturese; *npt*II and *hpt*II selectable marker genes; 35S and 2x35S are, respectively, the CaMV 35S and enhanced 35S promoters.

*DNA extraction*. Total DNA from young cassava leaves was isolated using a standard protocol. DNA was checked by 1% agarose electrophoresis and quantified.

*Primers design*. The primers sets for amplification of all genes were designed by using the PrimersQuest program (IDT® Coralville, Iowa), with the parameter set for Real Time PCR. All primers amplified portions of less than 200 bp. The same sets were useful for both PCR and Real Time PCR reactions.

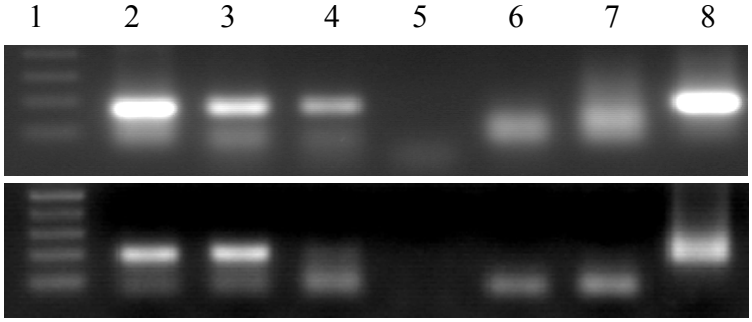
*Conventional PCR*. Amplification was carried out in 25 µl final volume containing 1X buffer 1X, 0.25mM dNTPs, $MgCl_2$ 3mM, 2 uM of each primer, 1 unit *Taq* polymerse and 200 ng of genomic DNA. PCR conditions consisted of an initial denaturation step of 95° C for 5 min, followed by 38 cycles of 95 C for 1 min, specific annealing temperature of each primer set for 1 min, and 72 for 1 min, whit a final extension of 72 C by 10 min.

*Real Time PCR.* Real Time PCR reactions was carried out in a fluorometric thermal cycler *Opticon 2* ( Mj Research) and whit the *DyNamo*® SYBR® *green* kit. The specific amplification of transgenes was detected by a melting curve analysis using *Opticon monitor 2* software. Each 20-µl reaction contained 1X *Master mix*, 0.1 µM of each primer and 50 ng of genomic DNA. The profile included a melting curve application started in 65 C to 95 °C increasing 0.2 °C each second.

**Results**

*Lines transformed with plasmid pCasPhyt*

Twelve lines of genotype TMS 60444 were regenerated. Figure 2 shows the conventional-PCR specific amplification of the transgenes *hpt* II and *Crt*B for four lines, for which enough tissue was available (from in vitro, regenerating plants). Although the signal in one of the lines (240206-122) was very faint, the results show the presence of both genes in at least three lines. There are actually 12 lines regenerated after transformation with this construct (see Table 1), but only four are reported here.




Wells:
1. 1kb plus
2. 240206-487
3. 240206-143
4. 240206-122
5. 240206-220
6. TMS (-)
7. $H_2O$
8. pCasPhyt

Fig 2. PCR amplification of the *hpt* II and *Crt*B fragments in cassava lines transformed whit pCasPhyt plasmid.

The melting curve analysis shows specific amplification of the *Crt*B gene in the four lines evaluated. The same test was done for the transgen *hpt* II in three of these lines and all showed amplification. The specificity of Real time PCR products are shown in fig 3. All melting curve picks overlap with those of the plasmid pCasPhyt used as positive control. Negative controls showed non-overlapping melting curves.

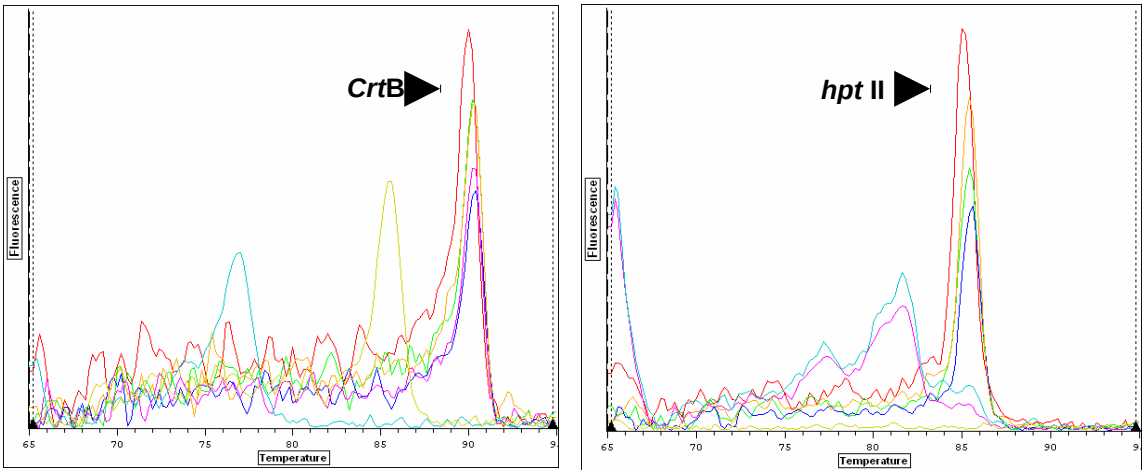



Fig 3. Detection of *Crt*B and *hpt* II transgenes by melting curve analysis using Real Time PCR. (red line = pCasPhyt)

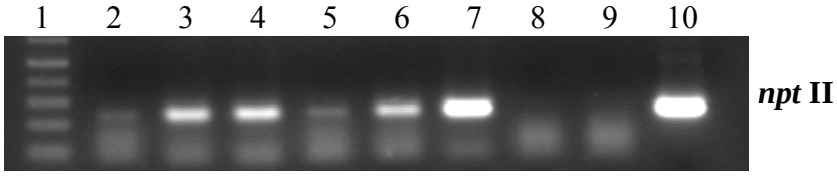
The correlation of the PCR and melting curve analysis is summarized in the Table 1. The RealTime PCR results indicated that the four TMS 60444 lines may be transgenic.

Tabla 1. PCR and Real Time PCR results of the lines transformed whit pCasPhyt (?= faint band; Ud= undetected; Nd= not done)

| | PCR | | Real Time PCR | |
|---|---|---|---|---|
| Line | *hpt* II | CrtB | *hpt* II | CrtB |
| 240206-487 | + | + | + | + |
| 240206-143 | + | + | + | + |
| 240206-122 | + | ? | + | + |
| 240206-220 | Ud | Ud | Nd | + |

*Lines transformed whit plasmid* pPAT-ERW II

Seven putatively transgenic lines were regenerated from two genotypes (Col = Mcol2215; CM = CM3306-4). There was enough tissue from regenerating plants to analyze six of them. In a first PCR screening made for the *npt* II transgen, all lines transformed whit pPAT-ERW II seemed to be transgenic. The amplified fragment in each line is shown in figure 4.




Wells:

1. 1kb plus
2. Col 10
3. CM1
4. Col 21
5. Col 17
6. Col 5
7. Col 14
8. NT (-)
9. $H_2O$
10. pPAT-ERW II plasmid

Fig 4. PCR amplification of the *npt* II fragment in cassava lines transformed whit pPAT-ERW II plasmid (NT = Not transgenic)

There were six lines evaluated, five of which belonged to the cultivar Mcol2215 (Venezolana) and the fifth one was obtained from the cultivar CM-3306-4 (Ica Negrita). All showed specific amplification for the four genes evaluated with conventional and RealTime PCR, although with the second technology the transgenic status of the lines was more evident. The specificity of the melting curve allows for distinction between amplified bands of the target genes and background . Figure 5 suggests that all six lines analyzed may be transgenic. A comparison between the results with conventional and Real Time is summarized in Table 2.

**Coclusions and future plans**

At this point of the analysis it is not possible to discard contamination of *Agrobacterium* in the tissues that were subject to DNA extraction and, therefore, false positives may be present. However, the tests based on PCR may de indicating that most lines evaluated are transgenic, for which Southern blot must be conducted to confirm stable integration of the carotenoid genes and to estimate copy numbers. The plants are now being propagated and moved to the biosafety green house. Expression analyses will be necessary to explain the functional molecular profile of the genes inserted in each transgenic line. Quantitative test of gene expression with Taq-Man Real Time PCR assays will give hints on the best promoter-gene combination to express carotenoid enzymes in cassava roots.

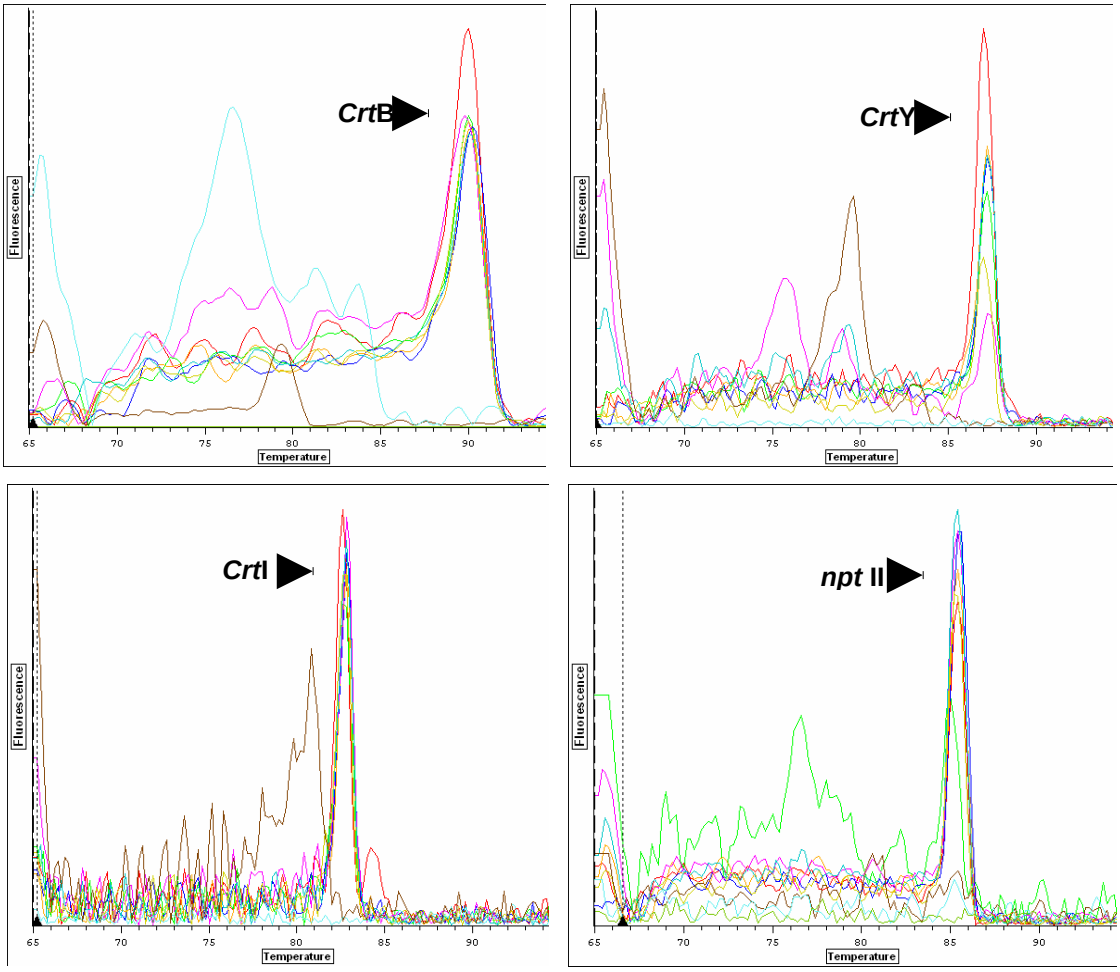



Fig 5. Detection of *Crt*B, *Crt*Y, *Crt*I and *hpt* II transgenes by melting curve analysis (red line = pPAT ERW II; light blue = non transgenic cassava; brown line = water; other six colored lines represent one putatively transgenic cassava line each, named as in figure 4).

**Table 2**. PCR and Real Time PCR results of the lines transformed whit pPAT-ERW II

| Line | PCR | Real Time PCR | | | |
|---|---|---|---|---|---|
| | *npt* II | *npt* II | CrtB | CrtY | CrtL |
| MCol2215-300606-10 | +? | + | + | + | + |
| CM3306-4-300606-1 | + | + | + | + | + |
| MCol2215-300606-21 | + | + | + | + | + |
| MCol2215-300606-17 | +? | + | + | + | + |
| MCol2215-210706-5 | + | + | + | + | + |
| MCol2215-300606-14 | + | + | + | + | + |

### 2.1.8 Final selection for RHBV resistance and yield potential of advanced breeding generation from crosses with transgenic rice resistant to RHBV in the field in 2006

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Funding The Rockefeller Foundation and CIAT

**Introduction**

The Rice Biotechnology Project had generated transgenic rice lines with resistance to RHBV (Lentini *et al*., 2003) an endemic disease and of the main constrains of rice of Tropical America. Resistant transgenic lines carrying different source of resistance than the one already deployed in commercial varieties would allow increased production to a lower cost by reducing the need of insecticide applications commonly used to control *Tagosodes oryzicolus* (the virus vector). After stepwise selection from the original transgenic plants, including elimination of un-stable inheritance of RHBV resistance and plants with undesirable agronomic traits, the best lines were crossed with various varieties and derived progenies had been subjected to a breeding selection process in the field including evaluation for yield potential, other disease resistance (*Rhizoctonia* and *Pyricularia*), agronomic and grain quality traits. This report describes the final selection of the best lines, step after which these lines should be evaluated by third party and decision should be made on the fate of these materials for potential deployment to farmer fields.

**Key Words:** transgenic rice, RHBV, yield potential, field

**Introduction**

The transgenic RHBV resistant rice was generated by splicing in genes from the RHBV virus into Cica 8 variety (Lentini *et al*., 2003), selected by the breeders as potential parent donor of the transgene for future conversion of modern varieties because of its good grain quality, high productivity, and broad adaptation including low soil humidity, and acceptance to large and small resource farmers. In spite of this variety is still of preference by small farmers due to its good agronomic qualities and resilience to stress conditions, this variety is not currently grown commercially because of its high susceptibility to RHBV. The transgenic resistance incorporated is RNA-mediated and some plants show hypersensitive reaction when challenged with the RHBV virus (Lentini et al., 2003). Some of the transgenic lines outperform in RHBV resistance some of most currently grown commercial rice varieties. Attempts to transfer this resistance

into other modern varieties through regular crossing indicated that the RHBV-N transgene is inherited and expressed independently of the genotype background. Thus this transgenic resistance could be used to complement the breeding resistance that has been deployed so far and does not protect plants when younger than 5 to 20 day-old. In addition to selection for RHBV resistance and yield potential, the advanced lines were evaluated for tolerance to *Rhizoctonia*, resistance to *Pyricularia*, and grain quality traits. Progress in previous years led to a shift from the testing of concepts towards the final steps for its potential release to farmers' fields. Previous reports described the stepwise breeding selection in the field. Last year we reported the selection of advanced 8 advanced progeny lines derived from the original transgenic lines, and 8 lines derived from crosses between selected transgenic lines and with commercial varieties, for a total of 28 individual-plant selections. This year we report the evaluation and selection for RHBV resistance in the field, and final selection based on their RHBV resistance, tolerance to sheath blight and blast, grain quality and agronomic traits, and yield potential of $T_7$ and $T_{10}$ advanced transgenic lines, and $F_6$ progeny plants derived from crosses between the transgenic lines and commercial varieties .

**Materials and Methods**

*Evaluation of RHBV resistance in the field in 2006*. Field evaluations were conducted using 12 $T_7$ and $T_{10}$ advanced transgenic lines, and 16 $F_6$ families derived from crosses between the transgenic lines and the commercial varieties Fedearroz 50 or Oryzica 1. The selection of the original lines was based on its agronomic performance in the field in 2005. A row with 40 plants per line with 4 replicates was used for the RHBV evaluation. Controls consisted of RHBV resistant plants (Colombia 1, and Fedearroz 2000) and susceptile checks (Bluebonnet 50, Cica 8, Oryzica 1, Fedearroz 50, a cross Cica 8/ Fedearroz 50, and Cica 8/ Oryzica 1). Eighteen days after planting, plants were infested with dosages of 1.5 insects per plant using viruliferous insects from the colony "Tolima-CIAT". Five days after the infestation, the insects were killed, and the plants were evaluated for disease symptoms development at 30 and 45 after infestation.

*Agronomic evaluation and selection of advanced generations of transgenic events and derived progeny plant from crosses.* The same lines evaluated for RHBV resistance were also evaluated for agronomic performance in the field. Plants were transplanted in the field using 8 rows per lines of 21 plants per rows with 3 replicates. Agronomic traits were evaluated throughout the life cycle up to maturity. Tiller number, plant height, plant vigor, days to flowering, fertility, and yield was evaluated. Agronomic traits were evaluated according the scale IRRI (1996).

**Results and Discussion**

Cluster analysis of advanced crosses or self-cross transgenic lines using principal coordinates were conducted using data from the RHBV resistance evaluations with one dosage of 1.5 insects per plant and 3 replicates. Five $F_6$ generation lines derived from crosses with Oryzica 1, and five $T_7$ and $T_{10}$ self progeny advanced transgenic lines were clustered in Groups 9 and 8 respectively, jointly with Fedearroz 2000 showing the highest level of RHBV resistance (score $\leq 3$) (Figure 1A and 1B, and Table 1). The RHBV resistance of these crosses and transgenic lines were significantly different from their corresponding non-transgenic controls (the controls clustered in other groups). The non-transgenic cross Cica 8/Oryzica 1 score $\geq 5.7$, and the non-transgenic control varieties Cica 8, score $\geq 7.7$ and Oryzica 1, score $\geq 7$ were clustered in other groups with susceptible materials likewise the controls Cica 8/Fedearroz 50, score $\geq 5.7$; and Fedearroz 50, score $\geq 7.7$(Figure 1A and 1B, and Table 1). The susceptible transgenic control line A3-78-24 (which does not contain the RHBV transgene, internal control for the transgenic procedure) show the highest susceptibility as in earlier evaluations (score = 9).

The lines listed in Table 1 showed RHBV resistance score $\leq 5$, have medium to long slender grains with low degree of white center and high amylose, tolerance to sheath blight (*Rhizoctonia sp)* and known resistance race-profile to *Pyricularia grisae* pathogen, as shown last year. Most lines with highest RHBV resistance level (score $\leq 3$) also showed promissory agronomic characteristics such as high plant vigor,

intermediate days to flowering initiation and days to 50% anthesis, plant type, plant height, and tillering capacity.

The crosses with Fedearroz 50 were discarded by its genetic instability over generations, although some plants showed the highest vigor and agronomic performance (data not showed). No significant differences for agronomic traits and grain yield were found between the transgenic materials selected and the non-transgenic controls (crosses or commercials varieties Fedearroz 2000, Fedearroz 50, Cica 8, Oryzica 1 and Colombia 1)(Tables 1). Some lines showed low plant fertility (70%) likewise some of the commercial varieties (Fedearroz 50), but most lines showed fertility above 90%. Only lines with high fertility ($\geq$ 95%) were selected. Some lines with good agronomic traits and grain quality were discarded (5 of total 28 lines, 18%) because of its inconsistency response to RHBV. Most instability in RHBV response was discarded in earlier generations. It is important to highlight that at these advanced generations which had been subjected to at least 5 cycles of selection for RHBV resistance, 82% of the evaluated lines showed stable resistance. This stability is also seen in experimental designs sowing small plots of 0.8 m X 0.8 m of each line infested with virouliferous insects at a density of 1.5 insects per plant, where clear-cut resistance level differences are noted between the original Cica 8 susceptible variety and the lines selected with resistance to RHBV score $\leq$ 3 (Figure 2)

Based on this RHBV resistance profile, the agronomic performance including yield potential, and previous evaluations for sheath blight and blast resistance as well as for grain quality traits, three F6 generation lines from the cross with Oryzica 1, one T7 line and four T10 transgenic lines composed the final selection (Table 1, lines in bold letters). Of the 3 lines selected from crosses with Oryzica 1, two of them are sister lines; likewise, of the 5 transgenic lines selected four of them are sister lines (Table 1). Results suggest that the stability of traits over generation is genetically determined by the transgenic lines selected in early $T_3$ generation (generation selected to initiate the pedigree process). The final selections are derived from lines: A3-49-60-4-5; A3-49-60-12-3; A3-49-60-13-69; and A3-49-101-18-19. Furthermore, 6 of the 8 lines selected are derived from the original $T_1$ transgenic line A3-49-60 (Lentini et al., 2003).

**Future Activity**

The selected lines are currently being processed through anther culture to generate doubled haploid (complete homozygous) lines, convenient material for seed multiplication of replicated multi-location field trials and molecular genotyping.

**References**

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IRRI (1996). Standard evaluation system for rice. 4th edition. 52p

**Table 1.** RHBV resistance and agronomic performance in the field in 2006 of selected families from crosses (total 9) derived from crosses between Cica 8-RHBV transgenic resistant lines and commercial variety Oryzica 1, and Cica 8-RHBV transgenic resistant progeny (total of 14) derived from self cross.

| **Pedigree** | **Cluster** | | **[1]Average RHBV** | **Yield (Kg/ha) Potential** | **[2]Vig** | **[3]DF** | **[4]DF 50%** | **Plant Height (cm)** | **[5]Tiller** | **F (%)** |
|---|---|---|---|---|---|---|---|---|---|---|
| | **C** | **L** | | | | | | | | |
| | | | | | | | | | | |
| A3-49-101-18-19/Oryzica 1-14-M-10-1-2 | 8 | | 4,3 | 10,912 | 3 | 98 | 105 | 105 | 3 | 95 |
| **A3-49-101-18-19/Oryzica 1-14-M-10-1-3** | **9** | | **2,3** | **10.975** | **3** | **98** | **103** | **110** | **3** | **95** |
| A3-49-101-18-19/Oryzica 1-14-M-10-2-4 | 8 | | 4,3 | 9,508 | 3 | 94 | 98 | 110 | 3 | 95 |
| **A3-49-101-18-19/Oryzica 1-14-M-7-2-M-1** | **9** | | **3,0** | **8,643** | **3** | **90** | **96** | **100** | **3** | **98** |
| A3-49-60-4-13/ Oryzica 1-13III-6-5-M-1 | 9 | | 3,0 | 6,864 | 3 | 99 | 104 | 120 | 3 | 70 |
| A3-49-60-4-13/ Oryzica 1-13III-6-5-M-2 | 9 | | 2,3 | 5,691 | 3 | 100 | 105 | 125 | 3 | 70 |
| A3-49-60-4-13/ Oryzica 1-13III-6-5-M-3 | 1 | | 5,0 | 7,431 | 3 | 98 | 105 | 120 | 3 | 70 |
| **A3-49-60-4-5/ Oryzica 1-15-15-11-M-3** | **9** | | **3,7** | **9,792** | **3** | **110** | **116** | **105** | **3** | **95** |
| A3-49-60-4-5/ Oryzica 1-15-15-11-M-6 | 10 | | 5,0 | 17,508 | 3 | 107 | 112 | 115 | 3 | 95 |
| | | | | | | | | | | |

Contin.....

**Cont. Table 1**. RHBV resistance and agronomic performance in the field in 2006 of selected families from crosses (total 9) derived from crosses between Cica 8-RHBV transgenic resistant lines and commercial variety Oryzica 1, and Cica 8-RHBV transgenic resistant progeny (total of 14) derived from self cross.

| Pedigree | Cluster | | [1]Average | Yield (Kg/ha) | [2]Vig | [3]DF | [4]DF | Plant Height | [5]Tiller | F |
|---|---|---|---|---|---|---|---|---|---|---|
| | C | L | RHBV | Potential | | | 50% | (cm) | | (%) |
| | | | | | | | | | | |
| A3-49-60-13-69-M-1-4-1 | | 5 | 3,7 | 9,002 | 3 | 109 | 114 | 110 | 3 | 95 |
| **A3-49-60-13-69-M-1-4-2** | | **8** | **3,0** | **14,543** | **3** | **109** | **114** | **115** | **3** | **97** |
| A3-49-60-13-69-M-1-4-3 | | 6 | 3,0 | 9,253 | 3 | 108 | 113 | 120 | 3 | 95 |
| A3-49-60-12-3-20-M-13-2-1-M-1 | | 7 | 4,3 | 8,534 | 3 | 98 | 105 | 115 | 3 | 97 |
| **A3-49-60-12-3-20-M-13-2-1-M-2** | | **8** | **3,7** | **12,814** | **3** | **98** | **105** | **118** | **3** | **97** |
| A3-49-60-12-3-20-M-13-2-1-M-3 | | 5 | 3,7 | 10,596 | 3 | 107 | 112 | 118 | 3 | 95 |
| A3-49-60-12-3-20-M-13-2-2-M-1 | | 6 | 3,0 | 5,766 | 3 | 106 | 112 | 115 | 3 | 95 |
| **A3-49-60-12-3-20-M-13-2-2-M-2** | | **8** | **4,3** | **6,301** | **3** | **109** | **114** | **117** | **3** | **95** |
| **A3-49-60-12-3-20-M-8-4-4-M-3** | | **8** | **3,0** | **15,141** | **3** | **105** | **110** | **125** | **3** | **95** |
| A3-49-60-12-3-20-M-8-4-4-M-4 | | 5 | 3,7 | 8,532 | 3 | 102 | 106 | 115 | 3 | 90 |
| **A3-49-60-12-3-20-M-8-4-4-M-5** | | **8** | **3,7** | **7,326** | **3** | **105** | **109** | **115** | **3** | **97** |
| A3-49-60-12-3-20-M-8-6-4-M-1 | | 5 | 4,3 | 5,507 | 3 | 102 | 106 | 125 | 3 | 95 |
| A3-49-60-12-3-20-M-8-6-4-M-3 | | 7 | 3,7 | 9,191 | 3 | 105 | 110 | 125 | 3 | 90 |
| A3-49-60-12-3-20-M-8-6-4-M-4 | | 6 | 5,0 | 8,987 | 3 | 105 | 109 | 125 | 3 | 90 |
| | | | | | | | | | | |
| Cica 8/ Fedearroz 50 | 5 | | 5,7 | 9,793 | 3 | 105 | 110 | 120 | 3 | 90 |
| Cica 8 / Oryzica 1 | 5 | | 5,7 | 7,883 | 3 | 96 | 110 | 115 | 3 | 80 |
| Cica 8 | 7 | 1 | 7,7 | 6,580 | 3 | 98 | 105 | 110 | 3 | 91 |
| **Fedearroz 2000** | **9** | **8** | **2,3** | **15,429** | **3** | **99** | **105** | **115** | **3** | **95** |
| Fedearroz 50 | 3 | 1 | 7,7 | 7,434 | 3 | 105 | 112 | 120 | 3 | 70 |
| Oryzica 1 | 5 | 1 | 7,0 | 9,555 | 3 | 94 | 101 | 100 | 3 | 95 |
| ***Colombia 1*** | ***10*** | ***6*** | ***4,3*** | ***10,085*** | ***3*** | ***101*** | ***105*** | ***155*** | ***3*** | ***85*** |
| A3-78-24 | | 4 | 9,0 | 7,490 | 3 | 107 | 113 | 110 | 3 | 95 |

[1] Average value score for resistance to the RHBV. [2] Plant vigor (1) higest vigor and (5) lowest vigor. [3]DF days to flowering initiation. [4] Days to 50% anthesis. F = mean fertility % per line. [5]Tillering ability (1) Extra vigorous and (9) Very weak plant.

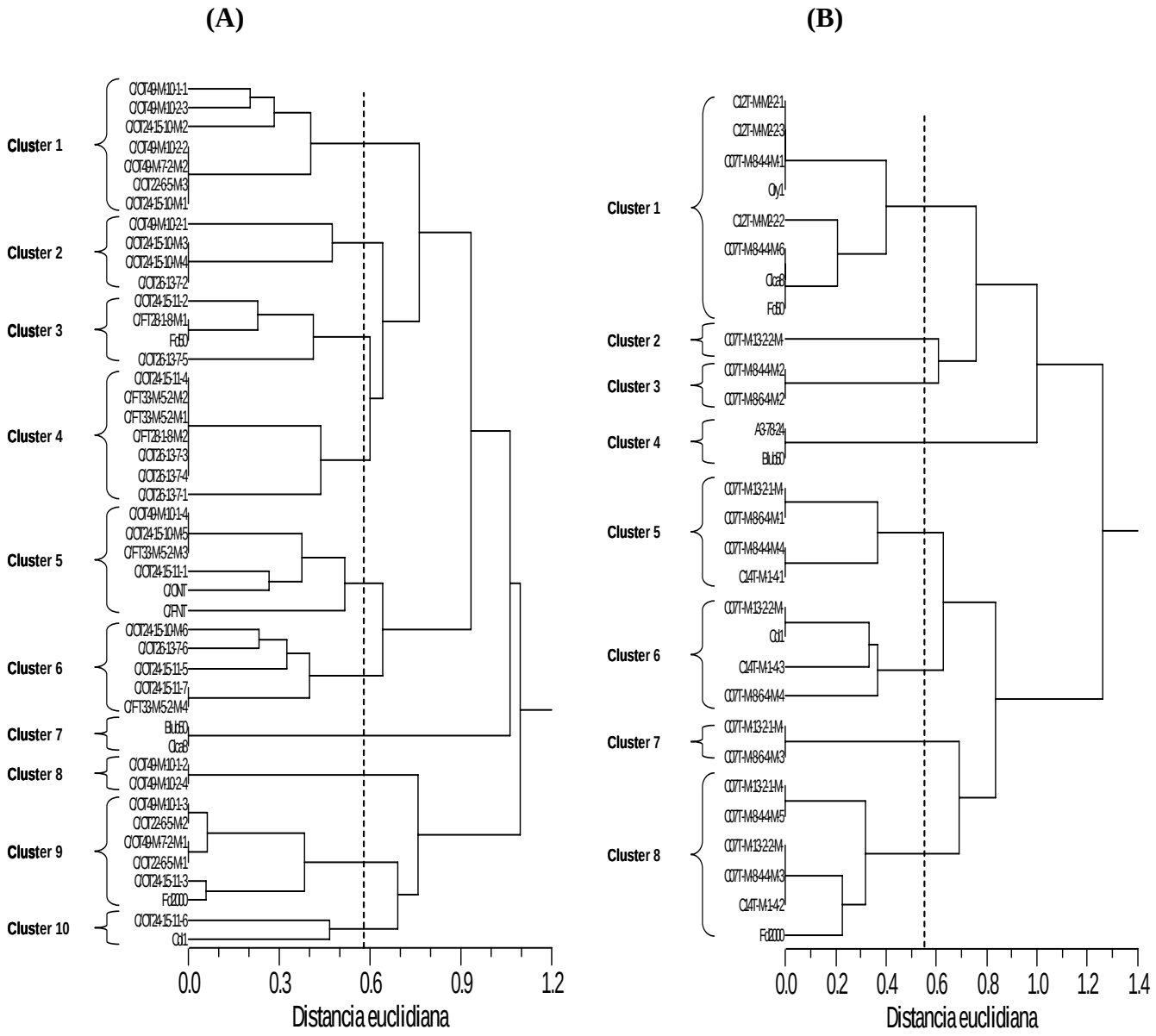


**Figure 1.** Similarity analysis for RHBV resistance response of 38 $F_7$ plants selected from crosses between selected Cica 8-RHBV transgenic resistant lines and Oryzica 1 (A), and 21 $T_9$ to $T_{12}$ plants Cica 8-RHBV transgenic resistant lines (B) selected progeny plants derived from self cross. Controls consisted of varieties Fedearroz 2000 (highly resistant, score ≤3); susceptible checks of Oryzica 1 (score ≥ 7); Cica 8 (score ≥ 7.7); Fedearroz 50 (score ≥ 7.7) and Bluebonnet 50 (highly susceptible, score = 9); and the non-transgenic cross Cica 8/Oryzica 1 and Cica 8/Fedearroz 50 (each score ≥ 5.7).

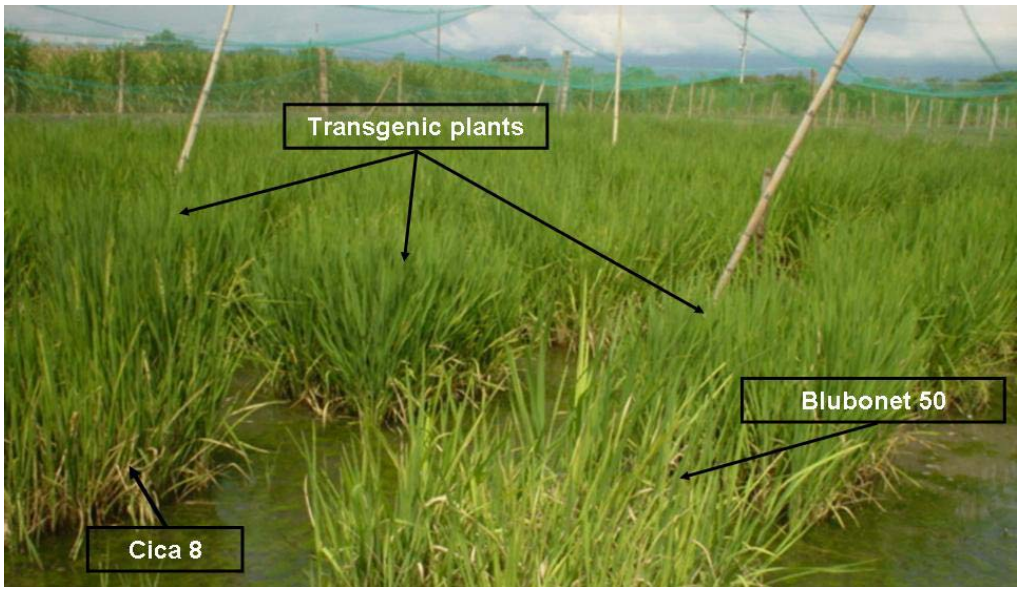


Figure 2. **RHBV resistant transgenic lines (score ≤ 3) and susceptible varieties Cica 8 and Bluebonnet 50 planted in the field in small plots of 0.8 X 0.8 m and infested with virouliferous insects at a density 0f 1.5 insects per plant.**

## 2.1.9 Increased frequency plant regeneration for various pretreatment combinations to callus derived from anther culture of indica rice (*Oryza sativa* L.) varieties

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Funding CIAT

**Abstract**

Previous year report indicated that three fold increased callus induction in *indica* rice is obtained when PAA 10mg/l is added to the induction medium and maintained in constant agitation in a shaker at 80rpm, and that improved in green plant regeneration efficiency is reached when callus were subjected to desiccation or subculture prior plant dedifferentiation induction (Quintero et al., 2005 in SB2 annual report 2005). This year, the work focused in three aspects to improve embryogenesis and regeneration from recalcitrant indica genotypes: 1) to evaluate of the interaction between PAA concentration in the induction medium and the aeration treatments using low cost procedures (shaker); 2) to test different desiccation period treatments and its interaction with various combinations of growth regulator compositions in the medium and/or sub-culture to induce green plant regeneration of *indica* rice; 3) to determine the effect various culture conditions to induce cell division from microspore cultures.

**Key Words:** anther culture, callus induction, plant regeneration

**Introduction**

Production of callus and its subsequent regeneration are the prime steps for the generation of doubled haploid (DH) plant to be manipulated by conventional breeding programs or by biotechnological means. The response to callus induction and plant regeneration are genotype (variety) dependant, and a highly efficient regeneration from *indica* rice still poses a major bottleneck for genetic manipulation through innovative approaches (Toki, 1997). However some *indica* rice genotypes are more amenable than others for callus induction, but plant regeneration of green plants still remains a major problem (Lentini et al., 1995). Strategies to improve plant regeneration frequency in cereals, including rice, have been steadily evolving during the last decade (Datta *et al*., 1992; Raman *et al*., 1994). The factors affecting callus formation and plant regeneration from *in vitro* cultured anthers of rice were initially studies by Niizeki and Oono (1968), Iyer and Raina (1972), Chand et al. (1977), Datta et al. (1992), and more recently by Saharan et al. (2004). Studies on green plant regeneration were centered in the medium composition and environmental conditions, nowadays it is focused on various types of pretreatment to the callus prior the induction of plant differentiation.

**Materials and methods**

*Anther Culture.* Anther culture of *indica* rice Cica 8, Fedearroz 2000, Fedearroz 50 and CT 11275 were used. Anther donor plants were grown in the field, and then rice panicles were collected, treated and cultured according to Lentini *et al*. (1995). Anther were cultured in liquid medium M1 (Quintero et al., 2003) or NL (Lentini et al., 1995) contained in baby food jars closed with perforated plastic caps with a foam plug in a hole for aeration, and placed either in shelves (stationary) or with in a shaker (agitation).

*Plant regeneration*. Three experiments were conducted to evaluate various treatments for plant regeneration of anther-derived callus of *indica* rice Cica 8, Fedearroz 2000, Fedearroz 50 and CT 11275. Control consisted of callus induced in M1 medium containing PAA 10mg/L, and cultured in a shaker at 80 rpm, then callus were transferred onto MS (Murashige and Skoog, 1962) solid medium to induce plant regeneration according to in Lentini et al. (1995). The treatments consisted of: *Experiment 1, Partial*

*desiccation,* where different desiccations times were tested (0, 12, 24, 48 and 72 hours) prior transfer to plant regeneration induction medium. Desire extent of desiccation was obtained by transferring 4 weeks old callus to sterile empty petri dishes containing two sterile Whatman-5 filter papers. The petri dishes were sealed with parafilm and kept at 25 ± 1°C in the dark for different periods of time to induce the callus desiccation. After pretreatments, stressed callus were transferred to MS medium for regeneration and incubated in light according to Lentini *et al*. (1995). *Experiment 2, Subculture calluses*: Induced callus were directly transferred to solid MS medium and after two weeks of culture, only those callus with green meristematic zones or buds were sub-cultured on the same medium. Experiment 3, *Combinations of experiments 1 and 2*: Callus were pretreated by desiccation for 48hr in Whatman paper and then cultured on solid medium MS; after eighth days, only those callus with green meristematic zones or buds were sub-cultured on the same medium. A factorial completely randomized experimental design was used. At least 10 replicates of 20 calluses each was evaluated per treatment.

*Microspore isolation*. Microspore donor plants from *indica* rice Cica 8, Fedearroz 2000, Fedearroz 50 and CT 11275 were grown at CIAT's experimental field. These indica rice genotypes were selected because their response to embryogenesis and green plant regeneration from anther culture. Rice tillers were harvested, sterilized and the microspores were isolated by a method similar to the described by Weiguo, et al. (2002). The boot were sterilized and then the spikes were aseptically cut in sections of 2 to 3 cm of length and placed on sterile flasks that contained 50 ml of pre-treatment solution 2HNA (0.18mM). The flasks were placed in a shaker at 80 rpm and incubated at 27±1ºC for 48h in the dark. Florets obtained from spikes section were blended in 50ml of autoclaved 0,3*M* mannitol solution for 20s. To eliminate the large debris, the resulting slurry was filtered through mesh of different pore sizes and recovered in a capped tube containing 5 ml solution of 21% maltose and centrifuged at 450g for 3 min. The microspores were rinsed with M1 medium and finally cultured in the same medium at a density of 4 x $10^3$/ml, in 60 mm plastic Petri dishes, each Petri dish contained 3 ml of MI medium.

**Results and discussion**

*Anther Culture.* An increase of two to three folds in callus induction was obtained when PAA was added to the induction medium. Interaction PAA and different culture conditions (stationary Vs shaker) showed that Shaker significantly increase callus induction and embryogenesis to the different genotypes as in previous experiments (Quintero et al., 2005 in SB2 annual report 2005).

*Partial desiccation.* Plant regeneration was lower in controls (without desiccation) than with desiccation treatments. Significant higher green plant regeneration was obtained when desiccation was applied for 48 hr. Twice as many green plants were obtained with this treatment respect to the control (Figure 1A). Similar results was reported by Saharan et al. (2004), who reported that shoot regeneration frequency was also higher by 1.2 to 5.6 fold in both cultivars in 48 h desiccation whereas in 72 h desiccation treatment regeneration frequency declined. These results were in conformation with the reported by Diah and Bhalla (2000) and Chand and Sahrawat (2001).

*Subcultured calluses.* Increase plant regeneration was seen after subculture of callus with meristematic green plant primordia. During subculture the shoot buds elongated further and multiplied vigorously. The regeneration frequency of 75.5%and 92.5% was obtained when callus were sub-cultured on MS without or with growth regulators, respectively. An increase of 3 to 4 fold in shoot regeneration frequency was obtained with callus sub-cultured as compared to control (Figures 1B). The results of these experiments indicate that the retransfer of callus was optimal for improvement of green plant regeneration in *indica* rice varieties.

*Combination experiments 1 and 2.* Partial desiccation and subculture showed to be the best combination to improve plant regeneration efficiency from callus of the different *indica* genotypes. Response of 53.8%,

77.2% and 90% on the average were obtained with partial desiccation, subculture or a combination of both pretreatments respectively. Significant higher increase in plant regeneration efficiency of 6 fold is achieved when the two pretreatments are combined for the *indica* rice Cica 8 recalcitrant to tissue culture (Figure 2). Results indicated that desiccation increased the number of callus with green buds primordial and that subculture promotes these buds to differentiate into plants. Results suggest that depletion of nutrient in the medium and /or accumulation of inhibitory substances in the medium such as phenolic compounds exuded by the callus may affect the plant differentiation process. Regenerated plants were transplanted into pots in the greenhouse. About 50 – 60% of the plants were doubled haploids, average which is in the range previously reported by Lentini et al. (1995).

*Microspore isolation and culture.* After isolation and culture, embryogenic microspores have typically eight or more small vacuoles immediately enclosed by the cell wall (Figure 3.). These vacuoles surround the condensed cytoplasm in the center, forming a fibrillar structure. This protocol allowed inducing initial cell divisions at 10 – 12 days after culture from *indica* rice Fedearroz 2000 and CT 11275. The chemical, 2-HNA has been previously reported to increase the efficiency of androgenesis in anther culture when applied to wheat spikes at a critical developmental stage (Konzak et al., 2000). The 2-HNA can be effectively and conveniently delivered to act on microspores by the described method. Results indicate that is possible to induce embryogenesis from micropore culture of indica Latin American genotypes. Microspore culture maybe an useful biological cell system to identify factors for promoting cell division response at early stage of culture, but this system to be efficient it is necessary to improve the protocol by increasing the yield of clean microspore suspensions and testing various modifications of the culture medium including different osmolality and co-culture treatments with ovaries for providing the nurse factors, which the embryogenic microspores apparently cannot efficiently synthesize.

**Future activities**

To test different concentrations of 2-HNA in the pretreatment of spikes sections and evaluate its effects on callus induction.
To test the effect of cool-pretreatment of panicles on the isolated microspore culture and the changes in amino acid ratio in anther cultures of japonica and indica rice, and the relationship between free amino acids change and culture response of isolated microspores.
To test the shed pollen culture system, in which anthers are cultured on the liquid /solid inter-phase medium

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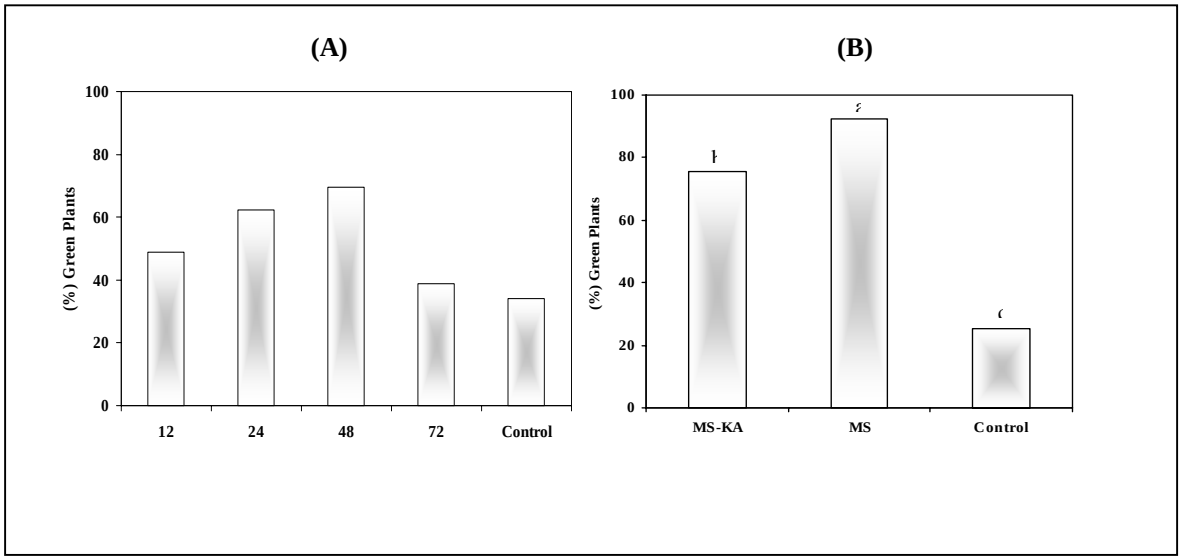

**Figure 1.-** Effect of desiccation (A) or callus subculture (B) pretreatments on the green plants regeneration of *indica* rice callus.

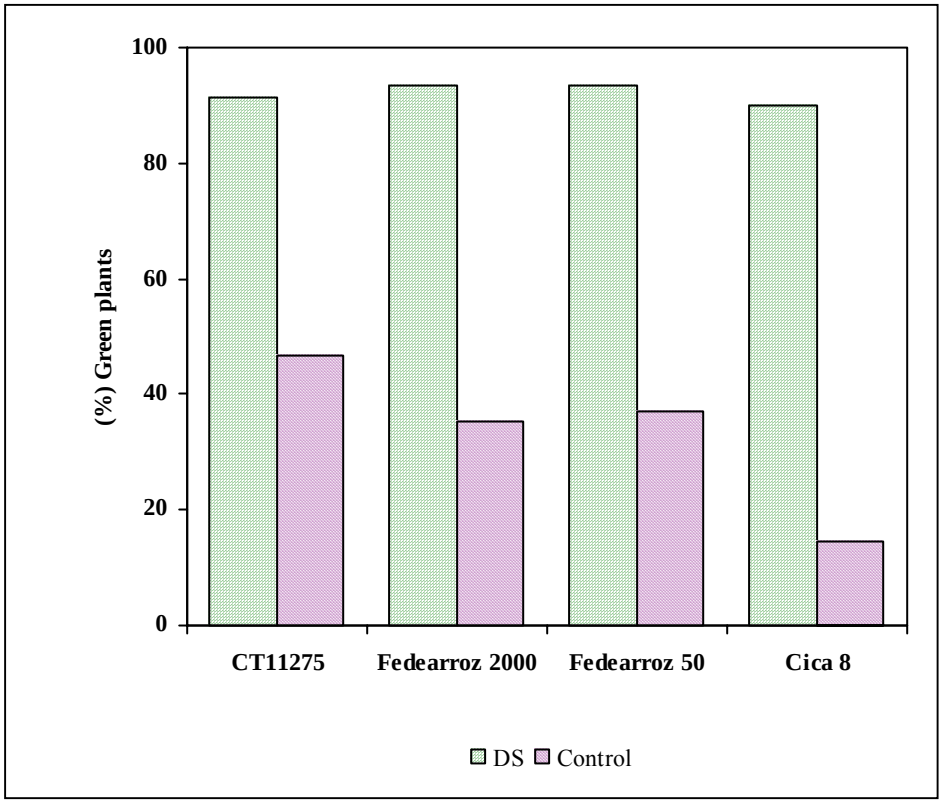

**Figure 2.-** Effect of combining desiccation and callus subculture on green plant regeneration of *indica* rice callus

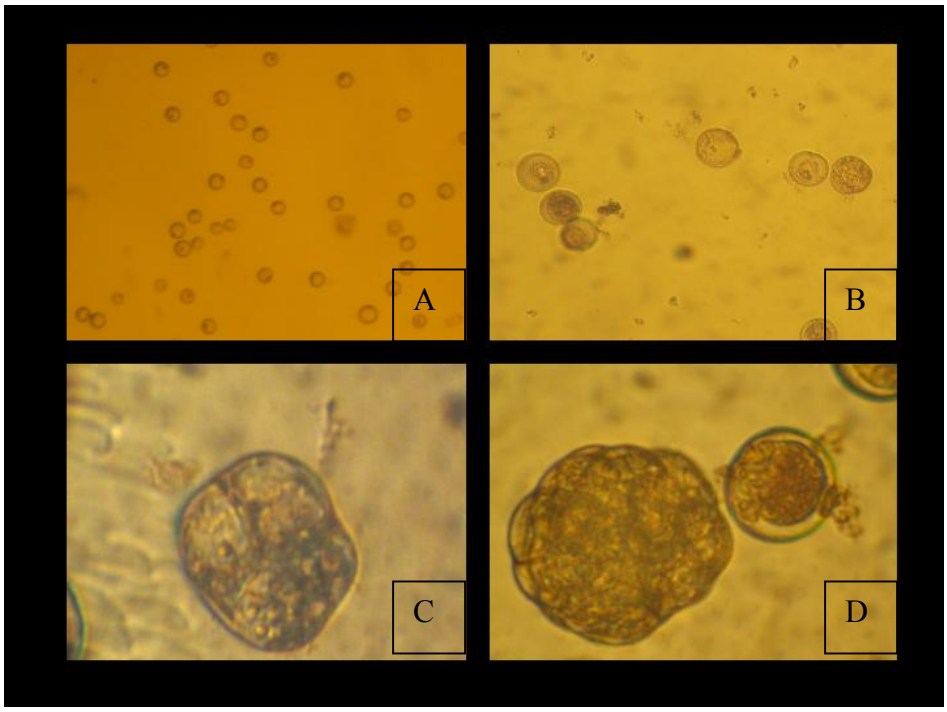


Figure 3.- Embryogenesis of Fedearroz 2000 microspores isolated, and then treated with 0.18 mM 2-HNA. (A) Microspores immediately after isolation; (B) Embryogenic microspores induced by 2-HNA after 48h; (C) Multi-cellular structures 7 days after culture; (D) Proembryoid structures at 14 days in induction medium.

## 2.1.10 Chromosome elimination in the diploid *Oryza sativa* L. x tetraploid *O. latifolia* Desv.

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**Abstract**

Meiotic behavior of *O. sativa* L. (2n=2x=24), *O. latifolia* Desv. (2n=4x=48) and their $F_1$ *O. sativa* x *O. latifolia*, $BC_2$ and $BC_3$ was evaluated. All meiotic phases were normal and pollen viability was high in both parents. Whole meiotic process of $F_1$ hybrids presented abnormalities in spindle formation, chromosome segregation and cytokinesis leading to polyads formation, which give rise to unviable pollen. In addition, chromosome elimination mediated by microcytes was observed. Some $BC_2$ and $BC_3$ plants were completely diploid (2n=24) and fertile, while others showed tendency to diploidy with one or two additional chromosomes, which were eliminated throughout segregation mechanism in the next generation. We discuss here meiotic behavior that take place for fertility restoration and the importance of chromosome elimination in this type of interspecific cross.

Key words: chromosome elimination, interspecific cross, meiotic behavior, *Oryza sativa*, *Oryza latifolia.*

**Introduction**

According to Vaughan *et al*. (2003), *Oryza* genus counts with 23 species formed by 10 genomes, six diploids (AA, BB, CC, EE, FF and GG) and four tetraploids (BBCC, CCDD, HHJJ, and one unknown genome). The interspecific cross between individuals with different genomes generates abnormal embryos and therefore abortion. The $F_1$ individuals are totally sterile, being necessary several backcrosses for fertility restoration (Brar and Khush, 1997). To avoid this, hybrids embryos are recovered in culture medium. Many works have been achieved in order to obtain alien introgression from wild species with different genomes to the cultivar *O. sativa* L. (AA). For instance, *O. officinalis* (CC) (Jena and Khush, 1989), *O. australiensis* (EE) (Multani *et al*., 1994), *O. minuta* (BBCC) (Mariam *et al*., 1996), *O. malapuzhaensis* and *O. punctata* (BBCC) (Kaushal and Ravi, 1998), *O. latifolia* (CCDD) (Multani *et al*. 2003), and *O. grandiglumis* (CCDD) (Kim *et al*., 2003) were used, but the sterility problem remained in all cases, which is surpassed by backcrossing. Despite it, efforts for fertility restoration is necessary since the pairing and recombination among distant genomes, although limited, are the mean for interest wild traits transfer (Voss *et al*., 2000; Chen *et al*., 2001; Multani *et al*., 2003).

Meiotic behavior defines the possibility of producing viable pollen. Therefore, its evaluation is important for understand the process that give rise to fertile and sterile individuals. It could be more crucial in the case of intergenomic crosses, where cell cycle is under different genomes control, in special in those between diploid and allotetraploid species. The extra chromosome load may be generate genetic unbalance and therefore prevents embryo development. In this view, these additional chromosomes are generally eliminated. This strategy has been reported in intergeneric hybrids of *Brassica napus* and *Orychophragmus violaceus* (Cheng *et al*., 2002), interspecific hybrids of *Hordeum* (Linde-Laursen and Von Bothmer, 1993), somatic hybrids of *Oryza sativa* and *O. punctata* (Shishido *et al*., 1998), and in polyploids as *Paspalum subciliatum* (Adamowski *et al*., 1998) and *Avena sativa* (Baptista-Giacomelli *et al*., 2000).

Meiosis evaluation in rice cultivars was widely described by Chen *et al.* (2005), while chromosome pairing in prophase and metaphase I in individuals arising from interspecific crosses of *Oryza* has been

focused by Gopalakrishnan and Shastry (1966) and Katayama (1995). On the other hand, meiotic behavior of progenies resulting from crosses of the wild tetraploid *O. latifolia* and the diploid *O. sativa* has been poorly studied (Li *et al*., 1962; Multani *et al*., 2003). We discuss here the meiotic behavior of *O. sativa*, *O. latifolia* and the progeny arising of crosses among them, and the implications of chromosome aberrations observed in these materials.

**Materials and Methods**

Seeds of wild tetraploid accession of *O. latifolia* were collected in the Colombian Pacific Coast South. The first cross was achieved using the Fedearroz 50 variety of *O. sativa* as pollen receptor and the wild tetraploid as pollen donator. $BC_1$ was obtained from the one $F_1$ individual named CT18228-8 as pollen receptor and two CIAT improved lines of *O. sativa* and the Fedearroz 50 variety as pollen donors. $BC_2$ and $BC_3$ were produced from only one $BC_1$ individual named CT18487, with BCF1720, Pi9, Fanny and Fedearroz varieties. All $F_1$ and BCs were obtained by embryo rescue in MS medium (1/4), about 12 days after pollination to avoid abortion.

Chromosome number was counted in mitotic cells from roots tips obtained from parents and $F_1$, pretreated with 8-hidroxiquinoline 2mM during six hours and fixed in Farmer's solution (ethanol–acetic acid 3:1) for 24 hours. Cell wall was digested in HCl 1M to 60ºC for 10 minutes. The cells were stained with modified fuchsine.

Meiotic behavior was evaluated in panicles of parents, $F_1$, $BC_2$ and $BC_3$, in the ideal stage for meiosis studies, which were fixed in Farmer's solution for 24 hours at room temperature and then transferred to a new fixer solution and stored under freezing. Anthers were removed. Meiocytes were released and stained with 1% acetic-carmine for evaluation under light microscope. A minimum of 100 meiocytes per phase was considered. Pollen viability was determined using the same 1% acetic carmine. Black and white photography was taking with a Wild MF 45 system in Leica microscope on Kodak T-Max 100 films, scanned in a Nikon Coolscan V ED system and edited with the Software Adobe Photoshop CS2.

**Results**

*O. sativa* and *O. latifolia* genotypes showed a normal behavior in all meiosis phases (Table 1, Figures 1a and b). Some cells of *O. latifolia* presented two nucleoli in both prophases, which did not affect fertility. Because seed development was impossible, $F_1$ plants obtained by embryo rescue showed intermediate morphology between their parents. Some traits such as purple stigma of *O. latifolia* were dominants, and the heterosis effect was seen in the awns size. In general plants structure didn't show important irregularities. However, all individuals were sterile, although a few mature seeds were obtained in $F_1$ plants. Chromosome number in mitotic metaphase was 36 in 20 cells of three individuals, which may indicate the presence of 12 chromosomes from *O. sativa* (A genome), and 24 chromosomes from *O. latifolia* (C and D genomes). The same 36 chromosomes were counted in all diacinesis, mainly as univalent. In this case, bivalents ranged from 1 to 8 per cell (Figure 3a). In prophase I and II a high number of cells had two nucleoli, but one of them disappeared before the other. Due chromosome accumulation in some regions, cytoplasm extensions involving cytoskeleton were observed in prophase I of some PMCs (Figure 3b).

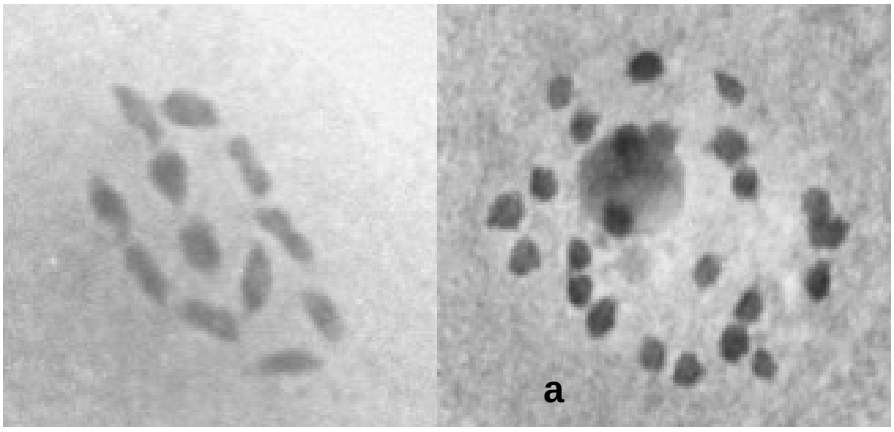


Figure 1. Chromosome number in *O. sativa* and *O. latifolia*. a. Prometafase in *O. sativa* exhibiting 2n=24. b. Diacinesis in *O. latifolia*, with 2n=48.

An abnormal chromosome arrangement throughout cytoplasm was observed in both metaphases of the $F_1$ PMCs. Although chromosomes may congregate in the mid cell plate, spindle formation was irregular and therefore segregation in both anaphases was altered. Due abnormal disposition of chromosomes, it can give rise to multiple spindles in metaphase I (Figure 3c). Abnormalities in the spindle no only was seen in the number but also in the orientation pattern during metaphase II, contrarily to the parallel disposition which is the common pattern in all metaphases of a normal meiosis rice. In these hybrids cells, convergent, perpendicular and lineal patterns were found (Figure 2a-c). These patterns were also observed, although in a low percentage, in aneuploids $BC_2$ and $BC_3$. In some $F_1$ meiocytes there is no spindle formation. Micronuclei resulting by abnormal chromosome segregation and microcytes originated by extra cytokinesis were observed from telophase I to metaphase II (Figure 3d). Microsporocytes and microcytes could present several micronuclei (Figure 3e). In addition, asynchronous cell behavior during meiosis II occurred at regular frequency. While some cells were in metaphase or anaphase II, others were in 'tetrad of microspores' stage, accompanied by microcytes rising from abnormal cytokinesis (Figure 3e). As a consequence of this type of cytokinesis, polyads presenting a varied number of microspores and microcytes were observed (Figure 3f). The number of microcytes ranged from one to four. Finally, pollen viability was very low (0.01%). Pollen grains were highly polymorphic.

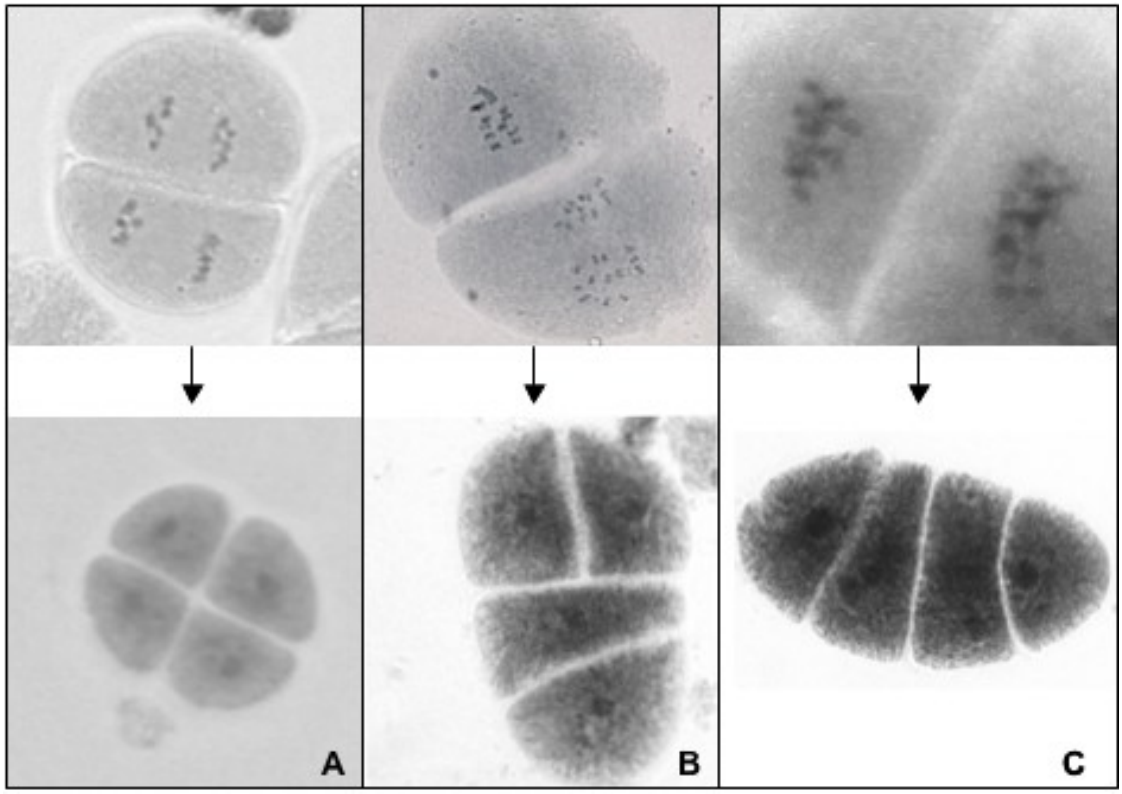


Figure 2. Polyads resulting by irregular spindle formation and cytokinesis. Upper meiocytes in metaphase II and anaphase II, down tetrads produced by the each type of segregation. A. Spindles in parallel disposition produce normal tetrad. B. Perpendicular disposition produce abnormal tetrad. C. Lineal disposition.

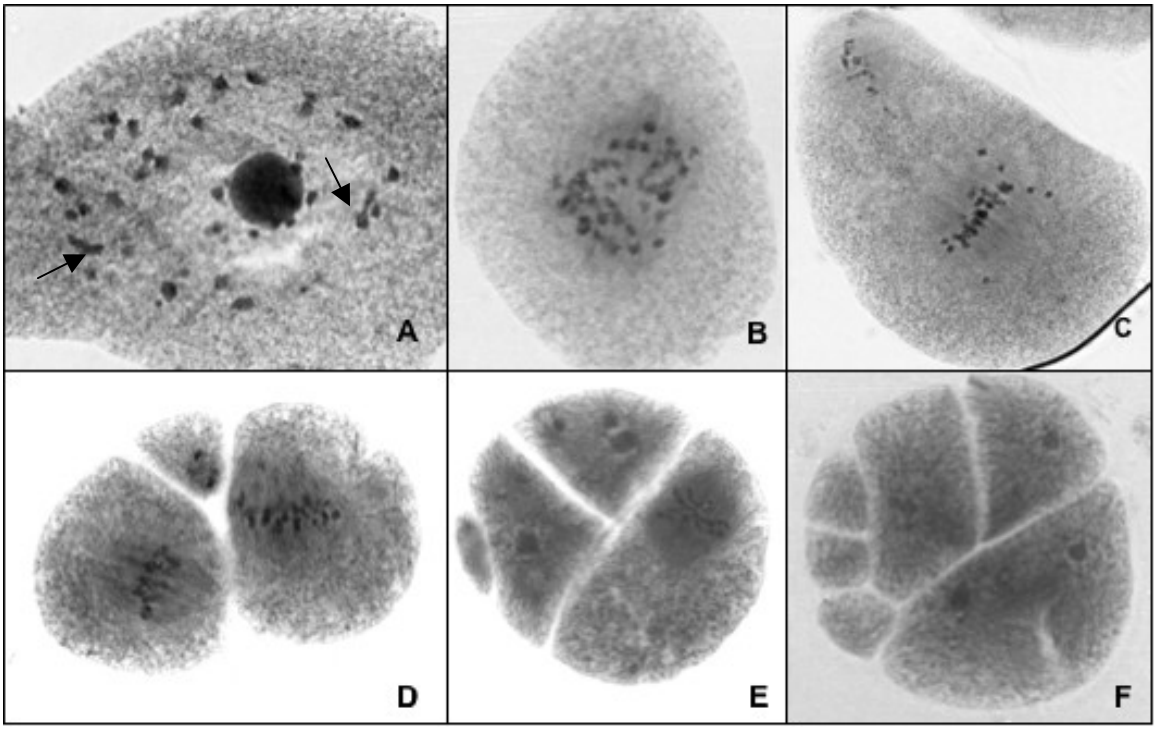


Figure 3. Meiotic abnormalities in $F_1$ *O. sativa* x *O. latifolia*. a. Diacinesis showing bivalents (arrows), while others chromosomes are distributed as univalent. b. Cytoplasmatic extensions in prophase I induced by random chromosome presence c. Asynchronous chromosome segregation during anaphase I. d. Multiple spindles and irregular chromosome segregation in the same metaphase II cell. e. Microcyte formed during metaphase II by additional cytokinesis induced by multiple spindles and irregular chromosome position. f. Abnormal cytokinesis leading to microcyte formation; micronuclei were observed in one microspore (arrow head). One of de cells exhibits phase asynchrony, as anaphase initial (arrow). Polyad resulting by irregular spindle formation and cytokinesis.

Twenty eight $BC_2$ and $BC_3$ individuals were very similar in its architecture to the varieties of *O. sativa*, although presented traits of *O. latifolia*, such as stigma purple, presence of awn, color of grain and height. Eleven plants of both $BC_2$ and $BC_3$ were 12 II+ 1I = 25, 10 were 12 II+ 2 I = 26 and seven were 12 II = 24 (Table 1). Meiotic behavior in these plants was normal except by segregation problems of additional chromosomes (Figures 3a-c). These showed precocious chromosome migration (Figures 3d-f). Individuals with two additional chromosomes showed pollen viability average of 37.85% (ranged from 9 to 65%), while individuals with one additional chromosome 33.22% (ranged from 12 to 72%). Diploid showed an average of 78.86% (ranged from 59 to 94%). Size polymorphism of pollen grain in $BC_2$ and $BC_3$ individuals with additional chromosomes was also observed.

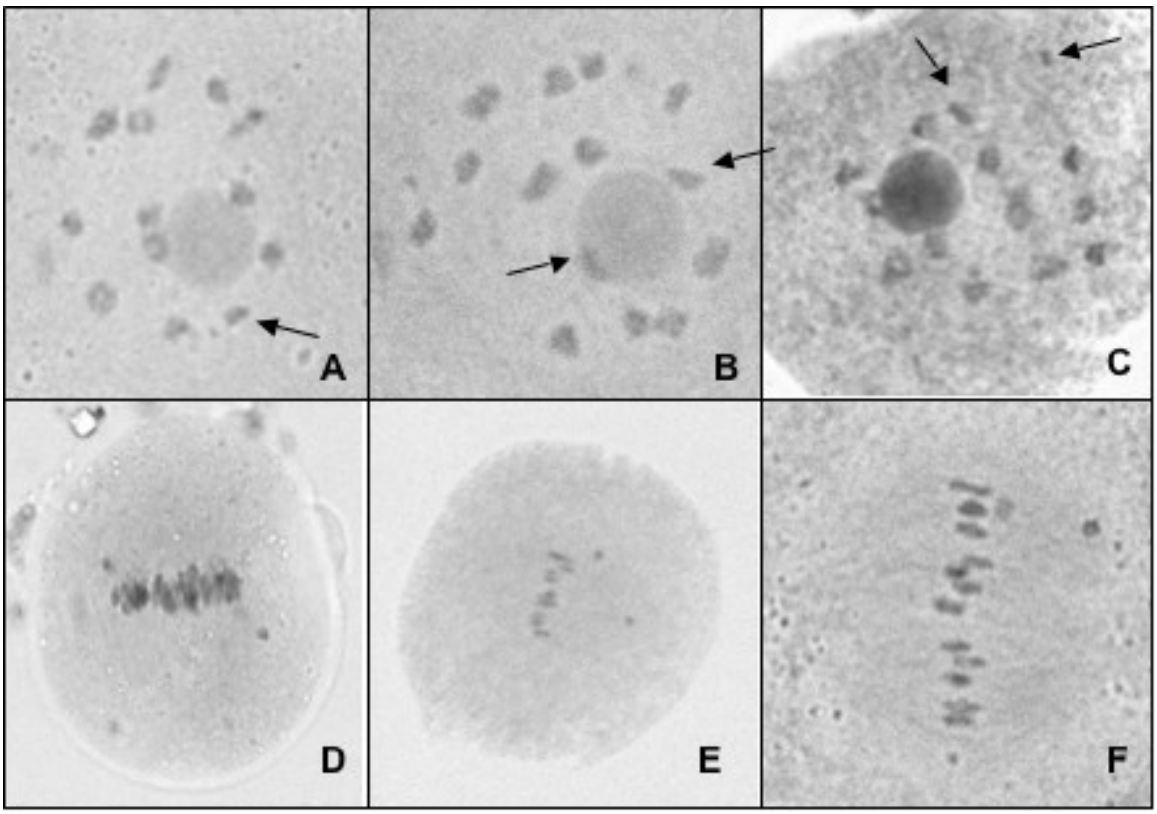


Figure 4. Additional chromosomes in $BC_2$ and $BC_3$ plants. Diacinesis with 12 II + 1 I (arrow, a), 12 II + 2 I (b), 12 II + 1I (arrow) + chromosome fragment (arrow head; c). Irregular segregation of additional chromosomes during metaphase I (d-f).

**Discussion**

*O. sativa* presented a higher meiotic stability, as such as described by Chen *et al*., 2005. Meiotic behavior in *O. latifolia* was normal, despite of its tetraploid condition, composed by two different genomes. Although homeologous pairing may reduce the fertility (Ma and Gutafton, 2005), this pairing in the first step of the development of allotetraploid species is quickly suppressed by changes in the genomes next to the early generations, after duplication (Kashkush *et al.*, 2002; Blanc and Wolfe, 2004).

The way to obtain $F_1$ individuals in the present case was embryo rescue in culture medium, by providing to embryo the nutrients that not were received by the fail in the endosperm development. According to Vinkenoog *et al*. (2003), difficulty to obtain embryos and impossibility to develop seeds in the first cross are caused by gene dosage in tetraploid - diploid hybrids, which affects the ratio of parental genomes, and therefore producing genomic imprinting. These could explain the reduced number of embryos obtained from the tetraploid *O. latifolia* and the diploid *O. sativa*.

Behavior of $F_1$ individuals was completely aberrant in all meiosis phases. A higher number of univalents and a variable frequency of bivalents were observed in diacinesis, which was hoped, because the presence of three different genomes. Several genes and proteins that act in the homologous pairing in rice are known, as PAIR 1 (Nonomura *et al*., 2004), PAIR 2 (Nonomura *et al.*, 2006), and cohesin protein OsRad21-4 (Zhang *et al*., 2006). However, the process of the homeologous pairing is no understood very well. In grasses, chromosome regions conserved among species maintain colinearity (Bowers *et al*., 2005), but chromosome arrangements and changes in the chromosome size generally prevent homeologous

recognition and therefore recombination process. The comparative mapping of *O. sativa* and *O. latifolia* has shown that exists a high conservation between both species (Huang and Kochert, 1994). This idea is reinforced by pairing and introgressions works from *O. latifolia* to *O. sativa* (Multani *et al*., 2003) and by the high detection of introgressions of species nearly to *O. latifolia*, as *O. grandiglumis* to *O. sativa* cv. (Kim *et al*., 2003). Moreover, preliminary results using molecular markers in the lines originated of our cross, confirm the introgression of *O. latifolia* in nine of the 12 chromosomes of *O. sativa* (unpublished data). It suggests that recombination could be affected by fails in the synaptonemal complex (SC) between homeologous chromosomes. Expression of two different genomes from the formation of SC could inhibit correct formation of this structure. In the case of co-expression, the structural protein subunits could fail in the recognition of subunits generated by the other genome, and in the recognition protein-DNA. Thus, recognition of homologous regions of both genomes would be given by others different mechanisms to SC or randomly encounters among chromosomes with homologous sequences. The random encounters could explain the variable number of bivalents by cell. This is possible since SC cannot be strictly required for meiotic exchange (Dernburg *et al*. 1998). May exist proteins involved in the homology searching such as Rec102 and mei4 in yeast, but in absence of these there exist alternative mechanisms that allow the pairing (Nag *et al*. 1995). Comparison among the protein involved in the SC formation in several species of *Oryza* genus with different genomes could contribute to understand homeologous pairing and genome evolution. In addition, comparison of physical maps of the *Oryza* genus, achieved in the Oryza Map Alignament Project (OMAP) (Wing *et al*., 2005) also would help.

The protein OsRad21-4 orthologue of yeast Rec8 protein is required for homologous pairing and for the joint of sister chromatids in rice, but contrarily to the mutants from this protein evaluated by Zhang *et al*. (2006) and in maize meiotic mutants by Chan and Cande (1998), which showed chromosomes of the interspecific hybrids between *O. sativa* and *O. latifolia* in metaphase I, does not fail in the cohesion of sister chromatid, and the segregation is give in a ramdom distribution of the univalents towards the poles according to the orientation of kinetochore (figure 2B), taking place at separation of sister chromatids in anaphase II, such it occur in haploids of durum wheat (Jauhar *et al*., 2000).

In many cases, meiotic division take place on acentriolar spindles. After to the nuclear envelope breakdown, microtubules are established as an array of microtubules around condensed chromatin in absence of centrosomes or discrete microtubule-organizing centers (McKim and Hawley, 1995). From here the microtubules are self-organized and is defined the polarity. In this way, the spindle formation independent of centrosomes could explain the appearance of multiples congregations of chromosomes in metaphase I, multiple spindles and later several micronuclei. The segregation in anaphase I depend of the interaction between the kinetochore and the microtubules. The normal segregation in meiosis I involved the kinetochores of both homologous chromosomes of a bivalent, each kinetochore is oriented towards one of the poles give rise to a bivalent bi-oriented, and therefore, equal distribution of chromosomes in the daughter cells. However, in the $F_1$ hybrids from *O. sativa* and *O. latifolia*, each univalent is oriented toward anyone side, only depending of the kinetochore positioning, this make that the distribution of homeologuos chromosomes be asymmetric in the daughter cells, except for the chromosomes that formed bivalents, while that in meiosis II the distribution among the daughter cells may be equal, since the distribution depend of the segregation of the sister chromatids. Is possible that these cytologic phenomen also are present in the first steps of natural polyploidization and would be surpassed by the duplication and later stabilization of the genomes.

Chromosome congression in the metaphase plate in mitosis is give when chromosomes begin to be bi-oriented, when the congression is finalized, begins the anaphase of a synchronous way towards each pole (Kapoor *et al*., 2006), is possible that in meiosis the transition among metaphase I and anaphase I work in the same way, but in meiocytes with a high number of univalents the bi-orientation is no possible. Therefore, metaphase and anaphase I transition does not exist, and the segregation is completely asynchronous, as well as the later phases (Figure 2E). This is also true in the individuals $BC_2$ and $BC_3$

with one or two additional chromosomes, when the univalents have irregular segregation (figure 3), although in some cases its segregation agree with the other chromosomes.

Incorrect orientation and the fail in the congression make that the chromosomes be distributed in a disorder way in the cytoplasm during metaphase I, the later accumulation of microtubules around of separate chromosomes groups can to form meiotic spindles for each group, generating multiple spindles for cell (figure 2C). This behavior is similar to the described one by Caetano-Pereira and Pagliarini (2001) in maize, however in this case the aberrant behavior possibly is give by the mutation in a gene that it controls the formation of the spindle, since there are a normal formation of bivalents. On the other hand, Risso-Pascotto (2005) has described multiple spindles in interspecific hybrids in *Brachiaria*, and this hybrids present problems in the segregation although the number of univalents is not as high as in the hybrids between *O. sativa* and *O. latifolia*. Would be possible to be thought that each spindle is involved with each genome of the parents, but the relatively low rate of this aberration in some meiocytes in metaphase I (8%), and the several accumulations of chromosomes in others as micronuclei in others cells, take to think to us that the organization of the chromosomes in groups in the cytoplasm is an event completely random.

Each event of chromosome accumulation creates a different system of microtubules generates from the chromatin in each point of the cytoplasm. In normal later anaphase I, is begin a reassembly of the microtubules of the spindles, followed by the synthesis of polysaccharids how a perpendicular line to the spindle line for the formation of fragmoplast and the later citokinesis (for review see Verma, 2001). In the $F_1$ hybrids this happens around of the several micronuclei generating a variable number of microcytes (figure 2F). However, this does not happens with all the micronuclei, since sometimes several micronuclei are remains in a unique daughter cell (figure 2E). Therefore is possible that some specific chromosomes inside the groups formed be involved with the cytokinesis. The cytokinesis and morphology of the tetrads is affected also by the orientation pattern of the spindle lines, this abnormality does not only was seen in $F_1$ individuals but also in $BC_2$ and $BC_3$ individuals with additional chromosomes.

Up to here is clear that the sterility in hybrids $F_1$ is caused by the high amount of aberrations in the meiotic process, but the really surprising is that from the pollen cells generates in a aberrant meiosis such as this, be possible grow new plants by means backcrossing and even be possible obtain some few seed $F_2$. But this can be explain by the high presence of random events of chromosomal distribution in the cytoplasm of hybrids meiocytes, each cell have a genome component that will be distributed of different manners in the daughter cells, thus, in the case of one backcross the sexual cell generate of this meiotic events that have major similarity in its genome component with the one of *O. sativa*, will have more probability of pairing with the gametes of this. On the other hand, the few events in which two cells, generates one by microsporogenesis and other by megasporogenesis in a same flower in $F_1$ individuals, have genomic components seemed also could give rise the seed $F_2$ that were obtained. However, in embryos generate by backcross is possible that the genome component extra (chromosomes of *O. latifolia*) continue affecting the development of the endosperm being required again for its development the embryo rescue in culture medium. Therefore, the elimination process that take place in the first steps is giving by the formation of micronuclei and later elimination of microcytes is very important for fertility restoration, as also is described recently in others interspecific hybrids by Qian *et al*. (2005) and Mendes-Bonato *et al*. (2006). Uniparental chromosome elimination also has been seen in somatic cells at mitosis in interspecific hybrids (Gernand *et al*, 2005). However, the evaluations achieved in root tips of $F_1$ individuals has not showed somatic elimination, as well as the fact of that the meiocytes always present 36 chromosomes distributed among univalents and bivalents, reinforce this observation. Although for recovery the diploidy in backcross is necessary the elimination of wild chromosomes, this does not mean that all elimination be uniparental, but only the cell with chromosome component similar to *O. sativa* can pairing for grow a new generation. Thus, in the individuals originated by backcrosses only show a few additional chromosomes, that although affect the fertility of the plants, are eliminated by segregation in one or two generations. The

results also has suggest that the fertility in $BC_2$ and $BC_3$ individuals, may depend of the additional chromosome present, since some plants with additional chromosomes present production of seed, but not others and the pollen viability is very variable, thus as the presence of some plants with polymorphic pollen. Some regulatory pathway may be controlled by genes located in specific chromosomes, presence of one or several additional chromosomes with genes involved in this regulatory pathway produce dosage effect that could inhibit them (Birchler *et al*., 2005), in this cases pathways or genes involved with the plant reproduction.

Finally, is possible obtain in $BC_2$ and $BC_3$, plants with a normal meiotic behavior and a high seed production as well as with interest traits of the wild rice, *O. latifolia*. After of a process of aberrant meiotic and chromosome elimination by mean of backcrossing, the generation of plants with wild introgressions of species of the *Oryza* genus with different genomes at A of *O. sativa* is possible, and is a important step to broad the rice gene pool using all the species pertaining to *Oryza* genus. However, is necessary understand better the process involved in the homeologous recombination, therefore, works in molecular cytogenetic and function and evolution of protein are required for this purpose.

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## 2.1.11 *In Vitro* Regeneration of *Solanum quitoense* (Lulo) Plants

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**Abstract**

The objective of this work was to improve the plant regeneration efficiency of lulo plants from leaf petioles. Two treatments were tested. Tissues were either sonicated or not prior *in vitro* culture. Sonication was tested as an alternative to increase the permeability of the plant tissue because the large trichome density in the leaves affect a direct contact with the culture medium.

**Key words:** lulo, *Solanum quitoense*, plant regeneration, sonication

**Introduction**

Naranjila or lulo (*Solanum quitoense*) as it is known in Colombia, has become a crop with great potential for opening markets and revenues to small farmers in the Andean region. A current industrial demand includes its use for the production of concentrated juice, yogurt, and fruit pulp for export markets, in addition to the traditional fresh market. Additionally, lulo has a great potential in the international market as exotic fruit (García and García, 2001). Despite all these advantages, the rate of adoption of this crop by farmers is greatly restrained by its high susceptibility to diseases and pests, post harvest deterioration, heterogeneity in the fruit quality, as well as low accessibility to clean (pathogen free) starting materials for planting. These factors contribute to high production costs and increase its vulnerability (Segovia & Lentini 2002). Genetic improvement is a key alternative to develop new resistant materials to diseases and pests. Biotecnology aided, including genetic transformation, could also facilitate splicing in genes for traits of interest. Plant regeneration is usually the main bottleneck for an efficient protocol of genetic transformation. CIAT with co-financing from the Colombian MADR (Ministerio de Agricultura y Desarrollo Rural), developed *in vitro* protocols to facilitate *(a)* the conservation of germplasm, *and (b)* the multiplication and distribution of healthy elite clones selected by farmers. Previous reports showed that farmers are interested in growing *in vitro* propagated plants in their fields. *In vitro* propagated plants in addition of providing a homogenous, true-plant type and clean starting material, reduced flowering and fructification time in about 1 month with respect to the highly heterogeneous plants propagated through botanicals seeds used by farmers without affecting crop productivity and fruit quality. This year report describes a plant regeneration system from petioles applicable to genetic transformation of this species.

**Materials and Methods**

Experiments were performed using *in vitro* propagated plants of clone No 19 previously selected by CIAT in the field; this clone has shown good response to plant regeneration (Ruiz *et al*., in SB2 Annual Report 2003). *In* vitro plants were propagated in 500 ml glass jars containing medium A, which consisted of MS salts, 0,3% of sucrose, calcium panthotenate (2.5mg/L), thiamine HCL (0,5 mg/L), pyridoxine HCL

(1mg/L), nicotinic acid (5 mg/L), solidified with Gel-rite® (3,5 g/L).Petioles were identified as the most responsive explant for plant regeneration as compared to leaves (Segovia, 2002). Fifteen day old *in vitro* plants were used as the petiole explants donors. Petiole explants were either sonicated or not, prior cultured. For sonication, the first un-expanded leaves were excised and immersed in liquid MS medium supplemented with Zeatine (2 mg/L), followed by sonication during 1 minute at 60 volts, using a Branson 450 Ultrasonic Corporation apparatus, followed by vacuum at 500 mmHg for 15 minutes. Eight petioles explants were cultured per Petri dish. A total of 120 explants were sonicated and 120 were cultured without sonication per experiment. Four experiments were conducted. Explants were placed axial side down on solid regeneration media (Ultzen, *et al*., 1995), consisting of MS salts, B5 vitamins, sucrose 0,1%, glucose 0,1%, zeatine 2 mg/L, Gelrite ® 1.5g/L and to agar g/L, and cultured in the dark at 21ºC during 2 days and then transferred to light for 16 weeks. Evaluations were conducted every week. A block design completely randomized with four repetitions was used; each repetition consisted of 15 experimental units by treatment.

**Results and Discussion**

Previous work reported plant regeneration up to 15% explants with at least one shoot, when using petioles of clone Not 19 with thorns (Segovia and Lentini, 2001). This work indicated that petioles were more responsive that the leaf blade and that sonication was a key treatment, however the regeneration rates were suboptimal for genetic transformation. Subsequently, Ruiz *et al*. (2005) initiated a systematic evaluation of the plant regeneration protocol to assess the possibility of eliminating the sonication treatment since sonicated tissues were more prone to deterioration and death during agro-infection for genetic transformation. Results reported in 2005 (Ruiz *et al*., 2005) indicated for the first time that it was possible to regenerate plants from petioles without sonication in contrast to results reported by Segovia (2002) which suggested that sonication was indispensable to induce plant regeneration.
Results reported this year indicate that results reported by Ruiz et al (2005) are reproducible with a large number of explants and repetitions. About 76% of the sonicated petioles explants showed plant regeneration and those without sonication showed 67% plant regeneration (Figure 1, significant difference at $p= 0.0176$). Clear differences were noted at 5 weeks of culture for both treatments (Figure 1). The explants with organogenesis generated a total of 1762 regenerated plants from sonication, and 1242 regenerated plants from treatments without sonication (Figure 2, significant difference at $p= 0.0045$). These results suggest that each sonicated explants may generate in average 3,9 plants whereas those tissues without sonication yield an average of 2,6 plants per explants.

**Conclusions**

Although plant regeneration from sonicated explants is significantly higher (76%), the response of the non-sonicated explants is close to 70% making this treatment an attractive and suitable protocol for genetic transformation since sonication treatment had shown to induce more rapid tissue deterioration after co-culture with Agrobacterium.

**Future Plans**

Currently, the protocol described herein is being used to develop a genetic transformation protocol of lulo by *A. tumefaciens*.

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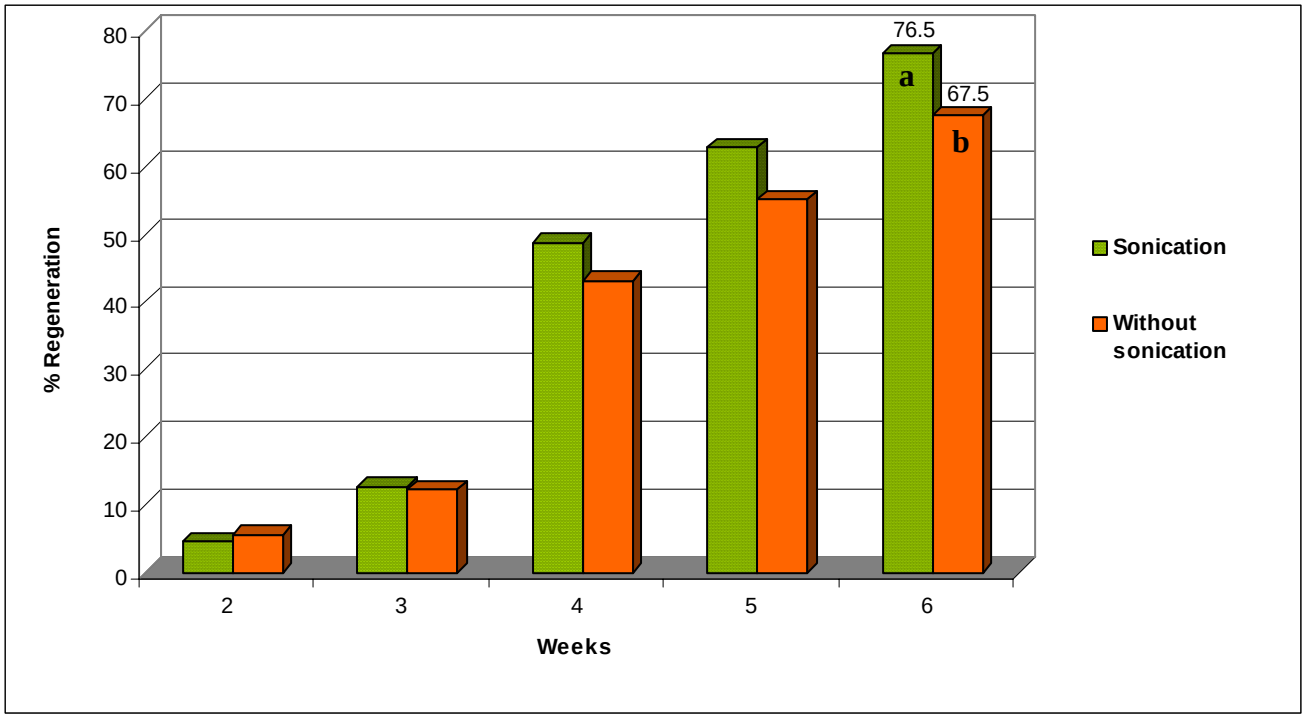


**Figure 1**. Plant regeneration from petiole explants of lulo with or without sonication prior *in* vitro culture.

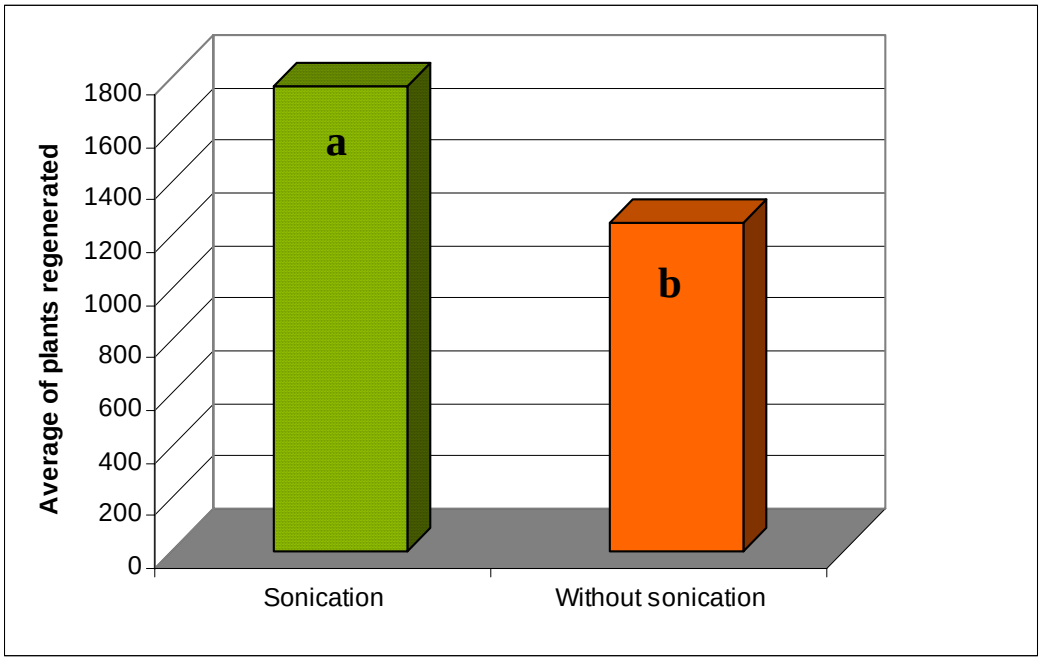


**Figure 2**. Total number of plants regenerated at 16 weeks after culture from treatments with or without sonication.

## 2.1.12 Somatic Embryogenesis and Plantlet Regeneration of Mango (*Mangifera indica* L.)

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**Abstract**

The lack of a reproducible and highly efficient plant regeneration protocol was identified as a key bottleneck for genetic transformation of mango at the onset of the execution of this project. The first report (February-December 2005, Florez et al., SB@ Annual Report 2005) described the progress attained on inducing somatic embryogenesis from nucellar tissues of immature fruits (48% induction with Keitt commercial cultivar), and the identification of key environmental and physiological factors to bear in mind when selecting the mother plants used as explants donors in order to reach reproducible results. The progress described in 2006 includes the identification of conditions for increased conversion of somatic embryos into complete developed rooted-plants *in vitro*, the successful transfer of plants from *in vitro* conditions and development of healthy growing plants in the greenhouse, and the first putatively transformed mango embryogenic masses with expression of the reporter *gus* gene. Results indicate that this component of the project delivered the agreed outputs in 18 months instead of 24 months as originally proposed.

**Key words:** mango, somatic embryogenesis, nucellar tissue, plant regeneration

**Introduction**

In the last 15 years there has been a tremendous increase in areas dedicated to mango production in the tropics and subtropics. Mango production accounts for an estimated 38 percent of total tropical fruit output in 2002 with a total production of 25 million tons. In Africa mango production is currently at the level of 2.6 million tons annually (FAO 2004) worth an estimated US$ 42 million (ICIPE 2004). Smallholders supply over 90% of mangoes produced (ICIPE 2004). Mango production is appealing because the fruit is nutritionally important and constitutes an attractive option to increase income and reduce poverty in the rural sector of developing countries. Despite its importance and worldwide distribution, mango suffers from a long juvenile period, erratic flowering and alternate bearing habits. Flowering and its control is one of the most important development aspects of crop plants. There are still many crop plants in which control of flowering is not optimal. Opportune flowering could greatly increase fruit crop productivity and income generation through a better synchronization of fruit production with market demand. In the tropics, fruits can be produced throughout the year; nevertheless fruit producers face major problems with the seasonality of their production due to uncontrolled flowering and the consequent difficulties in balancing supply and demand of high quality fruits. Biotechnology can potentially be used to manipulate existing cultivars by targeting specific genetic traits, such as flowering behavior. If farmers gain the ability to control flowering in fruit trees, they could better target their products to the highly competitive markets of fruit commercialization. The principal objective of this project is to manipulate the expression of a target set of developmental genes in mango that are known to modulate flowering in other species such as *Arabidopsis* and Citrus. To apply these tools, a consistent embryogenesis and regeneration protocol is required. In the case of mango, routine techniques for plant regeneration and genetic need to be established as first step. This report described the progress attained in the 2-year work plan approved by the Rockefeller Foundation.

## Materials and methods

*Induction of Somatic Embryogenesis*
Immature fruits of 'Keitt' (~ 4 cm long) and 'Mangdalena River' (~ 3 cm long) cultivars collected from the Varahonda and CIAT mango fields were used. Fruits surface sterilization, nucellar tissue extraction and culture on the induction medium MMSE (Pateña *et al*., 2002) were made following the same procedures detailed by Flórez *et al*. (2005). In addition, four media combinations were tested for somatic embryogenesis induction, which included different concentrations of growth regulators 2,4-D and thidiazuron (TDZ). After 3-5 days of culture, the explants were transferred onto fresh medium on daily basis to prevent accumulation of phenolic compounds. Subsequent subcultures were done every 20 days.

*Development of Somatic Embryos*
Pro-embryogenic masses (PEM) were isolated and cultured on MMSE medium (Pateña *et al*., 2002). Subsequently, masses of globular embryos were transferred to Recovery (REC) medium (DeWald *et al*., 1989 modified by Manzanilla, 2004). Until this stage, all cultures were cultured in the dark at 28±2$^{o}$C. Once embryos reached the torpedo stage, somatic embryos were transferred to culture vessels with aeration and incubated under a 16h photoperiod, on the Maturation (MAT) medium to reach the cotyledonary stage (DeWald *et al*., 1989 modified by Manzanilla, 2004). Embryos at advanced cotyledonary stage were incubated in germination medium (GER) (Ara *et al.*, 1999 modified by Manzanilla, 2004).

*Induction of Secondary Embryogenesis using Temporary Immersion (RITA)*
A new approach to induce secondary embryogenesis was tested. Germinated somatic embryos of 'Keitt' cultivar were selected for these experiments. The physical and biological factors as well as advantages of using temporary immersion method have already been described for other crops. Somatic embryos were cultured in the RITA system using 200 ml of MMSE liquid medium (Pateña *et al*., 2002) and immersion 1 minute every 8 h.

*Necrosis Apical Control*
Germinating somatic embryos with developed roots and green cotyledons (1-2 cm long) showed shoot tip necrosis, a typical bottleneck reported for mango at this stage of *in vitro* development. Serial experiments were conduced to overcome this problem. Treatments included: *(1)* application of a drop of MMPR liquid medium on the shoot tip, *(2)* periodical immersion of shoots in a double-calcium strength MMPR liquid medium; *(3)* culture in double phase medium (liquid/solid); *(4)* change of culture vessel to improve aeration including the culture in temporary immersion system (RITA) and *(5)* changes in nutrient composition of the embryo-germination medium, mainly in macronutrients, in order to obtain an improved ionic balance and to facilitate availability of nutrients, determinant factors for *in vitro* plant growth.

*Acclimatization of In Vitro Grown Plantlets to Greenhouse Conditions*
Healthy looking plantlets developed *in vitro* were transplanted to the greenhouse in bags containing a mixture of sand: soil: peat moss in the radio 3:1:1. The plantlets were covered to maintain high humidity and control exposure to sunlight, initially intermittent sunlight exposure of 30 min/day, and gradually increased time exposure until plastic-glasses were completely removed 15 days later.

*Genetic Transformation of Mango*
*Agrobacterium* mediated transformation was conducted using the C58C1Rif$^{R}$ strain harboring the binary vector pATARC3-B1b containing the *npt*II (neomycin phosphotransferase II), the *uidA* (GUS) and the *arc5-I* genes (Goossens *et al*., 1999). This *Agrobacterium* strain/vector combination has proven to be effective with other dicot species, and the kanamycin selection gene (*npt*II) was previously reported as effective selection for mango (Mathews *et al.*, 1992 and Cruz-Hernandez *et al.*, 1997). A single colony of *A. tumefaciens* C58C1 (pATARC3-B1b) from LB agar medium supplement with10 mg l$^{-1}$ rifampicin and

50 mg $l^{-1}$ kanamycin was inoculated into 5 ml of liquid broth of the same composition at pH 7. After 16 h, 1 ml was transferred to 50 ml of liquid broth supplemented with 100 □M acetosyringone and incubated at 250 rpm / 28ºC for 5-6 h, until *Agrobacterium* reached $O.D._{600}$ = 0.8 (log phase culture). 'Keitt' PEM were lightly abraded, cultured in MMSE medium plus 100 □M AS and dripped with a log phase culture of acetosyringone-activated *Agrobacterium* suspension. Young tobacco leaves were used as genetic transformation efficacy control. Plates were incubated at 21°C in darkness for three days. After the co-cultivation with *Agrobacterium*, PEM were rinsed with MMSE liquid medium plus 500 mg $l^{-1}$ cefotaxime, and transferred directly onto MMSE supplemented with 500 mg $l^{-1}$ cefotaxime (to eliminate the bacteria) plus 200 mg $l^{-1}$ kanamycin (as plant selection agent.) After two months on this selection medium, PEM were transferred to the same medium composition except with kanamycin 400 mg $l^{-1}$, used herein as lethal concentration (Mathews *et al.*, 1992 and Cruz-Hernandez *et al.*, 1997). Healthy growing tissues on kanamycin selection medium were tested for histochemical GUS expression (Jefferson, 1987).

**Results and Discussion**

Results suggest that a combination of low concentrations of 2,4-D (auxin) with TDZ (cytokinin) significantly improved the embryogenic response from nucellar tissue of 'Keitt' cultivar . Somatic embryogenesis induction was increased to about 50% compared to the control medium MMSE (Pateña *et al.*, 2002). M4 induced the highest embryogenesis response. Similar results were obtained by Córdoba (2005) using a combination of 2,4-D 1 mg $l^{-1}$ and TDZ 0.5 mg $l^{-1}$ with a different basal medium, improving the quality (reduced PEM phenolization) and the quantity of somatic embryos induced from 'Magdalena River' mango cultivar.

A large number of secondary somatic embryos were generated when using the immersion temporary (RITA) system. Secondary embryogenesis was generated from the hypocotyls. Results indicated that the number of embryos formed as well as their morphology is improved with RITA. Increased number of embryos with cotyledonary shape and two distinct cotyledons are noted. The standardization of embryo formation resulted in a more reproducible and synchronous development of proembryogenic masses and somatic embryos with normal shape.

Mango somatic embryos with the sequential developmental stages (globular, heart, torpedo and cotyledonary stages) were observed using the experimental cultivars (Figure 1f-i). Total of 262 somatic embryos with advanced cotyledonary stage were recovered, of these 226 embryos were derived from cultivar 'Magdalena River', 10 embryos from 'Keitt', 7 embryos from 'Manzano Vallenato', 9 embryos from '505-4' and 10 embryos from 'Yulima", respectively. Although some somatic embryos germinated their survival or conversion into plantlets was very low (~10%). The main problem associated with this low conversion rate was due to necrosis at the shoot tip.

Six different treatments were evaluated to reduce apical shoot necrosis in mango. A significant reduction in apical shoot necrosis was obtained by changing the macronutrient composition in the medium (liquid GER medium) used for inducing germination of somatic embryos. B5 and MS basal media are commonly used in mango tissue culture. These two basal media were originally developed for soybean and tobacco *in* vitro culture. Plant nutrition requirements vary from species to species. These differences are noted not only *in planta* but also *in vitro*. The elements and concentrations as well as the salt form in which they are supply affect their bio-availability, and the *in vitro* response. However, these factors are not usually studied in plant tissue culture and a common practice is to use basal medium reported elsewhere. For this reason, various combinations of macronutrients were tested aiming to find an optimal macro salt formulation for mango.

Calcium and potassium are essential for plant development, but they have antagonist effects. A balanced ratio of $Ca^{++}$: $K^{+}$ and in relation to nitrogen and magnesium have major effects on plant development. B5 and MS salt formulation were not developed for to woody plants. The major salts formulation tested were defined emulating those found in fertilizers. In the new formulation, $Ca^{++}$ concentration was increased almost four times compared to the concentration used in B5 medium. Calcium availability was improved by using calcium nitrate and reducing significantly potassium concentration in the medium. Additionally, the nitrogen source was supplied as nitrate instead of as ammonium salt. This new formulation is more similar to other tissue culture media used successfully in woody species. So far, mango somatic embryos continue a normal development into plantlet without shoot tip necrosis symptoms when transfer to this new salt formulation after root initiation. The evaluation of this new salt composition is still in progress.

None of the non-transgenic control survived the stepwise selection with kanamycin using a concentration of 200 mg $l^{-1}$ and then 400 mg $l^{-1}$. The entire non-transgenic callus cultures died after this selection scheme. In contrast, some PEM co-cultivated with *Agrobacterium* showed healthy callus formation from secondary embryogenesis after two months on kanamycin selection medium. Histochemical assay to detect β-glucuronidase activity demonstrated *gus* expression in these tissues suggesting they are putatively transformed. At present, somatic embryos resistant to kanamycin were transferred from MMSE kanamycin-selection medium to REC kanamycin-selection medium, in order to recover kanamycin-resistant plantlets and conduct the corresponding molecular analyses (Southern and Northern blots) to confirm the transgenic nature of these plants.

**Conclusions**

Protocols to induce proembryogenic masses of thirteen mango cultivars and the embryogenic response were tested. Current protocol generates somatic embryogenesis, and development of embryos into plantlets for a couple of commercial mango genotypes. The first healthy plants developed were successfully transplanted to greenhouse conditions. The use temporary immersion system proved to be ideal for proliferation of somatic embryogenesis. A new macronutrient basal medium composition containing increased levels of calcium and using nitrate instead of ammonium as the main source of nitrogen significantly reduced apical shoot necrosis, a common bottleneck reported in mango tissue culture. Some proembryogenic masses with *gus* expression were recovered after co-cultivation with *Agrobacterium* and a stepwise selection in medium containing kanamycin at lethal concentrations for control tissues. Plant regeneration from putatively transgenic PEM is in progress.

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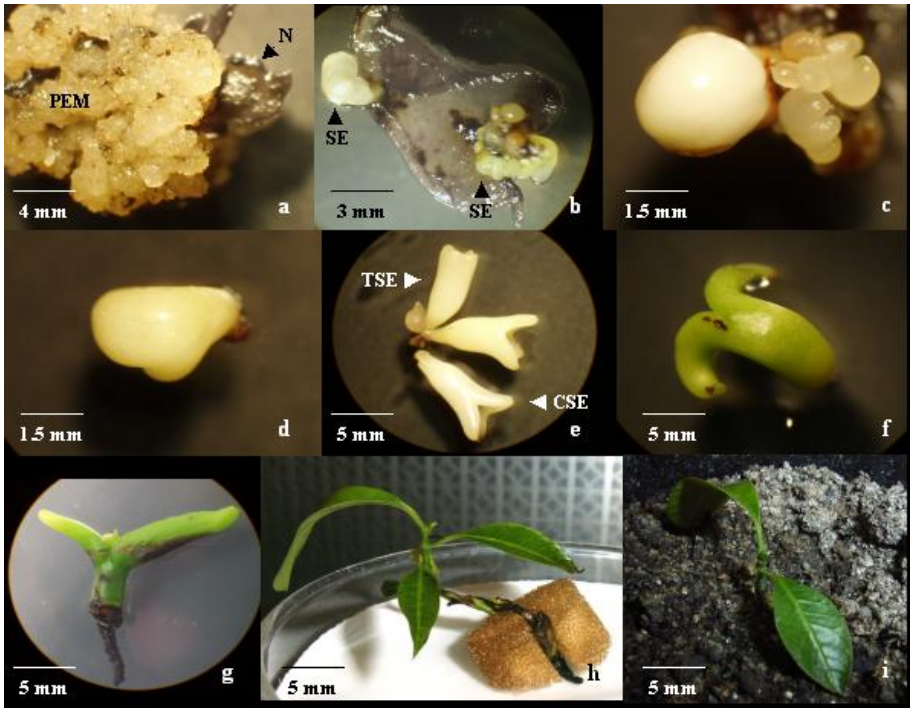


**Figure 1.** Mango somatic embryogenesis from nucellar tissues of 'Keitt' and 'Magdalena River' cultivars. *(a)* indirect somatic embryogenesis in 'Keitt' cultivar, PEM formation; (b) direct somatic embryogenesis from nucellar tissue of 'Magdalena River' cultivar; *(c)* somatic embryo at globular stage; *(d)* somatic embryo at heart stage; *(e)* torpedo and early cotyledonary somatic embryos; *(f)* advanced cotyledonary stage somatic embryo; *(g)* germinated somatic embryo; *(h)* plantlet in GER medium; (*i)* Plantlet acclimated in the greenhouse. N: nucellus; PEM: proembryogenic masses; SE: somatic embryos generated *in vitro*; TSE: somatic embryo at torpedo stage; CSE: somatic embryo at cotyledonary stage.

## 2.1.13 Assessment of gene flow in Maize's (*Zea mays*) at field commercial scale: A first step for testing and adapting gene modeling for tropical conditions as a tool for biosafety decision authorities

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Funding from GTZ (Germany), Colombian Biosafety project GEF-World Bank,the Colombian MADR (Ministerio de Agricultura y Desarrollo Rural), ICA and CIAT.

**Abstract**

The goal of this project is to generate baseline genetic information for the development of guidelines on the safe introduction and use of novel agriculture traits (biotechnology derived or not native from the place of introduction), while reducing potential environmental impact on native biodiversity in the Neotropics, in this case, using non-transgenic crops as models Gene flow models available and developed for European environmental conditions will be adapted for their use in the Neotropics. These models will also serve for anticipating a potential impact from a transgenic situation.

**Key Words:** gene flow, maize, landscape, commercial hybrids, non-transgenic tracer marker

**Introduction**

Gene flow via pollination is a natural process whereby genes, aided by wind or insects, are exchanged between plants. This process can occur between sexually compatible plants, land races, weedy and wild relatives, if the appropriate conditions are met. Unintended cross-pollination of different cultivars of a crop can sometimes occur with commercially improved varieties, developed through conventional breeding or biotechnology, or landraces, which are locally adapted varieties derived from thousands of years of seed selection by farmers. Nevertheless, pollen-mediated gene flow takes importance only when, viable pollen successfully out-competes with locally produced pollen to form viable seed; this process has received renewed attention with the need to ensure commercial coexistence of genetically modified (GM) crops and non-GM crops grain production (Timmons *et al*., 1996). The rapid development of plant genetic engineering has led the deployment of transgenic crops and simultaneously to the need of a thorough assessment of the risks associated with their environmental release. Among the commonly listed risks is the transfer of foreign genes through pollen dispersal from GM crop to other cultivars of the same species. Many factors determine the likelihood of gene flow between two genotypes, including: (a) amenability to cross pollination, which commonly occurs in crops and is species dependant; (b) synchronicity of pollen shedding and receptivity of the two varieties; (c) crop pollen longevity, which can be minutes for some crops but hours for others; (d) relative amounts of pollen produced by the varieties; (e) pollination agents and pollen transport via wind, insects or both; (f) the physical proximity of the varieties to each other, which is of primary importance; and, (f) weather conditions (Eastham and Sweet 2002). Physical proximity of two varieties depends not only on the distance between cultivated fields but also on the inadvertent planting of a few seeds of one variety within a field of a different variety. Such inadvertent, but unavoidable seed mixing can occur via a number of routes. Research is being done to determine effective methods for decreasing or preventing pollen-mediated gene flow. Some involve non-technical solutions, such as increasing required isolation by time, distances or using border rows of non-GM crops to decrease the presence of GM material, others involve the use of biotechnology to manage gene flow (Halsey et el., 2005; Luna et al., 2001; Ma, B.L. et al., 2004; Gustafson et al., 2006). In Latin-American, gene flow in maize has not been studied to fullness. Maize can be used as a model for other wind pollinated crops. In this paper we report the assessment of wind-mediated pollen gene flow using a planting designed selected by the European project Sigmea, as model design to analyze effects across

regions and conditions. The data generated in Colombia will allow testing the gene flow simulation model develop by Sigmea, test its applicability to tropical conditions and modify it according to needs.

**Materials and Methods**

*Plant Material.* Maize commercial hybrids DK4004 (yellow-seeded corn, pollen donor) and DK777 (white-seeded corn, pollen receptor) were used in this study.

*Experimental design.* Field plots were prepared following standard commercial practices. Certified hybrid seeds were sown in July 15 of 2006 at ICA experimental Station in Palmira. Hybrid DK4004 (yellow-seeded corn, pollen donor) was sown in 2.5 Ha. The material was planted twice in alternate rows with an interval of 3 days between the first and the second planting, in order to warrant coincidence in flowering with the white-seeded maize (pollen receptor). Hybrid DK777 (white-seeded corn, pollen receptor) was sown in an adjacent 8.5 Ha located at 24 meters down South following the main wind direction respect to the pollen donor field in order to favor pollen movement from the donor to the receptor (Figure 1A). This plot design allows to determine pollen flow up to 325 meters respect to the border from the pollen donor field (yellow maize) (Figure 1A). Standard commercial agronomical practices were applied to both fields.

*Data collection.* Plant height was evaluated at 15 days after planting. The number of days to 50% and 100% of flowering was registered in both plots. When the plants were 30 days old, each sampling point were marked with wood stakes at different distances from the pollen source (Figure 1a and 1C). The sampling points are indicated by each point of intersecting lines (Figure 1A and 1C). Each sampling point was represented by 30 plants collected around each point. An automated mobile meteorological station (generously lent by Cenicaña, Figure 1A) was installed between the pollen donor and pollen receptors fields to register the climatic conditions 24 hours for about 2 weeks during the anthesis cycle (starting when the first plant flowered until the last plant flowered), the parameters registered included wind speed and direction, temperature, relative humidity, rain precipitation and solar radiation. The monitoring of these meteorological and biological parameters will allow testing and modifying the gene flow simulation model according to needs.

*Data Analysis.* Gene flow was estimated by analyzing the number of white hybrid cobs containing yellow grains, and the number of yellow grains per white hybrid cobs were registered (Figure 1C). Samples were collected starting at 24 m, 51 m, or 78 m (the first front row of the white maize plot without maize border, Figure 1A), and spaced from that starting point following a horizontal transept at both sides at 5, 10, 20 m in width; and a perpendicular transept at 5, 10, 20, 40, 80, 100, 150, 200 and 250 m in depth from each starting point (Figure 1A). Strip split-plot design using a replicated mirror design at each side of the central plot axis (Figure 1A) was used.

**Results and Discussion**

There was a high synchronization (coincidence) of flowering between the pollen donor source and the pollen receptor plants. The first plants started flowering at 49 days for both hybrids, 50% flowering was between 57 and 63 days, and anthesis ended at about 72 after sowing. The main wind direction was from North-East (NE) and North-West (NW) (pollen source) towards the South (pollen receptor) with a frequency of 56% y 32% respectively, followed by minor winds (4%) from South-West (Figure 1B (Figure 1A y 1B). Wind speed between 9 am and 12 m (period for the highest pollen viability and stigma receptivity) ranged from 1 meter/ second to 2.8 meter/ second (Figure 1B). Mean temperature was 25ºC with maximum and minimum of 29.2 and 22.2 ºC respectively. Relative humidity varied from 52% to 89% and solar radiation 26 – 69 cal/cm$^2$. Meteorological conditions were favorable for cross pollination in maize.

A total of 8.850 maize cobs were harvested by the third week of December 2006. Simple processing ended at the end of January 2007. Statistical analyses are not completed yet. Preliminary data on gene flow [(# yellow grains/ # total grains) X 100) per block sampled indicate that the maximum gene (15%) is found in the first rows at 24 m from the pollen source, as expected (Figure 2). Gene flow rate decreases significantly wit the distance and rates of 0.9% (threshold set by the European Union as highest limit for GM and non-GM seed mixture) is reached at 51 meters with 27 meters of maize border, at 61 meters with 10 meters of maize borders, and at 83 meters with 5 meters of maize border (Figure 2). In general, the east side of the plot showed higher levels of out crossing than the west side, perhaps due to the higher predominance of winds from the North-East. The white maize plot could be divided into two main zones, a zone of specific depth where gene floe oscillate between 0.3% y $\geq$0.9% (Figure 2C), and a zone general depth where two areas can be distinguish, the first one with gene flow rates lower than 0.3% and up to 0.09%, followed by the other one after 278 meters with flow lower 0.07% reaching values closed 0.01% at the field border at 328 meters respect to the pollen source (Figure 2D). If a threshold of 0.9% GM ingredient within non-GM grains is set according to the EU, plots planted at a 100 meters with a 5 meter of maize border could comply with this specification. Plots planted at 200 meters may show gene flow below 0.01%, if there is not another potential source of maize pollen around. Final statistical analyses are underway.

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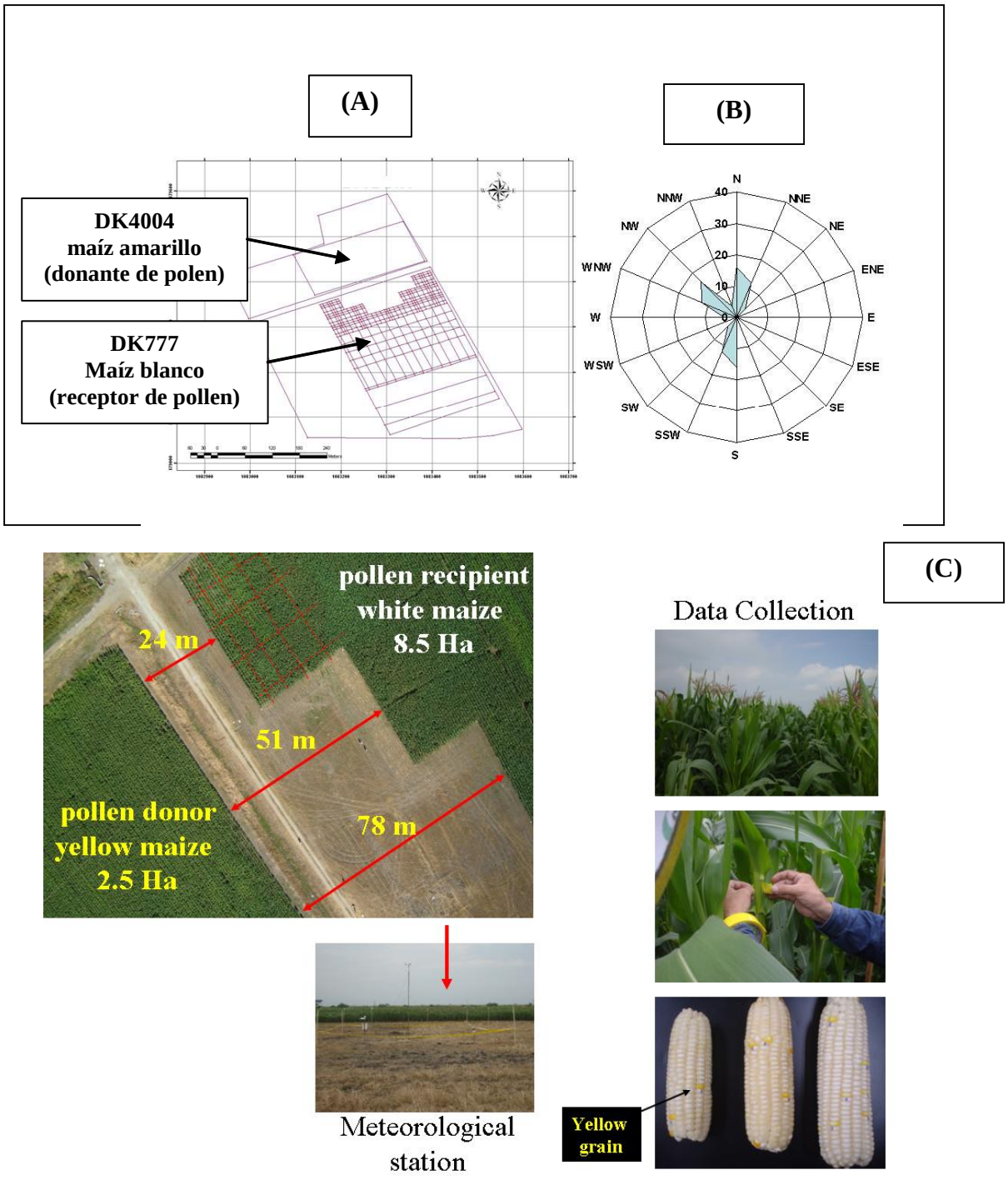


**Figure 1**. **(A)** Localization and field plot design to assess gene flow in maize. **(B)** Wind rosette indicating main wind directions during flowering. (**C**) Aerial view and details of data collection including mobile field meteorological station.

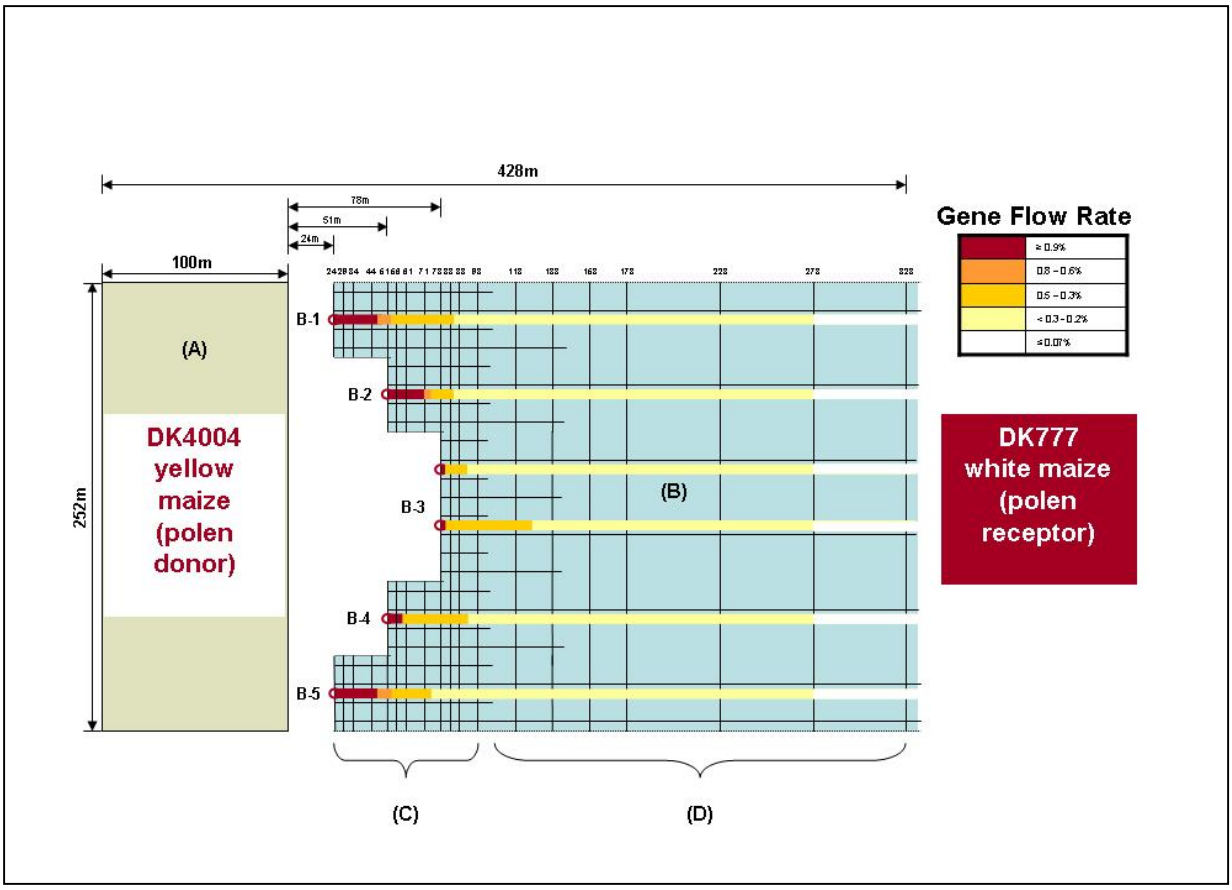


**Figure 2**. Scheme showing plot design to determine gene flow in maize: **(A)** Yellow maize, pollen donor; **(B)** White maize, pollen receptor; **(C)** Specific Depth Zone, gene flow >0.3% a ≥ 0.9%; **(D)** Common Depth Zone, gene flow <0.07% a ≤ 0.3%; [ red = ≥0.9%; orange = 0.6% a 0.8%; mustard = > 0.3% a 0.5%; light yellow = 0.2% a < 0.3%; white = < 0.07%] (B-1 to B-5) Sampling blocks. Intersecting lines refer to sampling points.

# Activity 2.2 Development of cellular and molecular techniques for the transfer of genes for broadening crops genetic base

## 2.2.1 Overcoming crossing incompatibility between selected genotypes of tepary and common bean through Double Congruity Backcrossing

A. Mejía-Jiménez, L. Galindo, H. Jaimes, A. Criollo, C. Cardona and J. Tohme
[1]Agrobiodiverstity-Biotechnology Project SB2; [2]Project IP1

The tepary bean (*Phaseolus acutifolius*) possesses multiple traits that are important for common bean breeding (for a review see Singh, 2001). Furthermore, this species has been the only one *Phaseolus* species, in which *in vitro* tissue culture methods could be reproducibly applied to produce transgenic plants through *Agrobacterium tumefaciens* (*Agro*-transformation. Dillen *et al.*, 1997; Mejía-Jiménez, 2000; Clercq *et al.* 2002; Zambre *et al.* 2005). Due to the difficulties encountered in the *Agro*-transformation of common bean cultivars, the tepary bean has been proposed as a bridging species for the introgression of transgenes to this species through sexual crosses (Dillen *et al.*, 1997).

Although difficult to cross, morphological and biochemical markers, as well as agronomic important traits, have been introgressed from tepary into common bean (Waines et al, 1988). Through recurrent and congruity backcrosses performed at CIAT (Mejía-Jiménez *et al.* 1994), the lines of common bean with the highest levels of tolerance to bacterial blight (*Xanthomonas campestris pv. phaseoli*) available have been produced (Singh and Muñoz, 1999). These lines are being used around the world to introgress the resistance to other local commercial cultivars (see Bean Improvement Cooperative reports at: www.css.msu.edu/bic/). Nevertheless the introgression of other agronomic important traits of tepary bean such as the resistance to drought, leafhopper (*Empoasca kraemeri*) or the bean weevil (*Acanthoscelides obtectus*), has not been possible applying the same backcross strategies as for bacterial blight. Incompatibility between most tepary bean accessions and common bean cultivars if crossed directly, self sterility of the hybrids, linkage between desirable and undesirable traits (Waines *et al.* 1988) and/or low levels of trait introgression from tepary bean (Muñoz *et al.* 2004) may be the constraints preventing this.
During 2005 we continued to apply a novel backcross strategy called Double Congruity Backcrosses (DCBC) which may help to overcome some of the above mentioned constraints.
This backcross strategy was initiated in 2000 to introgress into common bean the traits responsible for *Agro*-transformation competence, as well as resistance to *A. obtectus*. During 2005 a large number of fertile hybrids showing the presence and segregation of traits of both species were produced.

### Methodology

Our DCBC backcross program is described in http://gene3.ciat.cgiar.org/blast/docs/DCBCHybrids.pdf. The parentals used during 2006 were DCBC hybrids chosen using the following criteria: advanced DCBC hybrids showing high fertility and vigor, stem and flower pigmentation (male parents) or no pigmentation (female parents), good response to *in vitro* culture methodologies or high transient expression of the GUS intron transgene after *Agro*-transformation, resistance to the bruchid *Acanthoscelides obtectus*, a large seed size and/or fertility. All embryos resulting from interspecific and from most of the intraspecific DCBCs, were rescued and cultured *in vitro* (Mejia Jimenez *et al.* 1994). All plants resulting from a backcross on fertile hybrids (interspecific DCBCs), were verified as such by the presence of

morphological markers derived from the male parent (hypocotyl and flower color or primary leaf petiole size).

**Results**

**Production of hybrid lines using parental accessions with response to *in vitro* culture, plant regeneration and *Agrobacterium* mediated genetic transformation.**
Different accessions of tepary bean such as the wild genotype NI576 (Dillen *et al.* 1997; Clercq *et al.* 2002), the cultivated ones TB-1 and PI- (Zambre *et al.* 2005) and the intraspecific hybrids G40065 x NI576 and G40022 x NI576 (Mejia *et al.* 2000 and 2002) have been *Agro*-transformed. Methodologies used to transform these genotypes have been applied to common bean wild and cultivated accessions without success in the production transgenic regenerable tissues or plants (see bean transformation report and unpublished results).

As in other crops such as rice and maize have been demonstrated, the response to *in vitro* tissue culture methodologies for plant regeneration or genetic transformation is influenced by genetic traits. Traits for competence to *Agrobacterium* mediated transformation seem to be present in selected accessions of tepary bean that could be transformed using this vector, and absent in common bean.
In order to transfer these traits to common bean, a crossing program was started in 2000, in which a tepary bean accession, competent to *Agro*-transformation, and other tepary and common bean accessions selected for their response to *in vitro* culture were included.

The direct crossing of the selected common and tepary bean accessions using recurrent or congruity backcrosses yielded no fertile or cross-fertile plant. It was necessary to develop the DCBC backcross strategy in order to generate fertile progeny involving the selected accessions as parentals.
For accumulating alleles which may play an important role in competence to *Agro*-transformation, we transformed each fertile population developed, selected the best responding hybrids and then used them as parentals in the next DCBC (see report on bean genetic transformation). We are aiming at improving both, crossability between hybrids of both cytoplasms, and competence to Agro-transformation.

During 2006, 1 interspecific and 10 intraspecific DCBC hybrid generations with the common bean cytoplasm and 3 interspecific and 9 intraspecific DCBC hybrid generations with the tepary bean cytoplasm succeeded, and more than 500 fertile hybrid lines were produced. The number of crosses performed and the embryos rescued have not been yet evaluated to calculate and compare hybridization efficiencies among the different DCBC generations.
Several of the DCBC hybrids produced during 2006 involve in their pedigree, lines selected for improved competence for *Agro*-transformation (Tables 1 and 2). These lines will be tested for competence to *Agro*-transformation during 2007.

**Development of hybrid lines using parentals resistant to *A. obtectus***
During the past five years interspecific hybrid lines produced in our DCBC program were provided to CIAT's bean entomology for testing for resistance to the bruchid *A. obtectus.* At least 10 different lines have been identified as resistant (IPM report 2004). One of the resistant lines has the cytoplasm of common bean (code T7K-2E), the other (codes GNVAV, GVV, GKVGAG, NNIQLAC, and BWG) the cytoplasm of tepary bean.
The most stable resistance to *A. obtectus* has been found in DCBC hybrids with tepary bean cytoplasm. In order to pyramid different resistance genes in common bean lines we are attempting to cross the different lines that showed resistance, including the resistant progeny of the line T7K-2E with the most advanced V-DCBC lines. During 2005 some of this crosses succeeded in the production of fertile or cross fertile hybrids (Table 1 and 2 ). These lines are being multiplied or have been already provided to CIAT's bean entomology for resistance testing.

**Optimization of the screening methodologies for identifying lines resistant to *A. obtectus***

One of the bottlenecks of the screening methodologies for identifying resistant hybrids to *A. obtectus* are the long incubation periods under high temperature and relative humidity. During this time many of the resistant seeds loss their viability. Therefore, no progeny is obtained to confirm or stabilize the resistance . At the end of 2005 a M.Sc. thesis was started (Hugo Jaimes) at the National University of Palmira (Valle del Cauca, Colombia) with the main objective of developing faster and non-destructive methodologies for screening segregating progenies of interspecific hybrids for resistance to the insect. The methodology proposed in this research intends to screen individual halves of seeds, without embryo axes, while the other halves are to be used to grow the plant and produce the next progeny.

**Conclusions**

Double congruity backcrosses and advanced A-DCBC and V-DCBC hybrids used as bridge, are helping to overcome crossing incompatibility existing between genotypes of common and tepary bean carrying important traits (competence to *Agro*-transformation, resistance to bruchids). The fertile hybrid populations produced may be a source for other desirable traits of tepary bean.

**Future plans**

* To continue with the DCBC strategy to produce more advanced DCBC hybrid generations
* To study the introgression of DNA fragments, morphological and biochemical markers from the genotypes and species involved in the DCBCs in the different hybrid populations, using AFLP or other molecular techniques.

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### 2.2.2 Embryo rescue of Gene Tagging and MAS Breeding Populations

Luis G. Santos M., Adriana M. Alzate, Adriana Núñez, Diana L. Falla, Milciades Medina, Edgar Barrera, Martin Fregene.

Funding: CIAT and Rockefeller Foundation

**Introduction**

Embryo rescue of matured sexual seeds have become a key technique for the establishment of breeding populations *in vitro* to ease distribution and for the safe keeping and sharing of mapping populations with partners. Establishment of gene tagging projects as embryo axes culture has become a key activity of the cassava tissue culture facility.

**Methodology**

Embryo rescue of 3199 mature seeds of cassava from $F_1$, $BC_1$, and $S_1$ populations corresponding to 31 families of CIAT, 2 families from Tanzania and 1 family from USA were carried out this year following a protocol developed earlier at CIAT (CIAT 2003). The seeds represent mapping populations for either delayed post-harvest physiological deterioration (PPD), resistance to hornworm, beta-carotene, yellow roots, resistance to fly whites, harvest index or breeding populations for MAS of CMD resistance. Plants recovered were immediately micro-propagated and will be sent to the screen house for hardening, and subsequent evaluation here at CIAT. The new CMD resistance population will be evaluate by MAS in 2007.

Hardening of *in vitro* plantlets in screen house followed a slightly modified protocol again this year. A soil:sand mixture of 1:1 was used rather than the last year 2:1 mixture to provide for stronger root system of the plantlets. A stronger fungicide solution of Banrot, 1 gr/l was applied to the seeding the day of transfer to the soil. Fertilization using a solution 1.5 g/l of Plantafol (NPK 10:54:10) remained the same. On some occasions it was necessary to make applications against some fungi such as *Oidium* or against mites using Elosall (fungicide and acaricide) at a concentration of 0.5 ml/l or Vertimec at a concentration of 1 ml/l (acaricide).

After 60 to 90 days in the screen house and plants now had well lignified stems, and were placed outside the screen house in the shade for 1-2 weeks and then planted in the field that had previously been irrigated. Plantafol an organic fertilizer (Teravite) was applied to plants in the field on a weekly basis for the first month. The above modifications led to very high recovery of hardened plants in the screen house (>85%) and of plants established in the fields (90%).

**Results**

A total of 3199 seeds harvested from controlled crosses made up of mapping and MAS breeding populations here at CIAT and Tanzania were established *in vitro* from embryo axes. From CIAT 2420 sexual seeds representing mapping populations for post-harvest physiological deterioration (PPD), resistance to hornworm, beta-carotene, yellow roots, resistance to fly whites, harvest index or breeding populations for MAS of CMD resistance was established *in vitro* from embryo axes. Of these 958 genotypes were obtained, a plant recovery of 56% (Table 1). From Tanzania, 779 sexual seeds were

obtained and cultured as embryo rescue of which 503 plants were obtained, a plant recovery of 65% (Table 2).

In total 1461 genotypes with fully formed plants were obtained from culture of embryo axes and have been micro-propagated for transfer to the screen houses or shipments to partners. As at the beginning of March only 283 genotypes had been shipped to Tanzania. Many of the genotypes were lost due of the severe flooding that affected the growth room and consequent contamination suffered in the first semester of 2006. This year we will send 85 genotypes that were lost due to flooding. As at the time of submission of this report, the MAS populations have been micro-propagated and will be transferred to the screen house for hardening, and then it will be transferred to the field for evaluations. MAS will be conducted on them later in the year.

Earlier in 2006 we sent to the screen house a total of 3379 plants corresponding to773 genotypes segregating for delayed PPD, CW188, CR and some wild species

Table 1. Summary of the establishment of mapping and MAS breeding populations with different objectives from embryo axes

| CODE | MOTHER | FATHER | OBJECTIVES | Delivered No. Seeds | No. Sem. Shallow/lost | Planted No. Seeds | Formed No. Plants | % Formation of Plants |
|---|---|---|---|---|---|---|---|---|
| CBC1 | C-243 | AM320-145 | Beta-carotene | 329 | 45 | 284 | 243 | 86% |
| CCH5A | TAI 8 | NIG 11 | Resistance to hornworm | 532 | 310 | 222 | 171 | 77% |
| CCH5B | NIG 11 | TAI 8 | Resistance to hornworm | 30 | 8 | 22 | 19 | 86% |
| S1CH1 | NIG 11 | NIG 11 | Resistance to hornworm | 95 | 21 | 74 | 39 | 53% |
| S1IC1 | COL 1684 | COL 1684 | Harvest index | 164 | 87 | 77 | 42 | 55% |
| CR 85 | C-18 | CM523-7 | CMD resistance | 168 | 24 | 144 | 64 | 44% |
| CMB1 | CW67-125 | SM1406-1 | Resistance to Fly white | 50 | 3 | 47 | 2 | 4% |
| CMB2 | CW67-125 | TAI 8 | Resistance to Fly white | 46 | 1 | 45 | 6 | 13% |
| CMB3 | CW67-125 | SM629-6 | Resistance to Fly white | 49 | 10 | 39 | 5 | 13% |
| CMB4 | CW67-125 | CM2772-3 | Resistance to Fly white | 8 | 0 | 8 | 1 | 13% |
| CMB5 | CW67-125 | AM320-145 | Resistance to Fly white | 18 | 0 | 18 | 4 | 22% |
| CMB6 | CW67-130 | COL 2141 | Resistance to Fly white | 32 | 4 | 28 | 13 | 46% |
| CMB7 | CW67-130 | SM1406-1 | Resistance to Fly white | 3 | 0 | 3 | 1 | 33% |
| CMB8A | CW67-160 | TAI 8 | Resistance to Fly white | 119 | 23 | 96 | 24 | 25% |
| CMB8B | TAI 8 | CW67-160 | Resistance to Fly | 67 | 2 | 65 | 45 | 69% |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | white | | | | | |
| CMB9A | CW67-130 | TAI 8 | Resistance to Fly white | 24 | 5 | 19 | 12 | 63% |
| CMB9B | TAI 8 | CW67-130 | Resistance to Fly white | 276 | 121 | 155 | 111 | 72% |
| CMB10A | CW67-130 | TAI 1 | Resistance to Fly white | 6 | 0 | 6 | 4 | 67% |
| CMB10B | TAI 1 | CW67-130 | Resistance to Fly white | 6 | 0 | 6 | 0 | 0% |
| CMB11 | CW67-44 | TAI 8 | Resistance to Fly white | 150 | 10 | 140 | 28 | 20% |
| CMB12 | CW67-44 | SM629-6 | Resistance to Fly white | 1 | 0 | 1 | 0 | 0% |
| CMB13 | CW67-44 | AM320-145 | Resistance to Fly white | 8 | 1 | 7 | 0 | 0% |
| S1MB1 | CW67-44 | CW67-44 | Resistance to Fly white | 6 | 0 | 6 | 3 | 50% |
| CRA1 | C-4 | CM2772-3 | Yellow root | 113 | 27 | 86 | 69 | 80% |
| CRA2 | C-243 | CM2772-3 | Yellow root | 62 | 14 | 48 | 37 | 77% |
| CRA3 | C-18 | CM2772-3 | Yellow root | 9 | 0 | 9 | 3 | 33% |
| CRA4 | C-19 | CM2772-3 | Yellow root | 18 | 1 | 17 | 0 | 0% |
| CRA5 | C-413 | CM2772-3 | Yellow root | 6 | 0 | 6 | 3 | 50% |
| CRA6 | C-54 | CM2772-3 | Yellow root | 5 | 0 | 5 | 0 | 0% |
| CRA7 | C-6 | CM2772-3 | Yellow root | 7 | 0 | 7 | 2 | 29% |
| CRA8 | CM2772-3 | COL 2279 | Yellow root | 3 | 0 | 3 | 3 | 100% |
| S1WPD | WALKERAE | WALKERAE | PPD | 10 | 0 | 10 | 4 | 40% |
| TOTAL | | | | 2420 | 717 | 1703 | 958 | 56% |

Table 2 Summary of the establishment of mapping populations of Tanzania from embryo axes
Conclusion and perspectives

| CODE | MOTHER | FATHER | PRUPOSE | Delivered No. Seeds | No. Sem. Shallow/lost | Planted No. Seeds | Formed No. Plants | % Formation of Plants |
|---|---|---|---|---|---|---|---|---|
| CBH1 | NAMIKONGA | ALBERT | Mapping population in Tanzania | 410 | 0 | 410 | 286 | 70% |
| CBH2 | KALOLO | KIBAHA | Mapping population in Tanzania | 369 | 2 | 367 | 217 | 59% |
| TOTAL | | | | 779 | 2 | 777 | 503 | 65% |

A total of 3199 sexual seeds of cassava were established from embryo axes this year and 1461 of these have been multiplied. Percentage recovery of plants was low for some populations, this could be due to the wild origin of the parental lines. Perspectives include transfer of all micro-propagated materials to screen house and plants in the screen house to the field and the shipment of the some materials. Additional seeds pending for rescue of embryos will also be established *in vitro*.

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## 2.2.3 Sharing results of 30 years of cassava breeding: shipments of improved germplasm to Africa, Europe, Asia and Latin America

Luis G. Santos M., Adriana M. Alzate, Adriana Núñez, Edgar Barrera, Martin Fregene.

Funding: CIAT

Important output
Shipment of 1320 genotypes consisting of CIAT improved varieties and advanced breeding lines to countries in Africa, Europe and Latin America. We received 6 CIAT elite varieties with different irradiation doses from Austria for multiplication and field evaluation.

### Introduction

The cassava tissue culture facility was set up to facilitate easy and rapid sharing of improved varieties and advanced breeding lines from the genetic resources unit (GRU) and the cassava project to collaborators in Africa, Europe, Asia and Latin America. This year improved varieties at CIAT were shipped to 6 countries and then we describe below germplasm shipment and training activities undertaken by the cassava tissue culture laboratory.

### Methodology

The germplasm that was received from GRU and Austria (CIAT elite varieties with irradiation) were micro-propagated to obtain the number of plantlets per genotype requested. Micro-propagation using meristems or axillary nodes is as described by Roca et al. (1984) in17N (only apexes) for screen house hardening or 4E for conservation or screen house hardening.

Hardening of *in vitro* plantlets in screen house is as described in section 12.27. The above modifications led to very high recovery of hardened plants in the screen house (>85%) and of plants established in the fields (90%).

### Results

A total of 1320 genotypes consisting of CIAT improved varieties and advanced breeding lines were shipped to 4 African countries, 1 Europe and 1 Latin America, A list of the countries, shipments, and genotypes are shown in Table 1 between 6 and 10 plantlets per genotype were shipped. In Table 2 are presented tissue culture materials irradiated and in Austria and sent to CIAT; these materials were micro-

propagated and this moment are being hardened in the screen house for subsequent plant and evaluations in the field.

Table 1 List of shipments and destination of improved varieties and advanced breeding lines shipped from the cassava tissue culture facility between March and December 2006.

| COUNTRY | DATE | FAMILY | No. GENOTYPES |
|---|---|---|---|
| GHANA | March 16, 2006 | CR | 273 |
| UGANDA | March 16, 2006 | CR | 272 |
| NIGERIA | March 23, 2006 | CR | 271 |
| SOUTH AFRICA | April 21, 2006 | Elite materials, CR and AR | 8 |
| NIGERIA | June 29, 2006 | CR | 263 |
| BRAZIL | September 9, 2006 | Elite materials and AR | 35 |
| SWEDEN | December 11, 2006 | CW188-1and grandparents | 198 |
| TOTAL | | | 1320 |

Table 2 List of Elite varieties with different irradiation doses that were shipped from Austria.

| GENOTYPE | 0 GY | 5 GY | 10 GY | 15 GY | 20 GY | 25 GY | 30 GY | TOTAL OF TUBES |
|---|---|---|---|---|---|---|---|---|
| CM 1335-4 | 19 | 17 | 16 | 12 | 7 | 0 | 0 | 71 |
| CM 523-7 | 15 | 78 | 41 | 31 | 13 | 3 | 3 | 184 |
| CM 6119-5 | 9 | 74 | 26 | 40 | 21 | 8 | 2 | 180 |
| HMC-1 | 14 | 21 | 28 | 14 | 4 | 2 | 0 | 83 |
| MCOL 2215 | 14 | 0 | 0 | 0 | 0 | 0 | 0 | 14 |
| M TAI-8 | 0 | 5 | 5 | 3 | 1 | 0 | 0 | 14 |
| TOTAL | 71 | 195 | 116 | 100 | 46 | 13 | 5 | 546 |

Renewal of genotypes being conserved *in vitro* by the cassava tissue culture facility includes genotypes of the CR and AR families (resistance to CMD), the mapping population for cyanogenic potential and dry matter content (AM320). Others are the core collection, a group of 38 elite varieties, MNG11 (the model cassava regeneration genotype being used to produce FECs for genetic transformation), TME-3 (source of CMD resistance), a number of other materials including high protein content varieties, PPD, materials of Tanzania, irradiated materials, and 33 wild *Manihot* genotypes.. The above materials were micro-propagated for conservation purposed during the year.

Another principal activity of the tissue laboratory is training; a list of visiting researchers and students trained this year is presented in the section Training.
The above training mentioned training focused on:
Proper management of equipment, implement, and safety cabinet in a tissue culture lab.
Precautions that should be taken into account upon entering the tissue culture work area and growth rooms.
Importance of keeping an aseptic environment at all times
Multiplication spread of *in vitro* material, embryo rescue, transfer of screen house plant *in vitro.*
Preparation of tissue culture media and stock solutions.
Handling massive micro-propagation and flow chart of the process
Safe practices in a tissue culture laboratory
Screen house hardening of *in vitro* plants.
References and support material for tissue culture manipulation of plants

**Conclusion and perspectivas**

The cassava tissue culture laboratory has continued to provide invaluable services of micro-propagation, embryo rescue and shipment of elite materials to partners, propagation of germplasm required by CIAT projects as well as conservation of a large group of materials being held for cassava breeding and genetics. Members of the laboratory have also trained a large number of people this year. Future perspectives include micro-propagation and distribution of elite material, conservation of useful germplasm, and screen house hardening of *in vitro* materials.

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recovering cassava clones distributed *in vitro*. Centro Internacional de Agricultura Tropical (CIAT). Pp 8.

## 2.2.4 Hardening one-third of the in vitro accessions from the cassava germplasm collection

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Hernán Ceballos IP3/SB2 Project – CIAT

Financial Support: The HarvestPlus Program. The Rockefeller Foundation.

**Introduction**

One of the most important assets for cassava research at CIAT is the availability of the Worldwide Germplasm Collection. However, little screening has been made in search of useful traits that can be expected to be found in such a unique cassava population. In addition, because of the unsolved problem of frog skin disease and the limited resources available, it was decided not to grow the germplasm collection year after year as it was done in the past. Therefore, further evaluation and screening of the collection became much more difficult and expensive, because it involved the hardening of *in vitro* material.

In March 2006 CIAT organized a Coordination Meeting for the HarvestPlus Program together with colleagues from IITA and Brazil. As a result of this meeting it was decided that the screening of the germplasm collection in search of genetic variability, with particular emphasis in Fe and Zn content in the roots (but also nitrogren and carotenoids) was urgently needed. In addition, in spite of a recognized outstanding performance in the harsh environmental conditions of the eastern savannas of Colombia (characterized by acid soils and strong pressure for cassava by the cassava bacterial blight and the super elongation disease) a group of about 80 genotypes had never been grown in the region to confirm their superiority.

**Materials and Methods**

During the first semester of 2006 as the in vitro material underwent its routine renewal some tissue was left over and used for hardening. In vitro plants were hardened in different batches during a two months process. Hardening took place following established procedures (Segovia et al., 2002). Hardened plants were maintained in the greenhouse for about two months until vigorous growth was initiated. The plantlets were then transplanted to the field during the second semester of 2006 (from October 5 through November 15).

**Results**

Table 1 provides a list of the accessions hardened for general screening with emphasis in nutritional quality, as well as the number of plants that could be transferred to the field. Table 2 provides a summary of the germplasm that was hardened for further testing in the eastern savannas of Colombia.

The materials listed in Table 1 will be analyzed for morphological description as a contribution to completion of the data base for the germplasm collection. At harvest time, sample of roots will be screened for nutritional quality (total carotenoids, β-carotene, proteins, Fe and Zn contents and cyanogenic potential) as well as for useful commercial traits related to starch quality (amylase/amylopectin ratio, total reducing sugar contents, dry matter content, pasting properties, and starch granule size). In addition, eight vegetative cuttings will be obtained from each genotype, so that the whole set of genotypes are evaluated for agronomic performance in the sub-humid environment of the Northern Coast of Colombia and in the acid soil savannas of Eastern Colombia. Reaction to the main biotic and abiotic stresses in these locations will also be performed. It should be pointed out that only few of these genotypes have properly been evaluated for agronomic performance in these kinds of environments. Recent discoveries of resistance to whiteflies (Bellotti et al., 1999, Bellotti and Arias, 2001), high-protein content (Ceballos et al, 2006), as well as novel starch types (CIAT, 2006 Annual Report) have been very important to emphasize the importance of germplasm conservation and, more critical, the proper evaluation and screening of this germplasm.

Table 1. List of accessions from the cassava germplasm collection that were hardened from the in vitro condition and transplanted to the field during the second semester of 2006.

| Country of origin | Total number of accessions | Total number of plants |
|---|---|---|
| Argentina | 40 | 133 |
| Brasil | 568 | 2060 |
| CG (Elite Germplasm) | 10 | 21 |
| CM (Elite Germplasm) | 38 | 80 |
| Colombia | 580 | 1757 |
| Costa Rica | 43 | 154 |
| Cuba | 32 | 107 |
| Ecuador | 27 | 83 |
| Guatemala | 20 | 57 |
| Indonesia | 12 | 40 |
| Malaysia | 26 | 81 |
| Mexico | 28 | 80 |
| Nicaragua | 2 | 7 |
| Panama | 6 | 16 |
| Paraguay | 41 | 139 |
| Peru | 109 | 309 |
| Philippines | 1 | 3 |
| Puerto Rico | 1 | 3 |
| SG Elite (Germplasm) | 1 | 2 |
| SM Elite (Germplasm) | 4 | 9 |
| Thailand | 2 | 5 |
| *M. tristis* | 2 | 2 |
| USA | 20 | 50 |
| Venezuela | 28 | 86 |
| TOTAL | 1641 | 5284 |

The germplasm listed in Table 2 will follow a similar evaluation upon harvest at ten months after planting. However the emphasis will be in their evaluation in the target environment of the Eastern Savannas. In

addition to the overall performance, these genotypes will be particularly screened for resistance to bacterial blight and super-elongation disease. Potential use of these genotypes would be as a direct release to farmers (as it happened with MTAI-8 about three years ago, but for the sub-humid conditions) and also as progenitors in the production of new segregating populations adapted to these conditions.

Table 2. List of accessions from the cassava germplasm collection that were hardened from the in vitro condition and transplanted to the field during the second semester of 2006. These genotypes had been selected because of reported adaptation to the acid soil conditions of the eastern savannas of Colombia.

| Country of origin | Total number of accessions | Total number of plants |
|---|---|---|
| Argentina | 1 | 2 |
| Brasil | 1 | 2 |
| Colombia | 1 | 2 |
| Indonesia | 1 | 1 |
| CM | 50 | 117 |
| CG 165- 7 | 4 | 7 |
| SG 104- 13 | 10 | 20 |
| SM 301- 3 | 11 | 26 |
| TOTAL | 79 | 117 |

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## 2.2.5 Cloning the Phytoene Synthase gene from roots of *Ipomoea batata*s


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(2) Agrobiodiversity and Biotechnology Project, CIAT


**Introduction**

In transgenic crops it has been demonstrated that the source of a gene is important in the expression of the desired phenotype. In the case of Golden Rice, to increase the □-carotene content in the grain it was better to introduce the Psy gene that came from close relatives like maize, or from rice itself and, if possible, a gene that is expressed in an environment similar to the one being targeted (Paine et al., 2005). Following this reasoning, and thinking about increasing □-carotene synthesis in the root of cassava, we are cloning the Phytoene Sinthase gene in Sweet Potato, var. Tainung, a crop with high carotene content in starchy roots. The long-term objective is to introduce this gene into cassava, under the direction of a root-specific promoter, and to compare its expression with the bacterial heterelogous gene (crtB) already introduced in cassava (CIAT Annual Report 2007, in press).

## Methods

We constructed a cDNA library (Creator™ SMART™ library construction of Clontech) of sweet potato (*Ipomoea batatas*, Var. Tainung) roots, with approximately 1,140,000 clones, to search for genes of the carotenoid biosynthesis pathway, with emphasis on *Psy*. Using radioactive hybridization, we screened 100,000 clones with an enzyme-restricted probe, amplified by PCR from genomic cassava DNA of leaves. For PCR amplification we used specific primers designed from a cassava *Psy* sequence (J. Arango, University of Freiburg, pers. comm.).

## Results

A 2kb band was initially amplified from cassava genomic DNA from leaves (Figure 1). This band was cloned and sequenced. The sequence corresponded to the 5' and 3'ends of a Psy gene (Figure 2A). The middle region was not available due to the size of the insert. Figure 2B shows an alignment performed with sequences of the NCBI database.

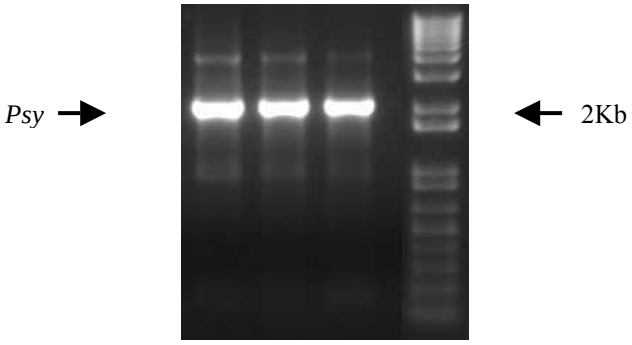


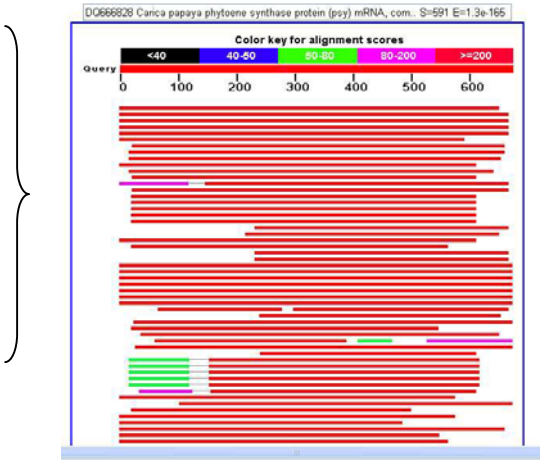

Figure 1. A 2 Kb band is visible on a gel after PCR amplification from genomic cassava DNA, using primers specific for a *Psy* gene.

The 2Kb PCR product was restricted with the enzyme RsaI to remove possible intronic regions that could interfere with cloning from a cDNA library. A 400 bp band was isolated from the gel (Figure 3), purified and labeled with dATP- $^{32}$P to be used as probe. This purified 400 bp fragment was not sequenced, although it was BLASTED against the NCBI database and the result showed that approximately 200 bp matched a psy sequence from several plant species (Figure 4).

TATGACCCCTGAAAGGCGAAGAGCTATTTGGGCAATATATGTGTG
GTGTAGAACGACAGATGAGCTTGTTGATGGACCTAATGCTTCACA
CATAACGCCAACAGCTTTAGATAGGTGGGAAGCAAGGTTGGGAG
ATATGTTTCGAGGTCGTCCCTTTGATATGCTTGATGCTGCTTTATC
AGATACAGTTACTAAATTTCCTGTTGATATTCAGCCATTCAAAGA
TATGATTGAAGGAATGAGGATGGATCTGAAGAAGTCCAGATATA
AGAACTTTGACGAGCTTTATCTTTACTGTCATTATGTTGCTGGGAC
GGTTGGATTAATGAGTGTTCCAGTGATGGGCATTGCACCTGAATC
ACAGGCATCAACTGAGAGTGTTTACAATGCTGCTTTAGCACTAGG
AATAGCCAATCAGCTGACCAACATATTCAGGGACGTCGGAGAGG
ATGCAAGAAGAGGAAGGATTTATTTACCACAGGATGAATTGGCA
CAGGCAGGGCTTTCAGATGATGACATATTTGCTGGAAAAGTGAC
AGACAAATGGAGAAATTTCATGAAGAACCAGATTAAAAGAGCAA
GAATGTTCTTTAACGAGGCAGAGAAAGGAGTGACAGAGCTGAGT
GCTGCAAGTAGATGGCCGGTATGGGCATCCTTGCTGCTGTACAGA
AGAA……

……ACGAGCTTTATCTTTACTGTTATTATGTTGCTG
GGACGGTTGGATTAATGAGTGTTCCAGTGATGGGCATTGCTCCTG
AATCACAGGCATCAACTGAGAGTGTTTACAATGCTGCTTTAGCAC
TAGGAATAGCCAATCAGCTGACCAACATACTCAGGGATGTCGGA
GAGGAGTAAGTACAGAGTTTCTTTTTTTCTGCTCAGTTCCCATTTG
CTTCCTCTATATGTCATCTATGTTCTTTTTAGCTCACATTTTCCTGG
GTTTCGCTGTGGGCGAGCTTTTCTCAGTGCAAGAAGAGGAAGGAT
TTATTTACCACAGGATGAATTGGCACAGGCAGGGCTTTCAGATGA
TGACATATTTGCTGGAAAAGTGACAGACAAATGAGAAATTTCAT
GAAGAACCAGATTAAAAGAGCAAGAATGTTCTTTAACGAGGCAG
AGAAAGGAGTGACAGAGCTGAGTGCTGCAAGTAGATGGCCGGTA
AAACATCAATATCCTTTGTCAAATGCAAATAGCCCTGTAATAGAA
AAAATGATTAAAAAATGTTTGCGGTTGTGGACATTGCAGGTATGG
GCATCCTTGCTGCTGTACAGAAGAATACTAGACGAGATAGAAGC
AAATGATTACAACAACTTCACAAAGAGGGCTTATGTGAGCAAAA
CCAAGAAGATAGCATCTTTGCCAATTGCATATGCAAGATCATTTG
TTGGGCCTTCAAGAATGTCATTTCCTGTGACAAAAGCTTGA

Figure 2A. Partial sequences of a cassava genomic clone of Psy. Secuencia parcial del clon genómico Psy. In green is the sequence of the probe used for screening the cDNA library.



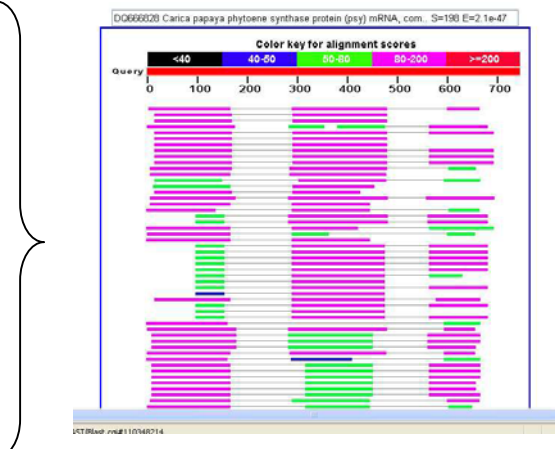




Figure 2B. Results of a BLAST comparison between the sequence shown in figure 2A and sequences in the NCBI database. (Sources: BLAST, NCBI, 2007)

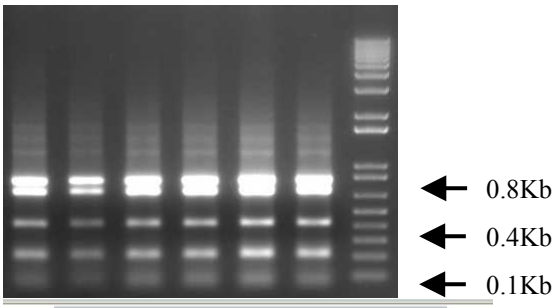



Fig. 3. Restriction pattern of the genomic Psy clone from cassava after cutting with Rsal. The 0,4kb fragment was purified, re-sequenced and used as probe to screen a cDNA of sweet potato roots.

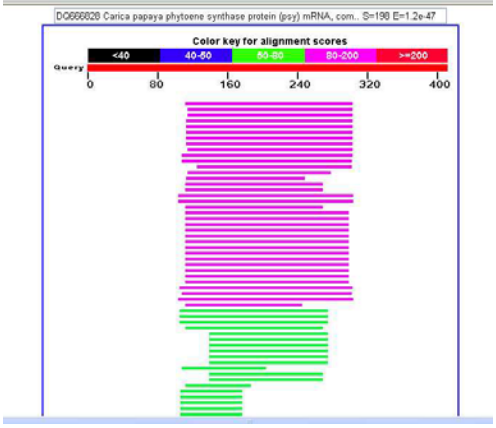



Figure 4. BLAST results against the NCBI database after querying with the sequence of a 411 bp shown in figure 3. (Sources: BLAST, NCBI, 2007)

The probing of putative positive clones for *Psy* was done twice using the same 411 bp fragment as probe, and using the protocol suggested by the University of Clemson for screening BAC libraries. Figure 5 depicts the results obtained with the second screening, after picking individual colonies from a first round of selection. Approximately 100,000 clones were screened. Fifteen clones were sequenced. Eight of them had no psy sequence. There was one with Sporamin-B, the major storage protein in sweet potato. For the other seven clones the sequences were mostly vector, suggesting that they should have been sequenced from the other side.

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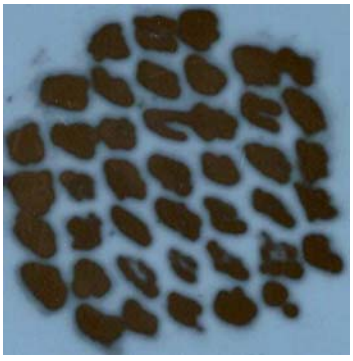

Figura 5. Radiography of putative psy clones of Ipomoea batatas, probed with a psy fragment isolated from cassava.

**Perspectives**

From these results it's clear that screening cDNA libraries with heterologous probes is not the most efficient way to clone a psy gene from *I. batatas*. We can think of several reasons to explain our findings: 1) psy may be of too little expression in roots, and therefore the chances of cloning it are quite small; 2) the expression of psy may depend on the developmental stage of roots, so, in this case, we did not use the right tissue at the right time; 3) the probe was not very specific, meaning that the 400 bp fragment, isolated from a gel, but not re-sequenced, could have contained introns that diminish the specificity of the screening.

A an alternative to clone a psy gene from sweet potato, we are performing RACE (Rapid Amplification of cDNA Ends) on mRNA extracted from three months old roots of the same cultivar. This method may be more efficient since it's based on PCR amplification, which enhances the chances of cloning an amplified cDNA of a psy gene expressed in roots. A spin off the search for a psy sequence in sweet potatoes is the finding of a Sporamin-B gene. It would be interesting to search and clone the promoter of this gene since it drives the expression of a protein (Sporamin) that represent between 60 and 80% of the total protein from roots (Maeshima et al 1985). A promoter of such strength would be ideal to drive carotenoid genes expression in cassava roots.

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## 2.2.6 Gene Expression of the Carotenoid Cleavage Dioxygenase I (CCDI) in Cassava roots

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**Introduction**

The content of β-carotene in the roots of cassava differs between genotypes. At the molecular level however, in terms of gene expression, the explanation of this difference remains unknown. Previous analyses of the expression of some of the genes involved in β-carotene synthesis did not show differences between yellow-rooted cultivars and those with white roots [1]. We hypothesize that the β-carotene catabolism pathway, that is the conversion of □-carotene into other metabolites, might be partially responsible for the contrasting accumulation of this carotene in roots.

One of the more important genes implicated in the catabolism of β-carotene is the *Carotenoid Cleavage Dioxygenase*1 (CCD1) [2]. Its activity has been tested in several species in different tissues where the *loss-of-function* mutants, for example, lead to increase of carotenoids including β-carotene [3]. This evidence constitutes the basis for the hypothesis in which the expression of CCD1 may correlate with the content of β-carotene in cassava roots. One would expect that yellow roots express less CCD1 than would white roots during the first six months of root filling, which, according to Howeler and Cadavid (1983; cited by Cunha. 2002) are the period of maximum dry mater accumulation for many genotypes in tropical

areas [10]. We present preliminary data on the expression of CCD1 in cassava roots of white and yellow cultivars.

**Methodology**

Primer Design to Clone CCD1 from Cassava:

Because the sequence for cassava CCD1 is not yet known, two alternatives were used to design primers. The first one was to compare the *Arabidopsis thaliana* AtCCD1 sequence with a database of 20,000 gene sequences from cassava available at CIAT [4]. We obtained one *EST (4901)* that was used to design two primer pairs. The second alternative was to align CCD1 sequences from different species from the NCBI database. The vector NTI ® software was then used to design eight primer pairs on consensus sequence (Figure 1).


Figure. 1. Vector NTI ® Platform. Example of the localization of one of the primers on the consensus sequences of CCD1 genes from NCBI.

RNA Isolation and cDNA synthesis:

Yellow roots from cassava clone MBRA-253, and white roots from CM3306-4, two varieties contrasting in β-carotene root accumulation, of 3 months of age, were collected and RNA extracted following Reilly *et.al* [5]. Creator™ Smart™ cDNA library construction kit was used to produce cDNA.

For RealTime PCR quantification, cDNA synthesis was made using random primers, Superscript III enzyme and other chemicals recommended by Invitrogene®.

Real Time PCR Quantification:

Expression of CCD1 in roots was carried out on four biological and three technical replicas. The relative standard curve method [6] was selected for quantification of gene expression, using as endogenous gene the ribosomal 18S sub-unit, and as calibrator the cDNA from the yellow clone. The reaction was performed in the *DNA Engine Opticon 2 MJ Research®* using the *Syber green® Kit.*

**Results**

Partial Cloning of CCD1:

The primers were tested in full–length cDNA of yellow roots (MBRA 253). Five fragments amplified and cloned using PgemT-Easy®Cloning system. The sequences were compared with NCBI database, showing high similarity to CCD1. All sequences were then assembled in one contig and the consensus was compared against the NCBI database. The highest hits are shown in Table 1.

| Best Hits NCBI-database | | |
|---|---|---|
| Score (Bits) | E Value | Sequences producing significant alignments: |
| gi\|76560796\| carotenoid cleavage dioxygenase 1 [Coffea canephora] | 712 | 0.0 |
| gi\|90991006\| carotenoid cleavage dioxygenase1 [Citrus unshiu] | 725 | 0.0 |
| gi\|76560804\| carotenoid cleavage dioxygenase 1 [Coffea arabica] | 709 | 0.0 |
| gi\|61654494\| 9,10[9',10']carotenoid cleavage dioxy [Vitis vinifera] | 710 | 0.0 |

Table 1. BlastX between the consensus contig of *Manihot esculenta* CCD1 and the NCBI database.

The consensus sequence of cassava CCD1 --MeCCD1-- was translated whit *Translate tools* - ExPASy Home Page and ORF Finder from NCBI. The most likely *Open Reading frame* ORF is showed below (fig.2).

**A** **B**

```
GGRGNSIGDGMIHGMRIKNGKATYVRRYVRTSRIQQEEF
FGGSKFMKVGDLKGLFGLFMVNMQILRAKLKVLDMSYGN
GTANTALIYHHGKLLALQEADKPYXVKVLEDGDLQTVGM
LDYDKRLKHSFTAHPKVDPNTGEMFTFGYAHEPPYITYR
VISKDGVMHDPVPITISDPIMMHDFAITENYAIFLDLPL
YFRPKEMVKDKKLIFTFDATKKARFGVLPRYAXDDXQIR
WFELPNCFIFHNANAWEEEDEVVLITCRLENPDLDMVSG
NVKEKLENFANELYEMRFNMKTGVASQKRLSAPAVDFPR
VNEXYTGRKQRYVYGTILDSIAKVTGIIKFDLHAEPQQG
KTKLEVGGNIKGIFDLGPGRFGSEAVFVPREPGTSSEED
DGYLIFFDAR-KHRKIISECD-RKNNVKRPCCSR-
ITPQGAIRLPRLLCDRGTTSSTVNAEIVQKPENMQLL
RHLSSGT-
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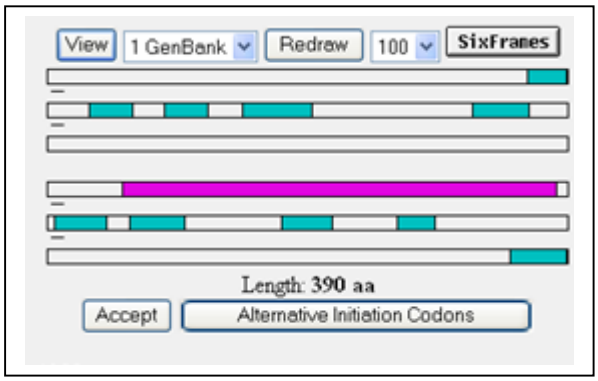


Figure. 2 MeCCD1 translation sequence (A) and graphic representation of the ORF from 208 to 1380 pb.

CCD1 gene expression in cassava roots of 3 months of age:

Primer sequences used for RealTime® PCR were Fw5´CAGGTCCCAGATCAAAGATGCCTT3´ Rv 3´TGGCAGGAAGCAAAGGTACGTCTA5´. The standard curve was constructed amplifying 2-fold dilutions of cDNA (100, 50, 25 and 12.5 ng/□l) from yellow roots (Figure 3).

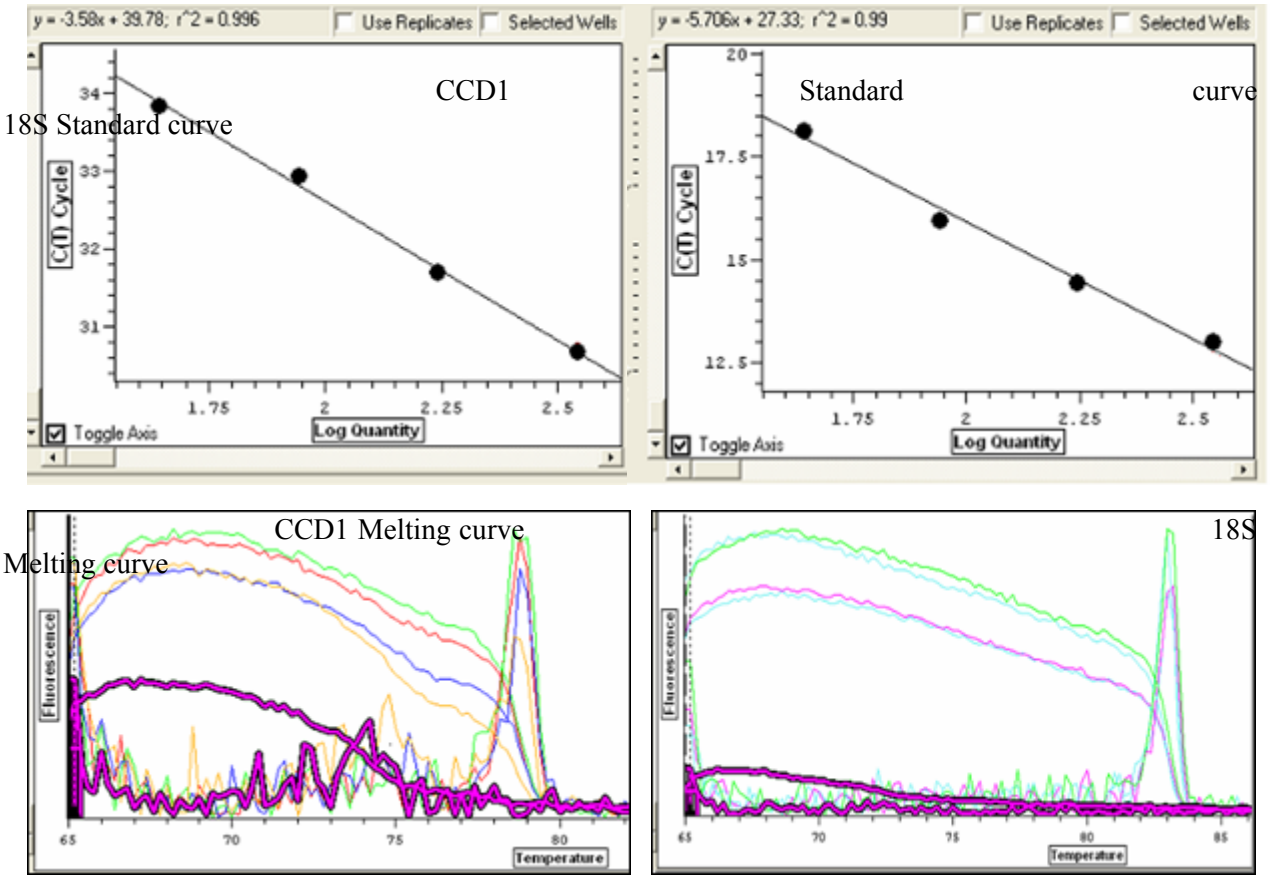


Figure 3. Top two graphs: Standard curves for cDNA dilutions from yellow roots to amplify CCD1 (left) and 18S. The linear regression showed good correlation between template dilutions. Bottom graphs: Examples of melting curves for the same two genes. Thin line picks represent specific gene amplifications while thicker curves are negative controls (water).

Table 2 summarizes the results for several Real-Time PCR assays. The relative expression of CCD1 is expressed as n-fold times over the sample with the least expression (P3YR) used as calibrator.

| SAMPLE | **CCD1 Normalized Values** | **CCD1 Relative expression with P3YR** |
|---|---|---|
| P1WR | 1.20 | 0.18 |
| P1YR | 1.12 | 0.10 |
| P2WR | 1.20 | 0.18 |
| P2YR | 1.22 | 0.20 |
| **P3WR** | 1.11 | 0.10 |
| ***P3YR*** | 1.02 | 1 |
| P4WR | 1.42 | 0.39 |
| P4YR | 1.04 | 0.03 |

Table 2. CCD1 expression levels in cassava roots. WR (white root) YR (yellow root). P1 to P4 # are biological replica.

**Conclusions and Perspectives**

The cloned sequence from MeCCD1 is relatively close in size, 1411 bp, to that of AtCCD1 (1617 bp), and it's also very similar to other CCD1 sequences from plant species. However, given that cassava is a tetraploid, and that CCDs are a gene family, the PCR products from which the contig was assembled for cassava may actually represent parts of several genes of the CCD family and/or different alleles of CCD1.

Assuming that the cloned sequence belongs to a CCD1 gene from cassava, and understanding that we still need to confirm the number of alleles of this gene and their expression patterns (tissue specificity), the preliminary data indicate that there is not difference between the varieties tested for CCD1 gene expression. This result suggests that the explanation for the differential accumulation of β-carotene in yellow and white roots of cassava resides somewhere else; it may be partially explained by the polygenic inheritance of the trait [7].

Our results however open new scientific perspectives for understanding the accumulation of β-carotene in roots. The analysis of changes in tissue age and expression of CCD-related genes [8] may also help to explain differences. There is evidence that the conversion of amyloplast into chromoplasts during flower development in tobacco provides metabolites (glucose) for the synthesis of carotenes [9]. It would be interesting to analyze how starch is accumulated and spent in yellow versus white roots of cassava. May be, in yellow roots, there might be more starch spent for carotene synthesis, while in white roots starch gets accumulated faster (early bulking). Preliminary observations on thickness and number of roots per cassava plant at three months of age indicate that yellow roots are thinner and less than white roots, although genotypic differences between yellow and white cultivars can not be ruled out.

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## 2.2.7 Induction of Somatic Embryos in cassava (*Manihot esculenta* Crantz) using Stationary Liquid Cultures.

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### Background

Modifying cassava through transgenesis requires a system of totipotent cells that can be genetically modified, and that regenerate complete and normal plants. In cassava the technology is fairly well established[1], but there is always room for improvement, especially when it comes to reducing costs, time and labor, without sacrificing the quality of the new plants. We report a new method to generate totipotent cells, Somatic Embryos, for genetic modification, which employs liquid instead of solid medium, and glass jars instead of Petri dishes.

### Methodology

In vitro plants of cultivars 60444, Mcol2215 (Venezolana) and CM3306-4 (Ica Negrita) were propagated on 4E medium (Roca and Mroginski 1991) for 45 days, at 28$^{o}$C and photoperiod 12/12. One-inch long cuttings, containing one axillary bud each, were precultured for 2-3 days on 4E medium under the same conditions to stimulate growth of meristems. The axillary buds were then dissected and placed floating on liquid MS2-50Pi medium in a baby-food jar. Each jar contained 4 ml of medium and 10 buds. Buds were left still, growing in darkness, at 28$^{o}$C ± 2$^{o}$C for 20-25 days. After this period, the SE produced were cleaned off non-embryogenic tissues, and either used for embryo maturation, cyclic embryogenesis or FEC induction. The percentage and quality of somatic embryos, the latter scored as high (H), medium (M) or low (L) as an indicator of amount of tissue obtained from each explant, was indicative of the success of the tested methodology.

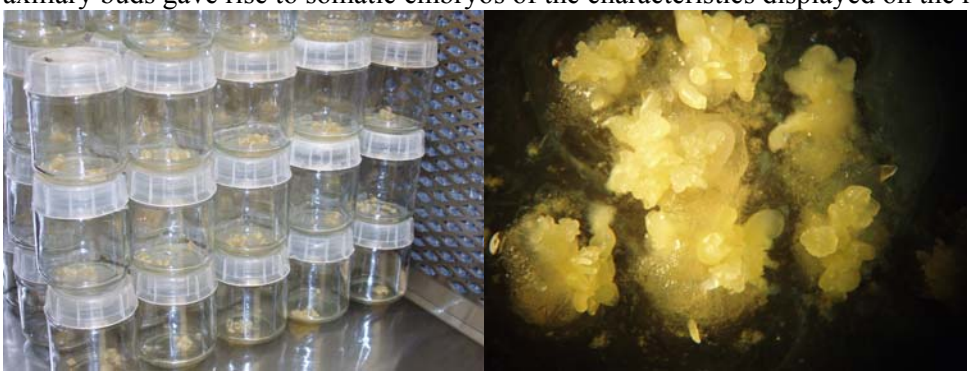
### Results

We used three cultivars to test the new method to obtain embryogenic tissues for genetic modification.. Table 1 summarizes the results. The liquid system always produced somatic embryos of high quality for at least two cultivars (60444 and Mcol2215), and with higher quality (Figure 1). For CM3306-4 however there seems to be no much change respect to the conventional methodology.

Table 1. Comparison of the efficiency (%) of production of somatic embryos in conventional medium (second column; solid MS with 2,4-D) and liquid media (third and fourth columns; liquid MS plus either 2,4-D or Picloram). In parenthesis there are the scores of the quality of somatic embryos produced under each treatment (H = high; M = medium; L = low).

| Variedad | Solid with 2,4-D | Liquid with 2,4-D | Liquid with Picloram |
|---|---|---|---|
| 60444 | 35.4 (M to L) | 67.4 (M to L) | 75.9 (H) |
| MCOL2215 | 70.0 (M to L) | 55.4 (M to L) | 78.4 (H) |
| CM3306-4 | 78.3 (M to L) | 67.3 (M to L) | 72.3 (M to L) |

Figure 1. On the left panel there are 60 baby-food jars containing 10 axillary buds each, of cultivar 60444, for a total of 600 explants to induce somatic embryos in liquid medium. Approximately 76% of them produced embryos after 20-25 days. The quality of the embryos was almost invariably high, where 7/10 axillary buds gave rise to somatic embryos of the characteristics displayed on the right panel.



**Conclusions and Future Plans**

In general, like for most characteristics of agronomic value in cassava, they do depend on the genotype. In vitro behavior, to produce somatic embryos amenable for genetic modification, is not an exception. In this case two of the clones tested behaved better on liquid medium while the third clone (CM3306-4) remained the same in terms of percentage and quality of embryogenic tissues produced.

We recommend using liquid media to induce Somatic Embryos since it reduces costs by avoiding using gelling agents like agar, and Petri dishes. Besides, the quality (amounts of somatic embryos produced per explant) is much higher, as confirmed by the embryos shown in Figure 1. One more advantage of this new method is that there are always many more initial explants per in vitro plant. In the conventional method – with solid medium- we used to use very young, unfolded leaves, which appear at one per plant only. With the new methodology each axillary bud is useful, and there are as many buds in one in vitro plant as leaves it has.

Given the success of this new method, we would like to, first, reproduce the experience to include statistical analysis and publish it. Second, produce other type of totipotent cells (Friable Embryogenic Callus; Taylor et al 2004) amenable for genetic modification, and to expand it to more genotypes of commercial value.

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Taylor N., Chavarriaga P., Raemarkers K., Siritunga D. & Zhang P. (2004) Development and application of transgenic technologies in cassava. Plant Molecular Biology 56: 671-688.

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## 2.2.8 Cloning Phytoene Synthase alleles from cassava

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[1]Universidad del Valle-Departament of Biology; [2]University of Freiburg, Freiburg, Germany; [3]Agrobiodiversity and Biotechnology Project, CIAT

### Introduction

The current strategies to improve the nutritional value of crops include the tissue-specific expression of genes of the same or related plant species, whenever possible; an approach that responds to consumer's concerns on the biosafety of novel genes introduced into eatable plant products. For cassava one should expect the same tendency. Therefore, an early effort to clone genes of the carotenoid synthesis pathway from cassava is advisable. Those genes may soon be considered for transformation of the crop to increase the content of β-carotene in roots. We described the approach currently followed to clone alleles of a phytoene synthase gene from cassava, backing up the effort of our collaborators in the University of Freiburg, Germany.

### Methods

Based on a 1057 bp sequence of a cloned psy allele from cassava –*Mepsy* (Figure 1; J. Arango, personal communication; University of Freiburg, Germany) we designed five sets of primers to amplify the entire gene. The platforms used to design the primers were Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi ) and *Pearlprimer* (http://sourc -eforge.net/). Total RNA was extracted from roots and leaves of 3 months old cultivars Mbra253 (yellow roots) and CM3306-4 (white roots), and a cDNA library constructed with SMART cDNA Library, Clontech.

Figure 1. Sequence of a *Me-psy* gene provided by the University of Freiburg. The green highlighted regions indicate the sequences selected for primer design. The start and stop codons are in red.

Gcccttagtttgtgctgagtatgccaagacttttacttgggaactctgttaatgacccctgaaaggcgaagagctatttgggcaatatatgtgtggtgtagaaggacagatgagctt
gttgatggacctaatgcttcacacataacgccaacagatttagataggtgggaagcaaggttggaagatatgtttcgaggtcgtccctttgatatgcttgatgctgctttatcagatac
agttactaaatttcctgttgatattcagccattcaaagatatgattgaaggaatgaggatggatctgaagaagtccagatataagaacttcgacgagctttatctttactgttattatgttg
ctgggacagttggattaatgagtgttccagtgatgggcattgcacctgaatcacaggcatcaactgagagtgtttacaatgctgctttagcactaggaatagccaatcagctgacca
acatactcagggatgtcggagaggatgcaagaagaggaaggatttatttaccacaggatgaattggcacaggcagggctttcagatgatgacatatttgctggaaaagtgacaga
caaatggagaaatttcatgaagaaccagattaaaagagcaagaatgttctttaacgaggcagagaaaggagtgacagagctgagtgctgcaagtagatggccggtatgggcatc
cttgctgctgtacagaagaatactagncgngatagaagcaaatgattacaacaacttcncaaagagggcttatgtgagcaaaaccaagaagatagcttnnttgccaattgcatatg
caagatcatttgttgggccttcaagaatgtcatntcctgtgacaaaagctgatatgtgcaaaccatgaacatcaatttcaaatgcccaattgatattaaaaagttaaatctttacttccg
atataatttttttaaaatgttgatataatagttcaaatttaaaaatttattgtcattttatataaatttaaaatttaatttttaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
agcttgtacaaaaaagggc

**Results**

The five sets of primers are depicted in Figure 2. They expand the entire ORF, and the cloned 5'and 3' UTRs of *Mepsy*. Table 1 shows the sequences and characteristics of primer sets for PCR amplification.

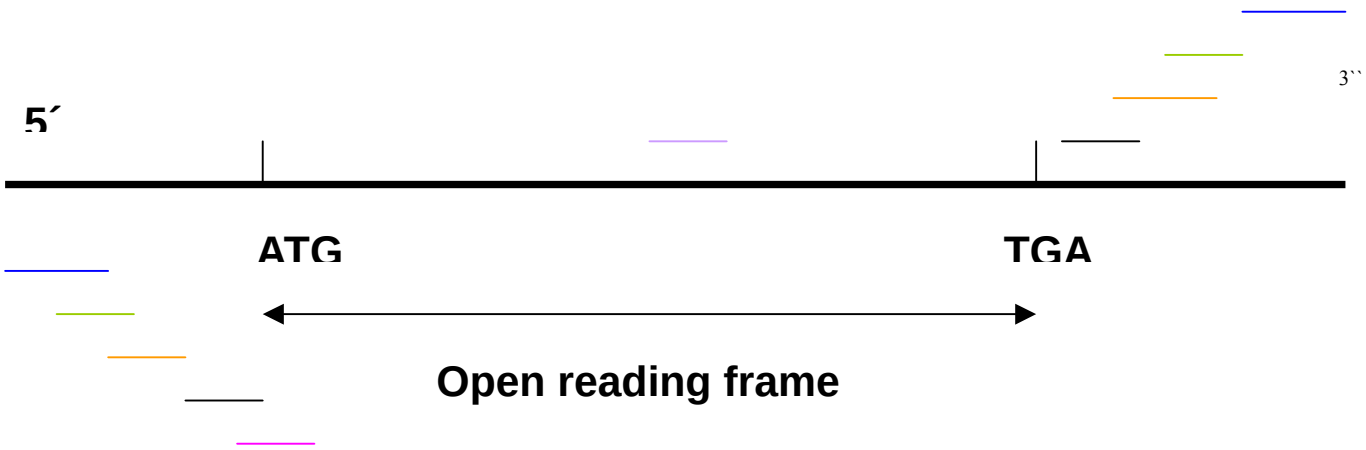


Figure 2: Location of the five primer sets designed to expand the entire Mepsy.

Table 1: Primer sequences and characteristics to amplify Mepsy.

| **set 1** | **Amplicon** | **len** | **tm** | **GC%** |
|---|---|---|---|---|
| GCCCTTAGTTTGTGCTGAGTATGC fw | 1 | 24 | 63.38 | 50.00 |
| TCAATTGGGCATTTGAAATTGATG | 892 | 24 | 64.84 | 33.33 |
| **set 2** | **start** | **len** | **tm** | **GC%** |
| TTTGTGCTGAGTATGCCAAGAC fw | 9 | 22 | 60.31 | 45.45 |
| AAATTGATGTTCATGGTTTGCAC | 900 | 23 | 60.99 | 34.78 |
| **set 3** | **start** | **len** | **tm** | **GC%** |
| CTTAGTTTGTGCTGAGTATGCC fw | 3 | 22 | 61.61 | 45 |
| TTGATGTTCATGGTTTGCAC | 896 | 20 | 59.62 | 40 |
| **Set 4** | **start** | **len** | **tm** | **GC%** |
| ACTTGGGAACTCTGTTAATGAC fw | 35 | 22 | 60.31 | 40 |
| TCATGGTTTGCACATATCAAGC | 889 | 22 | 61.89 | 40 |
| **set 5** | **start** | **len** | **tm** | **GC%** |
| GGGAACTCTGTTAATGACCC fw | 33 | 20 | 60.05 | 50 |
| CTTCAGGTCCATCCTCATTCC rv | 302 | 21 | 62.28 | 52 |

The amplification products run in agarose gels varied in size and did always agree with their expected mobility on the gel. All were around 800 and 900 bp, except for the band amplified with primer set 5 (purple on figure 2), which was between 200 and 300 bp in size. For primers set 3 and 4 it was not possible to obtain amplification products from yellow leaves. The primer set number 5 did amplify in white roots and leaves, although the product is not shown in figure 3.

**Discussion and Perspectives**

Most primers amplified products in all tissues, except for the primers sets 3 and 4 in leaves of the yellow-rooted Mbra253. It may be hypothesized that there are sequence differences between the *Mepsy* cDNAs of leaves and roots of this clone. All PCR products are now being sequenced to confirm if this is the case. We know that there are at least two copies of *Mepsy* in cassava, of which two different alleles have been cloned in Freiburg (R Welsch and J Arango). This opens the possibility of finding more alleles given the polyploidy origin and heterozygous nature of cassava. The gene for phytoene synthase is duplicated in tomato and cereals (Bartley and Scolnik 1993; Gallagher et al 2004). Both copies in tomato are specialized for tissue specific expression in leaves and fruits. Finding differences in allele sequences of psy genes cloned from cassava, and correlating those differences with gene expression patterns, spatially and timely regulated, would strongly support our effort of improving the β-carotene content of cassava roots.

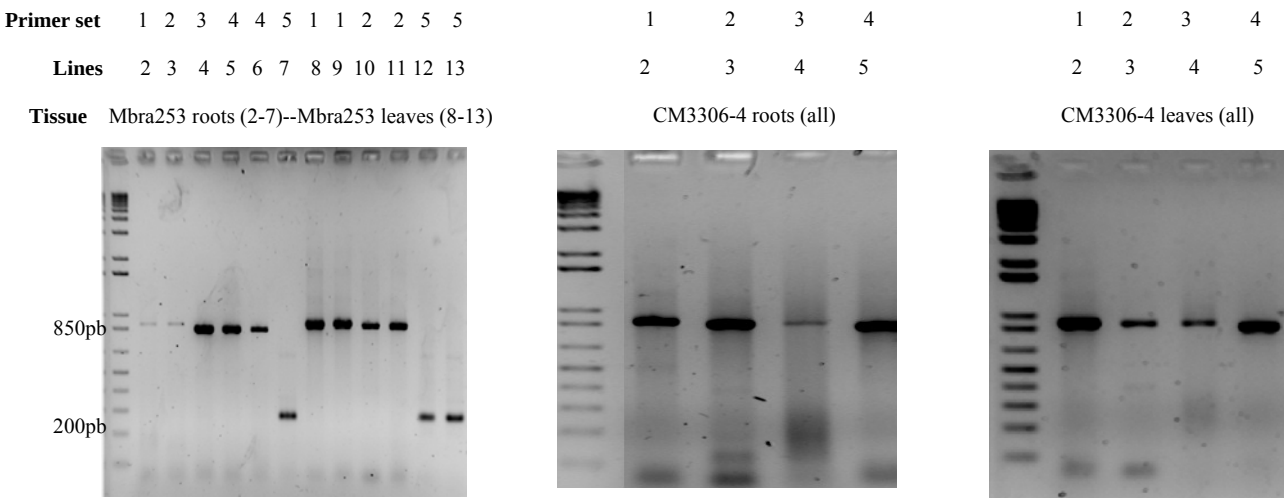


Figure 2. PCR amplifications of Mepsy from cDNA roots and leaves of cassava cultivars Mbra253 (yellow roots) and CM3306-4 (white roots)

**References**

Bartley GE, Scolnik PA (1993) cDNA cloning, expression during development and genome mapping of Psy2, a second tomato gene encoding phytoene synthase. J Biol Chem 268: 25718–25721

Gallagher C.E., Matthews P.D., Li F.Q. & Wurtzel E.T. (2004) Gene duplication in the carotenoid biosynthetic pathway preceded evolution of the grasses. *Plant Physiology* 135, 1776–1783.

## 2.2.9 Improvement of a bioluminescence assay to detect biological nitrification inhibition (BNI) activity from root exudates using *Nitrosomonas europea*

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[1]SB-2 and [2] IP-5 CIAT project

**Introduction**

Nitrification is a soil biological process where ammonium nitrogen is transformed into $NO_2$-N (by ammonia-oxidizing bacteria) and subsequently to $NO_3$-N (by nitrite-oxidizing bacteria). The $NO_3$ ion, unlike $NH_4^+$, is highly mobile in the soil, and thus is often leached from crop rooting zones by rain or irrigation. The nitrification process is performed by soil bacterial species including *Nitrobacter*. In this process, more than 70% of the fertilizer nitrogen used worldwide in agricultural systems is believed to be wasted due to nitrification-associated nitrogen losses. To minimize the loses, it is necessary to keep soil N in $NH^{4+}$ form as long as possible for the crops to absorb and utilize it as a nitrogen source.

Recently, JIRCAS has demonstrated that a tropical pasture grass *Brachiaria humidicola*, has the ability to inhibit nitrification using root exudates by a bioluminescence assay (Subbarao et al. 2006a). The original assay was developed by Iizumi's group (Iizumi et al., 1998) that used a recombinant *Nitrosomonas* strain which produced bioluminescence due to the expression of a *luxAB* gene. CIAT has been working with JIRCAS on this subject (Subbarao et al. 2006b).

To extend our capacity for screening a large number of plant materials for BNI activity we first developed an improved bioluminescence assay by modifying the bioassay that was reported by Subbarao et al. (2006).

**Materials and Methods**

Bacterial strain and growth conditions: The recombinant *Nitrosomas europea* ATCC 19178 harbouring a gene cassette containing a *luxAB* gene, a gift from JIRCAS in Japan, was grown aerobically at 30°C in the dark in HEPES culture medium containing 4.0 g $(NH_4)_2SO_4$, 1.0 g $KH_2PO_4$, 11.93 g HEPES, 1.0 g $NAHCO_3$, 200mg $MgSO_4.7H_2O$, 10 mg $CaCl_2.2H_2O$ and 600 mg Fe (III)EDTA per liter of distilled water. The medium pH was adjusted to 7.5. The production rate of NO2 during the bacteria growth period was monitored by a colorimetric method (Eaton, et al., 1995).

Bacterial cells for BNI assay: Bacterial cells from 500 ml of 4 day-old broth culture were separated by centrifugation at 10,000g, 4°C for 10 min and re-suspended in 100 ml of P-medium, then kept in the dark for 30 min before the bioassay. An aliquot (5 ul) of test sample (root exudates or synthetic inhibitor) was mixed with 200 ul doubled distilled water. 250 ul of bacterial cells were added to this mixture and stirred and incubated at 15 °C for 30 min. After the incubation period, bioluminescence was measured. For each test sample three measurements were made.

Bioluminescence measurement: Bioluminescence was measured using a TECAN Genios luminometer multiplate reader. The luminescence reaction was started by mixing 100 ul of the sample as prepared above with 2.5 ul of 10% (v/v) n-decyl aldehyde dissolved in ethanol. The relative light unit (RLU) was used as a ¨full integration value¨ which means an average light output during 5 to 15 seconds after the initiation of the reaction.

Growth of *B. humidicola* and *Panicum maximum* in a hydroponic system to collect root exudates: Shoots of *B. humidicola* accessions (CIAT 679, 16888, 26159) and *Panicum maximum* (16028) grown in soil were transferred in aerated nutrient solution consisting of 0.35mM $(NH_4)2SO_4$, 0.27mM $K_2SO_4$, 18µM $H_3BO_3$, 4.6µM $MnSO_4$, 1.5µM $ZnSO_4$ $7H_2O$, 1.5µM $CuSO_4$, 1µM $Na_2MoO_4$, 0.18mM $Na_2HPO_4$, 0.36mM $CaCl_2$, 0.46mM $MgSO_4$ $7H_2O$, 45µM Fe (III) EDTA. The shoots transferred to the nutrient solution were grown in 18 liter tanks on polyethylene blocks for two months. The pH of the solution was adjusted to 5.5. The nutrient solutions were replaced with fresh solution every four days. Root exudates were collected non-destructively as follows: 10-15 plants were briefly washed in 1mM $NH_4Cl$ and the roots were placed in 500 ml of 1mM $NH_4Cl$ solution where the root exudates were allowed to collect for 24 hour.

Preparation of root exudates for bioassay: Root exudates were filtered through a filter paper to remove any debris such as root tissues and the pH of the filtered root exudates was adjusted to 7.8. The filtrate was centrifuged at 1,000g at 4°C for 10 min. The supernatant was again filtered through 0.8 um and 0.22 um membranes to remove any contaminating organisms such as bacteria and then freeze-dried. The resultant pellet was dissolved in 10 ml methanol and further evaporated to dry using a rotary-evaporator at 40°C. The root exudates was extracted with 100-500 ul water and after centrifugation the supernatant (water-soluble compounds: WSC) was submitted to bioluminescence assay. The remaining pellet was dissolved in 50-200 ul DMSO and centrifuged to collect the supernatant as water-insoluble compounds (WIC).

**Results and Discussion**

We have improved the bioassay by modifying several steps described in the bioassay that JIRCAS has developed for plants. The incorporated steps such as additional centrifugations, filtrations and extraction with different solvents allowed us to 1) prevent biological contaminations from other sources in the process and 2) to test BNI activity in water-soluble and -insoluble fractions of root exudates. We standardized the method to calculate BNI activity as allylthiourea (AT) units using the synthesized inhibitor. Using water as control, various concentrations (0.01-0.22 uM) of AT were used to generate an AT standard curve for every set of experiments (Fig. 1). The inhibitory effect of 0.2 uM AT in the bioassay medium was about 80% and was defined as one AT unit of BNI activity. Using this standard curve, the inhibitory effect of the test samples was converted and indicated as AT units.

JIRCAS previously found that grasses including *B. humidicola* and Panicum have different levels of BNI activity. Based on their observations, CIAT16888 had the highest BNI activity among the plant genotypes used in this report. As shown in Table 1, significant differences in BNI were found among *B. humidicola* accessions. We found that all tested materials have higher BNI activity in WSC fractions than that in WIC fractions. This strongly suggested that BNI compounds released from roots are water-soluble.

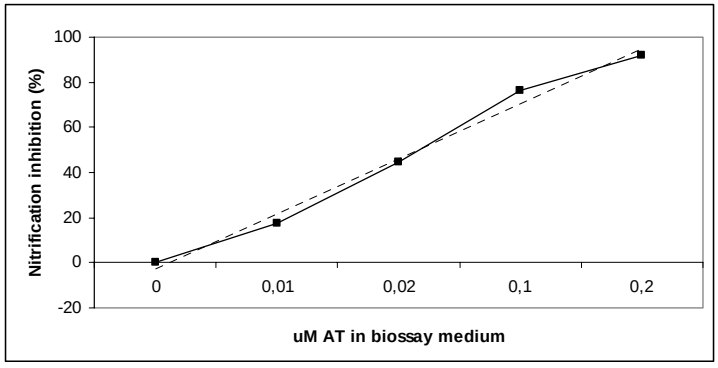


Fig 1. Transgenic *Nitrosomonas europea* response to synthetic nitrification inhibitor, allylthiourea (AT) in the bioassay medium

It is also found that CIAT16888 had the highest BNI activity (173 AT units $g^{-1}$ DW) compared with other materials tested in this work as JIRCAS reported previously. We believed that our bioassay is now operational to test root exudates from any genotype of interest including rice germplasm.

Table1. Biological Nitrification Inhibition (BNI) activity of root exudates of *B. humidicola* accessions and *Panicum maximum*

| Accession number | Specific BNI activity (AT units $g^{-1}$ DW) | | | |
|---|---|---|---|---|
| | WSC extract | STDV | WIC extract | STDV |
| CIAT 679 | 2.3 | 2.26 | -0.7 | 0.28 |
| CIAT 16888 | 173.08 | 33.6 | -12.14 | 16.6 |
| CIAT 26159 | 14.9 | 7.6 | 2.0 | 1.54 |
| *Panicum maximum* 16028 | 11.21 | 3.6 | 5.5 | 2.25 |

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# OUTPUT 3. Collaboration with public and private partners enhanced

## Activity 3.1 New collaborative arrangements, networks, training and workshops

### 3.1.1 Strengthening farmer seed systems in acute and chronic stress

L.Sperling

**Background: Seed Aid and Seed Security**

Seed aid is increasingly applied as an emergency response throughout Africa. The logic looks straightforward. Beyond free handouts, communities affected by emergency (for example, drought, flood, short-term conflict) should be given the means to sow (and harvest) their own food., through seed provision.

However the practice of seed aid, as increasingly analyzed from a research and development perspective, is raising a series fundamental concerns. *Inter alia,* studies show that seed delivered in emergency is often is not needed at all (as seed systems have proven remarkably resilient); and that the kind of aid given, repeatedly, is promoting farmer dependencies in a range of countries. In terms of markets, free seed delivery has also been shown to undermine local seed/grain markets and to compromise the development of longer-term more commercial seed supply.

Seed aid is a key issue for the CGIAR and CIAT as: a) it is frequently delivered among the more vulnerable populations, in marginal areas (i.e. among our prime client groups); b) it is being given in chronic stress areas, that is, areas of 'failed development' rather than of acute crisis *per se;* and d) such aid is often *the* main way small poor farmers get access to new varieties. Seed aid is given on a regular basis in many of the countries in which CIAT operates, particularly in East, Central, and Africa.

**CIAT and Partners' Roles**

CIAT, in partnership with relief, developmental and donor agencies, facilitates a multi-activity initiative to improve responses to foster seed security in acute and chronic stress contexts. The CIAT-led Seed Systems Under Stress Program (www.ciat.cgiar.org/africa.seeds.htm) :

Helps shape immediate emergency relief, particularly in terms of seed , germplasm and livelihood insight;

Analyzes the effects of different types of disaster (war, drought, flood, or crop plague) on the functioning of seed systems (including its crop and variety diversity);

Evaluates emergency operations to refine practices of seed system maintenance and strengthening;

Develops robust seed security assessment tools for use during and after disaster;

Works with policy makers to institutionalize "best practices";

Strengthens skills and raises awareness among donors, managers and field practitioners: to shape seed security programs and to build better assessment and evaluation skills.

Below, we briefly present milestones for 2006 and then present recent progress in several specific activity areas.

**Bulleted Activity Summary: 2006**

*Seed Aid for Seed Security Briefs* published in English. They are aimed at donors, project managers, and humanitarian practitioners and provide insight into the design, implementation and evaluation of emergency seed security projects. The briefs have had over 20,000 downloads.

Seed System Security Assessment (SSSA) manual tested in Douentza Circle, northern Mali. First-ever testing of a 'seed security' versus food security instrument

Field Long-term Seed Aid Analysis in Ethiopia near completed. (including organizational analyses, policy overview, farmer surveys). Ethiopia proves to be recipient of seed aid since 1974.

Awareness-raising of 'seed systems in stress program', through extensive publication, website posting and outreach seminars. Five of the top 10 downloads from the CIAT Africa website come from this program.

**Activity 1: Seed System Security Assessment (SSSA)**

Collaborators

| | |
|---|---|
| Louise Sperling | International Center for Tropical Agriculture, Italy |
| Tom Remington | Catholic Relief Services, Nairobi |

This activity is funded by the USAID -supported project: Putting Seed Security at the Heart of Agricultural Relief and Recovery Response (funded as of January 2006)

Overview: Why assessing seed security is different from assessing food security

Farm families are seed secure when they have access to seed and planting material of adequate quantity and acceptable quality in time for planting. As helping farmers obtain the planting materials they need will enable them to produce for their own consumption and sale, fostering seed security is an integral component for promoting food and livelihood security.

Achieving seed security is quite different from attaining food security, despite their obvious links. One can have enough seed to sow a plot, but lack sufficient food to eat, for example during the 'hungry season' prior to harvest. Simply, the quantities needed for seed are often very small compared to the grain amounts needed to fill many stomachs. Conversely, a household can have adequate food, but lack access to seed (or the right seed) for planting. This happens more rarely, but can occur if seed stocks kept in the house have become pest-infected, or if the kinds of seed needed can no longer be found on farm (for instance, disease outbreaks mean that new types of resistant materials are demanded).

Despite these important differences between food security and seed security, determinations of seed security are invariably based, implicitly or explicitly, on food security assessments. Evaluators may conduct food need assessments and then extrapolate that seed is also required as part of the aid package. Similarly, evaluators may assess possible food stocks by measuring harvests. If there is a sharp harvest

drop, we know that food availability will steeply decline. However, an automatic link is often made that a production shortfall also results in a shortage of seed. Simply, this is just not true ***(see Box 2, below)***.

CIAT with its close partner CRS is developing a seed system security assessment (SSSA) guide. The aims of publishing such a guide are threefold: :

To make available in the international arena a concrete method for assessing seed system security (as opposed to food security).

To present a practical 'how to' guide to help field practitioners work through seed security problem definition and response.

To ensure that seed aid proposal development and review are based on rigorous seed security data rather than inferred from food security assessments

A draft of the guide was used in field testing in Mali, March 2006 and an advanced version was reviewed by the USAID/OFDA donor late 2006.

The SSSA Guide.

A seven step process has been developed for assessing seed security (Box 1). The Seed System Security Assessment (SSSA) process is geared first to assessing the effects of acute shock (emergency), but can also be used to assess longer-term trends in seed security. The longer-term perspective examines the negative and positive effects of chronic stress on seed systems, by identifying the chronic stress patterns and by identifying emerging opportunities to address these problems (i.e. variety, agro-enterprise or other innovations).

**Box 1: Assessing Seed System Security; Seven Basic Steps**
**Identify and characterize zone of action**

Describe (construct profile) of crop and seed systems: normal times

Describe the effects of disaster on agricultural system: the broad overview

Determine the goals for seed relief and recovery, including farmer demand and needs, post-crisis

Assess seed channel functioning post-crisis (framed in relation to demand and needs set)

Look to the longer term: probe for more chronic (versus acute) stress manifestations as well as for emerging development opportunities

**Match possible responses to priority constraints, opportunities and demands**

Key in shaping this guide has been the development of positive assessment methods and well as the addressing of what is negative, falsely-grounded practice. Positively, the tool combines desk-based background work, and field assessments of all the seed channels small farmers may use (home production, local seed/grain markets; and formal seed sector supply). In terms of negative practice, we have had to tackle the widespread misconception that a sharp drop in harvest means a lack of seed. (Box 2) For many crops analyzed in African contexts, harvests can drop as much as 80-90%, and enough seed is potentially

on offer. We are cautious and add 'necessarily' as the quality of seed in the harvest has to be adequate and farmers have to be in the position to save sufficient stocks till sowing time. This may be particularly challenging in regions with just one agricultural season per year.

Basically, the percent of a normal harvest required to meet the sowing needs in the next season is the inverse of the multiplication rate. Small seeded crops generally have high multiplication rates and thus only a very small proportion of the harvest is needed as seed. For the dominant small grain crops of dryland Africa -- millet and sorghum – typically less than one percent of the harvest is needed for seed. Thus, for these crops, even in a bad year, the seed requirement is unlikely to be a significant drain on the harvest, unless there is almost total harvest failure. On the other hand, large-seeded crops (for example, groundnut) may require upwards of 10% of the harvest to be set aside as seed. For these crops therefore, seed availability is more likely to be an issue, especially in bad years. Box 2 walks through the logic of establishing the sowing basics for different scenarios (with resowing, and in good and bad years).

**BOX 2: Sowing Needs in Relations to Harvests:**
**(or--Why a production shortfall does not necessarily equal a seed shortfall)**

Table A gives the basic assessment framework, using beans and sorghum in Rwanda as an example. Most of this basic information is common knowledge among agronomists and farmers in a field area. One needs to obtain a rough idea of the average areas sown to a crop per household, and how much seed farmers use or the density of seed sown for that area. The return for these crops (i.e. multiplication rate) will then give an estimate of yields. Seed needed for sowing can then be matched against yields (or harvests).

A. EXAMPLE FROM MID-ALTITUDE RWANDA

| **Crop** | **Beans** | **Sorghum** |
|---|---|---|
| Surface Area per household | ¼ ha. | ¼ ha. |
| Seeding Rates | 100 | 10 |
| Sowing Needs | 25 kg | 2.5 |
| Multiplication rates of seed | 8 | 100 |
| Harvest | 200 | 250 |
| **% of harvest required to meet sowing needs** | **12.5** | **1.0** |

Table B, drawn from northern Mali, shortcuts the process. It also suggests how this quick calculation can be refined, factoring in both farmers' seed sorting (tossing out small or broken seeds) and farmers' re-sowing rates (that is, replanting when the first set of seeds may not sprout) .

B. EXAMPLE FROM NORTHERN MALI

| **CROP** | **Pearl Millet** | **Groundnut** |
|---|---|---|
| Sowing needs (kg/ by farmer area) | 10-20 | 15 kg (1/4 ha) |
| Harvest (on normal farmer area) | 430 | 125 kg (1/4 ha) |
| **% of harvest required to meet sowing needs** | **3.4** | **12.0** |

Table C, moves towards even greater precision, drawing on actual field data: for a higher and lower potential area in Ethiopia, and contrasting a good versus bad harvest year.

C. EXAMPLE FROM EASTERN ETHIOPIA

| **Crop** | **Chiro (highland)** | **Miesso (Lowland)** |
|---|---|---|
| Surface Area per Household | 1/2 ha. | 3/4 ha |
| Sowing needs (kg– for area) | 7-8 | 11-12 |
| Harvest/yield (good year) | 1.25 t | 1.6 T |
| **% Harvest required to meet sowing needs: good year** | **0.7** | **I0.75** |
| Harvest/yield (bad year) | 400 kg | 260 kg |
| **% Harvest needed for seed needs – bad year** | **2.0** | **4.6** |

In all scenarios, the key conclusion is that a production shortfall does not automatically lead to a seed shortfall.

Field Testing in northern Mali: some results

The SSSA guide was field tested with a team in northern Mali, at the request of Catholic Relief Services (with a grant provided by the Bill and Melinda Gates Foundation). Seed security in the immediate high stress zone was not threatened (despite two years of drought and subsequent threats). However, a range of chronic stress were identified (problems with legume storage, *striga* and a general need for early maturing varieties). Among the more interesting findings was the identification of specialized pearl millet seed production villages (Catholic Relief Services and Partners, 2006). A range of villages produce the specialized (narrowly-adapted) varieties needed for the more arid areas, but which prove also to be highly sought in others parts of Douentza Circle . Seed security in Douentza Circle partially depends on keeping these seed production villages functional. Two types of actions were recommended:

Seed stocks need to be maintained. In this vein, food security (and food aid) may be *the* key for promoting seed security (i.e. not seed aid *per se)* . Food aid can help farmers keep stocks for their own use, but also for sale.

Efforts should be made to intensify production in these renown seed villages (e.g. Tabi, Tega, Toupere, Sariegner). To assess what might best reinforce their capacity, further diagnostic work focused on seed village production strengths, weaknesses, and opportunities need best be carried out.

.Note, the full report assessed the immediate seed security situation and also outlined a 3 to 5 year agenda for action.

Future Plans

The SSSA guide will continue to be field tested in 2007. It will then be published in English, French and Portuguese.

**Activity 2: Seed Aid for Seed Security Briefs**

Collaborators

*Key inputs across briefs by:*

Louise Sperling International Center for Tropical Agriculture
Tom Remington Catholic Relief Services
Jon Magnar Haugen Consultant for Care Norway
Office of Foreign Disaster Assistance (Laura Powers and Julie March)

*Inputs on select briefs from:*

| | |
|---|---|
| Jean Claude Rubyogo | International Center for Tropical Agriculture |
| Robbert van der Steeg | International Instite for Plant Genetic Resources |
| Paula Bramel | International Institute for Tropical Agriculture |
| Tom Osborn | Food and Agriculture Organization of the United Nations (FAO) |
| Dennis Lattimer | FAO |
| Geoff Heinrich | Catholic Relief Services |

These briefs activity aimed to provide 'better practice' guidance for donors and managers designing emergency seed security projects, as well as field staff involved in on-the-ground humanitarian practice. This activity was supported by the USAID-supported project: Assisting Disaster-Affected and Chronically-Stressed Communities in East and Central Africa.

The contents of the briefs were shaped from extensive field experience, across different types of disasters, seed security constraints and opportunities. Aside from the writers (drawn from different institutions and disciplines), the briefs were reviewed by the key donor: The Office of Foreign Disaster Assistance/USAID.

Results

The ten briefs were published in English in March 2006. They have been widely distributed in hard copy and also via multiple weblinks (e.g. USAID government - emergency site, ReliefWeb, Eldis, Southern Africa Regional Poverty Network (SARPN) Via web, they have had over 20,000 downloads, and, in hard copy, organizations such as Oxfam have ordered substantial sets for their field teams (e.g.. for work in Timor).

The briefs can be downloaded at: (http://www.ciat.cgiar.org/africa/practice_briefs.htm)
They include themes of:

1. Who are we and what do we do?
2. Overview of seed systems under stress project case studies
3. Understanding seed security
4. Agrobiodiversity and seed relief
5. Using seed aid to give farmers access to seed of new varieties
6. Understanding seed systems used by small farmers in Africa: focus on markets
7. Assessing seed system security
8. Seed relief responses: an overview
9. The power of evaluation
10. Developing a seed-aid proposal: a rapid review checklist for practitioners

Future Plans

The 'seed aid for seed security' briefs' are now being translated into French and Portuguese. They will be published in hard copy and posted on websites in April 2007.

**Activity 3: Long-term seed aid and the most vulnerable: 'Classic Case' – Ethiopia**

Collaborators

(*Team leaders)*

| | |
|---|---|
| T. Assefa, B. Amsalu | Ethiopian Institute of Agricultral Research (EIAR) |
| L. Sperling, J.C. Rubyogo | International Center for Tropical Agriculture (CIAT) |
| A. Asfaw, A, Tenaye | Southern Agricultural Research Institute (SARI- Ethiopia) |
| W. Mulugeta | Amhara Agricultural Research Institute: Debre Birhan |

G. Negusse — Tigray Agricultural Research Institute (TARI)
S. McGuire — Overseas Development Group (ODG) (member from University of East Anglia (UEA)

Overview

This IDRC-funded project aims to examine the effects of longer-term seed aid in Ethiopia, a country which has received food aid and/or seed assistance for over 30 continuous years. The project has two primary thrusts: 1) an examination of the effects of seed aid on farming families in four regions of the country: West Hararghe, North Shoa, Wolayta and Tigray; and 2) an analysis of the policies which shape such aid *(inter alia*, seed, relief, development and agro-biodiversity laws and regulations.). The goals of this work remain quite practical: a) to better shape assistance during the emergency period; b) to influence the type of agricultural developmental assistance offered among the very vulnerable; and c) to stimulate reflection on whether existing national policies enable practitioners to address needs of populations experiencing repeated stress.

The project represents a collaboration among the Ethiopian Institute of Agricultural Research (EIAR), The Southern Awasa Research Institute, Amhara Agricultural Research Institute, Tigray Agricultural Research Institute; The International Center for Tropical Agriculture and the Overseas Development Group of the University of East Anglia (i.e. four NARS, one IARC and a university). The emphasis on NARS has been deliberate as research experience and skills, as well as a developmental perspective, are often absent from emergency planning and implementation.

Methods

Four different bodies of information have been collected, with analysis near complete. The aim has been to compare and contrast the short-term with the long-term view; federal versus regional seed aid variations; policy guidelines with field implementation; and implementer (provider) with beneficiary points of view. As far as we know this study represents the most comprehensive seed aid analysis conducted to date

| ***Tasks*** | ***Progress as of Dec 2006*** |
|---|---|
| Implementer analysis: 4 regions<br>Government and NGO perspective | complete |
| Field Surveys- On-farm beneficiaries (N=500) | complete |
| Overview of Seed aid History<br>analysis ongoing: 1974 to the present | data collection complete. |
| Policy analysis (seed aid related)<br>Seed policy<br>Rural development policy<br>Emergency regulation<br>Biodiversity acts<br>interview policy-makers- Addis- ongoing<br>document review (regional)- ongoing | document review (federal) complete |
| Seed/Grain market analyses | scheduled for planting season, Feb/Mar 07 |

The four overall regions of study (West Hararghe, North Shoa, Wolayta and Tigray ) were chosen a) to represent diverse political/administrative perspectives (which is key in this regionalized country); and b) to embrace a range of stresses: drought, drought/civil strife, flood, frost and 'green poverty' (that is high economic stress in areas with richer agro-ecologies but very small land holdings). In all specific districts chosen **(***woreda)* , seed aid has been delivered on repeated basis in the last 10 years.

Results

The full report will be issued in June 2007. Select findings are indicated below:

Seed aid seems to have started in Ethiopia in 1974. It has been more or less continuous since 1982 (in select regions).

Much of the *acute* seed aid delivered (for emergency) is not needed by farmers. Farmers have seed. Even in high-stress zone, Haraghe, farmers report that only half of their seed comes from aid—the rest is secured from their own stocks and local markets). Seeds needs calculations are extrapolated from food needs assessments and this food assessment method is simply incorrect for assessing seed security.

A real need for seed aid is often not established. Routinely, seed aid may be delivered because food aid is delivered. Emergency seed aid is also increasingly used as a vehicle to promote new varieties. More recently**, if** there is an assessment, it is one tied to crop yield loss (i.e. the food and crop assessment missions). These are not sufficient for determining seed security more generally or for determining specific seed needs. There already exist positive ways to move forward on seed security and seed need assessment.

A wide range of approaches are in use to address seed-related problems in Ethiopia
Direct Seed Distribution (DSD)
Seed Vouchers and Fairs (SVF)
Vouchers alone
Cash alone---for relief
Revolving Seed funds
Revolving Cash funds
Seed Banks
Seed given in food security-related programs ,
Seed given in HIV/aids programs

The content of such seed aid seems most directly tied to institutional philosophy and logistics (current capacity) rather than to well specified seed security needs. More evaluation is needed to compare and contrast the effectiveness of these approaches when and where.

Much of the seed aid given in emergency is not for "acute" for emergency stresses at all. (for example, farmers cite histories of receiving seed aid 10 times in as many years.) Seed aid is being given in "chronic" stress contexts, were such 'stop-gap' seed aid approaches may not be effective. There is a need to tailor strategies (including seed systems assistance) specifically to meet the needs of chronic stress areas.

In cases where emergency seed assistance *is* needed, (the minority of cases), evidence of seed aid badly delivered is common: for example, the wrong varieties, late delivery. "Direct Seed Delivery' (DSD) may not be the most effective seed assistance response for a variety of problems. In near all cases, seed was available on markets-- (suggesting that 'access', rather an seed availability may be a common problem for

farmers.) When seed aid is needed, the type of response should be matched with the type of problem encountered (for example, to distinguish problems of availability, access and quality).

**Future Plans**

The initial findings of this project were presented to the Minister of Agriculture early 2007. The result is that the Minister has called for National "Seed Aid for Seed Security' meeting (presently scheduled for October 2007). Among the objectives, this meeting will provide a venue to present the findings of the Longer-term Seed Aid Project. It will also aim set in motion a process for establishing a concrete code of conduct or 'Seed Aid Guidelines for Ethiopia."

**References**

Catholic Relief Services and Partners, 2006. Seed system security assessment (SSSA) Douentza Circle, Mali, 2006. Contributors: Louise Sperling, Eva Weltzien, Moussa B. Sangaré, Janine Scott Shines, Sekou Salla Boré, Abderahamane Bamba, Chéry Traoré ,Dogola Coulibaly, Soungalo Traoré, Cheik O Keita, Mohamed Ag Hamada, M'Pè Ballo, Fassory Sangaré, Moussa Kanouté, Baba Sanogo, Hamidou Guindo, Boureïma Konta, Seydou Sanogo, Abdoulaye Traoré, Margret Loeffen, Alassane Dembélé

### 3.1.2 The Cassava Genetics Information System (CGIS)

Juan G. Rozo, Luis G. Santos M., Edgar Barrera, Fernando Rojas & Martin Fregene (CIAT)

Funding: CIAT

**Introduction**

In the Cassava Genetics program all the information that is generated from the *in vitro* tissue culture bank, screen house, field and shipments are entered in a manual form and stored in Excel spreadsheets. This process doe not permit timely access to information leading to delay in the generation of reports. Furthermore, the manual form of data collection can also lead to mistakes during transfer of raw data to excel datasheets.
The main objective of this project is to design and develop a web information system that makes for faster capture, processing, and storing of information in generated by the Cassava Genetics program. The information system uses an integrated platform of computer science technology tools such as Bar Code, PDA, Databases, and Web Applications.

**Methodology**

An information system can be defined as a set of functions or interrelated components that form a whole. It can capture, store process, and distribute information to support decisions making processes and the control of flow of information in an organization. Also it enables the analysis of problems and overview of complex aspects

The technologies used to design the Cassava Genetics information system are listed below:

• PDA' s. (WORKABOUT PRO)
• Software of design of Code bar. (ZebraDesigner Pro)
• Database. (MySQL - HSQLDB)

Wireless devices such as PDA (Personal Digital Assistant) are designed to capture and store data. For the data capture, a bar Code reading system and PDA software WORKABOUT PRO is used.

The design of the code bar (ZebraDesigner-Pro) allows companies to customize bar codes making faster the processes of inventory and control of merchandise. This software has been created for the design of labels or bar codes which works with the last generation technology using identification by radio frequency (RFID).

Database is a data set stored systematically for later uses. As example of a database engine is MySQL for applications Web and desktop and HSQLDB for mobile applications.

Taking into account these concepts and the needs of the Cassava Genetics Program, we have designed an information system to handle the information generated in the tissue culture and molecular markers labs as well in screen houses and field.

**Results**

The tissue culture module is composed of:
Entry of sexual seed and plant material
Generation of genotypes,
Record of plantlets (status, loss, etc)
Micro-propagation,
Control of inputs.

This module is being developed and implemented as the first phase of the information system leaving the necessary interfaces for the subsequent modules (screen houses, field, shipments and molecular markers).

**Conclusion and perspectives**

An information system to collect, processing and store information generated by the cassava genetics system is in the process of being developed. It will improve the time to consult and display information as well as the generation of reports.

## References

P.D.A:
http://www.psionteklogix.com/public.aspx?s=com&p=Products&pID=3965
ZEBRA DESIGNER PRO (Code Bar technology):
http://www.zebra.com/id/zebra/na/en/index/products/software/label_design_software/zebradesignerpro.html
MySQL(Database Server):
http://www.mysql.org/
ECLIPSE IDE(Integrated Development Environment):
http://www.eclipse.org/

HSQLDB (Database for PDA):
http://hsqldb.org/

### 3.1.3 Architecture for the publication of research results using GCP Platform

Rojas, Fernando
AgroBiodiversity and Biotechnology Project,, Centro Internacional de Agricultura Tropical (CIAT), AA 6713, Cali, Colombia.

**Objective**

To design and to implement adaptable Software tools. To publish the information registered in its Databases; in a compatible format with the standards and Architecture of GCP by means of the use of Web services Technology.(Figure 1)

Milko Skofic (Bioversity Internacional)

**Introduction**

CIAT is one of the institutions that participate in *Generation Challenge Program* http://www.generationcp.org GCP, this "challenge Program" includes the capture, storage, integration, analysis and dissemination of substantial volumes of information of genomes, genetic resources and improvement of cultures.
The CIAT in its participation in *Generation Challenge Program*(GCP) at the moment has projects in execution in the subprograms (sp1, sp2, sp3); generating distinct class of data. The subproject sp4 (Genetic Resources, Genomic, and Crop Information Systems) of the GCP have become technological advances that can and must be capitalized in the CIAT. The technology of Web Services is the mechanism by means of which we can make available to the community our information; proceeding of any data source in a Standard way. The GCP Platform Architecture is based in Web Services Technology Using Bio Moby (www.biomoby.org).

BIOMOBY is an international research project on methodologies for the representation, distribution and discovery of biological data; *MOBY-S* is a solution of interoperability based on technology of Web Services. At the present time the information can be consulted at internal level using a local interface, the end users must be in capacity to easily make certain information public in the web using friendly interfaces by them self.

The main objective of the GCP Data Submission Templates is to provide simple templates for the temporary storing or distributing of the different data sets that are being produced within SP1, SP2, and SP3, for which there is no current provision in public or institutional databases. These templates must contain consistent but sufficient explanatory notes on how they should be used. The completed data sets should contain the necessary information to be stand-alone and should be simple enough to be understood. For example, enough description of material and methods used, and no use of acronyms or a coding system that only the data provider can understand. The templates will be consistent with the GCP Domain models being produced and will be used to store data in the central repository.
TAPIR maps your database fields to a list of concepts. With TAPIR you do not need to map your database to a schema, it can be a flat list of concepts, better, ontology. This is a very important feature, because schemas are constantly revised, whereas a list of concepts can describe a kind of information that will never change, although it might change in a schema structure. So, once you map your database, the only maintenance you might need to do is map additional fields, not change anything previously mapped.
The other big advantage is that schemas and mappings are served through a series of *concept servers*, which practically are files served from a central point that all TAPIR providers can share. This means, for the GCP, that we can all share the same schemas and mappings. (Figure 2)

When returning data from a query, TAPIR can return it into any schema you want, so you may have mapped your database to MCPD, but want the GCP passport schema. The big advantage here is that this kind of mapping is stored on the *concept servers*, so it can be done centrally and all intra-schema mappings are automatically available to everybody.
TAPIR uses these features to implement MOBY services. MOBY services in TAPIR are defined as a service template that has input and output MOBY data types. These data types are directly taken from the configured MOBY central. So a MOBY service is defined in the same way as other intra-schema mappings: the concepts you have mapped to your database are mapped to the MOBY input and output data types. Since this is also centrally stored, these MOBY templates are available to everybody. The bottom line is that TAPIR will:
a.) check what concepts you have mapped,
b.) check which MOBY data types have been mapped to those concepts,
c.) determine the list of potential MOBY services that can be resolved,
d.) present you that list and prompt you to implement the MOBY service by pressing a button; once pressed, the service will be registered into the MOBY central and your database will be discoverable as any other MOBY service.

**Methodology**

The participation becomes necessary of the users who are generating information in another GCP projects (SP1, SP2, SP3), and users populating the AxLIMS and generators of another's data sources so that they become familiar with the GCP's Domain Model; and the mapping between the Data Schema of each source and GCP model can be carried out.

To use Bio Moby , Biocase and GCP tools to construct, register and use the web services that facilitates the publishing process of the information.

The implementation according to the GCP platform must be done using programming languages like Java and Perl within the scope of the technology of Web Services Moby.
The resulting tools also will incorporate technology related to the controlled vocabulary and Ontology inspired by standards of the community such as: Gene Ontology Consortium (www.geneontology.org) and the Open Biological Ontology (obo.sourceforge.net).
It is necessary to consider UML like tool of modeling used in the GCP to establish its model. One is due to establish the platform and the standards, for the Development Registry and consumption of Services Web in the CIAT's AgroBiodiversity project.
To use the "*CIAT methodology for Development of Software*", for the construction of this tools.

**Results**

About Moby Services:
Data Sources identified ,designed and implemented

Currently in Implementation:

Web Services Designed, Developed, Registered and Documented for its consumption by the end users.
Client's GUI for the discovery and consumption of Services WEB
Client's GUI Integrated to the LIMS and another source of Data for the automatic publication of the information.
Trained users

About GCP Templates

Have data in GCP Central Registry for publish it.
Automatize the Filling of Templates (Design and Develop)
Use the GCP tools for Query and Download Datasets
http://www.generationcp.org/bioinformatics.php?da=0526518

About Tapir PyWrapper:
Installation and Tuning of TAPIR in our SERVER under LINUX.(Done): http://gene4.ciat.cgiar.org:8081/pywrapper/
Mapping and Exposing Databases as a Pilot Project (Cassava Est's and Cassava Passport Data)
Currently in implementation
Moby Services and TAPIR integration.
To have the Data from distinct Sources in the Publish Repository.
Concept definitions, "biodiversity ontology"
Mapping of DB to Concept Schema, Ontology
Links to the BioMOBY registry
Translates BioMOBY objects into TAPIR views
Views Shared

**Conclusion and future plans**

.
To design and to implement software tools that are in agreement with the GCP's Standards, to facilitate the publication of the information to the GCP partners and the international community.

To carry out the mapping process between the information and the Data modeling established by the GCP (and the ontology defined in it) to standardize the information to be published

To use the methodology of Web Services adopted by the GCP (Bio Moby, BioCASE), for the development of the services to publish and register; and build the data sources and the client's tools for users can consume these services

To train the Biotechnology's users so that easily they can use these products of Software

**References**

GCP: http://www.generationcp.org
TEMPLATES: http://www.generationcp.org/templates
TAPIR PYWRAPPER: http://trac.pywrapper.org/pywrapper/
http://rs.tdwg.org/tapir/1.0/rddl-2007-01-23.html
GCP PHANTEON: http://pantheon.generationcp.org
BIO MOBY: www.biomoby.org
GENE ONTOLOGY CONSORTIUM: www.geneontology.org
GCP CENTRAL REGISTRY: http://gcpcr.grinfo.net/

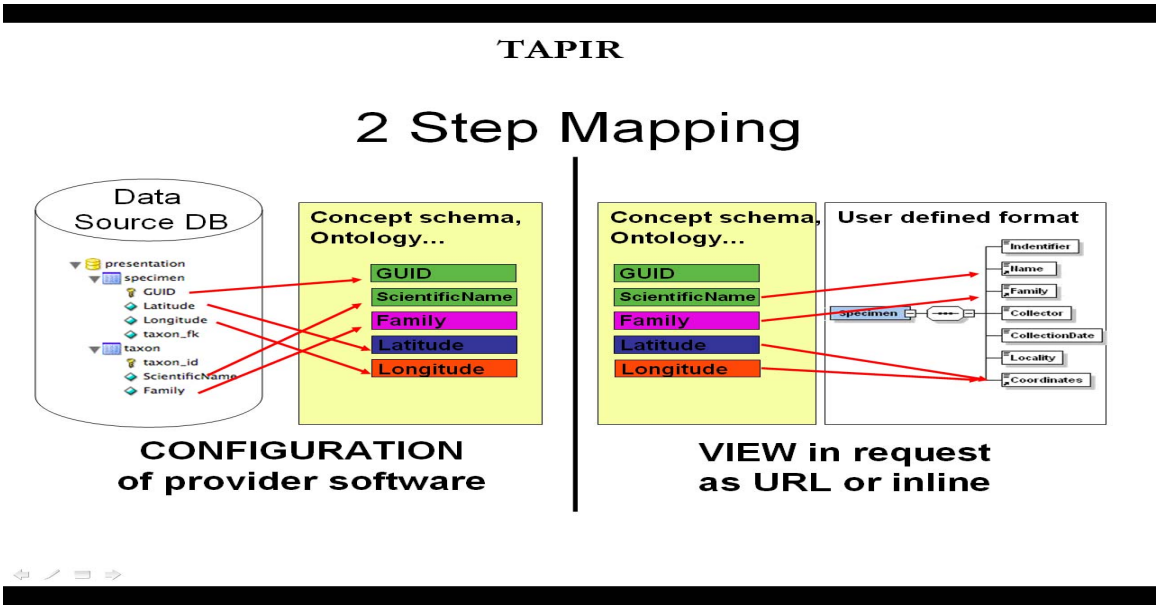


Figure 2

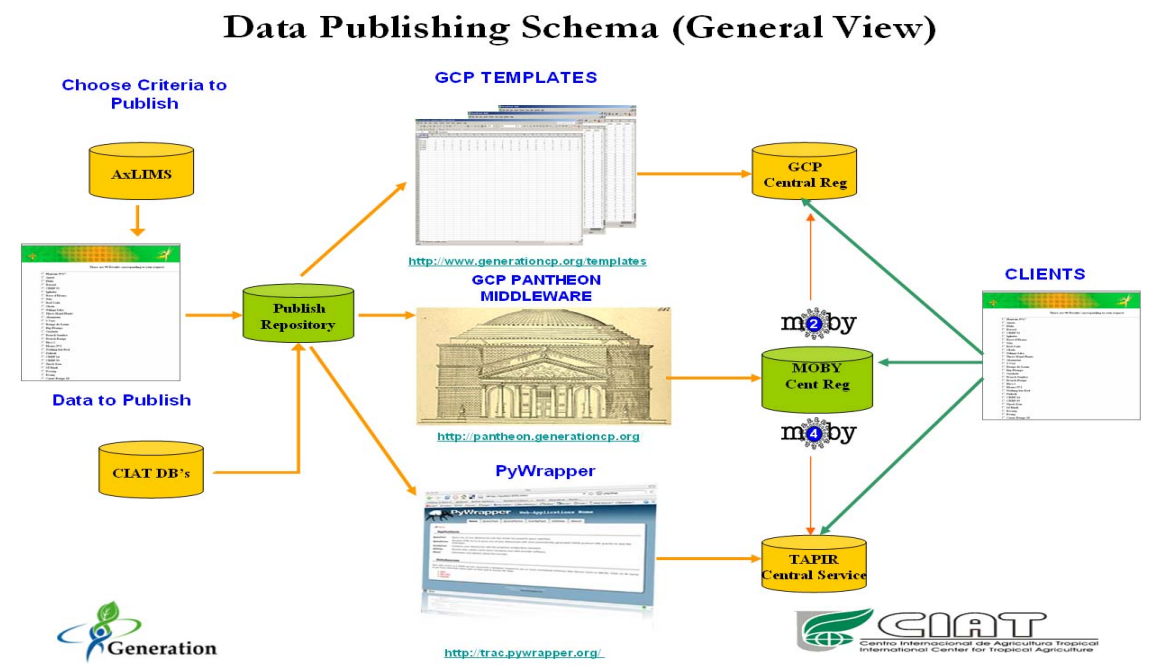


Figure 1

# Activity 3.2 Cassava Biotechnology Network's Activities

## Summary Final Technical Reports
## December, 06

### Project: Isolation and characterization of promoter sequences of genes possibly related to storage root formation in cassava.

**Researcher:** Prof. Dr. Claudia Regina Batista de Souza
Institution: Universidade Federal do Pará
Belém-PA, Brazil

**Project supported by Cassava Biotechnology Network, Small Grant program (US$ 8.000.00)**

#### Introduction

Cassava (*Manihot esculenta* Crantz), belongs to Euphorbiaceae family, is native from South America and one of the most important tropical foods crops for more than 600 million people worldwide. Basically every part of the plant can be utilized, but roots are the most commonly used product.
Cassava storage roots are an excellent source of starch (70-90% of their dry weight) but deficient in proteins, vitamins and other micronutrients [1]. Applications of transgenic technologies in cassava can be useful to increase nutritional value of roots, as well to solve losses in yield, such as those resulted from biotic and abiotic stresses, including disease, drought and acid soils, or from physiological deterioration during the post-harvest storage of roots [2].
Storage roots develop from primary roots through cell division and differentiation of parenchyma cells of the secondary xylem. Anatomical model revealed three tissue compartmentalization systems is being used in gene expression studies [3, 4]. According to this model; tissue system I is composed of phellogen and phelloderm, tissue system II of phloem and vascular cambium, and tissue system III of secondary xylem with its highly specialized starch rich parenchyma cells [3, 4]. Recent studies have been focused in the identification of proteins involved in storage roots formation using genomics and proteomics approaches [3, 4, 5, 6, 7, 8, 9]. Two cassava genes coding for glutamic acid-rich proteins, named Pt2L4 and C54, related to storage root formation were recently identified [4, 6, 8]. The Pt2L4 is an alcohol-soluble protein predominately expressed in the tissue system III, which contains secondary xylem and parenchyma cells with starch granules [4]. The deduced amino acid composition of the Pt2L4 protein shows that the most abundant amino acids are glutamic acid (31.6%), alanine (16.94%), valine (13.55%) and proline (11.29%) [8].
Pt2L4 and C54 proteins are 60% identical with similar molecular weights (16,7 to 18 kDa) and Isoelectric points (3.70 to 3.97) [6, 8]. There are two or more homologue genes coding for glutamic acid proteins in cassava genome according Southern blot analysis [6, 8]. Their transcripts are strongest expressed in vascular tissues and in parenchyma cells of storage roots indicating an important role on storage root formation [6, 8]. Besides, a strongest C54 promoter activity was detected in vascular cambium and starch-rich parenchyma cells of storage roots from cassava transgenic plants containing this promoter fused to the glucuronidase gene reporter [6]. Thus, this kind of promoter is a valuable candidate to drive a transgene expression in cassava storage roots.

Although recent advances in isolation and characterization of endogenous promoters of cassava, only few tissue and organ-specific promoters are available for genetic transformation of this crop. The identification

of genes with traits of biological, nutritional and agronomic importance and tissue-specific promoters is essential for genetic engineering of cassava.
In this work is reported the isolation of a promoter sequence of the *Mec1* gene coding for the Pt2L4 protein. Some putative transcription factor binding sites and *cis*-acting regulatory elements within this promoter sequence could be involved in storage root formation of cassava.

## Material and Methods

### Plant Material

Cassava (*Manihot esculenta* Crantz) leaves were kindly provided by Dr. Eloísa Cardoso from EMBRAPA Amazonia Oriental (EMBRAPA-CPATU, Belém-PA, Brazil).

### Genomic DNA extraction

Genomic DNA was isolated from cassava leaves using a Purelink Plant Total DNA Purification Kit and quantified using a Qubit fluoremeter, both supplied by Invitrogen Life Technologies, following manufacturer's instructions.

### Inverse PCR amplification

Samples containing about 10 μg of genomic DNA were partially digested with *HaeIII, EcoRI*, *HindIII*, *SphI*, *EcoRV*, *PstI* and *DraI* restriction enzymes. After phenol:chloroform:isoamyl alcohol (24:24:1) extractions and ethanol precipitation, DNA fragments were circularized by $T_4$ DNA ligase and used in PCR assays. Two reverse primers (Mec2-R and Mec3-R) and one forward primer (Mec4-F) were designed according to *Mec1* cDNA sequence (accession no. AY101376). DNA fragments were amplified using Mec2-R\Mec4-F primers in the primary PCR and Mec3-R\Mec4-F primers in the secondary PCR..

### Cloning and DNA sequencing

Amplified products were purified from an agarose gel using a QIAquick Spin kit (Qiagen), cloned into the pGEMTeasy vector system (Promega Corporation) and inserted into to electrocompetent bacteria cells (XL-1 blue, Stratagene). DNA sequencing was carried out with the Megabace Sequencer from Applied Biosystems following manufacturer's instructions.

### Sequence analysis

Nucleotide sequences were aligned using Blast algorithm [10]. TFSearch program was used for searching putative transcription factor binding sites [11]. PlantCARE and PLACE Databases were used to determining plant *cis*-acting regulatory elements [12, 13, 14].

## Results

In order to identify putative transcription factor binding sites and conserved plant *cis*-acting regulatory elements the 581 pb promoter sequence was analyzed using TFSearch algorithm and PlantCARE and PLACE databases. Sequence analysis showed that TATA box is located 111 pb upstream of the start codon. There are also several putative CAAT boxes, a common *cis*-acting element found in promoter and enhancer regions, upstream of the TATA box. Many known regulatory elements were also found in this promoter (Tab. 1).

**Tab. 1**. Putative Regulatory Elements found within *Mec1* promoter according to sequence analysis in PlantCARE and PLACE Databases. Positions given are relative to the 5'-end of the promoter. The orientation of motifs is indicated (+, forward; -, reverse).

| Motif | Organism | Position | Strand | Function |
|---|---|---|---|---|
| A-Box | *Petroselinum crispum* | 189 | - | *Cis*-acting regulatory element |
| Box-4 | *Petroselinum crispum* | 337 | + | Part of a conserved DNA module involved in light responsiveness |
| CAAT-box | *Hordeum vulgare*<br>*Arabidopsis thaliana* | 86<br>310 | -<br>+ | Common *cis*-acting element in promoter and enhancer regions |
| CAT-box | *Arabidopsis thaliana* | 564 | + | *Cis*-acting regulatory element related to meristem expression |
| CCGTCC-box | *Arabidopsis thaliana* | 189 | - | *Cis*-acting regulatory element related to meristem specific activation |
| CGTCA-motif | *Hordeum vulgare* | 209 | - | *Cis*-acting regulatory element involved in the methyl jasmonate-responsiveness |
| GARE-box | *Brassica oleracea* | 47 | - | Gibberellin-responsive element |
| MBS | *Arabidopsis thaliana* | 359 | - | MYB binding site involved in drought-inducibility |
| Skn-1 motif | *Oryza sativa*<br>*Oryza sativa* | 276<br>376 | -<br>- | *Cis*-acting regulatory element required for endosperm expression |
| Circadian | *Licopersicon esculentum* | 408 | - | *Cis*-acting regulatory element involved in circadian control |
| SPBF motif | *Ipomoea batatas* | 314 | + | SPBF binding site required by sporamin storage root expression |
| ATATT motif | *Agrobacterium rhizogenes* | 57<br>74<br>309<br>215<br>245 | +<br>+<br>+<br>-<br>- | *Cis*-acting regulatory element of rolD gene required for root expression in transgenic plants |
| AAAG motif | *Zea mays* | 178<br>324<br>14<br>24 | +<br>+<br>-<br>- | Core site required for binding of Dof proteins |
| CTCTT motif | *Glycine max* | 439 | + | Element characteristic of the promoters activated in infected cells of root nodules. |

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# Summary Final Technical Report
# December,06

## Project: Development of transgenic varieties of cassava resistanse to infection by *Xanthomonas axonopodis* pv *manihotis.*

**Researcher:** Francisco Campos, Federal University of Ceara, Brazil

**Submitted by Cassava Biotechnology Project (US$10,000)**

### Introduction

This document represents a summary of the Technical Report submitted to The Cassava Biotechnology Network (Small Grants Program) on the results obtained with the project "**development of transgenic varieties of cassava from Northeast of Brazil with resistance to infection caused by *Xanthomonas axonopodis* pv. *Manihotis***". Having established protocols for the induction of somatic embryogenesis (SE) in ten (10) cassava genotypes, we set out to improve the frequency of SE and embryo conversion of the explant. Parameters such as position of explant and explant size were considered relevant in this respect. In addition, we evaluated the effect of different concentrations the antibiotic kanamycin on secondary somatic embryogenesis using green cotyledons of cassava somatic embryos, in an attempt to establish a selective system for the explants. Experiments on transient expression of *uid*A (*GUS*) marker gene following the co-cultivation of green cotyledon pieces of with *Agrobacterium tumefaciens* were also carried out. This was followed by attempt to recover transgenic cassava plants from the co-cultured explants. Following the establishment and maintenance a number of Friable Embryogenic Callus (FEC) lines, we determined the concentration of the antibiotic paramomycin sufficient to select putative transgenic FEC lines in both proliferation and histodifferentiation medium. Furthermore, we carried out experiments on particle bombardment of cells with the plasmid pBI426, which harbors the *UidA* reporter gene fused with the antibiotic resistance gene *hptII*, under the control of *35SCaMV* promoter. Due to the difficulty of both embryo and plant recovery from cut pieces of cotyledons and FEC, we embarked on histological studies In order to examine the underlying histological changes associated with the induction and development of somatic embryos from green cotyledons of somatic embryos, which are the determining factors in plant regeneration.

### Materials and Methods

Primary somatic embryogenesis was induced by culturing isolated shoot apex isolated either from *in vitro* plants or from mature plants grown under field conditions on. These were cultured in petri-dish cassava induction medium (CIM) which is composed of Murashige e Skooge (1962) salts (MS) supplemented 8 mg/L picloram. Maturation of embryos was achieved by transferring the primary embryos into a medium containing 0.1mg/L BAP (CMM). A cyclic system of secondary somatic embryogenesis was established by subjecting cut pieces of cotyledons from mature somatic embryos CIM. Friable embryogenic callus was induced by transferring the fragments of somatic embryos to Gresshoff and Doy (GD) medium supplemented with MS vitamins, 2% sucrose and 12mg/l picloram. In the experiments to determine the phytotoxic levels of kanamycin and paramomycin, explants were incubated in medium containing filter-sterilized antibiotic and proliferation/tissue death of the individual explant was recorded over time.
*Agrobacterium tumefaciens* strain EHA105 containing the plasmid pBE2113-GUS was used to co-culture green cotyledons using a slightly modified protocol of *Zhang et al, 2000*. Both green cotyledons and FEC were bombarded using Particle Delivery System PDS1000/He (BioRad). For particle bombardment of FEC, cell suspension of the tissue was prepared at least 10 days before bombardment. Fractions of the cell suspension ranging in size between 100-500um were obtained from the suspension by first vortexing 15

seconds and passage through sieves. For each bombardment approximately 250mg of tissue was spread on the bombardment plate as a monolayer.
Where necessary, data obtained were analyzed using SAS (SAS INSTITUTE, 1992-1998).Histological sections of cotyledon pieces on CIM were made and analyzed at 0,1,3,6, 10, 16 and 20 days.

**Conclusion**

Results obtained indicate that higher frequency of SE and higher numbers of explants per embryo are obtained when explant is positioned with its abaxial side in contact with the culture medium. We have established cultures of independent FEC lines of FEC from different explants originating from different embryogenic cycles which demonstrated that somatic embryos can be recovered from these FEC lines at an average rate of 450 embryos/g of FEC tissues. Up to 50mg/L of Kanamycin is required to select green cotyledon of somatic embryos. When FEC tissues were subjected to both solid proliferation and histodifferentiation medium, the phytotoxic level of paramomycin was also found to be around 50mg/L. On the other hand, 5mg/L of the antibiotic was sufficient to arrest the proliferation of the cells in liquid medium. Results from *Agrobacterium*-mediated transformation experiments indicate that co-culture with the strain used reduces of embryogenic capacity of the explants, a trend that increases with time. We are presently screening a collection of *Agrobacterium tumefaciens* strains in order to identify more suitable for use in future transformation events. Particle bombardment of FEC showed that higher frequency of transformation is recorded when tissues were plasmolysed with equimolar concentrations of mannitol and sorbitol and the particle M5 used.

**Acknowledgements**

In the course of the development of the activities in this project, a number of collaborative works were carried out. Notably, I am collaborating with Jigawa Research Institute (Nigeria), whose staff is presently pursuing and MSc program under my supervision. Other collaborative works involve the laboratory of Prof. Richard Visser, University of Wageningen, and Holland, supported by the Brazilian Ministry of Education; Dr. Adilson Kobayashi of National Center for Cassava and Fruit Crops, EMBRAPA, Brazil and Dr. Francisco Jose Lima Aragão CENARGEN/EMBRAPA, Brasilia, Brazil.

## Summary Final Technical Report
## December, 06

### Project Title: Analysis of proteins, enzymes and genes related to tuberization and biosynthesis of starch from tuberous roots of cassava (*Manihot esculenta* Crantz)

**Researcher:** Agronomist Ricardo Daniel Medina

**Director of Training:** Dr. Luiz J.C. Branco Carvalho. Biochemistry and
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**Submitted by Cassava Biotechnology Project (US$5000)**

**Objectives**

1. Review and receive training in methods for analyzing proteins, enzymes and genes related to the formation of tuberous roots in cassava (*Manihot esculenta* Crantz).
2. Conduct a comparative analysis of fibrous and storage roots of cassava in plants grown under field conditions and regenerated by means of in vitro tissue culture. These studies involve analyses with optic

and scanning electron microscopes, evaluation of the activity of the principal enzymes of starch synthesis, and analysis by Northern blot of gene expression of candidate genes related to the formation of storage roots.

**Introduction**

Cassava (*Manihot esculenta* Crantz) is one of the principal crops worldwide, figuring among the 10 most important in the tropics and subtropics, where it constitutes one of the most widely used sources for providing carbohydrates for both human consumption and animal feed, as well as a raw material for diverse industries (Cereda *et al*., 2003). Cassava is cultivated in more than 60 countries, and it is a staple food for a population of some 500 million people (Cock, 1985). In SE Asia, Africa and South America, it is grown primarily for the production of its starchy tuberous roots (Cock, 1989). Traditionally it has been considered a subsistence crop; however, there is evidence in Africa, Asia and Latin America that documents its global emergence as a profitable crop (Scott *et al*., 2000).

The root system of this plant has different types of roots: assimilative or fibrous roots and storage or tuberous roots (Viégas, 1976; Domínguez *et al*., 1982). The latter are characterized primarily by their great capacity to store starch. The physiological process whereby a section of stem or root of a plant undergoes a morphological change to be converted into a special storage organ is known as tuberization (Melis & van Staden, 1984).

Different from other storage organs, little is known about protein patterns, enzymatic activity and gene regulation during the formation of the storage roots, especially the tuberous roots of cassava (Cabral *et al*., 2000; Carvalho *et al*., 2000; Cabral & Carvalho, 2001). Thus there is an urgent need to understand and explore in greater depth the research on the biology, physiology and biochemistry of tuberization in this species. Moreover, the lack of systematic information delays progress in relation to genetic engineering applied to plant improvement, a prerequisite being the establishment of efficient regeneration systems, the understanding of the metabolic processes involved, and the subsequent identification of promoter genes related to tuberization (Zhang *et al*., 2003). There is no doubt that these topics are linked to a theme of high priority for the Cassava Biotechnology Network, related to the study of useful genes and promoter genes within the area of "Biotech tools for the genetic improvement of cassava."

The foregoing was the basis for in-service training in methods for analyzing proteins, enzymatic activity and gene expression during the in vitro tuberization of cassava at the Biochemistry and Biophysics Laboratory of EMBRAPA-CENARGEN, developed under the direction of Dr. Luiz J.C. Branco Carvalho.

**Experiment 1:**

a. External morphology and anatomy of the cassava roots regenerated in vitro and in the field

b. Histological and anatomical analyses using optic and scanning electron microscopes

c. Morphology of the starch granules

In conclusion, the three field-grown cassava cultivars analyzed had three types of roots (primary fibrous roots, secondary fibrous roots and secondary storage roots). The secondary roots are similar both morphologically and anatomically although in the storage root, the reserve parenchyma stands out. The primary roots differentiated in vitro were similar to those obtained in the field, but not the in vitro storage roots, which had a primary state but showed signs of tuberization, which to a certain extent would make them comparable to their homonyms from the field. The experimental model used in this study to differentiate the in vitro storage roots can also simulate, with a high degree of fidelity, the formation of the

different types of starch granules synthesized in roots grown in the field, thereby opening up the possibility of advancing in studies related to their origin, growth and regulation.

**Experiment 2**

a. Analysis by means of standard radioactive methods of the ADP-glucose pyrophosphorylase (ADPGppase), soluble starch synthease (SSS) and branching enzyme (BE) activity in cassava roots regenerated in vitro.

b) Analysis by means of standard radioactive methods of the ADPGppase, SSS and BE activity in roots of different cassava clones grown in the field.

It was possible to measure the ADPGppase (EC 2.7.7.27), SSS (EC 2.4.1.21) and BE (EC 2.4.1.18) activity in the cassava roots grown under field conditions as well as in vitro.

The differences found between the fibrous roots (control) and those obtained in media with 5% sucrose plus naphthalene acetic acid and 6-benzylaminopurine would corroborate the inductive action of high concentrations of sucrose and the presence of growth regulators on the activity of the ADPGppase, a fundamental enzyme for synthesizing starch. The lower activity of the three enzymes studied in the storage roots as compared with the fibrous roots *in vitro* regenerated in media with 5% sucrose plus plant growth regulators could be explained by the assumption that these are occurring due to a declining phase of starch synthesis.

On the other hand, the biochemical characterization of different cassava clones made it possible to discriminate them by measuring the activity of these three enzymes, being able to associate them with their commercial use.

**Experiment 3**

Analysis by Northern blot of gene expression of candidate genes related to the formation of storage roots

In summary, it was possible to acquire experience and a greater knowledge of all the steps involved in analyzing gene expression by Northern blot although it was not feasible to obtain conclusive results. It is necessary to continue adjusting the protocol for extracting total RNA from in vitro material in order to obtain a good quality and quantity of RNA for running the blots. The use of guanidine thiocyanate in the extraction caused problems in pellet solubility so it is recommended to use guanidine hydrochloride or experiment with other denaturation buffers.

**Papers presented at scientific meetings**

**1.** 01-04/08/06: Biochemical characterization of clones of cassava (*Manihot esculenta* Crantz) with different commercial applications, by **R.D. Medina**, L.J.C.B. Carvalho, M.A. Valle Agostini; S.J. de Oliveira; M.M. Faloci and L.A. Mroginski. Abstract and panel, 17th Meeting of Scientific and Technical Communications from the School of Agrarian Sciences, National University of the Northeast (UNNE).

**2.** 01-04/08/06: Analysis of the activity of enzymes involved in the synthesis of starch in cassava roots regenerated in vitro, by **R.D. Medina**, L.J.C.B. Carvalho, R.E. Pinto da Silva, L.P. Barrueto Cid, M.M. Faloci and L.A. Mroginski. Abstract and panel, 17th Meeting of Scientific and Technical Communications from the School of Agrarian Sciences, UNNE.

**3.** 24-27/10/06: Activity of ADP-glucose pyrophosphorylase, soluble starch synthease and branching enzyme in roots of cassava (*Manihot esculenta* Crantz) regenerated in vitro, by **R.D. Medina**, L.J.C.B. Carvalho, L.P. Barrueto Cid, M.M. Faloci and L.A. Mroginski. Panel and abstract, 11th Meeting of Scientific and Technological Communications, organized by UNNE –Resistencia Campus.

# Summary - Final Technical Report
# December 2006

## Project: Production of high quality cassava planting material

**Researcher :** Teresa Losada Valle, Biological Institute, Sao Paulo, Brazil

**Submitted by Cassava Biotechnology Project (US$10,000)**

### Introduction

Annualy the Brazilian Central/Southern region grows and processes about 6 million tons of cassava roots per year. This production is done mainly by small farmers who sell the roots to flour or starch industries, or to the fresh market. Nine high-performance varieties cover almost the total area of approximately 300,000 ha. Several other varieties may have local importance and are cultivated in small areas. (IAC, 1998)

Many pathogens occur in this region, but just few are responsible for crop losses. The bacterial blight caused by *Xantomonas axonopodis pv. manihotis* (*Xam*), is the most important disease, causing direct losses. The bacterium occurs in an endemic manner, but the weather conditions (high discrepancies between the minimum and the maximum temperatures, and cool temperatures associated with rains) favor severe epidemics (Takatsu *et al*, 1978).

The cultivated varieties have a resistance level from middle to high, although they may permit some pathogen growth. Thus, the disease outbreaks rely on the genetic resistance level of the variety, contamination of the planting material and or weather conditions. The bacterial lodges in the vascular system and in the epiphytic bacterial population symptoms may not appear for years, It does not survive in other plants or in the soil. The disease spreads mainly through infected planting stakes, what stresses the importance of healthy material. Production of healthy planting material should be a complementary agronomic practice to the genetic resistance (Valle, 1996)

The Cassava Common Mosaic Virus (CsCMV) may infected all cultivated varieties. Although it does not to cause significant direct losses, field observations suggest that infected material may drop the resistance to the bacterium. The mosaic spreading by vectors is very low, and the major dissemination is due to planting material. Other viruses such as vein mosaic, frogskin and phytoplasma occur in an endemic manner.

Since several decades the Instituto Agronômico (IAC) has worked on the selection of varieties with good agronomic characteristics and resistance to *X. axonopodis* pv. *manihotis*. The "IAC-varieties" have gained widespread acceptance among the growers. In the last years, the IAC has developed a project with the aim of producing healthy, high quality planting material, with two purposes: a) to deliver a basic stock of healthy planting material to the farmers and make them capable to produce their own material; b) to warn the growers about the importance of the sanitary and physiological quality of the planting material for the crop success. However, this work has been made only by visual survey and by using planting areas, for seed purposes, under unfavorable micro-climates to the bacterium. These practices, however, have limited

success for many pathogens that induce no symptoms and areas under unfavorable micro-climates are small and far from the main growing areas.

The thermoterapy associated with meristem culture is a reliable and efficient method to eliminate *Xam* and asymptomatic pathogens. It is also utilizated to eliminate the CsMCV associated with other serological and biological tests to identify the healthy plants. The virus can be detected by ELISA in plants in *in vitro* culture, what hastens the production of basic planting material.

The cleaning process of pathogens may also destroy useful micro-organisms. Thus, the acclimatization of the plant material from a total asepsis until the field cropping should be gradual in order to permit a reinoculation with useful micro-organisms, in the excludents areas, where some pathogens are under control. From these practices it is possible to obtain a stock mother plants of varieties to feed a production system of certified planting material.

**Matherial and Methods**

Individual vigorous and apparently healthy plants of thirteen varieties (Fécula Branca, Espeto, Fibra, IAC 576-70, IAC 12, IAC 13, IAC 14, IAC 15, IAC 90, Branca de Santa Catarina, Olho Junto, IAC 14-18 e Ouro do Vale) were selected under field conditions. From each one, seven stakes were collected and submitted to thermoterapy for 30 days, under a regime of 16 hours with light and a temperature of $40^0$ C, and 8 hours without light and $28^0$ C (Figure 1), followed by another 30 days with the same light regime, but under a constant temperature of $40^0$ C. After the thermoterapy, several meristems were collected from each stake and cultivated *in vitro* during 30 days. Each plantlet originated of a single meristem was multiplied and a sample was collected and analised to verify the presence of the CsCMV virus by two methods. The first one was the ELISA test using a policlonal specific antiserum, and the second was the inoculation of the test plant *Chenopodium amaranticolor* (Palazzo *et al.,* 2006). The healthy plants were the basis of a stock lot kept *in vitro*. Another group of plantlets was multiplied and acclimated in a greenhouse, in a vermiculite substrate with nutritive solution irrigation. The adapted plants were transplanted to plastic pots filled with a non sterilized soil. From these plants, a basic stock is being maintained under greenhouse conditions, and non aseptic conditions. Plants originated from these stocks were integrated in the Instituto Agronômico seed production program, where the variety Secundina, an indicator variety for different cassava viruses, is used for the detection of the possible presence of these pathogens.

**Results and conclusions**

Cassava plants of thirteen varieties free from *Xam* and CsCMV, the most important cassava diseases transmited by the planting material, were obtained through thermoterapy and meristem culture. The combined methods freed 61% of the plantlets from the CsCMV, according serological and biological tests. The healthy plantlets were the basis of a basic stock kept in vitro or under greenhouse conditions, after acclimatization in a non sterilized soil. Plants originated from these stocks were integrated in the Instituto Agronômico seed production program.


Figura 1: Sprouting of cassava after thermoterapy

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Participantes
Teresa Losada Valle; Addoralata Colariccio; Jose Carlos Feltran; Antonio Lucio Mello Martins

# Summary - Final Technical Report
# December 2006

## Project: Isolation and identification of full-length cDNA clones from transcripts differentially expressed in cassava (*Manihot esculenta* Crantz) genotypes resistant to whitefly *Aleurotrachelus socialis* Bondar (Homoptera: Aleyrodidae)

Researcher : **Danilo Eduardo Moreta Mejía**

Participant Institutions: **International Center for Tropical Agriculture (CIAT), Colombia; and Instituto Nacional Autónomo de Investigaciones Agropecuarias (INIAP), Ecuador.**

Research advisors: **Dr. Martin Fregene (CIAT) and Dr. Julio Cesar Delgado (INIAP).**

**Submitted by Cassava Biotechnology Project (US$8,000)**

**Abstract**

Cassava *(Manihot esculenta* Crantz*)* is a traditional crop and an important source of carbohydrates for many developing countries, providing food for over 500 millions people worldwide (Allen, 1992). In the tropics, it is considered as the most important tuber crop for food security and economic value, ranging fourth as a source of calories in human diet after rice, sugarcane and corn. Cassava is mainly used as a source of food for human diet. Nevertheless, its use as animal food and raw material for starch production and byproducts is increasing very quickly (Best and Henry, 1992).

Whiteflies (around 1200 species) are considered as one of the most important plagues that affect worldwide to an extensive range of crops, resulting in substantial economic losses. In north south-America (mainly Colombia), whitefly *Aleurotrachelus socialis* Bondar is the species which cause the cassava´s most serious economic losses. It has been reported yield root reductions of up to 79% (Bellotti *et al.,* 2004). For this reason, cassava producers have increased the usage of chemical products, which generates induced resistance of the plague, and therefore it becomes more difficult to control it. Moreover, this strategy cause a negative impact to the environment and the cassava producer´s incomes.

In order to look for solutions to the whitefly constraint, CIAT has been conducting research focused on the search and develop of cassava resistant cultivars using cassava genotypes from its germplasm collection. Up to date, the major source of resistance is the MECU 72 cultivar. When *A. socialis* feeds on MECU 72, there is a reduction in its oviposition, its developing rate gets longer, and the mortality rate is higher than susceptible cultivars. These results suggest that MECU 72 has a combination of antibiotic and antixenosis resistance mechanisms. On the other hand, further phenotipical studies performed recently at CIAT report other genotypes of the *Manihot* genus (native and commercial cultivars) which have shown a high level of resistance to *A. socialis* quite similar to that showed by MECU 72 (Bellotti *et al.,* 2004).

At CIAT, molecular research has been carried out on the MECU 72 cultivar to develop molecular markers associated to whitely resistance (Bellotti *et al.,* 2004). However, it is also necessary to perform functional genomics studies to set up the probable ways of early response (differential of expression) for resistance to whiteflies through its different instars. Likewise, it is important to evaluate new cassava genotypes (native and commercial species) that have shown a similar resistance to MECU 72.

Due to the world significance of whitefly as a plague, it is really important to study cassava genotypes resistant to *A. socialis* and identify the genes involve in this trait. This will enable to study their function in the plant defense mechanism, and thus determine their potential utility in future cassava breeding programs for resistance to whitefly. Since so far it has not been reported cassava genes involve in the resistance to whitefly, this study intends to establish the basis to carry out in the near future deeper investigations related to this issue.

The objective of this research is to identify resistance genes to whitefly *Aleurotrachelus socialis* Bondar (Homoptera: Aleyrodidae) on cassava genotypes, and determine its differential of expression as a response to the plague infestation. To achieve this, a full-length cDNA library will be constructed from the evaluated resistant genotypes which have been subjected to pressure by *A. socialis* at a greenhouse bioassay.

The phenotipical response for *A. socialis* infestation of the cassava genotypes considered in this study has been studied in advance by means of bioassays. The obtained results shown a high level of resistance and hence they have been taken into account for the molecular analysis to be done in this study.

The cassava genotypes resistant to *A. socialis* that were evaluated are the follow:

MECU 72 (*Manihot esculenta*), and
MECU 64 (*Manihot esculenta*).

The MCOL 1468 genotype was used as a susceptible control.

In the same way, the genotypes CRA 013 and CW 67-147 (interspecific cross) were planted at the greenhouse for a subsequent molecular study since these genotypes have also displayed a high level of resistance to *A. socialis* (Fregene, 2005).

For the greenhouse bioassay, the genotypes to be studied of 35-45 days old were infested with adult individuals of *A. socialis,* which were previously grown up on the susceptible genotype (MCOL 1468). The infestation process was performed into wooden cages (1x1x1 m) covered with a tulle. Inside the cages, four plants cultivated on plastic pots (biological replications) were infested with 1000 *A. socialis* mature individuals (250 individuals per plant) using an buccal aspirator. Four independent cages were infected *with A. socialis*, each representing a particular instar of the plague and from which the total RNA from leave tissue will be isolated for each infestation stage. This bioassay was set up at 21 – 30$^{o}$C and at 60 – 90% of relative humidity.

In order to determine either the delayed or early response to *A. socialis* infestation by the genes involve in the resistance; the harvest times of the leaves for the total RNA isolation were done at 6 hours, 6, 25, and 33 days post-infestation corresponding to the instars of adult, egg, nymph, and pupae respectively (each infestation cage corresponded to each instar of the plague). Young cassava leaves were collected for the total RNA isolation because in this tissue has been observed the resistant genotypes´ defense response (antibiosis and/or antixenosis) to *A. socialis* infestation (Gómez, 2004).

The total RNA extraction was performed according to the protocol by Eggermont *et al.* (1996). Each total RNA isolation from cassava leaves infested with *A. socialis* was performed according to the plague´s four development stages (instares): adult (total RNA isolation 6 hour post-infestation), eggs, (total RNA isolation 6 days post-infestation), nymph (total RNA isolation 25 days post-infestation), and pupae (total RNA isolation 33 days post-infestation).

In order to establish if the isolated total RNA samples were contaminated with genomic DNA, a PCR was done with two cassava specific primers (BAC 35 and TAG1) and primers designed from conserved regions to amplify the rDNA 18S. The obtained results did not show amplification of the target genes when using the cassava primers likely due to RNA inhibition in the PCR. However, when the rDNA 18S primers were used, most of the RNA samples showed DNA contamination, so they were treated with DNase I enzyme before proceeding with the subsequent experiments.

The messenger RNA (mRNA) using the PolyATtract® mRNA Isolation System III with Magnetic Stand (Promega, Cat. No. Z5300) was isolated from total RNAs pool of the MECU 72 genotype which was previously subjected to *A. socialis* infestation. Afterwards, the mRNA was treated with DNase I and then converted to cDNA in order to construct the full-length cDNA library using the CloneMiner™ cDNA Library Construction Kit (Invitrogen, cat No. 18249-029). Such a library was already constructed, but there were some constraints with the process since the number of clones obtained was not high. In the same way, the characterization of this library by means of PCR to determine the size of the inserts has been quite difficult and remains to be standardized.

**Acknowledgments**

We thank Dr. Martin Fregene (Lead of the Cassava Genetics Program at CIAT) for his scientific supervision; Arturo Carabalí, M.Sc. for his guidance in the planning and establishment of the greenhouse bioassay, Isabel Moreno, M.Sc. for her great support in the lab experiments, and Adriano Muñoz, Carlos Ñañez and Gerardino Pérez for their help in the greenhouse activities.

Our research was financed by the Cassava Biotechnology Network (CBN) 2004 Small Grant Scheme.

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# Summary - Final Technical Report
# December 2006

## Project: Biochemical and molecular characterization of anti-immune compounds that suppress *Cyrtomenus bergi* Froeschner (Hemiptera: Cydnidae) immune response

Researcher **: Ana Milena Caicedo[1] -James Montoya Lerma**

**Submitted by Cassava Biotechnology Project (US6,000)**

### Introduction

Biochemical tools were used for the identification of the nature of the immune substance(s) produced by *C. bergi* (polyphagous pest that affects a diversity tropical crops) to explore and identify the proteins/peptides produced when is challenged with pathogenic (*Xenorhabdus nematophilus* and *Photorhabdus luminescens)* and non-pathogenic (*Escherichia coli)* bacteria, that help to understand its defence reactions.

Protease activities in the hemolymph and fat body infected with *Escherichia coli, Xenorhabdus nematophilus* and *Photorhabdus luminescens* were evaluated using SDS-polyacrylamide gel electrophoresis containing gelatin as substrate. Analysis of zymograms performed on samples of different tissues of controls and insects inoculated with pathogenic and non-pathogenic bacteria, demonstrated distintic patterns of proteases activities in both hemolymph and fat body of *C. bergi.*

The haemolymph pattern was similar for all treatments, so it was not possible to detect any *C. bergi* reaction to the bacteria. The activities had apparent molecular weights of 66, 45, 36 and 29 kDa (Fig 1.). In contrast, the fat body samples with the pathogenic bacteria (*Xenorhabdus* and *Photorhabdus*) showed a different pattern compared with the non-pathogenic bacteria (*E. coli*) and the control samples. It was clear that the *Xenorhabdus* activity displayed four proteases with 45, 36, 29 and 20 kDa of molecular weight while *Photorhabdus* showed one additional band of 33 kDa. *E. coli* treatment and the control share a similar pattern among them displaying very weak bands (Fig. 2).

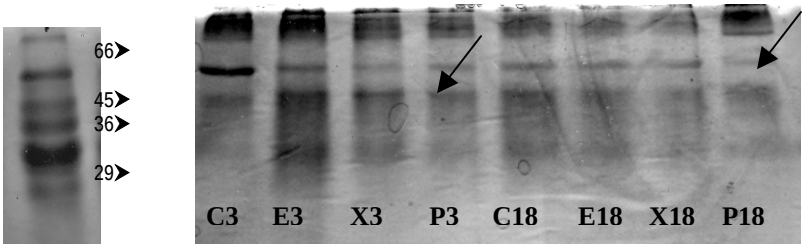



Figure 1. Proteolytic activity of *C. bergi* hemolymph challenged with bacteria. Lane C3 (hemolymph control, 3h), lane E3 (*E. coli*, 3h), X3 (*X. nematophilus* 3 h), P3 (*P. luminescens*, 3h), C18 (control 18h), E18 (E. coli 18h), (*X. nematophilus* 18h) and P18 (*P. luminescens*, 18h).

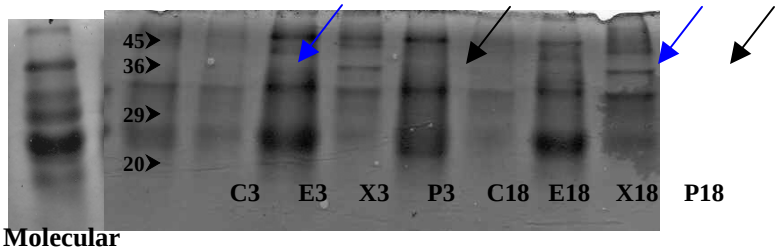



Figure 2.Proteolytic activity of *C. bergi* fat body challenged with bacteria. Lane C3 (fat body control, 3h), lane E3 (*E. coli*, 3h), X3 (*X. nematophilus* 3 h), P3 (*P. luminescens*, 3h), C18 (control 18h), E18 (E. coli 18h),(*X. nematophilus* 18h) and P18 (*P. luminescens*, 18h).

The proteases detected in hemolymph of *C. bergi* challenged with *E. coli, X. nematophilus* and *P. luminescens* belong to the class of cysteine proteinase since they are inhibited by E-64. Besides, EDTA inhibitor blocked all bands in all bacteria treatments in fat body (Fig 4), suggesting that blocking bands belong to the metallo-proteases class.

The significance of these proteases in the insects infected with pathogenic and non-pathogenic bacteria is discussed in relation to the effort to identify for the first time, specific biochemical markers related to immune response allowing understand the *C. bergi* defence reactions to specific bacteria. At the long term, we look to develop new strategies for the control of this important cassava crop pest.

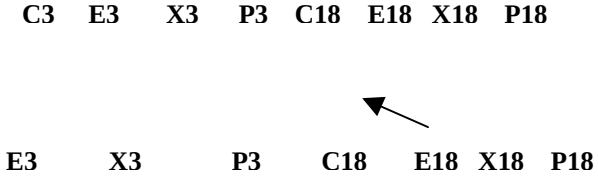



Figure 4. **Inhibitory pattern of proteolytic activity of**

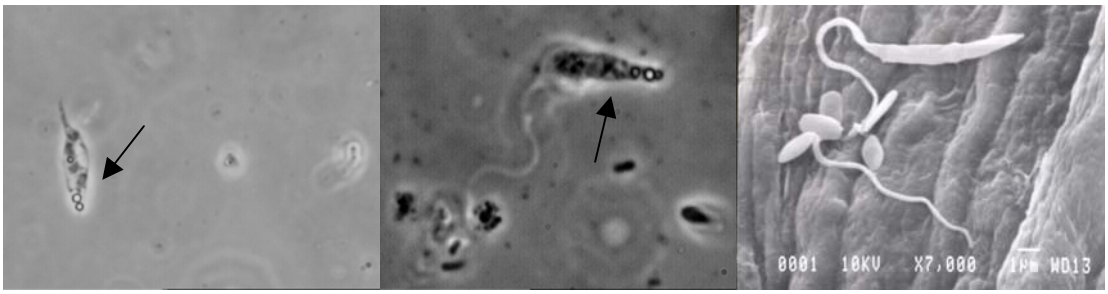
*C. bergi* fat body with EDTA. lane E3 (*E. coli*, 3h),X3 (*X. nematophilus* 3 h), P3 (*P. luminescens*, 3h),C18 (control 18h), E18 (E. coli 18h), (*X. nematophilus* 18h) and P18 (*P. luminescens*, 18h).

Aditionally, during the last year, we found an undetermined flagellate associated to the *C.bergi*, based on circumstantial evidence, it is thought that the flagellate confer resistance to the attack of the entomopathogenic nematode according with the results obtained during the evaluation of six species against *C. bergi* adults, producing only 10-30 percent of mortality by Caicedo et al (2004) evaluating but 100 percent of parasitism. Meaning that nematodes are able to penetrate but the associated bacterium is not able to disseminate.

At light microscopy, the flagellate appears to be present mainly in haemolymph and in the hindgut. However, no quantitative data have being obtained. Also, no data is available on its life cycle or on the mechanism of transmission of this potential bug symbiont.

In the present paper, we describe the successful isolation, *in vitro* culture of this flagellate, obtained from wild (two Colombian sites: La Florida and Santander de Quilichao) and laboratory *C. bergi* specimens. In addition, an attempt to genotype the flagellate is documented.

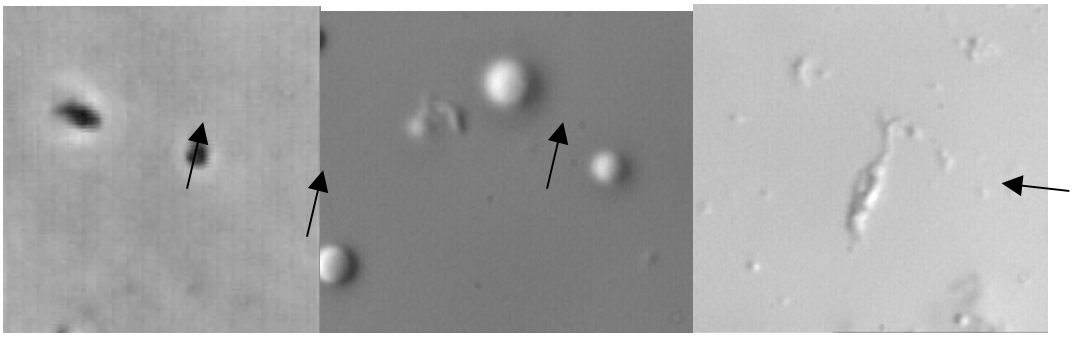
From both sites, all stages of the wild *C. bergi* were positive for flagellates when examined under light and electron microscopy and PCR assays. Parasites ranged from long to short forms and cyst stages are varied in numbers in the different stages of *C. bergi*. (Fig. 1). Heavy infections were detected in fifth nymphal and adult stages.




**A.** **B.** **C.**

**Figure 1. Different forms of flagellate observed on adults of *C. bergi* haemolymph and intestine from field population. A: Short form with short flagellum (light microscopy 100x1.6xDIC); B: Short form with long flagellum (light microscopy 100x1.6xDIC) and C: long form and short forms with long flagellum (scanning microscopy CIAT by Arroyabe).**

Under light microscopy exam, *C. bergi* laboratory populations showed the same forms also were detected from eggs to adult stages. From each egg smeared in PBS was possible to observe small cysts with a special movement (three to four for each). In the first and four instars was also possible to observe short and long flagellates (Fig 2). Measurements of the cyst eggs flagellates were performed and varied between 2.39 µm long to 1.88 µm wide.



**A.** **B.** **C.**

**Figure 2. A. Flagellates from eggs flagellum (light microscopy 100x1.6xDIC. B. flagellates from first instar flagellum (light microscopy 100xDIC) and C. flagellates form four instar of *C. bergi* laboratory population (light microscopy 100xDIC). (picture taken by Torres A., Univ. Cauca, 2006).**

The PCR assays performed to detect DNA from eggs to all stages of *C. bergi* from laboratory and field populations were positive for all pools of insects evaluated. The two genes 5S and SL rRNA were successfully amplified using the Dollet et al (2000) protocol from eggs to adult stages of *C. bergi*. The tandem arrangement of the 5S rRNA genes resulted in the amplification of one band in all cases of 0.08 kb (Fig. 6). For SL genes resulted in the amplification of two to three bands of 0.06 to 0.08 kb (Fig 7).

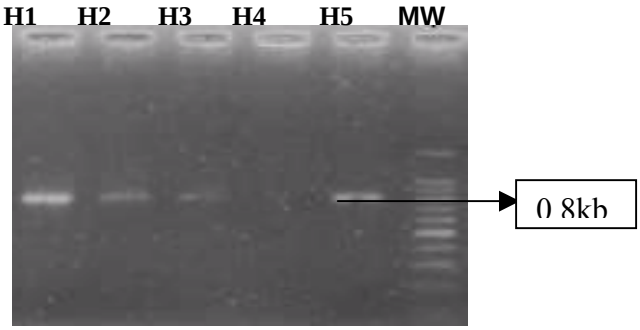



**Figure 6. Amplification of 5S rRNA band from eggs (H1), *in vitro* culture (H2), fifth nymph (H3), control (H4), adults (H5) and DNA molecular weight. (Agarose at 1.6%).**

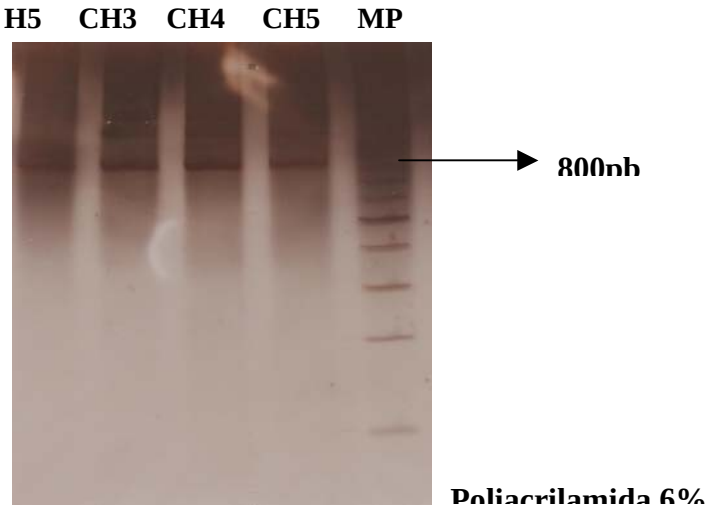



**Figure 7. Amplification of SL rRNA band from eggs (H1), *in vitro* culture (H2), fifth nymph (H3), fourth nymph (H4), adults (H5) and *in vitro* culture and DNA molecular weight (Poliacrylamide at 6%).**

Therefore, the amplification of two genes from all the stages of *C. bergi* from laboratory evaluated mean that transovarial transmission of flagellates in *C. bergi* is possible. This fact evidences the great amount of flagellates found in all stages and *C. bergi* populations evaluated until now.

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Dr. Alex Bustillo- Disciplina Entomología-CENICAFE.
Jorge W. Arboleda - Disciplina Entomología-CENICAFE.
Horacio Cadena-Laboratorio Entomología-CIDEIM.
Gustavo A. Vallejo-Laboratorio de Parasitología, Universidad del Tolima.

## Summary - Final Technical Report
## December 2006

### Project: Improving Cassava Drought Tolerance Through Enhanced Mycorrhizal Symbiosis

**Researcher :** Aldo Vilar Trindade

**Reporter: Institution:** Empresa Brasileira de Pesquisa Agropecuária (Embrapa Cassava and Fruit Crops)

**Submitted by Cassava Biotechnology Project (US10,000)**

Achievements and constraints:

Summarize the results of ongoing activities
Highlight achievements & breakthroughs
Describe constraints / set backs experienced (include comments on current capacity to carry out pre-established workplan)

According to timetable, the period was dedicated to arbuscular mycorrhizal fungi (AMF) isolation from diferent soils samples and accomplishment of first experiment with plants in condition of soil water deficit. This activities were programmed to be executed in coordination with Dr. Edmundo Barrios from CIAT, but this partnership was not accomplished due to organization changes in that institutuion. In Embrapa we have thirty five samples of soils in trap culture to produce fungi spores to future isolation. Some samples collected from Brazil semiarid were trap cultured one year early and now we could shorten the period of isolation. From this samples, spores were separated and groups of 60 were inoculated in *Sorghum bicolor* to produce pure inoculum. From the first pot culture evaluated we obtained two new isolates of *Gigaspora margarita* and *Glomus clarum*.

On other side, soil collected in Cruz das Almas, Brazil was prepared (drying, homogenizing and autclaving) to be used in the first experiment where we used one mycorrhizal fungi and diferent cassava genotypes to evaluate the contribution of FMA to plants under water stress. The experiment was set in april, 14$^{th}$. Six varieties ("Amansa Burro", "Cigana Preta", "Aipim Bravo", "Fio de ouro", "Cacau" e "Paraguai") with different tolerance to water stress were used. The first three are tolerant to water deficit and the others, are sensible. Seedlings were supported by Dr. Alfredo Alves. The experiment design was completed with two levels of water potencial and inoculations of AMF or not. After 40 days we begun to promote the two different water potencials by weighting the suite. This has been proved to be a challenge to set up.

Inoculum of mycorrhizal fungi from collection of our laboratory were cultivated with *Sorghum bicolor* for multiplication.

The project has the technical support of a undergraduating student. The main constraints were the time necessary to train this person in isolation techniques and the quit of Dr. Edmundo Barrios from our team, which turned unfeasiable some data. On other side, the obtention of cassava plants to set trap culture and the experiments is not so rapid and some time had to be spent. Also, we had to change the methodology to set water deficit in soil because of technical limitations in using probes, previously planed, in pots.

The results of the experiment showed that varieties have different growth capactity and responses to the factors evaluated. Variety Cigana Preta had no growth response do inoculation or water deficit (figure 5). The other varieties grew better when soil had no water limit and *Gigaspora margarita* was inoculated.

Water deficit was not limitant for plants without inoculation of AMF. As soil was autoclaved, we admit that these plants were not colonized by native fungi, also. When plants were inoculated, they grew better in soil with no water deficit, at least for two of the varieties evaluated, both, susceptible to soil with low water potential.

Water deficit reduced stomatal conductance of leaves, as expected, but this was influenced by the interaction of variety and inoculation. (figure 6). Under water stress, inoculation maintained a high stomatal conductance, but not in "Aipim Bravo", in which, caused a great reduction.

**Implications to workplan**

Explain how do the achievements and constraints from the previous section confirm or alter the established workplan (research design, partnerships, participatory and gender-sensitive methods, tools and approaches).

Fungi isolation is an activity time consuming and this was foreseen in the project, but we already have some soil samples, collected one year early in semiarid region that were submitted to trap cultured and now are been used to direct fungi isolation. This will probably garantee the accomplishment of activities in a good term.

With aid of the other participants of the project the obtention of cassava plants was assured.

**Communication and Dissemination of Information**

Summarize events held or attended during the reporting period (training programmes, workshops / seminars) and the activities undertaken at these events
Give details of peer reviews, scientists contacted and other mechanisms for successfully disseminating results to target groups

Results obtained were presented in July/2006, in a seminar of undergraduating activities supported by the project and by the CNPq (National Council for Scientific and Technological Development) . The project was included in a group of actions/projects of agroecological approach and presented in two events designed to cluster scientists with similar views. The events occured in Brasília, Brasil, in October/2005 and in Vitória da Conquista, Bahia, Brazil, in December/2005.

**Additional comments**

Any additional information the reporter feels should be included in the quarterly report

In attachment, some photographs of FMA isolation, trap culture (cassava and *Crotalaria juncea* + *Brachiaria decumbens*), and inoculum production of FMA.

Note: A limit of five pages excluding graphs and tables has been set for these reports.

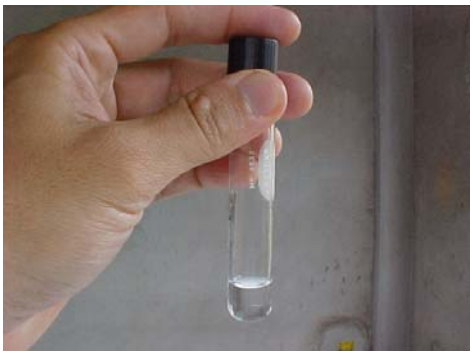


Figure 1. Photos from isolation of MA fungi

Figure 2. Trap culture of MA fungi using cassava micropropagated plants

Figure 3. Plants cultivated for inoculum prodution, isolation of MA fungi and trap culture, in greenhouse conditions.

Figure 4. Mounting of experiment with cassava plants. April, 2006

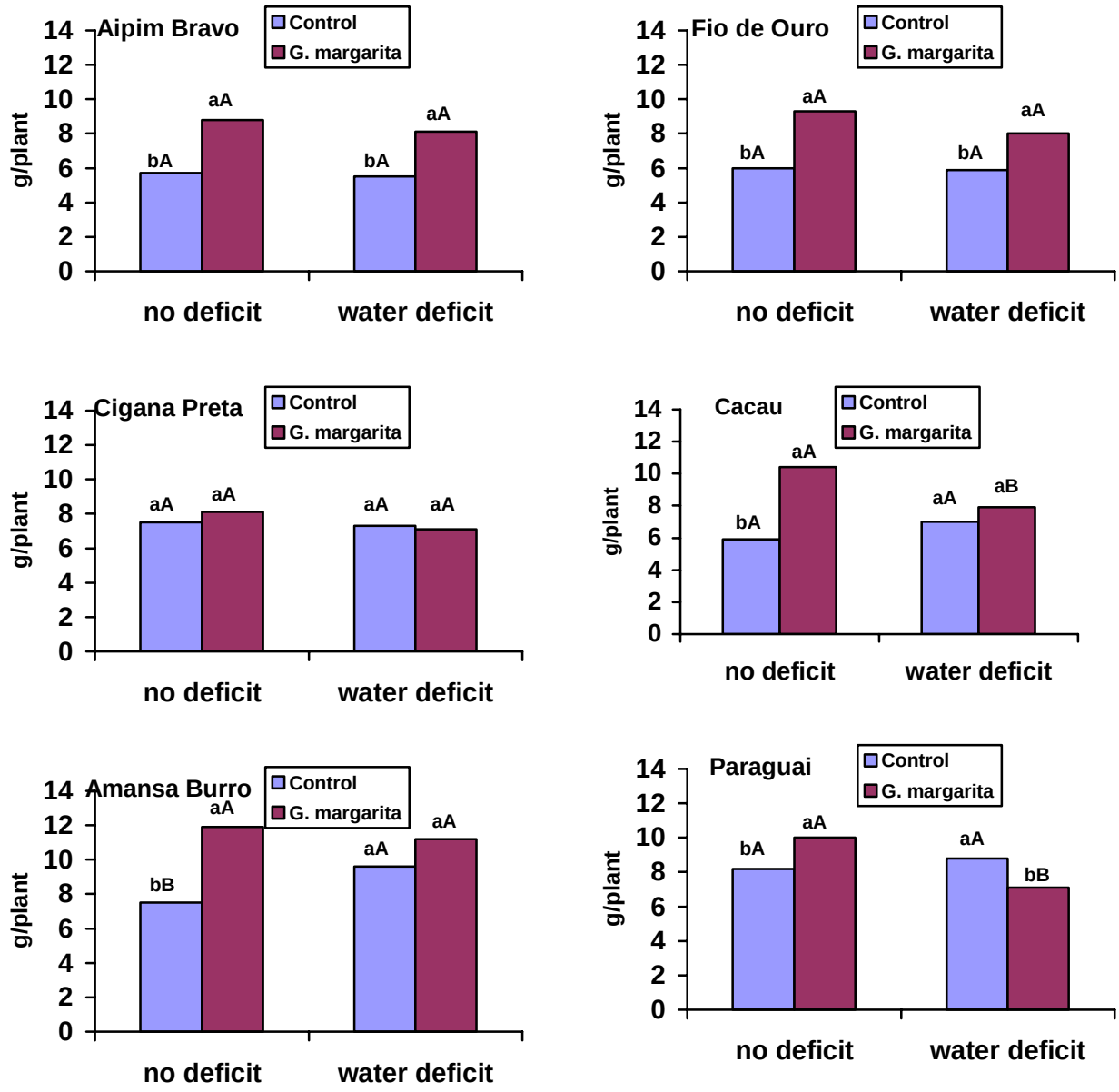
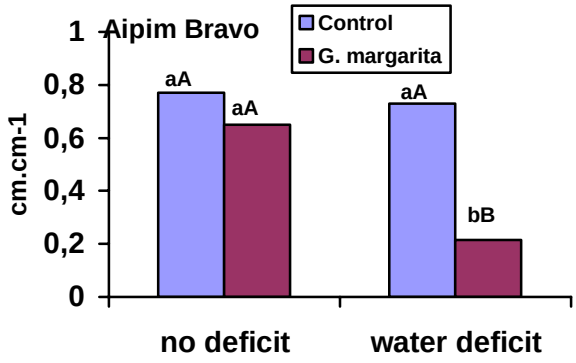



Figure 5. Shoot dry mass of cassava varieties submitted to water deficit and inoculation with mycorrhizal fungi. Small letters compare inoculation and capital letters compares water soil condition for the same inoculation treatmente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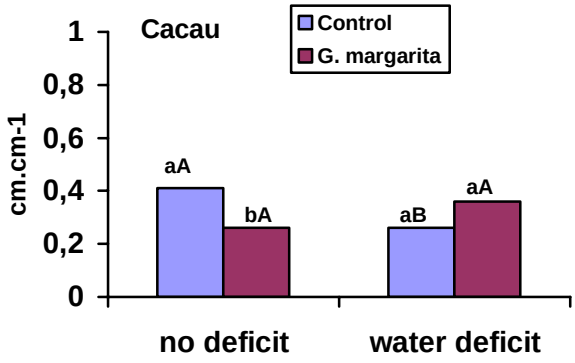
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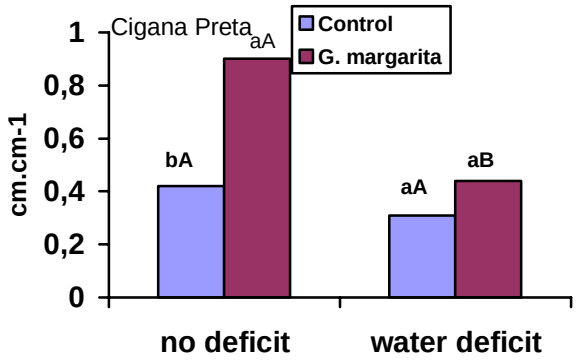
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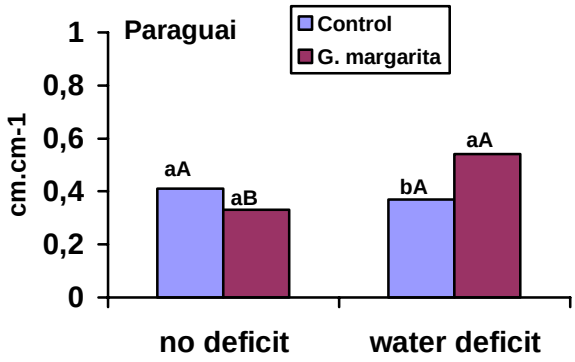
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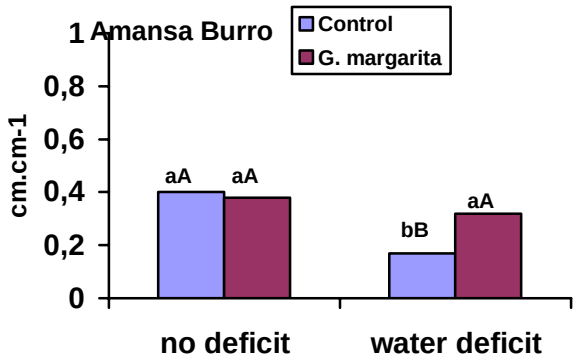
Amansa Burro
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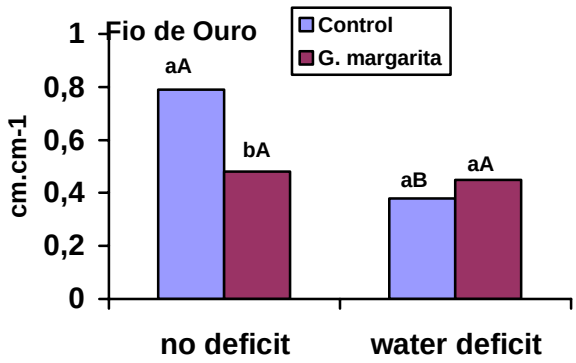
Fio de Ouro
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aB
aA
no deficit
water deficit

Figure 6. Stomatal conductance of leaves of cassava varieties submitted to water deficit and inoculation with mycorrhizal fungi. Small letters compare inoculation and capital letters compares water soil condition for the same inoculation treatmente.

## Summary - Final Technical Report
## December 2006

### Project: Rescue and Production of seed of high quality of local clowns of yuca and malanga by means of the employment of biotechnological technologies adapted to rural Cuban conditions (1st stage)

Implementation of Units of Microspread in rural zones of the Cuban west for the obtaining material of plantation of high quality in cultures of reproduction agámica. (2nd stage)

**Researcher :** Dra. Maria Margarita Hernandez Espinoza, Dr. C. Humberto Ríos Labrada

**Reporter: Institution:** National Institute of Agricultural Sciences (INCA)

**Submitted by Cassava Biotechnology Project (US10,000)**

### Introduction

In Cuba**,** there have been made many efforts to achieve high yields on some important crops as potato and bananas, which have very high yield potentials and, therefore, represent a guarantee of food to the people; this have been the reason for developing many research on them, and, on the other hand, to have left behind other tropical crops as cassava and taro; which however, have a great acceptance among rural people, who have maintained some local biodiversity, but they don't have the resources to reproduce a high quality planting material, because of it, many local cultivars are lost and the yields have been decreasing day by day because of the diseases and pest that have been accumulated on it through the years.

As a solution to the problem of producing planting material on cassava crop with a high physiological and phytosanitary quality, profiting a grant of CBN, it was proposed a Project, , which had two stages, the first was mainly dedicated to the familiarization of farmers with Biotechnology concept and knowledge building on micropropagation techniques, as well as on the characteristics and management of "in vitro" plantlets. A second part of the Project had as a principal objective the acclimatization of "in vitro" cassava plantlets and their planting on the field and the establishment of the Micropropagation Unit (MU) on a farm, as well as to qualify the actors to carry out this work, giving them the necessary knowledge to do it with the correspondent endogenous development of rural communities and the participation of women as an important fact of the process.

### The main objectives of the Project were:

To rescue and multiply cassava and taro high quality planting material according to the farmers criteria and needs.
To give access of farmers to cassava germplasm and allow them to select clones with characteristics adapted to their own conditions.

To qualify and train farmers on micropropagation techniques on cassava and other economical important crops.
To establish and carry on the Micropropagation Unit on the farm of a rural locality.
To give evidences to the actors involved on the cuban agricultural policy about the opportunities of a descentralized, sustainable and participatory concept of Biotechnology to the rural development.

**Methodology**

**To achieve the given objectives, were developed the following activities:**

**1.-** It was done a diagnosis about the problematic of planting material and grow of cassava and taro crops, their conservation and spread, varietal policy and the liders on seed management. This work had two steps:
A workshop with the involved and interested actors, it means farmers, agricultural policy makers, researchers, etc.
It was celebrated on the INIVIT installations and the main objective was to arrive, trhough the farmers criteria, to the principal problems they have with the cassava and taro cultivation and also give them the first evidences about the concept, potentiality and characteristics of the "*in vitro*" culture techniques, specifically the micro propagation, showing them all this on the laboratory and on the field.

It was developed an inquier on the selected rural locality. It was applied to 40 families and allow us to get a more exact idea about the problematic and characteristics of the community and the farms cassava varietal management and permit to select the local clones to be micro propagated.
**2.-** It was carried out the first training course to the selected farmers (3) on INCA laboratory. During this period they received the principal theoretical concepts and practical skills about micro propagation on cassava, taro and other crops.
**3.-** Production of "in vitro" plantlets of cassava and taro selected clones at the INCA and INIVIT laboratories.
**4.-** Second workshop about Experimental Methods. This was developed at the rural community of San Andrés and had as the main objective to give the involved farmers the necessary information to carry out the next stage (acclimatization of the material) and design the facilities to do it with local resources. On this workshop was created the first
" Experimental Farmers Group on Biotechnolgy"
**5**.- It were sent the first "*in vitro*" plantlets of cassava and taro clones to be adapted under the local facilities on two rural localities of the La Palma municipality ( San Andrés and La Jocuma).
**6.-** It was made the first Cassava Fair at La Jocuma locality where there are more of 25 cassava clones that had been given to the farmers at the beginning of the work and it had a great impact on the community and in general on the municipality and province, because it was the first time in which the farmers saw so many cassava clones and could select 5 of them to be grow on their farms. After that, the planting material was given to the farmers according with their selection.
**7.-** After that, the dissemination of cassava clones have continued and at the moment, more than 50 farmers have some of these clones, not only on the selected communities, but along of the island, as a result of the work on the Participatory Breeding Project that allow the dissemination of them through the country.
**8**.- The 3rd workshop was celebrated to evaluate the behavior of "in vitro" material on the field and to compare it with the traditional one on taro crop, which was practically extinguished on this area because of a disease that cause the destruction of the plants.
**9.-** There was celebrated the Second Cassava Fair on a farm of San Andrés locality and here also farmers were able to select the clones to grow on their farms. It was edited a video and also two papers that were published on national scientific publications.

**10.-** It was celebrated the 4th workshop, to select the farm in which will be placed the Micropropagation Unit and to discuss about the functioning and other aspects of it, as well as the farmers that will begin to work on it.
11.- Second training course to the farmers at INCA laboratory to give them the particular knowledges about micropropagation and "*in vitro*" culture techniques; this course also included young technical staff of the laboratory interested on this topic.
At the moment, are been done all the tramits to buy the equiment and other supplys to carry out the work on the Micropropagation Unit (MU), as well as the construction of facilities to it; anyway, research about the use of biologicaly active substances of national production and other natural ones on the culture media, to make the process more sustainable are developing at the INCA laboratory and will be employed on the MU.
It is important to remark that all this results are been used by pre and post graduated student to make their Diploma and MSc thesis. The pre graduated student belong to the Mountain Agronomical Faculty of Pinar del Río University, which are involved from the beginning to this work.

**Conclusions**

With the developing of the Projects financiated by CBN, it has given access to the farmers to the variability of cassava crop and allow them to grow new ones on their farms and that have done as a result the increasing of yields and sanitary quality of the crop, because of the source of the original material, with the corresponding impact on the local rural development and on the familiar economy.
Farmers have been familiarized with "*in vitr*o" culture and micropropagation techniques trough the diffrents workshops and practical experiences they have taken along the work on the Project.
Some farmers have been trained on micropropagation techniques and are capables to transfer their knowledges and skills to others to develop the micropropagation work at the MU. It is important to say that these work have involved the women, who are very active on it, so, they have increased their social and familiar rol.
Farmers have got experimental skills and knowledges that make them capables of take decisions on their farms and carry out some experimental work by themselves to achieve the results they need.
The stablishment of a MU will increase the rol of the community on the agricultural development to a local level and also the social recognition of farmers involved on this work, wich has already been increased with the results of the work on the Project.
As the firs experience of this type in Cuba, it has had a great impact on local actors, not only on farmers, but also on cuban agricultural policy decisors and political ones, who have participated on almost all the activities of the Project at the communities.

We want finally thanks to CBN because of the financial support of the Project, to CIAT staff mainly to Dr. Joe Tohme because his concern and support with the financial aspect of the project, to the Dr. Roosevelt Escobar, because of the training on cassava micropropagation and their help along all the activity, without which, we couldn't have got these results. We also want to thanks INIVIT, because the supply of diversity on cassava, and also because of the training on micropropagation of cassava and taro of technical staff from INCA involved on the Project, to the Participatory Plant Breeding Project and particularly to the head of it, Dr. Humberto Ríos, and also to Dr. Rodobaldo Ortíz, because of the transport facilities, wihout which, we never had made all this work and also because of the spread of cassava clones trough the island. We want to thanks also to the INCA Biotechnology Laboratory technical staff because of their work and finally to the General Director of INCA, Dr. José Roberto Martín Triana because to have given us always his confidence and support.

# Summary - Final Technical Report
# December 2006

## Project: Improvement of cassava crop through biotechnology, with grower participation

**Researcher :** Sergio Rodriguez,

**Reporter: Institution:** INIVIT - Cuba

**Submitted by Cassava Biotechnology Project (US5,000)**

### Introduction

In Cuba, high yielding clones are available, however, national mean yields are outstandingly low (6,5 t/ha) (Rodriguez, 2004) in comparison with the world average yield (10,9 t/ha) (FAO, 2006).

At the Laboratory of Biotechnology from INIVIT, tissue culture techniques have been applied in cassava since 1989, and protocols for micropropagation through meristems and somatic embryogenesis have been established. Vitroplants at different stages of the process have been obtained from commercial clones, and the superiority of this rejuvenated planting material has been tested at large-scale experiments (Medero *et al.*, 2000).

On the other hand, at worldwide level, several high yielding clones with tolerance to pests and diseases have been obtained in breeding programs; however, an important number of those clones hasn't been adopted by cassava growers. It means that high yields and resistance to pests and diseases are not enough for adopting new clones in this crop (Gonçalves *et al*., 2 000).

In the last 15 years, the participative plant breeding(PPB) program has brought interests and curiosity in a growing number of agricultural scientifics dedicated to rural development. However, there is a need to analyse the inter-phase within the breeding programs and the final users; that is, growers, processors, and consumers and to apply simple methods to be able to have a clear vision on the preference of such users and to traduce such preferences on selection criteria for breeding programs (Iglesias and Hernández, 2000).

At present, the most important cassava research programs in Latin America involve growers in the final breeding schedule. In our conditions, and taking into account the accumulated experience on the application of tissue culture to rejuvenate physiologically the commercial clones which have aged considerably and the possibility of having a mass multiplication of introduced or improved clones, the grower participation has to be considered to determine materials and genotypes to be tested and in the final validation of the results coming from the application of such techniques.

In order to give appropriate solutions to each problem, the following objectives were stated:
Refine the micropropagation method in cassava using selected genotypes.
Increase the clonal structure of growers with rejuvenated genotypes and/or improved genetically.

## Materials and Methods

Based on the most experienced growers and researchers in cassava crop, the following clones were selected to be tested: **'Señorita', 'CEMSA 74-725', 'CMC-40', 'CMC-76', 'CEMSA 74-6329' and 'INIVIT Y 93-4'.** Several experiments were carried out in order to refine an efficient and repetitive protocol for micropropagation. Auxin effects and their concentrations (2,4-D (4; 8; 12; 16 y 20 mg.L-1), optimal explant types (immature leaves, apical and axillary meristems from cutting shoots, apical and axillary meristems from vitroplants) and the effect of the physiological age were valued. Planting distances were 0,90 m x 1,10 m and 0,90 m x 0,90 m for 'CMC-76' and 'CEMSA 74-725', respectively. Soil preparation, fertilization and cultural practices were established according to the Cassava Technical Instructive (MINAGRI, 1998).

## Results and Discuss

During the working relations with growers to develop the project, 88 900 'seeds'(cuttings) were delivered as starting material to be planted in production areas for the establishment of 'Seed' enterprises (Annex 1). Significant productive results have been obtained at Santo Domingo Municipality, Villa Clara province by the following farmers: Virgilio Chávez from the Service and Credit Cooperative (SCC) "Quintín Banderas", Oliver Gutierrez from the SCC "Camilo Cienfuegos" and Filiberto Ruiz from the SCC "Cuba-Viet Nam". Yields per unit area were increased and local genotypes were substituted by rejuvenated commercial clones and/or genetically improved clones. On the whole, average yields were significantly higher than those obtained historically (less than 4 000 qq/cab (Annex 2).
Materials were first planted for multiplication at the Research Station from INIVIT at Camaguey. Later, they were distributed to growers in six localities for evaluation in comparison with clones they were used to plant traditionally (Annex 3).
Yields per unit area were considerably increased due to the introduction of rejuvenated and/or new genotypes compared with results obtained with traditional clones or physiologically aged clones. Besides, the clonal composition was high and it resulted in a greater availability of genotypes for planting according to specific edaphoclimatic conditions of each locality. The rejuvenated material delivered in small quantity has become 'seed' banks. Based on inquiries and interviews to crop leaders, the level of acceptance and adoption of clones given was appreciated, as well as, an important increment per unit area with the introduction of these materials. Crop leaders are involved in an important mission; that is, to monitor these materials in each municipality and province, so as to guarantee 100% of the material as 'seed' in the coming seasons.

## Conclusions

Micropropagation and plant regeneration for obtaining original 'seed'.
Original and basic 'seeds' were produced under controlled conditions and were delivered to growers to validate the effectiveness of the protocol applied to recover the potential yield in clones studied and in different edaphoclimatic conditions.
3.A greater link between growers and researchers was obtained, as well as, an increment in the adoption level of proposed technologies and in the introduction of new genotypes.

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**Acknowledge**

Executors of the project want to thank the financing support from the Cassava Biotechnology Network and the collaboration from to CIAT, Colombia.

Annex 1: Quantity of planting materials rejuvenated through biotechnological methods and delivered to growers within the project development.

| **Clones** | **Years** | | |
|---|---|---|---|
| | **2002** | **2003** | **2004** |
| 'Señorita' | 1 500 | 4 600 | 5 000 |
| 'CEMSA 74-725' | 2 000 | 5 000 | 6 000 |
| 'CMC-40' | 2 500 | 8 800 | 12 100 |
| 'CMC-76' | 6 000 | 10 000 | 8 000 |
| 'CEMSA 74-6329' | 2 000 | 5 000 | 5 000 |
| 'INIVIT Y 93-4' | - | 2 100 | 2 800 |
| **Totals** | **14 000** | **35 500** | **38 900** |

Annex 2. Average yield obtained at Santo Domingo municipality.

| **Clones** | **Yield (qq/cab.).** |
|---|---|
| CMC-40 e | 7 800 |
| CMC-40 | 4 100 |
| Señorita e | 9 200 |
| Señorita | 3 980 |
| CMC- 76 e | 5 974 |
| C. 74-6329 e | 7 086 |
| C. 74-725 e | 7 263 |
| I-Y 93-4 e | 6 320 |
| Blanquita | 3 650 |

e-Planting material rejuvenated through biotechnological methods.

Annex 3. Evaluation of the potential yield (qq/cab.) in localities from Camagüey province

| **Clones** | **S. Cubitas** | **Najasa** | **Jimaguayú** | **Camagüey** | **Guaimaro** | **Esmeralda** |
|---|---|---|---|---|---|---|
| C-74-725 e | 9 665 | - | 9 562 | 12 513 | 7 395 | 8 373 |
| C-74-725 | 8 804 | 9 936 | 9 123 | 11 631 | 9 997 | 4 638 |
| CMC-40 e | 8 151 | - | 8 213 | 8 427 | 6 436 | 10 906 |
| CMC-40 | 10 102 | 8 770 | 8 187 | 8 068 | 5 067 | 6 436 |
| I-Y93-4 e | 10 333 | 14 019 | 11 301 | 15 331 | 8 285 | 25 969 |
| CMC-76 e | 6 113 | 7 953 | 7 438 | 7 252 | 4 656 | 4 396 |
| J. Dulce | 3 889 | 9 545 | 10 400 | 8 938 | 3 423 | 13 194 |
| Señorita e | 9 659 | 8 970 | 9 072 | 8 676 | 8 365 | 6 749 |
| C74-6329e | - | 12 300 | 11 124 | 12 689 | - | - |

e-Planting material rejuvenated through biotechnological methods.

# Summary - Final Technical Report
# December 2006

## Project: Improving the competitiveness and productivity of cassava farmers in northern Department of Cauca, Colombia, by offering technology related to variety diversification and use of certified seed using *in vitro* propagation

**Researcher :** José Restrepo

**Reporter: Institution:** FIDAR , Colombia

**Submitted by Cassava Biotechnology Project (US12,000)**

### Introduction

Farmers who plant cassava in the southwestern Andes of Colombia have been using seed from different sources for their plantings. This situation has led them to plant varieties of very poor quality, accentuating their susceptibility to pests and diseases and obtaining low yields.

The slow multiplication to produce cassava seed is a bottleneck for the transfer and adoption of new varieties and slows down the recovery of regions where the local varieties have been devastated by natural or civil disasters. It also restricts the farmers` capacity to adapt to changes in the demand. The farmers point out that this slow multiplication is one of the causes of price instability.

To help solve these abovementioned problems, it was necessary to establish a seed production program that involves not research institutions but also farmer associations to improve cassava root production and strengthen the starch agribusiness which is of vital importance for the region.

This proposal made it possible to establish a basic cassava seed production scheme that incorporates biotechnological processes and rapid propagation schemes so that they can be implemented in other areas or regions that try to maintain a permanent seed regeneration system of their main varieties.

### General Objective

Establish a system to renew and conserve cassava seed with the participation of farmers, aiming to improve the productivity and competitiveness of small and medium producers of northern Cauca in Colombia.

### Methodology

The project was carried out in a coordinated manner between CIAT´s Agrobiodiversity and Biotechnology Project, the Foundation for Agricultural Research and Development (FIDAR), and the La Concepción Association of Indigenous Councils in the Municipality of Buenos Aires (60 families), the Farmers` Association of El Pital in Santander de Quilichao (45 families), and the La Independencia farmers´ group in the municipality of Piendamó (8 families).

The following activities were carried out:

Training of three farmer groups in production methodologies of vegetative cassava seed obtained by tissue culture.

Preparation of a multiplication plan for cassava seed obtained in vitro for 25 hectares of cassava in order to improve the availability and quality of the seed for starch producers of northern Cauca.

Establishment in the field of 37 native cassava varieties, which were cleaned and propagated by in vitro culture and heat therapy by CIAT`s Biotechnology and Biodiversity Project.

Field evaluation of native varieties with the participation of farmers.

Quality assessment of cassava starch and determination of the agroindustrial potential of cassava roots from lots planted with seed obtained by tissue culture.

Development of training topics that facilitate the incorporation of and the respect for women in activities aiming to improve in vitro cassava seed production.

**Results**

The main results obtained for this phase of the project were as follows:

Thirty-seven native materials were collected and cleaned by tissue culture and heat therapy and planted by farmers for their conservation and use.

An in vitro production scheme of cassava seed was adapted by farmers to improve the quality of planting material of native or improved cassava varieties.

Development of a multiplication system for seed obtained by tissue culture and increased by two rapid propagation systems to reduce seed production costs.

Establishment and evaluation by farmers of 22 hectares planted to seed from in vitro materials of five CIAT improved varieties.

A study that evaluated the quality of starch or its agroindustrial potential for several improved varieties that were planted with seed obtained in vitro.

**Conclusions**

This project made it possible to re-incorporate into the cassava production scheme a total of 37 certified and clean local materials in the low, middle, and high areas of the Department of Cauca.

Local banks of certified seed are available as input to planting material production schemes, especially for high areas.

A basic seed production scheme was developed that incorporates biotechnological processes and rapid propagation schemes that can be implemented in other cassava producing areas or regions that try to maintain a permanent seed regeneration system of its principal varieties.

The collaboration between different governmental and private entities as well as cassava farmers and processors in Cauca makes it possible to maintain a flow of certified material at the level of the small producer, provided that funds are available for their multiplication.

With the results obtained during these two phases of the project, cassava materials were identified and moderate amounts of seed produced to launch programs or agribusinesses related to the feed agroindustry.

The implementation of biotechnological seed production schemes in rural areas innovates and strengthens the production schemes of small producers and helps recover agrobiodiversity.

**Communications and dissemination of information**

The main training events and workshops held during 2005 and 2006 where project advances were reported include the following:

Use of tissue culture to improve cassava seed quality. Workshop held for biology teachers of the Fernández Guerra School in Santander de Quilichao. February 2005.

Tissue culture. Paper presented at the workshop on the support of biotechnological, genomic, and post-genomic techniques for plant improvement, CIAT, 1-3 March 2005.

Rapid cassava seed multiplication systems. Paper presented at the International Course on Modern Cassava Production and Processing Systems and Use. CIAT-CLAYUCA. CIAT, 18-27 April 2005.

Cassava Tissue Culture and its Applications. Paper presented at the Advanced Breeding Course for Cassava Breeders. CIAT, 11 April-8 May 2005.

Using in vitro culture techniques and rapid propagation methods to improve cassava seed quality. San Bosco and San Rafael CIAL Groups, Santander de Quilichao. July 2005.

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Use of tissue culture to propagate planting materials. Paper presented at the Workshop on Opportunities of Investment in Biotechnology in Agriculture. CIAT. 12 December 2005.

Production of good quality cassava seed for farmers of northern Cauca. CIDA-Canada-FIDAR Project. Puerto Tejada, Cauca. 22-23 February 2006.

**Acknowledgements**

Project executors express their thanks to the following farmer organizations of northern Cauca: La Concepciòn Association of Indigenous Councils in the Municipality of Buenos Aires, the Farmers´ Association of El Pital in Santander de Quilichao, and the La Independencia Farmers´ Group in the municipality of Piendamó for their support, leadership, and collaboration in the development of the project. We also present a special recognition to the Cassava Biotechnology Network (CBN) for providing the funds to carry out this work and for its collaboration and support for the project to participate in different CBN forums and congresses, spaces at which project member were able to exchange results and viewpoints with other researchers.

# Summary Technical Report
# December, 06

## Project: Development of molecular markers linked to dry matter content in cassava (*Manihot esculenta* Crantz) through genetic breeding techniques assisted by markers

**Author: Yoel Beovides García, Paula X. Hurtado, Wilson Castelblanco, Martin Fregene, Janneth P. Gutiérrez, Charles Buitrago, Constanza Quintero, Edgar Barrera, Marilys Milián y Sergio Rodríguez.**

**Budget: $ 5 000.00 USD**

**Funding:** Cassava Biotechnology Network (CBN)

### Introduction

Characterization, evaluation, storage and sustainable use of genetic resources is one of the priorities of researchers to develop cassava crop worldwide.

The development of microsatellite (SSR-*simple sequence repeats)*, as well as, other SSCP-SNP (*single-strand conformational polymorphism*) offer powerful tools that enhance the effectiveness of breeding programs on important crops for a modern economy and growers of low incomes. The SNPs are considered as the most common polymorphism within plant species (Bertin *et al*., 2005).
The use of microsatellite has permitted to carry out analysis on the structural diversity of 92 cassava Cuban cultivars(Beovides, 2005). After knowing the genetic structure of this working collection, it is possible to develop binding imbalance surveys to search for association within dry matter contents (DMC), an important characteristic for breeders and genes from starch biosynthesis.
This study is aimed to develop a functional analysis on diversity through gene analysis of starch biosynthesis in a 50 genotype group from Cuba. In this way, the sustainable use of cassava germplasm in breeding programs is increased, as well as, food production. This avoids genetic erosion in the crop and contributes to a more efficient exploitation of its genetic potential in Cuba and in the Caribbean region.
Materials and Methods

### Plant Material

50 Cuban cultivars collected from the national territory and maintained in the germplasm bank from the Research Institute of Tropical Root and Tuber crops were used and selected from the genetic structure obtained from a higher population survey with 36 micro-satellites in 2003.

### Evaluation of the dry matter content (DMC)

This study was developed in roots from 10 year old plants. Procedures included three replications of 200 g (fresh weight) well ground roots which were taken to constant weight in oven (Memmert, alemana) at $85^0$C and were expressed in percentage. Evaluations were done during three consecutive years and genotypes with high and low DMC were selected. Data were subjected to correlation analysis using the correlation coefficient of Pearson.

### Extraction of genomic AND and reactions of PCR

The total DNA was extracted with the method of Dellaporta *et al*. (1983). Its quality was checked in 0.8% agarose gel and quantification was developed by fluorometry. Starting from the initial concentration, dissolutions were prepared at 10 ng/ul to be used as tempering in the reactions of PCR

**Searching of homologous sequences**

Initially selected genes

SBE I, SBE II — Starch branching enzyme I and II

SS I, SS II, SS III — *Starch synthase*

aINV — *Acid invertase*

apoINV — *Apoplastic invertase*

SPS — *Sucrose phosphate synthase*

SUT — *Sucrose transporter*

HET — *Hexose transporter*

SUSY — *Sucrose synthase*

MEX 1 (RCP1) *Maltose transporter*

TBlastx was carried out on ESTs databases (*expressed sequence tags*) in cassava with different sequences found in the gene bank to identify those sequences having a significative *E-value*.

The SSI and SSII sequences did not result to be specific, as well as, the corresponding ones to apoINV and aINV; so, a primer set for invertase (INV) and another for SS isophorms (SS-isophorm) were designed.

Starting from the sequences with the highest homology, one or three primers ranging from 300 to 500 pb on the total RNA structure(*Primer-3 Program*) were designed according to gene sequence size. In this research, 16 primers sets from previously mentioned genes that codify to proteins related with starch synthase were used (Annex 1).

**PCR conditions and visualization of the final product**

Using as templado DNA dissolutions at 10 ng/ul, 25ul were used in each PCR reaction with the following final concentrations: DNA genomic-50 ng; Buffer- 1x; $MgCl_2$ - 1,5 mM: dNTPs- 0.2 mM; primers - 0.2 mM (of each one R and F) and 1.5 u *Taq* DNA polymerase.

The 16 designed primers (linked to 10 starch genes) were evaluated using control genotypes under standard conditions of amplification in the PCR reaction. After initial denaturalization at 95 $^{o}$C during two minutes, 30 cycles were performed: 94 $^{o}$C, 30 seconds (denaturalization); 55 ºC, 1 min (anneling); 72 ºC, 1 min (extension), and later, a final extension at 72 ºC during 5 minutes was carried out.

These conditions should be adjusted to obtain the highest efficiency in the amplification process of each primer (Table 1).

Only 2-SBE II, SS-isophorms and the two RCP1 variants do not amplify to the tested conditions, so, new conditions should be evaluated in further surveys. The following studies were performed using 12 sets of primers that amplified.

The quality of PCR products was confirmed in agarose gels at 1.5%.

The PCR product was denaturated and separated its fragment according to the conformational structure in a gel MDE (*Mutation Detection Enhancement*) (Martins-Lopes *et al*., 2001).

The gel mixture was prepared at a 70 ml final volume containing a final concentration of 0,5x gel solution and 0,6x TBE buffer and polymerized with the addition of 280 ul ammonium persulphate at 10% and 28 ul tetrametilendiamina (TEMED).

Table 1: PCR reaction conditions for evaluated primers.

| ***Primers*** | **weight** | Tm | Extension (72 ºC) | $MgCl_2$ | Notes |
|---|---|---|---|---|---|
| 1-SUSY | 523 pb | 53- 45 s * | 72- 45 s | 1.5 mM | 35 cycles |
| 2-SUSY | 806 pb | 53- 1 min | 72- 1:30 | 1.5 mM | 35 cycles |
| 1-HET | 484 pb | 55- 1 min | 72 – 1:30 min | 2.5 mM | 35 cycles |
| 2-HET | 412 pb | 55- 45 s | 72- 1:30 | 2.5 mM | 30 cycles |
| 1-SPS | 505 pb | 55- 45 s | 72- 1:30 min | 2.5 mM | *RoboCycler* |
| 2-SPS | 529 pb | 55- 45 s | 72- 1:30 min | 2.5 mM | *RoboCycler* |
| SSIII | 513 pb | 56.5- 45 s | 72- 1:30 min | 1.5 mM | 35 cycles |
| 1-SBE I | 513 pb | 55- 1 min | 72- 2 min | 1.5 mM | 35 cycles |
| 2-SBE I | 753 pb | 47- 1 min | 72- 1:30 min | 1.5 mM | 35 cycles |
| SUT | 445 pb | 52- 1 min | 72- 1 min | 1.5 mM | 30 cycles |
| 1-RCP1 | 580 pb | - | - | - | - |
| 2-RCP1 | 512 pb | - | - | - | - |
| 1-SBE II | 508 pb | 53- 1 min | 72- 1:30 | 2.0 mM | 35 cycles |
| 2-SBE II | 595 pb | - | - | - | - |
| INVERT | 509 pb | 51- 1 min | 72- 1 min | 2.0 mM | *RoboCycler* |
| SS-isof. | 309 pb | - | - | - | - |

* touching down (63-53 °C) *step by step*

Fragments were separated by electrophoresis at an 8 w constant voltage for 16-18 hours at room temperature and were visualized according to the staining protocol with silver (Bassam *et al*., 1991).

**Processing and data analysis**

**Phenotypic data**

Phenotypic data were gathered from the dry matter content (DMC) evaluation during three consecutive years, and genotype selection with high and low DMC was permitted. In order to identify the set of phenotypic data in this study, data distribution during the three-year period were determined and the correlation within them was calculated by the correlation coefficient of Pearson.

**Genotypic data (molecular)**

**Unbalanced Bond**

Taking into consideration, the generated genotype data and genetic structure observed in the diversity study of Cuban accessions(Beovides, 2005) that included 50 genotypes, the unbalanced bond was calculated using POPGENE program, version 1.32 (32 bits).

**Evaluation of primers and association analysis**

Visualized fragments using 12 pairs of primers related with starch synthesis were evaluated as presence (1) or absence (0) and fragments that could not be evaluated were recorded as missing data.

The association between genotype classes (1/0) and the phenotypic evaluation using the non-parametric independence test Mann-Whitney-U through software SPSS/PC was determined. The null hypothesis (no association between genotypic classes and DMC) was rejected when P <0,1.

**Results and Discussion**

**Analysis of phenotypic data**

In order to analyze the phenotypic data, an average of three replications evaluated each year (%) was used, and the average of these evaluations per each genotype was calculated. Data were divided into five categories and the genotype frequency was determined according to established ranges (Table 2). Later, the genotype frequency per category was shown in a graph (Figure 1).

Table 2. Frequency of genotypes included in each range (dry matter percentage)

| Category | Range (percentage of DMC) | First Evaluation | Second Evaluation | Third Evaluation | Average |
|---|---|---|---|---|---|
| 1 | 30.8 - 33.8 | 5 | 5 | 4 | 4 |
| 2 | 33.9 - 36.8 | 9 | 11 | 9 | 10 |
| 3 | 36.9 - 39.8 | 11 | 11 | 10 | 11 |
| 4 | 39.9 – 42.8 | 21 | 20 | 24 | 24 |
| 5 | 42.9 – 45.8 | 4 | 3 | 3 | 3 |

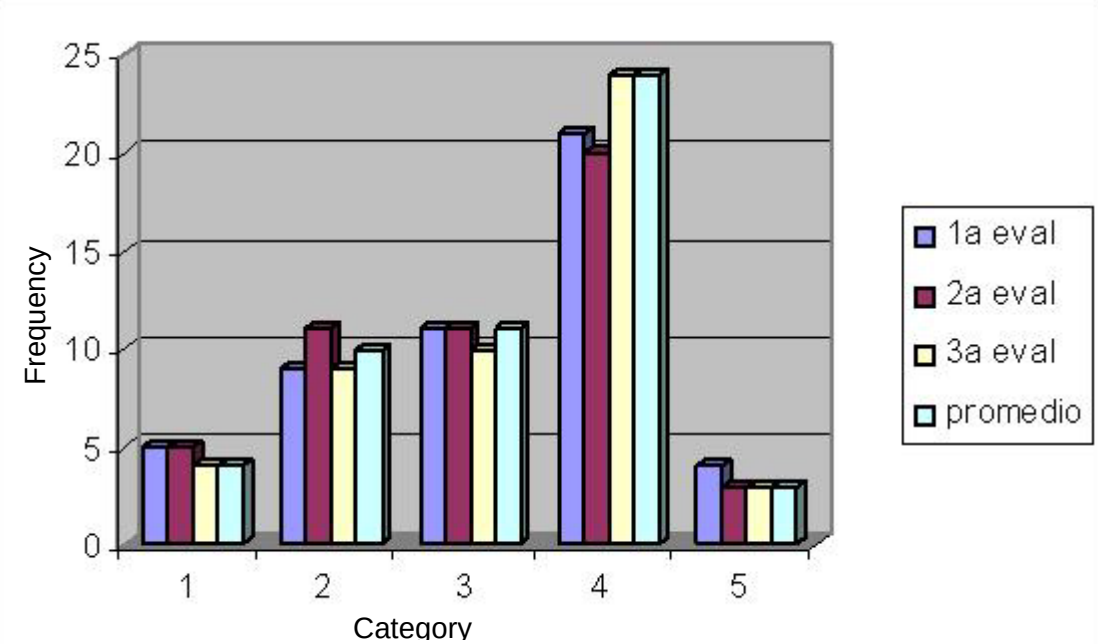


Figure 1. Comparison between frequencies of genotypes included in each category

To confirm results observed after comparing frequencies in each category, the correlation between evaluations was calculated, as well as, within each evaluation and means (Table 3) to define the data group used in the association analysis.

The results of the correlation analysis confirmed a high relation between evaluations carried out during three consecutive years, and within them and the three-year average. The DMC average percentage per each genotype during the evaluation in the three-year period was the most appropriate for the association analysis.

Table 3. Correlation per each pair of evaluation and for them in relation to the average.

| Comparison | Correlation |
|---|---|
| 1st evaluation – 2nd evaluation | 0.99 |
| 1st evaluation – 3rd evaluation | 0.98 |
| 2nd evaluation – 3rd evaluation | 0.97 |
| 1st evaluation - average | 0.99 |
| 2nd evaluation - average | 0.99 |
| 3rd evaluation - average | 0.99 |

Due to the high correlation between evaluations in the three years, evaluations were consistent, the evaluation method was appropriate and there is reliability in the type of evaluation carried out. These results may be associated to the fact that the procedure was the same every year, as well as, the team in charge of making evaluations.

**Analysis of genotypic data.**

**Unbalanced Bond**

Making use of the generated genotype data and genetic structure observed in the diversity study of Cuban accessions(Beovides, 2005) that included 50 genotypes, the unbalanced bond was calculated taking into the consideration the genotype division into two big groups (A and B). The microsatellite markers used in the diversity study permitted to gather together the genotypic information of 34 loci. The generation of 198 alleles was used to calculate the DL. In group A, a significant LD was found in nine allele pairs (Table 4) and only one of them belong to the same bond group (L) according to the cassava genetic map (Fegene *et al*., 1997; Mba *et al*., 2001). Group B showed significant LD in 40 allele pairs (Table 5) and only two of them belong to the same bond group (group G).

Table 4. Allele pairs with significant DL in genotypes from group A. The shaded line indicates the allele pair from the same bond group.

| **Locus** | **Allele** | **-** | **Locus** | **Allele** | **Burrows** | **Correlation** | **Chisq*** | **Probability.** |
|---|---|---|---|---|---|---|---|---|
| SSRY19 | H | - | SSRY21 | C | 0,0138 | 0,5 | 4,25 | 0,0393 |
| SSRY19 | F | - | SSRY105 | D | 0,026 | 0,5 | 4,25 | 0,0393 |
| SSRY20 | E | - | SSRY108 | D | 0,0519 | 0,5 | 4,25 | 0,0393 |
| SSRY20 | B | - | SSRY110 | A | 0,0138 | 0,5 | 4,25 | 0,0393 |
| SSRY20 | F | - | SSRY151 | D | 0,026 | 0,5 | 4,25 | 0,0393 |
| SSRY34 | C | - | SSRY69 | E | -0,04 | -0,5195 | 4,05 | 0,0442 |
| SSRY34 | A | - | SSRY100 | H | 0,0138 | 0,5 | 4,25 | 0,0393 |
| SSRY63 | F | - | SSRY155 | D | 0,0138 | 0,5 | 4,25 | 0,0393 |
|  | A | - | SSRY171 | F | 0,0311 | 0,5165 | 4 | 0,0454 |

Although, significant DL was found in a low number of allele pairs from the same bond group, it is important to take into consideration that the marker number used (34 SSR) is not sufficient to have a DL defined idea in this accession group.

So, the present study was guided to a genome region highly associated with the DMC to obtain a better idea on how DL is in 50 genotypes, and then, to be able to extrapolate results to cassava crop in general. After relating genotypic data of 34 loci with the DMC average of 50 accessions distributed in A and B groups, 80% of 17 accessions from group A showed DMC lower than 40% and from 32 accessions that

made up group B, 69% had a DMC above 40%. The results suggest that one of the cause of the genetic structure found in 50 Cuban accessions is the DMC, and it constitutes an ideal sample to start association surveys with molecular markers highly linked to this characteristic.

Table 5. Allele pairs with significant DL in genotypes that make part of group B.
Shaded lines indicate allele pair making part of the same bond group.

| Locus | Allele | - | Locus | Allele | Burrows | Correlation | Chisq* | Probability |
|---|---|---|---|---|---|---|---|---|
| SSRY4 | G | - | SSRY63 | E | 0,0156 | 0,5 | 7,75 | 0,0054 |
| SSRY4 | A | - | SSRY64 | G | 0,0083 | 0,5 | 7,25 | 0,0071 |
| SSRY4 | D | - | SSRY64 | G | 0,0083 | 0,5 | 7,25 | 0,0071 |
| SSRY4 | A | - | SSRY69 | E | 0,0083 | 0,5 | 7,25 | 0,0071 |
| SSRY4 | D | - | SSRY69 | E | 0,0083 | 0,5 | 7,25 | 0,0071 |
| SSRY4 | A | - | SSRY169 | C | 0,0078 | 0,5 | 7,75 | 0,0054 |
| SSRY4 | D | - | SSRY169 | C | 0,0078 | 0,5 | 7,75 | 0,0054 |
| SSRY9 | A | - | SSRY69 | G | 0,0155 | 0,4006 | 4,65 | 0,031 |
| SSRY12 | C | - | SSRY20 | E | 0,0669 | 0,4229 | 4,83 | 0,028 |
| SSRY12 | D | - | SSRY20 | F | 0,0381 | 0,4336 | 5,08 | 0,0243 |
| SSRY12 | F | - | SSRY20 | A | 0,0947 | 0,4928 | 6,56 | 0,0104 |
| SSRY19 | A | - | SSRY63 | D | 0,0073 | 0,3478 | 3,87 | 0,0491 |
| SSRY19 | H | - | SSRY63 | E | 0,0073 | 0,3478 | 3,87 | 0,0491 |
| SSRY19 | B | - | SSRY108 | E | 0,0073 | 0,3478 | 3,87 | 0,0491 |
| SSRY19 | E | - | SSRY161 | D | 0,0291 | 0,4011 | 4,99 | 0,0255 |
| SSRY20 | D | - | SSRY105 | D | -0,0389 | -0,3901 | 4,57 | 0,0326 |
| SSRY21 | G | - | SSRY161 | G | 0,0086 | 0,5 | 7 | 0,0082 |
| SSRY21 | E | - | SSRY177 | F | 0,0217 | 0,5113 | 5,75 | 0,0165 |
| SSRY34 | B | - | SSRY151 | B | 0,0328 | 0,355 | 3,91 | 0,0481 |
| SSRY38 | A | - | SSRY169 | A | -0,0564 | -0,4392 | 6,17 | 0,013 |
| SSRY38 | A | - | SSRY169 | B | 0,0576 | 0,4697 | 7,06 | 0,0079 |
| SSRY38 | B | - | SSRY169 | A | 0,0564 | 0,4392 | 6,17 | 0,013 |
| SSRY38 | B | - | SSRY169 | B | -0,0576 | -0,4697 | 7,06 | 0,0079 |
| SSRY51 | B | - | SSRY171 | D | 0,0092 | 0,5 | 6,5 | 0,0108 |
| SSRY59 | H | - | SSRY105 | D | 0,0407 | 0,4056 | 4,28 | 0,0386 |
| SSRY59 | E | - | SSRY161 | E | 0,0185 | 0,5 | 6,5 | 0,0108 |
| SSRY59 | E | - | SSRY179 | A | 0,02 | 0,5103 | 6,25 | 0,0124 |
| SSRY63 | E | - | SSRY100 | E | 0,0073 | 0,3478 | 3,87 | 0,0491 |
| SSRY64 | G | - | SSRY6 | E | 0,0086 | 0,5 | 7 | 0,0082 |
| SSRY64 | G | - | SSRY103 | B | 0,0163 | 0,3805 | 4,2 | 0,0404 |
| SSRY64 | G | - | SSRY169 | C | 0,0081 | 0,5 | 7,5 | 0,0062 |
| SSRY69 | E | - | SSRY169 | C | 0,0081 | 0,5 | 7,5 | 0,0062 |
| SSRY103 | B | - | SSRY106 | B | 0,0528 | 0,3889 | 4,08 | 0,0433 |
| SSRY103 | E | - | SSRY106 | F | 0,0432 | 0,4183 | 4,73 | 0,0297 |
| SSRY103 | G | - | SSRY148 | B | 0,0163 | 0,4519 | 5,92 | 0,015 |
| SSRY106 | C | - | SSRY108 | E | 0,0163 | 0,3753 | 3,94 | 0,0471 |
| SSRY110 | D | - | SSRY169 | B | 0,0146 | 0,3478 | 3,87 | 0,0491 |
| SSRY161 | E | - | SSRY179 | A | 0,0086 | 0,5 | 7 | 0,0082 |
| SSRY169 | A | - | SSRY179 | B | 0,0184 | 0,5224 | 7,91 | 0,0049 |
| SSRY169 | A | - | SSRY179 | C | -0,0202 | -0,6307 | 11,54 | 0,0007 |

**Evaluation of primers and association analysis**

Of the 16 primers designed from genes related with starch synthesis, the amplification of 12(1-SUSY, 2-SUSY, 1-HET, 2-HET, 1-SPS, 2-SPS, SSIII, 1-SBE I, 2-SBE I, SUT, 1-SBE II, INVERT) was standardized and 11 of them showed polymorphism and usefulness; in case of 1-SBE, it resulted to be monopolymorphic. From 3 to 10 allelic (bands) variants in the group of polymorphic primers were detected to generate a matrix of 65 alleles (Annex 2) that were evaluated separately in the genotypes included in A and B groups. The association between genotypic classes and the DMC was determined using the independence test Mann-Whitney-U when P<0.1 (Table 6).

Table 6. Association test between genotypic classes and DMC.Shaded lines indicate allelic markers associated with DMC

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 36 | 1 | 13 | 36,9 | 3,8 | 0,8874 | 95,5 | 36 | 1 | 20 | 41,2 | 1,8 | 0,1474 | 95,1 |
| | 0 | 5 | 36,9 | 3,2 | | | | 0 | 12 | 39,3 | 3,8 | | |
| 37 | 1 | 7 | 36,8 | 3,8 | 0,7583 | 95,7 | 37 | 1 | 20 | 40,9 | 2,6 | 0,457 | 95,1 |
| | 0 | 11 | 36,9 | 3,6 | | | | 0 | 12 | 39,7 | 3 | | |
| 38 | 1 | 17 | 36,9 | 3,6 | | | 38 | 1 | 30 | 40,6 | 2,8 | | |
| | 0 | 1 | | | | | | 0 | 2 | 38,1 | | | |
| 39 | 1 | 17 | 36,9 | 3,6 | | | 39 | 1 | 29 | 40,6 | 2,8 | | |
| | 0 | 1 | | | | | | 0 | 3 | 39,8 | 2,4 | | |
| 40 | 1 | 18 | 36,8 | 3,5 | | | 40 | 1 | 30 | 40,5 | 2,8 | | |
| | 0 | 0 | | | | | | 0 | 2 | 38,1 | | | |
| 41 | 1 | 6 | 36,3 | 4,3 | 0,631 | 95,9 | 41 | 1 | 11 | 39,7 | 3,2 | 0,2001 | 95,1 |
| | 0 | 12 | 37 | 3,2 | | | | 0 | 21 | 40,9 | 2,5 | | |
| 42 | 1 | 17 | 36,9 | 3,6 | | | 42 | 1 | 28 | 40,4 | 2,8 | | |
| | 0 | 1 | 35,2 | | | | | 0 | 4 | 41,3 | 3 | | |
| 43 | 1 | 8 | 36,6 | 3,9 | 0,8822 | 95,7 | 43 | 1 | 14 | 40,4 | 3,1 | 0,9766 | 95,2 |
| | 0 | 10 | 37 | 3,4 | | | | 0 | 18 | 40,5 | 2,5 | | |
| 44 | 1 | 1 | 39,6 | | | | 44 | 1 | 1 | 44 | | | |
| | 0 | 17 | 35,9 | 3,5 | | | | 0 | 31 | 40,1 | 2,8 | | |
| 45 | 1 | 4 | 38,2 | 2,4 | 0,1059 | 96,4 | 45 | 1 | 9 | 40,1 | 3,7 | 0,7524 | 95,4 |
| | 0 | 14 | 35,3 | 3,6 | | | | 0 | 23 | 40,3 | 2,5 | | |
| 46 | 1 | 13 | 36,2 | 3,6 | | | 46 | 1 | 23 | 40 | 2,9 | 0,2795 | 95,1 |
| | 0 | 5 | 34,9 | | | | | 0 | 9 | 41,6 | 2,6 | | |
| 47 | 1 | 11 | 36 | 3,7 | | | 47 | 1 | 26 | 40,2 | 2,9 | | |
| | 0 | 7 | 36,5 | 2,7 | | | | 0 | 6 | 41,5 | | | |
| 48 | 1 | 14 | 36,1 | 3,5 | | | 48 | 1 | 27 | 40,2 | 2,8 | | |
| | 0 | 4 | | | | | | 0 | 5 | | | | |
| 49 | 1 | 1 | 36,4 | | | | 49 | 1 | 1 | 34 | | | |
| | 0 | 17 | 36,1 | 3,6 | | | | 0 | 31 | 40,4 | 2,6 | | |
| 50 | 1 | 8 | 37,2 | 4,1 | 0,2824 | 95,7 | 50 | 1 | 11 | 39,9 | 3 | 0,799 | 95,6 |
| | 0 | 10 | 34,8 | 1,8 | | | | 0 | 21 | 40,4 | 2,8 | | |
| 51 | 1 | 8 | 34,6 | 2,9 | 0,0426 | 97,7 | 51 | 1 | 15 | 40 | 2,9 | 0,524 | 95,3 |
| | 0 | 10 | 38,2 | 3,2 | | | | 0 | 17 | 40,4 | 2,8 | | |
| 52 | 1 | 1 | 40 | | | | 52 | 1 | 18 | 40,3 | 3,2 | 0,7859 | 95,1 |
| | 0 | 17 | 36,6 | 3,5 | | | | 0 | 14 | 40,8 | 2,1 | | |
| 53 | 1 | 16 | 36,7 | 3,6 | | | 53 | 1 | 31 | 40,6 | 2,7 | | |
| | 0 | 2 | 37,8 | 3,7 | | | | 0 | 1 | 38,1 | | | |
| 54 | 1 | 0 | | | | | 54 | 1 | 1 | 37,3 | | | |
| | 0 | 18 | 36,8 | 3,5 | | | | 0 | 31 | 40,6 | 2,7 | | |
| 55 | 1 | 18 | 36,8 | 3,5 | | | 55 | 1 | 32 | 40,5 | 2,7 | | |
| | 0 | 0 | | | | | | 0 | 0 | | | | |
| 56 | 1 | 5 | 36,3 | 2 | | | 56 | 1 | 6 | 41,2 | 1,2 | 0,7232 | 95,3 |
| | 0 | 13 | 36,4 | 3,9 | | | | 0 | 26 | 40,4 | 3,1 | | |
| 57 | 1 | 16 | 36,4 | 3,4 | | | 57 | 1 | 29 | 40,5 | 2,8 | | |
| | 0 | 2 | | | | | | 0 | 3 | | | | |
| 58 | 1 | 16 | 36,4 | 3,4 | | | 58 | 1 | 29 | 40,5 | 2,8 | | |
| | 0 | 2 | | | | | | 0 | 3 | | | | |
| 59 | 1 | 2 | 35,4 | 0,3 | | | 59 | 1 | 3 | 39,8 | 5,2 | | |
| | 0 | 16 | 37 | 3,7 | | | | 0 | 29 | 40,6 | 2,5 | | |
| 60 | 1 | 13 | 35,9 | 3,4 | 0,0899 | 95,4 | 60 | 1 | 26 | 40,5 | 3 | 0,6483 | 95,6 |
| | 0 | 5 | 39,1 | 3 | | | | 0 | 6 | 40,4 | 1,6 | | |
| 61 | 1 | 15 | 36,8 | 3,6 | | | 61 | 1 | 23 | 40,6 | 2,9 | 0,4631 | 95,2 |
| | 0 | 3 | 36,6 | 3,6 | | | | 0 | 9 | 39,8 | 2,6 | | |
| 62 | 1 | 17 | 37 | 3,4 | | | 62 | 1 | 26 | 40,5 | 2,9 | 0,6265 | 95,6 |
| | 0 | 1 | 32,5 | | | | | 0 | 6 | 40 | 2,2 | | |
| 63 | 1 | 5 | 35,5 | 3,7 | 0,4865 | 95,4 | 63 | 1 | 5 | 40,9 | 1,7 | 0,9671 | 95,1 |
| | 0 | 13 | 37,3 | 3,4 | | | | 0 | 27 | 40,4 | 3 | | |
| 64 | 1 | 1 | 35,2 | | | | 64 | 1 | 3 | 41,3 | 3 | | |
| | 0 | 17 | 36,9 | 3,6 | | | | 0 | 29 | 40,4 | 2,8 | | |
| 65 | 1 | 12 | 35,6 | 3,3 | 0,0443 | 95,9 | 65 | 1 | 25 | 40,4 | 3 | 0,9241 | 95,1 |
| | 0 | 6 | 39,2 | 2,7 | | | | 0 | 7 | 40,7 | 1,5 | | |

According to results obtained in the independence test, in group A, the allelic markers 16, 29, 31, 60 and 65 corresponding to the alleles2 (*primer* 2 SPS), 5, 7 (*primer* 2-HET), 8 (*primer* 1-SUSY), 2 and 7 (*primer* 2 SBE I) are associated with DMC. It is important to stand out that group A is mainly (82%) made up by genotypes with a DMC lower than 40%, indicating that the association is present when the DMC is low. It is well-known that starch is the main carbon storage in plants and it is also responsible up to 80% of the DMC in cereals, bean, cassava and potatoes, among others. At the same time, amyl pectin is the polymer that makes up from 70 to 80% of the starch granules (Smith, Starch Update 2005).
These results are very interesting if gene characteristics from which these allelic variants were designed are considered. In case of primer 2-SPS, it is one of the two designed from the gene linked to the enzyme responsible for the sucrose synthesis, that some authors have associated to the synthesis process of cellulose and in the response to adverse factors in *Saccharum officinarum* y *Beta vulgaris* (Quick and Schaffer, 1996; Winter and Huber, 2000). . Tungngoen *et al*. (2005) suggested than in the cassava genome, several copies of the responsible gene can be found, as well as, in the regulation of starch conversion to sucrose in storage roots.
The rest of the genes that give rise to these primers has an essential participation in the conversion process to starch and in its degradation, which influences directly into the DMC and gives a better explanation in relation to the results found in its association with the low DMC.
No allelic markers associated with DMC higher than 40% was found in group B, as probability values for all genotypic classes were higher than 0.1.

**Final considerations**

Results permitted to identify markers associated to low DMC in cassava in the following alleles: 2 (*primer* 2 SPS), 5, 7 (*primer* 2-HET), 8 (*primer* 1-SUSY), 2 and 7 (*primer* 2 SBE I), so, the main objective of the project was fulfilled successfully.
However, it is important to emphasize that only a group of primers linked to some genes involved in the formation process and starch degradation has been studied. New surveys would be necessary to study in depth the subject, to evaluate new primers and genes, and it will help to find other associated markers that constitute an important aspect for the scientific community and cassava productivity.
In this sense, it would be essential to develop new research projects with a greater number of genes involved and to have the possibility to design and to use new primers.

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**ANNEX 1**

**GENE SEQUENCES AND STARCH SYNTHESIS (By Primer 3 Program)**

| Gene and weight *primers* | *Primers* | Sequences |
|---|---|---|
| cn1978 523pb | 1-SUSY-R | CTTGTCCCAGTGAGGTGGAT |
| | 1-SUSY-F | GCTAAAGGATCGCAACAAGC |
| cn1978 806pb | 2-SUSY-F | GACAAGGAGAGCTTGCATCC |
| | 2-SUSY-R | CGAGAGCATGAGCAATGGTA |
| m.01.M20.5 484pb | 1-HET-F | GGCTCATGGATATCTCTGGAAG |
| | 1-HET-R | TTGGTTTCCCACTCTCAGTTG |
| cn120 412pb | 2-HET-F | CAAGCCAAGGCAAACAACTA |
| | 2-HET-R | CCCAGAGATATCCTCAACCA |
| m.12.G10.5 505pb | 1-SPS-F | TCTTCAATATTCATTCACCAGCTT |
| | 1-SPS-R | CCTGCTGCATCTCCTTTTTC |
| cn1895 529pb | 2-SPS-F | AAGTGCAAAAGCCAGGAATG |
| | 2-SPS-R | GGAGATCTTGGCATGAAAGC |
| si.01.N9.5 513pb | SSIII-F | TGCAAACAAGACTCCCCTGT |
| | SSIII-R | TAATGGCAAATTGGAAGCTG |
| sg_ssh.01.C11.u 513pb | 1-SBEI-F | CCATCATCATGGGATCAACA |
| | 1-SBEI-R | TAGCCCTCACCTCCTAAAGC |
| X77012.1 753pb | 2-SBEI-F | GATGTCGAAAATTGCCTGGT |
| | 2-SBEI-R | CCCGTATACGAGGGAGAACA |
| rni.09.F10.5 445pb | SUT-F | GTTGCTGTGGCTGTTTTCCT |
| | SUT-R | CAGTGGTGTTTCCCTGTTCC |
| AF168390 580pb | 1-RCP1-F | TCTAAAGCGTGCGTCTATCG |
| | 1-RCP1-R | CCACAGTTGCCACACAGTTT |
| AF168390 512pb | 2-RCP1-F | CTGGGACAACTGCTTTTGGT |
| | 2-RCP1-R | GCTTGAACAAAGGGCGTTTA |
| Baguma *et al.*, 2003 508pb | 1-SBEII-F | GGATCGGATCATTGTCTTCG |
| | 1-SBEII-R | ATGGAAGCTGGCATGTAACC |
| Baguma *et al.*, 2003 595pb | 2-SBEII-F | ATGGTGGTGTTGGCTTTGAT |
| | 2-SBEII-R | GATCCGATCCCTTTCATCCT |
| rni.08.O6.5 509pb | INVERTASE-F | TCCATGAAGCTCTTCTTCTTCC |
| | INVERTASE-R | AATGGATTGTTGGGTGATTTC |
| m.04.F16.5 309pb | SS-isof. F | GGAATGGATATATTAAAGAGGATGG |
| | SS-isof. R | CAATGGGATCATGCAGTTTG |

**ANEXO 2**

| Primer | Alelo | No de alelo |
|---|---|---|
| SUT | 1 | 1 |
| | 2 | 2 |
| | 3 | 3 |
| | 4 | 4 |
| | 5 | 5 |
| | 6 | 6 |
| | 7 | 7 |
| | 8 | 8 |
| | 9 | 9 |
| | 10 | 10 |
| 1-SPS | 1 | 11 |
| | 2 | 12 |
| | 3 | 13 |
| | 4 | 14 |
| 2-SPS | 1 | 15 |
| | 2 | 16 |
| | 3 | 17 |
| | 4 | 18 |
| | 5 | 19 |
| | 6 | 20 |
| 1-HET | 1 | 21 |
| | 2 | 22 |
| | 3 | 23 |
| | 4 | 24 |
| 2-HET | 1 | 25 |
| | 2 | 26 |
| | 3 | 27 |
| | 4 | 28 |
| | 5 | 29 |
| | 6 | 30 |
| | 7 | 31 |
| | 8 | 32 |

| Primer | Alelo | No de alelo |
|---|---|---|
| INV | 1 | 33 |
| | 2 | 34 |
| | 3 | 35 |
| | 4 | 36 |
| | 5 | 37 |
| | 6 | 38 |
| | 7 | 39 |
| SSIII | 1 | 40 |
| | 2 | 41 |
| | 3 | 42 |
| | 4 | 43 |
| 1-SUSY | 1 | 44 |
| | 2 | 45 |
| | 3 | 46 |
| | 4 | 47 |
| | 5 | 48 |
| | 6 | 49 |
| | 7 | 50 |
| | 8 | 51 |
| 2-SUSY | 1 | 52 |
| | 2 | 53 |
| | 3 | 54 |
| | 4 | 55 |
| 1-SBE I | 1 | 56 |
| | 2 | 57 |
| | 3 | 58 |
| 2-SBE I | 1 | 59 |
| | 2 | 60 |
| | 3 | 61 |
| | 4 | 62 |
| | 5 | 63 |
| | 6 | 64 |
| | 7 | 65 |

# CBN ACTIVITIES

## Induction of Somatic Embryos in cassava (*Manihot esculenta* Crantz) using Stationary Liquid Cultures

Danilo López, Paul Chavarriaga, and Joe Tohme.

### Introduction

Modifying cassava through transgenesis requires a system of totipotent cells that can be genetically modified, and that regenerate complete and normal plants. In cassava the technology is fairly well established[1], but there is always room for improvement, especially when it comes to reducing costs, time and labor, without sacrificing the quality of the new plants. We report a new method to generate totipotent cells, Somatic Embryos, for genetic modification, which employs liquid instead of solid medium, and glass jars instead of Petri dishes.

### Methodology

*In vitro* plants of cultivars 60444, Mcol2215 (Venezolana) and CM3306-4 (Ica Negrita) were propagated on 4E medium (Roca and Mroginski 1991) for 45 days, at 28$^{o}$C and photoperiod 12/12. One-inch long

cuttings, containing one axillary bud each, were precultured for 2-3 days on 4E medium under the same conditions to stimulate growth of meristems. The axillary buds were then dissected and placed floating on liquid MS2-50Pi medium in a baby-food jar. Each jar contained 4 ml of medium and 10 buds. Buds were left still, growing in darkness, at 28°C ± 2°C for 20-25 days. After this period, the SE produced were cleaned off non-embryogenic tissues, and either used for embryo maturation, cyclic embryogenesis or FEC induction. The percentage and quality of somatic embryos, the latter scored as high (H), medium (M) or low (L) as an indicator of amount of tissue obtained from each explant, was indicative of the success of the tested methodology.

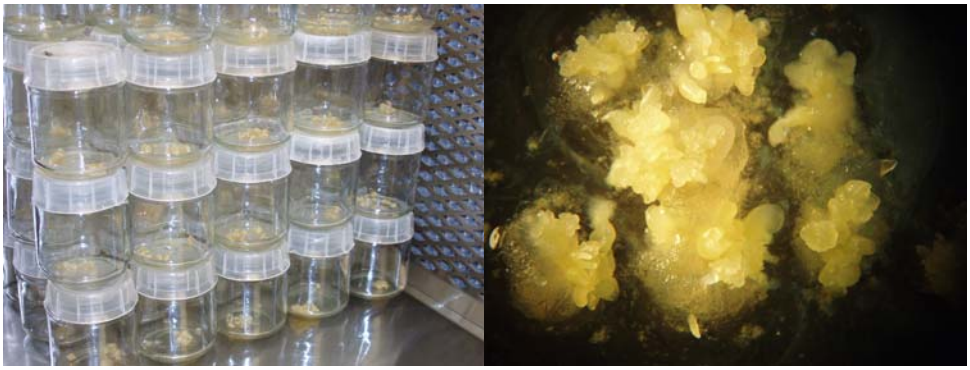
**Results**

We used three cultivars to test the new method to obtain embryogenic tissues for genetic modification.. Table 1 summarizes the results. The liquid system always produced somatic embryos of high quality for at least two cultivars (60444 and Mcol2215), and with higher quality (Figure 1). For CM3306-4 however there seems to be no much change respect to the conventional methodology.

**Table 1.** Comparison of the efficiency (%) of production of somatic embryos in conventional medium (second column; solid MS with 2,4-D) and liquid media (third and fourth columns; liquid MS plus either 2,4-D or Picloram). In parenthesis there are the scores of the quality of somatic embryos produced under each treatment (H = high; M = medium; L = low).

| Variedad | Solid with 2,4-D | Liquid with 2,4-D | Liquid with Picloram |
|---|---|---|---|
| 60444 | 35.4 (M to L) | 67.4 (M to L) | 75.9 (H) |
| MCOL2215 | 70.0 (M to L) | 55.4 (M to L) | 78.4 (H) |
| CM3306-4 | 78.3 (M to L) | 67.3 (M to L) | 72.3 (M to L) |

Figure 1. On the left panel there are 60 baby-food jars containing 10 axillary buds each, of cultivar 60444, for a total of 600 explants to induce somatic embryos in liquid medium. Approximately 76% of them produced embryos after 20-25 days. The quality of the embryos was almost invariably high, where 7/10 axillary buds gave rise to somatic embryos of the characteristics displayed on the right panel.



**Conclusions and Future Plans**

In general, like for most characteristics of agronomic value in cassava, they do depend on the genotype. In vitro behavior, to produce somatic embryos amenable for genetic modification, is not an exception. In this case two of the clones tested behaved better on liquid medium while the third clone (CM3306-4) remained the same in terms of percentage and quality of embryogenic tissues produced.

We recommend using liquid media to induce Somatic Embryos since it reduces costs by avoiding using gelling agents like agar, and Petri dishes. Besides, the quality (amounts of somatic embryos produced per explant) is much higher, as confirmed by the embryos shown in Figure 1. One more advantage of this new method is that there are always many more initial explants per in vitro plant. In the conventional method – with solid medium- we used to use very young, unfolded leaves, which appear at one per plant only. With the new methodology each axillary bud is useful, and there are as many buds in one in vitro plant as leaves it has.

Given the success of this new method, we would like to, first, reproduce the experience to include statistical analysis and publish it. Second, produce other type of totipotent cells (Friable Embryogenic Callus; Taylor et al 2004) amenable for genetic modification, and to expand it to more genotypes of commercial value.

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## Establishing mini-garden systems/preparing the site for training in low cost conventional and mass tissue culture/producing planting materials.

R.H. Escobar[1], L. Muñoz[1], A. Azcarate[2], C. Dorado[1] and J. Tohme[1]
[1]Agrobiodiversity and Biotechnology Project-CIAT; [2] CIAT's Maintenance Unit-CIAT.

**Introduction**

Planting material coming from tissue culture methodologies allow end-users reestablishment of cassava yield. CIAT in collaboration with the Municipal Farmers Association (AMUC) and NGO-FIDAR has shown that it was possible recover cassava in Northwest of Cauca, Colombia, using cassava clean material. In Colombia, other Foundation and NGO likes to replicate our experiences including other farmer's group, giving the opportunity to small-scale farmers to get access to these technologies.

Nevertheless, cost per plant not allows rapid diffusion of this technology. Based on Corpoica selling cost, each in-vitro plant are around 1000 Colombian pesos (0.48 US cents). Only, when an intermediate group make continuous propagation on field (II and II cycles) the price could be attractive for end-users.

This project will try to adjust to the cassava propagation schema a system that some forest companies has been developed as part of its business. Its known as mini-garden system, and it consist in a management of the mother-plants under greenhouse, with a nutritional plan and with a continuous harvesting of sprouting material. After this step, a root induction phase under non-control conditions allow scaling-up of the planting material to send to the field.

On the future we expect that low-cost tissue culture facilities join to mini-garden facilities allow cost reduction and farmer's access to planting material of first cycles. It could promote farmer's investment on this technology.

**Objective**

Develop alternative methods of propagation planting material focusing on cassava that allow cost reduction and scaling-up under farmer's conditions.
Write training manual as support for teaching courses and technical diffusion.
Technical meeting to diffuse low-cost technology among different end-users.

**Material and methods**

Objective 1:
Four channels were built under low-cost greenhouse that allows adjust the system using cassava a s a model crops.
Two varieties (CM 2177-2, MPer 183 and CM 6740-7) and three treatments (17N medium, MS (bud and shoots) using low cost methodology to determine better medium and explants.
Different substrates were implement to test fungal attacks, root formation and plants recovery.
Different irrigation rates are tested to determine best conditions that allow plant recovery under greenhouse reducing manpower.

Objective 2&3:
A draft of manual was discuses with different expert at Ciat that allow give about
Different activities are involved in this report: (1) Training manual development, (2) Establishment of an alternative propagation methods through mini-garden facilities and (3) Consolidation of training area to different end-user.

**Training activities**

*In-vitro* cassava's propagation methods: Low-cost and massive propagation systems. CIAT, June 29$^{th}$ – 1$^{st}$ July-2006. Address to two Corpoica's technician.
Intercambio de experiencias en la producción y manejo de material de siembra in-vitro de yuca, a nivel rural, en el Departamento del Cauca. CIAT, October 17$^{th}$-22$^{nd}$ –2006. Address to 5 farmers and two-technicians (one from PBA and Corpoica)
Participación, grupo y trabajo en equipo para los procesos de investigacion participativa y desarrollo. July 1$^{st}$-2006. Address to farmers, Corpoica technicians and CIAT's biotech team.

**Results**

Four channel with irrigation system working under greenhouse facilities as a mini garden at CIAT uses as pilot site.
Five small-scale farmers (3 women and two men), one technician from national program and one social research form PBA program trained on low cost propagation system.
Two technicians trained on RITA system.
Knowing of technical and logistical aspect involved in the establishment of rural Tissue culture laboratory and on local seed banks establishment.
A working plan concerned among farmers, technicians and researchers to replicated CIAT's experiences with North Coast farmers.

**Future activities**

Fine-tuning the mini-garden system that allows it cassava propagation under greenhouse set-up using tissue culture plants as sources.

Extrapolate this experiences to other regions and neglected crops
A concept note was submitted to make a theory-practical meeting at Mozambique with the National program (IIAM) and end-users to be involved in cassava program.
A final version of tissue culture manual on Spanish and English to be implemented as teaching tool.

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**Figure 1**: *Eucalyptus* vegetative propagation through mini-garden system established at Smurfit Carton de Colombia. It infrastructure support its clonal propagation scheme (1) Bed of cuttings, (b) Multi-branching stem uses as source of cuttings.

**Figure 2**: Mini-garden facilities that it has been building at CIAT under CBN's financial support. It will be use as pilot and training using cassava as a model crop. Four gutters will be implemented to test different substrates and solutions.

**Figure 3:** Cosmoagro's greenhouse. This company makes different comparison among greenhouse types, nutrient solutions and crops. (1) Tomatoes growing under Ecuadorian's greenhouse type. It was cheaper than confined greenhouse set-up, and (b) Technician applying Hoagland's solution to bean plants.

**Figure 4**: Cassava plot established with material coming from in-vitro plants. Those plot are under AMUC coordination's, and it alls them multiplied some clones to be released with its partners. These plot are part of Gines–Mera fellowship activities at Cauca..

**Figure 5:** Training activities made with different end-users. (a) Knowledge sharing –farmers their experiences and expertise. Farmer's meeting between Cauca and North-Coast Colombian regions. (b) Farmer from the north coast making tissue culture practices at Cauca rural laboratory (c) Special meeting supported by CIAT and PBA program How's work in-group? How could we join farmer and researcher's expectation? and (d) African researchers coming from four countries and MAFF coordinator visiting low-cost rural laboratory at Cauca.

# Activity 3.3 Databases and Libraries

## 3.3.1 GoMp - Gene Ontology and Metabolic Pathway Integration for the annotation of tropical crop genomes.

F. Rodríguez-Zapata[1], G. Plata[1] and J. Tohme[1].
[1] SB2 Project.

### Introduction

Functional annotation repositories like the Gene Ontology (GO) database (Harris *et al.*, 2004) and databases (DBs) of biological pathways such as MetaCyc (Caspi *et al.*, 2006) and KEGG PATHWAY (Kanehisa & Goto 2000) have become essential resources for gene discovery in non-model species. For instance, GO annotation of genes has been used to accelerate comparative mapping and discovery of candidate genes underlying quantitative trait loci in livestock (Harhay & Keele 2003) and crops (Pajerowska et al., 2004). Metabolic pathway annotations have also proven valuable in understanding cellular processes that can be the basis of desirable agronomical traits like insect antibiosis in maize (MacMullen *et al.*, 1998) and fruit color and size in tomato (Causse *et al.*, 2004). Gene Ontology (GO) and metabolic pathways databases are complementary for the annotation of genes and their products. However, access to Gene Ontology annotations and KEGG metabolic pathways is independent of each other in websites like the TIGR Gene Indices and the JGI. Nonetheless there are biological questions that require an integrated access to both annotation sources in order to be solved: Which metabolic pathways are involved in plant disease resistance? How many genes from a non-model organism might be involved in such pathways? Are these genes implicated in consecutive steps corresponding to known network motifs? Are the gene products catalyzing rate-limiting reactions? With the aim of helping to solve this kind of questions, and to assist gene discovery in tropical crops we designed and implemented GoMp, an annotation system and a relational database for the integration of GO and KEGG metabolic pathway maps.

### Methodology

*Datasets and Parsing*
GoMp is an extension of the Gene Ontology DB for the inclusion of KEGG GENES annotation. Consequently, the relational schema, data and documentation for the GO DB (http://archive.godatabase.org/) were downloaded and used as the base for further modelling. From the KEGG suite of resources we obtained the KEGG flat files and the file that contains the description and hierarchy of the KEGG Orthology (ftp://kegg.genome.ad.jp/pub/kegg). In or order to parse the KEGG GENES files and loading them into a MySQL schema we developed a bioperl *Bio::SeqIO::kegg* derived class, namely the *Gomp::DBloader* module. For representing the KO hierarchy in a nested data structure we wrote the perl module *Gomp::Tree::KO*. An additional module, *Gomp::Tree::KOParser*, relies on *Gomp::Tree::KO* objects for parsing not only the hierarchical content of KO but also the cross references between KO and other DBs. KO was fitted into the relational model using a transitive closure relation representing the hierarchy graph (http://www.oreillynet.com /pub/a/network/2002/11/27/bioconf.html). This hierarchical to relational mapping was implemented as *load_ko_graph_path*, a separate method in *Gomp::DBloader*.
GoMp Schema description
The GoMp database model is based upon the MySQL version of the GO DB and our *in house* relational implementation of both the KEGG GENES and the KEGG Orthology hierarchy (Fig. 1). Basically we added to the GO DB schema the *kegg_genes* and *kegg_gene_dbxref* tables for storing KEGG GENES. The

table *kegg_genes* contains the nucleotide and aminoacid sequences corresponding to each gene. The PATHWAY and ORTHOLOG fields from the KEGG GENES files were put into the *dbxref* table in the GO DB. The table *kegg_gene_dbxref* keeps the association between KEGG PATHWAY maps and the KEGG Orthology. Following the method for hierarchical to relational mapping of GO we designed the *ko_graph_path* table to represent the hierarchical structure of KEGG Orthology. In this way the GoMp schema is not only useful for simple keyword search but also allows the user to browse and query both DB sources in a hierarchical manner reporting annotation for each node in the hierarchies as well as the annotation for its children nodes.

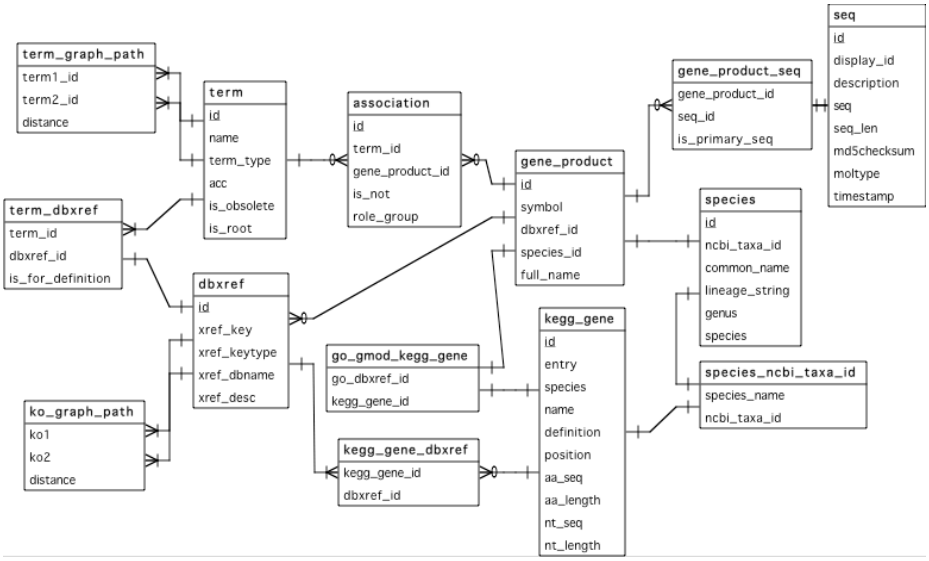


Fig. 1. GoMp Database Schema.

*Sequence annotation using GoMp*

A perl module, *Gomp::Annotation*, was developed to assign KOs and GO terms to new sequences based on their similarity to reference sequences in the KEGG GENES and GO DBs (Fig. 2). Similarity search of the chosen sequence sets was done running BLASTX, threshold e-value 1e-5, against a virtual database consisting of both GO DB lite and KEGG GENES aminoacid sequences. The search results for each query sequence could thus contain hits from the two separate DBs. In order to transfer the controlled vocabulary annotation we chose the best hit that belonged to the GO DB and the best hit coming from KEGG GENES when both ranked within the top five hits of the BLAST report, otherwise only the first hit was chosen. All the GO and KO annotations of best hits found were transferred to the query sequence and stored in the *dblink* table of the GoMp relational DB. Once KOs and the EC numbers corresponding to a set of DNA sequences were stored in the GoMp DB it was possible to draw hypothetical pathways for species lacking annotations in GO and KEGG by means of the KEGG web services API (http://www.genome.jp/kegg/soap/).

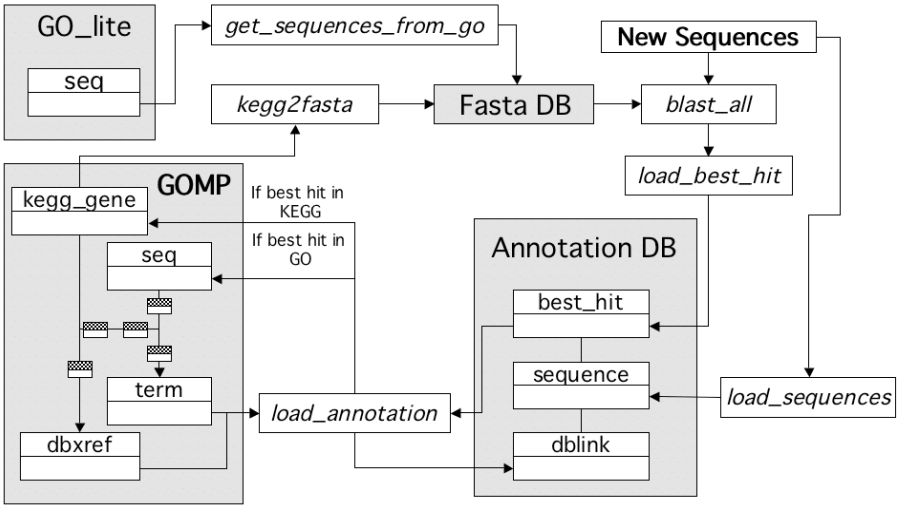


Fig. 2 Gomp::Annotation pipeline: sequences are aligned to amino acid sequences in the GO and KEGG GENES DBs using BLAST, the annotation of their best hit is then retrieved from GoMp and loaded onto an annotation DB. Methods in the perl module are shown in italics.

Four sets of sequences were annotated for this paper. Two EST assemblies for tropical crops and two reference sets for the model species *A. thaliana*, these are: the *Phaseolus vulgaris* Gene Index (PHVGI) release 1.0 EST assembly from TIGR; a set of 5704 *Manihot esculenta* EST Unigenes generated at CIAT (López *et al.*, 2004), a set of 31527 cDNA sequences from the *Arabidopsis* Information Resource (TAIR) and the *A. thaliana* KEGG GENES.

Results

The results of the annotation of bean and cassava EST assemblies along with that of the 2 reference sets, *A. thaliana* KEGG GENES and TAIR cDNAs, are shown in Table 4. The proportion of sequences annotated with KOs is greater in the EST assemblies than in the reference sets. Most of the annotated sequences have associated GO terms and almost all of the sequences annotated with KO were also annotated with GO. The number of pathways detected in both EST assemblies was similar; therefore, differences in the number of annotated KOs represent variations in the quantity of gene products within these pathways. This is consistent with the unequal sample size of the cassava and bean sets.

Table 1. Results of the GoMp automated annotation of 2 EST assemblies and 2 reference sets of sequences.

| | TIGR *P. vulgaris* | CIAT *M. esculenta* | KEGG *A. thaliana* | TAIR cDNAs |
|---|---|---|---|---|
| Sequences annotated with KO | 1703 (17.9%) | 1132 (19.8%) | 3007 (10.4%) | 3622 (11.5%) |
| Sequences annotated with GO | 7648 (81.0%) | 4439 (77.8%) | 26962 (93.5%) | 30652 (97.2%) |
| Sequences annotated with KO and GO | 1695 (17.9%) | 1125 (19.7%) | 2923 (10.1%) | 3612 (11.4%) |
| Total KOs | 720 | 530 | 1210 | 1205 |
| Total Pathways | 110 | 105 | 114 | 114 |
| Total GO terms | 1987 | 1429 | 2158 | 2705 |
| Total annotated | 7692 (81.1%) | 4446 (77.9%) | 27046 (93.7%) | 30662 (97.2%) |
| Total sequences | 9484 | 5704 | 28848 | 31527 |

While several GO terms in the 'molecular function' category have equivalent KOs, most other terms do not represent pathways or molecular activities and so they lack any counterpart in the KEGG Orthology. Some of the GO terms without equivalent KOs refer to processes or traits of agronomic interest, to find pathways associated with these terms it was necessary to rely on the transitive relationship of GO and KO. For instance, the GO terms in Table 2 are only transitively linked to pathways; nonetheless they refer to desirable traits in bean and cassava such as drought and heat tolerance and resistance to insects, bacteria or pathogenic fungi.

Table 2. Sample GO terms of agronomic interest.

| Term Name | Sequences with GO term | | Associated pathways | | Sequences within pathways | |
|---|---|---|---|---|---|---|
| | Pv | Me | Pv | Me | Pv | Me |
| GO:0042829 defense response to pathogen | 51 | 26 | 8 | 3 | 5 | 3 |
| GO:0009613 response to pest, pathogen or parasite | 110 | 78 | 6 | 3 | 12 | 5 |
| GO:0006950 response to stress | 287 | 176 | 28 | 24 | 73 | 34 |
| GO:0009628 response to abiotic stimulus | 308 | 172 | 39 | 31 | 93 | 37 |

Pv=P.vulgaris, Me=M.esculenta

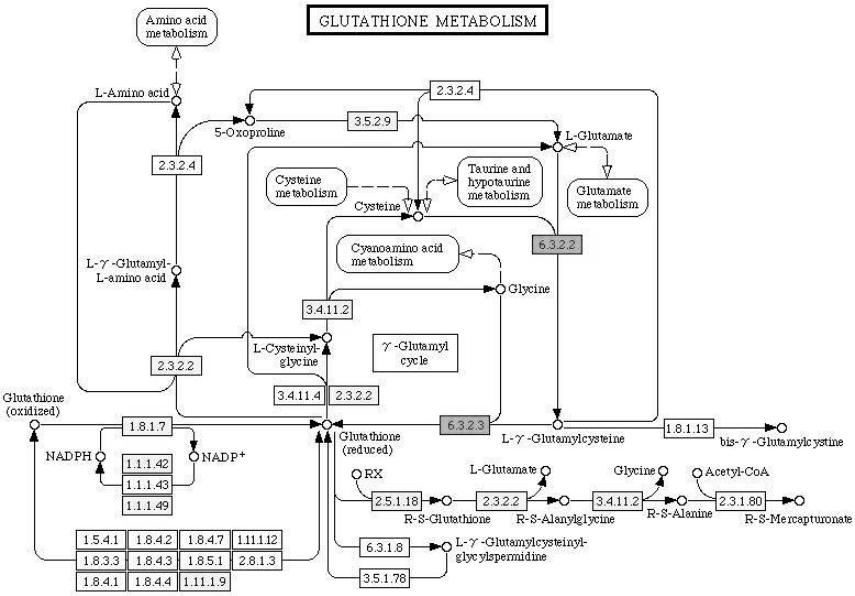


Fig 4. Glutathione metabolism pathway and enzymes related to GO term 'defense response to pathogen': glutamate cysteine ligase (EC:6.3.2.2), glutathione synthase (EC:6.3.2.3).

A closer inspection of this results for the *P. vulgaris* gene index shows that there are sequences annotated with the GO term 'defense response to pathogen, incompatible interaction' (GO:0009814) that are also related to the KEGG pathways 'Stilbene, coumarine and lignin biosynthesis' (00940) and 'Glutathione metabolism' (00480). While stilbene has been classified as a constitutive protectant against microorganisms or as an induced phytoalexin that protects plants from insects or fungi [38], glutathione levels can profoundly affect the poising of stress defenses in plants by modulating the activity of signaling proteins [39]. Within the KEGG pathway for glutathione metabolism (Fig. 3) we found the enzymes glutamate cysteine ligase (EC:6.3.2.2) and glutathione synthase (EC:6.3.2.3), which catalyse consecutive reactions leading to the synthesis of glutathione from L-Glutamate. These enzymes could be targets of further research efforts.

## Conclusions

The associations stored in GoMp between gene products of model organisms, Gene Ontology and KEGG Orthology are useful for EST sequence annotation, keyword search and hierarchical navigation.
The relations between GO and KO controlled vocabularios are not only biologically meaningful, but are also a tool for the rapid detection of candidate genes underlying desirable phenotypes.

## Ongonig Work

To integrate Cyc Databases (RiceCyc, AraCyc, Meta Cyc, BioCyc) to the current schema.
To improve the GoMp web based GUI.

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### 3.3.2 Paddy Genes Book, a database of rice T-DNA insertion lines phenotypes

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*These authors contributed equally to the work
*Partners:*
IRD; Cirad; Génoplante;
*Project funded by:* The Génoplante consortium; IRD

**Introduction**

In the framework of its work plan for functional analysis of cereal genomes, the Génoplante consortium decided to construct a rice T-DNA insertional mutagenesis collection (Sallaud et al 2003). Rice was chosen as a model species because of its small genome and because of all the genomic resources available for this species (ESTs, genetic maps, complete sequence, etc.). The lines were produced in Cirad laboratories, and grown in Cirad and IRD greenhouses, in Montpellier, France. The present work carried out at CIAT as a collaboration with Génoplante consists in: (i) a systematical phenotypic evaluation of the mutant collection, with production of an associated phenotypic database, and (ii) the multiplication of seeds for the entire collection, for later distribution to all laboratories interested in rice functional genomics. We focus here on the first topic.

**Methodology**

Screenhouse
Twenty thousand $T_0$ plants were produced at Cirad and grown in Cirad and IRD glasshouses in Montpellier, France. Twenty-five $T_1$ seeds per $T_0$ plant were received at CIAT and were sown in a screenhouse. Sowing was carried out in eight batches of 1,250 lines, with about three weeks delay between the batches. The seeds were pre-treated by heat for three days at 50 °C to break dormancy, and planted in plastic trays with a mixture of CIAT (67 %) and Santander de Quilichao (33 %) soils. Germination was determined at ten days after sowing (DAS). The first phenotypic observations were

carried out at 18-20 DAS, with counting of the number of individuals presenting the mutant phenotype. A list of possible phenotypic traits was established from data mining of several rice phenotypic databases (www.gramene.org, www.grs.nig.ac.jp/rice/oryzabase, www.irri.org/genomics), and was used as a guide for observations. An English-Spanish-French lexical of botanical and agronomic terms was established to facilitate phenotype identification.

Field

Two fields of two hectares each were prepared following the requirements of the ICA (Instituto Colombiano Agropecuario). The entire surface was covered by nets to avoid any damage or seed dissemination that could be caused by birds. The plantlets were transplanted at 25 DAS. A basic fertilization composed of Mono-Ammonium Phosphate, Iron Sulfate, Potassium Chloride and micro-elements was applied. The field was irrigated two times a week. Control lines of Nipponbare cv. were planted for each 10 T-DNA lines in order to facilitate the comparison with wild phenotype. Phenotypic analyses were carried out at different ages, using the list of possible traits as a guide. A first round of observation was done when the plants were approximately 45 days old. A second evaluation was done at flowering, while the ultimate observation was done at maturity. This maximized the chances to detect phenotypic variations, as various traits could be observed at only one of these stages. Moreover, this permitted to follow the evolution of a suspected phenotype at early stage and possibly confirm or invalidate it.

## Results

### Mutant Phenotypes

In the screen house, 8.5 % of the lines showed phenotype variation in comparison to the wild type. In the field, the rate was of 13.6%. The overall mutant phenotypes percentage was 16.7 %.

Numerous lines showed chlorotic or albino plantlets, with associated deficiency in leaf development. General abnormal development was also frequently observed. The most common phenotypes included several types of albinism, sterility, dwarfism more or less pronounced, chlorotic leaves, rolled leaves, awning, modified leaf shape, white streaks, lesion mimics, general abnormal development, late flowering, round hull, modified tillering.

Redundancy of phenotypes was frequently observed between two or more lines. This is probably due to the fact that these lines proceed from the same transformation event. We thus applied a correction to the calculation of the percentage of observed mutant phenotypes. If several lines proceed from the same callus and share at least one trait, only the first entry is retained. This leads to a corrected estimation of 12.2 % of mutant phenotypes (screen house + field).

### Database Set Up

A local database of all data relative to growth conditions, germination, flowering, and phenotypic observations was set up. This database, called *Paddy Genes Book*, is mainly used as a working tool to facilitate data entry and compilation. However, it also can be used for data browsing, as it permits the display of information by mutant bar code number or CIAT number. Several options for searching for lines or traits according different criteria are available. Moreover, the database offers tools for computing basic statistics over traits and lines.

This database also displays photographs of the mutant phenotypes (see screenshots for details). More than 36,000 photos are available.

A flat data file is regularly extracted form the database in order to fill the Génoplante Oryza Tag Line database available online (http://urgi.versailles.inra.fr/OryzaTagLine/).

## Conclusion

The overall mutation rate was higher than it is currently observed in other mutant collections, where visual phenotypic screening typically identifies about 3 to 5% of mutants. A part of that excessive mutation rate could be eliminated by clone redundancy analysis. T-DNA insertion is probably not responsible for all the variation observed. Indeed, it is well known that other sources of mutation like the Tos 17 retrotransposon are positively activated by in vitro culture of rice. Moreover, discrepancies in germination dates and seed quality, mainly due to the growth conditions of the T0 plants, may be responsible for apparent mutations, notably Retarded Growth (RG), tillering, height and delayed flowering. Also, in some cases we chose to include some doubtful data, as it is preferable to eliminate false-positive data after more detailed analyses for a specific trait than to miss real data.
The overall process of seed multiplication and phenotypic analysis worked very well. The timetable was respected, and valuable phenotypic data were produced. The phenotypic database will constitute a precious tool for selecting lines for functional genomics studies. We plan to extend the phenotypic analysis and seed multiplication to the entire collection (35,000 lines).

## References

C Sallaud, D Meynard, J van Boxtel, C Gay, M Bès, J P Brizard, P Larmande, D Ortega, M Raynal, M Portefaix, P B F Ouwerkerk, S Rueb, M Delseny, E Guiderdoni. 2003. Highly efficient production and characterization of T-DNA plants for rice (*Oryza sativa* L.) functional genomics. Theoretical and Applied Genetics 106:1396-1408.

**Figure 1.** Screenshots of the Paddy Genes Book phenotypic database
Figure 1a. Main menu

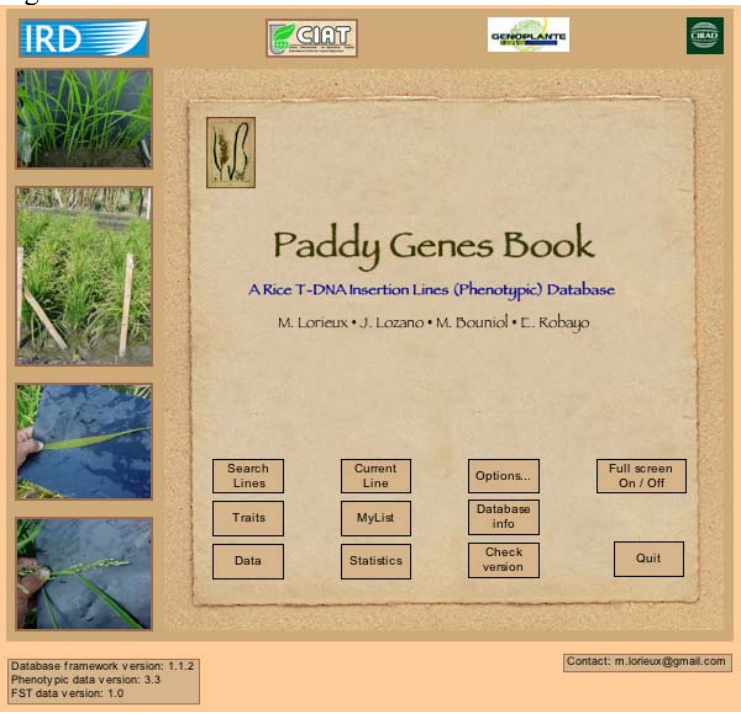



Figure 1b. Trait browsing

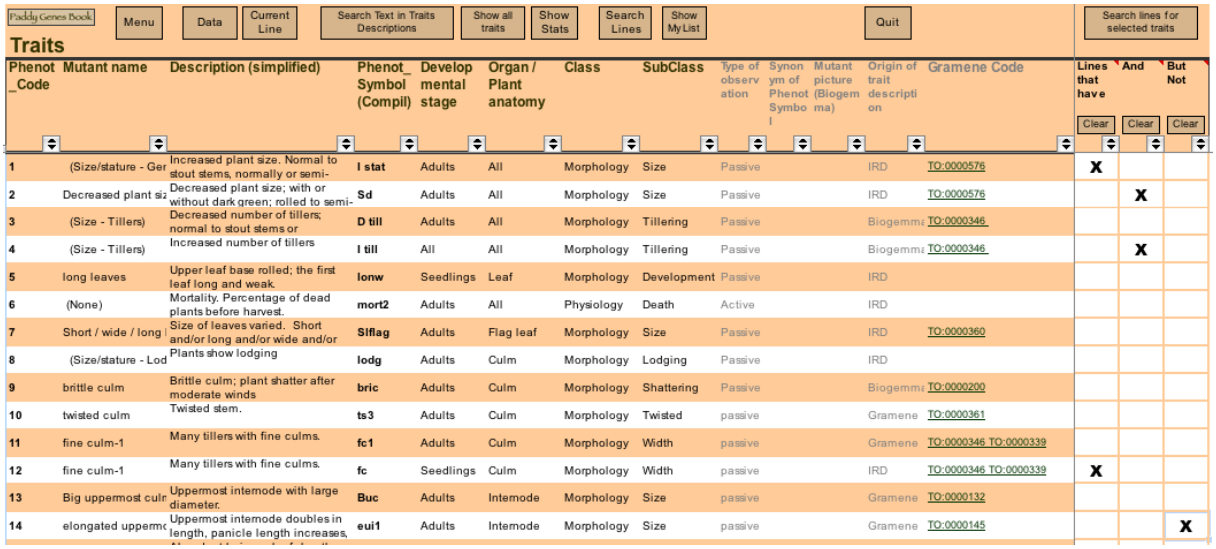



Figure 1c. Search for lines by trait code or full text

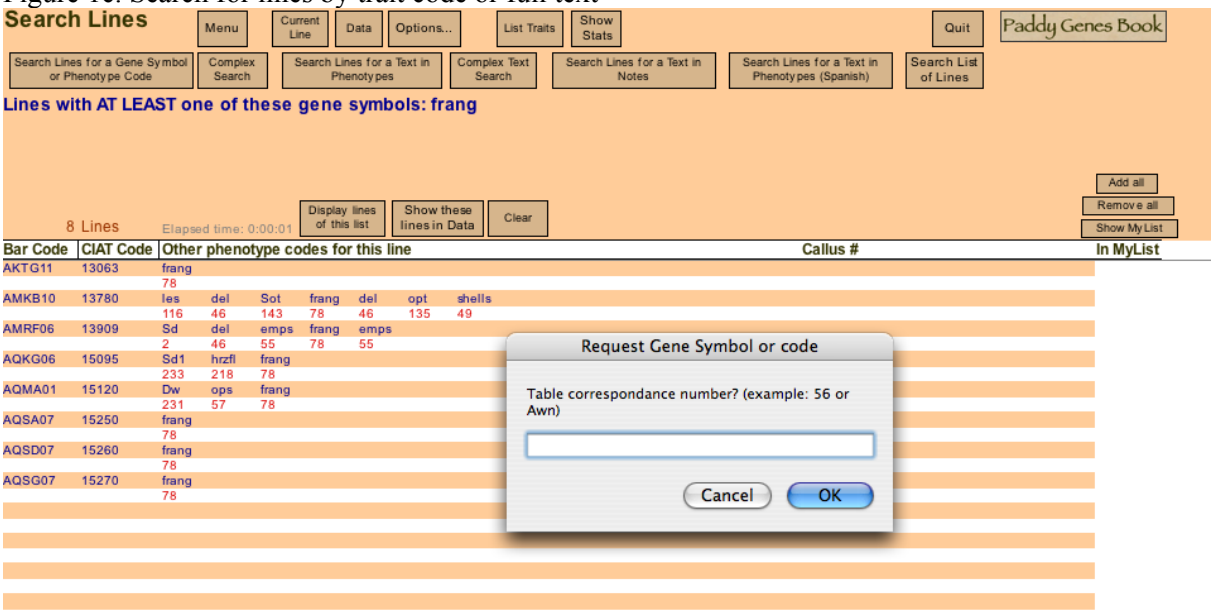



Figure 1d. Statistics on traits and lines

Menu | Data | List Traits | Search Lines | Quit

Paddy Genes Book

## Statistics on traits

Compute Stats on Traits | Correct for redundancy: 1 | Show details on a trait

## Statistics on lines

Compute Stats on Lines | Be strict: 1

Number of lines that show…

| TRAIT # | Mutant name | Description (simplified) | Gene Symbol (Compil) | y + y? + n? + n | y | y? | n? | n | Discrepancies |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 5319 | 1138 | 1679 | 2146 | 354 | 2 |
| 1 | (Size/stature - Gen | Increased plant size. Normal to stout s | l stat | 166 | 49 | 45 | 70 | 1 | 1 |
| 2 | Decreased plant siz | Decreased plant size; rolled to semi-r | Sd | 168 | 14 | 43 | 102 | 9 | |
| 3 | (Size - Tillers) | Decreased number of tillers; normal t | D till | 277 | 13 | 36 | 223 | 5 | |
| 4 | (Size - Tillers) | Increased number of tillers | I till | 150 | 15 | 33 | 100 | 2 | |
| 5 | (None) | Mortality. Percentage of dead plants ' | mort1 | 0 | 0 | 0 | 0 | 0 | |
| 6 | (None) | Mortality. Percentage of dead plants t | mort2 | 0 | 0 | 0 | 0 | 0 | |
| 7 | Short / wide / long l | Size of leaves varied. Short and/or lo | Slflag | 1 | 0 | 0 | 1 | 0 | |
| 8 | (Size/stature - Lod | Plants show lodging | lodg | 0 | 0 | 0 | 0 | 0 | |
| 9 | brittle culm | Brittle culm; plant shatter after moder | bric | 0 | 0 | 0 | 0 | 0 | |
| 10 | twisted culm | Twisted stem. | ts3 | 5 | 0 | 0 | 5 | 0 | |
| 11 | fine culm-1 | Many tillers with fine culms. | fc1 | 32 | 4 | 8 | 18 | 2 | |
| 12 | fine culm-1 | Many tillers with fine culms. | fc | 1 | 1 | 0 | 0 | 0 | |
| 13 | Big uppermost culm | Uppermost internode with large diame | Buc | 0 | 0 | 0 | 0 | 0 | |
| 14 | elongated uppermo | Uppermost internode doubles in lengt | eui1 | 0 | 0 | 0 | 0 | 0 | |
| 15 | hairy sheath* | Abundant hairs on leaf sheath | hsf | 0 | 0 | 0 | 0 | 0 | |
| 16 | slashed leaves* | Necrotic tissue and/or streaks. | necr2 | 0 | 0 | 0 | 0 | 0 | |
| 17 | slashed leaves* | Necrotic tissue and/or streaks. | necr1 | 18 | 9 | 6 | 2 | 1 | |
| 18 | (Leaves - Lesion m | HR-like spots observed on leaves; with | les mim1 | 4 | 2 | 1 | 1 | 0 | |
| 19 | (Leaves - Lesion m | HR-like spots observed on leaves; with | les mim | 68 | 16 | 28 | 22 | 2 | |
| 20 | slashed leaves* | Necrotic tissue and/or streaks. | necr | 52 | 5 | 28 | 19 | 0 | |
| 21 | (Leaves - Colors) | Plants remain green after maturity | gr | 1 | 0 | 0 | 1 | 0 | |
| 22 | yellow leaf margin | Yellowish stripes leaf margin; yellow n | ylm | 1 | 0 | 1 | 0 | 0 | |
| 23 | yellow leaf margin | Yellowish stripes leaf margin; yellow n | ylm1 | 4 | 1 | 2 | 1 | 0 | |
| 24 | yellow leaf margin | Yellowish stripes leaf margin; yellow n | ylm2 | 1 | 0 | 0 | 1 | 0 | |
| 25 | rough sheath*/ curl | Leaves curls, sheath of older seedling | rs | 0 | 0 | 0 | 0 | 0 | |
| 26 | grayish green* | Stripes between vascular bundle, tiss | stri/ wi | 0 | 0 | 0 | 0 | 0 | |
| 27 | brown midrib* | Brown pigment in vascular bundles of | bm | 0 | 0 | 0 | 0 | 0 | |
| 28 | clear patches* | Midrib and adjacent tissue lighter gre | pm | 0 | 0 | 0 | 0 | 0 | |

**Number of lines that show… (Corrected for clone redundancy)**

| | # | All % |
|---|---|---|
| Probable mutant phenotype(s) (y) | 800 | 28.9 |
| Mutant phenotype(s) to be confirmed (y?) | 668 | 24.1 |
| Unlikely mutant phenotype(s) (n?) | 529 | 19.1 |
| Invalidated mutant phenotype (n) | 137 | 4.9 |
| Total | 4983 | 77.0 |

**Number of lines that show… (Non corrected for clone redundancy)**

| | # | All % |
|---|---|---|
| Probable mutant phenotype(s) (y) | 799 | 28.8 |
| Mutant phenotype(s) to be confirmed (y?) | 938 | 33.8 |
| Unlikely mutant phenotype(s) (n?) | 867 | 31.3 |
| No or invalidated mutant phenotype (n) | 168 | 6.1 |
| Total | 2772 | 100.0 |

**Average number of traits per line (Corrected for clone redundancy)**

| | all traits |
|---|---|
| Probable mutant phenotype(s) (y) | 0.073 |
| Mutant phenotype(s) to be confirmed (y?) | 0.107 |
| Unlikely mutant phenotype(s) (n?) | 0.137 |
| No or invalidated mutant phenotype (n) | 0.023 |

Figure 1e. Display of phenotype, codes, statistics, heading date graphics and photographs for each line

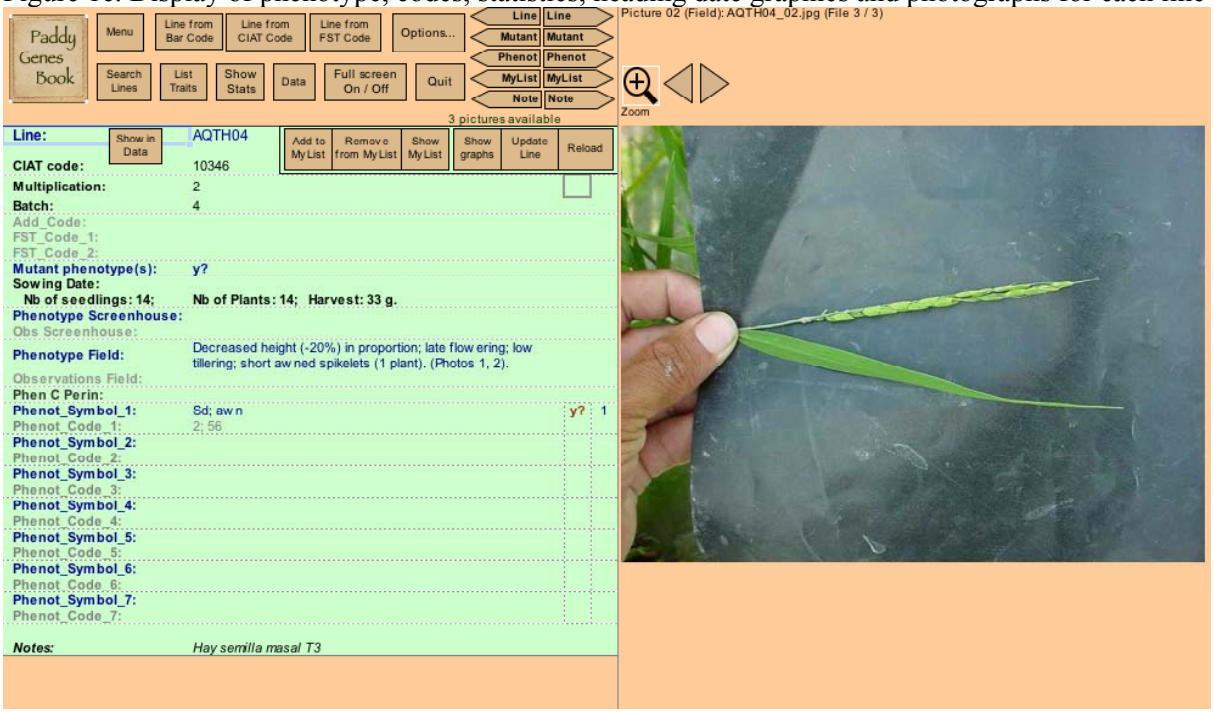
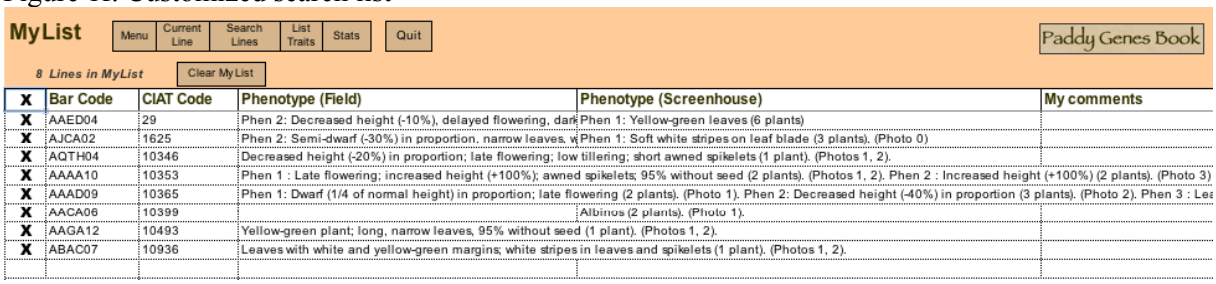


Figure 1f. Customized search list




| X | Bar Code | CIAT Code | Phenotype (Field) | Phenotype (Screenhouse) | My comments |
|---|---|---|---|---|---|
| X | AAED04 | 29 | Phen 2: Decreased height (-10%), delayed flowering, dar | Phen 1: Yellow-green leaves (6 plants) | |
| X | AJCA02 | 1625 | Phen 2: Semi-dwarf (-30%) in proportion, narrow leaves, w | Phen 1: Soft white stripes on leaf blade (3 plants). (Photo 0) | |
| X | AQTH04 | 10346 | Decreased height (-20%) in proportion; late flowering; low tillering; short awned spikelets (1 plant). (Photos 1, 2). | | |
| X | AAAA10 | 10353 | Phen 1 : Late flowering; increased height (+100%); awned spikelets; 95% without seed (2 plants). (Photos 1, 2). Phen 2 : Increased height (+100%) (2 plants). (Photo 3) | | |
| X | AAAD09 | 10365 | Phen 1: Dwarf (1/4 of normal height) in proportion; late flowering (2 plants). (Photo 1). Phen 2: Decreased height (-40%) in proportion (3 plants). (Photo 2). Phen 3 : Le | | |
| X | AACA06 | 10399 | | Albinos (2 plants). (Photo 1). | |
| X | AAGA12 | 10493 | Yellow-green plant; long, narrow leaves, 95% without seed (1 plant). (Photos 1, 2). | | |
| X | ABAC07 | 10936 | Leaves with white and yellow-green margins; white stripes in leaves and spikelets (1 plant). (Photos 1, 2). | | |

### 3.3.3 Contribution made towards protected areas in Latin America: databases about distribution of wild relatives of crops.

D.G. Debouck

We have continued with the establishment of databases about the geographic distribution of wild relatives for the so-called CIAT mandate crops. The objectives of that work are:

i. correct identification of materials collected and kept in *ex situ* conservation facilities (namely CIAT genebank, and other collaborating institutions). An output of this work is the taking of digital images of vouchers and to make them available through our web site (a service acclaimed by the Botanical Society of Colombia).

ii. geographic distribution of wild relatives of direct interest in breeding activities (namely acquisition of germplasm useful to the breeders).

iii. distribution of wild relatives genetically compatible with the crop, in view of introduction and management of transgenical crops.

iv. monitoring of modification/ destruction of natural habitats and disappearance of populations.

This year we have collated information in the following herbaria: A, BAA, BM, ECON, FHO, GH, HNMN, K, MA, NA, NEBC, OXF, and SI. These data will help us to build up the pilot for the component of threat analysis for a regional project in preparation with the Natural Resources Division of the World Bank. This pilot was agreed upon with the six partners of the project (CONABIO of Mexico, I. von Humboldt of Colombia, INBio of Costa Rica, CIAT, and Smithsonian Institute) at first meeting in February 2004. Agreements to 'repatriate' that information to CONABIO of Mexico and INBio of Costa Rica were also made. The information 'Cahiers de Phaséologie' has been put on CIAT web site for the sections: *Chiapasana, glabellus, microcarpus*, and *Revoluti*.

# Activity 3.4 Training, Trips, Workshops, Visiting Researchers and Students.

## 3.4.1 Trips and workshops of Principal Staff

Debouck, D. Gene flow project, University of Costa Rica, Costa Rica, 16-22 January 2006: visit to field trials in Quircot, Cartago and on the station Fabio Baudrit in Alajuela.

Lorieux Mathias. Attend Conference on Plant Genome XIV. USA . Jan 13, 06

Tohme, Joe. CIB Meeting. Medellín. Jan 19-06

Tohme, Joe. Monsanto and Danforth Meetings. USA. Jan 21-26,06

Tohme, Joe. Attendance a Cassava Meeting in Danforth. January 24-25,06

Sperling, L. Long-term Seed Aid Project: Government and NGO organizational analysis. Ethiopia. Jan 15-Feb 3,06

Martínez, César. February 2-11.2006. Santa Cruz, Bolivia. Visit to Ciat-Bolivia and Aspar to observe rice activities in several places and assess progres made.

Tohme, Meeting Republic Bank. Bogotá. Jan 20 – Febr.02,06

Tohme, Joe. Meeting HarvestPlus. Washington. Workshop "Crop Biofortification for Alleviating Micronutrient Malnutrition" Chenai – India. Febr.13-14,06

Sperling. L. 2006 Seed Security Assessment: Methods and Challenges. Crooss-Sector Seed Conference Bamako:Mali. March, 06. *Workshop presentation*.

Ishitani, Manabu. Meeeting with Dr. Okamato, Japan Embassy. Cassava Project. Bogotá. March 1-2,06

M. Blair – February 28 – March 2, 2006 – Bogotá, Colombia - Univ. Nacional de Colombia – thesis exam, planning for Colciencias project reports

Martínez, César. Feb.26-28. Participation in 31st Rice Technical Working Group Meeting in Houston, Texas . Poster presentation on "High iron and zinc rice lines".
April 3-4.2006. James Stangulis visited CIAT for discussions/suggestions on Fe/Zn analysis.

Fregene Martin. Trip to Ghana (February 2006). Participation in an IFAD meeting on a marketing initiative for cassava in West and Central Africa

M. Blair – March 3 – 10, 2006 – Santa Cruz and Cochabamba, Bolivia - Univ. Gabriel Rene Moreno and Centro Fitogenteico Pairumani – data analysis for Bolivian germplasm collection diversity and nutritional quality.

Sperling. L. Seed Security Assessment; Training and Assessment in zone of Douentza Circle. Bamako Mail Dounetza Circle. March 5-23,06.

Tohme, PBA Meeting. Bogotá, March 15-16,06

Debouck, D. Annual meeting of the System-wide Genetic Resources Programme of the CGIAR, 10-11 March 2006: visit to the national programme of plant genetic resources of Colombia, CorpoIca, Tibaitata, Bogotá, Colombia.

Ishitani, Manabu. HarvestPlus Meeting. USA. March 3-12,06

Sperling, L. April, 06. Seed Security Assessment: micro and macro methods/indicators. Food and Agricultural Organization, UN-Rome Italy. ***Workshop Presentation.***

Debouck, D. 2th annual congress of the Programa Cooperativo Centroamericano para el Mejoramiento de Cultivos y Animales (PCCMCA), invited conference: "Los recursos genéticos en construcción: flujo de genes y selecciones de campesinos", Montelimar, Nicaragua, 24 April 2006 (awarded as the best conference of the congress).

Lorieux, Mathias. Attended Conference on Challenge Program. Johannesburg, SouthAfrica. Attend Genoplant Conference. France March 23-April 10,06

Sperling, L. Seed Security Outreach Sessions at USAID/OFDA and Interaction.. Washington, D.C. USA. April 29 –May 7, 06

Tohme, Joe. HarvestPlus –Biotechnology Meeting. USA. March 29 – April 4,06

Tohme, Joe. HarvestPlus Meeting. USA April 5-7,06

Martínez, César. April 21-28,2006. Managua, Nicaragua. Attend and present a paper at LII Reunión Anual PCCMCA on activities related to High iron and zinc rice lines. A workshop was held to discuss the role and activities related to nutrition.

Sperling, L. May 2006. Seed Aid, Vulnerability and Funding More Effective Responses. Office of Foreign Disaster Assistance/USAID: Washington, D.C. *Workshop presentation*.

Sperling, L. May 2006. Guidelines for Better Seed Aid Practice. Interaction (coalition of 110 NG0s): Washington D.C. *Workshop presentation*.

Duque Myriam Cristina. Course Epidemiology. Corpoica, Bogotá. May,06

Garcia, Alexander, Workshop on Öntologias en Biologia, Mérida, Venezuela. May 21-28,06

Lorieux, Mathias. IRD Meeting. Montpellier. May22-June 6,06

Beebe, S. BMZ Aluminum project kick off meeting. Kigali, Rwanda. June 2006.

Sperling, L. Partner Meetings for Proposal Development: BMZ drought and aluminum toxicity project; BMGF drought, beans and impact-oriented seed delivery systems. Kigali, Rwanda. June 25-July 1,06

Debouck, D. Work in the Kew Herbarium, Royal Botanic Gardens, Kew, UK, 19-20 June 2006.

Fregene, Martin. Trip to Bellagio, Italy (May 2006). An RF sponsored meeting on partnerships in modern science to develop a strong commercial sector of cassava in Africa by the year 2020

Tohme, Joe. Visit Monsanto, Danforth Center, Chicago University and Meeting HarvesPlus – Washington. May 27-June 11,06

Martínez, César. June 28-30,2006. San Jose, Costa Rica. Present a paper on the CIDA –Agrosalud Project at the II International Rice Conference organized by CONARROZ.

Tohme. Joe. African Biotechnology. Visit to University of South Africa. Visit to Danforth Center. Visit Iowa University for HarvestPlus. June 26-July 09,06

M. Blair – July 26-27, 2006 – Bogotá, Colombia – IICA/Fontagro assessment meeting – presented results for the two Fontagro projects for beans: 1) "Voluble Alto Andino" and 2) "Fríjol/Maiz para mejorar las dietas de comunidades pobres en America Latina"

Debouck, D. Work in the NA Herbarium, National Arboretum, Washington, DC, 17-18 July 2006.

Debouck, D. Technical visit to the World Bank for the GEF Project on wild relatives of crops of Central America, 19-21 July 2006.

Debouck, D. Work in the Gray Herbarium, Harvard University, 24-28 July 2006.

Fregene, Martin. Trip to Nigeria, Ghana, Tanzania, and Mozambique. July 2006.
Consultation meeting with NARs partners on a concept note to the Gates Foundation on a cassava initiative in Africa

Garavito, Andrea – Alvarez Maria Fernanda. Course on Bioinformatics. Andes University. July, 06

Lorieux, Mathias. GCP Project. IRD-Montpellier, France. August 1-31,06

Tohme, Joe. Molconet Workshop. Bogotá. August 4-7,06

M. Blair – August 28 – Sept 2, 2006 – International Legume Genomics meeting – Asilomar Conference Center – discuss GCP/Gates Legume project.

Tohme, Joe. Attendance "The Tropical Crop Biotechnology Conference 2006. The 6$^{th}$ International Congress of Plant Molecular Biology. The Grand Challenges in Global, Australia. August, 10 –28, 06

Martínez, César. August 15-24,2006. Two workshops were held in Santa Rosa, Villavicencio, Colombia to present breeding activities in rice biofortification to participants coming from different institutions in Latin America. About 100 people attended and selected breeding lines for testing under local conditions.

Sperling. L. September. 06. Seed Relief and Seed Security. Conference: Seed Security : Nairobi (FAO/NGOs/EU) *Workshop Presentation*.

Debouck, D. International MolConnect workshop, invited conference: "Genetics of plant domestication: the basket and the clay pot challenging the PCR", Bogotá, Colombia, 5 September 2006.

Sperling, L. Partner Meetings for Proposal Development: BMGF drought, beans and impact-oriented seed delivery systems. Limuru, Kenya. Sept 10-12,06

Sperling, L. Long-term Seed Aid Project: Farmer surveys: SPSS training and data analysis. Nazret Ethiopia. Sept 14-23, 06

Pachón, Helena. Workshop: "Standarization of *in vitro* digestion/Caco-2 Cell Methods for Micronutrient Bioavailability", Columbus, Ohio. Sept 12-13,06

M. Blair – September 12-16, 2006 - Generation Challenge Program annual meeting – Sao Paulo, Brasil – present results of GCP projects in SP1 and SP2.

Fregene, Martin. Trip to Brazil (September 2006). GCP Annual Research Meeting in Sao Paulo, Brazil,

Martínez, César. September 17-22,2006. Brazil. Visit to CNPAF-Goiania ,and EMBRAPA-Meio-Norte in Teresina to observe field activities and discuss activities related to CIDA-Agrosalud Rice Biofortification project.

Tohme, Joe. HarvestPlus and Kansas State University Meetings. Sept 17-24,06

Ishitani, Manabu. Visit Nihon, Tsukuba, Tokyo and Nagoya Universities. Riken Plant Science Center. Oct 6-26,06

Fregene, Martin. Trip to Washington D.C., USA (Oct 2 - 6, 2006). Attendance of the Global challenges in global health as a member of the project oversight committee to the Biocassava plus project.

M. Blair – October 26 – Nov 1, 2006 - Ithaca and West Lafayette, USA - Cornell and Purdue Universities

Debouck, D. To represent CIAT and present an invited paper "Comentarios sobre Mecanismos de Protección de Propiedad Intelectual en Plantas", at the Regional Seminar "Derechos de Propiedad Intelectual en el ambito de los Recursos Fitogeneticos", organized by FAO, RedBio, and the Centro de Estudios Interdisciplinarios de Derecho Industrial y Económico, University of Buenos Aires, 18-20 October 2006.

Martínez, César. October 7-14, 2006. Attend International Rice Congress in New Delhi, India to keep up on new developments in rice research and HP+biofortification.

Lorieux, Mathias. Attend International Rice Functional Genomics Consortium and PhD. Thesis Andrea Garavito, IRD France. Oct 6-17,06

Tohme, Joe. Biointegra Meeting. Bogotá. October 24,06

Martínez, César. November 2-3, 2006. Workshop held in CIAT to discuss impact assessment on Agrosalud activities and future work plans.

Sperling, L. Long-term Seed Aid Project: Farmer surveys, West Hararghe. Asebe Teferi, Ethiopia. Oct 26-Nov 4, 06

Martínez, César. November 14-21,2006. La Habana . Cuba. Presented rice biofortification activities to 30 people attending the International Rice Breeding Course. Visit our AgroSalud collaborators from IIA, goverment officials and representatives of the nutrition and health sectors. Discussions on PhD thesis proposal to be carried out by Violeta Puldon.

Tohme, Joe. Visited Gates Foundation and Davis University, USA. Nov 13-16,06

Fregene, Martin. Trip to Abuja Nigeria (November 5-14 , 2006). Project briefing and fact-finding visit on the NNPC cassava to biofuels initiative (in collaboration with CLAYUCA

Tohme, Joe. Biointegra Meeting. Bogotá. Nov. 20-21,06

Tohme, Joe. Visited CENICAFE. Genoma del Café Project. Manizales. Dec 3-4,06

Martínez, César. December 4-5, 2006. Workshop held in CIAT with representatives from Venezuela interested in joining AgroSalud activities starting in 2007. Good possibilities for additional funding.

Tohme, Joe. Biolenguaje. Andes University. Bogotá, Dec 4-7,06

Ishitani, Manabu. Meeting with Japanese Embassy. Meeting at Equity Investment S.A. related to Cassava. Bogotá Dec 11-12, 06

Lentini, Z. Played a major role developing biosafety regional project including coordination with internal team at CIAT (SB2, SB1, Impact assessment, GIS, IPM), with support from CIAT Project Office, Finance, Human Resources, Legal Department and Procurement, with external task team at the World Bank (Matt McMahon, Indira Ekanayake, Teresa Roncal, Alexandra Horst, Danish Aryal, Anna Roumani, José Martínez) and regional partners (Costa Rica, Colombia, Peru and Brazil). Project Appraisal Document has been approved and final negotiation reached with the World Bank and now project is in final phase for receiving GEF CEO Endorsement for final approval (total USD 5 million).

Lentini, Z. 2006. Estimating Likelihood and Exposure (*Invited Moderator Session V*, *Key-note lecture*). Proceedings from the 9th International Symposium on Biosafety of Genetically Modified Organisms of the International Society for Biosafety Research (ISBR). Editor: USDA, USA. Jeju, South Korea; September 24th -29$^{th}$, 2006.

Lentini, Z*.2006. Estimating Likelihood and Exposure (Moderator Session V, *Invited Key-note lecture*). the 9th International Symposium on Biosafety of Genetically Modified Organisms of the International Society for Biosafety Research (ISBR). Jeju, South Korea; September 24th -29$^{th}$, 2006.

### 3.4.2 Training, trips and workshops of Staff personnel

Gallego, Gerardo. Work Colciencias –CIAT. Bogotá. Jan 24,06

Gallego, Gerardo. Visited IDIAF, Dominican Republic. March 12-17,06

Chavarriaga, Paul. Attend Meeting: "ZIL Progress Forum", ETH Zurichen. March 25-April 3/06

Chavarriaga, Paul. Follow up visit on collaboration with The Univesrity of Freiburg, Germany, on cassava genetic modification, March 25-31

Chavarriaga, Paul. HarvestPlus Biotechnology Meeting. Washington – USA. April 6-7/06

Chavarriaga, Paul. Visiting the Danforth Plant Sicence Center in St. Louis, MO, USA, to follow up collaborative work on cassava genetic modification, April 10-12

Duque, MC. Cenipalma. Bogotá, Colombia, March 2006 .

Duque, MC. Curso intensive análisis y usos de tecnicas microsatelites. **Tema:** Algunos elementos para el análisis de datos obtenidos con microsatélites. Universidad Nacional de Colombia, Facultad de Agronomía, Bogotá, March 22-24 de 2006

Duque, M.C. Curso-Taller bioseguridad ambiental, evaluación, manejo y monitoreo de OGM, Proyecto Interinstitucional GEF-BM ,"Desarrollo de Capacidades para Implementar en Colombia el Protocolo de Cartagena en Bioseguridad". **Tema**: Muestreo adecuado para la detección de materiales adventicios. CIAT, Palmira, Abril 2006

Gallego, Gerardo. Meeting FEDEGAN, Bogotá. April 4,06

Rescobar. International Course on Genetic and Forestry Biotechnology. March 27 - April 7 de 2006. Santiago de Chile. Con el auspicio del ICGEB, UNU-BIOLAC.

Gallego, G. Visited Andes University. Biological Sciences Department. Bogotá. May 10, 06

Garcia, Alexander. Lecturer in Ontology Plants Course. Merida, Venezuela. May 22-25,06

Duque, M.C. 4. Curso de Capacitación en MIP.
**Tema:** Patrones de disposición especial y su importancia en la definición de planes de muestreo en MIP. Corpoica, Tibaitatá. Mayo 2006

Gallego, G. Universidad Nacional Bogota. Extension Course. Theoric and Practics. Genómics and new concepts on molecular Phytopatology. June 5-9,06

Beltrán, Jesus. Course on Genomics. Bogotá. June 5-9, 06

1st meeting of the Governing Body of the International Treaty on Plant Genetic Resources for Food and Agricultura, Madrid, Spain, 13 June 2006: to represent CIAT and to give an invited presentation together with IPGRI: "Information build trust: how existing information systems can support the Multilateral System of the Treaty", and to present two posters:
(i) Mafla G., J.C. Roa, N.C. Flor, E. Aranzales & D.G. Debouck. 2006. Distribution of cassava germplasm from an international genebank: a service to the global agriculture. First meeting of the Governing Body, ITPGRFA, Madrid, Spain, 12-16 June 2006.

(ii) Torres A.M., A. Ciprian, O. Toro & D.G. Debouck. 2006. Distribution of bean and tropical forage germplasm from an international genebank: a service to the global agriculture. First meeting of the Governing Body, ITPGRFA, Madrid, Spain, 12-16 June 2006.

Moreno, Laura Tatiana. Training on Bioinformatics. Cornell University. June 21-27,06

Garcia, Alexander. Course Biological Ontologies, Javeriana University .June 27-30,06

Garavito, Andrea. Bioinformatics Introduction, Andes University. Bogotá, July 11-15,06

Garcia, Alexander, 9th International Protégé Conference, Stanford,California. July 23-26,06

Chavarriaga, Paul. Summer course on Biosafety assessment and regulation of agricultural biotechnology, offered by IPB, Ghent University, Ghent, Belgium, July 31 August 11

Duque, M.C. Capacitación para estudiantes de colegios de Cali. Genome, Biodiversity and Statistics. CIAT, Palmira, August, 2006

Duque, MolConnect "Un Puente entre Genómica y Biodiversidad" . Analisis estadístico como apoyo en Genómica y Biodiversidad. Bogotá, September, 2006

Bohorquez, Adriana. Genomica Funcional para la interacción planta-insecto. Molconnect. Bogotá, September, 06

Escobar, Roosevelt. Visited Witwatersrand. Cassava Projects. Johannesburg. Sept, 07-18,06

Beltran Jesus and Paul Chavarriaga attended the course on Bridging Genomics and Biodiversity, offered by The University of Potsdam and the Universidad Nacional de Colombia, Bogotá, Colombia, September 4-6/06

SB-2 Personnel. Molconnet, UNAL COLCIENCIAS. Sept, 4-6,06

Beltran, Jesus. Attended the XLVI Annual Meeting American Phytopathological Society Caribbean Division, XXVII Asociación Colombiana de fitopatología Annual meeting and III Universidad Militar Nueva Granada Phytopathology International Workshop. Cartagena, Colombia, 12 to 16 of September/2006

Gallego, G. Universidad de La Amazonía. Florencia – Caquetá. Apoyo institucional al fortalecimiento del plan de biología en técnicas moleculares y cultivo in vitro, mediante la formulación del proyecto: "Estudio de diversidad genética y conservación *ex situ* de árboles de importancia maderable para la amazonia mediante propagación *in vitro"* Septiembre12-13 de 2006

Gallego, G. COLCIENCIAS-Bogotá. Meeting to discuss the implementation Genomic platform. Establishing of the investigation centre of genomic and bioinformatic excelence. Septiembre 29 de 2006

Gallego, G. Cenicafé. Iniciativa para el Estudio del Genoma del Café, de la Broca y de su Agente Controlador *Beauveria bassiana.* September, 06/ Dec, 06

Garcia, Alexander, Bioinformatic National Network (NBN), SouthAfrica. August – October,06

Duque Myriam Cristina. Visited Valenciano Investigation's Institute. Dr. Emilio Carbonell. Oct. 24-26,06

Duque, MC. 1. GCP-LD workshop. (GCP: Generation Challenge Program- subprogram in Capacity Building LD: Linkage Desequilibrium)
Instituto Agronómico mediterráneo de Zaragoza, IAMZ, España, October 16-20 2006

Beltran, Jesus. Agrobio Award. Bogotá. November 9,06

Escobar, Roosevelt, Attend the Proceedings of the ASARECA/ECABIO Tissue Culture Workshop, 29 November-1st December 2006, Bujumbura, Burundi.

Garcia, Alexander, Attend Phenotype Representation Workshop in San Francisco, USA. Dec 1-2,06

Duque, MC. CIAT Internal Course. Todo lo que usted quería saber sobre mapeo genético y detección de QTLs y no se atrevía a preguntar. CIAT, Palmira, Diciembre de 2006

Gallego, Gerardo. Attend "Biotic and Abiotic Stress Responses in Plants". New Delhi. India. Dec. 19-06

Gómez, Marcela. Universidad del Tolima. Training in biochemical and molecular markers of *Phaseolus* ssp. Sept 2005-March 2006.

Trujillo, Iselen. Training in seed conservation and management. Universidad Nacional Experimental Simón Rodríguez, Venezuela. 11-15 September 2006.

Jiménez, Pablo Edgar . Training in conservation and management of *in vitro* cassava germplasm. CORPOICA, Tibaitatá, Colombia. 27-31 March 2006.

Triana, Alba Lucía. Training in conservation and management of *in vitro* cassava germplasm. CORPOICA, Tibaitatá, Colombia. 27-31 March 2006.

Gordillo, Erika Lucia. Training in conservation and management of *in vitro* cassava germplasm. Universidad de la Amazonía, Colombia. 24-28 July 2006.

Marchant, Alejandro. Training in conservation and management of *in vitro* cassava germplasm.Universidad Adventista de Chile. 24-28 July 2006.

Trujillo, Iselen. Training in conservation and management of *in vitro* cassava germplasm. Universidad Nacional Experimental Simón Rodríguez, Venezuela. 11-15 September 2006.

Wilfredo Pantoja. Training in the SDS-PAGE and 2D-IEF-SDS-PAGE techniques for phaseolin. CIAT, Bean Project. Novembre 2005-January 2006.

Prof. Erika Lucia Gordillo (Universidad de la Amazonia, Colombia) and Prof. Alejandro Marchant (Universidad Adventista de Chile). Training in biochemical and molecular markers. 27-28 July 2006.

Profesora Iselen Trujillo. Universidad Nacional Experimental Simón Rodríguez, Venezuela. Training in biochemical and molecular markers. 27-28 July 2006. 15 September 2006.

Ing. Walter Barrantes. University of Costa Rica, San José, Costa Rica. Training in molecular markers of *Phaseolus vulgaris* L. for the assessment of genetic diversity, gene flow events, and in seed conservation. 17 April- 16 June, 2006.

Sekou Salla Boré. Coordinateur du Project. DCC. CRS-Mopti. March,06

Dogola Coulibaly, Adjoint chef antenne, SSN-Mopti. March, 06

Bamba Abderahamane. Suivi Evaluateur. CRS-Mali

Chéry Traoré. Assistant Coordinateur DCC. CRS-Mali

Soungalo Traoré. Liaison – Recherche- Vulgarisat. DRA- Mali

Cheik O. Keita. Chercheur. CRRA-Mopti

Mohamed Ag Hamada. Chef axe biodiversité/semences. USC-SOS Douentza

M'Pé Ballo. Chef antenne. ONG Afrique Verte

Alassane Maiga. Chercheur. IER/URC-Bamako

Fassory Sangaré. Assistant chercheur. IER/ECOFIL

Moussa Kanouté. Technicien de recherché. ICRISAT

Baba Sanogo. Technicien de statistique. DRPSIAP-Mopti

Hamidou Guindo. Chargé de suivi des parcelles. USC/SOS-Douentza

Boureima Konta. Chef S/Secteur. Service Agri-Douentza

Seydou Sanogo. Chef Antenne Douentza. Service Local Prod.Ind. Anim. Douentza

Abdoulaye Traoré. Chargé de intensification veg. PSSA/Commisariat securité alimentaire

Margret Loeffen. APO socio-economist. ICRISAT - Bamako.

Janine Scott Shines. Chef de bureau. CRS-Bamako

Dembelé Alassane. Chef Antenne. Pastorale sociale du Diocése-Mopti

Sperling, L. Senior Scientist. ICRISAT.

Eva Weltzien. Principal Scientist. ICRISAT.

Moussa B. Sangaré. Chef programme. CRS/Mali

Training of Ms Ana M. Castro, Ms Amparo Rosero masters students intern of the National University of Colombia and Ms Eliana Macea intern of the Valle University, Colombia in cassava embryo rescue.

Training of Mr. Wilson Castelablanco PHD student at the Uppsala University, Sweden.

Training of Mr. Godwin Amenorpe from IAEA of Ghana in cassava rapid multiplication and embryo rescue.

Training of Ms Greisy Villafañe, Ms Ana L. Guevara, Mr. Sergio Ramirez and Mr. Cesar Aguirre from CASD, Colombia for the program ¨Biotechnology in the Classroom¨ in embryo rescue.

Training of Ms Wen Li Zhu from CATAS of China.

Improvement of the nutritional value of cassava: high storage protein content and zero cyanide cassava., DANIDA, US$120,000 for 3 years

A dataset on allele diversity at orthologous candidate genes in GCP crops (ADOC), GCP Commissioned grants, US$30,000 for 2 years

Development of Genomics Resources for Molecular Breeding of Drought Tolerance in Cassava. GCP, US$20,000 for 2 years.

Development of a feasibility study for the establishment of cassava plantations for the production of biofuels. NNPC, Nigeria. US$142,000 for 5 months

### 3.4.3 Courses and Training offered by SB-2 Staff in CIAT

Preparation of Final Biosafety Project GEF/WB, Feb 3.11,06

Biosafety in Centers of Biodiversity: Brazil, Colombia, Costa Rica, Mexico, and Peru. February 3, 2006.

Detección de Genes en Plantas Transgénicas, Taller Teórico-Práctico para el Sector Salud y Alimentos, CIAT, Palmira – Marzo 6 y 7 del 2006 – Sala Calima

Casa Abierta CIAT Mayo 2006

Casa Abierta CIAT October 2006

AgroSalud – Combating Hidden Hunger in Latin America: Biofortified Crops with Improved Vitamin A, Essential Minerals and Quality Protein, 21-23, March

HarvestPlus Meeting, 27-28, March ,06

Course/Workshop on Environmental Biosafety: Evaluation, Management and Monitoring of OGM, 26-28 March, 06

AgroSalud. March, 06

Final Project Review, Project Worldplan and operational Manual Regional Biosafety Project GEF/WB, 23-26, March, 06

Course Biosafety – MinAmbiente. CIAT April, 06

Practical training to PBA and Corpoica Technicians on RITA system. CIAT, Palmira. May 3,06

Adaptation and regional standardization of methodology for large scale monitoring of gene flow. May 24. 2006

Biotecnologia en el Salon de Clase. CIAT, May 26,06

Open House, May – October, 06 (5 universities – 2 institutes)

Biomoby – Biocase. CIAT, August 14-17,06

Training to IIAM (Mozambican Agriculture Institute) staff on Tissue culture. CIAT, Southafrica – Mozambique. Sept 17-18,06

HarvestPlus Eighth Project Advisory Committee (PAC) Meeting, 30,31 October, 06

Small-scale farmers interactions through low-cost tissue culture techniques. CIAT, Palmira, Oct 18-21, 06

AgroSalud Impact Meeting, 2-3 November, 06

Biopython Course, November 15-Dec 1, 06

Intercambio de experiencias – agricultura y Nutrición. CIAT Dic 5-6,06 IVIC - CIAT

Appraisal – World Bank Mission
Project Latin America: Multi-Country Capacity-Building in Biosafety, 11-14 December,06

Latin America: Multi-Country Capacity Building in Biosafety. Where do we stand?. Thematic Areas, Organizational Set up, and Implementation Arrangements. December 11, 2006

### 3.4.4 Visiting researchers

Amela, Francisco. MSc. Mozambique Natoinal Rice Program. 2006

Child Lydia. BSc. Cornell University. 2006

Ferreira, Oscar. Honduras. January, 06

Famoso, Adam, Cornell University. February,06

Lopez Camilo. Nacional University, Bogotá. February 10-17 , 06

Flanagan, Nicola Sian, University College Cork. Feb, 2006 – Febr,08

Victor Choque (March-April, 2006) – Univ. Autonoma Gabriel Rene Moreno – Santa Cruz, Bolivia - evaluation of Harvest Plus nurseries

Blanco, Andrés Eloy. UCV, Venezuela. March – April,06

Beovides Yoel. INIVIT. Cuba, Dec,05-March,06

Rosini Claudia. University of the Witwatersrand. May,06

Claudia Rosin from the School of Molecular and Cell Biology, University of Witwatersrand, Johannesburg, South Africa. Training in Genetic Transformation of Cassava. May 2-31/2006

Bouniol, Matheu Rene. May 2006-2007

Deguchi, Michihito. 2006

Benitez, Nicolás, CORPOICA. May 29-31,06

Bernal, Geraldine. IVIC, Venezuela.May – June,06

O'toole John. Consulting Gates Foundation. May 22-26,06

Molina, Yovanny de Jesus. CORPOICA, Santa Marta. May 29-31,06

Rossin, Claudia Beatrice. University of the Witwatersrand, SouthAfrica. May –June,06

Pieters Alejandro. IVIC, Venezuela. May – June, 06

Echeverry Morgan, Mayaguez University, Costa Rica. Jun-Aug,06

Rodriguez, Martin Emilio. Nacional University of Colombia. July-Dec,06

Del Villar Jorge Luis . IDIAF-Dominican Republic. July,06

Roca, Valentina. Universidad Libre. August,06

Luz Nayibe Garzon (August 2006) – Univ. de Cundinamarca, Fusagasuga – data analysis for Co-$4^2$ marker assisted selection and inheritance.

Lydia Mae Childs (since August 2006) – Cornell University Presidential Scholar – Ithaca New York – analysis of Phytates in raw and cooked rice and bean samples.

Sanger, Martin. European Bioinformatics Institute, August, 06

Carmenza Muñoz (August - October 2006) – University of Sasakatchewan – genetic mapping of tepary bean microsatellites.

Navas Muñoz, Jenny. Universidad Libre, August, 06

Duque Germán, Alexis. Universidad Libre. August, 06

Gonzalez, Pedro José. Universidad San Francisco de Quito, Ecuador. Sept,06

Teresa Avila (October 2006) – Centro Fitogenético Pairumani / Univ. San Simon – Cochabamba, Bolivia– evaluation of genetic diversity in Bolivian accessions of common bean with M. Blair.

Verdier, Valerie. IRD, France. November 27, 06

Gonzalez, Pedro Jose. San Francisco Quito University. Sept-Nov, 06

Soto, Mauricio. IRD, France. November 28- December 26,06

Yoshiaki Inukai. University of Nagoya, Japon. December,06

Garcia, Dora Yaneth. Technology University of Palmira. Dec, 06

Gonzalez Carolina . IRD, France. December 4-31, 06

Mauricio Suarez, IRD. France. December 4-31, 06

Michihito Deguchi. PhD. Tohoku University. Biochemistry. Jan,06 – Dec. 06

Suzuki Motofumi. PhD. The University of Tokyo. Jan- March, 06

### 3.4.5 Student/Thesis

Acuña Tiffany Grace, Universidad del Valle, Transformación genética de zanahoria.

Adeyemo Sarah, University of Cologne and Max Planck Institute for Plant Breeding, Cologne, Germany (expected finish date October 2008)

Arcia Moreno Kiliany Andrea. M.Sc. Tesis: Caracterización de especies silvestres del género *Oryza* colectadas en condiciones naturales y campos de arroz en el Estado Portuguesa, Venezuela. Tesis en Curso. Universidad Nacional, Sede Palmira.

Bengtsson Frida. MSc. Nov. 2006. Norway University of Life Sciences. The wider context of seed vouchers and fairs.

Blanco Andrés Eloy. M.Sc. Tesis: Caracterización de especies silvestres del género *Oryza* colectadas en condiciones naturales y campos de arroz en el Estado Guárico, Venezuela. Tesis en Curso. Programa sándwich Universidad Nacional, Sede Palmira/ Universidad Central de Venezuela, Maracay, Venezuela..

Bohorquez, Adriana. PhD. Candidate. Isolation of expressed sequences during the defense of Mecu72 to white fly attack using subtractive libraries and microarrays expression of cDNA. National University of Palmira. 2005-2006

BSc, MSc or PhD candidates supervised in 2006Yamid Sanabria (Universidad del Tolima), Olga Ximena Giraldo, Francisco Amela (Mozambique National Rice Program). Lydia Child from Cornell University (under-graduate) did some work on rice phytates: Supervisor: César Martínez

Bunmi Olasanmi, University of Ibadan and NRCRI, Umudike, Nigeria (Expected finish date January 2009)

Castro Anna Maria. M.Sc. Universidad de Valle, Cali, Colombia (expected finish date May 2007)

Cuambe Constantino. M.Sc. Universidad de Valle, Cali, Colombia (expected finish date October 2007)

Chavarro Carolina. Univ. Javeriana – Bogotá (graduated 2006) – studied heat tolerance in Andean common beans with M. Blair

Córdoba Adriana, Universidad del Cauca, Factibilidad del uso de la transformación genética como herramienta para introducir resistencia a la mosca blanca en la yuca (*Manihot esculenta* CRANTZ) en el Depto. del Cauca

Echeverry Morgan master student at the Puerto Rico University in cassava rapid multiplication and others aspects in tissue culture.

Hernandez Erick Giovanni B.Sc. Tesis: Caracterización genotípica y fenotípica de accesiones de arroz rojo *(Oryza sativa f. spontanea*) procedentes de los Departamentos de Tolima y Valle del Cauca. Universidad Francisco de Paula Santander Facultad de Ciencias Agrarias y del Ambiente Plan de Estudios de Ingeniería Producción Biotecnológica Cúcuta. Noviembre 2005. Tesis Meritoria.

Giraldo Olga Ximena. Current Student, MSc. Nacional University of Palmira, Colombia

Godwin Amenorpe. Ghana Atomic Commission sponsored by International Atomic Energy Agency, training on development of doubled haploid technology for cassava

Gonzalez Laura. Univ. Javeriana – Bogotá (since August 2006) – nutritional quality and diversity of Rwandan bean collection
Gonzalez Pedro José. Universidad Católica de Quito, training on molecular analysis of rice and gene flow.

Gomez Marcela. Univ. de Tolima – Ibague (since January 2006) – application of TILLING enzymes.

Gonzalez Laura – Univ. Javeriana – Bogotá (since August 2006) – nutritional quality and diversity of Rwandan bean collection

Gutierrez, Andrés. 2006. Biologist. Universidad del Tolima. Construcción y evaluación de líneas de introgression interespecífica de arroz (Oryza sativa L.) utilizando marcadores moleculares microsatélites.

Lopez Hernan – Univ. Antioquia – Medellin, Colombia (since January 2006) – inheritance of aluminum toxicity stress tolerance with M. Blair

Macia Eliana (Colombia) Universidad de Valle, Cali, Colombia (expected graduation May 2007)

Maldonado, Bibiana A, expresión del gen ccd1 y cuantificación de β-ionona en hojas y raíces de yuca de diferentes edades

Medina Juliana. Univ. Antioquia – Medellin, Colombia (since January 2006) – inheritance of nutritional quality in Andean beans with M. Blair

Medina Juliana. Univ. Antioquia – Medellin, Colombia (since January 2006) – inheritance of nutritional quality in Andean beans with M. Blair

Melgarejo, Laura Marcela. Universidad de los Andes. Desarrollar líneas de introgression avanzadas de arroz, utilizando una técnica de cruzamiento asistido por marcadores moleculares de tipo microsatélites.

Martínez, C.A. Evaluation of gene flow among populations of cultivated and wild Lima beans (*Phaseolus lunatus*) belonging to the Andean genepool. Thesis for biologist degree. Universidad del Tolima, Ibague, Colombia, January-December 2006.

Morales Mabel. B.Sc. Identificación y Caracterización de especies de los complejos *Oryza sativa* y *Oryza officinalis.* Tesis en Curso. Universidad Javeriana, Bogotá, Colombia.

Moreno Natalia. Univ. Javeriana – Bogotá (since January 2006) – nutritional quality and diversity of Eastern and Southern African common bean varieties

Moreno Natalia. Univ. Javeriana – Bogotá (since January 2006) – nutritional quality and diversity of Eastern and Southern African common bean varieties

Nuñez, Jonathan, Universidad del Valle. Obtención de Librerías de cDNA y clonaje del gen psy de yuca.

Moreno, Laura Tatiana. Universidad de los Andes. Seleccion y Mapeo de Marcadores Microsatélites SSR a partir de un cruce interespecífico entre *Oryza sativa* y *Oryza meridionalis,*. Jan,07

Moreta, Danilo Eduardo. MSc. INIAP. Isolation and identification of full-length cDNA clones from transcripts differentially expressed in cassava (Manihot esculenta Crantz).

Murillo Anna Cruz (Colombia) PhD. Universidad Nacional de Colombia (expected finish date October 2007)

Ojulong Henry (Uganda) Ph.D. student, University of the Free State, Bloemfontein, South Africa (Graduated February 2007 )

Okai Elizabeth (Ghana) Ph.D. student, University of the Free State, Bloemfontein, South Africa (expected finish date February 2008)

Olalekan Akinbo (Nigeria) Ph.D. student University of the Free State, Bloemfontein, South Africa (expected finish date February 2008)

Orjuela, Julie Alexandra. 2006. BSc. Biologists. Tolima University. First approximation to build a Universal Core Genetic Map of Rice (Oryza Sativa L.) using microsatellites markers.

Osmond Ndomba, University of Witswaterstrand, Johannesburg, South Africa (Expected Finish date January 2009)

Oviedo Tatiana, Intern (Colombia) Universidad de Tolima, Ibague, Colombia (Expected graduation date May 2007)

Padilla, Jonathan Mauricio, Univesridad del Quindío, Pasantía en cultivo de tejidos de yucca y frijol. Enero a Junio de 2006

Parra, Londoño Sebastian. Universidad del Valle. Obtención de Librerías de cDNA y clonaje del gen psy en batata.

Plata Germán. Feb. 2006. BSc. Biologist. Universidad Nacional de Colombia. Honor Grade. Theses: Polimorphism detection and development of molecular markers for angular leaf spot resistance gene candidates in cluster RGC7 of common bean.

Roda Federico. 2006. BSc. Biologist. Universidad Nacional de Colombia, Bogotá. "Isolation of Cysteine Protease Sequences Expressed Under Brachiaria Spittlebug Interaction".

Rodriguez Martin Emilio, MSc candidate. Universidad Nal de Colombia, 2006

Sanabria Yamid. 2006. BSc. Biologist. Thesis Title: Morphological, citogenetical and molecular characterization of an Oryza accesion and evaluation of introgressions on lineages F1, BC2 and BC3, arising from crosses with *Oryza sativa* L. Universidad del Tolima.

Sandoval Tito. Univ. Del Valle – Cali, Colombia (graduated 2006) studying phytate accumulation in common bean with M. Blair – the thesis was judged meritorious

Sakai, Tomoko. MSc. Candidate. Nacional University of Palmira. 2006

Suárez Barón, H.G. 2006. Evaluation of gene flow in wild –weedy- crop complexes in *Phaseolus lunatus* L., of the Mesoamerican gene pool, with help of morpho agronomic, biochemical and molecular markers. Thesis for biologist degree. Universidad del Quindio, Armenia, Colombia, January-December 2006.

Tsegaye Bayush. PhD. Norway Univerity of Life Sciences. Incentives for on-farm conservation in a centre of diversity: a case study of durum what (*Triticum turgidum* L.) landraces from East Shewa, central Ethiopia.

Vargas, Jaime. Dec, 2005. MSc. Title: Identification of molecular markers associated to Aleurotrachelus socialis BONDAR resistance genes in Cassava Manihot esculenta CRANTZ using AFLP, SSR, SSR-EST y RGA. Universidad Nacional de Colombia
Facultad de Ciencias Agropecuarias de Palmira.

Velásquez Alicia Milena. B.Sc. Rastreo de flujo de genes en campos comerciales de arroz utilizando marcadores moleculares y resistencia a herbicidas. Tesis en Curso. Universidad Javeriana, Bogotá, Colombia.

West Jennifer Joy. MSc. Nov. 2006. Norway Univerity of Life Sciences.

Zhu Wenli. CATAS. South China University for Tropical Agriculture, China), training on development of doubled haploid technology for cassava

# Activity 3.5 Publications

### 3.5.1 In Referred Journals


Andersson, M.S.; Peters, M.; Schultze-Kraft, R.; Gallego, G.J.; Duque E. M.C. 2006. Molecular characterization of a collection of the tropical multipurpose shrub legume flemingia macryphylla. Agroforestry Systems 144:237-248

Arce,K., Bonilla C.R., Sánchez M. S., and R. H. Escobar. 2006. Morfoanatomía y respuesta fisiológica de las semillas de chambimbe (*Sapindus saponaria var. drummondii (hook. & arn.) l. Benson* a condiciones de crioconservación.. Universidad Nacional de Colombia-Palmira.

Beebe, Stephen, Marcela, Rojas, Yan Xialong; Blair, M. Fabio Pedraza, Fernando Arboleda, Tohme, Joe, Jonathan Lynch. Quantitative trait loci for root architecture traits correlated with phosphorus acquisition in common bean. Crop Science 46 (1): 413-423

Beebe, S., M. Rojas-Pierce, X. Yan, M.W. Blair, F. Pedraza, F. Muñoz, J. Tohme, and J.P. Lynch. 2006. Quantitative trait loci for root architecture traits correlated with phosphorus acquisition in common bean. Crop Sci. 46:413-423.

Beebe SE, Rojas M, Yan X, Blair MW*, Pedraza F, Muñoz F, Tohme J, Lynch JP (2006) Quantitative trait loci for root architecture traits correlated with phosphorus acquisition in Common Bean. Crop Science 46: 413-423.

Blair MW*, Astudillo C, Restrepo J, Bravo LC, Villada D, Beebe SE (2005) Analisis multi-locacional de líneas de fríjol arbustivo con alto contenido de hierro en el departamento de Nariño. Fitotecnia Colombiana 5: 20-27.

Blair MW*, Giraldo MC, Buendia HF, Tovar E, Duque MC, Beebe SE (2006) Microsatellite marker diversity in common bean (*Phaseolus vulgaris* L.) Theor Appl Genet 113: 100-109.

Blair MW*, Iriarte G, Beebe S (2006) QTL analysis of yield traits in an advanced backcross population derived from a cultivated Andean x wild common bean (*Phaseolus vulgaris* L.) cross. Theor Appl Genet 112: 1149–1163.

Blair MW*, Muñoz C, Garza R, Cardona C (2006) Molecular mapping of genes for resistance to the bean pod weevil (*Apion godmani* Wagner) in common bean. Theor Appl Genet 112: 913–923.

Blair MW, Nin JC, Prophete E, Singh SP, Beaver JS (2006) Registration of Two Bean Golden Yellow Mosaic Virus Resistant, Large Red-Mottled Common Bean Germplasm. Crop Sci 46: 1000-1001.

Blair MW*, Rodriguez LM, Pedraza F, Morales F, Beebe SE. (in press) Genetic mapping of the bean golden yellow mosaic geminivirus resistance gene *bgm*-1 and linkage with potyvirus resistance in common bean (*Phaseolus vulgaris* L.) Theor Appl Genet.

Castelblanco W. and Fregene M. (2006). SSCP-SNP based markers as conserved orthologous set (COS) markers for comparative genomics in cassava (*Manihot esculenta* Crantz). Plant Molecular Biology Reporter 24:229-236

Ceballos, H. Teresa Sánchez, Chavez, AL; Iglesias, C. Debouck, D. Mafla, G. Tohme, J. variation in crude protein content in cassava (*Manihot esculenta* Crantz) roots journal of Food Composition and Analysis 19:589-593

Ceballos*, H., M. Fregene, Z. Lentini, T. Sánchez, Y.I. Puentes, J.C. Pérez, A. Rosero and A.P. Tofiño. 2006. Development and Identification of High-Value Cassava Clones. Acta Horticulturae 703:63-70. Xiong L and Ishitani M. (2006) Stress signal transduction: components, pathways and networking integration. Rai AK & Takabe T (eds), Abiotic Stress Tolerance in Plans, 3-29. Springer.

Ceballos, H., T. Sánchez, A.L. Chávez, C. Iglesias, D.G. Debouck, G. Mafla & J. Tohme. 2006. Variation in crude protein content in cassava (*Manihot esculenta* Crantz) roots. Journal of Food Composition and Analysis 19: 589-593

Ceballos, H., Fregene, M., Lentini, Z.J., Sanchez, T., Puentes, Y.I., Perez, J.C., Rosero, A., & Tofiño, A.P.( 2006). Development and Identification of High-Value Cassava Clones. Acta Hort. (ISHS) 703:63-70.

Checa O, Ceballos H, Blair MW* (2006) Inheritance of climbing ability in common bean (*Phaseolus vulgaris* L.). J of Heredity 97: 56-465

Díaz LM, Blair MW (2005) Race structure within the Mesoamerican gene pool of common bean (*Phaseolus vulgaris* L.) as determined by microsatellite markers. Theor Appl Genet 114: 143-54.

Díaz LM, Díaz JM, Blair MW* (2005) Diversidad genética de fríjol común (*Phaseolus vulgaris* L.) en Colombia. Fitotecnia Colombiana 5: 28-36

Dwivedi SL, Blair MW, Upadhyaya HD, Serraj R, Balaji J, Buhariwalla HK, Ortiz R, Crouch, JH. (2006) Using Genomics to exploit grain legume biodiversity in plant breeding. In Jules Janick (ed) Plant Breeding Reviews, Volume 26 394 pp

Dwivedi SL, Stalker HT, Blair MW, Bertioli D, Upadhyaya HD, Nielen S, Ortiz R (in press) Enhancing crop gene pools of cereals and legumes with beneficial traits using wild relatives. Plant Breeding Reviews (in press)

Escobar, Roosevelt, Carlos Hernández, Larrahondo, N.; Gloria Ospina, Restrepo, M., Muñoz Liliana, Tohme. J., Roca, W. 2006. Tissue Culture for farmers: Participatory adaptation of low-input cassava propagation in Colombia. Experimental Agriculture 42:103-120

Flórez-Ramos C.P., Z. Lentini***,** M.E. Buitrago, and J. Cock. 2006. Somatic Embryogenesis and Plantlet Regeneration of Mango (*Mangifera indica* L.). *Acta Horticulturae* (In Press)

Fregene M. Morante N., Sanchez T., Marin J., Ospina C., Barrera E., Gutierrez J., Guerrero J., Bellotti A., Santos L., Alzate A., Moreno S., and Ceballos H (2006). Molecular Markers for the Introgression of Useful Traits from Wild *Manihot* Relatives of Cassava; Marker-Assisted Selection of Disease and Root Quality Traits. Journal of Root Crops, Vol 32, No.1, pp 1-31

Gómez, OJ, Blair, MW, Frankow-Lindberg, BE, Gullberg U (2005) Comparative study of common bean (*Phaseolus vulgaris* L.) landraces conserved *ex situ* in genebanks and *in situ* by farmers. Genetic Resources and Crop Evolution 52: 371-380

Gonzalez, C.; Szurek, B.; Duque, E.; M.C. Manceau, C.; Mathieu, T.; Yacouba, S.; Verdier, V. 2006. Molecular and pathogenic characterization highlight substancial differences between Asian and Africa strains of Xanthomonas oryzae pathovars (abstract). Phyopathology 96 (6): S41

Iriarte G, Blair MW*, Hoyos A, Beebe S (2006) Evaluación agronomica de una retrocruza avanzada entre una accesion silvestre colombiana y la variedad cultivada de frijol común, ICA Cerinza. Fitotecnia Colombiana 6: 24-32.

Kizito E., Chiwona-Karltun L., Egwang T., Fregene M., Westerberg A. (2006). Genetic diversity and variety composition of cassava on small scale farms in Uganda: an inter- disciplinary study using genetic markers and farmer interviews. Genetica. Published online September 2006.

Miklas PN, Kelly JD, Beebe SE, Blair MW (2006) Common bean breeding for resistance against biotic and abiotic stresses: from classical to MAS breeding. Invited review paper for special issue on 'Legume resistance breeding' Euphytica: 147:105-131.

Muñoz C, Duque MC, Debouck D, Blair MW* (2006) Taxonomy of tepary bean (*Phaseolus acutifolius*) and wild relatives as determined by amplified fragment length polymorphism (AFLP) markers. Crop Science 46:1744–1754

Muñoz, L.C., M.C. Duque, D.G. Debouck & M.W. Blair. 2006. Taxonomy of tepary bean and wild relatives as determined by amplified fragment length polymorphism (AFLP) markers. Crop Science 46 (4): 1744-1754.

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Ochoa IE, Blair MW, Lynch JP. (2006) QTL analysis of adventitious root formation in common bean (*Phaseolus vulgaris* L.) under contrasting phosphorus availability. Crop Science 46: 1609–1621

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"Improving common bean productivity for marginal environments in sub-Saharan Africa" International Workshop on Genomics-Enabled Improvement of Legumes – Asilomar Conference Center, USA Aug 28-Sept 1, 2006

"Mejoramiento de la nutrición humana en comunidades pobres de América Latina utilizando maíz(QPM) y fríjol común biofortificado con micronutrientes" Proyecto FTG-05/03 – Matthew Blair – IICA/Fontagro assessment meeting – Bogotá, Colombia – July 26, 2006.

"*Phaseolus* / common bean genotyping: SP1 - Generation Challenge Program" – Matthew Blair - Annual General Meeting Generation Challenge Program, Sao Paulo, Brazil - Sept 13, 2006

"Race structure and inter gene pool diversity in common bean: the case for introgression" Institute for Genomic Diversity, Cornell University – October 27, 2006

"Selección de gametos para el mejoramiento de la resistencia a enfermedades en fríjol voluble autóctono de la región Alto Andina" Proyecto FTG-9/99 – Matthew Blair – IICA/Fontagro assessment meeting – Bogotá, Colombia – July 26, 2006.

"TILLING mutagenesis for common bean (*Phaseolus vulgaris* L.): tropical legume mutant resource" - Matthew Blair - Annual General Meeting Generation Challenge Program, Sao Paulo, Brazil - Sept 13, 2006

"Improvement of Climbing Beans: Resistance to Biotic Stresses, Tolerance to Abiotic Stresses and Nutritional Quality" VLIR workshop - November 22-25, 2006

"Improving common bean productivity for marginal environments in sub-Saharan Africa" International Workshop on Genomics-Enabled Improvement of Legumes – Asilomar Conference Center, USA Aug 28-Sept 1, 2006

"Mejoramiento de la nutrición humana en comunidades pobres de América Latina utilizando maíz(QPM) y fríjol común biofortificado con micronutrientes" Proyecto FTG-05/03 – Matthew Blair – IICA/Fontagro assessment meeting – Bogotá, Colombia – July 26, 2006.

Identificación de QTL para resistencia a sequía en líneas recombinantes (RILs) de la cruza MD 23-24 x SEA 5. S. Beebe, I. M. Rao, M. Blair, E. Tovar, M. Á. Grajales, and C. Cajiao. Paper presented at the LII meeting of the PCCMCA, Managua, Nicaragua. April, 2006.

### 3.5.5 Posters

Blair, M.W, L.M. Rodriguez, L. Galindo, M. Ishitani, S.E. Beebe, I.M. Rao "Characterization of DREB genes as drought tolerance candidates in Common Beans (*Phaseolus vulgaris* L.)" – Annual General Meeting Generation Challenge Program, Sao Paulo, Brazil 2006

Blair, M.W., C. Muñoz, R. Garza, C. Cardona "Molecular mapping of genes for resistance to the bean pod weevil (*Apion godmani* Wagner) in common bean" – CIAT board meeting, Cali, Colombia 2006

Blair, A. Frei, ir, C. Cardona, S.E. Beebe, H. Gu and S. Dorn "QTL Mapping of Resistance to *Thrips palmi* Karny in Common Bean (*Phaseolus vulgaris* L.) – CIAT board meeting, Cali, Colombia 2006

Blair M.W. , C. Galeano, C. Muñoz, C. Pankhurst, P. Lariquet, W.M. Broughton "TILLING Mutagenesis for Common Beans (*Phaseolus vulgaris* L.): A Tropical Legume Mutant Resource" – Annual General Meeting Generation Challenge Program, Sao Paulo, Brazil 2006

Brian M. Waters, Chee-Ming Li, Matthew W. Blair, Steve Beebe, and Michael A. Grusak "Influence of Rhizosphere pH on Whole-Root Ferric Reductase Activity in Diverse Accessions of *Phaseolus vulgaris*" – ISINIP Conference 2006

Diaz L.M. and M.W. Blair "Race structure within the Mesoamerican gene pool of common bean (*Phaseolus vulgaris* L.) determined by microsatellite markers." – Annual General Meeting Generation Challenge Program, Sao Paulo, Brazil 2006

Flórez-Ramos, CP; Lentini, Z*; Buitrago, ME; and Cock, J. 2006. Somatic embryogeneis and plant regeneration in mango. (*Mangifera indica* L.) First Congreso of the Colombian Society of Horticultura Science, SCCH. Bogotá, Colombia. p 77.

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# Activity 3.6 Projects

## 3.6.1 List of proposals active 2006

- A dataset on allele diversity at orthologous candidate genes in GCP crops (ADOC), GCP Commissioned grants.
- An Integrated Approach for genetic improvement of aluminium resistance of crops on low – fertility acid soils.
- Aplicacion de Microarreglos al studio de la interacción Café –Broca metabolic engineering of cyanogens metabolism in Transgenic cassava: Generation of safer more marketable cassava Food products for subsistence farmers. Ohio State University –OSU
- Bean genomics for improved drought tolerance in Latin America. BMZ-Germany. No-cost extension to Aug, 2007- Yearly contracts 2003-2006.
- Biofortified Crops for Improved Human Nutrition – Harvest Plus Challenge Program. World Bank DANIDA, Denmark Gates Foundation CIDA (AgroSalud). Developed with CIMMYT and CIP.
- Beta carotene Enhanced Mustard. USAID.
- Biotechnology RCSA. USAID.
- BMZ-Germany - "Bean genomics for improved drought tolerance in Latin America",
- Cassava Biotechnology Network. Ministry of Foreign Affairs Netherlands.
- Coffe Genome Studies. . FEDERECAFE.
- Colciencias – "Obtención de nuevas variedades de fríjol común con atributos de rendimiento y potencial para nuevos mercados, utilizando selección convencional y asistida por marcadores moleculares" Universidad Nacional with CIAT
- Cenicafe. Technical assistance to the Coffee Genome funded by MADR.
- Crop gene expression profiles and stress-gene arrays
  Donor: Generation Challenge Program; Project number SP2-2005-013
- Development and use of inbred lines in cassava breeding. Donor: The Rockefeller Foundation
- Development of Genomics Resources for Molecular Breeding of Drought Tolerance in Cassava. GCP.
- Development of a feasibility study for the establishment of cassava plantations for the production of biofuels. NNPC, Nigeria.
- Development of an *In Vitro* Protocol for the Production of Cassava Doubled-Haploids and its Use in Breeding. CIAT – ETH (Switzerland) - SCIB (China). Donor: ZIL, Switzerland.
- Development and evaluation of drought-tolerant rice transgenic plants. GCP SB3
- Ginés Mera Fellowship Fund por Postgraduate Studies in Biodiversity. IDRC
- EcoFondo – "Manejo del germoplasma local y aumento de la agrobidodiversidad de frijol y maiz con variedades biofortificadas para mejorar la nutricion en comunidades rurales del departamento de Nariño" – FIDAR with CIAT –
- Enhancing grain legumes' productivity, production and the incomes of poor farmers in drought-prone areas of sub-Saharan Africa and South Asia. Bill and Melinda Gates Foundation (BMGF).
- Exploring natural genetic variation: Developing genomic resources and introgression lines for four AA Genome Rice relatives. CIMMYT
- Evaluation and multiplication of 5000 lines de T-DNA mutants conservation and sustainable Use of Neotropical Native Crops and Wild Relative Crops

- Evaluation and Deployment of Transgenic Drought-Tolerant Varieties
  Donor: Generation Challenge Program; Project number SP3-19
- Fighting drought and aluminium toxicity: Integrating functional genomics, phenotypic screening and participatory research with women and small-scale farmers to develop stress-resistant common bean and Brachiaria for the tropics
  Donor: BMZ

- Fighting Drought and Aluminium Toxicity: Integrating Genomics, Phenotypic Screening and Participatory Research with Women and Small-Scale Farmers to Development Stress-Resistant Common Bean and Brachiaria for the Tropics. BMZ.
- Fontagro – "Mejoramiento de la nutricion humana en comunidades pobres de America Latina utilizando maiz (QPM) y frijol comun biofortificados con micronutrientes"
- Flowers, Fruits and Roots: Modification of Flowering to Improve Traits of Agricultural Importance. CIAT - Max Planck Institute, Germany. Donor: The Rockefeller Foundation.
- Generation Challenge Program - "TILLING mutagenesis and drought gene analysis"
- Gene Flow Analysis for Environmental safety in the Tropics. CIAT – University of Costa Rica – Hannover University and BBA, Germany.
- Harvest Plus Challenge Program – "Biofortified crops for human nutrition" Harvest Plust challenge program, various donors.
- High iron and zinc rice lines. AgroSalud. CIDA-Cananda US$230,000. Interspecific bridges to get full access to genetic diversity found in O. glaberrima: GCP
- Identification and Expression Analysis of Genes Important for Iron Translocation to the Rice Grain, HP.
- Impacto ambiental de la adopción del arroz resistente a las imidazolinoas en sistemas productivos contrastantes de América Latina (AL). INIA-UCV-CIAT. Donor: Fontagro.
- Improvement of the nutritional value of cassava: high storage protein content and zero cyanide cassava., DANIDA.
- Improving potato-bean-sweet potato (PBS) based rural livelihood systems through integrated soil ecosystem management (ISEM), market development and nutritional innovation in the highlands of Lake Kivu area. Sub-Saharan Africa Challenge Program. Project Rejected. 4 years.
- Introduction of inbreeding in cassava genetic improvement. Donor: The Rockefeller Foundation. Approved November 2006. January 2007-December 2009

- Japan Capacity Building Program for African Agriculture Researchers
  Donor: MAFF of Japan
- Latin America: Multi-country capacity-building for compliance with the Cartagena Protocol on biosafety (Brazil, Colombia, Costa Rica, Peru). Donor: GEF-World Bank
- Long-Term Seed Aid in Ethiopia. IDRC. Addresses chronic stress areas and extreme poverty.
- Lulo (*Solanum quitoense*, naranjilla) with added value: New alternatives for the small farmer. CIAT-CORPOICA.
- Mayze- Vit A Biofortification. USAID.
- Nutritional Genomics. USAID.
- Systematic evaluation of rice mutant collections for conditional phenotypes with emphasis on stress
  Donor: Generation Challenge Program

- Putting Seed Security at the Heart of Agricultural Relief and Recovery Response. USAID/OFDA. Focus on Seed System Security Assessment and Tool Development. January 2006-June 2007.
- Rice Functional Genomics consortium. Yale University
- Southern Africa Biotechnology Program.CGIAR.
- The Generation Challenge Program. IRD – CIAT
- Understanding the mechanism of Plant Resistante to Whitflies –USDA –
- Improving rural livelihoods in Rwanda: Promoting integrated crop, disease, and pest management (ICDPM) strategies for intensification and diversification of agricultural systems. Bilateral project for Belgium. Proposal approved pending to confirm. 3 Years.
- Tools for Cassava Breeding, Improvement, and Germplasm Exchange. Objective 5: Development of doubled Haploid and Induction of flowering.Donor: Bill and Melinda Gates Foundation.

### 3.6.2 List of Partners

Agrosalud network: Fedearroz-Colombia, IIA-Cuba, CIAT-Bolivia and Aspar, MAN-B(Mision Alianza Noruega), IDIAF- Dominican Republic, INTA-Nicaragua, CTA-Embrapa Brazil, IDIAP-Panama).
Centro Fitoecogenetico Pariumani – Bolivia – T. Avila
CIBIOGEM, UNAM, Mexico
CIMMYT
CIRAD– Génoplante
CGIAR Genebank Upgrading, phase 1, World Bank,
Clemson University Genomics Institute – J. Tomkins
CONAM, Peru
CORPOICA – G. Santana. M. Lobo
Cornell University
CNPAF-Embrapa
CTA-Embrapa
Chinese Academy of Agricultural Sciences – S. Wang
EMBRAPA-CNPAF
EMBRAPA, Brazil
ETH, Switzerland
Fedearroz
Federecafé
FIDAR – J. Restrepo
HP+ IRRI, Phil-Rice Philiipines,
ICA, Corpoica, Institute von Humboldt, Colombia
INIA – Lara – Venezuela – ME Morros
INIA – Peru – A. Valladolid, M. Gamarra
INIFAP-Mexico – R. Garza, J. Acosta – Ecuador – E. Peralta
IDRC
IRD
IRD/CIRAD
JIRCAS, Japan
LSU
PRI, University of Wageningen, The Netherlands
OSU
Purdue University – Scott Jackson

RiceTec,
Texas &AM Univ.,
TheGénoplante consortium
The French Ministry of Foreign Affairs
UAGRM – BOLIVIA- J. Ortubé
UCV, IVIC, INIAP, Venezuela
Universidad del Valle - A. Pradilla, C. de Plata, B. Gracían
Universidad Autonoma de México – G. Hernandez, M. Lara, F. Sanchéz
Universidad Nacional de Colombia – G. Ligareto
Universidad Rio Grande do Soul-Brazil,
University of Geneva – B. Broughton, P Lariquet
University of Costa Rica, Costa Rica
University of Arizona,
University of Hanover, BBA, University of Braunschweig, Germany
University of Yale
USDA – M. Grusak, T. Porch
WARDA

## Activity 3.7 Current SB-2 Investigators: Discipline, position and time fraction

| Name | Discipline | Time dedication% |
|---|---|---|
| Beebe Steve | Bean Breeding | 30 |
| Bellotti Anthony | Cassava Entomology | 20 |
| Blair Mathew | Bean Genetics and breeding | 70 |
| Ceballos Hernan | Cassava Breeding | 40 |
| Chavarriaga, Paul | Transgenesis, Cassava | 100 |
| Debouck Daniel | Botany | 20 |
| Fregene Martin | Cassava Genetics and breeding | 60 |
| Ishitani Manabu | Molecular Biologist | 100 |
| Lentini Zaida | Biology/Genetics | 80 |
| Lorieux Mathias | Rice Genetics and Biotechnology | 50 |
| Martínez César | Breeding | 49 |
| Mejía Alvaro | Cell Biology | 100 |
| Pfeiffer, Wolfgang | Coordinator HarvestPlus-LA | 100 |
| Sperling Louise | Seed Systems | 20 |
| Tohme Joe | Genomics, Project manager | 100 |

**Tissue Culture/Cryopreservation/Plant Transformation**

| | |
|---|---|
| Bolaños, Eugenio | Technician |
| Brito Marco | Technician |
| Buitrago Maria Eugenia | Research Assistant |
| Criollo Arturo | Technician |
| Dorado, Carlos | Technician |
| Escobar, Roosevelt | Research Assistant |
| Fory, Luisa | Rice Biotechnology Research Coordinator |
| Galindo, Leonardo F. | Research Assistant |
| González, Eliana | Research Assistant |
| Herrera, Pablo | Technician |
| Ladino, Yaneth Julieta | Research Assistant |
| Manrique, Norma | Research Assistant |
| Muñoz, Liliana | Research Assistant |
| López, Danilo | Research Assistant |
| Rios, Auradela | Technician |
| Ríos, Alexander | Technician |
| Rodriguez Fausto | Research Assistan |
| Tabares, Eddie | Research Assistant |

| | |
|---|---|
| Tigreros, Humberto | Technician |
| Vacca Orlando | Research Assistan |
| Villamizar Johnna Patricia | Research Assistant |

**Genome Diversity**

| | |
|---|---|
| Barrera, Edgar | Research Assistant |
| Bernal Diana | Research Assistant |
| Bohorquez, Adriana. | Research Assistant |
| Gallego,Gerardo. | Asociate – Laboratory Coordinator |
| Giraldo,Olga X. | Research Assistant |
| Gutiérrez, Janeth P. | Research Assistant |
| Londoño, Claudia | Technician |
| Plata, German | Research Assistant |
| Quintero, Constanza. | Research Assistant |
| Roda Federico | Research Assistant |
| Rodriguez Fausto | Research Assistant |
| Vargas, Jaime | Research Assistant |

**Plant-Stress interactions**

| | |
|---|---|
| Chaves Alba L. | Research Associate |
| Recio Maria Eugenia | Technician |
| Salcedo Andrés Felipe | Research Assistant |

**Administrative**

| | |
|---|---|
| Cruz, Olga L. | Administrative Assistant |
| Duque, Myriam C. | Statistical Consultant |
| Quiceno, Jorge | HarvestPlus – Administrative Assistant |
| Rojas Fernando | System Analist |
| Zuñiga, Claudia S. | Administrative Assistant |

**Institute A. von Humboldt**

| | |
|---|---|
| Barrios Dora Yovana | Visiting Research |
| Chavez Alejandro | Thesis Student |
| Estrada Oscar | Thesis Student |
| López Diana Carolina | Thesis Student |
| Palacio Juan Diego | Visiting Research |
| Rivera Hernándo | Thesis Student |
| Villafañe Carolina | Visiting Research |

**Bioinformatics**

| | |
|---|---|
| Garcia Alexander | Consultor |
| Plata German | Research Assistant |
| Rodriguez Fausto | Research Assistant |
| Rojas Fernando | System Analist |

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