# ANNUAL REPORT 2001

# PROJECT SB-02



# ASSESSING AND UTILIZING AGROBIODIVERSITY THROUGH BIOTECNOLOGY

## CIAT
## For Internal Use only

## October, 2001

# CONTENT

# PROJECT SB-2: ASSESSING AND UTILIZING AGROBIODIVERSITY THROUGH BIOTECHNOLOGY

## PROJECT OVERVIEW

**The Challenge:** The Project's approach to the challenges involved in increasing agricultural productivity, with probably less water and soil, and agricultural competitiveness in tropical developing countries, is based in the application of modern biotechnology to enhance our ability to develop improved strategies for the characterization and sustainable utilization of genetic diversity in crop improvement and conservation. Common bean, cassava and rice are vital to food security and human welfare and along with tropical forages, are grown in developing countries. Our research on the mandated crops expands to other crops of current or potential economic importance in Latin America

**Objective:** To employ modern biotechnology to identify and use genetic diversity for broadening the genetic base and increasing the productivity of mandated and selected nonmandated crops.

**Outputs:** Improved characterization of the genetic diversity of wild and cultivated species and associated organisms. Genes and gene combinations used to broaden the genetic base. Collaboration with public-and private-sector partners enhanced.

**Milestones:**
Cassava cryopreservation implemented. Screening with microarray technology initiated. Gene transfer used to broaden the genetic base and enhance germplasm of rice, cassava, and the forage grass *Brachiaria.*
Marker-assisted selection implemented with cassava and beans.
Marker-assisted selection implemented for rice, beans, cassava, and *Brachiaria.*
ESTs generated for cassava starch and CBB. Efficient transformation system devolved for beans. Transgenic cassava tested for resistance to stemborer. Bioreactor technology implemented for cassava and rice.
Collaboration with public and private partners strengthened.
Integration of genotype × environment GIS system with molecular characterization.
High throughput screening of germplasm bank and breeding materials implemented, using microarray technology. Marker-assisted selection for ACMV and whitefly resistance initiated. Transgenic rice resistant to a spectrum of fungal diseases.

**Users:** CIAT and NARS partners (public and private) involved in crop genetic improvement and agrobiodiversity conservation; AROs from DCs and LDCs, using CIAT technologies.

**Collaborators:** IARCs (IPGRI through the Systemwide Genetic Resources Program, CIP, and IITA through root and tuber crop research); NARS (CORPOICA, ICA, EMBRAPA, INIAs); AROs of DCs and LDCs; biodiversity institutions (A. von Humboldt, INBIO, SINCHI, Smithsonian); and corporations and private organizations.

**CGIAR system linkages:** Saving Biodiversity (30%); Enhancement & Breeding (60%); Training (10%).

**CIAT project linkages:** *Inputs to SB-2:* Germplasm accessions from the gene bank project. Segregating populations from crop productivity projects. Characterized insect and pathogen strains and populations from crop protection projects. GIS services from the Land Use project. *Outputs from SB-2:* Genetic and molecular techniques for the gene bank, crop productivity, and Soils (microbial) projects. Identified genes and gene combinations for crop productivity and protection projects. Methods and techniques for propagation and conservation for gene bank and productivity projects. Interspecific hybrids and transgenic stocks for crop productivity and IPM projects.

## WORK BREAKDOWN STRUCTURE

*PROJECT SB-02: ASSESSING AND UTILIZING AGROBIODIVERSITY THROUGH BIOTECHNOLOGY*

**PROJECT GOAL**

To contribute to the sustainable increase of productivity and quality of mandated and other priority crops, and the conservation of agrobiodiversity in tropical countries.

**PROJECT PURPOSE**

To ensure that characterized agrobiodiversity, improved crop genetic stocks, and modern molecular and cellular methods and tools, are used by CIAT and NARS scientists for improving using and conserving crop genetic resources.

**OUTPUT 1**. Genomes characterized: Genomes of wild and cultivated species of mandated and non-mandated crops, and associated organisms characterized.

- Molecular characterization of genetic diversity
- Identification and mapping of useful genes and gene combinations
- Development of molecular-genetic techniques for assessing genetic diversity.

**OUTPUT 2**. Genes modified: Genes and gene combinations utilized for broadening the genetic bases of mandated and non-mandated crops.

- Transfer of novel genes and gene combinations by means of cellular and molecular gene transfer techniques.
- Identification of points for genetic intervention in plant/stress interactions.
- Development of cellular and molecular techniques for genome modification.

**OUTPUT3**. Collaboration with public and private sector partners enhanced.

- Organization of conferences, networks, workshops and training courses.
- Assembling of data bases, genetic stocks, maps and probes, and related information.
- Publications, project proposal development and contribution to IPR and biosafety management.

## Log Frame - Work Plan for SB-2, 2002

**Area:** Genetic Resources Research
**Manager:** Joe Tohme

| Narrative Summary | Measurable Indicators | Means of Verification | Assumptions |
|---|---|---|---|
| **Goal**<br>To contribute to the sustainable increase of productivity and quality of mandated and other priority crops, and the conservation of agrobiodiversity in tropical countries. | • CIAT scientists and partners using biotechnology information and tools in crop research.<br>• Genetic stocks available to key CIAT partners. | CIAT and NARS publications.<br>Statistics on agriculture and biodiversity. | |
| **Purpose**<br>To ensure that characterized agrobiodiversity, improved crop genetic stocks, and modern molecular and cellular methods and tools are used by CIAT and NARS scientists for improving, using, and conserving crop genetic resources. | • Information on diversity of wild and cultivated species.<br>• Mapped economic genes and gene complexes.<br>• Improved genetic stocks, lines, populations. | Publications, reports, project proposals. | Pro-active participation of CIAT and NARS agricultural scientists and biologists. |
| **Output 1**<br>Genomes characterized: Characterization of genomes of wild and cultivated species of mandated and nonmandated crops and of associated organisms. | • Molecular information on diversity of mandated and nonmandated crops species, and pathogenic and beneficial organisms.<br>• Bioinformatic techniques. | Publications, reports, project proposals.<br>Germplasm. | Availability of up-to-date genomics equipment and operational funding. |
| **Output 2**<br>Genomes modified: genes and gene combinations used to broaden the genetic base of mandated and nonmandated crops. | • Transgenic lines of rice and advances in cassava, beans, *Brachiaria*, and other crops.<br>• Cloned genes and preparation of gene constructs.<br>• Information on new transformation and tissue culture techniques. | Publications, reports, project proposals.<br>Germplasm. | IPR management to access genes and gene promoters.<br>Biosafety regulations in place. |
| **Output 3**<br>Collaboration with public- and private-sector partners enhanced. | • CIAT partners in LDCs using information and genetic stocks.<br>• New partnerships with private sector. | Publications.<br>Training courses and workshops.<br>Project proposals. | Government and industry support national biotech initiatives. |

| Narrative Summary | Measurable Indicators | Means of Verification | Important Assumption |
|---|---|---|---|
| **OUTPUT 1: Genomes characterized** | | | |
| Activity 1.1.Molecular characterization of genetic diversity | • Characterization of core collections<br>• Identification of sources of resistance to disease<br>• Genetic structure of wild and cultivated beans and cassava available Phylogenic trees based on ITS sequences.<br>• Characterization of genetic diversity of endangered palms and soursop in Colombia | Report, articles, databases of molecular fingerprinting | • Availability of structure collections<br>• Material supplied by GRU |
| Activity 1.2 Identification and mapping, of useful gene and genes combinations | • Marker assisted scheme established for bean rice, and *Brachiaria*<br>• Linkage detected between markers and important agronomical traits<br>• QTL analysis for quality traits,disease resistance, and agronomic performance in bean, cassava and rice. | Draft articles, Annual Report, publications | • Availability of mapping populations and phenotypic characterization |
| Activity 1.3 Development of molecular techniques for assessing genetic diversity and mapping useful genes | • Bean, Cassava and *Brachiaria* microsatellites developed<br>• New technologies - SAGE, cDNA AFLP , and microarray implemented.<br>• Resistance genes analogues identified, characterized and mapped in bean and *Brachiaria*.<br>• Mapping resistance genes in *Brachiaria* rice. | Sequences available<br>Report, draft articles | • Access to facilities in advanced labs |
| **OUTPUT 2. Genomes modified** | | | • |
| Activity 2.1 Transfer of novel genes an gene combinations by cellular/molecular techniques | • Expression of insecticidal protein<br>• Isolation of lignin biosynthetic genes from *Brachiaria*<br>• Generation of transgenic *Brachiaria*, cassava, rice, tomato, sugarcane.<br>• Field test of transgenic rice with virus resistance<br>• Backcross conversion from transgenic rice. | Transgenic plants in biosafety field<br>Report, draft articles | • Biosafety regulation approved<br>• Biosafety greenhouse space available<br>• Collaboration with NARS |
| Activity 2.2 Development of cellular and molecular techniques for the transfer of genes for broadening crop genetic base | • Rapid propagation rates of cassava cultivars improved by bioreactors.<br>• Low cost cassava in vitro propagation method transferred to farmers association.<br>• Cost analysis of propagation and conservation of cassava germplasm by different methods.<br>• Use of bioreactors for rice anther culture | Farmer reports, level of adoption of technology | • Access to farmers association<br>• Access to RITA system |

| | | | |
|---|---|---|---|
| | • Adaptation and use of selection system for genetic transformation non dependent on antibiotic resistance<br>• Development of propagation, plant regeneration and transformation of naranjilla<br>• Cryopreservation of cassava and tree tomato<br>• *In vitro* propagation of soursop improved | | |
| Activity 2.3 Identification of points of genetic intervention and mechanism of plant stress | • Genomics tools used to understand and exploit diversity for cassava starch and post-harvest deterioration<br>• Characterization of genetic diversity and key pathway genes for carotene and mineral contents in cassava | | • |
| **OUTPUT 3. Collaboration enhanced** | | | • |
| Activity 3.1 Organization of Networks, Workshops, training courses in biotechnology | • The Cassava Biotechnology Network was re-established<br>• Contribution to training courses<br>• Organization of a legume genomics meeting between CG and US universities.<br>• Organization of the CG planning workshop on biofortification.<br>• At least 70 people received training<br>• Participation of team members to international, regional conferences | Reports | • Funding available |
| Activity 3.2 Data Base and Genetic Stocks | • Database for bean microsatellites established<br>• New version of Flora Map distributed<br>• Database for gene constructs, plasmid and vectors established | Number of register users, report, access to databases, publications | • Continued core support |
| Activity 3.3 Project proposals and Publications | • Five new projects approved and 11 proposals submitted | Number of projects approved | Continued core support |
| Activity 3.4 Donors contributing | • Twenty five donors contributed | Number of current donors | Continued core support |
| Activity 3.5 Project SB-2 staff | • 8.9 Senior Staff person/time<br>• 41 Support Staff<br>• 3 Administrative Support Staff<br>• 18 Graduate Students<br>• 14 Undergraduate Students | Total number of staff | Continued core support |

## PROJECT SB-2 HIGHLIGHTS 2001

### Output 1

- Diversity of *P. vulgaris*, *Phaseolus coccineus*, *P. polyanthus*, *P. acutifolius*, *P. parvifolius* and *P. tenuifolius* with AFLP and microsatelies provides insights on gene pool structure and taxonomic relationships.

- Genetic diversity of common bean for a range of important agronomic traits was assessed by microsatellite and incorporated into the Bean Gene Bank database.

- Assessment of genetic diversity of cassava land races from South America, Central America, and Africa using microsatellites identified substantial variation for CMD resistance, yield, and other agronomic traits.

- Microsatellite markers were identified to assess gene flow from transgenic rice into rice varieties and wild/weedy relatives.

- No correlation between geographical origin of *Xanthomonas* strains and DNA polimorphism was found in various populations within and between ecological zones in Togo.

- Molecular characterization of *Flemingia macrophylla* and *Cratylia argentea* was initiated to integrate with morphological and agronomical data for conservation and management purposes.

- Characterization of Colombia endangered palm species using microsatellite was initiated in collaboration with the von Humboldt Institute.

- High iron trait was transferred from wild bean into cultivated background.

- QTLs for iron and zinc were identified on chromosomes from the Andean and Mesoamerican bean populations used.

- MAS for CMD hs been initiated with IITA after identifying at CIAT molecular markers linked to a qualitative and high level of resistance gene source.

- Candidate genes for CMD has been identified.

- The use of Annotation of SAGE tags differentially expressed in CMD resistant and susceptible genotypes has allowed the identification of genes involved in SAR response to disease in plants.

- QTL from *O. rufipogon* associated with improved agronomic traits were identified in inter-specific hybrids with cultivated rice.

- The dissection and sequence analysis of a cluster of RGA associated with resistance to angular leaf spot was completed.

- The isolation of full-length RGAs in beans was initiated.

- The number of sequences available in the common bean Genebank public database was increased at minimum a 600% by adding over 3,000 microsatellite sequences.

- Microsatellite markers developed for soybean and cowpeas were screened to adapt microsatellite available for other *Phaseolus* legume crops to bean.

- Bean T y 1-copia group retrotransposon LTR sequences and 24 different sequences corresponding to RNAse-polypurine tract-long terminal repeat were isolated and characterized.

- The implementation of novel microarray based technology Diversity Array Technology (DarT) was initiated on bean and cassava.

- New set of 85 microsatellites were located on the cassava genome map. The integration analysis of another new set of 157 SSR on the current cassava map is in progress.

- Analysis using cDNA-AFLP technique allowed the identification of putative molecular markers linked to CBB resistance in cassava.

- The construction of a molecular *Brachiaria* map using grasses RFLP, RAPD, SCAR, AFLP and SSR was achieved.

- Two major QTLs for spittlebug resistance in *Brachiaria* were identified.

**OUTPUT 2**

- Scaling up genetic transformation of cassava was accomplished by broadening from one to four the cultivars used.

- Transgenic cassava lines containing Crylab insect resistance genes were established in the biosafety greenhouse.

- Two field trials with transgenic rice resistance to RHBV were evaluated. Transgenic rice with the highest level of resistance to RHBV were identified.

- A selection system for generating transgenic rice based on the use of mannose isomerase selection gene was developed. The system will aid the generation of transgenic plants non-dependant on antibiotic resistance selection marker genes.

- A cDNA library to clone key genes involved in linging biosynthesis in *Brachiaria* was accomplished.

- Lines of Friable Emrbyogenic Callus (FEC) from various cassava commercial cultivars were established.

- Novel backcross methodology for producing fertile common x tepary beans hybrids from otherwise incompatible genotypes was developed.

- Excellent agronomy and phenology performance in the field of 3-year-old soursop clones *propagated in vitro*, validated the *in vitro* micrografting as a alternative for multiplying pathogen free materials.

- A large scale test of the cryo-preservation cassava protocol was implemented. About 43% of the core collection was tested, and 82% of the accessions showed > 30% recovery rates after freezing.

- The cryo-preservation protocol has been extended to cassava wild relatives including *Manihot esculenta subsp flabellifolia, subsp. Peruviana* and *subsp. Carthaginensis.*

- FEC from two cassava cultivars were recovered after freezing allowing long term storage of suitable material for genetic transformation.

- Plants from the tropical fruit tree tomato were recovered after freezing.

- Propagation methods for cassava commercial clones and doubled haploid generation from rice anther culture were optimized using RITA bioreactors.

- *In vitro* cassava plants propagated by small farmers are under field testing and new clones selected by the farmers were included in a new cycle of propagation.

- A methodology for the reproducible plant regeneration of naranjilla fruit (i.e. lulo) was developed.

- Characterization of key genes involved in carotene biosynthesis pathway was initiated.

- Genotypes of cassava core collection with higher levels of Fe, Zn, and pro-vitamin. A were identified for improving cassava quality through breeding.

- The genetic variation of carotene content in leaves and roots of 682 cassava accesions was determined. Carotenes concentrate much more on leaves suggesting a higher nutritive value of cassava leaves.

- Two classess of secondary metabolites, hydroxyconmarins and flavan 3-ols, were identified as antioxidants and antimicrobials for the control of post-harvest deterioration in cassava.

**Output 3**

- CIAT obtained approval of a project from BMZ, to integrate approach for genetic improvement of aluminum resistance of crops on low-fertility acid soils.

- During period Oct 2000-2001 more than 70 people (researchers, journalists, visitors and others) received training with SB-2 Project Staff.

- In collaboration with two scientists from the Biotechnology Centers of Rutgers University, a training course was held at CIAT to upgrade knowledge of SB-2 staff on molecular approaches for disease resistance, and modulating gene expression in transgenic plants.

- A course on the use of microarray and Diversity Array Technology (DarT) was conducted at CIAT by CAMBIA (Australia) for 30 assistants from SB-2.

- A second workshop on Biotechnology and GMOs biosafety was given by CIAT to Colombian journalist.

- An updated version of Floramap was released in 2001. Some 200 registered users from several countries obtained a copy.

- Data base for bean microsatellites was established

- The cassava Biotechnology Network was re-established for Latin America with funding from DGIS and IDRC.

- The first Planning Workshop to develop a biofortification proposal at the CG level was organized and conducted at CIAT.

- In the period Oct 2000-Sept 2001, SB-2 members published 40 Scientific Papers in refereed journals and books, abstracts and posters in proceedings.

- In the same period, 5 new projects were approved and 11 proposal were submitted.

- A total of 24 donors contributed funding projects in SB-2

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# Output 1. Genomes of wild and cultivated species of mandated and non mandated crops, and associated organisms characterized

## Activity 1.1 Characterization of genetic diversity

### Main Achievements

- A core collection of *Phaseolus coccineus* and *Phaseolus polyanthus* was evaluated with AFLP markers, demonstrating that very little gene pool structure exists in these two species, although Mexican and Guatemalan accessions of *P. coccineus* separate slightly, and an ecotype of *P. polyanthus* exists in South America.

- Diversity in the CIAT collection of tepary beans (*Phaseolus acutifolius*) was analyzed with AFLP and microsatellites to distinguish taxonomic relationships with *P. parvifolius* as well as within the species between *P. a.* var. *acutifolius* and *P. a.* var. *tenuifolius.*

- Genetic diversity of microsatellite alleles was determined for two parental surveys of common bean that provide the basis for mapping and genetic tagging for a range of important traits; including biotic and abiotic stress resistance / tolerance, micronutrient accumulation. This information was incorporated into a new molecular genetics database constructed for microsatellite parental surveys and the Bean genes database.

- The study of cassava land races using microsatellites was extended to assessing genetic diversity and differentiation of cassava land races from 5 countries in South America, 2 in Central America, and 2 in Africa, and to African cassava genotypes resistant to the Cassava Mosaic Disease (CMD). The analysis showed a substantial amount of genetic diversity in CMD resistance germplasm appropriate for the genetic improvement of CMD resistance as well as of other traits, particularly yield.

- A set of microsatellite markers were identified detecting polymorphism between transgenic rice, other rice varieties, wild *Oryza* species and red rice. This tool is being used to monitor gene flow from transgenic rice into rice varieties and into wild/weedy relatives. With this project we initiated studies on environmental biosafety jointly with the generation of transgenic plants to set guidelines for their safe use in agriculture.

- The assessment of the origin and the genetic diversity of the *Xam* was extended to cassava populations within and between ecological zones in Togo. The cluster analysis revealed the existence of 7 groups at 70% similarity. No correlation between the geographical origin of the strains and DNA polymorphism was observed.

- The Genetic diversity of the multipurpose shrub legumes Flemingia macrophylla and Cratylia argentea was initiated using AFLPs. The molecular data will be integrated with morphological and agronomical data to improve the use and management, including conservation, of the collections.

- As a collaboration with the Humboldt Institute, the analysis of the genetic structure of Colombian endangered palm species was carried using microsatellite isolated at CIAT.

## 1.1.1 Evaluation of the *P. coccineus* and *P. polyanthus* core collections with molecular markers

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[1]SB-2 Project; [2]IP-4 Project, [3]IP-1 Project

### Introduction

In a project financed by the Belgian government to explore the potential of *P. coccineus* (PC) and *P. polyanthus* (PP), a core collection was formed as reported in past years. No previous study of the genetic structure of these two species had been performed, to determine if they display gene pools comparable to other species of the genus such as *P. vulgaris*. Therefore the core was studied with molecular markers to determine if gene pools exist within these species.

### Materials and methods

A total of 178 accessions of *P. coccineus* and *P. polyanthus* were studied, including the PC-PP core collection and others that had been designated as promising for specific traits, such as resistance to BGYMV or bean fly. The PC accessions included 66 cultivated types, 40 wild accessions, 1 intermediate and 1 hybrid (possibly with PP). Geographically, accessions originated in Mexico (57), Guatemala (30), Colombia (5), Yugoslavia (3), Rumania, Rwanda, and Turkey (2 each), Costa Rica, Germany, United Kingdom, Honduras, Puerto Rico, Holland and Portugal (1 each). The PP accessions included 59 cultivated, 7 wild types, and 3 hybrids. These originated in Mexico (26), Guatemala (29), Colombia (10), Costa Rica (2), and Venezuela (1). An accession of *P. vulgaris* (cv. 'ICA Pijao') and of *P. costarricensis* were included for comparison. The technique of AFLP was applied to the PC-PP core collection, using two primer combinations, selected from among 38 combinations of primers tested from a kit obtained from GIBCO BRL™. Bands were read as present or absent and were analyzed by Multiple Correspondence Analysis (MCA) and by Unweighted Pair Group Method with Arithmetic Averages (UPGMA). Initially the analysis was performed with only accessions produced under controlled pollination (type 1 and type 2 seed), and subsequently accessions with open-pollinated seed were added to the analysis.

### Results and discussion

The four species separated widely from each other in the analyses by UPGMA and by MCA, confirming the independent status of *P. polyanthus* as a species apart from *P. coccineus* (Figure1).

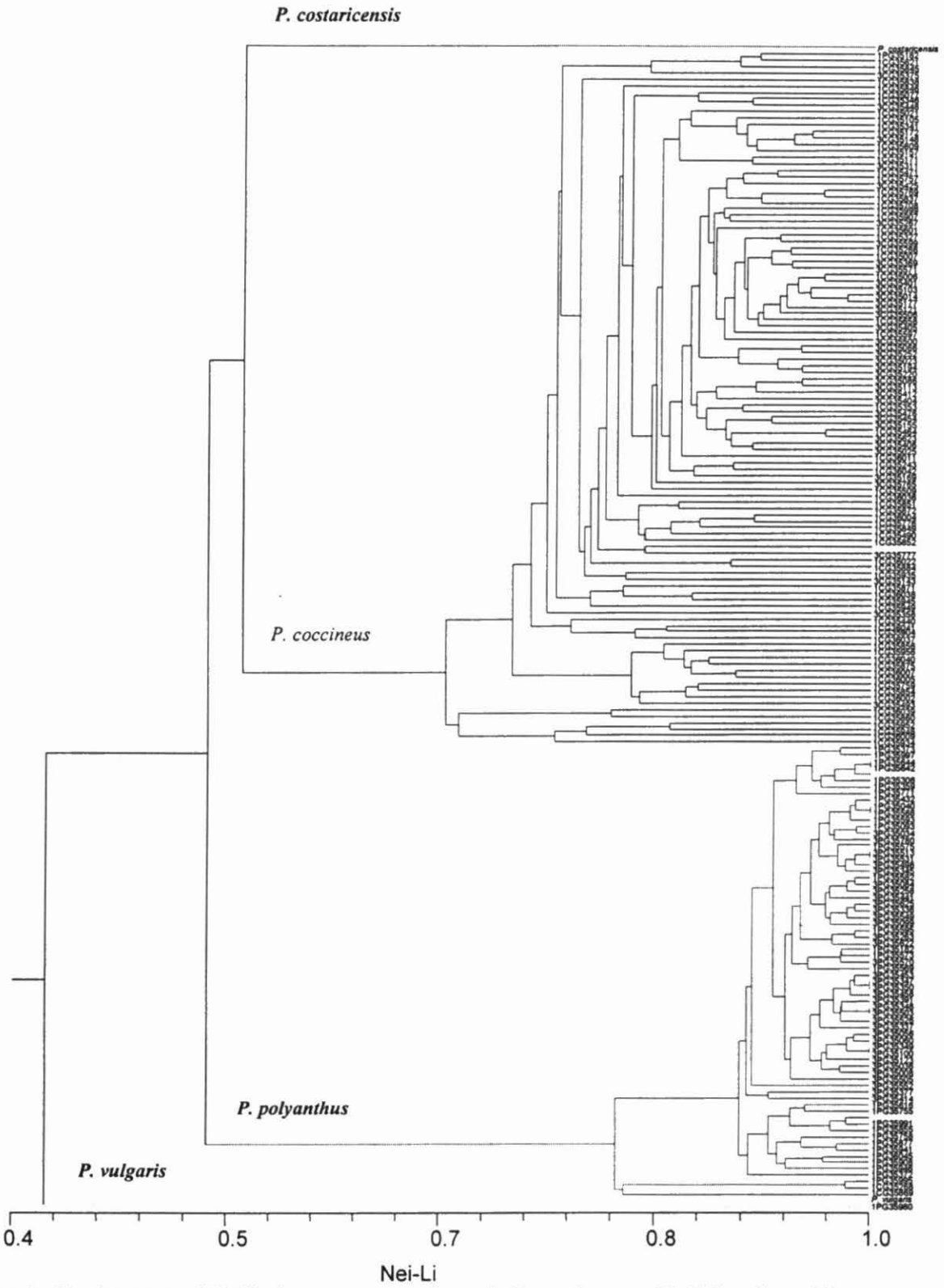


**Figure 1. Dendrogram of similarity among accessions of *P. coccineus y P. Polyanthus* with controlled pollination , including *P. vulgaris* and *P. costarricensis*, with two primer combinations.**

When wild and cultivated PC were analyzed together by MCA, the first dimension separated wild from cultivated (Figure 2). There was no unambiguous overlap between wild and cultivated groups to suggest that a certain wild population might have participated in a unique domestication event. Rather, only two wild accessions from Guatemala occupied an area on the margin of the cultivated. The second dimension showed a geographical gradient, whereby the wild PC from

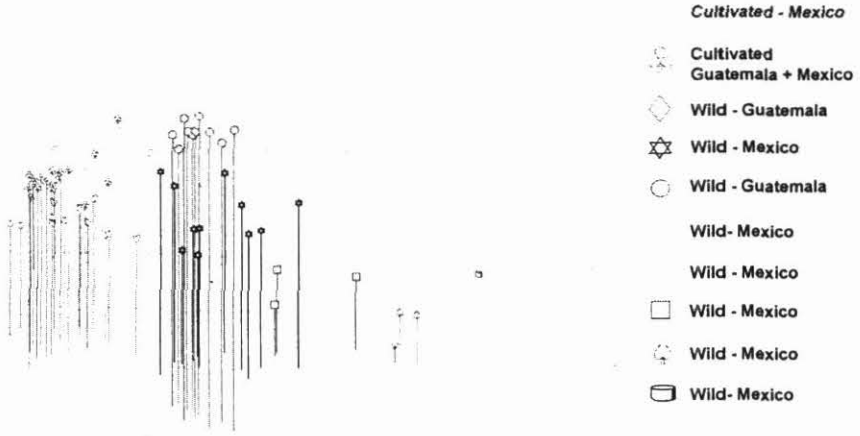


**Figure 2. Three dimensional graph derived from Multiple Correspondence Analysis (MCA) of controlled pollination accessions of *P. coccineus*, and excluding *P. vulgaris* and *P. costaricensis*. The first dimension shows the separation of accessions according to biological state: wilds (to the right) and cultivated (to the left).**

Guatemala and Mexico separated from each other (Figure 3). However, the wild accessions in general did not result in discrete groups, and the Mexican accessions in particular occupied a very dispersed space.

However, in the second dimension, the position of the cultivated was even more ambiguous. It fell in between the wilds from Guatemala and Mexico, being similar to the Guatemalan wilds on the first dimension and similar to the Mexican wilds on the second dimension. These results suggest that the cultivated PC might not have a direct relationship with any single wild population. It is possible that the allogamous nature of the species has resulted in cultivated PC that represents introgression from multiple wild populations, hence its intermediate position between wild populations from Mexico and Guatemala.

In the case of *P. polyanthus*, the first dimension of the MCA (Figure 3) again separated wild from cultivated accessions. Wild PP was surprisingly diverse, considering that all seven accessions of wild PP come from a restricted geographical area of Guatemala. Neither did these present any apparent structure. Among cultivated accessions, it was noted that those that graphed most distantly from the wild beans were those from South America (Figure 4). When these were viewed on the second dimension (Figure 5), their differentiation from all other accessions was even clearer. Last year we noted that these occupy an ecological niche that is very different from other PP. Molecular analysis confirms that there is a subtle difference of these accessions compared to others, although the genetic distance compared to other cultivated PP is in fact quite small.

When accessions with open-pollinated seed were included in the analysis, no significant changes occurred in the structure of the dendrogram, although the absence of a clear gene pool structure made it more difficult to analyze this effect and to estimate the effect of open-pollination on the genetic structure of the species. However, the allele frequencies of controlled vs, open-pollinated accessions were compared, resulting in a correlation of r=0.965 for PC and r=0.975 for PP. Therefore, the open-pollination technique has not changed allele frequencies and may not have altered the genetic structure as much as we might have feared.

Heterogeneity of different populations was compared (Table 1). In neither species were the wild accessions more diverse than the cultivated accessions. Therefore there does not appear to a serious founder effect in these two species. Neither was one country or region inherently more diverse than any other.

The foregoing results probably reflect the allogamous nature of the two species. Outcrossing has probably erased any possible tendency for the formation of gene pools by creating a relatively homogenous intermating population. Similarly, the allogamous nature has overcome any possible founder effect.

Given the ambiguity of results with nuclear DNA in our attempts to relate wild populations of PC to cultivated accessions, an additional study was undertaken with chloroplast DNA. Chloroplast-specific primers were used to amplify products from 30 wild and 10 cultivated accessions of PC. However, results continued to be ambiguous. Wilds from both Guatemala and Mexico grouped with cultivated PC. Surprisingly, PC presented very diverse chloroplast DNA, with some wild PC being identical with P. costaricensis, others grouping close to P. vulgaris, and others forming groups that were quite divergent among themselves. Neither did parsimony analysis reveal clear tendencies. In contrast to PC, a similar attempt with PP failed to find any polymorphism at all in chloroplast DNA.

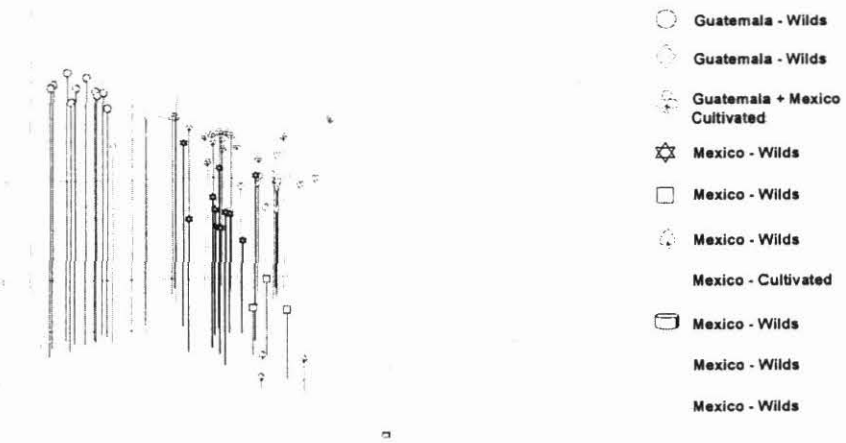


**Figure 3. Multiple Correspondence Analysis (MCA) of controlled pollination accessions of *P. coccineus* (excluding *P. vulgaris* and *P. costarricensis*), showing in the second dimension, separation of accessions by geographic origin.**

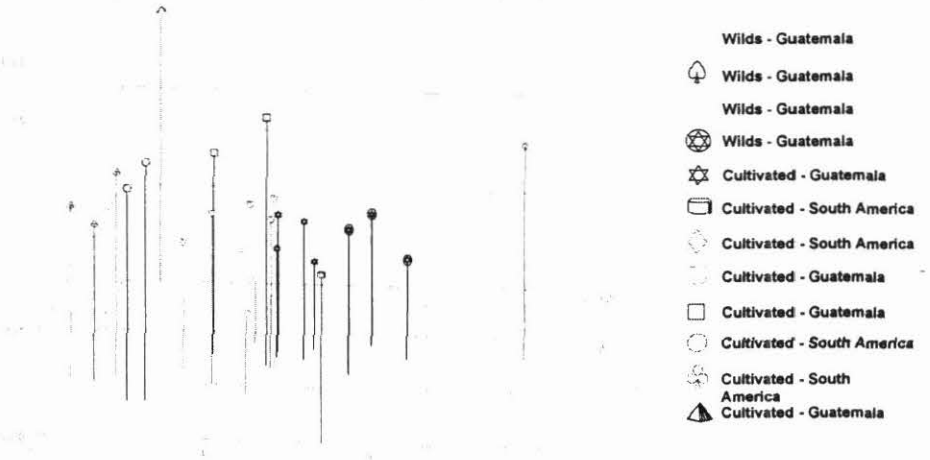


**Figure 4. Multiple Correspondence Analysis (MCA) of controlled pollination accessions of *P. polyanthus* (excluding *P. vulgaris* and *P. costaricensis*), showing in the first dimension the separation of wilds (to the left) and cultivated (to the right).**

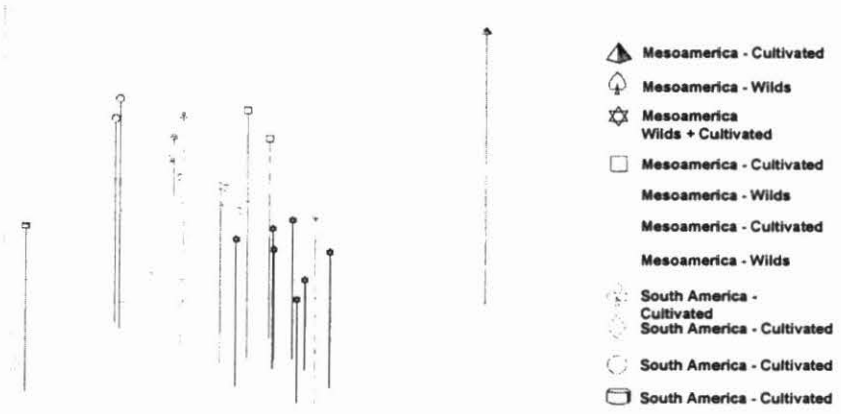


**Figure 5. Multiple Correspondence Analysis (MCA) of controlled pollination accessions of *P. polyanthus* (excluding *P. vulgaris* and *P. costaricensis*), showing in the second dimension the separation of accessions by geographic origin.**

**Table 1. Heterogeneity values of *P. coccineus* and *P. polyanthus* calculated within groups according to biological state and geographical zone.**

| | Heterogeneity | | | | | |
|---|---|---|---|---|---|---|
| Groups | H | Hs | Hst | Gst | Value t | Significance |
| *P. coccineus* | | | | | | |
| Wild | 0,162 | 0,152 | 0,01 | 0,07 | 0,725 | 0,469 |
| Cultivated | 0,139 | | | | | |
| Wilds GTM + SMEX | 0,124 | 0,147 | 0,015 | 0,093 | 0,995 | 0,322 |
| Wilds MEX | 0,166 | | | | | |
| Cultivated GTM + SMEX | 0,113 | 0,131 | 0,008 | 0,056 | 0,322 | 0,749 |
| Cultivated MEX | 0,132 | | | | | |
| Total species | 0,164 | | | | | |
| *P. polyanthus* | | | | | | |
| Wild | 0,031 | 0,048 | 0,006 | 0,122 | 0,897 | 0,372 |
| Cultivated | 0,054 | | | | | |
| Mesoamerica | 0,05 | 0,051 | 0,004 | 0,077 | 0,041 | 0,967 |
| South America | 0,051 | | | | | |
| Total species | 0,055 | | | | | |

H: total heterogeneity, Hs: heterogeneity with groups, Hst: heterogeneity between groups, Gst: proportion of heterogeneity due to differentiation between groups. GTM = Guatemala, SMEX = south of Mexico, MEX = rest of Mexico.

**Conclusions**

Neither *P. coccineus* nor *P. polyanthus* showed any clear gene pool structure, and on the contrary, the genetic variability within any groups formed was greater that that between groups. Therefore, we conclude that both species have only one gene pool. Neither did either species show any evidence of a founder effect. On the contrary, present-day cultivated *P. coccineus* appears to have resulted from ample introgression from different wild populations in Guatemala and Mexico. The allogamous nature of the species has probably contributed to all of these results. However, *P. coccineus* presents remarkable variability in chloroplast DNA. In the case of *P. polyanthus*, an ecotype exists in South America which, although it can be distinguished with AFLP markers, is in fact genetically very similar to other cultivated polyanthus.

## 1.1.2 Genetic diversity of the CIAT tepary bean (*Phaseolus acutifolius*) collection measured with AFLP and microsatellite markers

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SB-2 Project

**Introduction**

Tepary bean (*Phaseolus acutifolius* A.Gray) is in the tertiary gene pool of common beans (*P. vulgaris* L.) and as such represents a potential but difficult to use genetic resource for the improvement of common beans. The two species have been crossed, despite high embryo abortion, using congruity or recurrent backcrossing and these interspecific hybridizations have been used to incorporate common bacterial blight resistance into common bean. Notwithstanding their utility for the improvement of other species, tepary beans are a useful and interesting crop in their own right, especially for dryland agricultural systems. They are known to have high drought and salinity tolerance, good nutritional quality and a tradition of cultivation in Mexico, South western United States and Central America, that goes back 5000 years. What is needed is to create improved varieties as none exist. For this it is critical to have a baseline data on the diversity that exists within tepary beans. Previous authors (Scinkel and Gepts, 1988, 1989; Garvin and Weeden, 1994) have suggested that tepary beans seem to be less diverse than common bean or lima beans. They are thought to have had a single center of origin and to have been distributed from a few original sites across the present distribution. The objective of this research was to study the patterns of diversity within the species and its placement relative to other Phaseolus species that were used as an outgroup, using two types of molecular markers: 1) AFLPs which tend to be evolutionarily conserved markers and serve to reference different species relative to each other and 2) microsatellites which are by nature hypervariable loci that can distinguish between varieties. Additional objectives of the study were to determine if *P. acutifolius* and *P. parvifolius* merit being separate species and if molecular markers can distinguish between the botanical varieties var. *acutifolius* and var. *teniufolius* within the species *P. acutifolius*. AFLP markers have been applied before to study wild species of *Phaseolus* (Tohme et al., 1996) and lima bean, *P. lunatus*, accessions and their close relatives (Caicedo et al., 1999). We have developed new microsatellites that are beginning to be used in characterization studies. This is the first application of both marker systems to study tepary bean diversity.

## Materials and Methods

*Genotypes and DNA extraction:* A total of 127 genotypes from the Genetic Resources Unit of CIAT were analyzed in the experiments. The outgroup consisted in 10 genotype from the *Phaseolus* genus including 4 *P. vulgaris* (common bean); 4 *P. lunatus* (lima bean); 1 *P. coccineus* (scarlet runner bean); and 1 *P. glabellus* genotype. For both common and lima beans a wild and a cultivated representative from both the Andean and Mesoamerican genepools was included in the analysis. For the other species only a single representative was analyzed. A total of 117 tepary beans and their close relatives were analyzed, consisting in 49 cultivated *P. acutifolius* var. *acutifolius*; 44 wild *P. acutifolius* var. *acutifolius*; 12 *P. acutifolius* var. *tenuifolius;* and 12 *P. parvifolius* accessions. The genotypes were grown in the greenhouse and total genomic DNA was extracted from 2 g of fresh leaf tissue with a large preparation method (CIAT protocols).

*AFLP analysis:* Amplicon-template preparation, pre-amplification, and selective amplification were as described for the protocol of the Gibco BRL AFLP analysis system I kit for small genomes. In a previous study, we determined which were the best primer combinations based on the *Eco*RI (E) - *Mse*I (M) adapters and primers with 3 selective nucleotides each. One combination, based on E-AAG and M-CTT primers was analyzed for this study PCR products were run on 4% silver-stained polyacrylamide gels for 1, 1.5 and 2 hours to resolve as many fragments as possible. Bands were sized by comparison to a 50bp ladder molecular weight size standard. All the polymorphic AFLP bands between 100 and 400 bp were scored for presence or absence among the lines and used to calculate the similarity matrix. Larger or smaller bands were not considered.

*Microsatellite analysis:* A total of 10 microsatellites were amplified for the study; of which six were cDNA based (BMd1, BMd7, BMd10, BMd15, BMy1 and BMy6) and four were genomic (BMd11, BMd12, BMd36 and BM114) (Table 1). PCR product were run on 4% silver-stained polyacrylamide gels and the alleles sized by comparison with 10 and 25 bp molecular weight ladders. Alleles were considered separate taxonomic units for the purposes of calculating shared bands and similarity.

*Data analysis:* Genetic similarities between genotypes was determined with the Dice coefficient using the software packages SAS (SAS Institute, 1989) and NTSYS 2.02 (Rohlf, 1993).

## Results

*AFLP analysis:* The AFLP combination used in this study had a good polymorphism rate, clear amplification profile and well-distributed range in PCR product sizes. The AFLP combination produced a total of 167 bands. Of these 99.5% of the bands were polymorphic across all species although there was substantial monomorphism within the cultivated *P. acutifolius*. Both monomorphic and polymorphic band were used to determine the genetic similarity between genotypes. Figure 1 a shows the dendrogram created for the AFLP bands. The structure of the dendrogram agrees with known taxonomic relationships for the six species represented in the study. *P. lunatus* was the most distant group, followed by *P. glabellus* and *P. coccineus*. *P. vulgaris* was the closest to the *P. acutifolius* - *parvifolius* clade. The level of similarity was around 35% between the five groups. Within both *P. vulgaris* and *P. lunatus* the distinction between Andean and Mesomerican genepools was clear. The level of similarity between genepools was higher in *P. vulgaris* (68%) than in *P. lunatus* (62%).

Within the *P. acutifolius – parvifolius* clade, all the accessions shared up to 54% similarity. Five groups could be distinguished within the clade: 1) cultivated *P. acutifolius* from Central and North

America 2) cultivated *P. acutifolius* from North America (mainly Sonora and Sinaloa), 3) wild *P. acutifolius* var. *acutifolius* 4) wild *P. acutifolius* var *acutifolius* and *tenuifolius*; and 45) *P. parvifolius*. These five groups could be organized hierarchically into two supergroups, consisting of groups 1, 2 and 3 together and groups 4 and 5 together. The first supergrouping contained all the cultivated *P. acutifolius*, while the second supergrouping contained all the *P. acutifolius* var. *tenuifolius* and *P. parvifolius* accessions. The wild *P. acutifolius* accessions were distributed among the two supergroupings, with some more allied to the cultivated accesssions of the same species and others allied to the *P. parvifolius* group. Within the first supergroup, the two cultivated groups (1 and 2) were related at 80% similarity and these were related to the wild accessions (group 3) within that supergroup at 68% similarity. Within the second supergroup, the P. parvifolius and P. acutifolius (both var. acutifolius and tenuifolius) were related at 64% similarity. The supergroups were distinguishable at 54% similarity. A multiple correspondance analysis confirmed the groupings observed in the dendrogram, where five clusters could be found in the *P. acutifolius-prvifolius* clade, corresponding to the groupings described above.

*Microsatellite Analysis:* The ten microsatellites detected a total of 75 alleles which were scored as bands (present and absent) to determine genetic similarity between genotypes. The average number per locus of alleles produced across the range of genotypes was as high for the cDNA (7.8 alleles) as for the genomic (7 alleles) microsatellites. Figure 1b shows the dendrogram created for the microsatellites. Genetic similarities were much lower on average than with AFLP data. The structure of the dendrogram agrees with that of the AFLP results, however their were several important differences. In agreement, the microsatellite information predicted that *P. lunatus,* followed by *P. vulgaris* were the most distant groups from *P. acutifolius,* with low similarities of 9 and 15% similarity, respectively. Within both *P. vulgaris* and *P. lunatus* the distinction between Andean and Mesomerican genepools was clear as described above, and these group shared only 55% similarity. In contrast to the AFLP data, the *P. parvifolius* and *P. acutifolius* cluster were separate and shared only 32% similarity. Two *P. acutifolius* var. *tenuifolius* accessions were found within the *P. parvifolius* cluster, while the others were found mixed with the wild accessions of *P. acutifolius* var. *acutifolius.* Surprisingly, *P. coccineus* was found between the *P. parvifolius* and the *P. acutifolius* clusters. All the cultivated *P. acutifolius* formed a group with the highest genetic similarity of around 85%. One group of cultivars have similarity of 100% and are all accessions from Sinaloa. Despite the differences in scale and detail, correlation between the matrices generated for AFLP and microsatellite datasets was high ($r=0.703$) and significant as indicated by the approximate Mantel test ($t= 12.15$, $P=.0198$)

**Discussion and Future Plans**

Microsatellites detected much greater differences than AFLPs, probably because they were less conserved than AFLPs. The use of two marker systems to sample different part of the genome that evolve at different rates hopefully gave us a more accurate picture of the relationships within and between species. The high similarity among all the cultivated tepary beans, even with the microsatellites, seems to indicate that the crop may have arisen from a single domestication event that led to a genetic bottleneck which limits diversity within the cultivars. From this study, there is very little evidence for introgression from wild relatives into the cultivated genepool after the initial domestication event. Tepary beans are known to have a very low crossing rate that limits the creation of new diversity within the crop. The lack of diversity within the cultivated tepary bean is a serious limitation for improvement of the crop. The lack of diversity within the cultivated tepary bean belies some of the variability found for disease and insect resistance within the species. These are also fast evolving characteristics so could be expected to have been generated by mutation even without a lot of initial diversity or inter-crossing. However, that lack of diversity in other

characteristics such as plant morphology, adaptation range has serious implications for improving the species agronomically and using the species in inter-specific hybridization.

The relationships within the *P. acutifolius* – parvifolius clade has been controversial. The AFLP data presented here suggest that the *P. acutifolius* and *P. parvifolius* probably do not deserve to be different species, but could qualify as possible subspecies or varieties within the species. The microsatellite data meanwhile show that wild P. acutifolius accessions and the *P. parvifolius* accessions are the extremes of a continuum, with all of the *P. a.* var. *tenuifolius* accessions as intermediates between these two clusters. The high amounts of diversity found in the wild *P. acutifolius* and *P. parvifolius* accessions are an interesting resource for breeding tepary bean cultivars.

**Table 1. Outgroup used in the study of Tepary bean diversity**

| Identification | Species | status | region | country |
|---|---|---|---|---|
| G19889 | P. vulgaris | Wild | Jujuy | ARG |
| G23508 | P. vulgaris | Wild | Jalisco | MEX |
| ICA PIJAO | P. vulgaris | Cultivar | breeding line | COL |
| CALIMA | P. vulgaris | Cultivar | breeding line | COL |
| G24390 | P. vulgaris | Wild | na | COL |
| G19892 | P. vulgaris | Wild | na | ARG |
| G24404 | P. vulgaris | Wild | na | COL |
| G25704 | P. lunatus | Wild | Jalisco | MEX |
| G25914 | P. lunatus | Wild | Cajamarca | PER |
| G25832 | P. lunatus | Cultivar | Cajamarca | PER |
| G25577 | P. lunatus | Cultivar | Sn Martin | PER |
| G40594 | P. glabellus | Wild | Sl potosi | MEX |
| G35583 | P. coccineus | Cultivar | na | GTA |

**Table 2. Microsatellites used in the study of Tepary bean diversity.**

| Type | SSRs | No. alleles |
|---|---|---|
| CDNA | BMd1 | 11 |
| | BMd7 | 6 |
| | BMd10 | 7 |
| | BMd15 | 5 |
| | BMy1 | 8 |
| | BMy6 | 10 |
| Genomic | BMd11 | 7 |
| | BMd12 | 5 |
| | BMd36 | 9 |
| | BM114 | 7 |
| Total no. of alleles | | 75 |

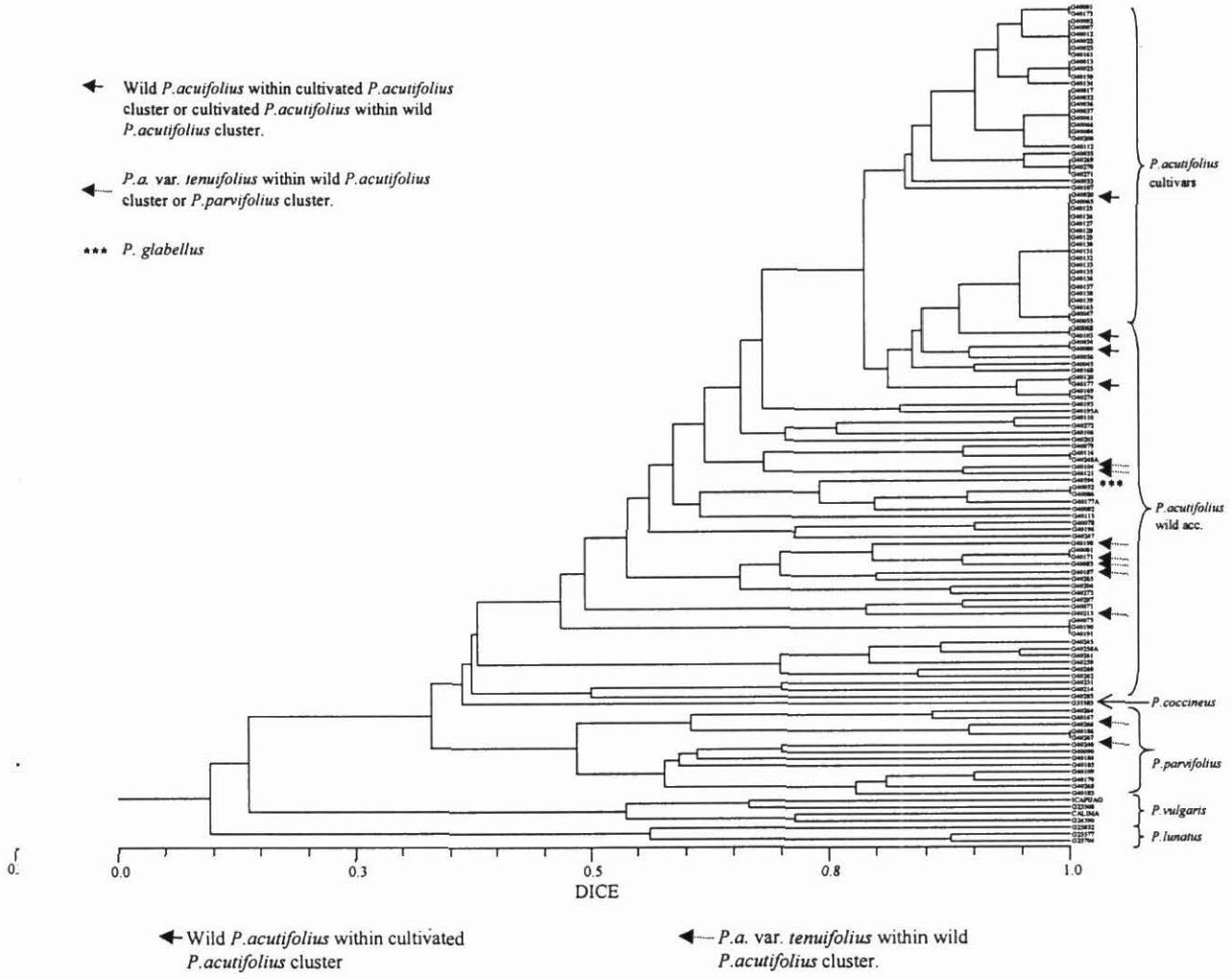


**Figure 1. Dendrograms showing the associations among 114 accessions of cultivated and wild tepary beans (*Phaseolus acutifolius* and *P. parvifolius)* and 10 other *Phaseolus* species, based on UPGMA clustering using the Dice genetic similarity coefficient for: AFLP banding pattern for the combination E-AAG, M-CTT Microsatellite alleles identified for 10 loci (6 cDNA-derived and 4 genomic)**

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## 1.1.3 Genetic diversity of microsatellites in common bean

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### Introduction

Microsatellites markers are based on short segments of DNA in which a specific simple sequence motif of 1-6 bases is repeated in tandem, multiple times. Due to the innate variability at microsatellite loci, these markers have been ideal for characterizing genetic diversity in crop species at the inter-specific, inter-subspecific, inter-varietal and even intra-varietal levels. Microsatellites have been found to vary in the polymorphism they detect depending on the length and sequence of the repeat motif they contain and their location along the chromosomes, specifically whether they reside in gene-coding or non-coding segments of the genome. The objective of this study was to evaluate all new *Phaseolus* microsatellite markers developed at CIAT and elsewhere for their allelic variability on two panels of 18 common bean genotypes representing diverse germplasm, both cultivated and wild, Mesoamerican and Andean, which have been used as parents in the bean breeding program.

### Materials and Methods

The genotypes consisted in 30 common bean genotypes arranged in two panels; The previous panel of 18 was described in last year's annual report (SB-02 report 2000). This year a new panel was instituted with 14 individuals, including two Andeans, nine mesoamericans and one tepary bean (Table 1) which are the parents of six mapping populations being studied at CIAT for the inheritance of disease resistance (common bacterial blight (CBB), bean golden mosaic virus (BGMV), angular leaf spot and anthracnose), insect resistance (Apion) and abiotic stress tolerance (low phosphorous adaptation, drought tolerance and adventitious rooting). Bulked segregant analysis (BSA) was carried out simultaneously with the parental survey for the disease and insect resistance mapping populations for CBB, BGMV and Apion. The parents of an additional mapping population (BAT93 x Jalo EEP558) were included because this population has been the basis for creating an integrated genetic map for the bean genome (Freyre et al., 1999). The populations included three intra-genepool Mesoamerican x Mesoamerican crosses and two inter-genepool Mesoamerican x Andean populations (Table 2). The genotypes were evaluated with a total of 131 microsatellite markers (of which 65 were derived from genomic libraries and 66 were derived from cDNA or gene sequences). The markers were amplified at different annealing temperatures according to the estimated melting temperatures of the primers. The PCR products were resolved on silver-stained polyacrylamide gels and microsatellite alleles were sized by comparison to the 10 and 25 bp molecular weight standards (Promega).

### Results and Discussion

Genomic microsatellites detected more polymorphism than cDNA microsatellites in the intra-genepool crosses but were about equally effective in uncovering polymorphism in the inter-genepool and interspecific crosses. The rate of polymorphism was much higher (77.9%) in the interspecific crosses than in the intra-specific crosses (37.5%). The average polymorphism rate between the parents of the inter-genepool crosses (44.3%) was higher than that of the intra-genepool crosses (33.1%). Among the Mesoamerican x Mesoamerican crosses, the intra-racial cross J117 (race Jalisco) x Jamapa (race Mesoamerica) was more polymorphic (43.5%) than the other crosses DOR476 x SEL1309 and VAX6 x MAR1 which were within race Mesoamerica.

Among the inter-genepool crosses BAT93 x Jalo EEP558 was more polymorphic than G2333 x G19839. The genomic microsatellites were more polymorphic markers than the cDNA derived microsatellites. Overall the average polymorphism rate for the genomic microsatellites was 51.5 % versus 37.2 % for the cDNA microsatellites.

Significantly fewer average alleles per locus were found for microsatellites from genes (3.3 alleles) than for microsatellites from non-coding sequences (4.5 alleles). The gene-derived microsatellites frequently were bi- or tri-allelic and distinguished the difference between Andean and Mesoamerican genepools and the difference between *Phaseolus vulgaris* and *P. acutifolius*. Meanwhile the genomic microsatellites detected more alleles and were thus able to resolve some within-genepool variation. The polymorphism information content (PIC) of the gene-derived microsatellites was lower (0.402) than for the genomic microsatellites (0.553). The PIC values were positively correlated with the number of alleles produced at the locus. Null alleles were uncommon in both types of microsatellites. The allele size range was generally a good predictor of the number of alleles present for a locus. The allele range was 67% wider for the genomic microsatellites (28.8 bp) compared to the gene-derived microsatellites (17.3 bp). However there were several microsatellites with large size ranges but few alleles.

The differences in allelic variability observed at specific bean microsatellite loci are probably due to the differences in the mutation rate inherent for each locus. Microsatellites mutate when they add or subtract a small number of perfect repeats or undergo changes in the flanking regions of the SSR. These changes can occur due to polymerase slippage, unequal crossing-over and/or insertion-deletion events. Although microsatellites are believed to have some of the highest mutation rates observed at any type of molecular loci, some microsatellites will evidently be more stable than others. In this study as in others before, microsatellite variability seems to be influenced by the structure, motif, SSR length and genomic context of the locus.

The more polymorphic genomic microsatellites may well become the mainstay of mapping studies since they will be useful even in narrow intra-genepool crosses. They will also be very useful for to analyzing recent changes in population structure and selection history in closely-related germplasm from a given area or from a specific commercial class. Meanwhile the more conserved and stable cDNA-derived microsatellites may find their greatest utility in mapping in wide inter-genepool or inter-specific crosses and in the phylogenetic analysis of the genus *Phaseolus*.

**Conclusions and future plans**

We plan to continue testing all new *Phaseolus* microsatellites on the existing panel and if the need arises, will create another panel of varieties to survey for polymorphisms in the parents of additional populations. In the future it will be very useful to genotype many of the common parents and genetic sources used at CIAT, as this will allow us to implement whole-genome marker assisted selection that is specific to the genetic crosses made in our bean breeding program.

**Table 1. Mapping parent genotypes used for assessment of genetic diversity of common bean microsatellites**

| | Variety | Genepool | Purpose | Origin |
|---|---|---|---|---|
| 1 | DOR476 | Mesoamerican | Disease resistance (BGMV) | CIAT line |
| 2 | SEL1309 | Mesoamerican | Disease resistance (CBB) | CIAT line |
| 3 | BAT93 | Mesoamerican | Integrated Map | CIAT line |
| 4 | Jalo EEP558 | Andean | Integrated Map | Brazil |
| 5 | ICA Pijao | Mesoamerican | Cultivar | Colombia |
| 6 | G40001 | Tepary bean | Abiotic stess | CIAT accession |
| 7 | VAX6 | Mesoamerican | Disease resistance (CBB) | CIAT line |
| 8 | MAR1 | Mesoamerican | Disease resistance (ALS) | CIAT line |
| 9 | J117 | Mesoamerican | Insect resistance | Mexico |
| 10 | Jamapa | Mesoamerican | Cultivar | Mexico |
| 11 | G2333 | Mesoamerican | Abiotic stess | CIAT accession |
| 12 | G19839 | Andean | Abiotic stess | CIAT accession |

**Table 2. Polymorphism rate among 11 parent combinations for 70 microsatellite loci (36 cDNA and 34 genomic).**

**by marker name**

| Population | BM All genomic | % | BMy All cDNA | % | BMd 9 genomic 35 cDNA | % | PV All cDNA | % | CLONES 17 genomic 14 cDNA | % |
|---|---|---|---|---|---|---|---|---|---|---|
| DOR 476 x SEL 1309 | 20 | 51.3 | 3 | 33.3 | 6 | 13.6 | 1 | 12.5 | 9 | 29.0 |
| BAT 93 x JALO | 31 | 79.5 | 8 | 88.0 | 17 | 38.6 | 3 | 37.5 | 10 | 32.3 |
| ICA PIJAO x G 40001 | 33 | 84.6 | 9 | 89.0 | 38 | 86.4 | 5 | 62.5 | 17 | 54.8 |
| VAX 6 x MAR 1 | 14 | 35.9 | 4 | 100 | 9 | 20.4 | 2 | 25.0 | 6 | 19.3 |
| J 117 x JAMAPA | 27 | 69.2 | 5 | 44.4 | 12 | 27.3 | 2 | 25.0 | 12 | 38.7 |
| G 2333 x G19839 | 31 | 79.5 | 6 | 55.6 | 6 | 13.6 | 2 | 25.0 | 9 | 29.0 |
| Total evaluated | 39 | | 9 | | 44 | | 8 | | 31 | |

**by marker class**

| Population | Cross | cDNA derived | % | Genomic | % | Total | % |
|---|---|---|---|---|---|---|---|
| DOR 476 x SEL 1309 | Mesoamerican x Mesoamerican | 13 | 19.7 | 25 | 38.5 | 38 | 29.0 |
| BAT 93 x JALO | Mesoamerican x Andean | 32 | 48.5 | 37 | 56.9 | 69 | 52.7 |
| ICA PIJAO x G 40001 | Interespecific | 52 | 78.8 | 50 | 76.9 | 102 | 77.9 |
| VAX 6 x MAR 1 | Mesoamerican x Mesoamerican | 16 | 24.2 | 19 | 29.2 | 35 | 26.7 |
| J 117 x JAMAPA | Mesoamerican x Mesoamerican | 23 | 34.8 | 34 | 52.3 | 57 | 43.5 |
| G 2333 x G19839 | Mesoamerican x Andean | 11 | 16.7 | 36 | 55.4 | 47 | 35.9 |
| TOTAL EVALUADOS | Total (no.) / Average (%) | 66 | 37.2 | 65 | 51.5 | 131 | 44.2 |

## 1.1.4 Identification of common bean genotypes using "Fingerprints" of metabolism enzymes and seed proteins: the case of the Enola variety

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### Introduction

Crop varieties must successfully fulfill the criteria of newness, distinctness, uniformity, and stability, in order to be registered at national or international level under any PBR regime. Traditionally, morphological data have been used to define the parameters of certification. However, morphological characters whose expression is affected by environment and wich exhibit continuous distribution are notoriously poor taxonomic descriptors. Therefore, there is growing interest in using biochemical and DNA-based tests to provide sharply defined and reproducible genotypic descriptions. On the other hand, the FAO-CGIAR agreement states that no designated germplasm can be protected under any PBR or patent. We are comparing a patented bean variety with 21 bean genotypes in order to check the condition of newness, using seed proteins (phaseolin) and 16 isoenzymes (with 21 monomorphic and 9 polymorphic loci). Our selection work was facilitated by existing prior art about yellow bean varieties selected in Mexico from Peruvian germplasm (Lépiz & Navarro 1983; Voysest 1983).

### Results

The biochemical data (enzyme bands) were interpreted as dominant markers and were compiled in a data matrix on the basis of presence (1) or absence (0) of selected bands. A pair-wise similarity matrix was calculated using the simple matching coefficient. This similarity matrix was employed to construct a dendrogram by the Unweighted pair group group method with arithmetical averages (UPGMA), using the SAHN-clustering and TREE program from the NTSYS-pc, version 2.02i package. The dendrogram obtained from the isoenzyme profile analysis (Figure 3) shows at the 0.87 similarity level six groups. The first and largest group was formed by 12 varieties, which includes Enola, while the other 10 varieties are separated from the main group. These results will help to select a group of designated germplasm for microsatellite analysis.

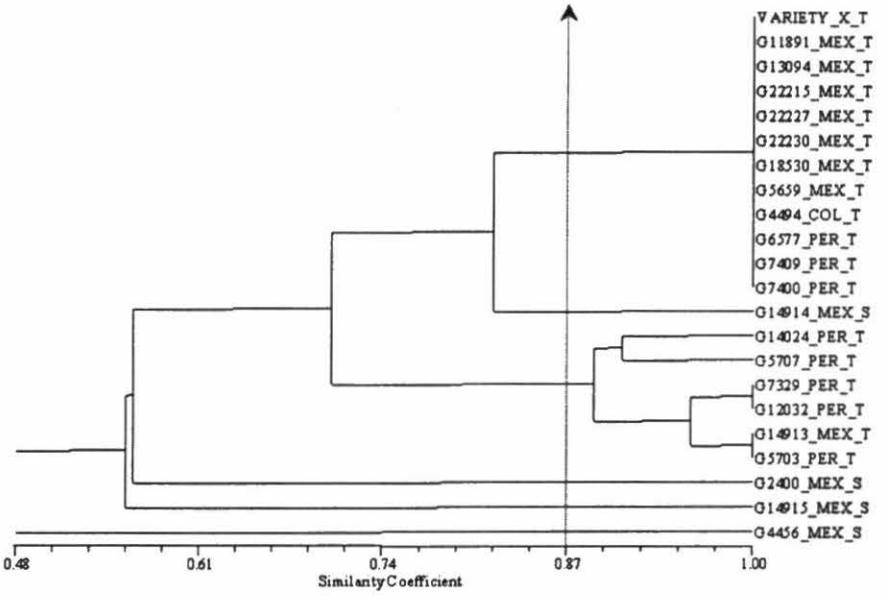


**Fig. 1. Dendrogram derived from a UPGMA cluster analysis, using the Dice similarity index based on isozyme banding patterns. Six clusters were resolved at the 0.87 similarity level.**

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## 1.1.5 Simple Sequence Repeat (SSR) marker diversity in cassava landraces: genetic diversity and differentiation in a predominantly asexually propagated crop

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### Introduction

The study of cassava land races from two Southern Tanzanian districts reported last year was extended to assessing genetic diversity and differentiation of cassava land races from 5 countries in South America, 2 in Central America, and 2 in Africa. A number of elite lines developed at CIAT and IITA were included in the analysis to evaluate the effect of breeding on genetic diversity. SSR marker variation at 67 loci was assessed in 314 accessions of cassava land races from Brazil, Colombia, Peru, Venezuela, Argentina, Guatemala, Mexico, Tanzania, and Nigeria. Accessions from the Neo Tropics were from the CIAT germplasm collections and those from Tanzania were the same field collection made in 1999 in a key introduction point of cassava into Africa (South Western Tanzania) and described in the annual report last year. The Nigerian Land races were from a collection held at IITA, Ibadan.

The main reason for the assessment of genetic diversity and differentiation found in cassava land races is to delineate heterotic pools for a more rational approach to choosing parents for cassava improvement and the exploitation of combining ability via reccurent reciprocal selection (Keeratinijakal and Lamkey 1993). The heterotic patterns found in maize populations at the turn of the century is the basis of a very successful maize hybrid industry and has raised maize yields 500% since 1928 (Shull 1952, Tomes 1998), a high level of genetic differentiation , as revealed by molecular markers, were later found between these populations (Melchinger et. al. 1990).

### Methodology

Plant materials, DNA isolation, and SSR marker analysis have been described elsewhere (CIAT 2000; Fregene et. al. 2001). Genetic diversity within and among accessions was estimated by the software package GEN-SURVEY (Vekemans and Lefebvre 1997) using the following statistics: percentage of polymorphic loci, mean number of alleles per polymorphic loci, average observed heterozygosity, $H_o$, and the average gene diversity, $H_e$ (Nei 1978). For all loci and for all accessions the total heterozygosity, ($H_T$) and the proportion of among accession differentiation ($G_{ST}$) were estimated according to Nei (1978). Standard deviations for the above parameters were estimated over loci and samples by Jackknife (Quenoille 1956; Efron 1982). Given the small evolutionary divergence times for the accessions, the infinite alleles model (IAM) (Goldstein et. al. 1995) was assumed for all calculations.

Genetic differentiation was quantified by the F statistics estimator $F_{ST}$ (theta) (Wright 1951) as described by Weir and Cockerham (1984) using FSTAT 2.9 (Goudet 1998). $G_{ST}$ gives the same estimate of genetic differentiation as $F_{ST}$ but takes into account variation in sample sizes, as is the case in this study. Confidence intervals were calculated per locus over samples, and over loci by Jacknife, and by bootstrapping over loci. Pairwise values of $F_{ST}$ between samples (land race group) was also estimated and the pairwise matrix analyzed by cluster analysis, using Ward's hierachical clustering of JMP (SAS Institute 1995).

To assess if random genetic recombination created by farmer selection from spontaneous seedlings have played a part in the evolution of genetic diversity, parent-offspring relations were sought in the SSR data from the Southern Tanzanian collection using the computer program CERVUS (Marshall et. al. 1998). CERVUS simulates a maternal and a paternal genotype from allele frequencies observed in the study population, and derives an offspring genotype by Mendelian sampling of the parental alleles. The simulation also alters the genotypic data to reflect the existence of un-sampled males, missing loci and incorrectly typed loci, according to the values of the simulation parameters. Each candidate parent is considered in turn as the alleged father, and LOD scores are calculated for all males for whom genetic data exists. Once all males have been considered, the most likely and second most likely males are identified and the Delta score (difference in LOD scores) calculated. The final stage of the simulation is to find critical values of Delta so that the significance of Delta values found in paternity inference in the study population can be tested.

**Results**

The large number of unlinked SSR loci employed in this study enabled a rigorous estimation of genetic differentiation and diversity structure of cassava land races from the primary and secondary center of diversity not previously carried out for cassava. The reliability of estimates for genetic variation, such as $H_e$, $H_o$, $F_{ST}$ and genetic distances, depend more on number of loci than the number of individuals sampled (Baverstock and Moritz 1996). Estimates of genetic differentiation ranged widely from loci to loci, underscoring the danger of assessing SSR diversity using a small set of SSR markers. The genetic diversity of maize as a sub-set of diversity found in its *teosinte* progenitors vary from 25% to 75% based upon what location of the genome the diversity analysis was based on (Eyre-Walker et. al. 1998).

Principal findings of the study is genetic diversity, as assessed by the average gene diversity, $H_e$, was high in all countries with an average heterozygosity of 0.5358±0.1184. (Table 1). Highest genetic diversity was found in Brazil and Colombian, although genetic diversity between Latin American and African land races is comparable. No unique alleles with a frequency of more than 25% was found within country samples with an exception of Guatemala and Nigeria. The genetic differentiation estimator $F_{ST}$ (theta), revealed a low level of differentiation ($F_{ST}$=0.091±005) between country samples compared to the average for crop species - $F_{ST}$ =0.34 (Hammrick and Godt 1997). Nonetheless pair-wise $F_{ST}$ data between countries reveals high genetic differentiation ($F_{ST}$ =0.26) between accessions from Nigeria and Guatemala, and a moderate to high differentiation between country accessions of the primary and a secondary center of diversity (Table 2 and Figure1).

A total of 51 parent-offspring relationships were found in the 96 accessions collected from Southern Tanzania using a delta threshold level of 1.0 (Fregene et. al. 2001, Appendix2). Analysis of parent-offspring are confounded by closely related offspring, the statistic delta calculated by CERVUS compares LOD scores of the two best putative parents to reduce the confounding effects of full- or half-sibs. Results of the parent-offspring relationship successfully identified a known parent of TMS 30572, an improved line from IITA, which was included as an internal control. The genotype 58308 from the Moor plantation, Ibadan, Nigeria, breeding program of the 1950s, served as a parent source of cassava mosaic disease resistance (CMD) for TMS30572.

The overall low level of genetic differentiation in cassava is comparable with that found in perennial forest trees, 0.084 on an average (Hamrick and Godt 1996; Le Corre et. al. 1997). Forest trees have experienced many foundation events after the expansion from a few Southern refuges 15,000 years ago after the last glacial period (Huntley 1990). Austerlitz et. al. (2000) demonstrated that the unexpected low differentiation and high genetic of trees events can be explained by high

gene flow, both seed and pollen flow, and the length of their juvenile phase. Cassava was likely domesticated from populations of *M.esculenta* sub spp *flabellifolia* along the Southern rim of the Amazonian basin within the last 10,000 years ago (Olsen et. al. 1999). Its expansion into other regions of Latin America, Africa and Asia would have led to founders effect of reduced diversity and an increase in genetic differentiation. The unexpected low level of genetic differentiation and the high genetic diversity of cassava land races in all countries may therefore be due to high genetic diversity of original populations, extensive movement of germplasm and spontaneous genetic recombination. The common practice of using volunteer plants and the circulation of woody planting material, often to replace varieties destroyed by herbivores, biotic and abiotic stresses would have lead to highly heterogenous cassava fields after domestication. Diversity found in a single farmer's field has also been shown to be equal to the core-of-the core collection of 38 accessions representative of the world cassava collection at CIAT (Elias et. al. 2000).

**Future Plans**

- A larger sample set of land races from Nigeria and Guatemala regions will be analyzed to confirm results obtained here.
- Genetic crosses between and within Guatamalan land races and Nigerian land races to test correlation between differentiation and heterosis.

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**Table 1. Genetic diversity within groups of cassava land races classified according to country of origin. Standard deviations were estimated by jack-knife over loci. Ht, Hs, Dst and Gst are given over loci and over populations (country collections).**

| Population | Sample size | No. of Loci | No. of Pol. Loci | Percent of Pol. Loc. | Mean No. of alleles/loc. | Mean No. of alleles/pol.loc. | Ho | He | He_p | Fis_p |
|---|---|---|---|---|---|---|---|---|---|---|
| Argentina | 3 | 67 | 57 | 85.1 | 2.6 | 2.9 | 0.5174 | 0.4635 | 0.5672 | 0.0596 |
| Brazil | 20 | 67 | 67 | 100 | 5.2 | 5.2 | 0.5311 | 0.6129 | 0.6285 | 0.146 |
| Colombia | 32 | 67 | 66 | 98.5 | 6 | 6 | 0.5012 | 0.6177 | 0.6277 | 0.1875 |
| GCA | 15 | 67 | 65 | 97 | 4.5 | 4.6 | 0.5244 | 0.5754 | 0.5952 | 0.1072 |
| Guatemala | 4 | 67 | 57 | 85.1 | 2.4 | 2.7 | 0.4925 | 0.396 | 0.4554 | -0.1269 |
| Mexico | 5 | 67 | 64 | 95.5 | 3.6 | 3.7 | 0.4915 | 0.56 | 0.6251 | 0.1987 |
| Peru | 7 | 66 | 62 | 93.9 | 3.7 | 3.9 | 0.4892 | 0.5596 | 0.6067 | 0.1771 |
| Venezuela | 5 | 66 | 64 | 97 | 3.5 | 3.6 | 0.4297 | 0.5692 | 0.634 | 0.2975 |
| Tanz-Mtwara | 84 | 67 | 65 | 97 | 5.3 | 5.4 | 0.543 | 0.558 | 0.5616 | 0.0295 |
| Tanz-Naliendele | 23 | 67 | 64 | 95.5 | 4.5 | 4.7 | 0.5448 | 0.5545 | 0.5667 | 0.034 |
| Tanz-Kibaha | 56 | 67 | 64 | 95.5 | 5.1 | 5.3 | 0.5274 | 0.5334 | 0.5382 | 0.0144 |
| Nigeria | 19 | 66 | 62 | 93.9 | 3.9 | 4 | 0.5965 | 0.5296 | 0.544 | -0.1245 |
| IITA | 6 | 67 | 61 | 91 | 3.2 | 3.4 | 0.4915 | 0.4866 | 0.534 | 0.061 |
| Moor | 4 | 67 | 63 | 94 | 2.7 | 2.8 | 0.51 | 0.4852 | 0.5596 | 0.0572 |
| Mean | | | | 94.23 | 4.03 | 4.17 | 0.5136 | 0.5358 | 0.5745 | 0.0799 |
| std deviation | | 4.45 | 1.11 | 1.06 | 0.0378 | 0.0602 | 0.0495 | 0.1184 | | |
| | Ht | Hs | Dst | Gst | | | | | | |
| Mean | 0.6499 | 0.5812 | 0.0687 | 0.1075 | | | | | | |
| std deviation | 0.1595 | 0.147 | 0.0318 | 0.0565 | | | | | | |
| 95% CI | 0.61 | 0.5463 | 0.0621 | 0.0953 | | | | | | |
| 99% CI | 0.6871 | 0.6136 | 0.0758 | 0.1195 | | | | | | |

Ho Average observed heterozygosity within country
He Average expected heterozygosity within country
He Average expected heterozygosity within country corrected for small sample sizes (Nei 1978)
Ht Total Heterozygosity in the entire data set
Hs Gene diversity within country averaged over the entire data set
Dst Average gene diversity between populations
Gst Coefficient of gene differentiation.

**Table 2. Pairwise estimator of Fst (theta) between pairs of country groupings of cassava land races**

| Population | Arg. | Brazil | Colombia | GCA | Gua. | Mexico | Peru | Ven. | Tanz-Mtwara | Tanz-Naliendele | Tanz-Kibaha | Nigeria | IITA | Moor |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Argentina | 0 | 0.0632 | 0.0708 | 0.068 | 0.2364 | 0.0429 | 0.1279 | 0.0283 | 0.1453 | 0.127 | 0.1654 | 0.2067 | 0.1447 | 0.1631 |
| Brazil | 0.0632 | 0 | 0.0572 | 0.0442 | 0.1454 | 0.0397 | 0.1119 | 0.0297 | 0.0791 | 0.0787 | 0.115 | 0.1076 | 0.1296 | 0.1054 |
| Colombia | 0.0708 | 0.0572 | 0 | 0.0114 | 0.1122 | 0.0379 | 0.0782 | 0.0123 | 0.0922 | 0.0827 | 0.129 | 0.129 | 0.1237 | 0.1137 |
| GCA | 0.068 | 0.0442 | 0.0114 | 0 | 0.1227 | 0.0283 | 0.0967 | 0.0119 | 0.0809 | 0.0682 | 0.1077 | 0.1388 | 0.1275 | 0.1117 |
| Guatemala | 0.2364 | 0.1454 | 0.1122 | 0.1227 | 0 | 0.1468 | 0.1914 | 0.1205 | 0.1682 | 0.1638 | 0.2103 | 0.2696 | 0.2689 | 0.2506 |
| Mexico | 0.0429 | 0.0397 | 0.0379 | 0.0283 | 0.1468 | 0 | 0.0853 | -0.0059 | 0.0984 | 0.082 | 0.1185 | 0.1405 | 0.117 | 0.1118 |
| Peru | 0.1279 | 0.1119 | 0.0782 | 0.0967 | 0.1914 | 0.0853 | 0 | 0.0538 | 0.1417 | 0.1212 | 0.1529 | 0.1877 | 0.1687 | 0.1612 |
| Venezuela | 0.0283 | 0.0297 | 0.0123 | 0.0119 | 0.1205 | -0.0059 | 0.0538 | 0 | 0.0567 | 0.0476 | 0.0839 | 0.1147 | 0.0795 | 0.0593 |
| Tanz-Mtwara | 0.1453 | 0.0791 | 0.0922 | 0.0809 | 0.1682 | 0.0984 | 0.1417 | 0.0567 | 0 | 0.0076 | 0.0452 | 0.1402 | 0.118 | 0.1081 |
| Tanz-Naliendele | 0.127 | 0.0787 | 0.0827 | 0.0682 | 0.1638 | 0.082 | 0.1212 | 0.0476 | 0.0076 | 0 | 0.0097 | 0.1358 | 0.0888 | 0.0919 |
| Tanz-Kibaha | 0.1654 | 0.115 | 0.129 | 0.1077 | 0.2103 | 0.1185 | 0.1529 | 0.0839 | 0.0452 | 0.0097 | 0 | 0.1625 | 0.1121 | 0.1215 |
| Nigeria | 0.2067 | 0.1076 | 0.129 | 0.1388 | 0.2696 | 0.1405 | 0.1877 | 0.1147 | 0.1402 | 0.1358 | 0.1625 | 0 | 0.1605 | 0.1142 |
| IITA | 0.1447 | 0.1296 | 0.1237 | 0.1275 | 0.2689 | 0.117 | 0.1687 | 0.0795 | 0.118 | 0.0888 | 0.1121 | 0.1605 | 0 | 0.0182 |
| Moor | 0.1631 | 0.1054 | 0.1137 | 0.1117 | 0.2506 | 0.1118 | 0.1612 | 0.0593 | 0.1081 | 0.0919 | 0.1215 | 0.1142 | 0.0182 | 0 |

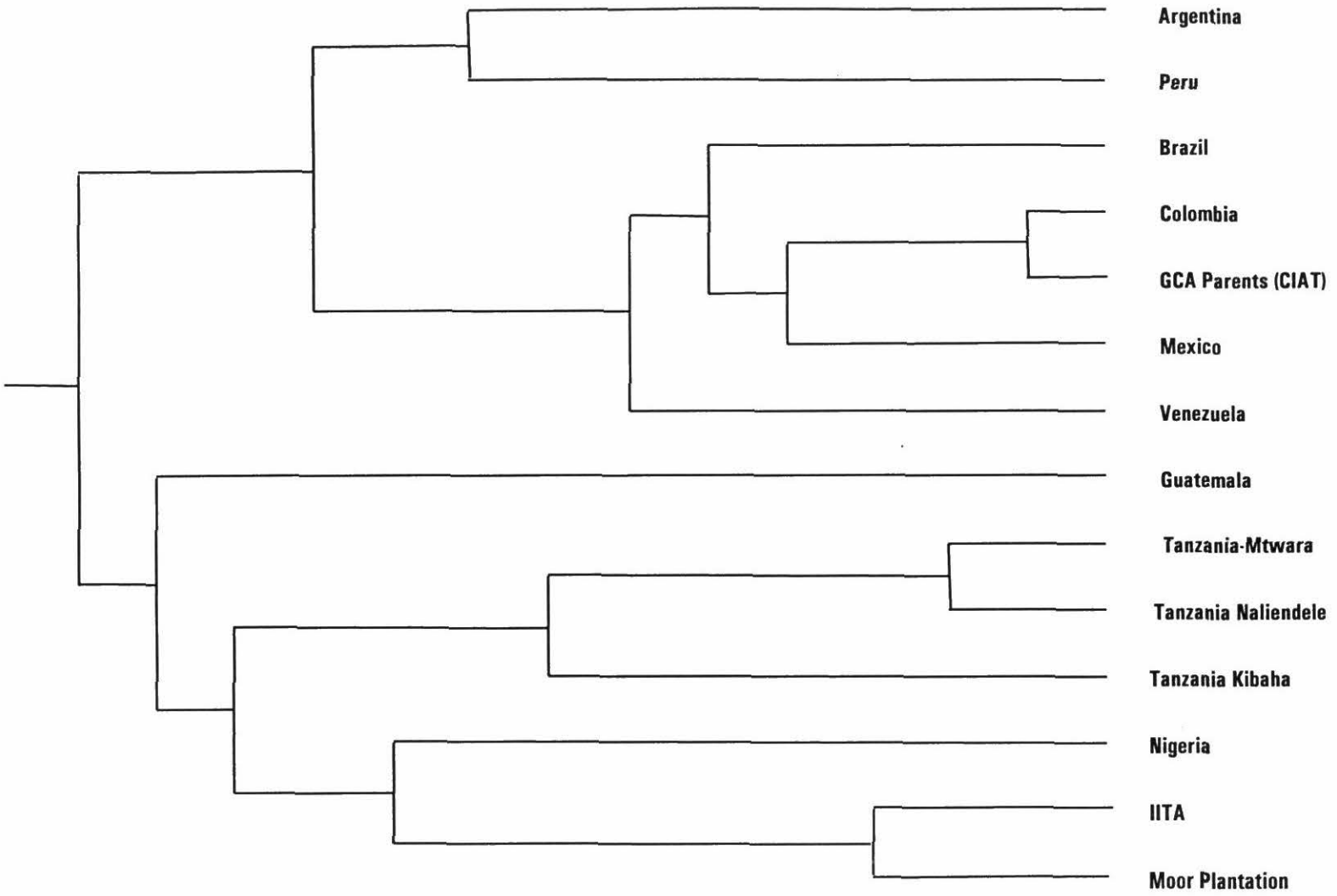


**Figure 1. Hierarchical clustering (Ward) of Fixation Index ($F_{ST}$) pair-wise distances between cassava land races, grouped by country, and elite parents by *source*.**

## 1.1.6 Assessment of genetic diversity among African cassava accessions resistant to the Cassava Mosaic Disease using SSR markers

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**Introduction**

The cassava mosaic virus disease (CMD) is considered the most devastating disease of cassava in Africa causing severe yield losses ranging from 20-95% (Thresh et al., 1994). It is caused by the cassava mosaic begmoviruses, which are transmitted by the whitefly (*Bemisia tabaci* Genn) and spread through propagation of infected vegetative propagules. It is estimated that total crop yield losses due to CMD cost the African continent about $2 billion per annum (IITA, 1997). The most effective means of controlling CMD is by host plant resistance and resistance was first identified in third back cross derivatives between cassava and its wild relative *Manihot glaziovii* Muller von Argau (Nicholas, 1947). Despite the progress made in resistance breeding, there still is the need to increase the levels of resistance, particularly against aggressive recombinant strains that can spontaneously occur (Zhou et. al., 1997). Recently a novel source of resistance controlled by a single dominant gene was found in some Nigerian land races (Mignouna and Dixon 1996) and this has lead to a more systematic evaluation of African land races.

To facilitate choice of parents for breeding more durable CMD resistance while maintaining a good level of genetic diversity, 18 SSR markers were used to evaluate genetic diversity within a collection of 78 African cassava accessions resistant and susceptible to cassava mosaic virus disease (CMD). The accessions include 5 improved accessions, 68 resistant and 10 susceptible land races A total of 18 SSR markers were employed to determine genetic relationships. The second objectives of this study was to predict possible novel sources of resistance to CMD based upon SSR marker clustering which can then serve as a basis for further genetic studies.

**Methodology**

The cassava accessions and their source used in this study are shown in Table 1. The land races had previously been evaluated in several location and years for their reaction to CMD based on their phenotypic expression of symptom severity using the standard five point scoring scale system for CMD (IITA, 1990). DNA isolation was from 1-3 g of young leaves per accession after Dellaporta et al., (1983). Thirty-six SSR markers, two each from 18 linkage groups of the cassava genetic map (Fregene et. al 1997; Mba et. al. 2000) (Table 2), were employed in the initial SSR analysis. SSR analysis was as described in Mba et al. (2000). Individual accessions were scored as diplotypic data "0102" and as haplotypic data "1" presence of a band, and "0" absence of a band for the SSR data. individually and the different alleles were recorded for each sample screened.

The haplotypic data was used to calculate genetic distances between pairs of cassava accessions, using the Dice algorithm, and to draw a dendogram using the Unweighted pair-group mean average (UPGMA) cluster method of Nei's genetic distances (Sneath and Sokal 1973). The genetic distances and dendogram were computed with the NTSYS-PC computer programme, ver. 2.02 (Rohlf 1997). The diplotypic data was employed to calculate estimates of genetic diversity estimates: percentage of polymorphic loci, mean number of alleles per polymorphic loci, average observed heterozygosity, $H_o$, and the average gene diversity, $H_e$ (Nei 1978), using the computer program Gen-Survey (Vekemans and Lefebvre, 1997).

Confidence intervals, at the 95% level, were obtained through 200 bootstraps over loci for the means of the above parameters.

**Results**

The overall level of polymorphism, 92%, is better than that found a previous AFLP study of CMD resistance and susceptible land races, 69%, (Fregene et al., 2000) confirming the superiority of SSR markers for genetic diversity studies. A dendogram of genetic distances grouped the 78 accessions into 5 groups at coefficient of similarity of 0.4. The first group has nine members including the line 58308, the principal parental line for the *M.glaziovii* source of CMD resistance, and its top progeny TMS 30572, the improved accession 91/02324, four resistant and one susceptible land race (see Table 1 for accession groupings). The next group, which was the largest, was made up of two improved accessions M94/0583 and 29 land races, including one susceptible accession. All the resistant land races from the Republic of Benin and the majority of resistant land races from Nigeria and the Togo were in this group. The group also included a resistant land race from Angola and one from Ghana. The third group consists of the improved accession TMS30001, 17 resistant and one susceptible land races. Group four was made up of seven susceptible and five resistant land races, and group five, made up of the improved accession M94/0121and eight other resistant land races. Duplicates were detected between some of the Nigerian land races such as TME581 and TME12, TME5 and TME3, TME62, TME6 and tME4, between TME242 and TME240, TME435 and TME288, TME479 and TME470 and between TME480 and TME225. The clustering pattern of the land races and the level of duplication is in agreement with the AFLP study of Fregene et al., (2000).

Overall genetic diversity of the land races was high, 0.512, comparable to that described for a larger set of land races from 7 African, South and Central American countries, although the large difference in number of markers makes this comparison inadequate (M. Fregene et. al. 2001, CIAT 2001, this report). Gene diversity was highest among the land races and accessions in cluster group 3 followed by those in group 4 then group 2 and the lowest was detected in cluster group 1 (Table2). Of the total genetic diversity, 0.47 was due to within cluster diversity and genetic differentiation between cluster was low (Gst = 0.096). The amount of genetic differentiation which has been reported for cassava, ($G_{ST}$=0.43, Fregene et al., 2000) is higher than that found in this study. Discrepancies in gene diversity estimates have been attributed to nature of markers systems (Djé, et al, 2000), but it may also be due to the small set of African land races and an inclusion of Latin American land races in the Fregene et. al. (2000) study.

Results of this study reveal a substantial amount of genetic diversity in CMD resistance germplasms appropriate for genetic improvement of CMD resistance as well as other traits, particularly yield. It also suggest that there maybe other sources of resistance to CMD other than the known ones based on the clustering pattern of the resistant accessions. The Nigerian land races that have the novel source of resistant cluster together away from land races from other African nations and from the older source of resistance, 58308. This result suggests that resistance to CMD may have arisen independently several times in the past. This result will be confirmed by genetic analysis of crosses between resistant and susceptible land races from clusters other than those with land races bearing currently known sources o resistance.

**Future Plan**

Marker-assisted genetic analysis of crosses between resistant and susceptible land races from clusters other than those with land races bearing currently known sources of resistance.

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**Table 1. List of Cassava accessions their pedigree/local name (or assigned code by Country collectors), country of origin CMD status (R= resistant, S= susceptible) and assigned cluster group of genetic similarity.**

| Accession | Pedigree/Local name | Country | Status | Group |
|---|---|---|---|---|
| 58308 | M.esculenta x M. glaziovii | IITA | R | 1 |
| M94/0583 | | IITA | R | 2 |
| I30001 | | IITA | R | 3 |
| 91/02324 | TME1 OP | IITA | R | 1 |
| M94/0121 | | IITA | R | 5 |
| TME638 | EJ 79 | Ghana | R | 4 |
| TME635 | MNN 55 | Ghana | R | 4 |
| TME631 | SE 210 | Ghana | R | 3 |
| TME630 | Amin | Ghana | R | 4 |
| TME581 | Oke Local | Nigeria | R | 2 |
| TME572 | Udoh Local | Nigeria | R | 5 |
| TME568 | Mundele Paco (ANG-3) | Angola | R | 4 |
| TME565 | Prescose de Angola (ANG-4) | Angola | R | 3 |
| TME546 | SS4 (T8) | Uganda | R | 4 |
| TME526 | Ka13 (Kenya Ostrom) | Côte d'Ivoire | R | 1 |
| TME498 | R.A 16 | Nigeria | R | 2 |
| TME480 | RB92/0119 | Benin | R | 3 |
| TME479 | Agric | Benin | R | 2 |
| TME478 | RB92/0123 | Benin | R | 3 |
| TME477 | RB92/0104 | Benin | R | 3 |
| TME474 | CAP94/064 | Benin | R | 5 |
| TME470 | CAP94066 | Benin | R | 2 |
| TME461 | RB92/0188 | Benin | R | 3 |
| TME456 | CAP94062 | Benin | R | 3 |
| TME455 | RB92/0116 | Benin | R | 2 |
| TME451 | CAP94067 | Benin | R | 2 |
| TME449 | RB92/0182 | Benin | R | 2 |
| TME446 | RB92/0204 | Benin | R | 2 |
| TME443 | CAP94090 | Benin | R | 2 |
| TME437 | RB92/0103 | Benin | R | 5 |
| TME435 | RB92/0175 | Benin | R | 2 |
| TME434 | RB92/0155 | Benin | R | 5 |
| TME431 | MAIN 11 | Togo | R | 1 |
| TME429 | MAIN 4 | Togo | R | 1 |
| TME419 | Gbazekoute | Togo | R | 3 |
| TME379 | Ofegbe | Nigeria | R | 2 |
| TME288 | Akano | Nigeria | R | 2 |
| TME287 | Power | Nigeria | R | 2 |
| TME282 | Alice Local | Nigeria | R | 2 |
| TME279 | Obasanjo | Nigeria | R | 2 |
| TME278 | Oko Warangbala | Nigeria | R | 2 |
| TME258 | 25 | Ghana | R | 1 |
| TME243 | Toma 26 | Togo | R | 3 |
| TME242 | Toma 76 | Togo | R | 2 |
| TME241 | Toma 136 | Togo | R | 3 |
| TME240 | Toma 75 | Togo | R | 2 |
| TME236 | Toma 37 | Togo | R | 5 |

| Accession | Pedigree/Local name | Country | Status | Group |
|---|---|---|---|---|
| TME232 | Toma 63 | Togo | R | 5 |
| TME230 | Toma 36 | Togo | R | 5 |
| TME229 | RB92/0130 | Benin | R | 5 |
| TME228 | Toma 97 | Togo | R | 2 |
| TME225 | 92/0099 | Togo | R | 3 |
| TME209 | 1254(880887) | Cameroon | R | 1 |
| TME204 | RB98/0113 | Benin | R | 3 |
| TME199 | RB89/59 | Benin | R | 3 |
| TTME5 | Bagi Wawa | Nigeria | R | 2 |
| TME62 | Yau Rogor | Nigeria | R | 2 |
| TME13 | MS-20 | Nigeria | R | 2 |
| TME12 | Tokunbo | Nigeria | R | 2 |
| TME11 | Igueeba | Nigeria | R | 3 |
| TME9 | Olekanga | Nigeria | R | 3 |
| TME8 | Amala | Nigeria | R | 3 |
| TME7 | Oko-Iyawo | Nigeria | R | 2 |
| TME6 | Lapai-1 | Nigeria | R | 2 |
| TME4 | Atu | Nigeria | R | 2 |
| TME3 | 2ND Agric | Nigeria | R | 2 |
| TME1 | Antiota | Nigeria | R | 2 |
| I30572 | 58308 X Branca de Santa Caterina OP | IITA | R | 1 |
| TME401 | Toma 141 | Togo | S | 4 |
| TME59 | Dandualla-2 | Nigeria | S | 4 |
| TME60 | Darazo Rogor | Nigeria | S | 2 |
| TME104 | Rogor-5 | Nigeria | S | 3 |
| TME107 | Danwara | Nigeria | S | 4 |
| TME117 | Isunikankiyan | Nigeria | S | 4 |
| TME123 | Panya | Nigeria | S | 4 |
| TME218 | 881260(882160) | Cameroon | S | 4 |
| TME382 | Suleja-5(92/0163) | Nigeria | S | 4 |
| TME557 | Lossakpleh | Côte d'Ivoire | S | 1 |

**Table 2. Gene diversity analysis within and among cassava accessions by cluster group**

| Cluster Group | PLP[a] | A[b] | $A_P$[c] | $H_o$[d] | $H_E$[e] | $H_{EC}$[e] |
|---|---|---|---|---|---|---|
| Cluster Group 1 | 88.9 | 2.7 | 2.9 | 0.4979 | 0.4165 | 0.444 |
| Cluster Group 2 | 94.4 | 2.6 | 2.6 | 0.6431 | 0.4369 | 0.4459 |
| Cluster Group 3 | 100 | 2.8 | 2.8 | 0.6446 | 0.4828 | 0.5006 |
| Cluster Group 4 | 94.4 | 2.7 | 2.7 | 0.6034 | 0.4378 | 0.4605 |
| Cluster Group 5 | 100 | 2.7 | 2.7 | 0.5663 | 0.4624 | 0.5193 |
| Mean | 95.56±4.65 | 2.68±0.06 | 2.73±0.09 | 0.591±0.061 | 0.4473±0.026 | 0.4741±0.034 |
| | Ht | Hs | Dst | Gst | Dm | Rst |
| Mean | 0.514 | 0.466 | 0.048 | 0.096 | 0.0634 | 0.1417 |
| Std | 0.138 | 0.135 | 0.041 | 0.083 | | |
| 95% CI | 0.446 | 0.404 | 0.032 | 0.062 | | |
| 95% CI | 0.583 | 0.533 | 0.065 | 0.135 | | |

[a] Percentage of polymorphic loci at the 5% level within accessions
[b] Mean number of alleles per locus within accessions
[c] Mean number of polymorphic alleles per locus within accessions
d. Average observed heterozygosity within accessions.
[e] Average gene diversity within accessions corrected for small populations

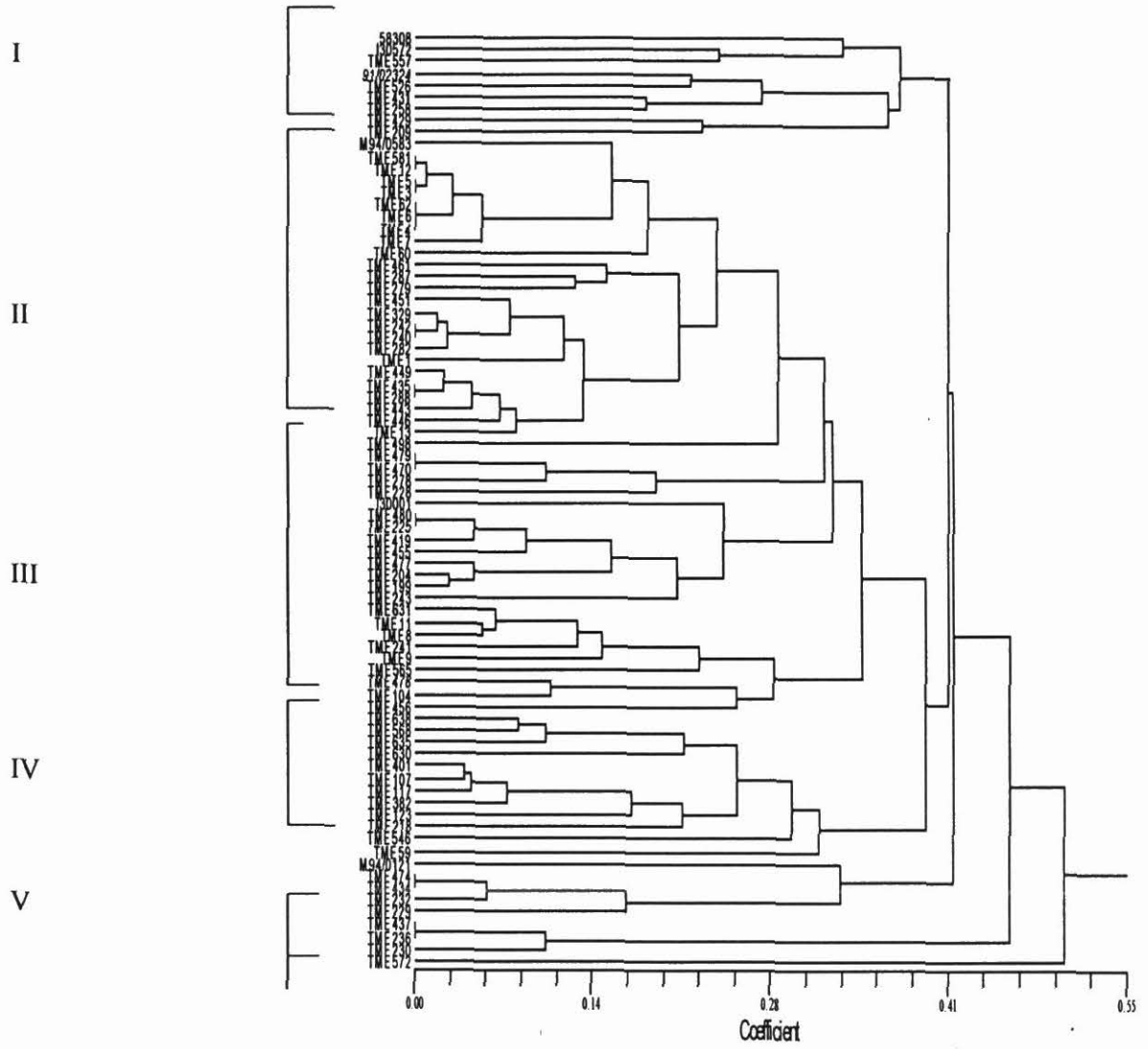


**Figure 1. Dendrogram of genetic distance showing the association between the 78 cassava accessions based on SSR using UPGMA cluster analysis.**

## 1.1.7 Analysis of genetic diversity in Cassava landraces from the coastal, andean and forest region of Peru

Jorge Alcantara[1] Luigi Guarino[2] Martin Fregene[3]
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### Introduction

Peru is considered one of several countries with enormous amounts of biodiversity and it is a center of diversity for numerous cultivated crop, including cassava. The National Genetic Resources and Biotechnology Program (PRONIRGEB, its Spanish Acronym) of the National Institute for Research (INIA, its Spanish acronym) has as its principal objectives the conservation of native germplasm for use by the scientific community and local farmers. PRONIRGEB has two cassava germplasm banks located in Donoso, Lima, with 240 accessions, and El Porvenir, Tarapoto, forest agroecology, having 260 accessions. These accessions are kept as field collections, with a significant portion as tissue culture collections, and they have been characterized morphologically.

Under the project "Models of diversity and genetic erosion of traditional cultivars in Peru: rapid assessment and early detection of risks using GIS tools" funded by the BMZ and executed by IPGRI and INIA, genetic diversity is being assessed using molecular tools combined with GIS methods to provide indicators of genetic erosion. In the first phase of this project a national laboratory for characterizing genetic resources has been set up and training of personnel, in the area of molecular markers for genetic diversity assessment, is being implemented to run the laboratories. The objective of this study was to train a national scientist from INIA in simple sequence repeat (SSR) analysis in cassava, one of crops addressed under the BMZ project.

### Methodology

One hundred accessions from the PRONIRGEB-INIA cassava germplasm collection was used in this study. The accessions were selected based upon the place collected: coastal, Andean or forest, to provide a representative sample of cassava grown in Peru (Table 1). About 100mg of young leaf tissue obtained from field grown plants was used to isolate DNA using a mini CTAB preparation (CIP1997). Eighteen SSR markers, one from each linkage group, and selected for to their high heterozygosity in a previous SSR study of cassava land races (Fregene et. al. 2001, CIAT 2001, this report) were used for SSR analysis as described by Mba et al. (2000). Gel analysis of PCR amplification product is also as described by Mba et. al. (2000).

Raw SSR data was scored as "1" and "0" for presence and absence of DNA bands respectively or haplotype data. The bands were then numbered and the data transformed by Excel to "0102" or diplotype data.. The haplotype data was used to calculate genetic distances between pairs of cassava accessions, using the Dice algorithm, and to derive principal components (PC) (Sneath and Sokal 1973). The first and second components were presented in a graphical form using Excel. The genetic distances were computed with the NTSYS-PC computer programme, ver. 2.02 (Rohlf 1997), while the PC analysis was done using SAS (SAS Institute). The diplotype data was employed to calculate estimates of genetic diversity estimates: percentage of polymorphic loci, mean number of alleles per polymorphic loci, average observed heterozygosity, $H_o$, and the average gene diversity, $H_e$ (Nei 1978), using the computer program Gen-Survey (Vekemans and Lefebvre, 1997). Confidence intervals, at the 95% level, was obtained through 200 bootstraps over loci for the means of the above parameters.

## Results

Average genetic diversity was very high 0.68, and there was no significant difference between diversity found in the three regions (Table1). Genetic differentiation as estimated by Gst was very low (0.0074) and confirms the same pattern found in the study of cassava land races from 7 countries (CIAT2001, this report) although the large difference in markers makes this comparison ineffective. PCA of land races from the coastal, Andean and forest region also did not reveal any distinct clustering pattern among the land races with the exception that a single accession from Brazil included in the analysis was separated from the Peruvian accessions (Figure1).

The present study reveals an unexpected low level of genetic differentiation and high genetic diversity of cassava land races in regions as diverse as the Andean, coastal and forest region of Peru. It strongly supports the hypothesis of extensive movement of germplasm between regions, the high genetic diversity of original populations, as well as the wide adaptation of cassava.

## Future Plans

A set of 36 primers, a gift from CIAT, will be used at PRONIRGEB-INIA to continue SSR analysis of all germplasm accessions held in the National collection.
Conduct a comparative analysis between Peruvian germplasm held at PRONIRGEB-INIA and at CIAT for exchange of land races currently not present in either collection.

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**Table 1. List of Cassava Accessions from the PRONIRGEB-INIA Germplasm Collection and Source**

| It. | Códi. | Región | Departamento | It. | Códi. | Región | Departamento |
|---|---|---|---|---|---|---|---|
| 1. | 181 | Selva | Ucayalí | 51. | 020 | Sierra | Cusco |
| 2. | 145 | Selva | San Martín | | 52. | 001 | Sierra Amazonas |
| 3. | 174 | Selva | Ucayalí | 53. | 037 | Sierra | Huánuco |
| 4. | 049 | Sierra | Junín | 54. | 002 | Selva | Amazonas |
| 5. | 085 | Costa | Lima | 55. | 024 | Sierra | Huánuco |
| 6. | 163 | Selva | Sap Martín | | 56. | 035 | Sierra Huánuco |
| 7. | 101 | Costa | Lima | 57. | 033 | Sierra | Huánuco |
| 8. | 157 | Selva | San Martin | | 58. | 038 | Sierra Huánuco |
| 9. | 180 | Selva | Ucayalí | 59. | 034 | Sierra | Huánuco |
| 10. | 153 | Selva | San Martín | | 60. | 029 | Sierra Huánuco |
| 11. | 143 | Costa | Piura | 61. | 030 | Sierra | Huánuco |
| 12. | 108 | Costa | Lima | 62. | 026 | Sierra | Huánuco |
| 13. | 104 | Costa | Lima | 63. | 027. | Sierra | Huánuco |
| 14. | 082 | Costa | Lima | 64. | 043 | Sierra | Junín |
| 15. | 048 | Sierra | Junín | 65. | 022 | Sierra | Cusco |
| 16. | 139 | Costa | Piura | 66. | 042 | Sierra | Junín |
| 17. | 112 | Costa | Lima | 67. | 031 | Sierra | Huánuco |
| 18. | 117 | Costa | Lima | 68. | 044 | Sierra | Junín |
| 19. | 097 | Costa | Lima | 69. | 023 | Sierra | Cusco |
| 20 | 138 | Costa | Piura | 70. | 036 | Sierra | Huánuco |
| 21. | 165 | Selva | San Martín | | 71. | 004 | Selva Amazonas |
| 22. | 175 | Selva | Ucayalí | 72. | 003 | Selva | Amazonas |
| 23 | 089 | Costa | Lima | 73. | 027 | Sierra | Huánuco |
| 24. | 142 | Costa | Piura | 74. | 045 | Sierra | Junín |
| 25. | 148 | Selva | San Martín | | 75. | 046 | Sierra Junín |
| 26. | 179 | Selva | Ucayalí | 76. | 113 | Costa | Lima |
| 27. | 155 | Selva | San Martín | | 77. | 116 | Costa Lima |
| 28. | 160 | Selva | San Martín | | 78. | 121 | Costa Lima |
| 29. | 144 | Costa | Piura | 79. | 125 | Costa | Lima |
| 30. | 047 | Sierra | Junín | 80. | 129 | Costa | Lima |
| 31 | 094 | Costa | Lima | 81. | 131 | Selva | Loreto |
| 32. | 183 | Selva | Ucayalí | 82. | 135 | Selva | Loreto |
| 33. | 178 | Selva | Ucayalí | 83. | 133 | Selva | Loreto |
| 34. | 173 | Selva | Ucayalí | 84. | 134 | Selva | Loreto |
| 35. | 182 | Selva | Ucayalí | 85. | 136 | Costa | Piura |
| 36. | 158 | Selva | San Martín | | 86. | 137 | Costa Piura |
| 37. | 164 | Selva | San Martín | | 87. | 140 | Costa Piura |
| 38. | 176 | Selva | Ucayalí | 88. | 141 | Costa | Piura |
| 39. | 086 | Costa | Lima | 89. | 146 | Selva | San Martín |
| 40. | 172 | Selva | Ucayalí | 90. | 149 | Selva | San Martín |
| 41. | 040 | Sierra | Huánuco | 91. | 150 | Selva | San Martín |
| 42. | 032 | Sierra | Huánuco | 92. | 151 | Selva | San Martín |
| 43. | 019 | Sierra | Cusco | 93. | 152 | Selva | San Martín |
| 44. | 005 | Selva | Amazonas | | 94. | 154 | Selva San Martín |
| 45. | 025 | Sierra | Huánuco | 95. | 156 | Selva | San Martín |
| 46. | 018 | Sierra | Cusco | 96. | 159 | Selva | San Martín |
| 47. | 081 | Costa | Lima | 97. | 161 | Selva | San Martín |
| 48. | 028 | Sierra | Huánuco | 98. | 162 | Selva | San Martín |
| 49. | 039 | Sierra | Huánuco | 99. | 199 | | Bolivia |
| 50. | 021 | Sierra | Cusco | 100. | 228 | | Brasil |

**Table 2. Gene diversity analysis of 100 accessions from the Peruvian Coastal, Andean and Forest regions**

| Region | Number | PLP[a] | A[b] | $A_P$[c] | $H_o$[d] | $H_E$[e] | $H_{EC}$[e] |
|---|---|---|---|---|---|---|---|
| Coastal | 27 | 100 | 5.2 | 5.2 | 0.7259 | 0.7031 | .7165 |
| Selva | 39 | 100 | 5.2 | 5.2 | 0.6178 | 0.6683 | 0.6770 |
| Sierra | 30 | 100 | 4.9 | 4.9 | 0.6879 | 0.6879 | 0.6994 |
| Mean | | 100 | 5.13±0.18 | 5.13±0.18 | 0.6718 ±0.0540 | 0.6864 ±0.0174 | 0.6976 ±0.0198 |
| | | Ht | Hs | Dst | Gst | | |
| Mean | | 0.7032 | 0.6980 | 0.0052 | 0.0074 | | |
| Std | | 0.0865 | 0.0866 | 0.0086 | 0.0124 | | |
| 95% CI | | 0.6573 | 0.6506 | 0.0009 | 0.0012 | | |

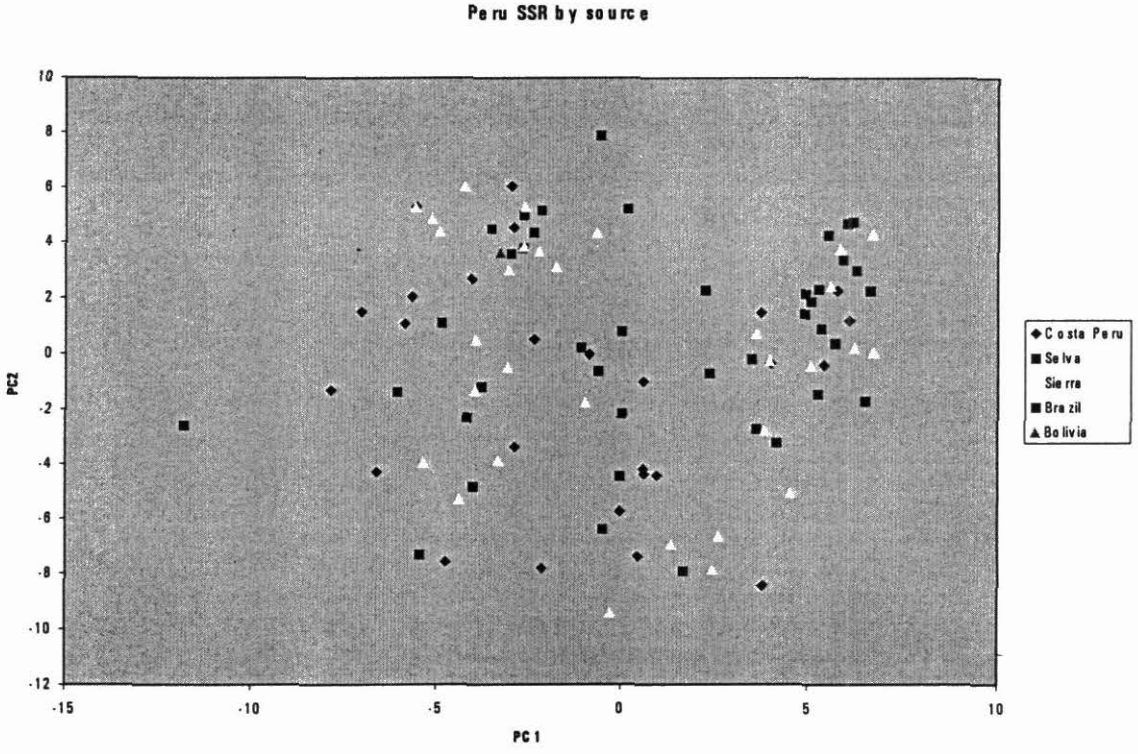


**Figure 1. Principal component analysis (PCA) showing the genetic relationship between 100 cassava accessions from the coastal, Andean and Forest regions of Peru.**

## 1.1.8 Root quality and pest resistance genes from wild relatives of Cassava for broadening the crop genetic base

Nelson Morante[3], Teresa Sanchez[3], Daniel Debouk[1], Antony Bellotti[2], Martin Fregene[2]
[1]SB-1 Project; [2]SB-2 Project ; [3]IP-3 Project

### Introduction

As a major staple food crop across the tropics, cassava can serve as a cheap means of deploying adequate protein requirement amongst the poor and for feeding animals. But cassava's starchy roots are very low in protein compared to other crops; less than 2% dry matter of protein in cassava compared to 9.1% in potato, there is therefore a need to increase the protein content in roots of cassava. Cassava is also an important source of starch, 70-90% of cassava dry root matter is starch, the rest being fibers. Raw or unmodified cassava starches are increasingly important raw materials in textile, alcohol, animal and human food industries world-wide and this is expected to grow (Henry 1995). An increase therefore in starch dry matter content (equivalent to starch content) translates into higher income per unit land, per unit labor (investment) for farmers growing cassava.

Several wild relatives of cassava are known to possess up to 15% protein and more than 50% dry matter in their roots. These germplasm resources are a useful source of genes for the improvement of protein in cassava. Reports of crosses between cassava and *M. tristis* revealed root protein content of more than 8% in $F_1$ hybrids (Bolhuis 1953; Asiedu et. al. 1992). Unfortunately the high protein content was lost during back crossing to recover the desired characteristics and high root yield of cassava (Asiedu 1992). For several years now, it has been shown that the "tremendous genetic potential locked up in germplasm banks can be released by shifting the paradigm from searching for phenotypes to searching for superior genes using molecular genetic maps and an advanced back cross mapping scheme (Tanksley and McCouch, 1997). An evaluation of protein and dry matter content, amylose/amylopectin ratio and white fly resistance was therefore conducted on germplasm resources of 7 wild *Manihot* species held at CIAT genetic resources unit (GRU).

### Methodology

More than 800 sexual seeds representing accessions of *M. esculenta* sub spp *flabelifolia*, *M. esculenta* sub spp *peruviana*, *M. tristis*., *M.carthaginensis*, *M. walkerae*, *M.brachyloba and M.fomentosa* were planted in seedling trays at CIAT. Of these number, 695 accessions were transplanted to the field at the Centro Experimental de la Universidad Nacional, Palmira (CEUNP). From six month after planting, sequential evaluation of white fly resistance was conducted on all genotypes. At 8 months after planting 3 roots were milked from 678 accessions and evaluated for protein content, dry matter percentage, amylose/amylopectin ratio, and storage root size according to standard procedures established at CIAT.

Another set of 400 sexual seeds of inter-specific hybrids between cassava and *M. esculenta* sub spp *flabelifolia*, *M. esculenta* sub spp *peruviana*, *M. tristis*., *M.carthaginensis*, *M. chlorosticta and M. pseudo glaziovii* were germinated. A total of 322 were successfully transplanted to the field and 3 roots evaluated at 8 months after planting for the above traits. To confirm results obtained in the first year, 6 woody stakes were obtained from wild species accessions and inter-specific hybrids high in protein, dry matter, white fly resistance or low in amylose/amylopectin ratio, and planted in single row clonal observation plots at CIAT. At the same time 6-10 stakes of

a selected sub set of these genotypes were planted in a hybridization block to initiate the advanced back cross QTL marker scheme to introgress favorable genes for the above traits into cassava. Due to the poor germination of some accessions, stakes were planted in the green house before transfer to the field.

**Result**

The first year evaluation of more than 1000 genotypes of 7 wild *Manihot* species and inter-specific hybrids revealed a moderate to very high levels for protein and dry matter content, waxy starches (low level of amylose), and white fly resistance. Table 1 shows the data for genotypes with the highest protein content. The best genotypes for white fly resistance were found in inter-specific hybrids with *M. chlorosticta*. (Table 2). A second year evaluation of six plants from the top genotypes will be conducted, but at the same time selections have been made from the top genotypes for genetic crosses. A selection index program developed by the cassava breeding unit (CIAT annual report 2000) was used to select the best 12 genotypes for protein content, dry matter content, and white fly resistance and the best 4 genotypes low amylose content (Table 3).

**Table 1. Best genotypes of wild *Manihot* accessions for protein content from evaluation of 3 roots.**

| Accession Number | Mother Plant | Bulking. | % dry matter | % Crude protein | %c Crude fibre | % ash | % Amylose |
|---|---|---|---|---|---|---|---|
| OW 27- 1 | CTH XXX- 1 | 3 | 10.17 | 7.00 | 4.27 | 3.10 | 10.33 |
| OW 62- 2 | FLA 433- 2 | 0 | 27.04 | 13.08 | | 2.74 | |
| OW 130- 4 | TST XXX- 42 | 2 | 17.92 | 11.77 | | 3.56 | |
| OW 132- 2 | TST XXX- 3 | 4 | 22.40 | 11.71 | 4.69 | 3.14 | 13.98 |
| OW 132- 4 | TST XXX- 3 | 1 | 43.82 | 13.12 | 12.52 | 1.85 | 16.31 |
| OW 139- 1 | TST XXX- 38 | 4 | 29.59 | 11.69 | 8.06 | 1.07 | 18.61 |
| OW 141- 1 | TST XXX- 41 | 0 | 30.43 | 12.15 | 14.72 | 3.08 | |
| OW 143- 1 | TST XXX- 54 | 0 | 34.30 | 12.17 | | 4.79 | |
| OW 145- 2 | TST XXX- 77 | 0 | 24.30 | 13.58 | | 5.41 | |
| OW 145- 3 | TST XXX- 77 | 0 | 34.88 | 14.71 | 22.91 | 2.99 | |
| OW 153- 4 | CTH 409- 1 | 0 | 16.94 | 13.17 | | 3.45 | |
| OW 170- 3 | CTH XXX- 106 | 1 | 29.03 | 12.12 | | 2.65 | |
| OW 172- 1 | CTH XXX- 121 | 1 | 6.47 | 18.99 | | 6.59 | |
| OW 180- 4 | FLA 423- 5 | 4 | 27.47 | 13.50 | 5.38 | | 14.56 |
| OW 183- 4 | FLA 423- 8 | 2 | 21.44 | 14.59 | 3.61 | 3.32 | 9.71 |
| OW 185- 2 | FLA 423- 10 | 1 | 28.97 | 11.77 | 6.97 | 3.56 | 17.19 |
| OW 231- 3 | FLA 444- 7 | 4 | 30.31 | 11.84 | 3.15 | 1.98 | 13.98 |
| OW 235- 1 | FLA 508- 1 | 1 | 35.46 | 12.07 | 5.84 | 1.91 | 13.94 |
| OW 276- 1 | TST XXX- 26 | 1 | 30.48 | 17.34 | 25.53 | 2.64 | |
| OW 278- 1 | TST XXX- 40 | 1 | 41.69 | 16.33 | 36.39 | 1.57 | |
| OW 284- 2 | TST XXX- 77 | 2 | 17.15 | 13.45 | 13.78 | 4.62 | |

Bulking
1=Fibrous roots
2=Poor storage root formation
3 = good but small sized storage root formation
4 = Commercial sized storage roots
5 = Very big commercial sized roots

**Table 2. Best 21 genotypes for white fly resistance evaluated in inter-specific hybrids. All hybrids are with *M.chlorosticta*.**

| | Extent of white fly on leaves | | | | Extent of Damage | | |
|---|---|---|---|---|---|---|---|
| Accession Number | ADULTS | EGGS | NYMPH | PUPAE | UPPER | MED. | LOWER |
| CW 14- 2 | 1.5 | 1.5 | 1.5 | 1.5 | 1.0 | 1.0 | 1.0 |
| CW 14- 3 | 1.0 | 1.0 | 1.0 | 1.5 | 1.0 | 1.0 | 1.0 |
| CW 14- 4 | 2.0 | 2.0 | 2.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| CW 14- 6 | 2.0 | 1.5 | 1.5 | 1.0 | 1.0 | 1.0 | 1.0 |
| CW 14- 7 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| CW 14- 8 | 2.0 | 2.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| CW 14- 9 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| CW 14- 10 | 2.0 | 2.0 | 2.0 | 2.0 | 1.0 | 1.0 | 1.0 |
| CW 14- 11 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| CW 14- 12 | 2.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| CW 14- 13 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| CW 14- 15 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| CW 14- 16 | 2.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| CW 14- 17 | 2.0 | 1.5 | 2.0 | 2.0 | 1.0 | 1.0 | 1.0 |
| CW 20- 1 | 2.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| CW 20- 2 | 2.0 | 1.5 | 1.5 | 1.0 | 1.0 | 1.0 | 1.0 |
| CW 21- 1 | 2.0 | 1.5 | 1.5 | 1.0 | 1.0 | 1.0 | 1.0 |
| CW 21- 2 | 1.0 | 1.0 | 1.0 | 1.5 | 1.0 | 1.0 | 1.0 |
| CW 21- 3 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| CW 21- 4 | 2.0 | 2.0 | 1.5 | 1.5 | 1.0 | 1.0 | 1.0 |
| CW 21- 5 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |

Extent of damage and white fly on leavesis in a scale of 1-5, 1 being no damage or presence and 5 being maximum damage.

**Table 3. Genotypes of wild *Manihot* accessions and inter-specific hybrids with high protein, dry matter content, low amylose (Waxy) and good white fly resistance selected for crosses to elite parents of cassava genepools.**

| Trait | Accession Number | Mother genotype | Number of plants |
|---|---|---|---|
| Waxy | | | |
| | OW 30- 3 | CTH XXX- 62 | 10 |
| | OW 179- 7 | FLA 423- 4 | 10 |
| | OW 183- 4 | FLA 423- 8 | 8 |
| | OW 262- 4 | PER 416- 1 | 10 |
| Protein and Dr Matter (combined) | | | |
| | OW 186- 2 | FLA 426- 3 | 10 |
| | OW 186- 5 | FLA 426- 3 | 8 |
| | OW 189- 1 | FLA 427- 3 | 10 |
| | OW 230- 3 | FLA 441- 5 | 10 |
| | OW 230- 4 | FLA 441- 5 | 10 |
| | OW 231- 2 | FLA 444- 7 | 10 |
| | OW 240- 7 | PER 406- 2 | 10 |
| | OW 257- 1 | PER 413- 5 | 10 |
| | OW 261- 1 | PER 415- 4 | 4 |
| | OW 262- 7 | PER 416- 1 | 10 |
| | OW 263- 4 | PER 416- 2 | 8 |
| | OW 263- 9 | PER 416- 2 | 10 |
| | OW 284- 1 | TST XXX- 77 | 10 |

| Trait | Accession Number | Mother genotype | Number of plants |
|---|---|---|---|
| Protein | | | |
| | OW 66- 5 | FLA 430- 5 | 10 |
| | OW 132- 2 | TST XXX- 3 | 10 |
| | OW 139- 1 | TST XXX- 38 | 10 |
| | OW 179- 1 | FLA 423- 4 | 10 |
| | OW 180- 1 | FLA 423- 5 | 10 |
| | OW 180- 4 | FLA 423- 5 | 10 |
| | OW 181- 2 | FLA 423- 6 | 10 |
| | OW 181- 3 | FLA 423- 6 | 10 |
| | OW 182- 8 | FLA 423- 7 | 10 |
| | OW 230- 6 | FLA 441- 5 | 10 |
| | OW 231- 3 | FLA 444- 7 | 10 |
| | OW 231- 4 | FLA 444- 7 | 5 |
| | OW 235- 3 | FLA 508- 1 | 5 |
| | OW 236- 2 | FMT XXX- 4 | 6 |
| | OW 248- 3 | PER 411- 5 | 10 |
| | OW 280- 1 | TST XXX- 51 | 10 |
| | OW 284- 2 | TST XXX- 77 | 10 |
| Dry Mattter content | | | |
| | OW 95- 1 | PER 412- 4 | 10 |
| | OW 146- 1 | TST XXX- 12 | 10 |
| | OW 213- 4 | FLA 437- 1 | 10 |
| | OW 213- 5 | FLA 437- 1 | 10 |
| | OW 234- 2 | FLA 496- 1 | 10 |
| | OW 240- 6 | PER 406- 2 | 10 |
| | OW 240- 8 | PER 406- 2 | 10 |
| | OW 248- 7 | PER 411- 5 | 10 |
| | OW 252- 2 | PER 412- 4 | 10 |
| | OW 262- 3 | PER 416- 1 | 10 |
| | OW 262- 5 | PER 416- 1 | 9 |
| | OW 269- 4 | PER 417- 6 | 10 |
| | OW 280- 2 | TST XXX- 51 | 10 |
| White fly resistance | | | |
| | OW 36- 2 | | 10 |
| | OW 61- 4 | | 10 |
| | OW 95- 2 | PER 412- 4 | 10 |
| | OW 96- 2 | PER 412- 8 | 10 |
| | OW 100- 2 | | 4 |
| | OW 103- 8 | | 10 |
| | OW 105- 6 | | 5 |
| | OW 105- 7 | | 10 |
| | OW 238- 1 | | 10 |

Although only 4 genotypes from each group will be used for crosses a larger number was selected to accommodate variation that may occur due to the environment. Evaluation for the above traits will therefore be conducted before crosses are made to cassava. ). At least 100 seeds are expected for each of the 48 families (cross combination). The cassava parents for crosses are the elite

parents of the 4 agro-ecological genepools (Table 4). Some of these lines are high carotene lines eg. SM 1433-4, and will lead to a combination of high carotene with high protein content.
The advanced back cross QTL identification and introgression scheme to be followed is briefly described. The $F_1$ families obtained from above will be evaluated twice at the seedling and clonal observation trial stage. The best 10 inter-specific hybrids, 3 for protein, dry matter content, white fly resistance, and one for low amylose, will be selected for each agro-ecology and backcrossed to their respective recurrent parent. At least 200 $BC_1$ seeds will be generated per family, or a total of 6000 seeds from 30 families. The $BC_1$ will be evaluated twice as above and marker genotyping will be for the best $BC_1$ families from each agro-ecology for each trait for QTL analyisis. $BC_1$ lines for the different agro-ecologies bearing favorable QTLs will be inter-crossed with one another,. Genotypes from the $BC_1F_1$ will be planted and evaluated in six-plant rows as described above. Lines found to have high protein and dry matter content will be selected and introduced into the normal breeding program at CIAT. More crosses will be made from the best families identified and sexual seeds will be shared with the International Institute for Tropical Agriculture (IITA), Ibadan and NARs collaborators in Latin America and Asia.

**Table 4. Elite Parents of Cassava Genepools for Advanced backcross QTL mapping scheme**

| Elite Parents by their prospective agro-ecologies |
|---|
| *CLONES FOR THE LOWLAND HUMID AGRO-ECOLOGY*: |
| MTAI -8 |
| SM 1411-5 |
| CM 3306-4 |
| SM 1433-4 |
| *CLONES FOR THE ACID SAVANNAH AGRO-ECOLOGY* |
| CM 523-7 |
| CM 6740-7 |
| CM 4574-7 |
| SM 1821-7 |
| *CLONES FOR THE MID ALTITUTDE AGRO-ECOLOGY* |
| MBRA383 |
| CM 7951-5 |
| SM 909-25 |
| SM 1219-9 |

**Future Plans**

- Genetic crosses of wild *Manihot* species accessions and inter-specific hybrids to elite parents of genepools by agro-ecology
- Second year evaluation of protein, dry matter, white fly resistance or low in amylose/amylopectin ratio in wild *Manihot* species accessions and inter-specific hybrids.

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## 1.1.9 Assessing the genetic variability of *Xanthomonas axonopodis* pv. *manihotis* in Togo by using RFLP

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SB-2 Project

### Introduction

*Xanthomonas axonopodis* pv. *manihotis* (*Xam*) is the causal agent of cassava bacterial blight (CBB). The disease was first reported in Brazil in 1912 and has been reported in Colombia and Venezuela and in most African countries. RFLP has been extensively used to evaluate *Xam* populations using different probes (Verdier et al., 1993; Restrepo and Verdier, 1997; and Restrepo et al., 1999). Based on previous work, it has been suggested that African strains originated from South America (Verdier et al., 1993). 235 strains collected in 14 localities representing the different ecological zones in Togo (West Africa) were analyzed using RFLPs with different probes: *pth*B (a probe containing a pathogenicity gene), pBS8 and pBS6 (two genomic and repetitive probes).

This study aimed to assess the origin and the genetic diversity of the *Xam* population within and between ecological zones in Togo.

### Methodology

*Bacterial isolation from infected leaves.*
Strain isolation was performed as described by Verdier et al. (1998). Briefly angular spots were cut out from leaves and resuspended into eppendorf tubes containing sterile water, then incubated at 4°C for 30min. 100µl were spread in agar plates and incubated at 30°C for 48h. Single colonies were purified and stored at -80°C into a 20% glycerol solution.

*Analysis with different probes.*
DNA purification, restriction and hybridization conditions were done as described by Restrepo and Verdier (1997).

*Statistical analysis.*
Banding patterns of hybridization obtained with the RFLP/*pth*B were used to compare the relatedness of each strain. Each band showing different electrophoretic mobility was assigned a position number after its size was determined in base pairs. The presence (coded 1) and absence (coded 0) of each fragment was recorded for each DNA sample. Similarity among strains was estimated by using NTSYSpc 2.01 program (Rohlf 1994).
The diversity of *Xam* strains from each locality and from the entire collection was calculated by the equation $H = [n/(n\text{-}1)](1\text{-}S X_i^2)$, where $X_i$ is the proportion of the *i*th distinct *pth*B haplotype within a group and $n$ is the number of strains in each group (Nei and Tajima, 1981). The percentage of total variance due to differences within and among localities was calculated using Arlequin 2.0 program (Schneider and Excoffier 2000).

**Results**

RFLP analysis
For the 218 *Xam* strains analyzed, 17 bands were observed using *pth*B fragment as a probe. The molecular weight ranged from 4.6 to 14 kb. Nine different haplotypes (Ht) were defined Of these groups, one included only one strain while two others are represented by 49 and 31 strains respectively (Table 1). Genetic diversities (H) varied from 0 (localities C and F) to 1 (locality I). The total diversity for Togo was 0.66.

**Table 1. RFLP analysis showing strains, number of strains, number of RFLP groups and genetic diversity found in each locality.**

| Locality | No of Haplotypes per locality | Haplotype [b] | No. of strains | H |
|---|---|---|---|---|
| A | 1 | T7 | 24 | 0 |
| B | 3 | T3, T5, T7 | 21 | 0.63 |
| C | 1 | T7 | 5 | 0 |
| D | 3 | T3, T7, T8 | 8 | 0.46 |
| E | 2 | T3, T7 | 13 | 0.28 |
| F | 1 | T3 | 6 | 0 |
| G | 4 | T1, T2, T3, T8 | 13 | 0.68 |
| H | 3 | T3, T6, T7 | 49 | 0.41 |
| I | 2 | T6, T9 | 2 | 1 |
| J | 5 | T1, T2, T3, T6, T7 | 23 | 0.75 |
| K | 3 | T1, T3, T6 | 16 | 0.64 |
| L | 1 | T7 | 1 | ND * |
| M | 5 | T2, T3, T4, T6, T8 | 6 | 0.93 |
| N | 4 | T3, T6, T7, T8 | 31 | 0.55 |

[a] Localities: A, Tove; B, Craf-Kpalime; C, Beme; D, Danyi-Apeyeme; E, Danyi-Apeyeme b; F, Zogbeguan; G, Adeta; H, SotovBova a; I, Blitta; J, SotovBova; K, Piya; L, Kpaka-Doutelbou; M, Landa-Pagouda; N, Davie.
[b] Designation of the haplotype, T = Togo.
[c] ND: not determined.

The cluster analysis revealed the existence of 7 groups at 70% similarity (data not shown). Clusters grouped strains collected from different localities, except cluster 2 that grouped strains from Craf-Kpalime and clusters with only one strain. In general, no correlation between the geographical origin of the strains and DNA polymorphism was observed.

No polymorphism among strains was obtained with pBS6 and pBS8 with all strains showing a unique haplotype with each probe.

*Analysis of molecular variance*
Percentage of total variation was determined among and within populations (localities). We observed that there was more variability within population, 63.6%, that among them, 36.4% (Table 2). Diversity is distributed in a microgeographical scale at the locality level.

**Table 2. Pairwise differences (AMOVA) calculated from RFLP results within and among populations (localities).**

| Source of Variation | d.f. | Variance components | Percentage of variation |
|---|---|---|---|
| Among Populations | 13 | 0.41268 Va | 36.41 |
| Within Populations | 207 | 0.72064 Vb | 63.59 |
| Total | 220 | 1.13332 | |
| Fixation Index FST: 0.36414 | | | |

## Future plans

- To test the pathogenicity of all the strains and characterize the pathotypes of representative strains.
- To recommend a set of strains representative of the genetic diversity to be used for breeding assays.

This work was done with the support of the European project on Cassava bacterial blight in Africa that will be finished at the end of 2001.

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## 1.1.10 Microsatellites to study genetic diversity in Indica and Japonica rice

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### Introduction

Microsatellites markers have become the markers of choice for a wide spectrum of genetic, population, and evolutionary studies (Jarne and Lagoda 1996, Powell et al. 1996). Microsatellite markers are highly informative and they can be rapidly and reliably visualized using silver staining. Although they are expensive to develop, once primers are available, they are technically easy and inexpensive to use. .In rice, microsatellites markers are distributed relatively uniformly throughout the genome and detect a high level of allelic diversity in cultivated varieties and distantly related species. The objective of this study is to initiate the establishment of a sub set of microsatellite primers that can be used to differentiate rice genotypes for selection of parentals in breeding programs.

### Methods

We selected 120 accessions as follows: 93 Indica, 24 Japonica and 3 of the Surinam type (Table 1). Most of the accessions are used Genetic Base of Irrigated Rice in Latin America and the Caribbean 1971 to 1989 for breeding programs . They were characterized on the basis of parentage coefficient (Cuevas et al 1992) and using RAPDs technique (Escobar 1994). We used 12 microstellite primer pairs (Table 2) to characterize accessions.

DNA extractions were carried out according to Dellaporta's modified method . PCR reactions were run according to BRU's methods and gels stained using silver staining. Data were collected in two ways: 1) reading presence (1) or absence (0) of alleles to obtain a binary matrix, or 2) using the concept of "pattern" for each primer pair, which finally constitutes a genotype. The statistical analysis was done using SAS, according to the criteria of Tessier et al. 1999. The tree (not shown) was developed using NTSYS.

### Results

Polymorphism of microsatellites may be analyzed with two parameters:

The Polymorphism Information Content (PIC), where each band corresponds to one allele. PIC is equivalent to H (heterozygosity) and equals = $1-\Sigma f_i^2$ . The average PIC may be calculated as the average of all bands analyzed for all microsatellites.

The parameter D (Discrimination capacity), which is actually derived from PIC. D estimates the discrimination power of primer pairs, by estimating the probability that two randomly chosen individuals have different band patterns. D may also be used for comparisons between different markers (Tessier et. al. 1999).

A total of 89 different bands, or alleles, were found using the 12 primer pairs. The number of different bands par primer pair ranged from 4 in RM7 to 10 in RM11, RM202 and RM225. The estimated average H, using PIC as estimator, was 16.7% for all 120 accessions. Within Indica, the average H was 14.3%, while for Japonica and Surinam it was 15.6% and 15.7% respectively. The results support a low variability within rice germoplasm used for breeding program in Latin America .

The most discriminatory band was number 51, corresponding to primers RM19, which showed 8 alleles. With the analysis of D we can conclude that most primers have a good discrimination capacity (Figure 1), except primers RM7 y RM167 which had low D (30 and 32% respectively). These primers have already already been used for genetic diversity studies among Cuban traditional varieties that are useful for the breeding program in Cuba (Ceaden- CIAT ,2001; data not shown).

**Ongoing Activities**

- Increasing the number of microsatellites in the sub set
- Establishment of a database for eventual selection of parental lines based on molecular diversity

**Table 1. Rice accessions.**

**Japonicas**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 1 | Altamira-7 | 24 | El Paso L-1444 | 47 | Porvenir | 70 | Icta Motagua |
| 2 | BR-IRGA410 | 25 | CR-5272 | 48 | Panama 1048 | 71 | Juma 61 |
| 3 | BR-IRGA412 | 26 | CR-1821 | 49 | Palizada A-86 | 72 | Juma 62 |
| 4 | Chancay | 27 | CR-1707 | 50 | IR665-23-3 | 73 | Metica 1 |
| 5 | Altomayo88 | 28 | CR-1113 | 51 | IRI529 | 74 | Morelos A-88 |
| 6 | Amazonas | 29 | Cimarron | 52 | Inti | 75 | Navolato A-71 |
| 7 | Amistad 82 | 30 | Cica 9 | 53 | INIAP 7 | 76 | Tocumen 5430 |
| 8 | Anayansi | 31 | Cica 8 | 54 | INIAP 6 | 77 | Viflor |
| 9 | Araure 1 | 32 | Cica 7 | 55 | INIAP 415 | 78 | CT6458-9-3-6-M Patselrec |
| 10 | Araure 2 | 33 | Cica 6 | 56 | INIAP 10 | 79 | CT5756-3-5-1-M Patselrec |
| 11 | Araure 3 | 34 | Cica 4 | 57 | ICTA Virginia | 80 | C46-15 |
| 12 | Araure 4 | 35 | Chetomal A-86 | 58 | ICTA Tempisque | 81 | DGWG |
| 13 | Bamoa A-75 | 36 | Juma 58 | 59 | ICTA Quirigua | 82 | Ecia 122-58-1-2-1 Patselrec |
| 14 | BR-IRGA 409 | 37 | Juma 57 | 60 | ICTA Polochi | 83 | P4076F3-2-2-4 Patinlen |
| 15 | BR-IRGA 415 | 38 | Juma 51 | 61 | PA-2 | 84 | P2851F4-145-9-58-1B-10 WC97 |

16 Camponi
17 Cea 1
18 Centa A-1
19 Cea 3
20 Empasc 103
21 Empasc 102
22 Empasc 101
23 El Paso L-227
39 J-104
40 IR8
41 IR84-63-5-18
42 Tanioka
43 San Pedro
44 San Martin 86
45 Saavedra
46 Rustic
62 Oryzica Llanos 5
63 Oryzica Llanos 4
64 Oryzica 2
65 Oryzica 1
66 Empasc 104
67 Empasc 105
68 Huarangopampa
69 IAC 1278
85 P3055F4-3-4P-1P-1B WC-106
86 IR35410-16-3-2-2-2-2- Patir BN
87 IR35353-94-2-1-3 Patir BN87
88 P4725F2-9-6-1X WC153
89 Khao Dawk Mali 105
90 L3
91 CO 39
92 MCVA
93 Carreon

**Indicas**

1 El Paso L-94
2 El Paso L-48
3 Diamante
4 Guay Quiraro
5 Villguay
6 CT6196-33-11-1-3-M WC 5177
7 Tox 340-1-7-3 (ITA 133)- Ace 4
8 CT6240-12-2-2-1-1P WC5178
9 Monolaya
10 Oryzica - Sabana 6
11 Bluerose1268
12 Bluerose1269
13 CT6261-5-7-2P-5-1P WC 5212
14 CT7242-16-9-2-M Patselrec
15 Irat 146 Acc 406
16 Tox 1859-102-GM-3 WC 5036
17 CT6393 M-9-2-5-M Patinlen
18 Marog Paroc Rexoro
19 Pachian Fortuna
20 Takao IKU 18
21 Irat 13
22 Colombia 1
23 Fanny
24 Lac 23

**Surinam**

1 Diwani
2 Ciwini
3 P5589-1-1-3 P-4-MP Patselrec

**Table 2. Microsatellite primers used for genetic diversity analysis. Primers were selected from the kit Primer for Genetic Mapping of the Rice Genome, Research Genetics.**

| Chromosome | Chr. 1 | Chr.2 | Chr.3 | Chr.5 | Chr.6 | Chr.7 | Chr.11 | Chr.12 |
|---|---|---|---|---|---|---|---|---|
| Primer name | RM5 | RM6 | RM7 | RM13 | RM225 | RM4 | RM167 | RM19 |
| | | | RM168 | | | RM11 | RM202 | |
| | | | | | | RM18 | | |

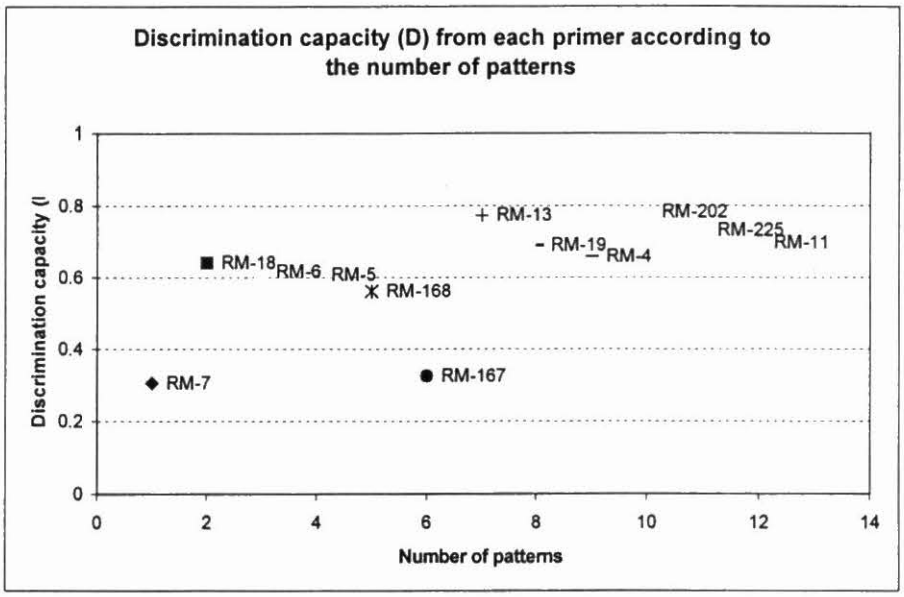


**Figure 1. Discrimination capacity (D) for each primer pair according to the number of band patterns observed**

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## 1.1.11 Gene flow analysis for assessing the safety of transgenic rice in the Tropics

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**Introduction**

Hybridization between crops and their wild relatives sometimes brings genes into wild populations, occasionally resulting in the evolution of aggressive weeds and/ or endangerment of rare species. Transgenic crops may result in similar outcomes. The likelihood of crop-to-wild hybridization depends on the out-cross rate, and on distance and direction between wild and crop populations. Cultivated rice, O. sativa L., is an autogamous plant, with a low outcrossing rate of 0-1%. In wild relatives of rice, rates as high as 56% have been reported (Roberts et al. 1961). Hybridization can be expected within the genomic group that includes O. sativa, viz., the AA group. The wild relatives of AA genome which are found in Central and South America and may hybridize with the rice crop include O. rufipogon (AA, hybrid seed set 19% without and 73% with embryo rescue), and O. glumaepatula (AA, hybrid seed set 39% without embryo rescue) (Oka and Chang, 1961;Vaughan and Tomooka, 1999). Gene transfer from O. sativa to O. rufipogon under field conditions has been documented in Asia and is not restricted by reproductive barriers. Spontaneous intermediates between cultivated rice species and their wild relatives occur frequently in and near rice fields when wild taxa are present. Natural rates of hybridization can be sometimes substantial, and the hybrids usually demonstrate heterosis (hybrid vigor) (Ellstrand et al., 1999). Red rice (Oryza sativa f. spontanea) is a weedy rice with a red pericarp and dark-colored grains. The seeds shatter readily and possess dormancy characteristics; the plants typically are tall, late maturing, and have pubescent leaves and hulls. In contrast to Asia where manual transplanting is still predominant, in tropical America direct seeding of red rice-contaminated seed source is common for a high proportion of rice farmers in Latin America, ensuring field re-infestations and making it one of the most serious weed problems in this region. There are indications that genes placed in cultivated varieties of rice have transferred quickly into red rice. The natural rates of hybridization can range from 1% (with early season variety, flowering at 72-76 days) to 52% (with late season variety, flowering at 82-96 days)( Langevin et al. 1990; Clegg et al. 1993). Thus, cultivated varieties that flower and mature late, like those mainly grown in Latin America, may enable hybridization with red rice to occur throughout several generations. This work is part of a project directed to analyze the gene flow from non-transgenic or transgenic beans and rice into wild/weedy relatives in the Neotropics, and its effect(s) on the population genetic structure of the recipient species. The research will provide guidelines for evaluating the potential risks of using transgenic plants in the tropics, and describe potential areas of gene(s) flow. The information will contribute to improve the risk assessment procedures in the region, in particular for the partner countries Colombia and Costa Rica, which both rank among the countries with the highest biodiversity in the world. The current report summarizes the progress attained during the initiation of the project, towards setting up the tools to assess gene flow from transgenic and non-transgenic rice into wild Oryza species and red rice under experimental plots or narural field conditions.

## Materials and Methods

A preliminary set of 50 microsatellite markers (at least 4 per each chromosome) were selected. Their selection was based on their location on the chromosome (McCouch et al., 1997). At least two markers located distal from the centromere per each chromosome arm were chosen to increase the likelihood of finding recombination between the experimental genotypes. The genotypes includes 9 rice commercial varieties (Cica 8, Cimarrón, Fedearroz 50, Fedearroz 2000, Fedearroz Victoria 1, Iniap 12, Oryzica 1, Oryzica Llanos 5, and Palmar). Sixteen homozygous transgenic Cica 8 rice lines carrying the RHBV-N transgene for rice hoja blanca virus resistance. Four hand made crosses each between one transgenic Cica 8 line and non-transgenic Cica 8, Iniap 12, Fedearroz 50 or Oryzica 1, respectively. One hand made cross each between non-transgenic Cica 8 and Iniap 12, Fedearroz 50 or Oryzica 1, respectively (controls). One hundred and sixty accessions of red rice collected from commercial rice fields in Saldaña, Tolima (Colombia). One accession each of O. rufipogon, O. barthii O. glaberrima. All these genotypes were included in order to select the microsatellites detecting the highest level of polymorfisms among genotypes, and the most polymorphic pairs from each class to conduct the gene flow study from transgenic Cica 8 under experimental conditions, and the corresponding gene flow analysis from commercial varieties under commercial rice field conditions. The markers were amplified at different annealing temperatures according to the estimated melting temperatures of the primers. The PCR products were resolved on silver-stained polyacrylamide gels and microsatellite alleles were sized by comparison to the 10 and 25 bp molecular weight standards (Promega).

## Results and Discussion

Microsatellite analysis is still in progress. Here is presented the preliminary results with a sub-set of the population using 23 microsatellite loci. The first data analysis was design to identify macrosatellites allowing to detect polymorphism generated by potential gene flow from transgenic Cica 8 lines into rice varieties, wild species and red rice. The average rate of polymorphism between Cica 8 and the different varieties ranged from 30% with the variety Fedearroz 50 to 87% with the variety Palmar (Table 1). Between 74% and 83% of the microsatellite markers analyzed were polymorphic between Cica 8 and O. rufipogon, O. galberrima, and *O. barthii*, respectively (Table 1). *O. barthii* and *O. glaberrima* were also included in this study to generate information that may be useful for Asia and Africa. The red rice accession analyzed showed a polymorhism of 56% respect to Cica 8. Only one entry of red rice has been analyzed so far because it was the only one fully characterized morphologically at the initiation of the work. The analysis of the other 159 accessions collected from Tolima (Colombia) is in progress. As expected, results indicate that no polimorphism is detected between non-transgenic Cica 8 variety and transgenic Cica 8 lines (Table 1). Results indicate that the transgenic lines are true-type Cica 8 with the exception of the transgenes introgress in the rice genome. Results also suggest that the change incorporated by transgenesis is so small that it is not resolved by microsatellite analysis. In order to detect gene flow from transgenic Cica 8 into non-transgenic Cica 8 will be by tracing the transgenes. In contrast, polymorfism from 30% to 39% is detected in hand made crosses between Cica 8 and the selected varieties (Table 1). One interpretation of these results is that more genetic changes are introduce by conventional crossing within *O. sativa*, and even more with wild species, than by transgenesis itself.

**Table 1.-Polymorphism rate between rice variety Cica 8 and various rice genotypes for 24 microsatellite loci**

| | T1 | T2 | C/ F50 | C/ I | C/ O | V | CM | P | F2000 | O | I | OL5 | Ob | Og | Or | RR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No | 0 | 0 | 7 | 9 | 8 | 12 | 10 | 20 | 13 | 11 | 11 | 11 | 19 | 18 | 17 | 13 |
| (%) | 0 | 0 | 30 | 39 | 35 | 52 | 43 | 87 | 57 | 48 | 48 | 78 | 83 | 78 | 74 | 56 |

T1= transgenic Cica 8 line A3-49-60-12-3 ; T2= transgenic Cica 8 line A3-49-60-4-13 ; C/ F50 = Cica 8 x Fedearroz 50 ; C / I = Cica 8 x Iniap 12 ; C / O = Cica 8 x Oryzica 1; V = Victoria 1; CM = Cimarron ; P = Palmar ; F2000 = Fedearroz 2000 ; O = Oryzica 1, I = Iniap 12 ; OL5 = Oryzica Llanos 5 ; Ob = Oryza barthii ; Og = Oryza glaberrima ; Or = Oryza ruffipogon ; RR = red rice

## Future Activities

Studies to define the red rice/ rice wild relatives complex in the crop contact zone is important to design biosafety guidelines for the Neotropical region. The spatial distribution of alleles will be used to study local gene flow, including pollen dispersal distances. Microsatellite will be used to trace crop-to wild/red rice gene flow and red rice/wild-to-crop hybridization rate under confined experimental settings as well as under natural conditions. Similar analyses will be conducted to assess transgenic-to non-transgenic variety gene flow. In order to select the red rice genotypes to use for the gene flow studies, a detailed phenology analysis of the 160 red rice accessions is underway to determine those genotypes more prompt to hybridize with cultivated rice. An accession of *O. glumaepatula* was just received from Costa Rica. Future analysis will also include individuals from this species.

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## 1.1.12 Genetic diversity and core collection approaches in the multipurpose shrub legumes Flemingia macrophylla and Cratylia argentea

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### Introduction

The work of CIAT on shrub legumes emphasizes the development of materials to be utilized as feed supplement during extended dry seasons. Tropical shrub legumes of high quality for better soils are readily available, but germplasm with similar characteristics adapted to acid, infertile soils is scarce. *Flemingia macrophylla* and *Cratylia argentea* have shown promising results in such environments and hence work on these genera is part of the overall germplasm strategy of the CIAT Forages team. *C. argentea* is increasingly adopted and utilized, particularly in the seasonally dry hillsides of Central America. However, research and development are based on only few accessions and hence activities to acquire and test novel germplasm of *C. argentea* is of high priority. *F. macrophylla* is also a highly promising shrub legume with excellent adaptation to infertile soils. In contrast to *C. argentea*, whose adaptation is limited to an altitude below 1200 m asl, *F. macrophylla* can successfully be grown up to altitudes of 2000 m asl. However, the potential utilization of *F. macrophylla* is so far limited by the poor quality and acceptability of few evaluated accessions. The project aims to investigate the genetic diversity of *F. macrophylla* and *C. argentea* with three main objectives. Firstly, to identify new, superior forage genotypes based on conventional germplasm characterization/evaluation procedures (morphological and agronomic traits, forage quality parameters, including IVDMD and tannin contents). Secondly, to optimize the use and management, including conservation, of the collections. For this, different approaches to identify core collections for each species will be tested and compared based on, respectively: (a) genetic diversity assessment by agronomic characterization/ evaluation; (b) germplasm origin information; and (c) molecular markers (AFLPs). Thirdly, to create a planning basis for future germplasm collections with respect to methodology, geographical focus and genetic erosion hazards.

### Methods

*Agronomic characterization and evaluation:* Space-planted, single-row plots in RCB design with three replications were established in Quilichao in March 1999 (*Cratylia argentea*, 39 accessions) and March 2000 for (*Flemingia macrophylla*, 73 accessions). Additionally two replications were sown for seed production and morphological observations. The following parameters will be measured in the trials: vigor, height and diameter, regrowth, incidence of diseases, pests and mineral deficiencies, dry matter yield during wet and dry seasons. For the morphological evaluation qualitative and quantitative parameters are measured, such as days to first flower, days to first seed, flower color, flowers per inflorescence, flowering intensity, pod pubescence, seeds per pod, seed color, branching capacity, leaf length and width, ratio, peduncle length, etc. For the analysis of nutritive value, crude protein content and *in vitro* dry-matter digestibility (IVDMD) of the entire collections will be analyzed.

For *F. macrophylla*, a more detailed analysis will be conducted of a representative subset which will include high nutritive value accessions (high crude protein content, high IVDMD) as well as intermediate and low nutritive value accessions. The subsequent analysis will comprise fiber

(NDF, ADF), condensed tannin and hydrolysable tannin contents, tannin purification, calcium, phosphate, ash and organic matter contents.

Based on data referring to the morphological, agronomic and feed quality variation of all accessions a core collection will be created, using multivariate statistic tools (Principal Component Analysis and Cluster Analysis).

*Analysis of available origin information:* Based on ecogeographical information of accession origins, a core collection will be created, hypothesizing that geographic distances and environmental differences are related to genetic diversity. The analysis will be conducted with FloraMap™, a GIS tool developed by CIAT, which allows the production of climate probability models using Principal Component Analysis (PCA) and Cluster Analysis.

*Genetic analysis by molecular markers (AFLPs):* The genetic analysis is conducted using AFLP molecular marker technique (Vos et al, YEAR). Based on the results a core collection will be created, using multivariate statistic tools (PCA and Cluster Analysis).

*Data analysis and synthesis:* Individual and combined data analyses of all generated information will be performed, including the use of GIS tools and multivariate statistics. In the analysis of each of the different approaches (agronomic characterization, origin information, molecular marker analysis), PCA and Cluster Analysis is utilized to create core collections. Eventual correlation between the different approaches and clusters obtained is evaluated. The resulting concept is expected to help deciding which of the three methods or which combination is most appropriate (time and cost efficient) to create a core collection, depending on availability of time and financial resources. E.g., if an agronomic evaluation is not feasible because of time constraints, a core collection may be created using origin information and/or molecular marker analysis.

Based on molecular marker similarities and the GIS analysis, suggestions will be provided for focussing future collections on areas with particularly high diversity, and for collection (= sampling) strategy improvements (e.g., regarding sampling frequency; roadside collections). Accession duplicates in the world collections will be identified.

The project is expected to last two growing seasons and to be terminated at the end of 2003.

**Results and Discussion**

*Agronomic characterization and evaluation:* Preliminary data of one evaluation cut in the dry season and one in the rainy season show considerable phenotypic and agronomic variation for *Cratylia argentea* (Table 1) and *Flemingia macrophylla* (Table 2).

For *Cratylia argentea* IVDMD varied between 61 and 67% and crude protein content between 18 and 21%. Mean dry matter production of *Cratylia argentea* was 45 g/plant in the wet and 60 g/plant in the dry season. According to these initial results the accessions 18674, 22375, 22406, 22408 and 22409 had the highest dry matter yields with between 68 and 100 g/plant. Productivity of these accessions were substantially higher than yields of the material advanced for cultivar release in Costa Rica - an accession mix of 18516/18668. The trial is on-going and quality analyses are pending but preliminary results indicate the potential to identify materials of superior performance to accessions 18516/18668.
Principal component analysis performed with the agronomic data of 39 accessions of *Cratylia argentea* revealed high correlations between total dry matter production, diameter, rebrotes and

vigour (>70%). Cluster analysis (UPGMA) resulted in 9 clusters. 5 of the clusters contained only one accession, among them three of the most productive accessions (18674, 22406 and 22408) (Table 3).

For *Flemingia macrophylla* IVDMD varied between 31 and 51% and crude protein content between 16 and 24%. Mean dry matter production of *Flemingia macrophylla* was 60 g/plant in the wet and 42 g/plant in the dry season. The most productive accessions were C 104890, 21090, 21241, 21529 and 21580 with a total dry matter production > 100 g/plant.

Principal component analysis performed with the agronomic data of 73 accessions of *Flemingia macrophylla* revealed high correlations between total dry matter production, hight, diameter and vigour (>70%). Cluster analysis (UPGMA) resulted in 7 clusters. Two of the clusters contained only one accession, among them one of the most productive accessions (21090) (Table 4). Based on these preliminary feed quality results the following subset has been chosen for subsequent analysis of NDF, ADF, condensed tannin and hydrolysable tannin contents, tannin purification, calcium, phosphate, ash and organic matter contents: 17403, 17407, 18437, 18438, 19457, 20065, 20616, 20621, 20622, 20744, 20975, 20976, 21083, 21087, 21090, 21092, 21249, 21529, 21580, 21982, 21990, 21992, 22082, J001 (total of 24 accessions; 9 erect, 11 semierect, 4 prostrate).

*Analysis of available origin information:* Cluster analysis (UPGMA) was performed with FloraMap™ on the data of 37 accessions of *Cratylia argentea* and 62 accessions of *Flemingia macrophylla* (Figure 1). A first comparison with the clustering according to agronomic data at the level of 9 (*Cratylia argentea*), resp. 7 clusters (*Flemingia macrophylla*) showed no correlation.

*Genetic analysis by molecular markers (AFLPs):* Samples of 5 g of young leaves have been taken of all *Cratylia argentea* and *Flemingia macrophylla* accessions and the DNA has been extracted and quantified (Table 5). To identify efficient primers for the AFLP analysis, 2 supposedly genetically contrasting accessions of each *F. macrophylla* and *C. argentea* (21990, 21529 and 18672 and 18516 respectively) have been tested with different primer combinations and the resulting polymorphic bands have been counted (Table 6).

**Table 1. Agronomic evaluation of a collection of *Cratylia argentea* in Quilichao. Preliminary data of four cuts (two in the dry season and two in the wet season).**

| Treatment | Height | Diameter | Regrowing points | Mean dry matter yields | | | IVDMD | Crude protein |
|---|---|---|---|---|---|---|---|---|
| | | | | Wet | Dry | Total | | |
| No. CIAT | (cm) | (cm) | (No.) | (g/pl) | | | (%) | (%) |
| 18516 | 112 | 105 | 19 | 55 | 78 | 66 | 65.03 | 20.68 |
| 18667 | 112 | 101 | 18 | 45 | 68 | 56 | 64.62 | 20.36 |
| 18668 | 106 | 110 | 17 | 48 | 68 | 58 | 65.20 | 19.88 |
| 18671 | 111 | 106 | 20 | 54 | 55 | 54 | 64.30 | 18.26 |
| 18672 | 96 | 83 | 13 | 34 | 39 | 37 | 62.12 | 20.10 |
| 18674 | 118 | 122 | 23 | 91 | 109 | 100 | 63.88 | 19.98 |
| 18675 | 112 | 97 | 15 | 47 | 63 | 55 | 63.34 | 19.03 |
| 18676 | 105 | 93 | 14 | 46 | 50 | 48 | 61.16 | 19.66 |
| 18957 | 111 | 102 | 16 | 50 | 76 | 63 | 62.47 | 20.08 |
| 22373 | 109 | 93 | 15 | 38 | 57 | 48 | 64.37 | 20.18 |
| 22374 | 116 | 102 | 17 | 55 | 71 | 63 | 66.39 | 19.57 |
| 22375 | 125 | 98 | 16 | 59 | 76 | 68 | 67.03 | 21.18 |
| 22376 | 95 | 70 | 11 | 23 | 36 | 29 | 64.14 | 19.61 |
| 22378 | 103 | 81 | 12 | 34 | 39 | 36 | 61.75 | 19.76 |
| 22379 | 111 | 89 | 16 | 47 | 65 | 56 | 63.51 | 19.60 |
| 22380 | 107 | 90 | 11 | 31 | 43 | 37 | 61.31 | 20.42 |
| 22381 | 105 | 85 | 11 | 34 | 46 | 40 | 63.98 | 19.15 |
| 22382 | 110 | 92 | 12 | 41 | 62 | 52 | 64.16 | 20.44 |
| 22383 | 99 | 90 | 13 | 34 | 43 | 39 | 62.57 | 18.60 |
| 22384 | 113 | 91 | 9 | 43 | 47 | 45 | 64.52 | 18.89 |
| 22386 | 111 | 86 | 12 | 39 | 47 | 43 | 64.67 | 18.62 |
| 22387 | 111 | 90 | 12 | 41 | 57 | 49 | 62.55 | 19.10 |
| 22390 | 99 | 92 | 13 | 45 | 47 | 46 | 64.81 | 18.49 |
| 22391 | 108 | 96 | 15 | 44 | 62 | 53 | 63.36 | 18.88 |
| 22392 | 114 | 83 | 13 | 33 | 53 | 43 | 63.19 | 21.04 |
| 22393 | 110 | 92 | 17 | 41 | 58 | 49 | 63.52 | 20.65 |
| 22394 | 112 | 88 | 13 | 33 | 46 | 40 | 63.96 | 20.52 |
| 22396 | 101 | 79 | 10 | 30 | 43 | 36 | 63.78 | 21.32 |
| 22399 | 102 | 86 | 13 | 35 | 42 | 39 | 66.13 | 19.85 |
| 22400 | 119 | 104 | 16 | 52 | 74 | 63 | 61.66 | 20.72 |
| 22404 | 110 | 97 | 13 | 42 | 68 | 55 | 67.00 | 20.91 |
| 22405 | 111 | 96 | 16 | 41 | 61 | 51 | 62.86 | 19.91 |
| 22406 | 113 | 112 | 20 | 63 | 86 | 74 | 62.59 | 20.96 |
| 22407 | 111 | 99 | 16 | 46 | 59 | 53 | 65.28 | 20.81 |
| 22408 | 120 | 109 | 18 | 69 | 88 | 79 | 67.16 | 20.06 |
| 22409 | 113 | 115 | 17 | 57 | 81 | 69 | 66.46 | 21.25 |
| 22410 | 116 | 96 | 14 | 42 | 60 | 51 | 64.28 | 19.80 |
| 22411 | 103 | 88 | 14 | 37 | 58 | 47 | 64.46 | 20.23 |
| 22412 | 116 | 90 | 11 | 42 | 63 | 52 | 64.91 | 18.66 |
| Mean | 110 | 95 | 15 | 45 | 60 | 52 | 64.06 | 19.93 |
| Range | 95-125 | 70-122 | 9-23 | 23-91 | 36-109 | 29-100 | 61-67 | 18-21 |

**Table 2. Agronomic evaluation of a collection of *Flemingia macrophylla* in Quilichao.**

| Treatment | Height | Diameter | Regrowing points | Mean dry matter yields | | | IVDMD | Crude protein |
|---|---|---|---|---|---|---|---|---|
| | | | | Wet | Dry | Total | | |
| No. CIAT | (cm) | (cm) | (No.) | (g/pl) | | | (%) | (%) |
| J 001 (e) | 125 | 85 | 30 | 102 | 58 | 80 | 40.07 | 22.33 |
| 801 (e) | 125 | 90 | 29 | 103 | 62 | 82 | 36.27 | 22.89 |
| 7184 (e) | 124 | 95 | 34 | 101 | 82 | 92 | 33.97 | 21.35 |
| C 10489 (e) | 108 | 99 | 34 | 121 | 79 | 100 | 33.65 | 22.74 |
| I 15146 (e) | 98 | 70 | 24 | 103 | 58 | 80 | 39.88 | 22.85 |
| 17400(s) | 63 | 98 | 33 | 55 | 52 | 53 | 33.23 | 21.5 |
| 17403 (s) | 67 | 96 | 32 | 68 | 57 | 62 | 35.84 | 22.17 |
| 17404 (s) | 58 | 79 | 32 | 46 | 45 | 45 | 32.91 | 22.46 |
| 17405 (s) | 65 | 94 | 36 | 71 | 67 | 69 | 36.12 | 21.93 |
| 17407 (s) | 78 | 106 | 39 | 87 | 62 | 74 | 32.85 | 21.87 |
| 17409 (s) | 56 | 109 | 35 | 87 | 66 | 77 | 33.00 | 20.21 |
| 17411 (s) | 55 | 86 | 33 | 56 | 54 | 55 | 35.55 | 22.36 |
| 17412 (s) | 73 | 96 | 39 | 61 | 63 | 62 | 38.63 | 20.21 |
| 17413 (s) | 58 | 93 | 35 | 51 | 39 | 45 | 35.19 | 20.1 |
| 18048 (s) | 32 | 43 | 19 | 12 | 8 | 10 | 42.84 | 20.38 |
| 18437 (s) | 54 | 101 | 37 | 57 | 55 | 56 | 47.85 | 22.55 |
| 18438 (s) | 58 | 71 | 31 | 36 | 22 | 29 | 51.46 | 23.46 |
| 18440 (s) | 59 | 87 | 38 | 65 | 44 | 55 | 33.39 | 21.35 |
| 19453 (e) | 105 | 78 | 20 | 65 | 33 | 49 | 36.03 | 21.64 |
| 19454 (e) | 115 | 82 | 24 | 73 | 52 | 63 | 39.14 | 19.66 |
| 19457 (e) | 116 | 85 | 25 | 52 | 64 | 58 | 33.09 | 21.3 |
| 19797 (s) | 57 | 90 | 22 | 58 | 46 | 52 | 38.50 | 20.98 |
| 19798 (s) | 55 | 95 | 27 | 61 | 55 | 58 | 38.27 | 20.92 |
| 19799 (s) | 50 | 69 | 19 | 28 | 39 | 33 | 37.34 | 21.68 |
| 19800 (s) | 65 | 85 | 29 | 34 | 48 | 41 | 32.00 | 20.71 |
| 19801 (s) | 82 | 91 | 40 | 68 | 57 | 63 | 35.74 | 21.75 |
| 19824 (e) | 62 | 93 | 35 | 54 | 61 | 58 | 35.57 | 21.33 |
| 20065 (p) | 15 | 21 | 4 | 0 | 1 | 1 | 32.14 | 18.86 |
| 20616 (s) | 67 | 108 | 34 | 86 | 54 | 70 | 32.10 | 21.99 |
| 20617 (s) | 72 | 92 | 27 | 51 | 44 | 48 | 30.64 | 20.14 |
| 20618 (s) | 74 | 95 | 31 | 57 | 60 | 58 | 33.23 | 21.57 |
| 20621 (e) | 84 | 88 | 32 | 58 | 54 | 56 | 31.59 | 21.57 |
| 20622 (e) | 146 | 88 | 30 | 105 | 76 | 91 | 42.81 | 22.86 |
| 20624 (s) | 74 | 122 | 39 | 101 | 91 | 96 | 34.51 | 19.83 |
| 20625 (e) | 128 | 86 | 26 | 105 | 69 | 87 | 42.49 | 22.81 |
| 20626 (e) | 115 | 92 | 28 | 88 | 70 | 79 | 39.55 | 22.3 |
| 20631 (e) | 121 | 90 | 25 | 97 | 75 | 86 | 41.46 | 20.92 |
| 20744 (e) | 125 | 87 | 27 | 102 | 65 | 84 | 42.95 | 23.09 |
| 20972 (p) | 24 | 56 | 31 | 12 | 14 | 13 | 39.65 | 23.42 |
| 20973 (p) | 24 | 45 | 17 | 4 | 10 | 7 | 34.17 | 19.65 |
| 20975 (s) | 52 | 83 | 45 | 44 | 24 | 34 | 45.28 | 20.31 |
| 20976 (s) | 45 | 57 | 27 | 17 | 11 | 14 | 40.79 | 20.01 |
| 20977 (s) | 33 | 35 | 9 | 5 | 4 | 4 | 46.14 | 18.46 |
| 20978 (s) | 52 | 56 | 24 | 21 | 11 | 16 | 46.61 | 22.08 |
| 20979 (s) | 48 | 76 | 38 | 27 | 25 | 26 | 38.90 | 21.17 |
| 20980 (s) | 43 | 55 | 26 | 27 | 18 | 22 | 41.80 | 20.95 |
| 20982 (s) | 49 | 61 | 28 | 26 | 23 | 25 | 40.96 | 19.94 |

| Treatment No. CIAT | Height (cm) | Diameter (cm) | Regrowing points (No.) | Mean dry matter yields | | | IVDMD (%) | Crude protein (%) |
|---|---|---|---|---|---|---|---|---|
| | | | | Wet (g/pl) | Dry | Total | | |
| 21079 (s) | 47 | 78 | 44 | 51 | 25 | 38 | 37.87 | 20.17 |
| 21080 (s) | 41 | 58 | 13 | 32 | 11 | 21 | 39.21 | 15.51 |
| 21083 (e) | 93 | 79 | 36 | 71 | 43 | 57 | 45.78 | 21.43 |
| 21086 (s) | 27 | 29 | 4 | 0 | 3 | 3 | - | - |
| 21087 (s) | 64 | 66 | 46 | 47 | 32 | 39 | 42.32 | 20.37 |
| 21090 (s) | 88 | 106 | 48 | 135 | 66 | 100 | 50.02 | 21.33 |
| 21092 (s) | 72 | 81 | 23 | 57 | 39 | 48 | 49.15 | 18.01 |
| 21241 (e) | 133 | 93 | 27 | 134 | 66 | 100 | 36.17 | 20.19 |
| 21248 (e) | 127 | 92 | 30 | 106 | 77 | 91 | 33.52 | 23.58 |
| 21249 (e) | 129 | 104 | 34 | 167 | 85 | 126 | 40.86 | 21.95 |
| 21519 (e) | 127 | 101 | 28 | 109 | 67 | 88 | 39.52 | 22.32 |
| 21529 (e) | 132 | 102 | 31 | 145 | 71 | 108 | 42.01 | 23.11 |
| 21580 (e) | 131 | 101 | 32 | 184 | 86 | 135 | 39.08 | 19.83 |
| 21982 (p) | 19 | 62 | 38 | 26 | 11 | 19 | 42.13 | 20.86 |
| 21990 (p) | 35 | 66 | 43 | 27 | 19 | 23 | 31.94 | 19.13 |
| 21991 (p) | 29 | 52 | 24 | 13 | 10 | 11 | 37.47 | 22.59 |
| 21992 (p) | 29 | 50 | 24 | 12 | 9 | 11 | 48.52 | 20.24 |
| 21993 (s) | 42 | 77 | 45 | 34 | 24 | 29 | 43.90 | 19.88 |
| 21994 (p) | 24 | 42 | 9 | 8 | 7 | 8 | 34.53 | 16.43 |
| 21995 (p) | 29 | 50 | 26 | 11 | 8 | 9 | 40.80 | 19.6 |
| 21996 (p) | 23 | 44 | 14 | 7 | 6 | 6 | 42.49 | 21.8 |
| 22058 (e) | 84 | 58 | 13 | 41 | 29 | 35 | 37.20 | 18.48 |
| 22082 (s) | 79 | 82 | 58 | 69 | 37 | 53 | 48.35 | 20.01 |
| 22087 (p) | 27 | 51 | 17 | 15 | 4 | 10 | 40.34 | 17.78 |
| 22090 (s) | 44 | 47 | 10 | 10 | 5 | 7 | 41.10 | 17.52 |
| 22285 (s) | 43 | 75 | 42 | 32 | 21 | 27 | 38.72 | 20.43 |
| 22327 (s) | 41 | 62 | 33 | 20 | 21 | 21 | 48.41 | 19.28 |
| Mean | 70 | 78 | 29 | 60 | 42 | 51 | 39.01 | 20.91 |
| Range | 15-146 | 21-122 | 4-58 | 0-184 | 1-91 | 1-135 | 31-51 | 16-24 |

Preliminary data of two cuts (one in aech season). Growth habit: e = erect, s = semierect, p = prostrate.

**Table 3. Cluster analysis for *Cratylia argentea* according to agronomic and climatic (Floramap) data.**

| ID | CIAT-No. | Elevation | Longitude | Latitude | Cluster (agron. data) | Cluster (Floramap) |
|---|---|---|---|---|---|---|
| 10 | 22373 | 780 | -46.3833 | -14.0833 | 1 | 3 |
| 12 | 22375 | 620 | -46.6167 | -13.0000 | 1 | 3 |
| 36 | 22411 | 550 | -46.8833 | -13.1167 | 1 | 4 |
| 24 | 22392 | 240 | -56.0000 | -15.6333 | 1 | 6 |
| 26 | 22394 | 270 | -57.8167 | -15.8667 | 1 | 6 |
| 27 | 22396 | 510 | -51.0500 | -16.3833 | 1 | 6 |
| 25 | 22393 | 210 | -56.1333 | -15.7000 | 1 | 8 |
| 31 | 22405 | 700 | -46.8833 | -13.1167 | 1 | 8 |
| 33 | 22407 | 500 | -46.8833 | -13.1167 | 1 | 8 |
| 35 | 22409 | 540 | -45.0000 | -20.0167 | 1 | 8 |
| 1 | 18516 | 800 | -46.4167 | -13.3667 | 2 | 1 |
| 4 | 18671 | 230 | -56.9333 | -14.6833 | 2 | 3 |
| 11 | 22374 | 660 | -46.4167 | -13.2833 | 2 | 3 |
| 2 | 18667 | 460 | -55.6667 | -15.6333 | 2 | 5 |
| 3 | 18668 | 180 | -56.2167 | -15.4167 | 2 | 5 |
| 15 | 22379 | 390 | -49.2000 | -13.4167 | 2 | 5 |
| 22 | 22390 | 300 | -54.8833 | -16.0167 | 2 | 5 |
| 5 | 18672 | 140 | -55.2333 | -3.7500 | 3 | 3 |
| 9 | 18957 | 350 | -48.6167 | -6.5000 | 3 | 3 |
| 14 | 22378 | 390 | -49.0333 | -13.6333 | 3 | 3 |
| 7 | 18675 | 380 | -52.3167 | -14.9167 | 3 | 5 |
| 16 | 22380 | 360 | -49.5167 | -13.2333 | 3 | 5 |
| 17 | 22381 | 365 | -50.0667 | -13.2500 | 3 | 5 |
| 18 | 22382 | 330 | -50.2000 | -13.2833 | 3 | 5 |
| 20 | 22386 | 320 | -52.3500 | -14.5667 | 3 | 5 |
| 19 | 22384 | 360 | -52.1667 | -14.2333 | 3 | 7 |
| 23 | 22391 | 660 | -55.5000 | -15.8167 | 3 | 7 |
| 21 | 22387 | 370 | -52.4167 | -15.8333 | 3 | 8 |
| 29 | 22400 | 560 | -46.6667 | -13.1667 | 3 | 8 |
| 37 | 22412 | 400 | -51.7333 | -15.8667 | 3 | 9 |
| 28 | 22399 | 660 | -46.4000 | -13.5000 | 4 | 8 |
| 30 | 22404 | 700 | -46.8833 | -13.1167 | 4 | 8 |
| 13 | 22376 | 580 | -49.1500 | -14.3833 | 5 | 5 |
| 6 | 18674 | 320 | -52.3333 | -14.5667 | 6 | 5 |
| 32 | 22406 | 780 | -46.8833 | -13.1167 | 7 | 8 |
| 34 | 22408 | 810 | -46.8833 | -13.1167 | 8 | 8 |
| 8 | 18676 | 450 | -51.6333 | -16.5667 | 9 | 2 |

**Table 4. Cluster analysis for *Flemingia macrophylla* according to agronomic and climatic (Floramap) data.**

| ID | CIAT-No. | Elevation | Longitude | Latitude | Cluster (agron. data) | Cluster (Floramap) |
|---|---|---|---|---|---|---|
| 10 | 18048 | 150 | 109.1000 | 19.3833 | 1 | 3 |
| 19 | 19799 | 150 | 102.0333 | -2.1333 | 1 | 3 |
| 32 | 20972 | 50 | 110.4333 | 18.9167 | 1 | 3 |
| 58 | 22087 | 160 | 103.6833 | 18.3333 | 1 | 3 |
| 35 | 20976 | 330 | 109.5000 | 18.7500 | 1 | 4 |
| 36 | 20977 | 220 | 109.4667 | 18.9167 | 1 | 4 |
| 51 | 21991 | 40 | 108.8333 | 15.4000 | 1 | 4 |
| 54 | 21994 | 700 | 108.2167 | 14.0000 | 1 | 4 |
| 55 | 21995 | 800 | 107.7667 | 12.4167 | 1 | 4 |
| 59 | 22090 | 170 | 104.2500 | 17.8833 | 1 | 4 |
| 33 | 20973 | 70 | 110.3333 | 18.7833 | 1 | 5 |
| 39 | 20980 | 200 | 109.1000 | 19.3833 | 1 | 6 |
| 40 | 20982 | 140 | 109.5667 | 19.5000 | 1 | 6 |
| 42 | 21080 | 370 | 99.1333 | 17.6500 | 1 | 6 |
| 56 | 21996 | 450 | 107.4000 | 11.9333 | 1 | 6 |
| 1 | 17400 | 160 | 102.8000 | 16.6833 | 2 | 1 |
| 5 | 17407 | 50 | 99.9167 | 8.3000 | 2 | 1 |
| 17 | 19797 | 180 | 100.0167 | 0.5667 | 2 | 1 |
| 2 | 17403 | 40 | 99.0667 | 10.0667 | 2 | 2 |
| 3 | 17404 | 50 | 99.1833 | 9.4167 | 2 | 2 |
| 4 | 17405 | 70 | 99.3833 | 8.9833 | 2 | 2 |
| 6 | 17409 | 70 | 100.2833 | 6.6500 | 2 | 3 |
| 7 | 17411 | 110 | 102.4333 | 3.3333 | 2 | 3 |
| 8 | 17412 | 50 | 102.4167 | 5.7667 | 2 | 3 |
| 9 | 17413 | 30 | 102.1833 | 6.0500 | 2 | 3 |
| 13 | 18440 | 30 | 102.4833 | 12.2833 | 2 | 3 |
| 18 | 19798 | 190 | 100.8333 | -0.7333 | 2 | 3 |
| 20 | 19800 | 140 | 103.6000 | -3.7833 | 2 | 3 |
| 21 | 19801 | 520 | 103.4167 | -3.9500 | 2 | 3 |
| 22 | 19824 | 130 | 100.6000 | -0.6500 | 2 | 3 |
| 23 | 20616 | 690 | 96.7500 | 4.7333 | 2 | 3 |
| 24 | 20617 | 560 | 96.7000 | 5.0167 | 2 | 3 |
| 25 | 20618 | 250 | 95.4833 | 5.4000 | 2 | 3 |
| 26 | 20621 | 1100 | 97.6167 | 3.7000 | 2 | 3 |
| 28 | 20624 | 30 | 99.7333 | 1.1833 | 2 | 3 |
| 38 | 20979 | 370 | 109.3833 | 19.2333 | 2 | 4 |
| 49 | 21982 | 70 | 107.5333 | 16.4000 | 2 | 4 |
| 50 | 21990 | 80 | 108.3667 | 15.4667 | 2 | 4 |
| 60 | 22285 | 80 | 108.9833 | 12.5500 | 2 | 6 |
| 41 | 21079 | 250 | 99.4500 | 14.7667 | 2 | 7 |
| 14 | 19453 | 1100 | 146.5667 | -6.9500 | 3 | 1 |
| 15 | 19454 | 650 | 146.5833 | -7.2167 | 3 | 1 |
| 16 | 19457 | 1630 | 145.3667 | -6.0333 | 3 | 3 |
| 27 | 20622 | 1350 | 98.5333 | 2.5667 | 3 | 3 |
| 29 | 20625 | 270 | 100.0833 | 0.2500 | 3 | 3 |
| 31 | 20631 | 550 | 98.7000 | 3.1000 | 3 | 4 |
| 62 | 22058 | 370 | 101.2000 | 17.0667 | 3 | 4 |
| 30 | 20626 | 250 | 99.2500 | 2.7667 | 3 | 5 |
| 48 | 21529 | 750 | 110.3333 | -7.7500 | 3 | 6 |
| 11 | 18437 | 190 | 100.8667 | -0.4833 | 4 | 3 |

| ID | CIAT-No. | Elevation | Longitude | Latitude | Cluster (agron. data) | Cluster (Floramap) |
|---|---|---|---|---|---|---|
| 12 | 18438 | 40 | 101.7333 | 12.7833 | 4 | 3 |
| 57 | 22082 | 190 | 103.1667 | 18.2333 | 4 | 3 |
| 45 | 21087 | 700 | 98.8667 | 18.8500 | 4 | 4 |
| 53 | 21993 | 150 | 109.0000 | 14.4000 | 4 | 4 |
| 61 | 22327 | 80 | 106.6000 | 11.0833 | 4 | 4 |
| 34 | 20975 | 230 | 109.2833 | 18.8167 | 4 | 6 |
| 37 | 20978 | 250 | 109.4667 | 19.1667 | 5 | 4 |
| 43 | 21083 | 620 | 97.9333 | 18.6667 | 5 | 6 |
| 47 | 21092 | 500 | 99.9000 | 20.4333 | 5 | 6 |
| 52 | 21992 | 40 | 108.8667 | 14.8500 | 5 | 6 |
| 46 | 21090 | 550 | 99.5167 | 19.2000 | 6 | 4 |
| 44 | 21086 | 490 | 98.8667 | 18.8333 | - | 7 |

**Table 5. Total DNA concentrations (ng/ml) of *Cratylia argentea* and *Flemingia macrophylla*.**

| *Cratylia argentea* | | *Flemingia macrophylla* | | | |
|---|---|---|---|---|---|
| No. CIAT | Total DNA | No. CIAT | Total DNA | No. CIAT | Total DNA |
| 18516 | 82 | 801 | 430 | 20977 | 328 |
| 18667 | 888 | 7184 | 165 | 20978 | 275 |
| 18668 | 318 | 17400 | 630 | 20979 | 413 |
| 18671 | 275 | 17403 | 620 | 20980 | 280 |
| 18672 | 233 | 17404 | 495 | 20982 | 282 |
| 18674 | 148 | 17405 | 235 | 21079 | 584 |
| 18675 | 138 | 17407 | 281 | 21080 | 222 |
| 18676 | 608 | 17409 | 271 | 21083 | 334 |
| 18956 | 456 | 17411 | 443 | 21086 | - |
| 22373 | 245 | 17412 | 370 | 21087 | 230 |
| 22374 | 339 | 17413 | 411 | 21090 | 548 |
| 22375 | 200 | 18048 | 603 | 21092 | 361 |
| 22376 | 529 | 18437 | 596 | 21241 | 246 |
| 22378 | 197 | 18438 | 601 | 21248 | 403 |
| 22379 | 100 | 18440 | 347 | 21249 | 191 |
| 22380 | 357 | 19453 | 167 | 21519 | 726 |
| 22381 | 247 | 19454 | 411 | 21529 | 321 |
| 22382 | 607 | 19457 | 443 | 21580 | 299 |
| 22383 | 147 | 19797 | 294 | 21982 | 267 |
| 22384 | 427 | 19798 | 357 | 21990 | 413 |
| 22386 | 282 | 19799 | 417 | 21991 | 131 |
| 22387 | 222 | 19800 | 572 | 21992 | 397 |
| 22390 | 127 | 19801 | 379 | 21993 | 286 |
| 22391 | 177 | 19824 | 389 | 21994 | 185 |
| 22392 | 181 | 20065 | 159 | 21995 | 129 |
| 22393 | 361 | 20616 | 339 | 21996 | 442 |
| 22394 | 180 | 20617 | 354 | 22058 | 352 |
| 22396 | 83 | 20618 | 255 | 22082 | 491 |
| 22399 | 215 | 20621 | 467 | 22087 | 237 |
| 22400 | 476 | 20622 | 329 | 22090 | 375 |
| 22404 | 208 | 20624 | 265 | 22285 | 330 |
| 22405 | 242 | 20625 | 442 | 22327 | 472 |

| *Cratylia argentea* | | *Flemingia macrophylla* | | | |
|---|---|---|---|---|---|
| No. CIAT | Total DNA | No. CIAT | Total DNA | No. CIAT | Total DNA |
| 22406 | 234 | 20626 | 193 | C-104890 | 518 |
| 22407 | 306 | 20631 | 316 | I-15146 | 625 |
| 22408 | 438 | 20744 | 393 | J001 | 463 |
| 22409 | 115 | 20972 | 469 | | |
| 22410 | 242 | 20973 | 381 | | |
| 22411 | 476 | 20975 | 447 | | |
| 22412 | 282 | 20976 | 304 | | |

**Table 6. Polymorphic bands of different primer combinations for *Flemingia macrophylla* (accessions 21990 and 21529) and *Cratylia argentea* (accessions 18672 and 18516).**

| Primer combination | Polymorphic bands | | |
|---|---|---|---|
| | *F. macrophylla* | *C. argentea* | Total |
| E-AAC / M-CAA | n.a. | n.a. | n.a. |
| E-AAG / M-CAA | n.a. | n.a. | n.a. |
| E-AAG / M-CAT | 28 / 24 | 2 / 4 | 58 |
| E-ACA / M-CAT | 18 / 20 | 7 / 9 | 54 |
| E-ACA / M-CTG | 15 / 8 | 4 / 5 | 32 |
| E-ACT / M-CTG | 13 / 8 | 4 / 5 | 30 |
| E-ACC / M-CAG | 20 / 15 | 9 / 8 | 52 |
| E-ACG / M-CAG | 16 / 24 | 2 / 21 | 62 |
| E-ACG / M-CAC | 26 / 24 | 19 / 12 | 81 |
| E-AGC / M-CTA | 11 / 18 | 3 / 3 | 35 |
| E-AGG / M-CTC | 24 / 21 | 9 / 12 | 66 |
| E-AAC / M-CTT | 45 / 20 | 18 / 3 | 86 |

## 1.1.13 Use of molecular techniques for the studies of the genetic diversity and conservation studies of endangered palms in Colombia

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### Introduction

There are approximately 200 genera and 1500 species of palms in the world. The palm genera are endemic to major continental areas, none of them being pantropical. In the Americas 67 genera and 550 species occur naturally (Henderson et al., 1995). A broad spectrum of uses have been described, ranging from foods and nutritional beverages, sugar and starch to construction materials, oil, fuels, fibers, rattan and ornamentals. It is precisely because of their usefulness that some of these species have become endangered. Examples of this are three endangered species in Colombia. Two of these species belong to the *Ceroxylon* genus: *C. alpinum* and *C. sasaimae*. The genus *Ceroxylon* contains some of the most spectacular American palms, including some of the tallest palms in the world and those growing at the highest elevations. Ceroxylon has 11 species, distributed throughout the Andes—from Venezuela and Colombia to Ecuador, Peru and Bolivia. The young leaves of *C. alpinum* and *C. sasaimae*, known as "*palma de cera, palma de*

*ramo* or *palma real*," are used for religious celebrations on Palm Sunday. The habitat of these species has been extensively deforested and transformed into agricultural land, mainly coffee plantations. Given the foregoing, the survival of these species is severely threatened. The third endangered species is *Attalea amygdalina* or "*almendrón del Río Cauca*," whose habitat is found in the Cauca River Valley of Colombia. Its seeds, which are edible, were recommended by Ruiz (1984) as promising economically because of their high oil content. Most of its habitat has been converted into coffee plantations, and the species is endangered (Bernal, 1989).

In collaboration with the Universidad Nacional de Colombia-Bogotá and the Instituto Alexander von Humboldt we started a project to generate microsatellites makers for palms and to study the diversity of these three species.

**Materials and Methods**

Construction of an enriched microsatellite library of *C. alpinum, C. sasaimae* and *A. amygdalina*. Enriched microsatellite libraries for all three species were constructed as described by Edwards et al. (1996). This involved the digestion of 200 ng of genomic DNA with *Rsa* I. An *Mlu*I adaptor was ligated to the digested fragments. Filter-immobilized oligonucleotides representing the $CT_{20}$ and $GT_{20}$ SSR marker classes were used to select for the genomic fragments containing SSRs. Enriched fragments were amplified by PCR, using the 21-mer adaptor primer. The enriched DNA was then digested with *MluI* and ligated into a modified pUC19 vector, pJV1 containing a *BssH*I site (K.J. Edwards, unpublished). Plasmids were transformed into DH5α. Genomic libraries were screened with a mixture of radio-labeled oligonucleotides ($CT_{20}$ and $GT_{20}$). Putative positive colonies were cultured, and plasmid DNA was isolated from the culture with a QIAGEN plasmid purification kit. Sequencing of the purified plasmid DNA fragments was done on the Applied Biosystems' ABI 377 sequencer model. This was from the M13 primer sites, using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions. Each sequence was aligned against all the others, using the SEQUENCHER program in order to eliminate redundant clones. Primers were then designed for the unique clones using the PRIMER3.0 software program (available at http://waldo.wi.mit.edu/cgi-bin/primer/primer3).

Microsatellite primer characterization. The SSRs were first amplified from plasmid DNA and the source genotype to standardize the PCR conditions. The PCR reaction was carried out in a 20-μl final volume containing 20 ng of genomic DNA, 0.1 μM of each of the forward and reverse primers, 10 mM Tris-HCl (pH 7.2), 50 mM KCl, 1.5-2.5 mM $MgCl_2$ (depending on the primer combination), 250 mM of total dNTP, and 1 unit of Taq DNA polymerase. The temperature cycling profile involved an initial 2-min denaturation step at 94°C. This was then followed by 35 cycles, each of which consisted of denaturation at 94°C for 15 s, an annealing phase of 48-65°C (depending on the annealing temperature for the given primer pair) for 15 s, and an extension at 72°C for 15 s. The PCR products run on 6% denaturing polyacrylamide gels (19:1 acrylamide: bis-acrylamide) contained 5 M urea and 0.5 XTBE. Electrophoresis was at 100-W constant power for 2-2.5 h. PCR amplifications were visualized by silver staining according to the manufacturer's guide.

**Results of Isolating SSR clones and PCR evaluation**

*C. alpinum* library. Of the 1152 clones screened with oligonucleotide probes $CT_{20}$ and $GT_{20}$, a total of 198 putative positive colonies (17.2%) were isolated, 30 of which were sequenced. Of these, only 17 were suitable for designing primers. We found 50% for both CA and GA. On

average, dinucleotide motifs had a maximum number of repeats (30). A total of 27 primer pairs including some primer pairs from the *C. sasaimae* library are suitable for amplification with 123 individuals. Currently, 10 of them have been amplified in a whole population, but only 3 of them show polymorphism.

*C. sasaimae* library. Of the 1152 clones screened with oligonucleotide probes $CT_{20}$ and $GT_{20}$, a total of 99 putative positive colonies (8.5%) were isolated, and 27 were sequenced. Of these, only 11 were suitable for designing primers. We found 50% for both CA and GA. On average, dinucleotide motifs had a maximum number of repeats (38). Ten primer pairs were suitable for PCR amplification. A total of 99 genotypes of a *C. sasaimae* population from Sasaima (Cundinamarca) were amplified with these 10 primer pairs and another 10 from the *C. alpinum* library. Four primer pairs were monomorphic for this population, and 17 were polymorphic (from 2-7 alleles), with a total of 56 alleles in a whole population.

*A. amygdalina* library. Of the 1152 clones screened with oligonucleotide probes $CT_{20}$ and $GT_{20}$, 290 putative positive colonies (25%) were isolated. Of the 29 sequenced, 23 were suitable for designing primers. We found 50% for both CA and GA. On average, dinucleotide motifs had a maximum number of perfect repeats (30). We have started the evaluation of a set of 123 individuals.

**Ongoing Activities**

- Continue evaluating the rest of the primer set in 123 individuals of *C. alpinum*
- Evaluate a set of 23 primer pairs obtained from *A. amygdalina* on 123 individuals
- Establish a database for the molecular data to be linked with ecological information

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## 1.1.14 Molecular and agro-morphological characterization of the genetic variability of Soursop (*Annona muricata* L.) accessions and related Annonaceus species

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[1]SB-2 Project; [2]Corporación BIOTEC; [3]CORPOICA; Project funded by Colciencias

### Introduction

Soursop is the most tropical of the 60 species of the genus *Annona* (Morton, 1987). It has a horticultural value comparable to those of "chirimoya" (*Annona cherimola*), "anón" (*A. squamosa*), "papauca" (*A. diversifolia*) and "atemoya" (*A. squamosa* x *A. cherimola*) (Escobar y Sánchez, 1992). It's origin is northern South America, possibly Colombia or Brazil (León, 1968). This is why Colombia may have the world's greatest genetic diversity of soursop, which has not being used in breeding programs to improve agronomic traits. As a matter of fact in Colombia there is only one commercially available clone, fairly characterized (Ríos Castaño *et al.*, 1996). C.I. Corpoica – Palmira has the only one Colombian annonas germplasm bank with 36 soursop accessions and 7 annonaceus species accessions, which have not been characterized at all.

This project is the first attempt to study the genetic diversity of annonaceus species from Colombia. The objective is to know the genetic variability of the germplasm bank by applying DNA molecular marker technology (AFLP). We would like to know if this variability is representative of Colombian diversity. The genetic variability analysis will identify possible duplicates and/or misidentified accessions. Corporación Biotec, Corpoica and CIAT (SBII project) are in charge of the molecular characterization. Corpoica- Palmira will carry out the agro-morphological characterization.

### Methodology

We planned a careful sampling throughout the most important annonas growing zones in Colombia and germplasm bank. We will evaluate 60 soursop samples and 45 samples of other annonaceus species. Farmers selected 26 soursop clones based on their productivity and fruit quality. We used three soursop clones and three accessions from "anon amazónico" (*Rollinia spp.*) for standardization of DNA extraction and AFLP fingerprinting. We applied a rice DNA extraction protocol (McCouch *et al.*, 1988), using PVP 1 g $l^{-1}$ in the extraction buffer and a chloroform / isoamylalcohol (24/1) cleaning step before a second precipitation. For DNA fingerprinting, we used AFLP Analysis System I (Gibco BRL), according to Vos *et al.* 1995, protocols.

### Results

We standardized a DNA extraction protocol for the recovery of high-quality DNA for AFLP fingerprinting (Figure 1). Four out of fifteen selective primers evaluated (primers E, F, G, H, Figure 1) showed clear polymorphism between soursop groups (samples 4, 5 and 6, Figure 1) and "anon amazónico" group (samples 1, 2 and 3, Figure 1). Preliminary results showed that primers E, F, G and H displayed 44, 48, 52 and 50 polymorphic bands between both groups, respectively. Primer E showed 12 polymorphic bands between anones amazónicos, and three polymorphic bands between soursop samples. Primer H showed 11 and 6 polymorphic bands, respectively, for

the same groups. Primer F showed the largest number of polymorphic bands (13) between anones amazónicos.

**Conclusions**

A DNA extraction methodology for obtaining high-quality DNA from annonaceus species for fingerprinting was establsihed. Four AFLP primer pairs were selected for the genetic variability evaluation of our target population. It is necessary to evaluate more selective primers to get ones that display polymorphism between soursop accessions.

**Future plans**

- Evaluate 20 new selective primer pairs.
- Evaluate the reproducibility of selected primers.
- Evaluate genetic variability of soursop and other annona species trough genetic distance dendrograms (NTSYS and SAS statistics programs)

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## Activity 1.2 Identification and mapping of useful genes and gene combinations

### Main Achievements

- Yield data was carried out on a population of a Mesoamerican cultivar x a Mexican wild bean, with the possible identification of lines with yield higher than the recurrent parent.

- The high iron trait was transferred from a wild bean through backcrosses to a cultivated background, although wild traits were still observed. QTLs were found for iron and zinc content. The most significant QTLs explained up to 12% of the variance in mineral content. In some cases the QTLs for both minerals occurred jointly at the same marker, in other cases there were QTLs specific for each mineral. Positive QTLs for both iron and zinc were found on chromosomes B5, B6, B8, B9 and B10 for the Andean population and on chromosomes B6, B8 and B10 for the Mesoamerican population.

- The discovery of a qualitative and high level of resistance to the devastating cassava mosaic disease (CMD) and molecular markers linked to it have made conceivable marker-assisted breeding for CMD resistance at CIAT. Progress made this year includes the establishment of a sexual hybridization scheme between resistant donor lines received last year from IITA and CIAT elite parents. A marker-assisted selection (MAS) scheme has also been initiated at for the rapid verification of CMD resistant selections in resistance breeding to contain and prevent the spread of the epidemic.

- Progress is being made on the identification of candidate genes that may mediate the molecular basis of CMD resistance.

- Results obtained through the use of the advanced backcross -QTLs analysis in two inter-specific populations, Caiapo/*O.rufipogon* and Bg90-2/*O. rufipogon* provided increasing evidence that *Oryza rufipogon* possesses traits -enhancing QTLs that can be used for the genetic improvement of cultivated rice.

## 1.2.1 Wild QTL pursued in population of DOR 390 and G10022

S. Beebe[1], M. Blair[1], H. Terán[2] J. Tohme[1]
[1]SB-2 Project; [2] IP-1 Project

### Introduction

Wild relatives of several crops have been investigated as sources of QTL for complex traits including yield. Wild *P. vulgaris* has been demonstrated to have much broader genetic variability than cultivated bean and feasibly could serve as a source of useful variability for broadening the base of the cultivated bean.

### Materials and methods

Several populations involving wild bean have been explored using the advanced backcross or backcross selfing method, whereby a large number of lines are generated by backcrossing a wild x cultivated hybrid twice to the cultivated parent. Yield and mineral analyses were initiated in the advanced backcross population of DOR 390 and G10022. DOR 390 is a widely used Mesoamerican, small black-seeded cultivar resistant to BGYMV, and G10022 is a wild bean from México that presents Mesoamerican DNA patterns and high iron concentration in seed.

Yield trials were established in the field in Santander de Quilichao under moderate fertility stress in the spring of 2001. A population of 144 lines was planted in a lattice of 12 x 12.

### Results

In the field no line presented a statistically significant yield advantage over DOR 390. However, three lines presented iron levels above the rest of the population (Table 5), and one of these had iron almost at the level of the wild parent (98 mg/kg) which is about 78% above the average for common bean varieties. Unfortunately, this line did not recover all of the desirable traits of the cultivated parent, although its yield was only slightly below that of DOR 390. Thus, it was possible to transfer the high iron trait from the wild bean to a much more acceptable genetic background for future use in the breeding program.

**Table 5. Yield and iron concentration of advanced backcross progeny of DOR 390 and G10022.**

| Line (NH 21153-...) | Iron (mg/kg) | Yield (kg/ha) |
|---|---|---|
| 67-1-1 | 98 | 1407 |
| 11-1-1 | 85 | 1098 |
| 13-1-1 | 81 | 1059 |
| 31-1-1 | 62 | 1647 |
| 31-2-1 | 66 | 1626 |
| 37-1-1 | 66 | 1647 |
| 37-1-2 | 63 | 1783 |
| DOR 390 | 62 | 1569 |
| LSD (0.05) | | 365 |

**Table 6. Linear correlations among yield, seed size and mineral content in advanced backcross lines of DOR 390 and G10022.**

| Traits | Correlation |
|---|---|
| Yield - seed size | +0.27 * |
| Yield - iron | -0.13 ns |
| Yield - zinc | -0.19 ns |
| Seed size - iron | -0.07 ns |
| Seed size - zinc | -0.11 ns |

Correlations among relevant parameters in this group of lines appear in Table 6. The only significant correlation was between seed size and yield. Although mineral content showed a very slight negative tendency in relation to other critical traits, these correlations were very weak and non-significant. Thus, there should not be any problem with recovering high mineral content with acceptable grain size and yield.

**Conclusions**

Although we did not achieve a line that had all of the desirable traits of the cultivated DOR 390 with high iron, we confirmed in this population that the high iron trait can be transferred from the wild bean. Line NH 21153-67-1-1 ought to be a useful parent for additional crosses to increase iron concentration of grain. Given the fact that mineral content was not correlated with yield, it ought to be possible to recover high iron and high yield.

## 1.2.2 QTL analysis of an Andean advanced backcross population for yield traits derived from wild *P. vulgaris*

M.W. Blair[1], G. Iriarte[1], S. Beebe[1]
[1]SB-2 Project

**Introduction**

The genetic diversity of cultivated *P. vulgaris* is thought to be larger than that of wild common bean due to a genetic bottleneck that occurred during domestication. Yield increasing alleles may still reside untapped in the wild accessions that could be exploited to improve cultivated beans. The advanced backcross method has been shown to be a useful method for incorporating wild germplasm into cultivar breeding programs for tomato and rice. Although wild beans have been used before to transfer resistance to diseases and insects, as in the noted case of the Arcelin gene that provides resistance to bruchids, the studies presented here are among the first to attempt to obtain a higher yield potential from wild beans. The objective of this research was to conduct a molecular analysis of an Andean advanced backcross population to find quantitative trait loci from a wild bean that could be useful in the improvement of cultivated beans.

**Materials and Methods**

The population that was analyzed was derived from derived from the Mexican wild accession G24404 and the Colombian, large red-seeded, "Radical" type variety, ICA Cerinza and represented the BC2F3 generation. The population consisted of 95 selected lines that were evaluated in three environments and an 62 lines that were evaluated in one of the locations. The

population Cerinza /2/ G24404 had a total of 157 lines. The experiment was grown in two seasons in Popayán and one season in Darien. Three repetitions in a lattice design were used for each of the experiments. A total of 65 microsatellite markers were used to evaluate the introgression level in the full set of lines. Individuals with unexpected alleles were eliminated from the population for the sake of the quantitative analysis. Quantitative trait loci (QTL) were identified through single-point regression of the phenotypic data onto the marker genotypes using the software program qGENE, assuming the BC2S1 model. Marker order was inferred for the 50 markers with known locations on other genetic maps of beans. Linkage analysis was used to estimate the genetic distance between markers and to place 10 unmapped markers that were linked at 10cM or less from a mapped marker.

**Results and Discussion**

In the BC2F1 generation, 25% of plants are expected to contain an introgressed allele for a given locus, but all of these plants are expected to be heterozygous. Upon self-pollination, the loci are expected to segregate in a Mendelian ratio whereby in each generation heterozgosity is lost and the loci are fixed to homozygosity. In this study we analyzed B2F3 derived families, in which the total amount of introgression for a given locus is expected to be 15.675% of which, 9.375% should be homozygous and 6.25% heterozygous for the introgression.

The microsatellites were ideal for this study because they had the advantage of being co-dominant and single copy and therefore could diagnose the families derived from heterozygous genotypes and differentiate them from those derived from homozygous individuals. Introgression could be scored for families that were derived from both genotypes. The results showed that the selected lines had a significantly lower introgression rate over all loci than the additional lines indicating that selection had eliminated some amount of introgression. However together the selected and additional lines had rates of introgression over all loci that were not significantly different from those expected. For example the chi-square test for the average number of introgressed individuals across all loci was not significantly different than expected. However, segregation distortion was variable depending on the region of the genome that was assayed as indicated by significant chi-square tests showing deviation of the observed ratio from the expected ratio as described above. The chi-square tests, showed that for the chromosomes that were mapped, selection was most intense in the middle of B2 and on one arm each of B4 and B10. Mild segregation distortion occurred on chromosome B5 and on one arm of B3 and B9. Selection was always against the wild allele and always led to a predominance of the cultivated allele. Since selection was made for return to recurrent parent plant architecture and seed type this was presumed to be due to selection against genes in these regions that had a negative phenotypic effect on these traits. Alternatively the wild alleles could encode other domestication traits (non-shattering, large seed size, etc.) as outlined by Gepts (2000), that would be undesirable in the advanced backcross lines. Lack of any introgressions on chromosome B1 could have been a result of fewer microsatellites being screened for this chromosome or that introgressions for this chromosome were eliminated due to linkage drag with the *Fin* and *Ppd* genes. Wild alleles at these loci would condition indeterminate growth habit and photoperiod sensitivity. Both of these traits would be agronomically negative factors that were selected against in this cross, since the desired plant type was a early-flowering, bush bean like the recurrent parent, Cerinza.

The microsatellites allowed unexpected alleles to be diagnosed and individuals carrying these alleles to be eliminated from the study. A total of 113 individuals were used in the QTL analysis. Significant QTLs for yield were found on chromosome B4 and at an unmapped locus during the seasons in 1999B Darien and 1998B Popayan, however in these cases the significant positive loci were associated with the recurrent parent Cerinza allele and the wild alleles were negative in their

effect. This indicates that there were still a large number of the alleles transmitted from the wild parent that had negative effects on yield and that remained to be eliminated from the progeny. One QTL for yield in 1999A Popayan was found on chromosome B3 associated with the wild allele. This QTL was not detected with yield data from any other season, indicating that there is a significant QTL x environment interaction for this trait as would be expected. Several QTLs from the wild parent were found associated with unmapped markers in all three seasons. Both Darien and Popayan are good growing environments for the Cerinza variety and therefore were appropriate testing sites for advanced backcross progeny derived from Cerinza. The yield QTLs were not associated with later maturity and flowering or reduced seed size, since independent QTLs were located for these traits. Days to maturity and days to flowering as well as number of pods per plant and number of seed per plant were correlated. QTLs for these component traits were less variable across seasons. The results presented here, suggest that wild beans can be a source of genes for higher yield in cultivated beans and that advanced backcross strategies can be successful at transferring these genes into commercial seed -types.

**Future Plans**

We will compare the location of QTLs found for this population with other advanced backcross populations that are being studied.

## 1.2.3 QTL mapping of micronutrient content in two populations of common bean

M.W. Blair, C. Astudillo, S. Beebe
SB-2 Project

**Introduction**

Legumes provide essential micronutrients that are found only in low amounts in the cereals or root crops. An ongoing project, has shown that bean seeds are variable in the amount of minerals (iron, zinc and other elements), vitamins and sulfur amino acids that they contain and that these traits are likely to be inherited quantitatively. The objective of our most recent studies has been to tag some of the quantitative trait loci (QTLs) controlling mineral content in beans.

**Materials and Methods**

Two populations, representing an Andean x Andean cross (G 21657 x G 21078); and a Mesoamerican x Mesoamerican cross (G14519 x G4825) were selected for genetic mapping. Each population contained 95 recombinant inbred lines in the F5:7 generation. Miniprep DNA was extracted from all the lines in both populations and all the individuals were genotyped for RAPDs and microsatellites. The marker information was used to construct separate genetic maps for each of the populations, using the software program MAPMAKER. The phenotypic data was obtained by analyzing both the parents and the recombinant inbred lines for iron and zinc content by ICP (Inductive coupling plasma). Quantitative trait locus (QTL) analyses were conducted by regressing the phenotypic data for micronutrient content onto marker genotypes with the software program qGENE.

**Results and Discussion**

In general the rate of polymorphism was low in these crosses since they were from within a single genepool, however both RAPD and microsatellite markers detected sufficient polymorphism to allow us to proceed. RAPD primers were used to generate a large number of markers, while microsatellites were used because they have several advantages including occupying unique mapped positions in the genome and a somewhat higher rate of polymorphism. The two genetic maps were constructed based on 119 markers in the Andean cross and 98 markers in the Mesoamerican cross. Fewer microsatellites were polymorphic and could be mapped in the Mesoamerican population (13) than in the Andean population (28). The number of microsatellites per chromosome varied from 0 to 5 across both populations. However, the single locus nature of microsatellite markers made it especially useful for anchoring the RAPD markers to known chromosomes. RAPD bands were also cross-referenced between populations and with the main mapping population at CIAT, to provide additional markers for comparative mapping. For both populations, 10 out of the 11 chromosomes of common bean could be identified based on the microsatellites or homologous RAPD bands that they contained. The order of markers along each chromosome was generally the same across all three populations. Unlinked markers were evident for both populations (16 for Mesoamerican and 5 for Andean). One additional linkage group was found for each population, that could not be tied to a chromosome because they lacked a microsatellite or a cross-referenced RAPD.

QTLs were found for iron and zinc content in both populations. The positive markers varied in their level of significance and the proportion of variance in mineral content that they explained (R-squared value). The most significant QTLs explained up to 12% of the variance in mineral content. In some cases the QTLs for both minerals occurred jointly at the same marker, in other cases there were QTLs specific for each mineral. Positive QTLs for both iron and zinc were found on chromosomes B5, B6, B8, B9 and B10 for the Andean population and on chromosomes B6, B8 and B10 for the Mesoamerican population. The QTLs were generally found in similar locations of the same chromosomes in both populations. A QTL specific for iron was found on chromosome B11 in the Andean population, while one that was specific for zinc was found on chromosome B7 for the Mesoamerican population.The majority of the positive QTLs are associated with alleles from the high mineral parent. It can be concluded that some of the QTLs for the accumulation of both minerals may be genetically linked or pleiotropic, controlling both traits at once. If the same QTLs contribute simultaneously to both iron and zinc content, it may be easy to select for these traits jointly. It also appears that high mineral content parents provide most of the genes for high mineral content to their progeny, while low mineral parents provide only a few additional genes for mineral content.

**Future Plans**

We have made progress in detecting QTLs for micronutrient accumulation and linking the two maps derived from both populations using microsatellites and homologous RAPD bands. The present work will hopefully permit us to focus on certain parts of the genome to determine if desirable alleles for higher mineral content are located at the same loci in additional populations developed specifically for this purpose. Future analysis will also include the detection of QTLs for the amount of sulfur containing amino acids (SAA), as well as for the amount of the other minerals analyzed in the ICP study, which include Mn, Ca, Mg, K, P and S. The QTL analysis will help us to consider the possibility of genetically increasing SAA (methionine or cysteine) or Lysine as uptake promoters of iron or decreasing antinutritional factor such as tannins or lectins that reduce the uptake of iron. We also plan to integrate the information about the map locations

of QTLs for micronutrients with those for other agronomic traits that we have been studying, so that we can select for the best recombinants from crosses between high micronutrient lines and the best varieties using marker assisted selection.

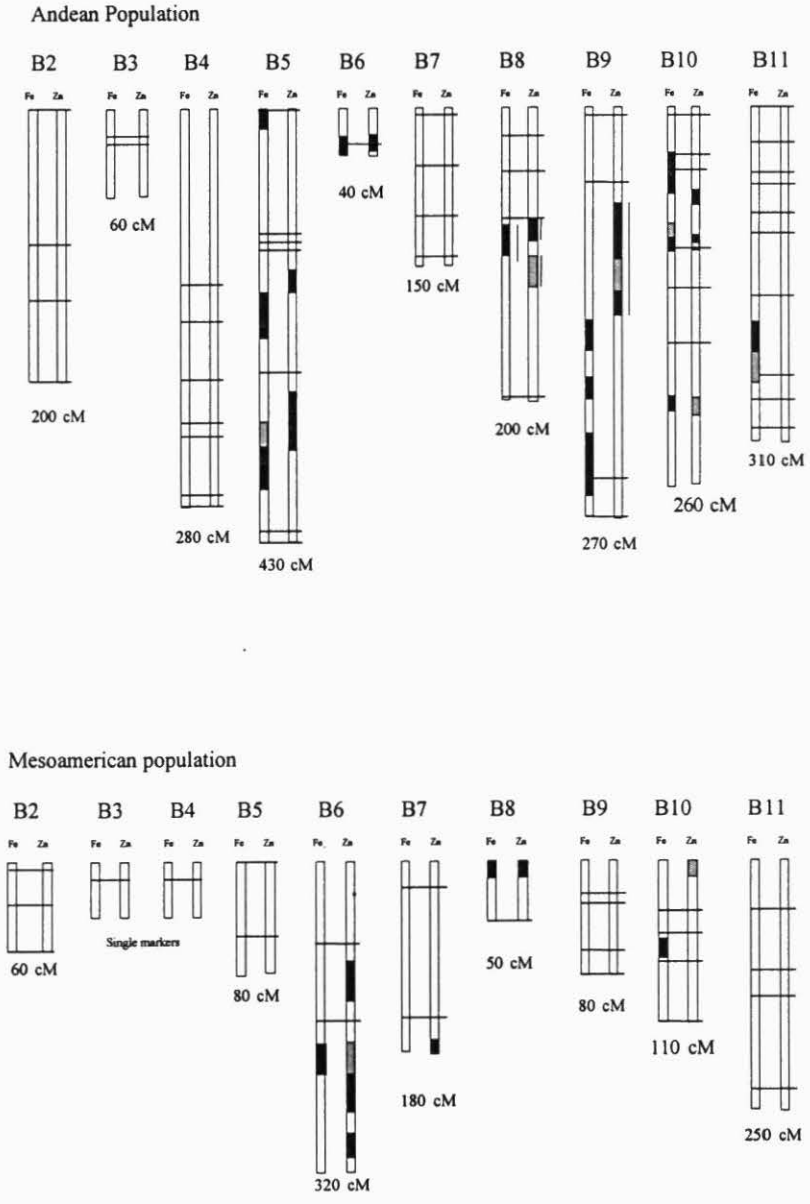


**Figure 1. Comparative mapping across three populations of common bean and analysis of quantitative trait loci (QTLs) for iron (Fe) and zinc (Zn) micronutrient content in the Andean x Andean population (G 21657 x G 21078); and the Mesoamerican x Mesoamerican population (G14519 x G4825).**

## 1.2.4 Analysis of ferritin and other candidate genes for micronutrient accumulation in common bean seed.

M.W. Blair[1], C. Astudillo[1], A. Hernandez[2], D. Debouck[2]
[1]SB-2 Project ; [2]SB-1 project

### Introduction

The common bean genes involved in getting iron and zinc from the root zone to the grain have not been studied. Several genes from other legumes or from the model species, peas and *Arabidopsis*, or *Medicago*, could qualify as candidate genes for the control of micronutrient accumulation and storage in the bean seed. Among the most likely is phytoferritin which is the major storage form of iron in all tissues including seeds. Iron in phytoferritin is generally thought to be of low bioavailability, however definitive studies with radioactively labeled protein would need to be done for bean seed ferritin, since some recent results contradict this assumption for (pers. comm, Ross Welch, Cornell University). Other candidate genes that we considered were involved in the production and processing of Nicotianamine, a polyamine that is known to function in scavenging iron from the root zone in grasses and may be involved in other iron related functions in plants more generally, including possibly the transport of iron. The enzymes, nicotianamine synthase and nicotianamine amino transferase are involved in making this substrate and are possible candidate genes for this study. The other candidate genes being considered are ones for an iron responsive element (IRE) binding protein, an iron-deficiency specific clone and a putative zinc transporter from soybean. The objective of this research is to analyze whether these genes are involved in mineral uptake, transport and seed-loading.

### Materials and Methods

The parents of the populations used for micronutrient mapping were prepared for both protein and DNA assays. Protein was extracted from scarified seed that was dehydrated on silica gel at 45 C for one hour by standard methods used for Phaseolin analysis. The samples were run on SDS-polyacrylamide gels (at concentrations of 5, 6, 8, 10 and 12% polyacrylamide) with a 4% stacking gel separation for approximately 2 hours each. Gels were stained for 24 hours with Coomassie blue in water, methanol and acetic acid. DNA was extracted by a large-scale phenol-chloroform extraction protocol for standard RFLP analysis. A total of 16 soybean cDNA probes were obtained from Incyte Genomics Inc. for the candidate gene evaluation. These had been sequenced from the 5'end by the National Science Foundation sponsored EST project. At CIAT, we obtained full length sequence for the clones to identify the full coding region. Primers were designed in the conserved exons of the ferritin gene which had homologues from both common bean and soybean.

### Results and Discussion

*Protein results:* The 5 and 6% polyacrylamide gels gave the best resolution of the high molecular weight ferritin proteins. Figure 1 shows the protein extracts from the four parents as well as purified apo-ferritin used as a control that were stained with Coomassie blue. Three bands could be observed in both control and sample lanes and corresponded to approximately the same size as ferritin (450kD) and had a similar pattern of staining observed for ferritin in a previous study (Van der Mark, 1985). In that study, ferritin was identified by staining with potassium ferrous cyanide however this solution did not work with the gels analyzed here. None of the putative ferritin bands identified with Coomassie blue presented any polymorphisms and therefore were

not able to be mapped genetically. The 8, 10 and 12% polyacrylamide gels gave the best resolution for detecting phaseolin bands. The high molecular weight proteins, such as ferritin were not evident in these higher concentration gels. The phaseolins detected ranged in size from 43kD to 54kD. Phaseolin bands were monomorphic for both the Andean parents (G21242 and G21078), which both had "T" type phaseolin and the Mesoamerican parents (G14159 and G4825), which both had "S" type phaseolin.

*DNA results:* The primers developed for ferritin amplified several bands indicating that this is likely to be a gene family. None of the primer pairs amplified consistently but one band was mapped as a sequence tagged site in the populations described in the micronutrient QTL analysis. The results for other genes are still pending.

*Sequencing of soybean ESTs:* Full-length sequence was obtained for all 16 soybean clones to confirm gene identification. Use of the clones and sequence information to obtain common bean homologues is pending.

**Future plans**

We will continue to analyze ferritin and the other candidate genes in an attempt to define the underlying factors responsible for micronutrient QTLs. The possibility of detecting and quantifying bean seed phytoferritin with commercially available ferritin immuno-detection systems will be explored. Proteomic analysis of other seed proteins varying between high and low mineral varieties might also reveal which genes are influencing overall mineral content. For the other candidate genes, degenerate primers will be made for the conserved domains identified in soybean and used to amplify the homologues from common bean. Alternatively, the soybean genes will be used as probes to isolate their homologues from root and leaf cDNA libraries made for common bean. Once isolated, the common bean genes will be sequenced. Specific primers will be designed to the common bean genes to map them as sequence tagged sites. If the primers fail to detect polymorphisms, the candidate genes will be mapped as restriction fragment length (RFLP) probes. We may try to sequence the promoters of the candidate genes to see if variability in these regions explains the accumulation of mineral content.

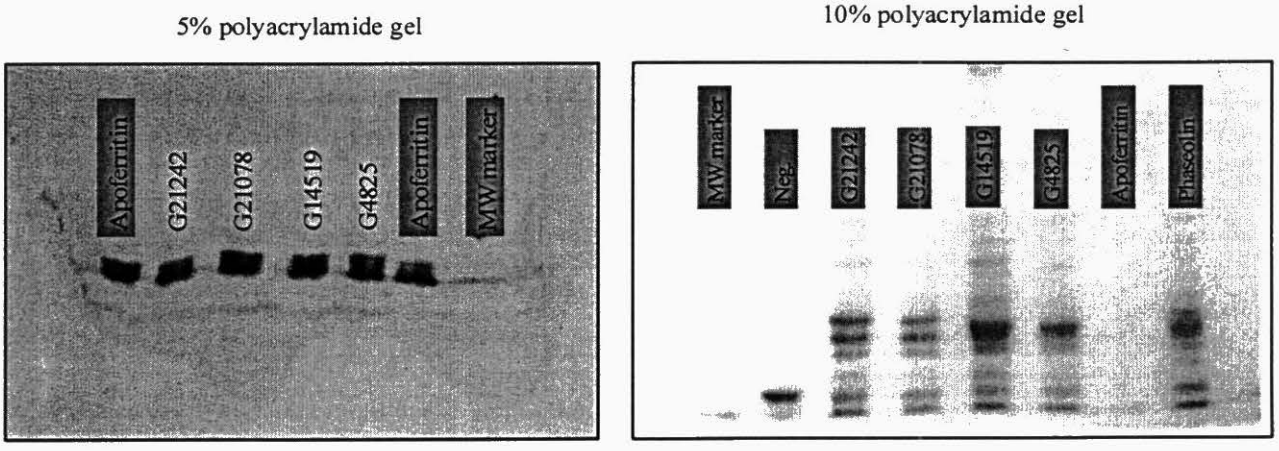


**Figure 1. Protein extracts from micronutrient mapping parents run on 5 and 10% SDS-polyacrylamide gels, showing ferritin and phaseolin standards.**

## 1.2.5 Tagging genes for resistance to *Apion goodmani* in common bean

M.W. Blair[1], C. Cardona[2], C. Muñoz G[2]., H. F. Buendia[2]
[1]SB-2 Project; [2]IP-1

### Introduction

The bean pod weevil (*Apion goodmani* Wagner) damage beans grown in Mexico and Central America by laying its eggs in the pods, from which larvae develop and attack the seeds, reducing their quantity, quality and viability. Yield loss caused by the insect is variable depending on the climatic conditions of the previous year but can be as high as 90%. Sources of resistance to the pest have been identified in Mexican germplasm and include Amarillo154, Amarillo155, Amarillo169; APN18, APN83, Mexico332, Pinto168, Puebla36, Tlaxcaca76 as well as others. Resistance is thought to be conditioned by two epistatic genes, *Agm* and *Agr*, both of which are needed for high levels of resistance (Garza et al., 1996). One mechanism of resistance to the pod weevil is a hypersensitive response in the pod wall that affects the tissue around the oviposition site and effectively encapsulates the insects' recently laid eggs. The objective of this research was to locate the genes controlling resistance in the recombinant inbred line (RIL) population derived from Jamapa x J117.

### Materials and Methods

The Jamapa x J117 population, consisting in 50 F5 derived lines was evaluated over four seasons (1994 – 1997) at a field site in Texoco, Mexico. The lines were evaluated for level of infestation and percent seed damage in every year. DNA was extracted for all of the lines with standard protocols from plants in the F5:10 generation. Susceptible and resistant bulks were made with four lines each based on the phenotypic data. The bulks and the parents were compared for RAPD and microsatellite marker polymorphisms. A total of 132 RAPD primers and 131 microsatellite markers were evaluated. In addition the SCAR that was developed for Apion resistance was used in the screening. The RAPD primers were ones reported previously to produce bands that were linked to insect resistance genes in other crops. The microsatellites are a set developed at CIAT for common beans. PCR conditions for RAPDs and microsatellites were as described in standard CIAT protocols. The SCAR was amplified with 35 cycles of 1 minute denaturation at 94 C, 2 minutes annealing at 65 C and 2 minutes extension at 72C. RAPD products were run on 1.5% agarose gels stained with ethidium bromide, while microsatellites were run on 4% polyacrylamide gels and stained with silver nitrate. All markers detecting a difference between the parents and the same difference with the bulks were run on all the individuals in the population and the segregation information was analyzed to construct a genetic map using the software program MAPMAKER. RAPD markers had the following designation based on the primer that amplified the band, the size in nucleotides of the band and the parental source of the band (either from the resistant (R ) or susceptible (S) parent). Quantitative trait loci (QTL) were identified through single-point regression analysis of the phenotypic data onto the marker genotypes using the software program qGENE.

### Results and Discussion

A total of 20 RAPD and 9 microsatellite markers were polymorphic for the bulks and parents of the population and were run on the entire population. Figure 1 shows the bulk segregant analysis for a significant and a non-significant microsatellite. These were mapped and for the most part were linked to each other in three tight linkage groups with four or more markers each. The

single locus microsatellites were especially useful for anchoring these linkage groups to known chromosomes and the three linkage groups could be identified as being part of chromosomes B1, B8 and B11. All three of these linkage groups were associated with important QTLs that were consistent across all four seasons of evaluation. The microsatellites BMy4 and BM189 were the most important markers on chromosome B1 and B8, respectively, explaining 20 and 24% of the variance respectively. The QTL on chromosome B11 was tagged with the SCAR K16 as well as two highly significant RAPD markers, F13.850S and Z4.800R/Z4.850S, both of which explained 40% of the variance in resistance. Of these two RAPD markers, the second was interesting because it consisted in a co-dominant pair of bands. Theis highly significant QTL was also tagged with the mmicrosatellite BMd22. Another important QTL was identified with two markers on chromosome B7. For all the positive markers, the resistant parent J117 provided the resistant allele. Additional single markers on chromosomes B3, B4 and B5 were significant for one or two seasons only and were not considered to be important given their low R-square values. Three unliked RAPD clusters were also detected that also were less significant and only important in a few seasons. The inheritance of resistance appeared to be fairly simple, which is in agreement with the hypothesis that two genes condition the best resistance. From the QTL studies, we could postulate several additional modifier genes that provide resistance, but the hypothesis of two major genes is sound.

**Future Plans**

Additional microsatellites will need to be mapped on the three positive chromosomes to pinpoint the location of the QTLs found in the present study. Once we have more complete maps for the regions, two-way interaction tests will be done to confirm epistasis between the genes. We will also try to fine map the genes with additional markers. For that we will need to increase the size of the segregating population that is being studied. Plans are to implement marker assisted selection for Apion resistance at CIAT. For this we will need to test the validity of using the microsatellite markers. Another possibility is to develop SCAR marker from some of the positive RAPD markers. A good candidate for SCAR development would be the pair of co-dominant RAPD bands located on chromosome B11.

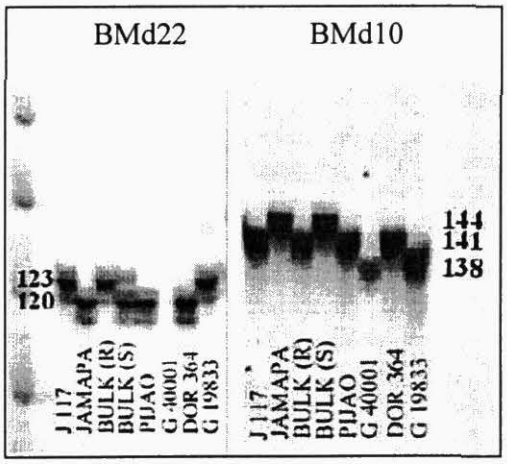


**Figure 1. Bulked segregant analysis of the cross Jamapa x J117, showing the microsatellites BMd22 and BMd10 that tagged unlinked QTLs for Apion resistance on chromosomes B11 and B1, respectively. Molecular weight and size standard are shown, as well as alleles for the DOR364 x G19833 mapping population.**

## 1.2.6 Identification of relevant traits and development of segregating progenies in Cassava for further molecular marker analysis

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[1]SB-2 Project ; [2]IP-3 Project

**Introduction**

The breeding scheme utilized for cassava breeding has changed in the last three years (Figure 1). As a result of these modifications the project has been able to identify some key traits that are relevant for cassava productivity and which are expected to be ideal targets for molecular marker identification. The modifications introduced in the breeding project are not described in detail in this report but are described in detail in the annual report of project IP3. However, the most important modifications relevant for this activity are described in methods.

**Methods**

The first evaluation in the target environment is carried out with several plants per genotype (not only one plant, as before). This is important because a drastic selection process that reduced the number of segregating progenies from 4000 to less than 700 genotypes is now based on more representative samples. It happen to be also relevant because having several plants per progeny allowed for the identification of new key traits that can significantly improve our breeding capacity.
Data is taken and recorded for every plot, not only on those that have been selected. This modification implies that more balance data sets are produced, particularly at early stages of the selection process. This, in turn, means that estimates of general combining ability (closely related to additive genetic effects) can be obtained.

In addition to shortening the length of each breeding cycle by almost a year, it is also very important that the segregating populations reach at the second year of evaluation the replicated trial status. The previous selection scheme required three years of single-replication evaluation. In other words, from the original 4000 genotypes the selection narrowed down the number of progenies to about 160 materials based on single replication observations. The current system allows that the second year of evaluation is already using replicated trials.

This situation meant that seed (produced from only three plants) was limited and, as a result, the following evaluation stage could not be made with replicates. This year, for the first time, the procedure for conducting the Clonal Evaluation Trial was modified considerably. First, the number of plants representing each clone was increased to eight. Of these eight plants, two were harvested in March, mainly to measure dry matter content during the optimal time for taking this measure. When the rains arrive, the cassava plant reinitiates its growth, thus extracting energy that had been accumulated in the roots. As a consequence, dry matter content drops to the extent that starch and chip-drying industries usually either reject the roots or pay low prices for them.

The remaining six plants stayed in the field until the rains arrived and were finally harvested in mid-May, at which time root yield and dry matter content were measured again. Despite the duplication of work, the advantages of this procedure are great:

Six plants are available as seed source, making available sufficient seed for the following evaluation to be conducted with three replicates.

Genotypes whose dry matter content does not diminish drastically upon the arrival of the rains can be identified. This has enormous impact for areas with bimodal rainfall distribution.

Since data is taken and recorded on all genotypes (not only selected ones) it is possible to estimate general combining ability effects.

**Results and Discussions**

*Table 1 summarizes* the results of the Clonal Evaluation Trial at Santo Tomás. For yield, data from the March (two plants) and May (six plants) harvests were combined. Plant type was classified on a visual scale where 1 = plant with outstanding traits and 5 = highly undesirable plant. Yield of dry matter content, averaged across the experiment (1350 clones), was 5.77 t/ha, and across the selected fraction (215 genotypes) was 9.76 t/ha. To compare, the average dry matter yield of eight checks (including the best clone for the area, MTAI 8) was 7.29 t/ha. The best check yielded a maximum of 11.02 t/ha, while the best of the selected clones produced 18.15 t/ha.

The final section of Table 1 presents the results of the best 10 clones across the three strata. As mentioned above, clones located in intermediate areas presented a markedly lower performance than those of the upper and lower areas. The last clone listed in Table 1 came from intermediate areas of the plot, thus showing a poorer performance. Some materials with relatively low dry matter content were selected because they could meet certain needs of the fresh market, which usually requires intermediate levels of dry matter content.

The new procedure, for the Clonal Evaluation Trial permitted the measuring of dry matter content in each clone on two occasions: during the dry season (March) and after the rains arrive (May). Figure 2. illustrates the relationship between dry matter content in March and that in May for the 1350 genotypes evaluated.

From the information provided in Figure 2, one concludes that a close relationship exists between the two sets of dry matter contents, corroborated by a correlation coefficient of 0.689. However, observations made in March do not allow us to efficiently predict those materials that will present high dry matter content in May. For example, clone "A" in Figure 2 had high dry matter content in March (>40%), but very low content in May (<25%). In contrast, clone "B" showed a mediocre performance in March (<35%), but it was outstanding (about 37.5%) after the rains arrived.

Because the crop needs to maintain high dry matter content after the rains arrive to supply the industrial sector of the North Coast, we proceeded to select a group of materials that stood out for this trait. Many were already among the 215 genotypes selected by their good general performance, but others were characterized only for their high dry matter content, even after the rains arrived (Table 2). The average dry matter content across all clones evaluated was 32.41% and 26.74% for March and May, respectively, thus indicating the significant progress expected for this trait.

## On going work

The group of selected clones will be evaluated again for their dry matter content in march and may 2002. In the mean time the plants brought back to headquarters will be used to generate progenies for further genetic analysis along with their agronomic performance.

**Table 1. Results of the selection carried out in the Clonal Evaluation Trial at Santo Tomás, Department of Atlántico, from 1350 families evaluated during May 2000 to May 2001.**

| Parameter o Genotye | Yield (t/ha) | | Harvest Index (0 to 1)[¶] | Plant type (1 to 5)[§] | Dry matter content (%) | |
|---|---|---|---|---|---|---|
| | Fresh roots | Dry matter | | | March | May |
| **Results from the 1350 clones evaluated** | | | | | | |
| Minimum | 0.00 | 0.00 | 0.00 | 1.00 | 0.00 | 0.00 |
| Maximum | 57.02 | 18.15 | 0.84 | 5.00 | 45.96 | 36.76 |
| Mean | 20.23 | 5.77 | 0.45 | 3.02 | 32.41 | 26.74 |
| **Results from the 215 clones selected** | | | | | | |
| Minimum | 19.76 | 6.23 | 0.39 | 1.00 | 25.96 | 16.64 |
| Maximum | 57.02 | 18.15 | 0.84 | 5.00 | 45.96 | 36.76 |
| Mean | 32.20 | 9.76 | 0.54 | 2.59 | 34.41 | 29.05 |
| **Results from the 8 checks included in the trial** | | | | | | |
| Minimum | 6.90 | 1.84 | 0.40 | 3.00 | 0.00 | 21.83 |
| Maximum | 34.17 | 11.02 | 0.69 | 5.00 | 37.45 | 36.76 |
| Mean | 23.77 | 7.29 | 0.55 | 3.88 | 31.62 | 29.25 |
| **Best 10 clones selected across the three strata for high, medium and low areas in the field** | | | | | | |
| SM 2546-44 | 53.09 | 18.15 | 0.64 | 2.00 | 34.31 | 35.00 |
| SM 2546-32 | 54.88 | 16.24 | 0.61 | 2.00 | 32.95 | 28.53 |
| SM 2771-5 | 51.55 | 16.21 | 0.64 | 2.00 | 35.49 | 30.12 |
| SM 2615-13 | 47.62 | 14.82 | 0.48 | 4.00 | 38.57 | 28.22 |
| SM 2615-28 | 51.31 | 14.77 | 0.64 | 2.00 | 35.12 | 26.73 |
| SM 2629-36 | 38.09 | 12.17 | 0.60 | 2.00 | 37.07 | 30.21 |
| SM 2621-1 | 34.29 | 11.34 | 0.45 | 2.00 | 38.38 | 31.33 |
| SM 2769-15 | 39.64 | 10.95 | 0.62 | 3.00 | 31.81 | 26.19 |
| SM 2775-2 | 41.43 | 9.71 | 0.69 | 3.00 | 27.08 | 22.20 |
| SM 2769-11 | 30.24 | 8.64 | 0.63 | 1.00 | 33.04 | 27.09 |

[¶] The harvest index is obtained by dividing the production of commercial roots by total biomass (roots + aerial parts). Preferred harvest indexes are > 0.5.

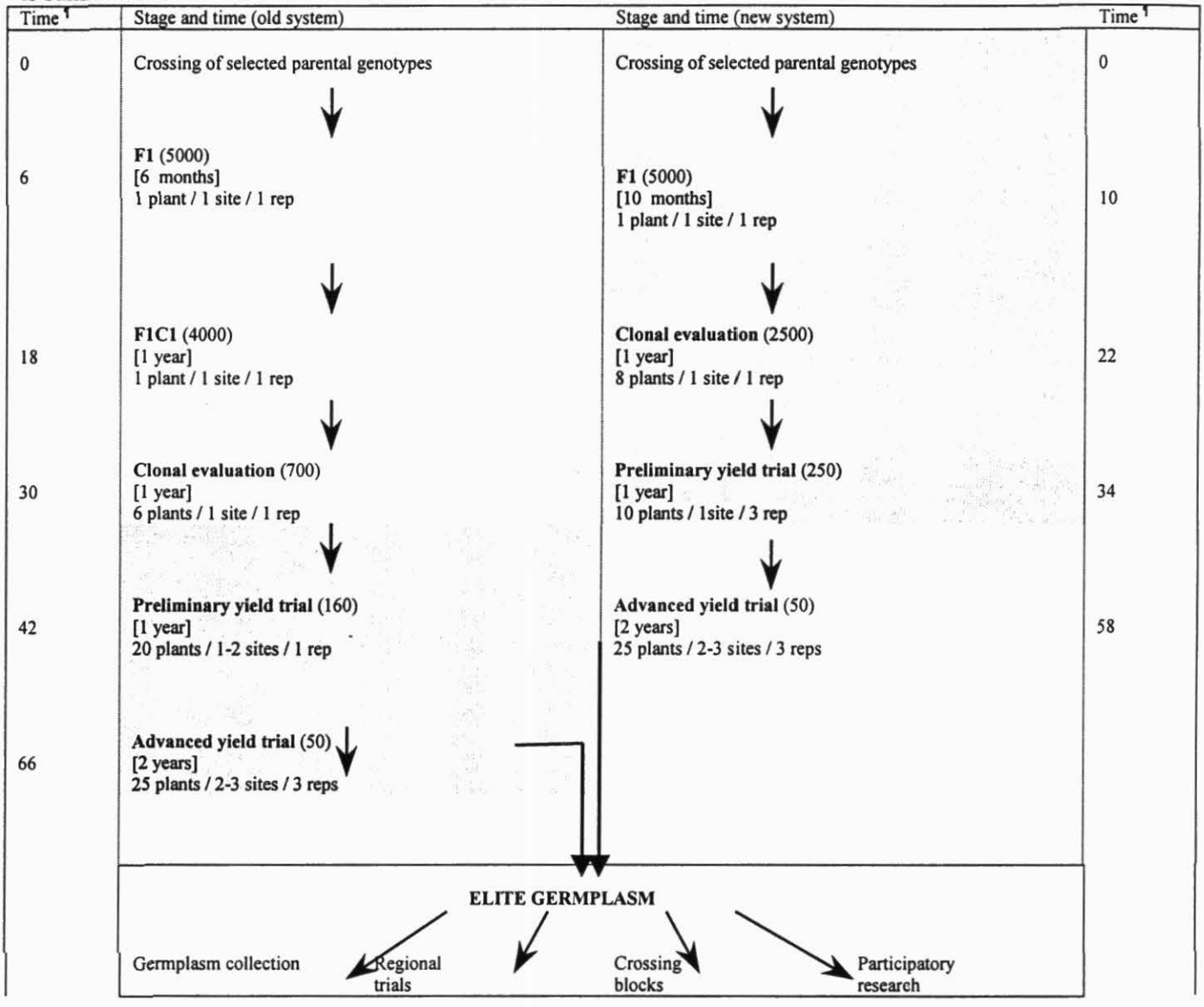
[§] Plant type integrates under one value, plant architecture, leaves health, and capacity to produce stakes on a scale where 1 = excellent and 5 = very poor is used.

**Table 2. Dry matter content as measured in March and May 2001, of a group of clones selected for their good performance in this trait from the Clonal Evaluation Trial.**

| Pedigree | Dry matter content (%) | | Percentage of Retention [1] |
|---|---|---|---|
| | In March | In May | |
| SM 2545-20 | 39.73 | 35.77 | 0.90 |
| SM 2546-52 | 37.16 | 35.96 | 0.97 |
| SM 2546-54 | 37.36 | 34.74 | 0.93 |
| SM 2618-16 | 40.13 | 36.70 | 0.91 |
| SM 2619-1 | 37.97 | 34.00 | 0.92 |
| SM 2619-5 | 39.78 | 36.52 | 0.92 |
| SM 2619-6 | 38.46 | 34.58 | 0.90 |
| SM 2619-12 | 37.68 | 34.02 | 0.90 |
| SM 2621-4 | 43.16 | 34.60 | 0.80 |
| SM 2621-14 | 39.54 | 35.16 | 0.89 |
| SM 2621-25 | 37.45 | 33.85 | 0.90 |
| SM 2621-28 | 38.74 | 36.71 | 0.95 |
| SM 2622-1 | 40.92 | 36.46 | 0.89 |
| SM 2623-1 | 37.01 | 33.92 | 0.92 |
| SM 2772-2 | 40.41 | 33.74 | 0.83 |
| SM 2772-7 | 38.54 | 35.89 | 0.93 |
| SM 2772-8 | 39.30 | 34.20 | 0.87 |
| SM 2773-46 | 34.31 | 37.98 | 1.11 |
| SM 2775-17 | 38.07 | 34.54 | 0.91 |
| SM 2603-9 | 40.86 | 34.67 | 0.85 |
| Promedio | 38.83 | 35.20 | 0.91 |
| Mínimo | 34.31 | 33.74 | 0.80 |
| Máximo | 43.16 | 37.98 | 1.11 |

[1] Ratio between measurement in may over that of march.

Below some interesting results and observations which are relevant to both SB2 and IP3 projects is summarized.




[1]Time in months after germination of botanical seed.

**Figure 1. Basic cassava breeding schemes applied for each of the priority ecosystems. On the right is the new scheme currently under implementation (shaded area). Later stages of selection are made following the old system (shaded area on left).**

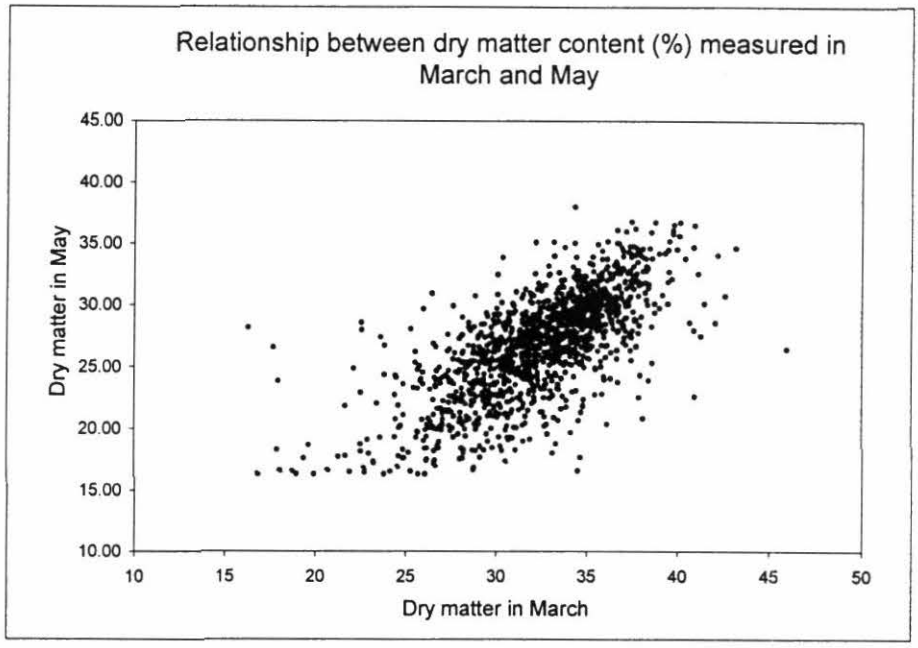



**Figure 2. Dry matter content (%) measured for 1350 genotypes in March and again in May. To provide points of reference, averages are marked, together with a group of materials that maintained dry matter content of more than 33% after the rains arrived. Clones A and B are discussed in the text.**

## 1.2.7 Identification of relevant traits and development of segregating progenies for further molecular marker analysis: leaf retention during plant growth in the absence of water stress.

F. Calle[1]; J.I Lenis[1]; N. Morante[1]; G. Jaramillo[1]; J.C. Perez[1]; H. Ceballos [1-2]
[1]SB-2 Project ; [2]IP-3 Project

**Results and Discussions**

Another significant result obtained from the Clonal Evaluation Trial was the capacity of some genotypes to retain leaves for longer periods during plant growth. Data were obtained during 25-27 October, when the crop was 5½ months old and a differential capacity to retain leaves was already obvious. Although in most materials (1225 or 90.7%), leaf abscission had already occurred in the lower 2/3 of the plant, the remaining 125 (or 9.3%) clones had still retained their leaves.

**Table 1. Effect of leaf retention in 5½-month-old cassava on traits measured 5 months later (at harvest) in the Clonal Evaluation Trial, Santo Tomás, Department of Atlántico, Colombia.**

| Leaf retention | Dry matter content (%) | | Harvest index (0 to 1) | | Fresh roots yield (t/ha) | | Dry matter yield (t/ha) | | Fresh root yield[¶] |
|---|---|---|---|---|---|---|---|---|---|
| | March | May | March | May | March | May | March | May | (t/ha) |
| Yes | 32.15 | 28.51 | 0.55 | 0.50 | 27.05 | 24.12 | 9.16 | 6.95 | 24.96 |
| No | 31.48 | 26.27 | 0.48 | 0.44 | 21.91 | 18.89 | 7.08 | 5.10 | 19.75 |

[¶] Weighted average of fresh roots yield taking into account the number of plants harvested in March and May.

Table 1 presents the averages of different traits for the 1225 clones that did not retain their leaves and for the 125 that did. The notable difference observed between the performance of the two groups suggested that the capacity to retain leaves at 5 months of age (over a period when no marked water stress has yet occurred in the region) has, indeed, a profound effect on overall performance. The materials that retained leaves yielded, on the average, 26% more fresh roots (24.96 versus 19.75 t/ha), which represents an addition of about 2 t/ha of produced dry matter. Furthermore, leaf retention was also observed to associate with higher dry matter content (between 1% and 2% more, depending on when it was measured) and with a higher harvest index (by about 10%). These results are significant in that a trait has been identified that is most likely to be of high heritability (i.e., easy to find a closely linked molecular marker and therefore more amenable for marker assisted selection) and has a profound effect on the agronomic performance of cassava in this region.

This trait will be monitored again this season particularly in the diallel crosses, and not only for the North Coast but also for the other two environments where these experiments are currently underway.

*Identification of relevant traits and development of segregating progenies for further molecular marker analysis: leaf retention during plant growth in the absence of water stress.*

Table 2 shows the most relevant results from the Clonal Evaluation Trial in the Orinoquía Region, for which 1525 clones were planted, each represented by seven plants. Because of the prevalence of foliar diseases such as cassava bacterial blight (Xanthomonas axonopodis pv. manihotis) and superelongation (induced by the fungus Sphaceloma manihoticola), evaluations must be carried out to ensure optimal pressure from these diseases to eliminate as early as possible in the improvement process those genotypes susceptible to these diseases. Thus, in the Clonal Evaluation Trial, the furrows were located, one behind each other, in a single band and separated by plants that served as spreaders of these diseases. Figure 4 shows an observation field and surroundings at about 1½ months after planting. These spreader plants permitted not only high pressure, but also ensured uniform distribution of the diseases. Planting material for spreader plants were stakes chosen from plants that had been discarded, precisely for being susceptible to these diseases, during the previous cycle.

Good development of leaf diseases could be observed early, together with a wide range of variation for both cassava bacterial blight and superelongation. The fraction selected, as a result, reacted well to leaf diseases (average of 2.61), compared with the average for the whole

population (2.92). Similarly, the dry matter productivity improved (8.57 versus 5.47 t/ha), as did dry matter content (32.33% versus 30.41%), plant type (2.67 versus 3.64), and harvest index (0.50 versus 0.43). Finally, although, for this trial, checks were not inserted among the clones evaluated, the variety Reina (CM 6740-7) was planted in lateral furrows and in one central throughout the experiment. The general performance in the trial compared favorably with the performance of Reina.

**Table 2. The most relevant results from Clonal Evaluation Trial evaluated at CORPOICA—La Libertad, Villavicencio, Department of Meta, Colombia, during June 2000 to March 2001.**

| Data set | Disease Ratings (1 to 5) | Plant type[1] (1 to 5) | Yield (t/ha) | | Dry matter Content (%) | Harvest Index (0 to 1) | Selection Index |
|---|---|---|---|---|---|---|---|
| | | | Fresh roots | Dry matter | | | |
| **Results from the selected fraction (240 clones)** | | | | | | | |
| Mean | 2.61 | 2.67 | 26.67 | 8.57 | 32.33 | 0.50 | 26.07 |
| Std.Dev. | 0.77 | 0.92 | 4.15 | 1.10 | 2.41 | 0.06 | 5.88 |
| Minimum | 1.00 | 1.00 | 17.71 | 5.89 | 24.23 | 0.38 | 18.54 |
| Maximum | 4.00 | 5.00 | 43.06 | 11.61 | 47.82 | 0.66 | 46.72 |
| **Results from the complete data set (1525 clones)** | | | | | | | |
| Mean | 2.92 | 3.64 | 17.74 | 5.47 | 30.41 | 0.43 | 0.00 |
| Std.Dev. | 0.80 | 1.13 | 6.57 | 2.16 | 3.22 | 0.09 | 20.07 |
| Minimum | 1.00 | 1.00 | 0.00 | 0.00 | 0.00 | 0.00 | -124.37 |
| Maximum | 5.00 | 5.00 | 43.06 | 11.61 | 47.82 | 0.66 | 46.72 |

[1] Leaf diseases and plant type were classified visually on a scale where 1 = excellent and 5 = very poor. A score of 3 represents a performance similar to the average of the population being evaluated.

Table 3 illustrates the concept of the general combining ability, mentioned above. A group of families is chosen ("SM" with only one progenitor in common, "CM" with two progenitors in common) in terms of the number of clones that constitute each family and of each family's contrasting responses to variables of agronomic importance. Of the 1525 clones evaluated, 240 were selected, that is, about 16%. Any family presenting an index higher than 0.16 of selected clones probably performed better than the overall population. As a result, the progenitor that identifies them had a better performance (in terms of its progeny) than did the average progenitor.

Those families with a higher proportion of selected clones were CM 9459 (i.e., CM 6370-2 × CM 4574-7), CM 9460 (CM 6740-7 × CM 4574-7), CM 9461 (CM 6921-3 × CM 4574-7), and SM 2786 (CM 6438-14). Note that among the parents is clone CM 6740-7, recently released as 'CORPOICA Reina'. These parents tend to produce progeny superior to that of the other progenitors in the same evaluation. Precisely this information led to the inclusion of all these materials (except clone CM 6370-2) as parents for crossings to be carried out during the current agricultural year. Likewise, those clones whose progeny did not stand out (SM 1210-10, CM 8275-2, SM 1282-2, SM 673-1, SM 737-38, and CM 3997-1 were excluded. MPER 183 was included as progenitor because of the good culinary quality of its roots. Such a trait may have good industrial potential.

This type of analysis can give rise to additional information. For example, with regard to reaction to leaf diseases, the progenies of MPER 183, SM 1282-2, SM 1210-10, and SM 1411-5 (a clone adapted to coastal conditions, where disease pressure is not as heavy as in the Acid Soil Savannas) were clearly more susceptible than the overall population. Contrasting progenies were those of SM 1565-15 and CM 6438-14—the latter is to be released soon and will be precisely characterized for its tolerance of diseases per se). Similar comparisons can be done for other variables, such as dry matter yield or dry matter content.

A fundamental advantage of identifying parents with good general combining ability for disease resistance is the higher likelihood that they will posses higher levels of horizontal (polygenic, non race-specific) resistance. This would be particularly relevant for the case of the bacterial blight due to the large genetic variation demonstrated for the causal agent.

Similar data has been obtained for other relevant trait in cassava research: resistance to white flies. In a similar Clonal Evaluation Trial for the mid-altitude valleys, data was taken on all the progenies regarding their reaction to white flies. Although cassava has been found to posses the only recorded source of resistance (antibiosis) to this pest from a cultivated crop, CIAT is interested in expanding the alternatives available to deal with this formidable problem. As in the case of bacterial blight we are hopeful that some genotypes will prove to produce better progenies (regarding reaction the flies) but based on mechanism(s) different from antibiosis.

**Table 3. Averages of some families (with either one (SM) or two (CM) progenitors in common) that participated in the Clonal Evaluation Trial evaluated at CORPOICA—La Libertad, Villavicencio, Department of Meta, Colombia. Contrasting families were selected to illustrate the concept of general combining ability.**

| Family | N° of Clons | Mother (father) | Proportion selected (0 to 1)[1] | Disease rating (1 to 5) | Plant type (1 to 5) | Dry matter content (%) | Yield (t/ha) | | Harvest Index (0 to 1) | Selection Index |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Fresh roots | Dry matter | | |
| SM 2743 | 15 | MPER 183 | 0.00 | 3.88 | 4.59 | 24.02 | 10.82 | 2.84 | 0.35 | -35.80 |
| SM 2733 | 46 | SM 1210-10 | 0.00 | 3.07 | 4.70 | 26.46 | 10.82 | 3.01 | 0.34 | -29.27 |
| SM 2725 | 35 | CM 8275-2 | 0.00 | 2.95 | 4.48 | 27.67 | 16.14 | 4.59 | 0.48 | -8.35 |
| SM 2609 | 38 | SM 1282-2 | 0.03 | 3.36 | 4.48 | 28.59 | 12.06 | 3.61 | 0.41 | -18.34 |
| SM 2607 | 18 | SM 1210-10 | 0.00 | 3.56 | 4.22 | 28.89 | 12.60 | 3.72 | 0.41 | -17.08 |
| SM 2220 | 12 | SM 673-1 | 0.25 | 2.92 | 3.35 | 28.89 | 20.40 | 5.85 | 0.51 | 6.26 |
| SM 2600 | 30 | SM 737-38 | 0.00 | 2.77 | 4.11 | 28.89 | 12.95 | 3.88 | 0.40 | -13.33 |
| CM 9459 | 24 | CM 6370-2 (CM 4574-7) | 0.50 | 3.12 | 3.13 | 29.50 | 24.66 | 7.22 | 0.50 | 13.63 |
| SM 2590 | 31 | CM 3997-1 | 0.00 | 3.04 | 4.26 | 31.02 | 14.01 | 4.43 | 0.43 | -7.71 |
| CM 9449 | 12 | CM 4574-7 (CM 4365-3) | 0.25 | 3.01 | 3.09 | 31.02 | 18.98 | 5.91 | 0.44 | 6.08 |
| SM 2792 | 75 | SM 1565-15 | 0.21 | 2.34 | 3.13 | 31.02 | 18.63 | 5.85 | 0.41 | 5.88 |
| SM 2632 | 26 | CM 4574-7 | 0.23 | 2.51 | 2.91 | 31.32 | 20.22 | 6.35 | 0.42 | 8.86 |
| CM 9460 | 42 | CM 6740-7 (CM 4574-7) | 0.40 | 2.89 | 3.20 | 31.32 | 23.24 | 7.28 | 0.43 | 11.79 |
| CM 9456 | 16 | SM 1411-5 (CM 4365-3) | 0.13 | 3.45 | 3.24 | 31.63 | 15.61 | 5.03 | 0.46 | 0.55 |
| SM 2634 | 21 | CM 6438-14 | 0.24 | 2.48 | 3.64 | 31.63 | 20.22 | 6.35 | 0.42 | 6.52 |
| SM 2635 | 12 | CM 6740-7 | 0.25 | 3.24 | 3.68 | 31.63 | 22.00 | 6.89 | 0.47 | 10.15 |
| SM 2636 | 44 | SM 593-5 | 0.30 | 2.74 | 3.20 | 31.93 | 19.69 | 6.29 | 0.43 | 7.88 |
| CM 9461 | 56 | CM 6921-3 (CM 4574-7) | 0.39 | 2.98 | 3.42 | 31.93 | 22.18 | 7.06 | 0.43 | 10.91 |
| SM 2791 | 17 | SM 1562-11 | 0.29 | 3.18 | 3.42 | 31.93 | 17.39 | 5.58 | 0.48 | 5.34 |
| SM 2642 | 34 | SM 1565-15 | 0.12 | 2.54 | 2.84 | 31.93 | 18.27 | 5.85 | 0.37 | 5.20 |
| CM 9464 | 41 | SM 1411-5 (CM 4574-7) | 0.22 | 3.33 | 2.58 | 32.54 | 20.58 | 6.67 | 0.41 | 10.27 |
| SM 2786 | 19 | CM 6438-14 | 0.37 | 3.07 | 3.06 | 33.45 | 19.51 | 6.51 | 0.46 | 12.86 |
| **Mean of all progenies** | | | **0.16** | **2.92** | **3.64** | **30.41** | **17.74** | **5.47** | **0.43** | **0.00** |

[1] Proportion of selected clones over the total number of clones making up a given family.

## 1.2.8 Identification of relevant traits and development of segregating progenies for further molecular marker analysis: 'root bullking capacity

F. Calle[1]; J.I Lenis[1]; N. Morante[1]; G. Jaramillo[1]; J.C. Perez[1]; H. Ceballos [1-2]
[1]SB-2 Project ; [2]IP-3 Project

**Introduction**

An interesting trait, which perhaps bears a more obvious relationship to yield, is the capacity to accumulate starch in the roots. In the same crossing, one can observe that some clones present a normal root development, whereas others do not accumulate starch. Obviously, the incapacity to accumulate starches in roots is a highly undesirable trait. Nevertheless, this 'mutation' offers an interesting option, because we can eventually identify the gene responsible for starch accumulation in roots. A major achievement of the genetic improvement of crops has been to increase what is known in physiology as the 'sink strength'. Briefly, this concept defines a plant's productivity in terms of (1) its capacity to photosynthetize at the source (i.e., leaves) of photosynthates, and (2) the demand for photosynthates by storage organs (e.g., ears, spikes, and roots). In many cases, limited crop productivity has been demonstrated to reside, not in its capacity to produce photosynthates, but in the ability of storage organs to absorb them.

Many factors affect the bulking capacity in cassava, even when proper canopy has developed (i.e. absence of foliar diseases and pests, or environmental stresses such as drought). Frog skin disease is known to restrict the flow of photosynthesis products to the root system. Also, some plants in segregating progenies have been observed to lack the bulking capacity required for economic yields in cassava. These plants are the subject of the current interest.

**Specific objectives**

- Identify progenies with large number of individual genotypes in which a few show lack of bulking capacity.
- Recover stakes from each member of such family and plant them again to confirm preliminary observations.
- Ultimately, if segregation of bulking capacity is confirmed, develop populations for molecular markers.

**Results**

The practical usefulness of this study is the possibility that it offers for identifying, through molecular markers, the gene (or genes) responsible for defining sink strength in roots (or bulking capacity) so they accumulate starch. Although this allows the elimination of plants that do not have the molecular marker(s) that identify them as having sufficient sink strength. More interesting is the possibility of inserting multiple copies of the gene(s) within an individual with the expectation that this will result in increased sink strength.

Currently, segregating families have been selected from Clonal Evaluation Trials harvested in the first semester of 2001, and planted again. After repeating observations in the current planting for

the incapacity to accumulate starch in roots, we will initiate crossings for the respective genetic studies.

## 1.2.9 Improving the experimental error in the measurements of post harvest physiological deterioration.

G. Zapata[2]; H. Ceballos [1-2]; T. Sánchez[1]
[1]SB-2 Project ; [2]IP-3 Project

### Introduction

Postharvest physiological deterioration (PPD) represents a major problem of storage and trade in cassava roots. Once harvested, cassava roots are difficult to manage. The lack of infrastructure to control PPD and the roots' high water content hamper transportation and raise costs, making the product more expensive. CIAT, therefore, has been involved on PPD research for a long time. On one hand, a collaborative project with the University of Bath (UK) is elucidating the biochemical pathways involved in PPD, on the other recent studies were conducted in search of molecular markers that can be associated with tolerance to PPD. However, no proper research can be conducted until the experimental errors associated with the measurements of PPD are reduced to a reasonable level. Part of the problem is that for PPD reaction, the roots are left under shade in open-air conditions. Since environmental conditions vary (particularly in relation to temperature and relative humidity) they are likely to have a profound effect on the readings of PPD.

### Specific objectives

Determine the effects of temperature and gas characteristics of the atmosphere on PPD.
Develop a chamber that will allow, under controlled environmental conditions, a better determination of the reaction to PPD of different cassava genotypes.

### Materials and methods

Two contrasting varieties were evaluated for their reaction to PPD: MPER 183 (tolerant) and CM 523-7 (susceptible). The methodology used permitted the (1) evaluation of increase in shelf life of cassava roots when stored under low temperatures, and (2) design, on an experimental scale, a chamber for controlled atmospheres to evaluate PPD incidence under three distinct mixtures of gases with compositions low in $O_2$ and high in $CO_2$. The trials were carried out in paired observations so that one treatment comprised controlled storage (positive control) and another was the check, with storage under environmental conditions (negative control). When storage times for both treatments were completed, each were analyzed and evaluated according to three variables: percentage of dry matter in roots on the day of harvest, PPD, and percentage of dry matter on the day of evaluation.

### Results

The evaluations showed that roots stored at temperatures between 8°C and 10°C, with a relative humidity at 80%, had delayed appearance of PPD, increasing shelf life from 2-4 days to at least 14 days. The controlled atmosphere treatments, with gas mixtures in compositions different from that of air, established two mixtures that delayed the appearance of PPD symptoms for 96 h. These mixtures were 5% $O_2$, 5% $CO_2$, and the balance in $N_2$; and 2.5% $O_2$, 5% $CO_2$, and the

balance in $N_2$. The knowledge generated by this may reduce costs of raw material to the precooked and frozen croquette industry by 30% to 40%. This work constitutes a Thesis research proposal by Gloria Zapata Otalvaro, who recently obtained her degree with distinction.

## 1.2.10 Progress Towards a PCR-Marker Based Map of Cassava and its in Cassava Breeding

Angela Zarate[1], Chikelu Mba[1], Graciela Mafla[2], Martin Fregene[1]
[1]SB-2 Project; [2]SB-1 Project

### Introduction

Gene mapping projects in cassava at CIAT have proliferated in the past 2 years from a single mapping population to about 5 at the moment. Genotyping these mapping population in a realistic manner, with respect to time and costs, require molecular markers other than RFLPs, currently the most predominant marker on the cassava map. The need for a PCR-based map of cassava is all the more urgent considering that an advanced back cross QTL (ABC-QTL) mapping project has been initiated to introgress higher protein and dry matter content from wild *Manihot* species into cassava. The success of any ABC-QTL relies heavily on a reliable frame-work map amongst other criteria.

A concerted effort to develop SSR markers was initiated 3 years ago and has yielded more than 500 markers at the moment. Mba et. al described the development of 186 SSR markers from genomic libraries and another 132 SSR markers were obtained from a cassava root and leaf cDNA (Mba et. al. 2000 unpublished data). A third set of 158 SSR markers was also generated from the previous genomic library by another round of screening (CIAT 2000, Fregene et. al. 2001 unpublished data). We describe here the genetic mapping of 58 SSR markers from the third set of 158 markers polymporphic in the parents of the $F_1$ mapping progeny. In addition we report on the progress in converting mapped RFLP markers to sequence tagged sites.

### Methodology

The development of the enriched SSR library, screening, sequencing of SSR containing clones, and primer design for the third set of SSR markers have been described elsewhere (CIAT 2000). The 158 primers were screened in the parents, TMS 30572, and CM2177-2, of the mapping progeny using standard methods for cassava (Mba et. al. 2000). Polymorphic markers were then analyzed in the entire progeny of 147 individuals. The source of plant tissue for DNA isolation of the mapping progeny was exclusively from a set of *in vitro* cultures obtained from the CIAT genetic resources unit or a copy kept in the green house. Linkage analysis of the SSR markers will be with the MAPMAKER computer software as described by Fregene et. al. (1997).

Due to cost considerations, particularly the cost of sequencing kits at CIAT, the sequencing of RFLP clones for the development of STSs clones was contracted out to the Washington University Genome Sequencing Center. Bacteria clones of RFLP probes were cultured overnight in LB media in special culture plates (COSTAR Inc, California USA) with the appropriate antibiotic. Culture plates were sealed and shipped on dry ice to the Washington University Genome Sequencing for sequencing.

**Results**

A total of 58 SSR markers were found to be polymorphic between the parents of the mapping progeny. At the moment 33 SSR markerss have been scored in the $F_1$ mapping progeny of 147 individuals. The gel images of the SSR analysis was captured and stored as JPG files for onward transfer to the cassava genome data base in the ACDB format. The raw SSR data was entered in microsoft excel text files in preparation for chi square analysis of segregation ratios and eventual linkage analysis using MAPMAKER. Results from sequencing of the RFLP clones, contracted out to the Washington University Genome Sequencing Center, is being expected. Once received primers will be designed and synthesized from close to the 3' and 5' ends. Genetic mapping of the STSs will be by straight-forward PCR amplification, cleaved amplified product polymorphisms (CAPs) and single sequence conformation polymorphism (SSCP).

**Future Plans**

Linkage analysis of single dose SSR markers scored in the mapping population; complete sequencing of mapped RFLP markers; implement a single sequence conformation polymorphism (SSCP) protocol for the mapping of sequence tagged sites (RFLP markers) and cDNAs in cassava.

**References**

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## 1.2.11 Marker-Assisted Breeding of Resistance to CMD in Latin American Cassava Gene Pools

N. Morante[1], E. Barrera[1], H. Ceballos[1-2], L. Calvert[1-4] and M.Fregene[1]
[1]SB-2 Project; [2]IP-3 Project ;[3]IP-4 Project

**Introduction**

In order to protect cassava production in Latin America and Asia from an accidental introduction of the Cassava Mosaic Disease (CMD), and to continue to provide improved cassava germplasm for gene pool enhancement in Sub-Saharan Africa, a marker-assisted CMD resistance breeding project was initiated two years ago at CIAT. There are two sources of resistance to CMD, the currently deployed source from *M.glaziovii* and a novel source controlled by a single dominant gene, *CMD2,* found in Nigerian cassava land races. The older source is already available at CIAT, having been introduced more than a decade ago, while progeny bearing the new source were introduced last year from IIITA. In addition, last year, an SSR and RFLP marker were identified that flank *CMD2* at 8 and 9cM respectively (Akano et. al. 2001, CIAT2000). Efforts

have continued to seek for additional SSR markers from two newly available sets of SSR markers and also to begin generating breeding populations for MAS.

The two principal tools for marker-assisted breeding of CMD resistance are a reliable source of the trait and easily assayed markers tightly associated with genes controlling the traits. Both are now available therefore setting the stage for MAS for CMD resistance at CIAT. Particular emphasis has been placed on combining CMD resistance with high carotene content (yellow cassava) for shipment to collaborators in IITA and India.

**Methodology**

Twenty CMD resistance cassava lines from an $F_1$ mapping population derived from TME3, one of the land races with the new source of CMD resistance, were shipped to CIAT from IITA as *in vitro* plantlets with permission from the Colombian plant quarantine authorities. The plantlets were sub-cloned and tested for the presence of virus by PCR, using gemini virus specific primers and ELISA (CIAT 2000). All tests were negative for the presence of virus. With the permission of the plant quarantine authorities, the plants were transplanted to the screen house, and after inspection by the plant quarantine officials, they were transferred to the field for genetic crosses.

Discovery of an SSR marker, from a set of186 SSR markers developed at CIAT (Mba et. al 2000), linked to *CMD2* have been described earlier (Akano et al. 2001, CIAT 2000). A second set of 132 SSR markers, obtained from a cassava root and leaf cDNA (Mba et. al. 2000 unpublished data), and a third set of 154 SSR markers, generated from the previous genomic library by another round of screening (Fregene et. al. 2001 unpublished data) have become available recently. Bulk segregant analysis, using the CMD susceptible and resistant parents, and two pools of 40 susceptible and 40 resistant genotypes from the mapping progeny, was conducted with the two new sets of markers. PCR amplification and gel analysis were as described by Mba et. al. (2000).

**Results**

More than 50 plants representing 20 progeny of TME3 introduced from IITA have been established in the CIAT hybridization block in preparation for genetic crosses. The CMD resistant lines will be crossed to elite parents of the respective genepools from 3 agro-ecologies and high carotene lines (Table 1). Genetic crosses are expected to begin the early part of next year and seeds will be ready by the middle of next year. Seeds harvested from inter-population improvement of the ACMD populations will be shared with IITA and Indian collaborators and the rest will be planted in germination trays and transferred to the field. Marker genotyping will be carried out on these plants and only those with the marker alleled tightly linked to *CMD2* will be transferred to the regular CIAT selection scheme.

BSA with the two new sets of SSR markers identified two markers NS158, from the enriched genomic library, and SSRY339, from the cDNA library, associated with CMD resistance. The two markers were analyzed in the 80 individuals of the resistant and susceptible bulks and 1 recombinant was found for NS158 and 10 for SSRY339. Although these results are preliminary and need to be confirmed in a large size progeny, they suggest that NS158 is much closer to CMD2 than SSRY28, the closest marker identified to date. The precise distance of NS158 is being determined in a large progeny of about 2500 genotypes, developed at IITA for comparison between marker assisted selection and conventional breeding of CMD resistance.

**Table 1. List of CMD resistance donor lines from IITA, elite CIAT parental lines and high carotene content lines for marker-assisted breeding of CMD resistance.**

| | Genotype | No. of plants | Genotype | No. of plants |
|---|---|---|---|---|
| | CMD Resistant donor lines from IITA derived from the TME3 land race | | | |
| 1 | C 6 | 2 | C 127 | 3 |
| 2 | C 18 | 10 | C 227 | 5 |
| 3 | C 19 | 6 | C 243 | 10 |
| 4 | C 22 | 0 | C 373 | 10 |
| 5 | C 24 | 4 | C 377 | 10 |
| 6 | C 35 | 4 | C 400 | 1 |
| 7 | C 39 | 4 | C 413 | 2 |
| 8 | C 43 | 10 | C 4 | 10 |
| 9 | C 54 | 2 | C 33 | 10 |
| 10 | C 101 | 4 | C 41 | 8 |
| | Elite Parental Lines for crosses to CMD resistant lines | | | |
| | *Clones for the Lowland Humid Agro-Ecology:* | | | |
| 1 | CM 3306- 4 | 20 | | |
| 2 | CM 6754- 8 | 20 | | |
| 3 | SM 1411- 5 | 20 | | |
| 4 | MTAI 8 | 20 | | |
| | Clones for the Acid Savannah Agro-Ecology | | | |
| 1 | CM 523- 7 | 20 | | |
| 2 | CM 4574- 7 | 20 | | |
| 3 | CM 6740- 7 | 20 | | |
| 4 | SM 1821- 7 | 20 | | |
| | Clones for the Mid Altitutde Agro-Ecology | | | |
| 1 | CM 7951- 5 | 20 | | |
| 2 | SM 909- 25 | 20 | | |
| 3 | SM 1219- 9 | 20 | | |
| 4 | SM 1741- 1 | 20 | | |
| | Yellow cassava lines low in cyanogenic potential for crosses to CMD resistant lines | | | |
| 1 | CM 489- 1 | 20 | 7 MCOL 2056 | 20 |
| 2 | CM 2772- 3 | 20 | 8 MCOL 2061 | 20 |
| 3 | CM 3750- 5 | 20 | 9 MCOL 2206 | 20 |
| 4 | SM 1433- 4 | 20 | 10 MCOL 2316 | 20 |
| 5 | MBRA 1A | 20 | 11 MCOL 2564 | 20 |
| 6 | MCOL 1734 | 20 | 12 MMAL 66 | 20 |
| | | | 13 MTAI 2 | 20 |

## Future Plan

Genetic crosses between CMD donor lines from IITA and CIAT elite parental lines as well as high carotene lines Marker genotyping of populations with the SSR marker NS158 and selection of lines carrying the resistant allele for introduction into the regular cassava breeding program Sharing of seeds with IITA, particularly those from crosses with high carotene content.

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## 1.2.12 The novel CMD resistance gene (*CMD2*) confers high levels of resistance to the aggressive Ugandan strain (UgV)

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### Introduction

There are at least three geminivirus species that are causal agents of CMD in Africa and one in India: the African cassava mosaic virus (ACMV), the East African mosaic virus (EACMV), and the South African cassava mosaic virus (SACMV), and the Indian cassava mosaic virus (ICMV) in the Indian sub-continent. In addition some strains of EACMV are recombinant like the one associated with the Ugandan epidemic; which consists of the EACMV containing the coat protein of ACMV (Zhou et. al. 1997). This is the virus, the Ugandan variant, (UgV) causing the current epidemic that swept through Uganda and is now spreading into the Democratic Republic of Congo, Kenya, Tanzania and Rwanda.

The new source of CMD resistance controlled by a single dominant gene, *CMD2*, has been shown to confer high levels of resistance to ACMV and EACMV (Akano et. al. 2001, Ogbe 2000, personal communication). The necessity to evaluate the new resistance of CMD against more strains, particularly the aggressive Ugandan variant (UgV) before deployment in African and Latin American genepools led to the shipment of *in vitro* plants of 3 CMD mapping populations to Uganda. The mapping populations have been evaluated for CMD resistance over a period of one year in the field.

### Methodology

Three CMD resistance mapping populations, including two with the novel source of resistance, were multiplied *in vitro* and shipped to IITA Eastern regional center sub-station at the NARO station in Namulonge in early 2000, at least 3 plantlets were shipped per genotype. The plantlets were hardened and transferred to the field in June 2000. CMD resistance evaluation was carried out at 3 and 6 months after planting by staff of IITA East and South Africa regional center, and jointly with Lee Calvert and Martin Fregene at 12 months after planting in June 2001. Disease pressure is very high in Namulonge and susceptible genotypes are easily noticed by to their stunted growth and general deformation. The severe symptom is usually attributed to the Ugandan variant (UgV variant).

To confirm that *CMD2* gene also confers resistance against UgV, SSR analysis was conducted on leaf tissue harvested from 40 resistant and 40 susceptible progeny of the C population (TME3 X TMS30555) in the field. DNA isolation was by a miniprep version of Dellaporta et. al. (1983) using about 200mg of leaf tissue. DNA isolation was conducted at the Medical Biotech Laboratories, Kampala, Uganda and shipped to CIAT. The SSR markers, SSRY28, and NS158 were analyzed in the 80 genotypes as described by Akano et. al. 2001.

## Results

Variation in CMD resistance in the B and C mapping population was qualitative, i.e. all plants of resistant genotypes showed no visible symptom, even on regrowth, while all plants of susceptible genotypes were heavily infected. The chi square of the ratio of resistant to susceptible plants gave a value of 1.1. This is not significantly different from a 1:1 ratio at a probability level of 0.05. This fits the expected segregation ratio for a single dominant gene heterozygous in the CMD-resistance parent, as was observed from earlier evaluations in Nigeria. SSR marker analysis revealed an allele of SSRY28 was present in the resistant progeny but absent in the susceptible progeny. (Figure 1) as found earlier for SSR analysis of evaluations done in Nigeria. Analysis with NS158 again showed similar results with the exception that no recombinant was found, with SSRY28 one recombinant was observed among the resistant genotypes.

The discovery that *CMD2* confers resistance to UgV is of strategic importance in efforts to contain the Ugandan epidemic that has now spread to the Democratic republic of Congo, Kenya, Tanzania and other regions around the great lakes of East Africa. A concerted effort should be made to deploy *CMD2* in local germplasm from these and surrounding areas. IITA is currently distributing seeds to NARs in the region obtained from crossing TME 3, and other donor parents, to local cultivars, this process can be made more efficient by adding a marker pre-screening step of the germinated seeds to ensure that only CMD resistant lines are introduced into the breeding scheme and subsequent multiplication exercises. This is particularly important in adjacent countries with low disease pressure where the UgV has not made its debut but may arrive in the near future.

## Future Plan

- Work with IITA to deploy *CMD2* in cassava gene pools around the great lakes of East Africa under the auspices if the RF CMD project.
- Evaluate TME3 and its progeny in India against the ICMV.

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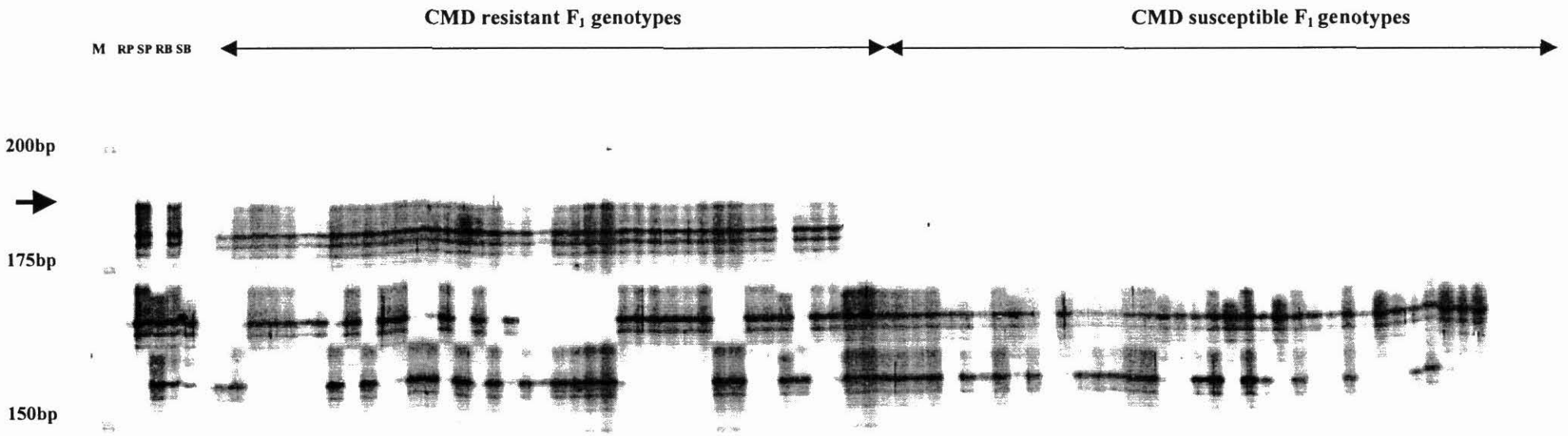


**Figure 1. Polyacrylamide gel image of SSR marker SSRY28 analyzed in the CMD resistant parent (RP), susceptible parent (SP), resistant bulk (RB), susceptible bulk (SB), 40 resistant and 40 susceptible genotypes from Uganda. Arrow points to the SSR allele that is associated with CMD resistance. Molecular weight marker (M) is 25bp ladder.**

## 1.2.13 QTL mapping in an $F_1$ population from non-inbred parents in cassava: yield, yield related and root quality traits.

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### Introduction

Most characteristics of agronomic importance such as yield and related characters as well as quality traits are inherited quantitatively. The number of genes and their interactive effects controlling expression of quantitative traits are poorly understood. The observed phenotypes of these traits are also influenced by the environment. Mapping genes controlling quantitative characters may elucidate the genetics of these traits and help design rationale breeding schemes. Studies on mapping quantitative trait loci (QTL) have been conducted in a number of crop species and have helped to dissect the inheritance of complex traits (Paterson et al. 1988; Stuber et al. 1992).

Cassava breeding strategies can be made more efficient if the inheritance of complex yield and yield related traits are known, particularly in the choice of parents for this long growth cycle crop. An $F_1$ population intra-specific cross from non-inbred parents that has been analyzed with more than 400 RFLP, SSR, and RAPD markers was evaluated in replicated multi-locational trials for yield, yield related, and quality traits over a period of two years in two environments. We describe here the final results of QTL analysis of these traits and discuss insights gained from the study.

### Methodology

Twelve agronomically important traits were evaluated in the $F_1$ mapping population (CM7857) of 144 progeny at the CIAT research station in Palmira and Quilichao in 1998 and 1999. Traits evaluated include fresh root yield (FRY), dry root yield (DRY) and starch yield (STY). Yield related characters evaluated were dry matter percentage (DMC), fresh shoot weight (FSW), harvest index (HI) number of storage roots (NSR), starch content (STC), and leaf area index (LAI). Quality traits such as culinary quality (CQ), amylose content (AML) and post harvest physiological deterioration (PHD) were also scored. The experimental design in both locations was a partially balanced triple lattice design, with three replicates of twelve blocks each, and twelve plots per block (12 by12), with 144 individuals of the $F_1$ population. The plot size of 20 $m^2$, comprising of twenty plants, was arranged in five rows of four plants each, resulting in fourteen border plants and six central plants. The trials were planted on ridges in 1 x 1 m arrangement for a population density of 10,000 plants $ha^{-1}$. Field experiments were not fertilized, but were kept free of weeds and insects as much as possible. Phenotypic evaluation of the 11 traits is as described in Okogbenin and Fregene (2001). These traits were measured on six central plants in a plot and values were taken per plot, with means calculated over replications. All evaluations were at eleven months after planting (11 MAP), in both locations.

Nine genotypes in the $F_1$ mapping population provided insufficient amount of good quality stem cuttings due to the poor plant vigor of the mother plants from which planting stakes were made and resulted in missing data. Our data were therefore analyzed as RCB experiment (Cochran, 1957). Combined analysis of variance across trials in the two environments and years was calculated on phenotypic data of the $F_1$ progeny. The analysis was done considering all variables as random. Variation in the traits measured in two years, were partitioned into sources

attributable to genotype, location, year, replications, interactions and error by analysis of variance (ANOVA), according to the model:

$$Y_{ijkl} = \mu + \gamma_i + \epsilon_j + \gamma\epsilon_{ij} + r(\gamma\epsilon)_{ijk} + g_l + \gamma g_{il} + \epsilon g_{jl} + \gamma g\epsilon_{ijl} + \varepsilon_{ijkl}$$

Where $\mu$ is the population mean; $\gamma_i$ is the effect of the ith year; $\epsilon_j$ is the effect of the jth environment; $\gamma\epsilon_{ij}$ is the effect associated with the interaction of the ith year and jth environment; $r(\gamma\epsilon)_{ijk}$ is the effect of the kth replication within the interaction of the ith year and jth environment; $g_l$ is the effect of the lth genotype; $\gamma g_{il}$ is the effect of the interaction of the ith year and lth genotype; $\epsilon g_{jl}$ is the effect associated with the interaction of the jth environment and lth genotype; $\gamma g\epsilon_{ijl}$ is the effect of the interaction between the ith year, lth genotype and jth environment; and $\varepsilon_{ijkl}$ is the error associated with measuring the phenotype, Y, of the genotype lth genotype grown in the kth replication of the jth location in the ith year; i = 1, 2; j = 1, 2; k = 1, 2, 3; l = 1,2 3, .....144. Tests of significance of F-ratios were obtained using SAS procedures GLM (SAS Institute, 1996). Type III sums of squares were used in these analyses because our data was unbalanced. Traits evaluated in one year (CQ, NR, STY, STC LAI and AML) were analyzed in a combined analysis using a similar model above excluding year as a variable.

Genetic variances were calculated by solving the expected mean squares from ANOVA, and then used in estimating heritability according to the formula of Fehr (1987, p. 257). Pearson correlation coefficients were calculated for each trait/environment combination.

QTL analyses were performed on untransformed data. Normalizing data through transformation may misrepresent differences between individuals by pulling skewed tails toward the center of the distribution. For QTL analysis, we used two framework linkage maps that were derived from the segregation of gametes in the female and male parents of 150 individuals. The female derived map (1208.3 cM) is based on the segregation of 143 molecular markers in the gametes of the female parent, while the male derived map spanning 1475 cM in length is based on the segregation of 135 markers in the male gametes (Fregene et. al. 1997). Association between markers and trait was determined by a simple linear regression of phenotypic data on marker genotype marker class means (single point analysis) using the computer package Q-GENE 2.30B (Nelson, 1997) running on a G3 Power Mcintosh. The amount of phenotypic variance explained by each marker was considered significant if the probability of observing an $R^2$ value was less than 0.005 (Lander and Bostein, 1989). This stringent threshold was adopted in order to reduce Type 1 errors (Dudley, 1993). We also re-analyzed our data using the PGRI computer package based on the t-test conditioning analysis (Liu, 1995) to identify more potential loci associated with evaluated traits. Where three or more linked makers were found significantly associated to a trait, such markers were subjected to further analysis in a multiple regression model according to the equation:

$$Y_{ij} = \mu + A_i + B_i + C_i + \ldots. + Z_i + \varepsilon_{ij}$$

Where $Y_{ij}$ is the trait value of phenotype j with marker score i, $\mu$ is the overall mean for the trait, $A_i + B_i + C_i + \ldots.$ and $Z_i$ represent the linked markers associated with the trait loci and $\varepsilon_{ij}$ is the random error. Using multilocus model can reduce collinearity among markers.

**Results**

Mean phenotypic values, standard errors and ranges for the 11 traits are shown in Table 1.

**Table 1. Performances of the twelve traits in different trials.**

| Trait[a] | Trial[b] | Mean | SD | Range | Kurtosis | Skewness | W -statistic |
|---|---|---|---|---|---|---|---|
| DRY | Q-98 | 925.55 | 267.15 | 1480.46 | 0.35 | 0.12 | 0.99 ns |
| | P-98 | 1179.73 | 343.50 | 1922.31 | 0.41 | -0.12 | 0.99 ns |
| | Q-99 | 465.45 | 178.40 | 917.39 | -0.12 | 0.13 | 0.98 ns |
| | P-99 | 388.92 | 157.99 | 700.69 | -0.26 | 0.46 | 0.96* |
| DM% | Q-98 | 32.99 | 3.50 | 16.75 | -0.34 | 0.00 | 0.98 ns |
| | P-98 | 38.16 | 3.40 | 19.58 | 1.06 | -0.64 | 0.97* |
| | Q-99 | 31.15 | 3.39 | 19.41 | 1.63 | -0.94 | 0.95* |
| | P-99 | 32.77 | 2.30 | 10.71 | 0.08 | -0.50 | 0.96* |
| FRY | Q-98 | 3089.42 | 741.63 | 4962.5 | 1.08 | -0.20 | 0.99 ns |
| | P-98 | 2778.50 | 874 | 5043.33 | 0.19 | 0.00 | 0.99 ns |
| | Q-99 | 1462.93 | 571.79 | 2811.11 | 0.05 | -0.29 | 0.98 ns |
| | P-99 | 1088.36 | 470.06 | 2100 | -0.17 | 0.44 | 0.96 |
| NSR | Q-99 | 9.06 | 3.22 | 16.56 | 0.21 | -0.10 | 0.98 ns |
| | P-99 | 5.75 | 1.84 | 10.27 | 0.60 | 0.32 | 0.98 ns |
| FSW | Q-98 | 1165.13 | 425.13 | 2181.94 | -0.18 | 0.48 | 0.97* |
| | P-98 | 2757.15 | 825.33 | 4454.17 | 1.01 | 0.58 | 0.97* |
| | Q-99 | 903 | 391.32 | 2067.78 | 0.16 | 0.42 | 0.98 ns |
| | P-99 | 904.8 | 526.14 | 2456.94 | 0.84 | 1.05 | 0.91* |
| HI | Q-98 | 0.71 | 0.06 | 0.31 | 0.51 | -0.70 | 0.96* |
| | P-98 | 0.52 | 0.08 | 0.43 | 0.16 | -0.22 | 0.98 ns |
| | Q-99 | 0.60 | 0.10 | 0.60 | 2.15 | -1.10 | 0.94* |
| | P-99 | 0.53 | 0.11 | 0.56 | 0.11 | -0.55 | 0.96* |
| LAI | P-99 | 1.23 | 0.49 | 2.39 | -0.08 | 0.34 | 0.98 ns |
| ST% | Q-98 | 82.92 | 1.79 | 9.67 | -0.03 | -0.02 | 0.98 ns |
| | P-98 | 82.87 | 2.29 | 11.33 | -0.29 | -0.15 | 0.98 ns |
| STY | Q-98 | 768.86 | 226.20 | 1222.64 | 0.27 | 0.14 | 0.98 ns |
| | P-98 | 977.17 | 283.38 | 1624.29 | 0.39 | -0.15 | 0.99 ns |
| AML | Q-98 | 14.98 | 0.98 | 4.61 | -0.26 | -0.04 | 0.98 ns |
| | P-98 | 13.08 | 1.24 | 8.95 | 3.30 | 0.83 | 0.97* |
| CQ | Q-98 | 3.91 | 0.66 | 3.11 | -0.49 | -0.40 | 0.95* |
| | P-98 | 1.94 | 0.76 | 4 | 1.80 | 1.29 | 0.89* |
| PHD | Q-98 | 9.26 | 9.38 | 48.1 | 2.60 | 1.60 | 0.81* |
| | P-98 | 37.50 | 17.34 | 97 | 0.37 | 0.47 | 0.98 ns |
| | Q-99 | 9.51 | 9.90 | 47.18 | 4.43 | 2.12 | 0.74* |
| | P-99 | 10.23 | 13.69 | 84.45 | 3.72 | 1.56 | 0.89* |

[a]DRY,dry root yield; DMC, dry matter content, FRY, fresh root yield, NSR, number of storage roots; FSW, fresh shoot weight; HI, harvest index; LAI, leaf area index (5 MAP), STC, starch percentage content; STY, starch yield; AML, amylose content; CQ, culinary quality; PHD, post harvest deterioration.

[a] Q = Quilichao, P = Palmira, 98 = 1998.

ns Distribution is not significantly different from normal; * Distribution is significantly different from normal

Analysis of variance revealed significant differences between the genotypes for all traits with the exception of PHD (Table 2).. Significant year by environment interaction was observed for five (FRY, DRY, HI, DMC and FSY) of the six traits evaluated in two years (Table 2). All six traits (STY, ST%, NR, LAI, AML and CQ) which were evaluated only in one season showed significant genotype by environment interaction. Broad sense heritability estimates were 87% for

HI, 71% for FSW, 70% for AML, 62% for DMC, 60% for DRY, 50% for FRY, 36% for NR, 44% for STY, 30% for ST% and 7% for PHD. A low heritability estimate for PHD suggests high non-genetic influence in the expression of PHD in the population, which is in agreement with our observation of non-significant variation between the genotypes for PHD. Heritability was not estimated for culinary quality because it was qualitatively scored.

**Table 2. Mean squares from the analysis of variance of six traits evaluated both in 1998 and 1999 in two locations**

| Source of variation[a] | *Df* | Fresh root yield | Dry matter percentage | Dry root yield | Fresh shoot yield | Harvest index | Post harvest physiological deterioration |
|---|---|---|---|---|---|---|---|
| Yr | 1 | 462919875.21**** | 7481.52**** | 155989869.82**** | 1150135473.76**** | 0.965**** | 94971.08* |
| Loc. | 1 | 243704324.26* | 5729.69**** | 2190308.44**** | 1816531.04**** | 6.393**** | 236704.96*** |
| Yr x Loc | 1 | 259387114.12**** | 660.50**** | 12599434.65**** | 52433740.52**** | 1.118**** | 79882.42* |
| Rep x (Yr x Loc) | 8 | 6169239.88**** | 49.52**** | 662439.27**** | 6849731.98**** | 0.072**** | 18940.97 |
| Gen. | 143 | 2169912.54**** | 63.63**** | 321104.09**** | 2804802.47**** | 0.060**** | 14818.32 |
| Yr. x Gen[b] | 142 | 432370.52 | 18.00** | 102450.16 | 769424.95 | 0.011**** | 14481.94 |
| Loc x Gen[b] | 143 | 622342.50 | 18.08** | 128509.07 | 800730.68 | 0.007 | 14575.65 |
| Yr x Loc x Gen | [c] | 520630.64**** | 11.65 | 107287.64**** | 803714.69**** | 0.005*** | 15335.84 |
| $R^2$ | | 0.83 | 0.73 | 0.82 | 0.85 | 0.83 | 0.35 |

[a]Abbreviations under sources of variation are: Yr (year), Loc. (location), Rep. (replication), and Gen (genotype).
[b] Yr x Gen. and Loc. x Gen were tested using Yr x Locx Gen as the error term under random model used for ANOVA.
[c] The degrees of freedom *df* was 136 for fresh root fresh shoot yield and harvest index, 129 for dry matter yield and dry matter content and 135 for post-harvest physiological deterioration.
*, **, **, **** F-test significant at the 0.05, 0.01, 0.001and 0.0001 probability levels

A positive and significantly high correlation was found between dry root yield and fresh root yield (r = 0.91 to r = 0.97). Fresh root yield was positively correlated with fresh shoot yield (r = 0.51 to r = 0.67). This positive correlation is in agreement with findings that top growth is critical for yield production (El-Sharkawy and Cock,). Negative correlation was found between fresh shoot yield and harvest index (r = -0.45 to –0.58) indicating that, the shoot system is a major sink which could strongly compete with storage roots for available carbohydrate in the plant. Higher number of storage roots resulted in higher dry root yield (r = 0.76 to r = 0.84) in agreement with previous findings that root yield is associated with number of roots (Pelliet and El-Sharkawy, 1994). Number of roots was also positively and significantly correlated with dry matter percentage (r = 0.50 to r = 0.53), dry root yield (r = 0.74 to r = 0.79) and fresh shoot weight (r = 0.41 to r = 0.49). Starch yield was highly correlated as expected with dry root yield (r = 0.99). Starch is the major component of yield and constitutes about 70% to 85% (on dry weight basis) of root yield (IITA, 1994; Rickard et al., 1991). Starch yield was negatively and significantly correlated with culinary quality. High culinary quality (given as low scores in the evaluation scale) was associated with increase in starch. Thus, it should be possible to increase starch yield and improve culinary attributes of cassava simultaneously. Culinary quality declined with increase in post harvest deterioration resulting in a negative correlation (r = -0.24 to r = -0.44). Post harvest deterioration was found to be positively correlated with dry matter percentage (r= 0.40 to r = 0.50). This suggests that while high dry matter percentage (increase in starch) is important to improvement in culinary quality, it is undesirable for post harvest deterioration. Leaf area index was significantly and positively correlated with DMP (r = 0.32), DRY (r = 0.38), FRY (r = 0.41), FSW (r = 0.33) and NSR (0.40). The growth, development and final yield of the cassava plant, is dominated and determined by, the relation of LAI to total biomass produced and its distribution (Cock and El-Sharkawy, 1988).

Because all the traits, with the exception of PHD, exhibited highly significant genotype by environment interaction or genotype by location by year interaction, QTL analysis was done separately for each trial. Putative QTLs for each trait are listed in Table 3. A total of eighty-one QTLs were found for the twelve traits analyzed. QTLs detected in this study were found distributed in all but two linkage groups (B and Q). Between four and eleven QTLs were detected per trait, with each explaining between 6 and 19 % of the observed phenotypic variances. The number of QTLs detected should be taken as the minimum. The stringent threshold chosen ($p < 0.005$) while reducing the chance of Type-I errors (false positives), would have also led to the possibility of Type-II errors (not detecting valid QTLs) (Dudley, 1993). Though QTL-marker linkages have been found to remain reproducible across environments specific QTLs are often expressed under particular environmental conditions (Patterson et al, 1988). Our results showed that majority of the QTLs associated with traits were found in one trial. This could be due to the very significant genotype x environment interaction observed in our experiment across sites and years for most of the traits evaluated.

Thirteen QTLs (16%) were found in common for with yield and related traits that were correlated (Table 4). The observed congruence of some of the QTLs, in classical quantitative genetics, is assumed to be due to pleiotropy or close linkage. Zhuang et al (1997) explained that if pleotropism is the major reason for the coincidence of the QTLs, for related traits, the directions of their phenotypic effects could be expected. However, if close linkage was the major reason, the direction of the effects of the QTLs may be different, although the coincidence of the locations of the QTLs can still be expected. A general coincidence of the locations and directions of the effects of the QTLs for related traits were observed in this study suggesting that pleiotropism rather than close linkage of the QTLs might be the reason for the correlation of related traits. The distinction between linkage and pleiotropy is important for breeding purposes as well as for scientific reasons.

QTLs analysis of important agronomic traits provides an insight into the inheritance of these traits that can guide breeding decisions. However the usefulness of this information depends on how good the QTLS study was and what percentage and magnitude of key QTLs were successfully detected The mating design and method of analysis becomes important here. Single marker analysis used in our study could inflate the phenotypic effects of the QTLS if the genes are very closely linked to the markers, or could underestimate the effects if these QTLs lie far away from the marker locusassociated them. The approach does not define the likely position of a QTL, and cannot distinguish between tight linkage to a QTL with small effect and loose linkage to a QTL with large effect. Further activities have been initiated to improve QTL mapping studies of these traits in cassava. The activities include the use of $F_2$ populations and adding more highly polymorphic markers to the genetic map to increase saturation. Markers such a simple sequence repeats (SSR) are particularly useful because of their high levels of polymorphisms.

**Table 3. Summary of single marker analysis , percent phenotypic variance attributable to a marker, linkage group membership of markers in an $F_1$ segregating population from the cassava CM 7857cross (TMS 30572 x CM 2177-2)**

| Trait[a] | QTL[b] | Map | Linkage group | Marker[c] | Trial | % PVE[d] | Effects | Probability |
|---|---|---|---|---|---|---|---|---|
| DRY | dryD.1 | Female | D | GY 42 | Q-98 | 15 | -199.90 | 0.0005 |
| | dryD.2 | Female | D | GY 181 | Q-98 | 12 | -179.34 | 0.0018 |
| | dryG.1 | Male | G | GY 6 | Q-99 | 11 | -121.60 | 0.0040 |
| | dryJ.1 | Male | J | GY 34-1 | Q-99 | 17 | -152.62 | 0.0030 |
| | dryUM.1 | Female | UM | Ai18b | Q-98 | 10 | -85.76 | 0.0044 |
| | dryUM.2 | Female | UM | ri14b | P-98 | 15 | -263.85 | 0.0025 |
| DMC | dm%A.1 | Male | A | GY28 | Q-98; P-99 | 16 | -0.87 | 0.0017 |
| | dm%D.1 | Female | D | GY 50 | P-98 | 10 | -2.19 | 0.0030 |
| | dm%D.2 | Female | D | GY 42 | Q-99 | 12 | -2.22 | 0.0.003 |
| | dm%J.1 | Male | J | rM8b | Q-99 | 12 | -1.31 | 0.0020 |
| | dm%J.2 | Male | J | GY34-1 | Q-99 | 11 | -1.20 | 0.0021 |
| | dm%L.1 | Female | L | rAF14a | Q-98 | 11 | -2.34 | 0.0047 |
| | dm%M.1 | Female | M | rGY 192 | P-98; Q-99 | 9 | 2.16 | 0.0004 |
| | dm%O.1 | Male | O | GY29 | Q-98 | 11 | -2.35 | 0.0034 |
| | dm%UM.1 | Female | UM | ONI.C2 | P-98 | 6 | -1.81 | 0.0024 |
| FRY | fryD.1 | Female | D | GY42 | Q-98 | 10 | -327.67 | 0.0051 |
| | fryE.1 | Male | E | r14a | P-98 | 10 | 267.23 | 0.0044 |
| | fryG.1 | Male | G | GY6 | Q-99 | 11 | -327.67 | 0.0050 |
| | fryH.1 | Male | H | GY77 | Q-98 | 15 | 327.67 | 0.0004 |
| | fryJ.1 | Male | J | GY7 | Q-99 | 12 | -327.67 | 0.0020 |
| | fryM.2 | Female | M | rGY215 | Q-99 | 6 | -277.74 | 0.0040 |
| | fryN.1 | Male | N | GY109-1 | Q-99 | 10 | -327.67 | 0.0050 |
| | fryUM.1 | Female | UM | r14b | P-98 | 18 | -327.67 | 0.0008 |
| NSR | nsrC.1 | Female | C | GY23 | Q-99 | 10 | 2.06 | 0.0050 |
| | nsrG.1 | Male | G | rGY62 | P-99 | 10 | -0.55 | 0.0046 |
| | nsrJ.1 | Male | J | GY7 | P-99; Q99 | 14 | -1.38 | 0.0010 |
| | nsrJ.2 | Male | J | GY34-1 | Q-99 | 19 | -2.88 | 0.0001 |
| FSW | fswC.1 | Male | C | rGY89-1 | Q-99 | 13 | -279.27 | 0.0010 |
| | fswC.2 | Male | C | GY23-1 | Q-99 | 12 | -274.94 | 0.0040 |
| | fswD.1 | Female | D | rGY180 | P-98 | 11 | 327.67 | 0.0001 |
| | fswF.1 | Female | F | GY203 | Q-98 | 8 | 238.46 | 0.0008 |
| | fswG.1 | Male | G | rCDY16-1 | P-98, Q-98 | 14 | -327.67 | 0.0009 |
| | fswG.2 | Male | G | GY41 | P-98 | 18 | 327.67 | 0.0001 |
| | fswH.1 | Male | H | GY77 | Q-98 | 12 | 280.58 | 0.0019 |
| | fswI.1 | Female | I | rD5a | Q-98 | 11 | 260.82 | 0.0040 |
| | fswJ.1 | Female | J | rCDY74 | P-99 | 12 | -327.67 | 0.0040 |
| | fswN.1 | Male | N | GY20 | Q-99 | 14 | 283.94 | 0.0010 |
| | fswP.1 | Female | P | rGY220 | Q-99 | 11 | 247.05 | 0.0042 |
| HI | hiC.1 | Male | C | GY81-1 | P-98 | 13 | 0.05 | 0.0026 |
| | hiD.1 | Female | D | rGOT-2 | P-98 | 11 | -0.04 | 0.0029 |
| | hiF.1 | Female | F | GY 194 | P-99 | 11 | -0.07 | 0.0001 |
| | hiUM.1 | Female | UM | GY 212 | Q-99 | 7 | -0.05 | 0.0020 |
| | hiUM.2 | Female | UM | GY 142 | Q-99 | 7 | -0.06 | 0.0028 |
| | hiUM.3 | Female | UM | ri14b | P-98 | 19 | -0.07 | 0.0005 |
| LAI | laiA.1 | Female | A | ACP-1 | P-99 | 11 | 0.44 | 0.0020 |
| | laiC.1 | Female | C | rO11a | P-99 | 20 | -0.33 | 0.0001 |
| | lai.C2 | Male | C | rGY18 | P-99 | 11 | -0.19 | 0.0043 |
| | laiG.1 | Female | G | AGPaseB | P-99 | 13 | 0.15 | 0.0028 |
| | laiG.2 | Female | G | AM18 | P-99 | 18 | -0.26 | 0.0001 |

| Trait[a] | QTL[b] | Map | Linkage group | Marker[c] | Trial | % PVE[d] | Effects | Probability |
|---|---|---|---|---|---|---|---|---|
| STC | st%A.1 | Male | A | rGY75-1 | Q-98 | 12 | -1.22 | 0.0039 |
| | st%C.1 | Male | C | rK9a | P-98 | 12 | 1.53 | 0.0022 |
| | st%F.1 | Male | F | GY80 | Q-98 | 11 | -1.17 | 0.0032 |
| | st%O.1 | Male | O | rGY91 | Q-98 | 15 | -1.44 | 0.0004 |
| STY | styD.1 | Female | D | GY42 | Q-98 | 15 | -169.09 | 0.0006 |
| | styD.2 | Female | D | GY181 | Q-98 | 12 | -152.83 | 0.0017 |
| | StyUM.1 | Female | UM | ri14b | P-98 | 15 | -218.45 | 0.0022 |
| | StyUM.2 | Female | UM | Ai18b | Q-98 | 10 | -144.19 | 0.0048 |
| AML | amlD.1 | Female | D | GY50 | P-99 | 7 | -0.66 | 0.0014 |
| | amlD.2 | Female | D | GY179 | P-99 | 7 | -0.65 | 0.0016 |
| | amlI.1 | Female | I | rD5a | Q-99 | 11 | -0.69 | 0.0044 |
| | amlK.1 | Male | K | GY82 | Q-99 | 12 | 0.78 | 0.0025 |
| | amlN.1 | Female | N | rGY145 | Q-99 | 7 | 0.53 | 0.0015 |
| CQ | cqA.1 | Male | A | GY28 | Q-98 | 14 | 0.49 | 0.0033 |
| | cqD.1 | Female | D | GY50 | Q-98 | 8 | 0.35 | 0.0010 |
| | cqE.1 | Female | E | rS2 | Q-98 | 12 | -0.45 | 0.0017 |
| | cqE.2 | Female | E | rGY118 | P-98 | 14 | 0.46 | 0.0010 |
| | cqF.1 | Female | F | GY196 | P-98 | 6 | -0.36 | 0.0044 |
| | cqH.1 | Male | H | rQ11 | Q-98 | 16 | 0.55 | 0.0008 |
| | cqP.1 | Male | P | r13b | P-98 | 12 | -0.52 | 0.0016 |
| | CqUM.1 | Female | UM | rONI.C2 | Q-98 | 6 | 0.32 | 0.0031 |
| PHD | PhdD.1 | Female | D | O19 | P-99 | 15 | -10.80 | 0.0006 |
| | phdE.1 | Female | E | rS2 | P-99 | 11 | 9.18 | 0.0040 |
| | phdE.2 | Female | E | GY217 | P-99 | 10 | -9.11 | 0.0050 |
| | phdF.1 | Male | F | rV20a | Q-99 | 11 | 7.22 | 0.0050 |
| | PhdG.1 | Female | G | rK16d | Q-99 | 10 | -8.42 | 0.0007 |
| | phdL.1 | Female | L | CDY131 | P-98 | 12 | 13.84 | 0.0025 |
| | phdL.2 | Male | L | rCDY131-1 | P-99 | 12 | 9.55 | 0.0040 |
| | PhdO.1 | Female | O | GY138 | Q-98 | 8 | 5.23 | 0.0024 |
| | PhdUM.1 | Male | UM | GY120 | P-98 | 11 | -11.36 | 0.0028 |
| | PhdUM.2 | Female | UM | Ai18b | P-99 | 13 | -5.25 | 0.0014 |
| | PhdUM.3 | Female | UM | GY202 | P-98 | 6 | 8.05 | 0.0049 |

[a]DMC = dry matter content, DRY = dry root yield, FRY = fresh root yield, FSY = fresh shoot yield, NR = number of storage roots per plant, HI = harvest index, STC = starch content in the roots, STY = starch yield, CQ = culinary quality, AML = amylose content (%), PHD = post harvest deterioration; LAI = leag area index.

[b]Individual QTLs are designated with Q indicating QTL, abbreviation of the trait name and the linkage group. When more than one QTL affecting a trait was identified on the same linkage group, they are distinguished by different number.

[c]Marker significantly associated with trait variation

[d] Percentage phenotypic variance explained by the individual marker locus-trait associations based on regression analysis

[e]Significance levels determined for F-tests based on each pairwise comparison of a quantitative trait and marker locus ($p < 0.005$)

**Table 4. Marker loci associated with pleiotropic/linkage effects**

| Marker | Linkage group | QTL[a] | No. of traits |
|---|---|---|---|
| GY28 | A | dm%A.1, cqA.1 | 2 |
| GY42 | D | dryD.1, fryD.1, styD.1 | 3 |
| GY181 | D | DryD.2, styD.2 | 2 |
| GY50 | D | dm%D.1, amlD.1, cqD.1 | 3 |
| rS2 | E | cqE.1, phdE.1 | 2 |
| GY6 | G | dryG.1, fryG.6 | 2 |
| GY77 | H | fryH.1, fswH.1 | 2 |
| rD5a | I | fswI.1,amlI.1 | 2 |
| GY7 | J | fryJ.1, nsrJ.1 | 2 |
| GY34-1 | J | dryJ.1, dm%J.2, nsrJ.2 | 3 |
| r14b | UM | dryUM.2, fryUM.1, styUM.2 | 3 |
| Ai18b | UM | dryUM.2, styUM.1, phdUM.2 | 3 |
| ONI.C2 | UM | dm%UM.1, cqUm.1, | 2 |

[a]Individual QTLs are designated with Q indicating *QTL, abbreviation* of the trait name and the linkage group. When more than one QTL affecting a trait was identified on the same linkage group, different numbers are used to distinguish them. DMC, dry matter content; DRY, dry root yield, FSW, fresh shoot weight; NSR, number of storage roots per plant; HI, harvest index; STC starch content in the roots; STY, starch yield; AML, amylose content; PHD, post-harvest deterioration; CQ, culinary quality.

## Future Plans

1.Test the effectiveness of other kinds of crosses, including F2 populations and wide crosses, involving wild relatives, in detecting QTLs.

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## 1.2.14 QTL mapping in an $F_1$ population from non-inbred parents in cassava: morphological traits

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### Introduction

Plant architecture is an important agronomic feature in cassava production particularly under particular farming systems and cultural practices such as in intercropping. This is most evident in sub-Saharan Africa where monoculture is rarely practiced due to the security and efficient use of space and resources crop diversification provides small-scale holder farmers. Plant architecture has implications for yield and weed control through canopy information, incident light absorption by leaves, and branching habit (Nweke et al., 1994). A strong association has also been found between length of stem with leaves and dry matter yield in certain genotypes (Ceballos 2001, personal communication).

The use of the linkage map of cassava to study the inheritance of morphological traits of cassava, such as plant height, branching height and levels, leaf morphology (shape and size), and length of stem with attached leaves is described here. A number of these traits have been observed to possess high broad sense heritability and some are thought to be controlled by single genes. Finding molecular markers tightly associated with genes controlling these traits will permit marker-assisted negative selection at the seedling stage to "weed" out undesirable morphologies

and make the selection at consequent stages more cost-effective and efficient. Negative selection at the seedling stage to eliminate inferior genotypes, combined with indirect selection for yield, using harvest index, is most the efficient breeding scheme for yield in cassava (Kawano et. al. 1998).

**Methodology**

The 144 individuals of the mapping progeny were evaluated for morphological traits in a partially balanced triple lattice design in CIAT headquarters at Palmira and at another CIAT station in Satander de Quilichao. The study was conducted over a period of two years, 1998 and 1999. The $F_1$ population was planted on ridges, in plot sizes of 20 $m^2$ (of 5 rows x 4 columns), by this arrangement, each plot had fourteen border plants and six central plants. The six central plants in each plot were evaluated for plant height (PH), length of stem with attached leaves (LSL), and Leaf shape (LS, a ratio of leaf length LL to leaf width LW). Other traits include first branching height (BH), branching levels (BL), leaf area index (LAI) and the derived trait, branching index (BI) expressed as the ratio of BH to PH.

Separate analyses of variance for progeny data were conducted, for all traits evaluated. The final data were analyzed as in RCB experiment due to missing data in a random effects model. Combined analysis of variance (SAS ANOVA procedure) was as described earlier for yield and yield related traits (CIAT 2001, this report). $H^2$, on entry mean basis, was estimated for each trait using the variance components of the expected mean squares (Fehr, 1987, p. 257) from our analysis of variance of the $F_1$ population. Spearman's rank correlation coefficients were calculated for each trait/location combination based on progeny data. Phenotypic data were subjected to QTL analysis using untransformed data based on markers from the male- and female- derived maps of the $F_1$ mapping population as described earlier. A significant association between the traits and the marker ($p < 0.005$) was considered an evidence of a QTL in the region of the marker.

**Results**

With the exception of leaf shape, where a bimodal distribution pattern was observed, all other traits analyzed exhibited continuous distribution in the $F_1$ population, typical of quantitative traits. The correlation between the two environments for the traits ranged from 0.38 in 1998 and 0.57 in 1999 for length of stem with leaves to 0.97 for leaf shape in 1998 (Table 1). Analysis of variance detected significant differences between the genotypes for each trait (Table 2). Analysis of variance showed that, of the four traits evaluated in two years (PH, BH, BL and LSL), only LSL was not significant for genotype by location by year interaction (Table 4). It would suggest that PH, BH, and BL are much more variable traits compared with LSL across locations and or years.Broad sense heritabilty estimates based on plot entry means were 91% for plant height, 83% for branching levels, 94% for branching height, 80% for leaf length, 90% for leaf width and 27% for length of stems with leaves.

Leaf shape was measured only in 1998 and a marker locus, GY99, explained phenotypic variation of 61% for LS suggesting it to be a major gene. This result was confirmed in a $BC_1$ cross derived from the mapping population. (Okogbenin and Fregene 2001). Earlier reports have described leaf shape as being controlled by a single gene with broad leaf shape recessive gene to narrrow leaf shape effect at this locus (Graner 1942). With the female derived map, no significant effect was found for leaf shape at this locus, but with male derived map, this locus explained 61% of the observed phenotypic variance for leaf shape. This observation underscores the need to analyze both male- and female-derived maps QTL mapping experiments in $F_1$ progeny from non-inbred

parents to capture the effects of dominant genes. QTLs found for the other morphological traits are summarized in Table 3.

Results of QTL analyses show that these morphological traits, with the exception of LS, are mainly under quantitative genetic control as expected. The small QTL effects found for most of the traits, particularly for stem with leaves (STL), a trait with a suggested simple mode of inheritance, disagrees with the high broad sense heritabilities and suggests the CM6857 mapping progeny may not be the most appropriate cross for QTL mapping of these traits or the population structure is inadequate. Another factor that could confound QTL analysis is the low level of polymorphism between genomes of the parents, major QTLs in a monomorphic region of the genome will fail to be detected. These factors will be addressed in subsequent studies.

**Table 1. Phenotypic correlation coefficients of morphological traits across Environments**

| Quantitative trait | Correlation coefficient between sites | |
|---|---|---|
| | 1998 | 1999 |
| Leaf length (LL) | 0.71*** | - |
| Leaf width (LW) | 0.90*** | - |
| Leaf area (LA) | 0.70*** | - |
| Leaf shape (LS) | 0.97*** | - |
| Plant height (PH) | 0.55*** | 0.76*** |
| Branching height (BH) | 0.83*** | 0.86*** |
| Branching levels (BL) | 0.46*** | 0.67*** |
| Length of stem with leaves (LSL) | 0.38*** | 0.57*** |

[1] Heritability estimate was based on data from two locations (Palmira and Quilichao) in two years except for leaf width and length evaluated in one year.
*** Indicates that the correlation coefficient (r) is significant at $P < 0.001$.

**Table 2. Mean squares from the analysis of variance of four traits evaluated both in 1998 and 1999**

| Source of variation[a] | *df* | Plant height | Branching height | Branching levels | Length of stems with leaves |
|---|---|---|---|---|---|
| Yr | 1 | 695180.53**** | 264118.87**** | 33.45**** | 30488.08**** |
| Loc. | 1 | 119310.32**** | 8.40[ns] | 2.60** | 280233.17**** |
| Yr x Loc | 1 | 893041.08**** | 87469.82**** | 51.84**** | 29362.45**** |
| Rep x (Yr x Loc) | 8 | 8376.52**** | 1564.48**** | 1.57**** | 3219.11**** |
| Gen. | 143 | 7457.05**** | 5121.28**** | 4.72**** | 1038.00**** |
| Yr. X Gen[b] | 142 | 701.09 [ns] | 533.74[ns] | 0.50[ns] | 601.25[ns] |
| Loc x Gen[b] | 143 | 710.00 [ns] | 201.50[ns] | 0.79** | 694.01[ns] |
| Yr x Loc x Gen | 137[c] | 716.56**** | 409.01**** | 0.50**** | 660.50[ns] |
| $R^2$ | | 0.90 | 0.93 | 0.78 | 0.56 |

[a] Abbreviations under sources of variation are: Yr (year), Loc. (location), Rep. (replication), and Gen (genotype).
[b] Yr x Gen. and Loc. x Gen were tested using Yr x Locx Gen as the error term under random model (McIntosh, 1983) used for ANOVA.
[c] The degrees of freedom *df* for plant height, branching height, branching levels was 137, but 136 for length of stems with leaves.
*, **, **** F-test significant at the 0.05, 0.01, and 0.0001 probability levels
[ns] not significant

**Table 3. List of putative QTLs detected from data collected in the $F_1$ mapping trials at Palmira and Quilichao in two years**

| Trait[a] | QTL[b] | Map | Linkage group | Marker | Trial | | | | % PVE[c] | Effects[d] | P-value[e] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Q-98 | P-98 | Q-99 | P-99 | | | |
| LW | lwB.2 | Female | B | GY197 | ns | * | - | - | 6 | 0.35 | 0.0040 |
| | lwL.1 | Female | L | CDY131 | * | * | - | - | 12 | 0.27 | 0.0017 |
| | lwH.1 | Male | H | rGY99 | * | * | - | - | 61 | -0.76 | 0.0000 |
| LL | llH.1 | Male | H | rGY99 | * | * | - | - | 15 | 2.00 | 0.0005 |
| LA | laF.1 | Female | F | GY37 | * | ns | - | - | 8 | 2.59 | 0.0011 |
| | laH.1 | Male | H | rGY99 | * | * | - | - | 33 | -12.98 | 0.0000 |
| | laJ.1 | Female | J | CDY76 | ns | * | - | - | 12 | -7.36 | 0.0023 |
| | laL.1 | Female | L | CDY131 | ns | * | - | - | 11 | 7.73 | 0.0037 |
| | laN.1 | Female | N | rGY148 | * | ns | - | - | 7 | -2.59 | 0.0010 |
| | laN.2 | Female | N | AD4b | * | ns | - | - | 10 | -3.28 | 0.0038 |
| | laO.1 | Female | O | GY138 | * | ns | - | - | 8 | 2.65 | 0.0009 |
| LS | lsD.1 | Male | D | ri18b | * | * | - | - | 13 | 0.026 | 0.0035 |
| | lsH.1 | Male | H | rGY99 | * | * | - | - | 61 | -0.10 | 0.0000 |
| | lsL.1 | Female | L | rAF14a | * | ns | - | - | 11 | 0.04 | 0.0037 |
| | lsL.2 | Female | L | CBB1 | * | ns | - | - | 8 | 0.019 | 0.0050 |
| PH | phD.1 | Female | D | rGY180 | * | ns | ns | ns | 6 | 15.56 | 0.0031 |
| | phF.1 | Female | F | GY196 | ns | ns | ns | * | 8 | 14.45 | 0.0008 |
| | phF.2 | Female | F | GY203 | ns | * | ns | * | 7 | 16.44 | 0.0010 |
| | phG.1 | Male | G | rCDY16-1 | ns | ns | ns | * | 14 | -19.90 | 0.0017 |
| | phG.2 | Male | G | GY121 | ns | ns | ns | * | 9 | -8.92 | 0.0030 |
| | phI.1 | Female | I | R1 | ns | ns | * | ns | 10 | 21.30 | 0.0050 |
| | phJ.1 | Female | J | rCDY74 | ns | ns | ns | * | 14 | -20.24 | 0.0010 |
| | phJ.2 | Male | J | GY34-1 | ns | ns | * | ns | 11 | -21.66 | 0.0040 |
| | phN.1 | Male | N | GY10 | ns | ns | * | ns | 11 | 11.82 | 0.0044 |
| BH | bhA.1 | Female | A | GY28 | ns | * | * | * | 7 | -16.06 | 0.0030 |
| | bhJ.1 | Male | J | GY34-1 | ns | ns | * | ns | 13 | -18.87 | 0.0031 |
| | bhM.1 | Female | M | rGY192 | ns | * | ns | ns | 7 | 16.32 | 0.0028 |
| BI | biD.2 | Female | D | rGY167 | ns | * | * | ns | 8 | -0.03 | 0.0008 |
| | biD.3 | Male | D | rGY57 | * | ns | * | ns | 14 | 0.08 | 0.0017 |
| | biJ.1 | Male | J | K10 | ns | * | ns | ns | 10 | -0.08 | 0.0042 |
| | biM.1 | Female | M | GY154 | ns | ns | ns | * | 6 | 0.05 | 0.0035 |
| BL | BlD.1 | Female | D | rGY167 | ns | * | ns | ns | 7 | 0.38 | 0.0026 |
| | blD.2 | Female | D | rGY180 | * | ns | ns | ns | 6 | 0.34 | 0.0040 |
| | blD.3 | Male | D | GY57 | ns | * | * | ns | 11 | -0.49 | 0.0002 |
| | blG.1 | Male | G | GY97 | * | ns | ns | ns | 10 | 0.45 | 0.0043 |
| | blO.1 | Male | O | nGY138 | ns | ns | * | ns | 12 | -0.43 | 0.0020 |
| | blR.1 | Male | R | AE2 | * | * | ns | ns | 10 | -0.44 | 0.0030 |
| | blS.1 | Female | S | GY142 | * | ns | ns | ns | 12 | 0.46 | 0.0028 |
| LSL | lslC.1 | Male | C | GY23-1 | ns | ns | ns | * | 12 | -8.40 | 0.0050 |
| | lslF.2 | Female | F | GY196 | * | ns | ns | ns | 8 | 2.64 | 0.0009 |
| | lslG.1 | Female | G | rGA-131 | ns | ns | ns | * | 11 | 4.26 | 0.0048 |
| | lslG.2 | Male | G | GY41 | ns | ns | ns | * | 10 | 7.60 | 0.0050 |

| Trait[a] | QTL[b] | Map | Linkage group | Marker | Trial | | | | % PVE[c] | Effects[d] | P-value[e] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Q-98 | P-98 | Q-99 | P-99 | | | |
| | lslI.1 | Female | I | rD5a | * | ns | * | ns | 13 | 1.96 | 0.0007 |
| | lslM.1 | Female | M | rGY192 | ns | ns | * | * | 7 | 1.38 | 0.0008 |
| | lslUM.1 | Female | UM | r14 | ns | ns | ns | * | 12 | 8.21 | 0.0030 |
| | LslUM.2 | Female | UM | GY212 | * | ns | ns | ns | 6 | 1.29 | 0.0015 |

[a]LL, leaf length; LW, leaf width, LA, leaf area; LS, leaf shape; PH, plant height; BH, branching height; BI, branching index; LSL, length of stem portion with leaves.
[b] Individual QTLs are designated with Q indicating QTL, abbreviation of the trait name and the linkage group. When more than one QTL affecting a trait was identified on the same linkage group, they are QTLs are distinguished by different number.
[c] Phenotypic variance explained.
[d] phenotypic effects of the QTL, being the difference between homozygote and heterozygote genotypes at a locus.
[e] Probability of the association between a QTL and marker. When a QTL – marker association is significant at more than one trial the most significant P value is declared and corresponding PVE and phenotypic effects of QTLs are given.

## Future Plans

- Development of new crosses with CM6754-8, SM1068-10, and SM1257-7, three genotypes that show a simple mode of inheritance for stem with leaves (STL) in previous crosses.
- Analysis of some of these traits in inter-specific crosses with a higher level of genome level polymorphism.

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## 1.2.15 Marker Fidelity Study of QTLs identified for early bulking in Cassava

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[1]IP-3 Project ; [2]SB-2 Project

***Introduction***

The QTL mapping early bulking at CIAT has identified a number of major QTLs for this important trait (Okogbenin and Fregene 2001; CIAT 2000). Comportment and inheritance of tentative markers identified to be associated with QTLs for early bulking would need to examined in advanced breeding populations and different genetic backgounds to propose a model of genetic control and estimate genetic interactions. This will lead to better exploitation of existing variability and fine mapping of earlier mapped QTLs. To validate the authenticity, magnitude and action of these QTLs a new $F_2$ mapping population was developed from $F_1$ individuals bearing positive alleles of the identified QTLs. This population is being genotyped with SSR toward the development of a frame work genetic map for QTL analysis. A field trial to evaluate early bulking in the $F_2$ population has also been set up.

Based on asymptotic theory, the type of progeny developed in an experiment will affect the power to identify QTL using marker trait methods (Beavis, 1998). An $F_2$ population has increased power for QTL mapping of traits as well as elucidating genetic action and interactions of genes involved. In addition, the development of $F_2$ population is a step further at improving QTL mapping through use of more advanced statistical tools such as interval mapping and composite interval mapping in detecting the precise locations of QTLs, and their genetic effects actions and effects.

**Methodology**

To confirm putative QTLs for early bulking and to determine gene actions involved in the control of this trait, 3 $F_2$ populations were developed by selfing three profusely flowering genotypes in the $F_1$ population in the last quarter of 1999. The individuals selected for -self-pollination, were CM 7857-68, CM 7857-145 and CM 7857-150 resulting in 396, 725 and 519 seeds respectively. Seeds from CM 7857-145 were established from embryo axes as described earlier (CIAT 2000). Seeds from CM 7857-68 and CM7857-150 were however planted in germination trays in the screen house to reduce cost associated with *in vitro* culture. A total of 473 plantlets were obtained for CM 7857-145. Of the 396 seeds for CM 7857-68, 245 or 62% germinated, while 72% (or 372 seeds) of the total 519 seeds for CM 7857-150 germinated. Resulting seedlings were then transferred to the field for planting after two months in the screen house. Results from a preliminary evaluation of traits related to early bulking indicate that CM 7857-150 showed high wide variability for these traits and therefore only $F_2$ progeny from this individual were selected for further marker analysis and phenotypic evaluation.

For SSR marker analysis, total genomic DNA was isolated according to a miniprep version of the Dellaporta (1983) extraction procedure. Total DNA was extracted from fully expanded young leaves of green house-or field grown plants of a total of 268 genotypes of the $F_2$ population produced by selfing CM 7857-150. Leaf samples of 0.15 – 2.0g was powdered in liquid nitrogen using a mortar and pestle. The powder was transferred to a frozen 1.5ml eppendorf tubes using a frozen spatula. The powder was resuspended in 800 µl of extraction buffer (100mM Tris-HCl,

50mM EDTA and 500mM Nacl) and 50 µl of 20% SDS. The ground tissue was shaken vigorously in the buffer and then vortexed intermittently for 15min at 65°C. To this solution, 250 µl of ice-cold 5M potassium acetate was added and homogenised by gently inverting 5-6 times. The mixture was incubated in ice for 20 minutes and then centrifuged at 12000rpm for 10 min. The aqueous solution was transferred to a new 1.5ml eppendorf tube and the nucleic acids was precipitated by adding one volume of ice-cold isopropanol (approximately 700µl), and then mixed by gently inverting 8-10 times before incubating at –80°C for one hour, followed by centrifugation at 12000rpm for 10 min. The resulting supernatant was poured off and the pellet resuspended in 500µl of 50mM Tris-HCl/10Mm EDTA. The precipitation process is then repeated for a second time to eliminate salts in the DNA Electrophoresis and fluorometry were used in determining DNA quality and DNA concentration respectively.

The development of 186 SSR markers for cassava have been described by Mba et. al. 2000. Another 132 SSR markers were obtained from a cassava root and leaf cDNA (Mba et. al. 2000 unpublished data). A third set of 154 SSR markers were also generated from the previous genomic library by another round of screening (Fregene et. al. 2001 unpublished data). A total of more than 500 SSR markers now exist for cassava and were the source of markers for genotyping the $F_2$ population. SSR Markers were first of all screened in the parents of the $F_1$ mapping progeny and 3 $F_1$ progenies - parents of the $F_2$ families, if this information was not already available from previous studies. PCR amplification and PAGE gel analysis were as described by Mba et. al. (2000)

A field trial of the selected $F_2$ populations was established at Santa Elena on 18 May 2001, in an RCB design of single row plots of 6 plants each and two replications. Of the 268 genotypes of the F2 population, only 207 genotypes had sufficient stem cuttings (12 stakes) could be planted. Germination percentage ranged from 16 – 100% amongst the genotypes. The trial will be harvested at 7 MAP (December 2001) and evaluated for early bulking by measuring dry matter yield, dry weight of foilage, harvest index and number of roots.

**Results**

Of the 500 SSR marker results reveal that 200 are heterozygous in CM7857-150 and will segregate in the $F_2$ population. One hundred markers have so far been screened in the $F_2$ population. Seventy three markers (73%) segregated as single dose markers and have yielded segregation data in the $F_2$ progeny. Eight markers (8%), which were polymorphic in the $F_1$ parents and K150, did not segregate in the $F_2$ progeny, revealing these markers as double dose markers. Results also revealed two markers, which though were heterozygous in K150, segregated into two genotypic classes only such markers are not useful in the construction of a $F_2$ framework map. Nine other markers, which though were polymophic in the $F_1$ parents and K150, selectively amplified only few individuals in the $F_2$ progeny. Such markers are being repeated with changes in $MgCl_2$ concentrations and annealing temperatures to enhance DNA amplification in the $F_2$ progeny. Only markers with goodness of fit to the expectation of 1:2: 1 will be utilized for map construction using the computer package MAPMAKER. Marker genotyping of the $F_2$ population continues and a target of 180 markers have been set. The phenotypic evaluation of the cross later in the year will provide the other component required for QTL analysis.

**Plans for Next Year**

- Completion of marker genotyping of the $F_2$ population
- QTL analysis of the $F_2$ population

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## 1.2.16 QTL mapping of cyanogenic potential (CNP) in cassava

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**Introduction**

The food safety of cassava is compromised by the presence of cyanogenic glucosides that break down on mechanical damage of cassava tissues and results in the release of a poison, hydrogen cyanide (HCN). Cassava varieties vary in their potentials to produce HCN, a fact that could be exploited to control its level through breeding. The cyanogenic potential (CNP) of a cassava genotype is assessed at the end of its growth cycle and it is affected by the environment. Improvement of CNP will therefore benefit from marker assisted selection via a reduction in the time for breeding and an increase in the selection efficiency. The goal of this project is to develop markers for marker assisted breeding for reduced cyanogenic glucoside content in cassava.

**Methodology**

CNP is a quantitative trait and many genes are expected to control the biosynthesis, transport and storage of cyanogenic glucosides. Quantitative Trait Loci (QTLs) mapping will be therefore be used to identify markers associated with key genes controlling CNP as has been described for casssava (Okogbenin and Fregene 2001, CIAT2001, this report). A high CNP cassava line Gomani from Malawi was crossed as pollen parent to a low CNP line Mbundumali as staminate parent and about 100 $F_1$lines were generated. After SSR marker analysis to confirm true hybrid nature of progeny, 3 $F_1$ lines were selfed to obtain about 200seeds each. Seeds were tested for viability by soaking in water. After the viability test, embyro culture was carried out for 600 seeds

in the 17N culture medium (1/3 medium, supplemented with 0.01 mg $l^{-1}$NAA, 0.01 mg $l^{-1}$ $GA_3$, 1.0 mg $l^{-1}$ thiamine-HCL, 100 mg $l^{-1}$ inositol, 2% sucrose, 0.7% agar (Sigma Co.) and 25 mg $l^{-1}$ of a commercial fertilizer containing: N 10, P 52, K 10, pH 5.7-5.8. Culture of embryo axes are as described by Fregene et. al. (1998). The embryo cultures were incubated in darkness for three days to promote radicle growth and then transferred to growth chambers with a 12hr photoperiod. A total of 240 progeny were hardened and established in the field.

**Result**

Three $F_2$ populations segregating were obtained by selfing 3 $F_1$ progenies obtained from a cross between high and low CNP lines. About 200 seeds were obtained from each cross and embryo axes from each seed cultured in vitro. The seeds were cultured in the Med Biotech Laboratories, Kampala, and will be transferred to the green house at NARO, Namulonge after 1 month They will the be taken to the field in a low pressure site in Uganda for the production of woody stakes.

The following year the $F_2$ populations will be evaluated for CNP in a replicated fashion. At the same time an SSR marker survey of the Mbundumali and Gomani grand parents, and $F_1$ parents, using all 500 available SSR markers will be conducted at CIAT. Polymorphic markers from the parental survey will be used to genotype an $F_2$ population selected based upon the highest number of heterozygous markers in the $F_1$ parent. Single marker and interval marker analysis will be conducted to identify markers associated with genes controlling CNP using the computer software packages Q-gene and PRGI. Molecular markers found associated with CNP will be tested in a marker-fidelity study to evaluate its usefulness for cassava breeding.

**Future Plan**

- Clone the $F_2$ family and establish a replicated CNP trial in the field
- Produce a map with 120 genome-wide markers selected from the cassava genetic map available at the International Center for Tropical Agriculture (CIAT).

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Fregene, M. A., Ospina J.A., and Roca, W.M. (1999). Recovery of cassava (*Manihot esculenta* Crantz) plants from culture of immature zygotic embryo. Plant Cell, Tisuue, and Organ tissue culture 55(1): 39-43

Okogbenin E. and Fregene M (2001) Genetic Analysis and QTL Mapping of Early Bulking in an $F_1$segregating Population from Non-inbred Parents in Cassava (*Manihot esculenta* Crantz) (Theor and Appl Genet in review)

## 1.2.17 Identification of marker linked genes conferring resistance to white fly in cassava

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[1]SB-2 Project ; [2] IP-3 Project ; [3]IP-4 Project

**Introduction**

Whiteflies, as direct feeding pests and virus vectors, are one of the most important agricultural pests in the world. They cause major damage in cassava-based agroecosystems in the Americas,

Africa and to a lesser extent in Asia. In cassava (*Manihot esculenta* Crantz), in the Americas, the whitefly species, *Aleurotrachelus socialis*, has caused crop losses greater than 70%. Stable host plant resistance (HPR) offers a practical, low cost, long-term solution for maintaining reduced whitefly populations.

HPR studies initiated at CIAT > 15 years ago has identified several sources of resistance to *A. socialis* (CIAT, 1999). The clone MEcu 72 has consistently expressed the highest levels of resistance. *A. socialis* feeding on resistant clones had less oviposition, longer development periods, reduced size and higher mortality than those feeding on susceptible ones. Whitefly-resistant clones, in field trials, showed no significant differences in yield between insecticide-treated and non-treated plots (Bellotti et. al. 1999).

Whitefly resistance in agricultural crops is rare; therefore, given the importance of these pests, there is a need to understand the genomics of the resistance that we are observing in MEcu 72 and other resistant clones. It would be especially advantageous to map whitefly resistance genes and understand their segregation in F1 progeny. Crosses were, therefore, made between MEcu 72 and a susceptible genotype to map resistance genes by using molecular markers. This will aid in a more rapid selection of resistant germplasm and also isolate those genes involved in resistance.

**Materials and Methods**

A cross was made between the resistant genotype, MEcu 72 and the susceptible genotype MCol 2246. The latter cultivar was selected because of its high level of susceptibility to *A. socialis*, but also having tolerance to mites and thrips, two additional important pests of cassava. In addition MCol 2246 has good floration, an advantage in obtaining the high numbers of progeny necessary for genetic studies. This cross produced 282 F1 individuals.

The sexual seeds produced in the cross were grown in sterile soil, in 67 plastic trays, and held in the screen house for 6 to 8 weeks (Temp. ± 30°C). Seedlings were subsequently planted in the field for multiplication.

Greenhouse evaluations were done by in vitro multiplication consisted of cutting plant species, removing to the laboratory, and disinfecting by washing in deionized sterile water, then 70% alcohol, then 0.25% hypochlorite and finally three additional washings in deionized sterile water (Escobar, 1991). The apices were planted in 4E media (Roca, 1984), in 16mm test tubes. The growth period was 60-80 days and a second propagation in 4E media resulted in 5 tubes per clone. Later, apices of each clone were cut and planted in 17N media (Roca, 1984) to obtain root growth; a period of 30-40 days. The plants were then ready for removal to the greenhouse for evaluation.

The afore-mentioned methodology permits maintaining plants in optimal sanitary conditions, in addition to having sufficient material available on need in a reduced area or space. Greenhouse evaluations were done with the parents MEcu 72 and MCol 2246, and the progeny using the leaf snap-cages and infected with *A. socialis* adults from the CIAT colony.

Field trials were carried out at two sties, CIAT, Palmira, and in Nataima, El Espinal, Tolima. The parents and progeny were planted 1 x 1 meter in the field and exposed to natural whitefly infestations.

Cassava microsatellite (Simple Sequences Repeat, SSR) were used. DNA visualization was done by the tincture of silver nitrate technique to observe allelic segregation of the markers.

**Results and Discussion**

*In vitro* propagation: Through in vitro propagation, 224 genotypes from the MEcu 72 x MCol 2246 cross, were obtained and grown in test tubes on a 4E media. From each of these genotypes, 5 clones were multiplied and propagated in a 17N media in the greenhouse. The 58 remaining genotypes are being collected for multiplication. The resistant (MEcu 72) and susceptible (MCol 2246) cultivars were evaluated with 343 cassava microsatellites, including 116 new SSRs of c DNA (Mba et. al., submitted). Approximately 60% of the microsatellites were polymorphic (Table 1); thereby obtaining 180 polymorphic microsatellites from the two parents. With the extraction of DNA from the 282 individuals progeny, polymorphic microsatellites were run (Figure 1).

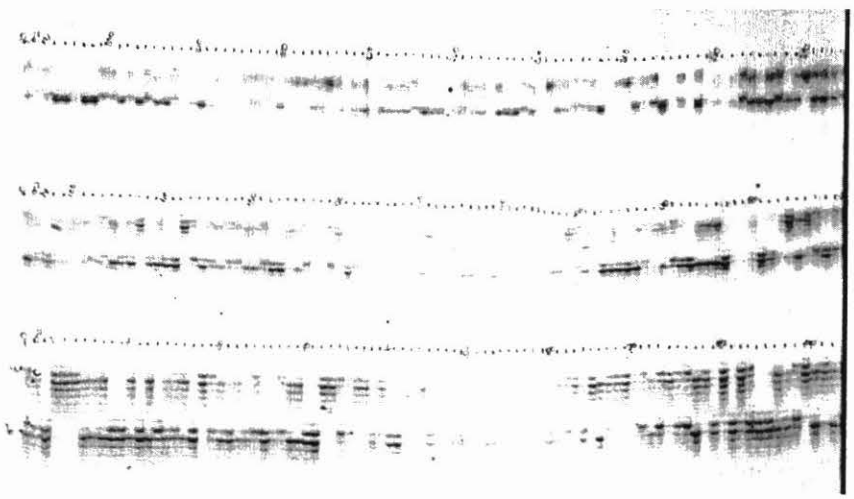

**Figure 1. Microsatellite 7 (F) of 282 F1 individuals from an MEcu 72 x MCol 2246 crosses.**

**Table 1. Cassava microsatellites fro the parental cultivars Mecu 72 and MCol 2246.**

| SSR # | Size (bp) | T. Anneal °C | Polymorphic | SSR # | Size (bp) | T. Anneal °C | Polymorphic |
|---|---|---|---|---|---|---|---|
| SSRY1 | 197 | 45 | X | SSRY51 | 298 | 50 | X |
| SSRY2 | 225 | 55 | X | SSRY52 | 266 | 55 | X |
| SSRY3 | 247 | 45 | X | SSRY53 | 138 | 55 | Monomorphic |
| SSRY4 | 287 | 45 | X | SSRY54 | 151 | 55 | X |
| SSRY5 | 173 | 55 | X | SSRY55 | 145 | 50 | X |
| SSRY6 | 298 | 45 | X | SSRY56 | 137 | 50 | Monomorphic |
| SSRY7 | 250 | 45 | X | SSRY57 | 293 | 55 | X |
| SSRY8 | 288 | 45 | X | SSRY58 | 217 | 55 | X |
| SSRY9 | 278 | 55 | Monomorphic | SSRY59 | 158 | 55 | X |
| SSRY10 | 153 | 55 | X | SSRY60 | 137 | 55 | X |
| SSRY11 | 265 | 55 | X | SSRY61 | 233 | 55 | Monomorphic |
| SSRY12 | 266 | 55 | Monomorphic | SSRY62 | 250 | 55 | Monomorphic |
| SSRY13 | 234 | 50 | X | SSRY63 | 290 | 55 | Monomorphic |
| SSRY14 | 300 | 55 | Monomorphic | SSRY64 | 194 | 55 | X |
| SSRY15 | 215 | 50 | Monomorphic | SSRY65 | 299 | 55 | X |
| SSRY16 | 218 | 55 | X | SSRY66 | 261 | 55 | Monomorphic |
| SSRY17 | 277 | 50 | X | SSRY67 | 278 | 55 | Monomorphic |
| SSRY18 | 198 | 44 | Monomorphic | SSRY68 | 287 | 55 | X |
| SSRY19 | 214 | 50 | X | SSRY69 | 239 | 55 | X |
| SSRY20 | 143 | 55 | X | SSRY70 | 249 | 55 | X |
| SSRY21 | 192 | 55 | X | SSRY71 | 217 | 55 | X |
| SSRY22 | 299 | 43 | Monomorphic | SSRY72 | 141 | 55 | X |
| SSRY23 | 247 | 45 | X | SSRY73 | 265 | 50 | Monomorphic |
| SSRY24 | 100 | 45 | Monomorphic | SSRY74 | 114 | 55 | X |
| SSRY25 | 296 | 45 | Monomorphic | SSRY75 | 284 | 55 | X |
| SSRY26 | 121 | 55 | X | SSRY76 | 273 | 55 | X |
| SSRY27 | 277 | 50 | X | SSRY77 | 275 | 55 | X |
| SSRY28 | 180 | 55 | Monomorphic | SSRY78 | 248 | 55 | X |
| SSRY29 | 281 | 55 | Monomorphic | SSRY79 | 210 | 55 | X |
| SSRY30 | 220 | 50 | X | SSRY80 | 299 | 55 | X |
| SSRY31 | 188 | 50 | X | SSRY81 | 204 | 55 | Monomorphic |
| SSRY32 | 298 | 50 | Monomorphic | SSRY82 | 211 | 55 | X |
| SSRY33 | 273 | 50 | Monomorphic | SSRY83 | 239 | 55 | Monomorphic |
| SSRY34 | 279 | 55 | X | SSRY84 | 203 | 55 | X |
| SSRY35 | 282 | 55 | Monomorphic | SSRY85 | 292 | 50 | X |
| SSRY36 | 134 | 55 | X | SSRY86 | 296 | 50 | X |
| SSRY37 | 187 | 50 | Monomorphic | SSRY87 | 102 | 55 | X |
| SSRY38 | 122 | 55 | X | SSRY88 | 243 | 55 | X |
| SSRY39 | 293 | 50 | X | SSRY89 | 120 | 55 | X |
| SSRY40 | 231 | 50 | X | SSRY90 | 193 | 55 | Monomorphic |
| SSRY41 | 271 |  | X | SSRY91 | 300 | 55 | Monomorphic |
| SSRY42 | 221 | 50 | X | SSRY92 | 171 | 55 | Monomorphic |
| SSRY43 | 255 | 43 | Monomorphic | SSRY93 | 289 | 55 | X |
| SSRY44 | 194 | 50 | Monomorphic | SSRY94 | 268 | 55 | X |
| SSRY45 | 228 | 50 | X | SSRY95 | 282 | 55 | X |
| SSRY46 | 268 | 50 | Monomorphic | SSRY96 | 149 | 55 | X |
| SSRY47 | 244 | 55 | X | SSRY97 | 194 | 55 | X |
| SSRY48 | 178 | 50 | Monomorphic | SSRY98 | 209 | 55 | Monomorphic |
| SSRY49 | 300 | 50 | Monomorphic | SSRY99 | 192 | 55 | X |

| SSR # | Size (bp) | T. Anneal °C | Polymorphic | SSR # | Size (bp) | T. Anneal °C | Polymorphic |
|---|---|---|---|---|---|---|---|
| SSRY50 | 271 | 50 | X | SSRY100 | 210 | 55 | X |
| SSRY101 | 213 | 55 | X | SSRY153 | 117 | 45 | X |
| SSRY102 | 179 | 55 | Monomorphic | SSRY154 | 318 | 55 | X |
| SSRY103 | 272 | 55 | X | SSRY155 | 158 | 55 | X |
| SSRY104 | 258 | 52 | Monomorphic | SSRY156 | 160 | 44 | Monomorphic |
| SSRY105 | 225 | 55 | Monomorphic | SSRY157 | 500 | 45 | Monomorphic |
| SSRY106 | 270 | 55 | X | SSRY158 | 224 | 45 | Monomorphic |
| SSRY107 | 120 | 45 | X | SSRY159 | 159 | 45 | Monomorphic |
| SSRY108 | 203 | 55 | X | SSRY160 | 151 | 50 | X |
| SSRY109 | 125 | 55 | X | SSRY161 | 220 | 55 | X |
| SSRY110 | 247 | 55 | Monomorphic | SSRY162 | 126 | 43 | X |
| SSRY111 | 235 | 55 | Monomorphic | SSRY163 | 231 | 44 | Monomorphic |
| SSRY112 | 117 | 55 | X | SSRY164 | 187 | 55 | X |
| SSRY113 | 187 | 45 | X | SSRY165 | 243 | 55 | X |
| SSRY114 | 167 | 55 | X | SSRY166 | 244 | 55 | X |
| SSRY115 | 296 | . | Non-amplified | SSRY167 | 183 | 45 | X |
| SSRY116 | 167 |  | Non-amplified | SSRY168 | 277 | 55 | Monomorphic |
| SSRY117 | 142 | 55 | X | SSRY169 | 100 | 55 | X |
| SSRY118 | 169 | 55 | Monomorphic | SSRY170 | 299 | 55 | X |
| SSRY119 | 155 | 55 | X | SSRY171 | 291 | 55 | X |
| SSRY120 | 139 | 55 | X | SSRY172 | 201 | 55 | X |
| SSRY121 | 168 | 43 | X | SSRY173 | 281 |  | NO |
| SSRY122 | 273 | 45 | X | SSRY174 | 136 | 43 | X |
| SSRY123 | 136 | 55 | X | SSRY175 | 136 | 55 | X |
| SSRY124 | 146 | 55 | Monomorphic | SSRY176 | 112 | 45 | Monomorphic |
| SSRY125 | 247 | 55 | Monomorphic | SSRY177 | 268 | 55 | X |
| SSRY126 | 245 | 55 | Monomorphic | SSRY178 | 104 | 55 | Monomorphic |
| SSRY127 | 130 | 44 | Monomorphic | SSRY179 | 226 | 55 | X |
| SSRY128 | 243 | 45 | X | SSRY180 | 163 | 55 | X |
| SSRY129 | 205 | 55 | Monomorphic | SSRY181 | 199 | 55 | X |
| SSRY130 | 223 | 55 | X | SSRY182 | 253 | 50 | Monomorphic |
| SSRY131 | 111 | 45 | Monomorphic | SSRY183 | 221 | 50 | X |
| SSRY132 | 196 | 45 | Monomorphic | SSRY184 | 163 | 50 | X |
| SSRY133 | 295 | 55 | Monomorphic | SSRY185 | 243 | 50 | X |
| SSRY134 | 213 | 55 | Monomorphic | SSRY186 | 297 | 55 |  |
| SSRY135 | 253 | 55 | X | SSRY187 | 160 | 55 |  |
| SSRY136 | 296 | 55 | Monomorphic | SSRY188 | 198 | 55 | Monomorphic |
| SSRY137 | 157 | 55 | Monomorphic | SSRY189 | 185 | 55 | X |
| SSRY138 | 129 | 50 | Monomorphic | SSRY190 | 164 | 55 |  |
| SSRY139 | 129 | 44 | Monomorphic | SSRY191 | 186 | 55 | Monomorphic |
| SSRY140 | 212 | 43 | Monomorphic | SSRY192 | 183 | 55 | X |
| SSRY141 | 262 | 55 | X | SSRY193 | 218 | 55 | X |
| SSRY142 | 206 | 55 | X | SSRY194 | 196 | 55 |  |
| SSRY143 | 153 | 55 | Monomorphic | SSRY195 | 186 | 55 | X |
| SSRY144 | 117 | 55 | X | SSRY196 | 188 | 55 |  |
| SSRY145 | 143 | 45 | X | SSRY197 | 209 | 55 | X |
| SSRY146 | 139 | 45 | X | SSRY198 | 219 | 55 |  |
| SSRY147 | 113 | 45 | Monomorphic | SSRY199 | 205 | 55 |  |
| SSRY148 | 114 | 55 | Monomorphic | SSRY200 | 205 | 55 | X |
| SSRY149 | 500 | 45 | X | SSRY201 | 197 | 55 | X |
| SSRY150 | 175 | 45 | Monomorphic | SSRY202 | 191 | 55 |  |

| SSR # | Size (bp) | T. Anneal °C | Polymorphic | SSR # | Size (bp) | T. Anneal °C | Polymorphic |
|---|---|---|---|---|---|---|---|
| SSRY151 | 182 | 55 | X | SSRY203 | 246 | 55 | X |
| SSRY152 | 233 | 45 | X | SSRY204 | 182 | 55 | X |
| SSRY205 | 201 | 55 | X | SSRY257 | 280 | 55 | Monomorphic |
| SSRY206 | 219 | 55 | | SSRY258 | 400 | 55 | Monomorphic |
| SSRY207 | 199 | 55 | | SSRY259 | 220 | 55 | Monomorphic |
| SSRY208 | 198 | 55 | | SSRY260 | 100 | 55 | |
| SSRY209 | 195 | 55 | | SSRY261 | 210 | 55 | X |
| SSRY210 | 219 | 55 | Monomorphic | SSRY262 | 140 | 55 | Monomorphic |
| SSRY211 | 202 | 55 | Monomorphic | SSRY263 | | n.a. | |
| SSRY212 | 238 | 55 | | SSRY264 | | n.a. | |
| SSRY213 | 199 | 55 | | SSRY265 | 230 | 55 | X |
| SSRY214 | 234 | 55 | | SSRY266 | 220 | 55 | Monomorphic |
| SSRY215 | 204 | 55 | X | SSRY267 | 265 | 55 | Monomorphic |
| SSRY216 | 210 | 55 | | SSRY268 | 215 | 55 | solo SSR55 |
| SSRY217 | 181 | 55 | X | SSRY269 | 200 | 55 | |
| SSRY218 | 203 | 55 | X | SSRY270 | 220 | 55 | |
| SSRY219 | 190 | 55 | X | SSRY271 | 280 | 55 | Monomorphic |
| SSRY220 | 190 | 55 | X | SSRY272 | 220 | 55 | |
| SSRY221 | | n.a. | | SSRY273 | | n.a. | |
| SSRY222 | 150 | n.a. | | SSRY274 | 280 | 55 | |
| SSRY223 | 170 | 55 | X | SSRY275 | | 50 | X |
| SSRY224 | | n.a. | | SSRY276 | 260 | 55 | X |
| SSRY225 | | n.a. | | SSRY277 | 210 | 50 | Monomorphic |
| SSRY226 | | n.a. | | SSRY278 | 210 | 55 | Monomorphic |
| SSRY227 | 200 | 55 | Monomorphic | SSRY279 | 170 | 55 | Monomorphic |
| SSRY228 | 210 | n.a. | | SSRY280 | 180 | 55 | Monomorphic |
| SSRY229 | 200 | 55 | X | SSRY281 | 195 | 55 | Monomorphic |
| SSRY230 | 185 | 55 | X | SSRY282 | 200 | 55 | X |
| SSRY231 | 260 | 55 | Monomorphic | SSRY283 | 215 | 55 | X |
| SSRY232 | | n.a. | | SSRY284 | 210 | 55 | Monomorphic |
| SSRY233 | 205 | 55 | Monomorphic | SSRY285 | 290 | 55 | X |
| SSRY234 | | n.a. | | SSRY286 | 220 | 55 | Monomorphic |
| SSRY235 | 250 | 55 | X | SSRY287 | 220 | 55 | Monomorphic |
| SSRY236 | 220 | 55 | X | SSRY288 | 180 | 55 | Monomorphic |
| SSRY237 | 200 | 55 | X | SSRY289 | 195 | 55 | Monomorphic |
| SSRY238 | 225 | 55 | X | SSRY290 | 300 | 55 | Monomorphic |
| SSRY239 | 220 | 55 | X | SSRY291 | 210 | 55 | X |
| SSRY240 | 200 | 55 | X | SSRY292 | | n.a. | |
| SSRY241 | 220 | 55 | X | SSRY293 | | 50 | Monomorphic |
| SSRY242 | 280 | 55 | X | SSRY294 | 175 | 55 | Monomorphic |
| SSRY243 | 400 | n.a. | | SSRY295 | 185 | 55 | X |
| SSRY244 | 220 | 55 | Monomorphic | SSRY296 | 175 | 55 | X |
| SSRY245 | 300 | 55 | Monomorphic | SSRY297 | 180 | 55 | X |
| SSRY246 | 210 | 55 | X | SSRY298 | 170 | 55 | Monomorphic |
| SSRY247 | 300 | 55 | Monomorphic | SSRY299 | 190 | 55 | X |
| SSRY248 | 250 | 55 | X | SSRY300 | 260 | 55 | Monomorphic |
| SSRY249 | 400 | 55 | Monomorphic | SSRY301 | 265 | 55 | Monomorphic |
| SSRY250 | 200 | 55 | X | SSRY302 | 200 | 55 | X |
| SSRY251 | 220 | 55 | | SSRY303 | 190 | 55 | Monomorphic |
| SSRY252 | 220 | 55 | X | SSRY304 | 240 | 55 | Monomorphic |
| SSRY253 | 190 | 55 | X | SSRY305 | 300 | 55 | X |

| SSR # | Size (bp) | T. Anneal °C | Polymorphic | SSR # | Size (bp) | T. Anneal °C | Polymorphic |
|---|---|---|---|---|---|---|---|
| SSRY254 | 220 | 55 | Monomorphic | SSRY306 | 265 | 55 | X |
| SSRY255 | 190 | 55 | Monomorphic | SSRY307 | | n.a. | |
| SSRY256 | 210 | 55 | Monomorphic | SSRY308 | 280 | 55 | Monomorphic |
| SSRY309 | 220 | 55 | Monomorphic | SSRY327 | | n.a. | |
| SSRY310 | | 50 | Monomorphic | SSRY328 | 240 | 55 | X |
| SSRY311 | 200 | 50 | Monomorphic | SSRY329 | 210 | 55 | X |
| SSRY312 | 200 | 55 | X | SSRY330 | | 52 | X |
| SSRY313 | 205 | 55 | X | SSRY331 | | 52 | X |
| SSRY314 | 190 | 55 | Monomorphic | SSRY332 | | 52 | X |
| SSRY315 | 230 | 50 | X | SSRY333 | | 52 | Monomorphic |
| SSRY316 | | 50 | Monomorphic | SSRY334 | | 52 | Monomorphic |
| SSRY317 | | 50 | Monomorphic | SSRY335 | | 52 | Monomorphic |
| SSRY318 | | 50 | Monomorphic | SSRY336 | | 52 | Monomorphic |
| SSRY319 | | 50 | X | SSRY337 | | 52 | Monomorphic |
| SSRY320 | | 50 | Monomorphic | SSRY338 | | 52 | Monomorphic |
| SSRY321 | | 50 | Monomorphic | SSRY339 | 220 | 55 | X |
| SSRY322 | | 50 | X | SSRY340 | | 55 | Monomorphic |
| SSRY323 | | 50 | Monomorphic | SSRY341 | 200 | 55 | X |
| SSRY324 | 200 | 55 | X | SSRY342 | 210 | 55 | Monomorphic |
| SSRY325 | 240 | 55 | Monomorphic | SSRY343 | 300 | 55 | Monomorphic |
| SSRY326 | | n.a. | | | | | |

## Conclusions

A high percentage of polymorphism (more than 60%) was obtained from the two parents, MEcu 72 and MCol 2246; this guarantees a high number of markers for the construction of a legitimate map. However still lacking are the in vitro propagation of some individuals (58) progeny, and field and greenhouse screening with *A. socialis*. Upon completing the running of the polymorphic microsatellites with the 282 individual progeny, a comparison will be made with greenhouse and field data.

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## 1.2.18 Mapping and pursuit of QTLs for yield and yield components in rice populations derived from backcrosses between wild species and cultivated rice

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### Introduction

*Oryza* wild species represent a potential source of new alleles for improving yield, quality and stress resistance of cultivated rice. Still, effective use of wild species genes remains largely unexplored. Advanced backcross-breeding schemes (Tanskley and Nelson, 1996) using molecular mapping techniques represent an alternative to reduce the genetic background from wild species parentals and to rapidly discover and transfer valuable alleles from the wild species into elite rice varieties. Even though wild and unadapted germplasm have phenotypically less desirable than modern varieties in their overall appearance and performance, breeders have long recognized the intrinsic value of wild species for the improvement of simple inherited traits (Xiao et al. 1998). The possibility of selectively introgressing useful genes from *O. rufipogon* to elite rice cultivars suggests a way for improving the performance of *O. sativa* while simultaneously broadening the genetic base of cultivated rice (Moncada, P. et al. 2001). For this reason, advanced backcross QTLs analysis is proposed as a method to discover and transfer valuable QTL alleles from wild species into established elite inbred lines. BC2 or BC3 populations are used along with negative selection of undesirable characters to reduce the frequency of deleterious alleles present in the donor parental. QTL-NILs can be derived from advanced backcross populations in one or two additional generations and used to verify QTL activity. These same QTL-NILs also represent potential commercial inbreeds improved for one or more quantitative traits. If successfully employed, advanced backcross QTL analysis can open the door to exploiting unadapted and exotic germplasm for the quantitative trait improvement of a number of crop plants (Tanksley, S. D. and Nelson, J. C. 1996).

This report focuses on progress made in identifying quantitative traits loci (QTLs) associated with yield increase in populations derived from crosses with *Oryza rufipogon, O. barthii* and *O. glaberrima*, and the introgression of trait-enhancing QTLs in near-isogenic lines (NILs).

### Materials and methods

Experiments to identify segregating alleles in advanced backcrossed populations were set up in the field, greenhouse and the CIAT Biotechnology laboratory.

*Development of NILs from a BC2F2 population derived from the cross BG90-2 x Oryza rufipogon .*

Eighty-five BC2F2 families were selected for NIL development based on the molecular and quantitative analysis (Nelson 2000.Qgene 3.0 software) of the BC2F2 population derived from the cross BG90-2 x *O. rufipogon*. As reported earlier (Ann.Report 2000) 66 SSRs were found associated with putative QTLs

determining yield and yield components. These putative QTLs were derived from *O.rufipogon.* Selected families were evaluated with known SSR markers and homozygote or heterozygous individuals for alleles derived from O.rufipogon were crossed with the recurrent parent (BG90-2 male parent) to generate the BC3F1 population. Based on field performance and agronomic data only 75 families out of the 85 initially selected were chosen for NIL development.

A total of 70 BC3F1 plants in each family was planted in the green house for molecular characterization. Leaf discs (5 mm in diameter) were collected from each plant (25-30 day-old seedlings) for DNA extraction using the Alkali method (Klimyuk et al. 1993). So far 11 SSRs have been analyzed in a total of 100 PCR assays done with 63 out of 75 families to verify which of the *O. rufipogon* alleles has been introgressed in this population (Figure 1). Afterwards plants were transplanted in the field to gather agronomic data.

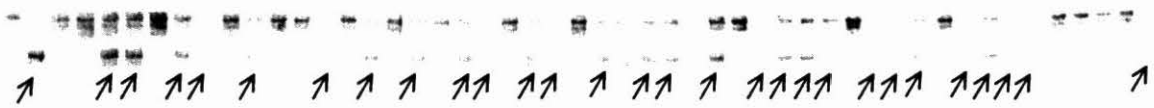

**Figure 1. CT17211-12 pedigree line evaluated with RM234. Parentals (BG90-2 and *O. rufipogon*) are placed on the first two lanes on the left, followed by the individuals of the family.**

*BC3F2 population derived from the cross Lemont x Oryza barthii .*

It was reported earlier that the BC3F2 population derived from the cross Lemont x *O. barthii* had been evaluated with 85 SSRs. This year we completed 126 SSRs markers in this population. Currently, we are selecting the appropriate statistical analysis method to generate the QTL map.

*BC3F1 Double haploid population derived from the cross Caiapo x Oryza glaberrima.*

Anthers from BC3F1 plants were harvested and run through anther culture (Ann. Report 2000). The response was very good and 695 doubled-haploid plants were obtained. Based on agronomic data 312 DH lines were selected for molecular characterization along with the parental lines. Five plants from each family were planted in the greenhouse for tissue collection and DNA extraction to be used in the development of molecular probes and the posterior QTL map for yield and yield components.

Based on the screening of 280 SSRs in the parents ( Caiapo and *O. glaberrima* ) following the method described by Temnykh et al. 2000, the PCR assay protocols for 120 SSRs of them were standardized for use in the screening of the 312BC3F1 DH lines (Figure 2). DNA of young leaves from the parental genotypes and segregating population was extracted using the Dellaporta Method (McCouch et al. 1988) and modified for the PCR assay by the CIAT Biotechnology Research Unit.

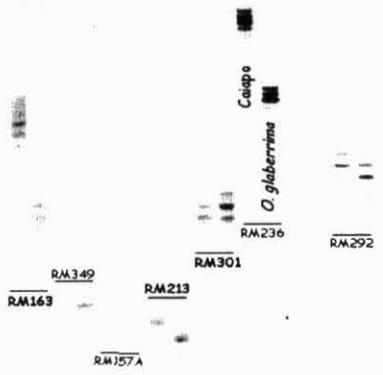


**Figure 2. Screening of SSRs in the parents Caiapo and *O. glaberrima***

## Results and discussion

We are following the molecular behavior of each QTL in BC2F2-NILs and BC3F2-NILs generations, developed from the cross BG90-2 x *O. rufipogon*. Currently, 93 assays were completed corresponding to evaluation of 11 SSRs (Table 1). In 62 cases there was previous molecular data available with regard to the genotype of the BC2F2 plant used as a female parent in crosses with BG90-2 (male-recurrent parent). However, molecular data was not available at crossing time in 31 cases and plants were selected based on phenotype.

A test for goodness of fit (Chi-square test) was used to determine whether the observed data conforms to a specified probability distribution. A 50% homozygous and 50% heterozygous segregation was expected when the BC2F2 plant crossed to BG90-2 was heterozygous for the "wild allele" at a given locus. On the contrary, 100% of the BC3F1 plants were expected to be heterozygous when the BC2F2 plant crossed to BG90-2 was homozygous for the "wild allele". Table 1 shows the observed, adjusted and expected values whilst Table 2 shows data corresponding to segregation obtained with RM242. The segregation pattern for each SSRs was kind of similar to that of RM242 in terms of the Chi-square results. A summary of the Chi-square test for all SSRs is shown in Table 3. In 51 cases (55%) the observed values did not differed significantly (5%) from the expected values, whilst in 42 cases (45%) there was a significant difference and the hypothesis was rejected. Table 3 also indicates that in cases (31) where molecular data was not available at crossing time, in 19 cases (61%) the resulting BC3F1 progenies segregated in an expected ratio whilst in 39 % of the instances did not.

Over all it can be seen that results differ a lot from expectations, since in only 55% of the instances results agreed with expectations. A much greater efficiency is expected when molecular markers are used to follow up introgression of specific alleles in a given background. As illustrated with RM242 none of the SSRs gave 100% accuracy suggesting that the problem has to do with the entire population. This is further supported by the behavior of case where no previous molecular data was available.

There are several factors that could explain the 45% inaccuracy observed in this study due to experimental errors of various nature, namely field and laboratory conditions. There are several steps where mistakes can occur under greenhouse/field conditions such as errors in plant identification for crossing, selfing, pollen contamination, etc. In terms of the laboratory protocols for DNA extraction and stability, PCR conditions, silver staining procedures, etc could produce unclear signals difficult to read. Several quality control measures are being implemented to reduce the experimental error.

Another important factor has to do with the genetic distance between the putative QTL and the SSR marker. The greater the distance between them the higher the probability of getting a crossover which could give rise to individuals with non-expected genotypes.

**Table 1. Chi-square test for goodness of fit in 93 BC2/BC3 assays carried out in the development of NILs from the cross BG90-2/O.rufipogon.**

| PEDIGREE | SSR | observed values (%) | | | | ajusted observed values (%) | | | expected values (%) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | B | H | R | - | B | H | R | B | H |
| CT17172-3 | RM1 | 100 | 0 | 0 | 0 | 100 | 0 | 0 | - | - |
| CT17175-10 | RM122 | 100 | 0 | 0 | 0 | 100 | 0 | 0 | - | - |
| CT17184-18 | RM122 | 0 | 98.6 | 0 | 1.43 | | 100 | | - | - |
| CT17204-56 | RM122 | 5.55 | 90.7 | 1.85 | 1.85 | 5.66 | 92.45 | 1.88 | - | - |
| CT17143-20 | RM13 | 81.03 | 19 | 0 | 0 | 81.03 | 18.97 | 0 | - | - |
| CT17143-21 | RM13 | 30 | 55.7 | 14.28 | 0 | 30 | 55.71 | 14.28 | - | - |
| CT17144-19 | RM13 | 38.57 | 60 | 0 | 1.43 | 39.13 | 60.86 | | - | - |
| CT17145-14 | RM13 | 91.43 | 7.14 | 0 | 1.43 | 92.75 | 7.24 | | - | - |
| CT17145-29 | RM13 | 54.29 | 41.4 | 0 | 4.28 | 56.71 | 43.28 | | - | - |
| CT17148-23 | RM13 | 55.38 | 43.1 | 0 | 1.54 | 56.25 | 43.75 | | - | - |
| CT17148-27 | RM13 | 61.43 | 27.1 | 0 | 11.4 | 69.35 | 30.64 | | - | - |
| CT17163-20 | RM13 | 87.14 | 0 | 0 | 12.9 | 100 | | | - | - |
| CT17165-10 | RM13 | 0 | 58.5 | 12.31 | 29.2 | | 82.6 | 17.39 | | 100 |
| CT17171-9 | RM13 | 15.94 | 81.2 | 0 | 2.9 | 16.41 | 83.58 | | - | - |
| CT17172-3 | RM13 | 91.67 | 8.33 | 0 | 0 | 91.67 | 8.33 | 0 | 50 | 50 |
| CT17174-7 | RM13 | 17.65 | 82.4 | 0 | 0 | 17.65 | 82.35 | 0 | | 100 |
| CT17178-44 | RM13 | 0 | 94.3 | 0 | 5.71 | | 100 | | | 100 |
| CT17181-3 | RM13 | 80 | 0 | 0 | 20 | 100 | | | - | - |
| CT17183-2 | RM13 | 100 | 0 | 0 | 0 | 100 | 0 | 0 | - | - |
| CT17185-11 | RM13 | 100 | 0 | 0 | 0 | 100 | 0 | 0 | - | - |
| CT17186-8 | RM13 | 0 | 44.3 | 0 | 55.7 | | 100 | | | 100 |
| CT17189-55 | RM13 | 82.86 | 0 | 0 | 17.1 | 100 | | | | 100 |
| CT17142-23 | RM212 | 7.14 | 90 | 2.86 | 0 | 7.14 | 90 | 2.86 | | 100 |
| CT17142-32 | RM212 | 35.21 | 64.8 | 0 | 0 | 35.21 | 64.79 | 0 | 50 | 50 |
| CT17143-20 | RM212 | 100 | 0 | 0 | 0 | 100 | 0 | 0 | - | - |
| CT17143-21 | RM212 | 92.86 | 7.14 | 0 | 0 | 92.86 | 7.14 | 0 | - | - |
| CT17146-32 | RM212 | 0 | 100 | 0 | 0 | 0 | 100 | 0 | | 100 |
| CT17146-34 | RM212 | 0 | 100 | 0 | 0 | 0 | 100 | 0 | 50 | 50 |
| CT17146-8 | RM212 | 34.78 | 58 | 2.9 | 4.35 | 36.36 | 60.6 | 3.03 | 50 | 50 |
| CT17159-2 | RM212 | 52.17 | 47.8 | 0 | 0 | 52.17 | 47.83 | 0 | 50 | 50 |
| CT17162-20 | RM212 | 50 | 48.5 | 0 | 1.52 | 50.76 | 49.23 | | - | - |
| CT17172-12 | RM212 | 54.29 | 45.7 | 0 | 0 | 54.29 | 45.71 | 0 | 50 | 50 |
| CT17182-21 | RM212 | 98.08 | 0 | 0 | 1.92 | 100 | | | - | - |
| CT17208-12 | RM212 | 0 | 100 | 0 | 0 | 0 | 100 | 0 | - | - |
| CT17139-12 | RM215 | 0 | 90 | 10 | 0 | 0 | 90 | 10 | | 100 |
| CT17139-14 | RM215 | 0 | 92.9 | 7.14 | 0 | 0 | 92.86 | 7.14 | | 100 |
| CT17146-32 | RM215 | 20 | 80 | 0 | 0 | 20 | 80 | 0 | 50 | 50 |

| PEDIGREE | SSR | observed values (%) | | | | ajusted observed values (%) | | | expected values (%) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | B | H | R | - | B | H | R | B | H |
| CT17146-34 | RM215 | 0 | 98.6 | 1.43 | 0 | 0 | 98.57 | 1.43 | | 100 |
| CT17146-8 | RM215 | 40.58 | 21.7 | 1.45 | 36.2 | 63.63 | 34.09 | 2.27 | 50 | 50 |
| CT17148-23 | RM215 | 0 | 75.4 | 0 | 24.6 | | 100 | | | 100 |
| CT17148-27 | RM215 | 46.27 | 50.8 | 2.99 | 0 | 46.27 | 50.75 | 2.99 | | 100 |
| CT17149-20 | RM215 | 28.57 | 52.9 | 7.14 | 11.4 | 32.25 | 59.67 | 8.06 | 50 | 50 |
| CT17159-2 | RM215 | 0 | 71.2 | 7.58 | 21.2 | | 90.38 | 9.61 | | 100 |
| CT17168-14 | RM215 | 15.15 | 80.3 | 0 | 4.55 | 15.87 | 84.1 | | 50 | 50 |
| CT17172-12 | RM215 | 90 | 0 | 0 | 10 | 100 | | | 100 | |
| CT17193-16 | RM215 | 0 | 98.5 | 1.54 | 0 | 0 | 98.46 | 1.54 | | 100 |
| CT17170-12 | RM234 | 42.86 | 35.7 | 0 | 21.4 | 54.54 | 45.45 | | - | - |
| CT17192-2 | RM234 | 100 | 0 | 0 | 0 | 100 | 0 | 0 | 100 | |
| CT17196-59 | RM234 | 98.44 | 0 | 0 | 1.56 | 100 | | | 50 | 50 |
| CT17206-48 | RM234 | 0 | 95.4 | 4.61 | 0 | 0 | 95.38 | 4.61 | | 100 |
| CT17211-12 | RM234 | 48.57 | 51.4 | 0 | 0 | 48.57 | 51.42 | 0 | | 100 |
| CT17138-14 | RM242 | 1.64 | 96.7 | 1.64 | 0 | 1.64 | 96.72 | 1.64 | | 100 |
| CT17138-9 | RM242 | 0 | 97.1 | 2.94 | 0 | 0 | 97.06 | 2.94 | | 100 |
| CT17139-12 | RM242 | 97.14 | 1.43 | 0 | 1.43 | 98.55 | 1.44 | | - | - |
| CT17139-14 | RM242 | 100 | 0 | 0 | 0 | 100 | 0 | 0 | - | - |
| CT17143-20 | RM242 | 53.45 | 44.8 | 1.72 | 0 | 53.45 | 44.83 | 1.72 | 50 | 50 |
| CT17143-21 | RM242 | 0 | 81.4 | 18.57 | 0 | 0 | 81.43 | 18.57 | - | - |
| CT17147-22 | RM242 | 0 | 94.3 | 0 | 5.71 | | 100 | | | 100 |
| CT17147-37 | RM242 | 23.88 | 26.9 | 5.97 | 43.3 | 42.1 | 47.36 | 10.52 | | 100 |
| CT17148-23 | RM242 | 0 | 95.4 | 1.54 | 3.08 | | 98.41 | 1.58 | | 100 |
| CT17148-27 | RM242 | 50 | 44.3 | 5.71 | 0 | 50 | 44.29 | 5.71 | 50 | 50 |
| CT17149-20 | RM242 | 32.86 | 51.4 | 5.71 | 10 | 36.5 | 57.14 | 6.34 | 50 | 50 |
| CT17149-7 | RM242 | 2.86 | 91.4 | 2.86 | 2.86 | 2.94 | 94.11 | 2.94 | | 100 |
| CT17150-50 | RM242 | 3.08 | 95.4 | 1.54 | 0 | 3.08 | 95.38 | 1.54 | | 100 |
| CT17153-14 | RM242 | 18.06 | 81.9 | 0 | 0 | 18.06 | 81.94 | 0 | 50 | 50 |
| CT17153-8 | RM242 | 47.14 | 51.4 | 0 | 1.43 | 47.82 | 52.17 | | 50 | 50 |
| CT17155-30 | RM242 | 40 | 1.54 | 0 | 58.5 | 96.29 | 3.7 | | | 100 |
| CT17158-21 | RM242 | 0 | 95.7 | 0 | 14.3 | | 100 | | | 100 |
| CT17160-2 | RM242 | 0 | 58.6 | 4.29 | 37.1 | | 93.18 | 6.81 | | 100 |
| CT17165-10 | RM242 | 35.38 | 3.08 | 1.54 | 60 | 88.46 | 7.69 | 3.84 | 50 | 50 |
| CT17165-39 | RM242 | 100 | 0 | 0 | 0 | 100 | 0 | 0 | | 100 |
| CT17172-12 | RM242 | 68.57 | 0 | 0 | 31.4 | 100 | | | 100 | |
| CT17179-12 | RM242 | 0 | 22.9 | 4.28 | 72.9 | | 84.21 | 15.78 | | 100 |
| CT17187-9 | RM242 | 51.79 | 30.4 | 10.71 | 7.14 | 55.76 | 32.69 | 11.53 | - | - |
| CT17188-19 | RM242 | 0 | 90 | 0 | 10 | | 100 | | | 100 |
| CT17189-55 | RM242 | 0 | 84.3 | 1.42 | 14.3 | | 98.33 | 1.66 | | 100 |
| CT17146-32 | RM257 | 30 | 67.1 | 2.86 | 0 | 30 | 67.14 | 2.86 | | 100 |
| CT17146-34 | RM257 | 51.43 | 48.6 | 0 | 0 | 51.43 | 48.57 | 0 | 50 | 50 |
| CT17146-8 | RM257 | 47.83 | 37.7 | 13.04 | 1.45 | 48.52 | 38.23 | 13.23 | 50 | 50 |
| CT17177-4 | RM257 | 100 | 0 | 0 | 0 | 100 | 0 | 0 | - | - |
| CT17195-10 | RM257 | 0 | 92.8 | 2.9 | 4.35 | | 96.96 | 3.03 | | 100 |
| CT17201-20 | RM257 | 52.78 | 47.2 | 0 | 0 | 52.78 | 47.22 | 0 | | 100 |
| CT17154-20 | RM27 | 0 | 93.9 | 0 | 6.06 | | 100 | | | 100 |
| CT17139-14 | RM44 | 78.71 | 14.3 | 0 | 10 | 84.12 | 15.87 | | - | - |
| CT17142-23 | RM44 | 47.14 | 41.4 | 0 | 11.4 | 53.22 | 46.77 | | 50 | 50 |
| CT17142-32 | RM44 | 40 | 38.6 | 1.43 | 20 | 50 | 48.21 | 1.78 | 50 | 50 |
| CT17144-29 | RM44 | 35.29 | 38.2 | 2.94 | 23.5 | 46.15 | 50 | 3.8 | 50 | 50 |
| CT17148-23 | RM44 | 50.77 | 0 | 0 | 49.2 | 100 | | | 100 | |
| CT17148-27 | RM44 | 85.71 | 7.14 | 0 | 7.14 | 92.3 | 7.69 | | 100 | |

| PEDIGREE | SSR | observed values (%) | | | | ajusted observed values (%) | | | expected values (%) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | B | H | R | - | B | H | R | B | H |
| CT17159-2 | RM44 | 97.22 | 2.78 | 0 | 0 | 97.22 | 2.78 | 0 | 100 | |
| CT17167-31 | RM44 | 92.42 | 0 | 0 | 7.58 | 100 | | | - | - |
| CT17168-14 | RM44 | 96.97 | 0 | 0 | 3.03 | 100 | | | - | - |
| CT17203-23 | RM44 | 7.15 | 57.1 | 35.71 | 0 | 7.15 | 57.14 | 35.71 | - | - |

B:Homozygotes like Bg90-2; H:Heterozygotes ; R: Homozygotes like O.rufipogon; "-" missing data

**Table 2. Chi-square test to probe the hypothesis of 50-50% segregation of heterozygote genotype in BC3-NILs families. RM 242.**

| PEDIGREE | SSR | observed values (%) | | | | Ajusted observed values (%) | | | expected values (%) | | X^2 <= 3.84146 | | ACEPTABLE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | B | H | R | - | B | H | R | B | H | 0.05, 1 DF | | |
| CT17138-14 | RM242 | 1.64 | 96.72 | 1.64 | 0 | 1.64 | 96.72 | 1.64 | | 100 | | | YES |
| CT17138-9 | RM242 | 0 | 97.06 | 2.94 | 0 | 0 | 97.06 | 2.94 | | 100 | | | YES |
| CT17139-12 | RM242 | 97.14 | 1.43 | 0 | 1.43 | 98.55 | 1.44 | | - | - | | | YES[2] |
| CT17139-14 | RM242 | 100 | 0 | 0 | 0 | 100 | 0 | 0 | - | - | | | YES |
| CT17143-20 | RM242 | 53.45 | 44.83 | 1.72 | 0 | 53.45 | 44.83 | 1.72 | 50 | 50 | 0.772628 | NS | YES |
| CT17143-21 | RM242 | 0 | 81.43 | 18.6 | 0 | 0 | 81.43 | 18.57 | - | - | | | NO[2] |
| CT17147-22 | RM242 | 0 | 94.29 | 0 | 5.71 | | 100 | | | 100 | | | YES |
| CT17147-37 | RM242 | 23.88 | 26.87 | 5.97 | 43.3 | 42.1 | 47.36 | 10.52 | | 100 | | | NO[3] |
| CT17148-23 | RM242 | 0 | 95.38 | 1.54 | 3.08 | | 98.41 | 1.58 | | 100 | | | YES[3] |
| CT17148-27 | RM242 | 50 | 44.29 | 5.71 | 0 | 50 | 44.29 | 5.71 | 50 | 50 | 0.652082 | NS | YES |
| CT17149-20 | RM242 | 32.86 | 51.43 | 5.71 | 10 | 36.5 | 57.14 | 6.34 | 50 | 50 | 4.664592 | * | NO |
| CT17149-7 | RM242 | 2.86 | 91.43 | 2.86 | 2.86 | 2.94 | 94.11 | 2.94 | | 100 | | | NO |
| CT17150-50 | RM242 | 3.08 | 95.38 | 1.54 | 0 | 3.08 | 95.38 | 1.54 | | 100 | | | YES |
| CT17153-14 | RM242 | 18.06 | 81.94 | 0 | 0 | 18.06 | 81.94 | 0 | 50 | 50 | 40.806544 | * | NO |
| CT17153-8 | RM242 | 47.14 | 51.43 | 0 | 1.43 | 47.82 | 52.17 | | 50 | 50 | 0.189226 | NS | YES |
| CT17155-30 | RM242 | 40 | 1.54 | 0 | 58.5 | 96.29 | 3.7 | | | 100 | | | NO |
| CT17158-21 | RM242 | 0 | 95.71 | 0 | 14.3 | | 100 | | | 100 | | | YES |
| CT17160-2 | RM242 | 0 | 58.57 | 4.29 | 37.1 | | 93.18 | 6.81 | | 100 | | | NO |
| CT17165-10 | RM242 | 35.38 | 3.08 | 1.54 | 60 | 88.46 | 7.69 | 3.84 | 50 | 50 | 65.386154 | * | NO |
| CT17165-39 | RM242 | 100 | 0 | 0 | 0 | 100 | 0 | 0 | | 100 | | | NO |
| CT17172-12 | RM242 | 68.57 | 0 | 0 | 31.4 | 100 | | | 100 | | | | YES |
| CT17179-12 | RM242 | 0 | 22.86 | 4.28 | 72.9 | | 84.21 | 15.78 | | 100 | | | NO |
| CT17187-9 | RM242 | 51.79 | 30.36 | 10.7 | 7.14 | 55.76 | 32.69 | 11.53 | - | - | | | NO |
| CT17188-19 | RM242 | 0 | 90 | 0 | 10 | | 100 | | | 100 | | | YES |
| CT17189-55 | RM242 | 0 | 84.29 | 1.42 | 14.3 | | 98.33 | 1.66 | | 100 | | | YES |

Ns: SSR without statistical significance for the goodness of fit test; ***: SSR with statistical significance
B: Homozigotes like BG90-2; H: Heterozigotes; R: Homozigotes like *O. rufipogon*; "-": missing data
[1]: Ajusted observed values, without missing data.
[2]: Instead of unknown genotypes of the parents, the segregation is compared with the three expected genotypes of the possible parents.
[3]: Maximum level of tolerance: 5%, respect to the expected genotype according to the parent.

*Table 3. Summary of Chi-square test obtained in BC3F1-NIL population developed from the cross BG90-2 and O. rufipogon.*

| | | Acceptable | | | |
|---|---|---|---|---|---|
| Parents information | | **50-50 (a)** | **100-0 (a)** | **0-100 (a)** | **total** |
| With information | **62** | 10 | 5 | 17 | 32 |
| Without information | 31 | 4 | 13 | 2 | 19 |
| TOTAL | 93 | 14 | 18 | 19 | 51 |
| | | Rejected | | | |
| Parents information | | **50-50** | **100-0** | **0-100** | **total** |
| With information | **62** | 12 | 1 | 17 | 30 |
| Without information | 31 | - | - | - | 12 |
| TOTAL | 93 | | | | 42 |

a. Expected percentages of B and H

## Ongoing activities

- Complete the molecular analysis in BC3-NIL population derived of BG90-2 x *O. rufipogon.*
- Follow up introgression of QTLs in BC4-NILs families derived from crosses between BC3-NILs families and Bg90-2.
- Complete the statistical analysis of BC3F2 Lemont x *O. barthii* population.
- Map F2 populations derived from BG90-2 x *O. rufipogon* and Lemont x *O. barthii* crosses.
- Initiate the characterization of agronomic and molecular data, and QTL analysis to determine the number of QTLs associated with yield and yield components for Caiapo x *O. glaberrima* cross.

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## 1.2.19 Evaluation and selection of inter-specific populations via conventional breeding methods

CP Martínez[1], J.Borrero[2] , James Carabali[2]
[1]SB-2 Project; [2]IP-4 Project

**Introduction**

Genetic variability is an essential requirement to make progress in plant improvement programs. Traditionally, plant breeders make use of diverse genetic resources to come up with improved varieties. Most of the time, rice- breeding programs are under high pressure to deliver superior varieties to meet demands coming from diverse users. Crosses using good and well-known progenitors have better probabilities of producing the kind of breeding populations from where superior genotypes could be selected. Unfortunately, not very many good donors are available and the continued use of them lead to reshuffling of genes reducing at the same time the genetic variability.

It has been estimated that around 25% of the total genetic variation available in rice are actually used by rice improvement programs. The use of un-improved gene pools, like wild rice species, represents a difficult task to conventional breeding programs; however, this kind of research is more appropriate to programs more strategic in its scope like the CIAT Rice Project.

Therefore, activities described in this section deal with the introgression of alleles from wild rice species into the Latin America gene pool using conventional breeding methods. The main objective is to develop potential parents to be used by national rice programs.

A backcross scheme to diverse improved breeding lines or varieties is used. Starting in the $F_2$ generation single plant selections are made for further evaluation in pedigree rows. Around 250 crosses were made of which 189 were evaluated under upland conditions in Villavicencio. Many populations (119) were discarded because of high sterility and/or poor plant type, low yield potential, and susceptibility to major diseases. Only 476 plant selections for evaluation as $F_3$ lines were made.

Table 4 presents a summary of inter-specific populations evaluated in CIAT-Palmira and Villavicencio. Advanced lines with good plant type, early vigor, and excellent grain type were selected in the cross Oryzica 3/*O. rufipogon*. It was shown (Rice Program Annual Report 2000) that both Oryzica 3 and *O. rufipogon* had a good level of resistance to Rhizoctonia sp, a disease that is becoming more important in several areas in Latin America. In collaboration with Rice Pathology and FEDEARROZ, evaluations are underway, under greenhouse and field conditions, for resistance to Rhizoctonia.

Advanced lines ($F_6$-$F_7$) with good plant type and yield potential and tolerance to some diseases were selected in the cross Bg90-2/O.rufipogon. Some of these lines have good grain quality and 25 were selected for yield tests in diverse environments through collaboration with national rice programs, including Colombia, Argentina, Venezuela, Costa Rica and Honduras.

High sterility has been observed in crosses with *O. glaberrima*, even after 2-3 backcrosses. However, $F_4$-$F_5$ progenies with high fertility, early plant vigor, good tillering, strong stems and

good grain type have been selected. Several accessions of *O. glaberrima* possess high levels of resistance to the rice stripe virus disease (Rice Program Annual Report 2000); consequently, selected lines are being screened for resistance to rice stripe virus in collaboration with Rice Pathology and FEDEARROZ.

Crosses between *O. barthii* and improved cultivars have resulted in progenies with low yield potential, poor plant type and high sterility. Nevertheless, 2-3 backcross to Lemont have produced advanced lines with excellent grain type, cooking and milling quality, early maturity, and, tolerance to major diseases, except hoja blanca virus disease. Some lines have better yield potential than Lemont. Several lines were included in the VIOAL nurseries for distribution to national rice programs.

Two populations (Caiapo/*O. glaberrima* and Progresso/*O. barthii*) were evaluated under upland savanna conditions in Villavicencio, in collaboration with the CIRAD-CIAT program. Caiapo/ *O. glaberrima* showed better performance in terms of adaptation to acid soils. Besides, doubled haploid lines were obtained, which will be used to identify and characterize alleles derived from *O. glaberrima* that are associated with traits of agronomic importance.

In addition, 2704 $F_4$ lines from a collaborative project between CIAT and Peru were evaluated for resistance to rice hoja blanca virus in Palmira and tolerance to major diseases in Santa Rosa, Villavicencio. In spite of the high disease; pressure 1097 single plant selections were made for further evaluation as $F_5$ pedigree rows.

**Table 4. Inter-Specific Populations Evaluated in the Pedigree Method for the Development of Improved Germplasm**

| Population | Target Ecosystem | Generation | Lines Evaluated | No.Plants Selected | Observations |
|---|---|---|---|---|---|
| Oryzica 3/*O. rufipogon* | Irrigated | $BC_2F_4$ | 1.123 | 613+26 | Excellent plant type, good grain quality, potential source of R genes for Rhizoctonia |
| | | $BC_2F_5$-$F_6$ | 10 | 55 | |
| Bg90-2/*O. rufipogon* | | $BC_2F_5$ | 952 | 1.970 | 25 Advanced lines selected for yield tests outside CIAT |
| | | $BC_2F_6$ | 93 | 63 | |
| Bg90-2/*O. glaberrima* | | $BC_3F_2$ | 338 | 203 | Excellent plant vigour, good tillering and grain type. Some sterility. |
| | | $BC_3F_4$ | 203 | 124 | |
| | | $BC_2F_4$ | 1.224 | 1.014 | |
| | | $BC_2F_5$ | 1.014 | 234 | |
| Bg90-2/*O. barthii* | | $BC_2F_4$ | 533 | 53 | Low yield potential |
| O. llanos 5/*O. barthii* | | $BC_2F_3$ | 304 | 20 | Poor vigour, low yield potential |
| | | $BC_3F_3$ | 111 | 9 | |
| Lemont/*O. barthii* | | $BC_2F_4$ | 633 | 613 | Excellent cooking and milling earliness, long panicle |
| | | $BC_2F_5$ | 614 | 561 | |
| | | $BC_3F_5$ | 119 | 154 | |
| | | $BC_3R_4$ | 354 | 17 | |
| Caiapo/*O.glaberrima* | Upland Acid Soils | $BC_2F_3$ | 488 | 45 | Good variability, DH lines to be used for mapping purposes |
| | | $BC_2F_2$ | 29 | 224 DH | |
| | | $BC_3F_1$ | 49 | 695 DH | |
| Progresso/*O. barthii* | | $BC_2R_2$ | 422 | 24 | Late maturing, low yield |

## 1.2.19 Influence of wild rice species on the grain quality, nutritional value, the eating and cooking quality of inter-specific progenies

CP. Martínez[1], J. Borrero[2], MC Duque [1-2]
[1]SB-2 Project; [2]IP-4 Project

### Introduction

The impact of modern agriculture in improving the life and well being of billion of people around the world is impressive. The prospect of mass starvation was avoided due to increased food production through the Green Revolution's push for food security. However, little thought was given to nutritional value and human health, and almost none to the concentration of iron and other micronutrients in the new cereal varieties bred (Welch and Graham, 2000). Research at IRRI has shown genetic variation for iron and zinc concentration in brown rice; improved cultivars contain about 12 mg of iron and 25 mg of zinc per kilogram, while some traditional cultivars have doubled these amounts (Gregorio et al, 2000). Results from WARDA (1998) indicates that inter-specific crosses with *O. glaberrima* gave rise to progenies with higher protein content, good eating quality, and high nutritive values.

As mention earlier, we at CIAT are looking at wild rice species as potential sources of new alleles associated with traits of agronomic importance. Emphasis was given to alleles associated with grain yield and its components. In this section, we are dealing with the nutritional and grain quality aspects.

### Materials and Methods

Seed of the advanced lines CT14938-30-5-M-3 and CT14938-36-1-M-1 derived from the cross Lemont/*O. barthii* was harvested, dried and milled. Samples were taken to the quality lab for evaluation. Remnant seed was bulked up and milled and 2-kg samples were given to 64 people for cooking and eating evaluations. People were advised to cook the rice sample the same way they used at home and to compare its behavior with that of the rice they usually buy and eat.

Eleven rice cultivars (Table1) including *O. barthii*, *O. glaberrima* and *O. rufipogon* were choosen to determine its iron and zinc content. Brown and milled rice samples were obtained from field plots grown at CIAT and 5 gram each were sent to the lab for chemical analysis using the method proposed by Isaac and Kerber, 1971. The experiment was replicated three times.

### Results

Data from the quality lab showed that both lines had long and slender translucent grains (0.2 white center), with amylose content around 26-29 %, and excellent milling recovery (60% head rice). Data from the cooking/eating tests are presented in Figures 1. Forty-seven (75%) people reported that the rice sample was dry and fluffy after cooking whilst 96% of people said that the grain appearance was good before cooking; 4% reported that the appearance was fair (Figure 1). Only 4% found the rice sample to be sticky. It is important to keep in mind that the ratio rice/water used by people was different (it ranged from 1/2, 2/3, 3/4 to 1/1).

Figure 2 shows that 34% of people detected some kind of aroma after cooking and a different taste compared to the rice they usually consume. Besides 51 % of surveyed people reported that the sample given produced more cooked rice than the one they usually consume whilst 41% was willing to pay a little bit more for that kind of rice(data not shown).

Results suggest that *O. barthii* did not affect in a negative way the eating and cooking quality of rice; on the contrary, some people detected special features in the quality of the rice derived from an inter-specific cross. Data also confirm differences in people's preference in terms of grain quality opening up opportunities for the development of special types of rice.

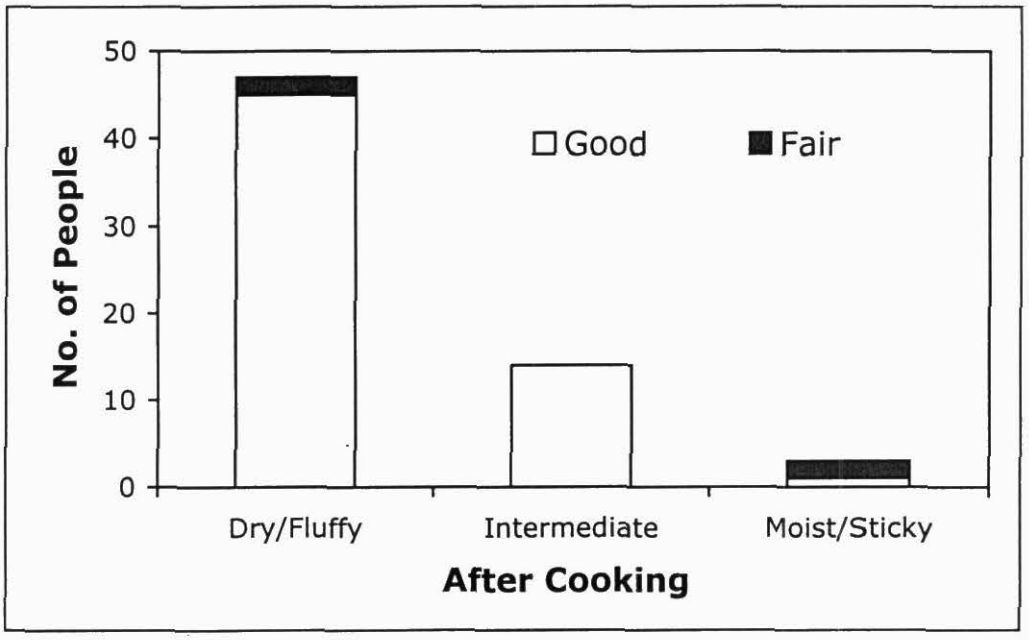


**Figure 1. Grain Appearance Before and After Cooking**

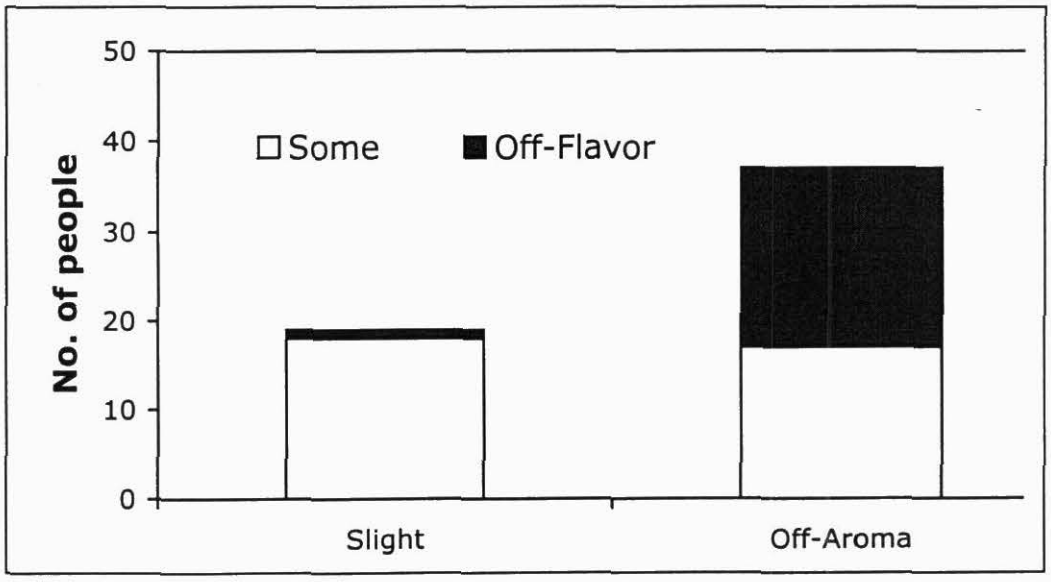


**Figure 2. Assessment of Milled Cooked Rice by Consumers Influence of Wild Rice on the Nutritional Value**

Data are presented in Table 1. Duncan's multiple test was used to determine statistical differences at the 1% level of significance. There were significant differences among cultivars with regard to iron and zinc content in both brown and milled rice, as well as in the effect due to milling.

As expected, brown rice contained higher amount of both iron and zinc than milled rice. *O. glaberrima* had the highest content of iron with regard to brown rice followed by Fedearroz 50 and Oryzica 1, whilst *O. barthii* had the highest content of zinc, followed by Fedearroz 50 and three accessions of *O. glaberrima*.

The effect of milling in reducing the contents of both iron and zinc is seen in Table 8. Milling reduced by 59 and 26%, respectively the content of iron and zinc. However, there were genotype differences. *O. glaberrima* lost 88% of its iron followed by the breeding line CT 13956-29-29-M (Bg90/*O. glaberrima*), IG10 (an acc. *O.glaberrima*), and the line P1274-6-8-M-1-M. It is encouraging to see that CG14 (different acc. *O. glaberrima*), *O. rufipogon* and Oryzica 1 had the highest content of iron after milling, suggesting its potential use as parents in a breeding program to increase the content of iron in commercial rice varieties. In terms of zinc, *O. barthii*, CG14, IG10 and *O, glaberrima* had the highest content after milling.

Results suggest that wild rice species can contribute to improve the nutritional quality of commercial rice varieties. It is noteworthy to mention that Oryzica 1 and Fedearroz 50 are good examples of improved varieties developed in Colombia out of the CIAT/FEDEARROZ breeding programs and no breeding effort was made to improve its nutritional value. However, given the fact that these varieties posses a good level of iron and zinc suggest that it should not be difficult to develop improve varieties with a better nutritional value. This is supported by the fact that the correlation coefficient to breed simultaneously for iron and zinc was 0.53. These results are in agreement with data from IRRI (Gregorio et al 2000) and WARDA.1998.

**Table 1. Effect of milling on grain Iron and Zinc content of selected rice cultivars**

| Cultivar | Iron | | | Zinc | | |
|---|---|---|---|---|---|---|
| | Brown Rice | Milled Rice | Milling Effect % | *Brown Rice* | Milled Rice | Milling Effect % |
| **Bg90-2** | 7.2 | 5.1 | **29.1** | 17.3 | 13.9 | **19.5** |
| Barthii | 10.4 | 4.2 | **60.1** | 27.9 | 22.0 | **21.1** |
| CG-14 | 10.8 | 6.3 | **41.3** | 24.8 | 19.7 | **20.4** |
| CT13956-29-M-3-M | 10.8 | 3.0 | **72.1** | 18.4 | 11.9 | **35.3** |
| Fedearroz 50 | 14.0 | 4.8 | **65.9** | 25.6 | 16.7 | **35.0** |
| IG10 | 12.3 | 3.7 | **70.1** | 24.8 | 18.1 | **27.0** |
| *O. glaberrima* | 30.4 | 3.6 | **88.0** | 25.0 | 19.2 | **23.3** |
| Oryzica 1 | 13.5 | 6.1 | **61.8** | 16.5 | 11.0 | **24.2** |
| Oryzica Llanos 4 | 13.0 | 4.9 | **54.4** | 20.8 | 15.7 | **33.3** |
| P1274-6-8-m-1-3-M4452 | 12.3 | 3.2 | **74.2** | 13.7 | 10.5 | **23.3** |
| *O. rufipogon* | 10.5 | 6.2 | **41.3** | 20.5 | 15.7 | **23.6** |
| **TOTAL** | 13.2 | 4.6 | **59.3** | 21.4 | 15.9 | **26.0** |

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## 1.2.20 M.A.S. for BGMV resistance in the common bean

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[1]SB-2 Project; [2] IP-1 Project

**Introduction**

In October 2000, $F_1$ plants derived from multiple crosses (BGMV 222 to BGMV 268) were planted at Santander de Quilichao (Cauca, Colombia). These plants were screened for the presence of the *bgm-1* marker (DOR21). A number of 1191 plants out of 2971 were found to have the marker and were then selected for generating new crosses and generation advancing.

From January to August 2001, three separate plantings were done. In January five nurseries were screened for the presence of the *bgm-1* marker. The first was a group of 12 $F_1$ multiple crosses (BGMV 269 to BGMV 280), in which 536 plants out of 904 had the marker and were used to generate new crosses.

The rest of the nurseries were screened in order to evaluate the segregation of this DNA fragment. The main purpose was to introduce the *bgm-1* gene in those bean varieties that are widely planted in Central America. Then eight plants per row of each $F_4$ backcrosses with DOR 364, DOR 390, DOR 500, ICTA OSTUA or A801 were sampled individually. Families that had a high proportion of the *bgm-1* marker were harvested and advanced to the next generation. Also 14 $F_3$ populations including the variety MD-2324 (Bribri) were evaluated. This time four individual plants of each population were screened with the *bgm-1* marker. Only the two that were positive (marker present) were harvested.

A wide number of $F_5$ families bred for Central America were also selected with the BGMV marker. Small red (168) and small black lines (166) were sampled for DNA amplification. Four plants per line were sampled as a bulk, and DNA was extracted using the alkali method with slight modifications in the amount of solutions used. The volumes of 0.25M NaOH and 0.25M HCl were increased from 40 to 60 µl, and 30 µl of 0.5M Tris-HCl (pH 8) was used instead of 20 µl. The rest of the protocol remained the same. After PCR, 94 small red families and 41 small black families having the *bgm-1* marker were selected. In addition, 84 $F_4$ families of G 22041 (Garbancillo Zarco) were selected among a group of 157.

In March another nursery of $F_1$ multiple-cross hybrids was planted. High-throughput M.A.S. was improved with the evaluation of the BGMV *SCAR* in a nursery of 7085 plants. A total of 3253

(46%) plants were found to have the *bgm-1* marker and selected for drought evaluation in the next generation.

A total of 28.7 days was spent in screening the marker in this nursery (7085 plants) (Table 1) in order to compare the efficiency of screening twice the usual number of plants at one time. Given that a different number of people are involved in each trial, we defined person-days (p-d) as the amount of time (days) that one person would spend while carrying out each activity (no. of people times no. of days). The screening of a set of 7000 plants took 55.2 p-d (or 23.7 p-d for 3000 plants). Thus, in comparison to a previous trial of 3000 plants that required 30.7 p-d, the p-d required to evaluate the BGMV *SCAR* was reduced significantly (Table 1). This was accomplished through the utilization of high-technology equipment that accelerated all laboratory processes such as electronic pipettes (Finnpipette Biocontrol) and the Hydra 96 micro-dispenser (Robbins Scientific) often used for large-scale genomics and high-throughput screening.

**Table 1. Improving large-scale screening for selecting *bgm-1* marker.**

| Task | Previous Trial (N=3000) | | | Last Trial (N=7000) | | |
|---|---|---|---|---|---|---|
| | Persons (no.) | Time (Days) | (p-d)[1] | Persons (no.) | Time (days) | (p-d) |
| Preparation of stickers and labels | 4 | 2 | 8 | 4 | 3 | 12 |
| Labeling plants in the field | 3 | 2 | 6 | 6 | 2 | 12 |
| Field sampling | 7 | 0.6 | 4.2 | 6 | 1.5 | 9 |
| DNA extraction | 1 | 2 | 2 | 1 | 3.2 | 3.2 |
| DNA dilution | 1 | 2 | 2 | 1 | 2.7 | 2.7 |
| PCR | 1 | 2 | 2 | 1 | 5.1 | 5.1 |
| Electrophoresis | 2 | 3 | 6 | 1 | 8.5 | 8.5 |
| Reading gels | 1 | 0.5 | 0.5 | 1 | 2.7 | 2.7 |
| Total | | 14.1 | 30.7 | | 28.7 | 55.2 |

[1] p-d = person-days or the amount of time (days) that one person would spend during each activity.

In July 71 $F_2$ families derived from $F_1$ were screened (4 plants/family) with the *bgm-1 SCAR*, and 34 were found to have the marker. Also 104 individual selections of small red beans that were evaluated simultaneously for drought stress showed the BGMV marker.

Some efforts were made to use microarray technology for M.A.S. in beans in order to speed up the screening. It was evident that more work has to be done in the design of more specific primers that would produce only the resistance allele; otherwise automatization of M.A.S. would not be possible.

## 1.2.21 M.A.S. in Rice

C. Quintero
SB-2 Project

### Introduction

A new, simple DNA extraction method (Nobuyuki et al., 2000) was tried in *Brachiaria*, common beans and rice. It did not work for the first two crops; however, good results were obtained for

rice. In a preliminary assay, no differences were found when comparing PCR products of the alkali DNA extraction, the Nobuyuki method and pure DNA. Then the protocol was modified as follows so that it could be utilized in microtiter plates for M.A.S purposes. Briefly, rice leaves were cut(6-mm diameter disks), placed into microtiter plate wells and stored at –20°C. The DNA was extracted by adding 60 µl of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8) to each sample and then boiling for 10 min. in a water bath. Then 120 µl of 10 mM Tris-HCl with 0.1m M EDTA (pH 8) were added. The extracts were stored at –20°C until their utilization. Five µl were used to amplify microsatellite markers, and stability of the DNA extraction was evaluated. No differences were found between the amplification of fresh extracts and DNA stored for one and two months (Figure 1). Therefore, as the DNA extracted proved to be stable for at least two months and no DNA dilution was needed, the method is suitable for M.A.S.

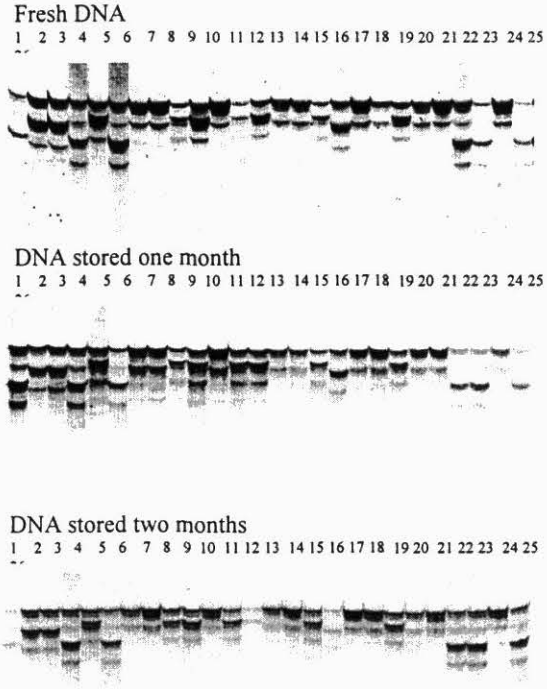


**Figure 1. Amplification of the microsatellite RM 107, using a new DNA-extraction protocol.**

## Ongoing Activities

- Try to use other BGMV markers besides DOR21 for M.A.S. in beans
- Continue making M.A.S. processes faster for other crops

## Reference

Nobuyuki, I.; Bautista, N.S.; Yamada, T.; Kamijima, O.; Ishii, T. 2000. Ultra-simple DNA extraction method for marker-assisted selection using microsatellite markers in rice. Plant Molecular Biology Reporter 18:1-6.

## Activity 1.3 Development of molecular techniques for assessing genetic diversity and mapping useful genes

### Main Achievements

- The dissection and sequence analysis of a cluster of resistance gene analogs associated with resistance to angular leaf spot in common bean was achieved opening the way to clone the gene cluster. The isolation of full-length Resistance Gene Analogs (RGAs) was initiated

- Microsatellite repeats in common bean were isolated and characterized over a wide range of wild and cultivated common bean and wild and cultivated accession from *P. acutifolius*, *P. coccineus*, *P. polyanthus* and *P. lunatus*.

- Four new libraries were screened for mircosatellites. Over 3000 sequences were collected, representing at minimum a 600% increase in the number of sequences available for common bean in the Gene bank public database. We have also screened a large number of microsatellite markers that were developed for soybean and cowpeas to try to adapt the microsatellites available for other *Phaseoleae* legume crops to common bean.

- *Phaseolus vulgaris* Ty1-Copia group retrotransposon LTR sequences were isolated and characterized. 24 different sequences corresponding to RNAse-PPT-LTR (ribonuclease- polypurine tract-long terminal repeat) sections of Ty1-copia retrotransposons were isolated. A study using primers derived from LTR sections also initiated. Primers were also identified for Sequence-Specific Amplification Polymorphisms (SSAPs) and Inter-Retrotransposon Amplified Polymorphisms (IRAPs) on accessions G19833 and DOR364.

- The implemetation of a novel microarray based technology Diversity array Technology (DarT) was initiated for bean and cassava. DarT, which is not reliant on DNA sequence information, has several potential applications including germplasm characterization, genetic marker-assisted breeding and tracking genome methylation changes

- A new set of Cassava microsatellites was developed from a cassava root and leaf cDNA library and whole genomic libraries. From the latter, 85 have been located on the cassava genome. A new set of 157 SSR-containing cDNA fragments was characterized and the integration of the new set with the current cassava map was initiated.

- The Annotation of SAGE tags (Transcripts) differentially expressed in CMD resistant and susceptible genotypes was pursued. So far tag annotation has identified genes known to be involved in systemic acquired resistance (SAR) response to disease in plants. They include a WRKY transcription factor, catalases, a pectin-esterase and reductases. Other genes were also found implicated in plant response to disease but are part of the cell mechanism known to aid virus replication including elongation factor alpha-1.

- A MAS project for CMD resistance was initiated with IITA to enable the testing of CMD markers developed at CIAT.

- The cDNA-AFLP technique was implemented to identify differentially expressed bands between two different cassava cultivars, one resistant and one susceptible to CBB. The cDNA-AFLP analysis at different times post inoculation with a *Xam* strain allowed the identification of putative molecular markers linked to disease resistance genes

- The construction of a molecular genetic map of *Brachiaria* using grasses RFLP RAPD, SCAR, AFLP and microsatellites was achieved. QTL analysis of spittlebug resistance revealed two major QTLs explaining up to 37% and 15 % of the variance

## 1.3.1 Dissection and sequence analysis of a cluster of resistance gene analogs associated with resistance to angular leaf spot in common bean

I.F. Acosta and J. Tohme
SB-2 Project

### Introduction

We have followed a candidate gene approach using PCR with degenerate primers to identify loci associated with resistance to diverse pathogens in common bean (BRU, Annual Report 1998, 1999). This approach takes advantage of the fact that the coding sequences of cloned plant disease resistance genes (R-genes) contain conserved structural motifs (Hammond-Kosack and Jones, 1997). The majority of R-genes encode a nucleotide-binding site (NBS) and leucine rich repeats (LRRs). Thus, it is possible to hypothesize that disease resistance in common bean follows this trend. We characterized a set of NBS-type sequences (RGAs, Resistance Gene Analogs) from common bean. Genetic mapping and QTL analysis showed that they are part of loci containing resistance specificities to angular leaf spot (ALS), anthracnose and Bean Golden Yellow Mosaic Virus (BGYMV) (BRU, Annual Report, 1999; López et. Al., manuscript in preparation). One of these sequences, RGA7, was particularly attractive as it explained 69% of resistance to two strains of the ALS pathogen in the parental line G19833. RGA7 is also part of a multigene family, a common feature of R-genes. There is a good probability that one or several members of the RGA7 family is an effective R-gene in common bean. Now, our goal is to determine the complete sequence of the members in the RGA7 family. This will be useful to clone R-genes that benefit breeding programs and eventually may be used to generate transgenic lines. Additionally, it will contribute to the increasing amount if information about the evolution of R-gene clusters that, may lead to the engineering of new disease resistance specificities.

### Materials and methods

The common bean BAC library has an average insert size of 90 Kb (Genome size: 635 Mb) covering 3-5 genome equivalents. It was constructed by S. MacKenzie using a cv Sprite snap derived (Fr restored CMS Sprite) and contains 33,792 clones (88 plates). The library was screened by hybridization in high-density BAC filters using RGA7 as a probe and the protocol From Clemson University (http://www.genome.clemson.edu/groups/bac/protocols/protocols2new.html). Identified BAC clones were subjected to Southern hybridization and BAC End Sequencing to confirm their overlaps using the protocols reported before (BRU, Annual Report, 2000).

BAC clone 57-M14 is being sequenced by a transposon insertion strategy using a kit from Epicentre that randomly inserts an EZ::Tn <KAN-2> Transposon, containing a selectable marker (kanamycin resistance) and sequencing primer binding sites, into BAC DNA. *In vitro* reaction conditions have been optimized to maximize transposon insertion efficiency while minimizing multiple insertion events. In brief, about 200 ng of BAC DNA were subjected to transposition with the EZ::Tn <KAN-2> Transposon as recommended by the manufacturer, and 0.5 µl of the reaction were used to electroporate 20 µl of DH10B *E.coli* competent cells. After recovery, aliquots of the cells were grown on plates containing chloramphenicol (Cm, 12,5 µg/ml) and kanamycin (Km, 50 µg/ml) to select for transposon insertion BAC clones. These were grown in LB broth with Km and Cm for BAC DNA isolation using the same protocol as before (BRU,

Annual Report, 2000). BAC DNA was used for sequencing employing primers annealing the EZ::Tn <KAN-2> transposon. Sequencher (Gene Codes, Ann Arbor, MI).) is used to edit sequences and assemble contigs. Database searches are performed with the BLASTX and BLASTN algorithms (Altschul et al., 1997).

**Results and discussion**

Molecular geneticists have seen multi-copy probes as disadvantageous molecular markers; however, as they may constitute complex gene families, their importance has been vindicated because they are more abundant than expected (M. Delseny, seminar at CIAT). This is the case for several of the common bean RGAs. Assuming that the multiple genes containing RGA7 are physically linked, we used it as a unique probe to screen the common bean BAC library expecting the identification of overlapping clones. Seventeen clones were identified and subjected to fingerprinting with *Eco*RI, southern hybridization with RGA7 and BAC End Sequencing. Two contigs were assembled using data from these procedures. One of the contigs included 11 out of the 17 BAC clones and 7 members of the RGA7 family (Figure 1). BAC clone 57-M14 contains four of these members and was selected for complete sequencing by the transposition insertion approach. Complete sequence of the BAC is important to unveil the structural organization of the cluster. As shown by BLASTX homologies of BAC End sequences, the genomic region containing the RGA7 family is especially rich in retroelement-type sequences. Association between transposable elements and a cluster of R-genes, the *Xa*21 from rice, has already been reported and evidence for the involvement of transposition in the evolution of the cluster provided (Song et al., 1997).

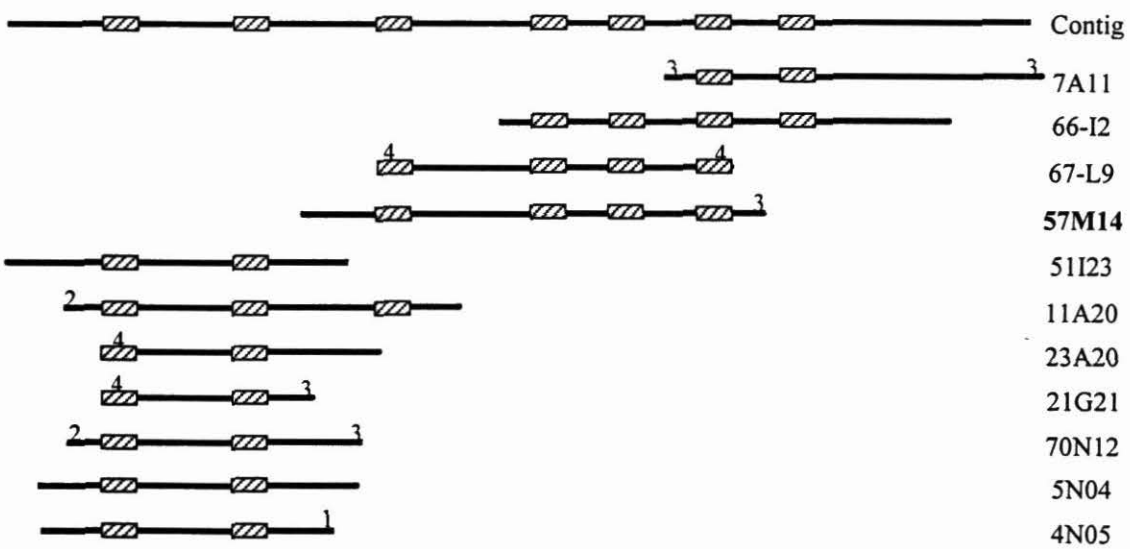


**Figure 1. A contig of 11 BAC clones containing 7 members (*stripped boxes*) of the RGA7 cluster was constructed based on fingerprinting, hybridization and BAC End Sequencing data. Key for BLASTX homologies of BAC End Sequences: 1 Maize transposon En/Spm; 2 Malate deshidrogenase; 3 Retroelement-type sequences; 4 Resistance Gene Analog. Ends without a number do not have significant homologies. BAC clone names are shown at right.**

We have successfully sequenced 179 different transposed BAC clones either by one or both ends with an average reading of 400 bp per sequencing reaction, that account for 47.5 Kb of total sequence achieved. Nineteen contigs have been assembled. The majority of the sequences are part of retroelements as indicated by BLAST searches. Another portion corresponds to sequences with no significant homologies. Finally, four contigs of 5.3, 3.5, 1.1 and 1.1 kb encode for R-genes. Interestingly, the R-gene contig of 5.3 kb contains a retroelement-type sequence in the middle of the coding sequence. About a half of the BAC remains to be sequenced. This work is currently under way.

**Future Work**

- Finishing and annotation of complete sequence of BAC clone 57-M14.
- Identification and comparison of the complete sequence of the four RGA7 members contained in BAC clone 57-M14. Design of primers to isolate by long-range PCR the remaining members of the family contained in other BAC clones.
- Isolation of members of the RGA7 family from parental line 619833 which contains resistance specificities in this family.

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## 1.3.2 Screening of a common bean cDNA library to isolate full-length Resistance Gene Analogs (RGAs)

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SB-2 Project

**Introduction**

Our set of Resistance Gene Analogs (RGAs) from common bean has proven to be useful landing markers of resistance loci (BRU, Annual Report, 1999; López et al., manuscript in preparation). As RGAs may correspond to a truly functional Resistance Gene (R-gene), we are interested in obtain the full-length coding sequence of as many RGAs as possible. Screening of a cDNA library with RGAs is not only a way to obtain their full-length sequences but also to establish if an RGA is actually expressed in the plant. We present the results of screening a custom-made cDNA library of bean (GIBCO-BRL) using RNA from young leaves.

## Results

Last year, attempts were made to screen the custom-made cDNA library of common bean (BRU, Annual Report, 2000). However, tested approaches were not apparently efficient to isolate full-length clones. This year, 55296 clones were picked to 384 plates and arrayed on high-density filters (M. Muñoz and M. Blair). A set of these filters was kindly provided to our group by M. Blair and we screened them with three multiplexes of our set of 15 RGAs as hybridization probes using the same protocols to screen BAC library high-density filters (BRU, Annual Report, 2000). A total of 14 clones were identified. Their 5' and 3' sequences were obtained and compared to the GenBank database. Seven clones were considered as false positives because they were homologous to sequences other than R-genes. The remaining 7 clones corresponded to RGAs. Figure 1 depicts the expected structure of a cDNA clone corresponding to a typical R-gene from the NBS-LRR class. 5' and 3'sequences of our cDNA clones have been located according to their sizes and homologies in this scheme. As shown, none of the 7 clones corresponds to a full-length cDNA. The same observation was made last year using different screening protocols. Therefore, we can conclude that the presence of truncated cDNA clones is a characteristic of this library. An R-gene as the one shown in Figure 1 may be 4 kB long. Thus, obtaining a full-length cDNA is a complicated task and high-quality procedures must be done.

Surprisingly, all 7 clones corresponded to only two out of the 15 RGA probes used for the screening: RGA1 and RGA2. Creusot et al. (1999) also isolated members of this family in their cDNA screenings. These RGAs probably constitute a large family of sequences in common bean that expressed at higher levels if compared to other R-genes. Indeed, R-genes are probably expressed at low levels (Scott Hulbert, pers. comm.). This may explain why it was not possible to isolate cDNA clones for every RGA used as probe. Screenings in the order of 10,000-100,000 clones are far of representation for rare transcripts.

### On-going work

We are currently designing a strategy to isolate full-length cDNA clones corresponding to R-gene homologues using rapid amplification of cDNA ends (RACE, Frohman, 1988). This method does not require the construction and screening of libraries and is based on PCR amplification procedures.

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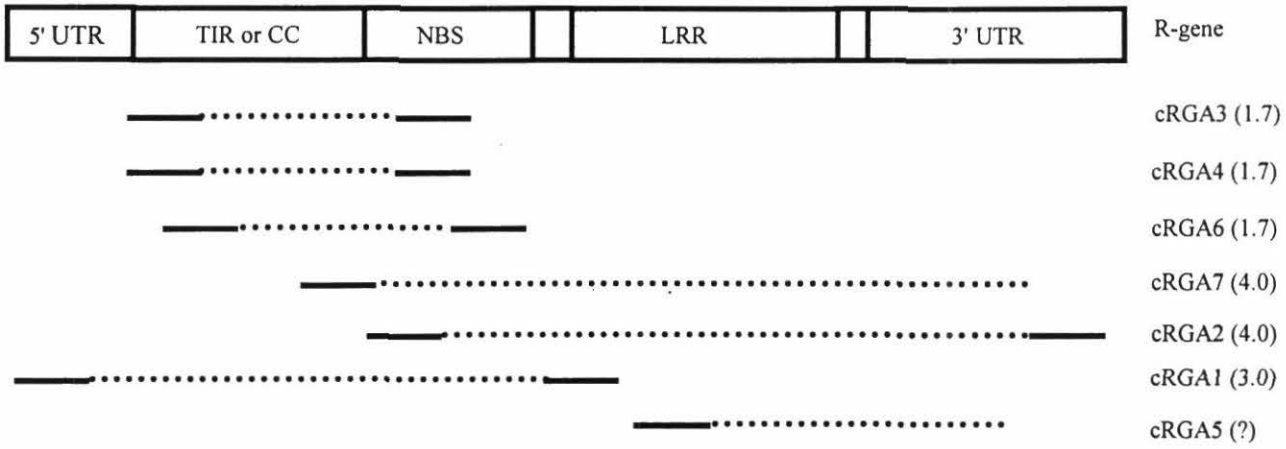
**Figure 1.** Schematic representation of an NBS-LRR type R-gene and location of the 5' and 3' sequences (black bars) of 7 cDNA clones corresponding to RGAs. Dotted lines represent portions of the clones that have not been sequenced. Numbers in parenthesis besides clone names are sizes in kb. Abbreviations in the R-gene scheme are as follows: 5' UTR, 5' untranslated regions; TIR, toll and interleukine-1 receptor domain; CC, coiled-coil motifs; NBS, nucleotide-binding site; LRR, leucine rich repeats; 3' UTR, 3' untranslated region.




## 1.3.3 Microsatellite repeats in common bean (*Phaseolus vulgaris*): Isolation, characterization and cross-species amplification in *Phaseolus*

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[1] SB-2 Project; [2] IP-4 Project ; [3] IACR, UK

### Introduction

*Phaseolus* beans are distributed worldwide and are cultivated in the tropics, subtropics, and temperate zones. However, while more than 30 Phaseolus species have been described, only four are cultivated for human consumption. Of these, the common bean, *P. vulgaris*, is the most widely grown, the remaining four being the runner bean (*P. coccineus*), year-long bean (*P.polyanthus*) lima bean (*P. lunatus*) and tepary bean (*P. acutifolius*). In the present study we evaluated 68 primers pairs on a 21 *P. vulgaris* genotypes. In addition to these, 9 accessions of *P. acutifolius*, *P. coccineus*, *P. polyanthus P. lunatus* were also analyzed to check cross-specific amplification,.

### Materials and Methods

Microsatellite primer characterization
A total of 21 *P. vulgaris* genotypes were chosen for the evaluation of the primer pairs. This was made up of 14 wild accessions from CIAT's wild core collection and 7 cultivated accessions from the Mesoamerican and Andean pools. In addition to these, 3 and 2 accessions each of wild and cultivated *P. acutifolius* and *P. coccineus*, respectively, were also evaluated. Also, two genotypes of wild and cultivated *P. polyanthus* and two wild genotypes from *P. lunatus* were also analyzed

to check cross-specific amplification, bringing the total number of bean accessions use in the study to 30.

The PCR reaction was carried out in a 20µl final volume containing 20ng of genomic DNA, 0.1µM of each of the forward and reverse primers, 10mM Tris-HCL (pH 7.2), 50mM KCL, 1.5mM to 2.5mM MgCL2 depending on the primer combination, 250mM of total dNTP and 1 Unit of Taq DNA Polymerase. Temperature cycling profile involved an initial denaturation step of 2 min duration at 94°C. This was then followed by 35 cycles with each cycle made up of denaturation at 94°C for 15 sec, an annealing phase of between 48°C and 65°C (depending on the annealing temperature for the given primer pair) for 15 sec and extension at 72°C for 15 sec. A volume of 6µl formamide containing 0.4% w/v bromophenol blue and 0.25% w/v xylene cyanol FF were added to each reaction, denatured and then 4µl loaded on 6% denaturing polyacrylamide gels (19:1 acrylamide: bis-acrylamide), contained 5M urea and 0.5XTBE. Electrophoresis was at 100-W constant power for between 2 and 2.5 hours. PCR amplifications were visualized by silver staining according to the Manufacturer's guide (Promega Inc., USA) with some modifications.

**Data analysis**

Gels containing PCR amplifications were scored for the presence or absence of alleles generated by each pair of primers for all 30 individuals.

In this study, we used the Discriminating Power, (D) to compare the efficiency of the microsatellites to differentiate between genotypes . "D" value represents the probability that two randomly chosen individuals show with the microsatellite locus different allelic patterns, and thus are distinguishable from one another. If *Pi* is the proportion of the population carrying $i^{th}$ banding pattern, and calculated for each microsatellite locus (Tessier, et al, 1999), then $D=1-\Sigma p_i^2$. This is an extension of the Polymorphism Information Content or PIC (Anderson ET. Al., 1993) available from the frequencies of the different banding patterns (or genotypes) generated by a primer.

**Results**

Characterization of selected microsatellite sequences into *P. vulgaris*

Sixty-eight primers were used to investigate the polymorphism detected among 21 individuals of *P. vulgaris*. Fourteen loci were monomorphic in the materials tested. A total of 584 alleles were detected at the 68 microsatellite loci. The number of alleles per microsatellite locus ranged from 1 to 14 with an average of 6.3 alleles per primer pair. Also, 1-19 different banding patterns were generated. We used the data from the microsatellite loci and their corresponding alleles and patterns per loci to calculate the Discrimination Power (D) in order to examine the extent of information on diversity that these markers can provide for *P. vulgaris*. The D value in the present study ranged from 0.09 to 0.94, with an average of 0.73 for loci with more than 1 banding pattern. We observed that 73% of the loci had more than 50% probability discriminating between two individuals. As reported by Tessier, et. al., 1999, we found that the analysis of discrimination power (D) revealed that the efficiency of a given primer does not depend only on the number of patterns it generated. For example, primers BM170 and BM140 produced the same number of patterns and alleles, but they also had different discriminating powers. On the contrary, primers BM153 and BM164 with quite a different number of patterns (7 and 11, respectively) had similar discrimination powers. Primers BM188, GATS 91 and BM 143 have D values higher than 0.90, which means that they would be very useful for genotyping *P. vulgaris* germplasm accessions.

The high Discriminating power values exhibited by microsatellites make them the markers of choice to saturate the bean map.

**Conservation of microsatellite loci across *Phaseolus* species**

The conservation of these 68 loci in *Phaseolus* was examined by evaluating them with *P. coccineus, P. polyanthys, P. acutifolius* and *P. lunatus* . The first two species are more closely related to *P. vulgaris* as they, belong to the same lineage than to *P. lunatus*, which is more distant and belongs to a different lineage (Fofana, et. al., 1999). Primers BM16, BM 138 and BM195 failed to amplify any non-source; GATS11B, BM150, BM167, BM188, BM195, BM199 and BM 200 amplified in almost all species, just 50% or less of the total of individuals.

Thirty three microsatellite markers produced amplification products in all samples with some of them showing length variability between species. This indicates that a considerable level of sequence conservation exists within the primer regions flanking the microsatellite loci. Eighteen microsatellite loci produced non-specific amplification products in *P. lunatus*. This occurred mainly with primers BM16, GATS11B, BM167, BM195, BM138, BM25, BM150 and BM200 that failed to amplify in 50% or less of the tested species. Nevertheless, the utility of the bean primers in producing PCR–amplified products across the genus has been demonstrated. It must be noted however that just as has been pointed out in other studies on SSR loci conservation in plant species, a decline of amplification success was observed with increase of genetic distance (White and Powell, 1997, Roa, 2000).

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## 1.3.4 Microsatellites isolated from common bean cDNA and small-insert genomic libraries

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### Introduction

Microsatellites based on simple sequence repeats have been developed for a wide range of plant species. Various techniques exist for discovering new microsatellite markers from anonymous genomic sequence. All of these techniques rely on the availability of DNA libraries. It is best if these libraries consist of small-insert clones (< 1 kb) because they are easier to screen for microsatellites and to sequence if they are shown to be positive. Various enrichment procedures have been used to increase the prevalence of simple sequence repeats in genomic libraries. One method relies on the selective capture of small fragments with oligonucleotides on hybond membranes (Edwards et al., 1996). An enrichment method has been used at CIAT to develop two GAn and CAn microsatellite-containing libraries for common beans (CIAT Annual Report 1999-2000). In this study, we were interested in screening non-enriched libraries with simple sequence repeat motifs that have not been screened in the enriched libraries. The screening of non-enriched random genomic libraries will allow us to investigate the normal frequency with which different microsatellite motifs occur in common bean and to develop a new set of genomic microsatellites for mapping and tagging projects in common bean.

### Materials and Methods

*Libraries:* Four libraries were screened for microsatellites. The largest library was a leaf cDNA library (pCMV Sport 6.0 vector) consisting of 64,128 clones. In addition, three small-insert genomic libraries were screened. These are named after the restriction enzyme that was used to generate them. They were: (1) *Rsa*I library (19,584 clones) 2) *Alu*I library (18,432 clones); and 3) *Hae*III library (19,968 clones). These libraries were made with frequently cutting restriction enzymes as described in more detail in the 2000 Annual Report. All three small insert libraries were made with DNA from the genotype DOR364 that was digested with the appropriate restriction enzyme and size selected to the range of 0.4-1.2 kb. The small-insert libraries for *Alu*I and *Hae*III were made with the standard pBluescript II KS+ vector while the *Rsa*I library was made with pGEMt-easy vector. The clones for each library were picked by a Q-bot robot and spotted onto gridded Hybond N+ membrane filters with six fields each with a 96 position double-replicate 4 x 4 pattern. Each of the small insert libraries was contained on a single filter while the cDNA library was spread over a set of four filters. All the clones were stored in 384-well plate format glycerol stocks that were copied twice into working and master copies of the library.

*Filter hybridization and library screening:* Six simple sequence repeat motif oligonucleotide probes were used to screen the cDNA and small-insert library filters. The probes included two that targeted dinucleotides repeat motifs, namely $(CA)_{20}$ and $(GA)_{20}$; and four that targeted trinucleotide repeat motifs, namely $(AAT)_{14}$; $(CAG)_{14}$; $(CAA)_{14}$; and $(ACG)_{14}$, where the sequence within the parenthesis indicates the repeat and the number outside the parenthesis indicates the number of copies of that repeat. Hybridization consisted in end labeling the simple sequence repeat motif probes with T4 DNA Kinase and hybridizing this probe to the DNA contained on the filters with standard protocols (Edwards et al., 1996). Briefly the filters were pre-hybridized in 100 mL of hybridization buffer for 4 – 6 hours at 60°C. Meanwhile, 10 pmoles of probe was end labeled with 1 ul of T4 polynucleotide kinase and 5 ul of $\gamma P^{32}$-ATP in a total

reaction volume of 20 ul that was incubated at 37°C for 80 minutes and stopped at 65°C for 20 minutes. The labeled oligonucleotide was added directly to the pre-hybridizing filters and incubated at 60°C for 12 hours. After the hybridization step, the filters were washed twice at 60°C with 6X SSC / 0.1 % SDS for 5 minutes each. Longer washes were used when signal was intense (> 100,000 cpm). The filters were blotted dry, covered with saran-wrap and arranged face-up in cassettes along with three sheets of X-ray film. The films were taped to each other so that they would not shift during the 0 to 8 day exposure in a –80°C freezer. Films were removed at three exposure intervals: after 2 hours, after 1 day and after 1 week to identify high, medium and low signal positive clones, respectively. Filters were re-used for sequential screening of different oligonucleotide repeats by stripping them between each use. Stripping consisted in washing the filters at room temperature in 100mM NaOH, 10mM EDTA, 0.1% SDS twice, followed by a 5X SSPE rinse for 10 min.

*Clone identification:* Positive clones were identified by which filter they were on; which field within the filter they were in, and what address they had within the field. Each filter contained six fields and each field contained the equivalent of eight 384-well plates worth of clones, for a total of 48 plates per filter. Clones could be identified by their position in the double-replicate 4 x 4 pattern found at each grid axis in the address system. Only double-spotted clones were selected. Any spots for which the replicate did not hybridize were considered false positives and were not selected. Putative simple sequence repeat-containing clones were picked from their appropriate position in the 384-well plate format glycerol stocks

*Clone sequencing:* The positive clones were sequenced initially from one end of the insert. In the case of the cDNA library, the 5'side of the clone was sequenced using either Sp6 or M13 Reverse primer, while in the case of the small insert libraries the clones were sequenced with either T7 or T3 high temperature primers. The sequences were searched for vector segments to check for insert integrity and were screened for simple sequence repeats with the program Sequencher. Any cDNA clone that did not contain an SSR in the 5'end was sequenced again from the opposite end with the T7 or M13 Forward primers.

**Results and *Discussion***

We were interested in calculating the overall frequency of di- and tri-nucleotide repeats in coding versus non-coding DNA and in the frequency of GA versus CA repeats in common beans. Therefore our hybridization strategy was the following: the small insert library filters were hybridized with each dinucleotide probe separately, the cDNA filters were hybridized with a mix of both dinucleotides probes; and the trinucleotides probes were hybridized simultaneously in both libraries. We limited the comparison between the di-nucleotide probes to the small-insert genomic libraries rather than the cDNA library, because we reasoned that di-nucleotide repeats occur with greater frequency in non-coding DNA than in coding regions. This screening technique allowed us to calculate the frequencies of di- and tri-nucleotide motifs in each library. Table 1 shows the number of positive clones found in each hybridization experiment with each of the libraries and with each set of oligonucleotide probes. The total number of clones screened in the three small insert libraries was 57,984, while the total number of clones in the cDNA library was 64,128. Surprisingly the CA probe hybridized to more clones (190) than the GA probe (24) in the two libraries that were evaluated for this purpose, *Alu*I and *Hae*III. These two libraries were chosen because they would be contrasting, given that the *Hae*III library represents the GC rich fragment of the genome due to its restriction enzyme recognition site, while the *Alu*I library does not. The results indicate that either GA motif is more common than CA motif in the bean genome or that the hybridization worked better for this probe. Neither hypothesis is satisfactory,

since the first possibility contradicts the higher frequency found for GA motifs in many other plant species, while the second possibility is unusual given that both oligonucleotides have the same melting temperature. The frequency of the tri-nucleotide positive clones was as high as that of the di-nucleotide positive clones, except in the RsaI library and in the cDNA library, were trinucleotide positive clones were more frequent than di-nucleotide positive clones. These results may indicate that trinucleotide repeat are more common in coding sequences than across the entire genome.

Sequencing results confirmed whether the hybridizing clones from each library were false positives or not and the number of repetitions that each positive clone contained. The rate of false positives was higher for clones selected by placing the hybridized filters on film for one week of exposure versus those that were on film for two hours (Figure 1). Meanwhile, the sequencing confirmed that as might be expected, the clones with the highest intensity signal had a larger number of repeats. The sequencing results showed that dinucleotide clones were more common than trinucleotide clones in the small insert libraries and about equal in the cDNA library (Figure 2), contradicting the earlier results based solely on hybridization. Among the 264 dinucleotide repeat containing clones identified so far, the CA motifs were 50% more common than the GA repeat in the small insert library, but only 30% as common as the GA repeat in the cDNA library (Figure 3). The AT motifs were more frequent in the small insert library than in the cDNA library. When AT microsatellites did occurr in the cDNAs they were more likely to be found in the 3' end than in the 5'end of the clones.

This was in contrast to the GA and CA microsatellites which were three times more frequent in the 5'end than in the 3'end of the cDNA. The cDNA library had a higher proportion of tetra- and penta-nucleotide simple sequence repeats than the small insert libraries, although these motifs had not been screened for specifically in the hybridization experiments. We believe that the screening of the small insert libraries has given us an accurate picture of the relative frequency of different SSR motifs in the bean genome based on the fact that these libraries each represent a total of 10 Mb of DNA. Considering that the genome of common bean is 650 Mb in size, each library is equivalent to 0.015 X genome equivalents and taken together the three libraries contain approximately 30 Mb of bean DNA which should be equivalent to 5 % of the total bean genome. An estimate of the relative abundance of microsatellites on an absolute scale per kb of genomic sequence could therefore be obtained.

Similarily these results give us a good idea of which motifs are common in cDNA sequences and where these are located. The large majority of the repeats occurred in the 5'untranslated region (UTR) upstream of the start codon for the open reading frame (ORF) where these could be identified. Another proportion of the SSRs occurred in the 3' UTR, while fewer were found within the ORF. The total percentage of ESTs that might be expected to contain SSRs based on this study is approximately 0.7 %. Non-SSR containing cDNA sequences are a useful resource in their own right since they add to the body of ESTs available for the crop.

The present work brings to a total of approximately 2000 the number of ESTs for beans generated by this project. The sequences represent both 5' and 3' ESTs, since they come from a directionally cloned cDNA library. The sequences have been BLAST searched against each other to check for sequence redundancy (redundant clones consist in 90% identity over half the nucleotides in a sequence) and the vast majority of the sequences were unique. The sequences were also compared to the Swissprot database and to all the soybean proteins downloadable from Genbank. This collection of new gene sequences for common bean represents many more individual sequences than are currently in the Genbank public database for all *Phaseolus* species together.

**Table 1. Number of positive clones detected by hybridization of each library, at each exposure time and with each oligonucleotide probe.**

| | PROBE | EXPOSURE[1] | LIBRARY | | | |
|---|---|---|---|---|---|---|
| | | | *Alu*I | *Hae*III | *Rsa*I[2] | cDNA[2] |
| 2nt | CA | 1 | 6 | 1 | - | - |
| | | 2 | 63 | 54 | - | - |
| | | 3 | 22 | 44 | - | - |
| | GA | 1 | 0 | 1 | 29 | 55 |
| | | 2 | 15 | 3 | 11 | 231 |
| | | 3 | 3 | 2 | 28 | 87 |
| | total 2nt | | 109 | 105 | 68 | 373 |
| 3nt | | 1 | 1 | 0 | 6 | 25 |
| | | 2 | 21 | 10 | 4 | 95 |
| | | 3 | 8 | 32 | 53 | 13 |
| | total 3nt | | 30 | 42 | 63 | 133 |
| TOTAL | | | 139 | 147 | 131 | 506 |

1/ exposure times: 1=2 hours; 2=a day; 3= a week 2/ both oligo probe were added in the same hyb reaction

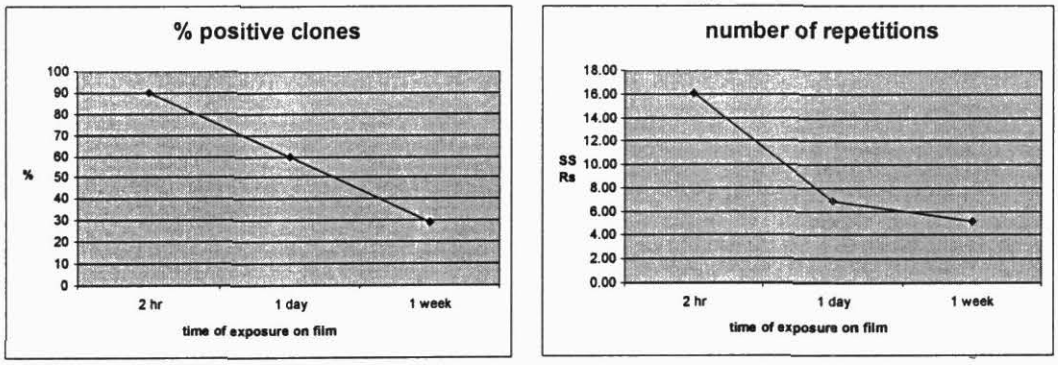


**Figure 1. Percentage positive clones and number of repetitions for clones selected from hybridized filters that were left for three exposure times of 2 hours, 1 day and 1 week.**

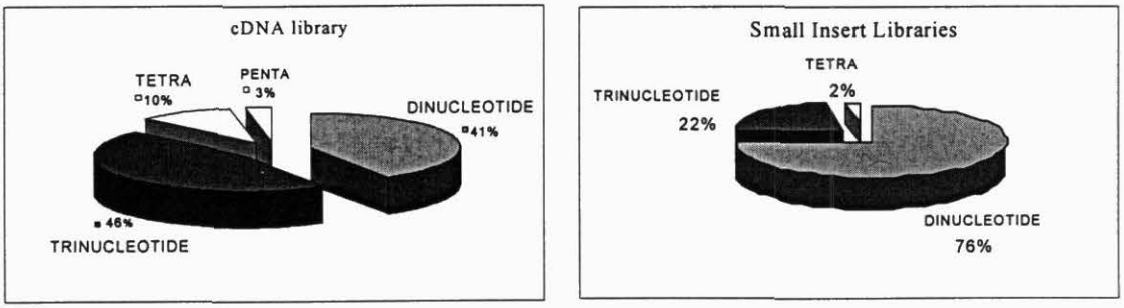


**Figure 2. Percentage positive clones from the cDNA and small insert libraries that contained di-, tri-, tetra- and penta-nucleotide simple sequence repeats upon sequencing.**

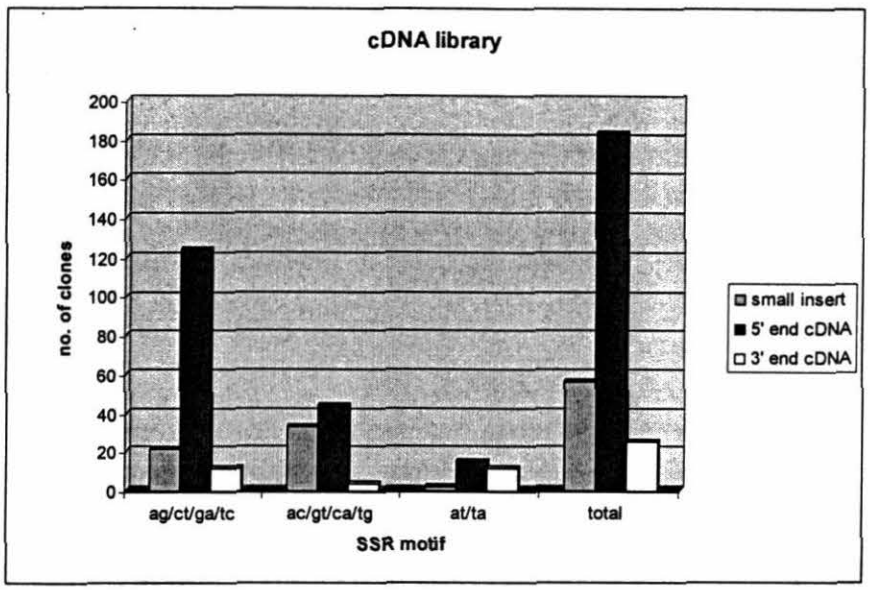


**Figure 3. Number of GA, CA and AT motif dinucleotide simple sequence repeats found in the sequenced small insert library clones and in the 5'and 3' ends of the cDNA clones.**

### Future plans

We are sequencing all positive SSR containing clones to design microsatellite primers for the development of new markers and hope to recover a large number of polymorphic markers for the crop. The sequenced cDNA clones represent the first substantial number of EST sequences in beans. Ultimately many of these cDNA sequences can be genetically mapped as RFLPs or SNP (single nucleotide polymorphism) based assays, especially as simple procedure such as dense chips, become available.

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## 1.3.5 Legume microsatellites tested in common bean

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### Introduction

A large number of microsatellite markers have been developed for soybean by the USDA (Cregan et al., 1999) and at companies such as Dupont (Peakall et al., 1998). Relatively fewer microsatellites have been developed for other tropical legume crops. However at IITA an effort is underway to develop cowpea microsatellites (C. Fatokun, pers. Comm.), and an initial set of peanut microsatellites is also available (Hopkins et al., 1999). The large number of common bean

microsatellites made at CIAT recently would also be useful for mapping in other species of legumes. Our objective in this study was to try to adapt the microsatellites available for other Phaseoleae legume crops (especially soybean and cowpea) to common bean and to test common bean microsatellites in a panel of these other legumes.

**Materials and Methods**

The microsatellites tested included 423 from *Glycine max* (408 with ATTn motif, 3 with GAn motif and 12 from coding sequences) and 118 from *Vigna* (mixture of GA, CA, AT and compound motif, genomic microsatellites and two cDNA based markers). An additional four cowpea microsatellites were designed from sequences in Genbank (Table1). The markers were tested against a panel of nine legumes, that included the soybean, Mesomerican and Andean common bean genotypes (Williams, DOR364 and G19833, respectively) that were the sources of microsatellite libraries made for these crops. For the other legume species we used genotypes that were representative of varieties grown in the Andean region. Primer amplification was tested with a range of conditions. The soybean microsatellites were tested initially with lax amplification conditions using 45 to 47°C for annealing temperature and 2.0 to 2.5 mM final concentrations of MgCl2. The cowpea microsatellites were amplified with 52°C annealing temperature and 2.5 MgCl2. Both sets of markers were analyzed on 2.0% agarose gels with ethidium bromide staining. The microsatellites with single amplification products were analyzed on 6% polyacrylamide gels with silver staining.

**Results and Discussion**

Soybean and common bean appeared to be especially divergent in regard to their microsatellite loci. The soybean genomic ATTn microsatellites generally did not amplify well in common bean. The cDNA based microsatellites were also poorly conserved. Table 2 shows the nine most transferable microsatellites and the molecular weight of the amplified products. Most other microsats amplified multiple bands from common bean, tepary bean, lima bean, cowpea or mung bean DNA that were completely different in size compared to the soybean allele. The likelihood that these represent homoelogous microsatellite loci was deemed low and these microsatellites were not investigated further. The total rate of transferability to the six species was between 2 and 0.5%. Therefore, it seems that soybean primers may be less useful in common bean than we thought because of the evolutionary distance separating these species. This is in marked contract to studied with animals where microsatellites have been successfully transferred among related species such as birds, tortoises, primates (eg. gorillas/apes), ungulates (eg. horses/cattle) and rodents (eg. rats/mice). Ultimately it is the genetic distance between species and genera that determines the ability of SSR primers to amplify in different genomes and the ability to transfer microsatellites between species must be determined empirically. And it seems that in plants, unlike animals, microsatellite loci are not well conserved over large genetic distance between species. Cowpea genomic microsatellites were slightly more useful than the soybean genomic microsatellies for amplification in *Phaseolus* species. The transferability rates were between 7.6 and 11.0 %. The gene-derived microsatellites were more conserved than the microsatellites from non-coding sequences, and four out of six primer pairs from Table 1 amplified well across the subtribe. For example one cowpea microsatellite, VM21, amplified well in a range of legumes and represented a gene from *Vigna radiata* for ACC oxidase that contained an ATn repeat in the 3' untranslated region. These results suggest that the close phylogenetic relationship between cowpea and common bean allowed us to exchange some of the genomic microsatellites and most of the cDNA based microsatellites. Based on these promising results, we tried the reciprocal experiment of amplifying the cDNA based microsatellites we have developed for common bean in cowpeas other species. In these experiments, we found that transferability was much high

especially within the *Phaseolus* genus as expected, but also across into *Vigna*, *Glycine* and *Cajanus*. In summary, we found that the pattern of amplification of legume microsatellites from soybean, cowpea and common bean, agreed well with the tribe, subtribe and genus designations of the legumes being tested. Why some related lineages maintain intact microsatellites over evolutionary time or why microsatellites in certain location such as upstream of genes are more conserved is not known. However, we can postulate that mutation rates for microsatellites are constrained around genes as compared to non-coding regions, just as they are for other forms of sequence variation.

**Future studies**

A set of common microsatellites that amplify across a range of tropical legumes, would be useful to study the synteny between species. Many legume genera are thought to have a conserved gene order, however this has been less well studied than in the grass family. Soybean, common bean and mung bean have been shown to have regions of macrosynteny across much of their genomes, however there has been no analysis of microsynteny or sequence conservation at homoelogous loci in the genomes of these related legumes.

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**Table 1. Microsatellites developed for cDNAs from Vigna.**

| SSR | Genbank number | gene | Predicted location | Species | Motif | Repetition | Predicted MW |
|---|---|---|---|---|---|---|---|
| VMd1 | U08140 | Ca-dep. protein kinase | 5`UTR | *V. radiata* | CAA | (CAA)2-(CAA)5 | 116 |
| VMd2 | AB030294 | CPRD86 cds parcial | ORF | *V.unguiculata* | GAA | (GAA)5-(GAA)4 | 120 |
| VMd3 | Y08624 | Ted2 | 5`UTR | *V.unguiculata* | CTT | (CTT)6 | 115 |
| VMd4 | Z23083 | Endo-1,4-beta-glucanase | ORF | *V. radiata* | TCTTC | (TCTTC)4 | 127 |
| VM21 | U06047 | ACC oxidase | 3`UTR | *V. radiata* | AT | (AT)7-(AT)10 | 179 |
| VM22 | M99497 | proteina kinasa | 5`UTR | *V.aconitifolia* | GA | (GA)12 | 217 |

**Table 2. Amplification products of soybean, cowpea and common bean microsatellites on a panel of economically-important legumes from the *Phaseoleae* tribe[1].**

| | | Family | *Fabacea* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Tribe | *Phaseoleae* | | | | | | | |
| | | Sub-tribe | *Phaseolinae* | | | | | | *Glycinin.* | *Cajanin.* |
| | | Genus | *Phaseolus* | | | | *Vigna* | | *Glycine* | *Cajanus* |
| | | Species | P.v (M) | P.v (A) | P.a | P.l | V.u | V.r | G.m | C.c |
| | | Variety | DOR364 | G19833 | G40001 | Peru | Molina | Mungo | Williams | IS-10 |
| Source of Microsatellite | Marker Type | Primer | | | | | | | | |
| Soybean | cDNA | SoyPRP1 | 240 | 240 | - | - | 240 | 240 | *120-240* | 240 |
| | cDNA | SoySc514 | 260 | 260 | - | - | 260 | 260 | *120* | - |
| | Genomic | Satt 511 | 360 | 360 | - | 540 | - | 270 | *250* | 470 |
| | Genomic | Satt 401 | 160 | 160 | - | - | 500 | 670 | *170* | - |
| | Genomic | Satt 206 | 140 | 140 | - | 220 | - | - | *220* | - |
| | Genomic | Satt 237 | 320 | 320 | 320 | 320 | 320 | 320 | *250-320* | 320 |
| | Genomic | Satt 305 | - | - | - | 290 | - | - | *200* | - |
| | Genomic | Satt 411 | - | - | - | - | - | 290 | *100* | - |
| | Genomic | Satt 275 | 140 | 140 | 140 | - | - | - | *160* | - |
| | Transferability (%) | | 1.89 | 1.89 | 0.47 | 1.18 | 1.18 | 1.66 | 100.00 | 0.71 |
| Cowpea | Genomic | VM26 | - | - | 90 | 160 | *160* | 140 | 140 | 140 |
| | Genomic | VM63 | *300* | *300* | *300* | *300* | *300* | *190* | *190* | 190 |
| | cDNA | VM21 | 208 | 202 | 208 | 208 | *240* | 240 | 260-202 | - |
| | Genomic | VM91 | 330 | 330-510 | 330 | 330 | *330* | 150 | 330 | 330 |
| | Genomic | VM98 | 160 | 160 | 160 | 160 | *160* | 160-90 | 160 | 160 |
| | Genomic | VM114 | 270 | 270 | - | - | *270* | 270 | 270 | - |
| | Genomic | VM118 | 280 | 280 | 280 | 280 | *280* | 280 | 280 | 300 |
| | Transferability (%) | | *11.02* | *11.02* | *10.17* | 9.32 | *7.63* | 100.00 | 11.02 | 5.93 |
| Common Bean | cDNA | BMd1 | *204, 164* | *204, 164* | 162, 166 | 174 | 204 | - | 204, 166 | 204 |
| | cDNA | BMd2 | *108* | *104* | - | 100 | 108 | - | - | - |
| | cDNA | BMd3 | *226* | *226* | - | - | - | - | 226 | - |
| | cDNA | BMd6 | *122* | *122* | 122 | 122 | - | - | 122 | - |
| | Genomic | BMd11 | *160* | *160* | 158 | 164 | 150 | - | - | - |
| | Genomic | BMd12 | *162, 125* | *164, 125* | - | 155, 125 | 164 | - | 170, 160 | - |
| | cDNA | BMd13 | *192* | *192* | - | 192 | 192 | - | 192 | 192 |
| | cDNA | BMd14 | *188* | *188* | - | 188 | 205 | - | 200 | - |
| | cDNA | BMd16 | *148, 86* | *136* | 180 | 240, 122 | 205, 185 | - | 180 | 276 |
| | cDNA | BMd17 | *100* | *106* | 106 | 274, 100 | 106 | - | 222, 170 | - |
| | cDNA | BMd18 | *242 156* | *242 158* | - | 242, 148 | 242, 204 | - | 248, 104 | 250, 235 |
| | cDNA | BMd19 | *154* | *154* | 154 | 302, 164 | 172, 154 | - | 308, 148 | 154 |
| | cDNA | BMd20 | *128* | *124* | - | 124 | 126 | - | 138 | 188, 174 |
| | cDNA | BMd22 | *118* | *130 , 118* | - | 130, 118 | - | - | | |
| | cDNA | BMd23 | *138, 126* | *138, 126* | - | 138, 126 | 138, 126 | - | 138, 126 | 138, 126 |
| | cDNA | BMd26 | *140* | *148 136* | - | 148, 136 | 160 | - | - | - |
| | cDNA | BMd30 | *145* | *145* | 150 | 115 | - | - | 150 | - |
| | cDNA | BMd32 | *112* | *112* | - | 106 | 270, 224 | - | 312 | 222 |
| | cDNA | BMd33 | *100* | *110* | - | 95 | - | - | - | - |
| | Transferability (%) | | 100.00 | 100.00 | 22.58 | 58.06 | 41.93 | na | 45.16 | 22.58 |

1/ P.v. (M) : *Phaseolus vulgaris* – Mesoamerican; P.v. (A): *Phaseolus vulgaris* – Andean; P.a: *Phaseolus acutifolius*; P. l: *Phaseolus lunatus*; V.u: *Vigna unguiculata*; V.r: *Vigna radiata*; G.m: *Glycine max* and C.c: *Cajanus cajan.*

## 1.3.6 Enhanced microsatellite map developed for common bean

M.W. Blair, F. Pedraza, E. Gaitán, J. Tohme
SB-2 Project

### Introduction

Microsatellites are polymerase chain reaction (PCR) based markers that detect length polymorphisms at loci with simple sequence repeats. They are also single-locus markers that are specific to a given place in the genome. Microsatellites are advantageous because they are readily amenable to relatively high throughput marker assisted selection strategies. The specificity of microsatellite marker for use in MAS selection depends on tight genetic linkage of the marker with a gene that produces a reliable phenotype. Genetic maps are needed to determine where microsatellites are located in the genome and what genes they may be linked to. Microsatellites have been found to be distributed densely throughout the genomes of higher plants, making them very appropriate for genetic mapping. Nearly-saturated microsatellite maps are now available in several crop plants including soybeans, rice, wheat, barley, etc. It would be tremendously useful to have a genetic map for common bean consisting entirely of microsatellites. These second-generation markers would be easy to assay and would enable a large number of segregating individuals to be analyzed in gene and QTL tagging studies. For now, we have implemented a set of over one hundred and fifty microsatellite markers in genetic mapping studies for common bean at CIAT. These microsatellites come from genomic sequences, gene or cDNA sequences and database searches mostly of common beans but also of other legumes as described in other sections of this annual report.

### Materials and Methods

The parents of the DOR364 x G19833 RIL population were surveyed for polymorphism with 153 microsatellites. The markers belonged to several different classes as shown in Table 1. BM microsatellites are genomic, while BMy and BMc microsatellites are based on cDNA. The BMd and Clone microsatellites are based on both genomic and cDNA sequences. The microsatellites are in varying stages of development. Clone microsatellites will be given a new designation under the BM naming system once they have been further tested. The polymorphic microsatellites were used to amplify DNA from the 87 recombinant inbred line progeny and the parents of the population. PCR product were run on silver-stained polyacrylamide gels and scored for the parental allele that they represented. Segregation data was used to place the microsatellites on a genetic map constructed with RFLP, RAPD, AFLP and SCAR markers (CIAT annual Report, 1998) using the software application Mapmaker.

### Results and Discussion

A total of 81 microsatellites (52.9% of those tested) were polymorphic for the parents of the DOR364 x G19833 population and could be located on the genetic map of common bean. An additional 5 microsatellites from Yu et al (1999) could be placed by comparative mapping between the DOR364 x G19833 and BAT93 x JaloEEP58 (Freyre et al., 1998) maps. Each chromosome was tagged with at least three microsatellites (Table 1). Two chromosomes, B02D and B04B had a relatively greater number of microsatellites, 14 and 12 markers respectively, placed on them. The average number of markers per chromosome was 7.4 microsatellites each. Considering that the total genetic distance of the entire map was 2453 cM, the average distance

between microsatellites was 30.3 cM. However many larger gaps remain and these will need to be filled with additional microsatellites. Other AFLP, RAPD and RFLP markers are available between the microsatellites, Since the entire map consists of 434 genetic markers, the average distance between markers was 5.7 cM. The markers BMy-11, BMd-28 and BM205 consistently amplified two polymorphic bands per reaction, which are suspected to represent duplicate loci. In another mapping population, the two BMy-11 loci co-segregate and are presumed to be very tightly linked (under 1 cM). Several microsatellites designed for related phytohemagglutinin and lectin gene sequences amplified single bands but were found to co-segregate, indicating that this gene family clusters at a single location in the genome. The microsatellites mapped during the course of this research will be invaluable for marker assisted selection because they are simple to analyze, specific for single genes of interest and diagnostic in most crosses due to their high level of polymorphism.

**Future Plans**

We will be studying the potential of specific microsatellites to be used in MAS selection for specific genes with which they are linked. The mapped microsatellites will provide a good set from which to chose markers for diversity studies and future QTL analysis. We plan to develop a set of fluorescent microsatellites for accurate allele calling and high-throughput mapping. A full set of anchor markers for the bean genome will probably require 300 or more working microsatellites, therefore the work of developing and mapping new microsatellites will continue. Of the 153 microsatellites evaluated, a total of 43 were monomorphic (28.1%) and 29 did not amplify well. We will try to recover these microsatellites by amplifying with variable PCR conditions and by mapping the monomorphic markers in well-integrated populations such as BAT93 x Jalo EEP558 or in populations where polymorphism is high, such as wild x cultivated crosses across genepools.

**Table 1. Number of microsatellites from each marker designation mapping to each chromosome in the CIAT genetic map based on the cross (DOR364 x G19833).**

| | B01 | B02 | B03 | B04 | B05 | B06 | B07 | B08 | B09 | B10 | B11 | UNLINKED |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BM | 1 | 8 | 7 | 1 | 2 | 1 | 6 | 2 | 5 | 3 | 1 | 2 |
| BMd | 1 | 4 | 2 | 4 | 5 | 1 | - | 2 | 1 | 1 | 3 | - |
| Bmy | 1 | - | - | 5 | - | - | - | - | - | - | 1 | - |
| BMc | - | - | - | - | - | - | - | - | - | 2 | 1 | - |
| Clone | - | 2 | 1 | 2 | 1 | 2 | - | 1 | 1 | - | - | - |
| TOTAL | 3 | 14 | 10 | 12 | 8 | 4 | 6 | 5 | 7 | 6 | 6 | 2 |

## 1.3.7 Isolation and characterization of Ty1-Copia group retrotransposon LTR sequences in *Phaseolus vulgaris*

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SB-2 Project

**Introduction**

Ty1-copia retrotransposons are mobile genetic elements that seem to be ubiquitous in higher plants (Flavell et al., 1992; Voytas et al., 1992). They also show high insertional polymorphism

(Flavell et al., 1992; Hirochika et al., 1992; Pearce et al., 1996) and preference for euchromatic regions (Hirochika et al., 1996; Flavell et al., 1998; Garber et al., 1999). In the last few years there has been a growing interest in exploding retroelements for molecular marker-based studies (Waugh et al., 1997; Ellis et al., 1998; Flavell et al., 1998; Kalendar et al., 1999; Pearce et al., 2000), however, most research in the field of transposable elements is aimed at diversity, expression or structural analysis because isolation and characterization of retrotransposons for marker studies imply a long process. High levels of polymorphism, necessary for developing different techniques, are usually encountered in long terminal repeats (LTRs), which cannot be amplified directly as they are specific for each retrotransposon.

The technique developed by Pearce et al. (1999) makes it possible to isolate RNAse-PPT-LTR (ribonuclease-polypurine tract-long terminal repeat) sequences from Ty1-copia retroelements for molecular marker-based studies such as Sequence-Specific Amplification Polymorphisms (SSAPs), Retrotransposon-Based Insertional Polymorphisms (RBIPs), Inter-Retrotransposon Amplified Polymorphisms (IRAPs) or Retrotransposon-Microsatellite Amplified Polymorphisms (REMAPs). We used this technique to isolate the aforementioned fragments using *Phaseolus vulgaris* DNA from CIAT accession G19833.

**Materials and Methods**

Previously extracted genomic DNA from CIAT accession G19833 was used to isolate RNAse-LTR sequences by the method of Pearce et al. (1999) with several modifications. Sequence analysis was carried out using the programs Sequencher 3.0, Blast x 2.0, Clustal W 1.8 and Clustal X 1.62.

**Results and Discussion**

From a library of 1152 clones, 45 were selected for sequencing. Of these, 24 were homologous to previously isolated sections of retrotranscriptase, ribonuclease or polyproteins when entered into Blast X using an E value of 10.

Alignment of isolated sections of ribonuclease using Clustal W showed conserved aminoacids (Figure 1), which have been previously reported by Pearce et al. (1999). High conservation might be important for enzymatic function, confirming ribonuclease's central role for reverse transcription and reintegration. In addition to the RNAse sequence, representative sections for the polypurine tract and a fraction of the long terminal repeat were determined for each sequence (some sequences are shown in Figure 2). Variability seems to be low at ribonuclease, but the PPTs and LTR sections show high plasticity due to mutations created by retroelement enzymes on nonessential transposon regions (Blusch et al., 1997).

A dendrogram constructed by Clustal X using neighbor-joining and bootstrap analyses helped determine the relationships among the RNAse sequences (Figure 3). Five different groupings, supported by bootstrap and high percentages of sequence identity between its members were defined, providing support for the existence of several Ty1-copia retrotransposon families. Sequences were also compared to homologous retrotransposon sections from other legume species, indicating a highly heterogeneous population of retroelements in legume species, but smaller differences between *Phaseolus vulgaris* RNAses (result not shown).

| | |
|---|---|
| P6 | ADILTKALGKERFLTLRHKLGVLDLHLPT |
| Bt1004 | ADILTKALGKERFLTLRHKLGVHDIHPPT |
| Bt764 | ADMLTKALGKERFLTLRHKLGVLDLHHP- |
| P11 | ADMLTKAVGKERFLTLRHKLGFMIFTYQL |
| Bt737 | ADMLTKPLPK-RFFFLRNELGILDLNNLS |
| Bt1136 | ADMLTKPLPKERFFFLRNELGILDLNNLS |
| Bt602 | ADMLTKPLPKERFFFLRNELGILDLNNLS |
| Bt490 | ADMLTKPLPKERFFFLRNELGILNLNNLS |
| Bt731 | ADILTKPLPKERFFFLRNELGILDLNNLS |
| Bt1126 | ADILTKPLPKERIFFLRNELGILDLNNLS |
| Bt966 | ADILTKPLPKERFFFLRNELGILDLNNLS |
| Bt59 | ADIFTKPLPKYRFFLSRNELGILYSHNIS |
| Bt454 | ADILTKHLPKDRFFLLRNELGIINSHTLS |
| Bt261 | ADILTKPLPKNRFLLLRNELGIVDSKNLS |
| P17 | ADMLTKGLPTKQFEDLTCKLGMIDIHSPT |
| Bt1094 | ADMLTKGLPTKQFEDLTCKLGMIDIHSPT |
| Bt438 | ADMLTKPLPSAKFDHCLNLAGIIHT---- |
| Bt443 | ADMLTKPLPSAKFDHCLNLAGIIHT---- |
| Bt1112 | ADMLTKPLPSAKFDHCLNLAGIIHT---- |
| Bt781 | ADMLTKPHPSAKFDHCLNLAGIIHT---- |
| Bt293 | ADILTKPLPSAKFDHCLNLAGIIHT---- |
| Bt877 | ADMLTKPLPPAKFDHCLNLASIIHT---- |
| Bt814 | ADMLTKVVRAKFEHCLDLVNILHI---- |
| Bt829 | ADILTKVVTRTKFEHCLDLVNILHI--- |

**Figure 1. Alignment of isolated fragments of RNAse sequences (* = identical or conserved aminoacids, : = conserved substitutions, . = semiconserved substitutions).**

| Element | aa | nb | PPT | LTR | Nd |
|---|---|---|---|---|---|
| P11 | 29 | 0 | GAGGAGAG | **TA**TTAAATAAAT | 56 |
| P17 | 29 | -2 | GAGGGGGAG | **TG**TTGCATAATC | 83 |
| Bt59 | 29 | 40 | GAAGAAAAA | **TA**GGGGGAGAT A | 128 |
| Bt261 | 29 | 55 | AGGGAGAAAAT A | **TG**TTGGTTTATT | 200 |
| Bt438 | 25 | 15 | GAGAGAG | **TG**TTCCAGTCAA | 182 |
| Bt602 | 29 | 11 4 | GAAAG | **TG**TTTTCTGTTG | 42 |
| Bt814 | 25 | -2 | GAAG | **TTG**GCGCTCGAA G | 282 |

**Figure 2.Regions in isolated sequences: aa = no. of aminoacids from RNAse, nb = nucleotides between RNAse and polypurine tract, PPT = polypurine tract, LTR = long terminal repeat, nd = remaining no. of downstream isolated nucleotides.**

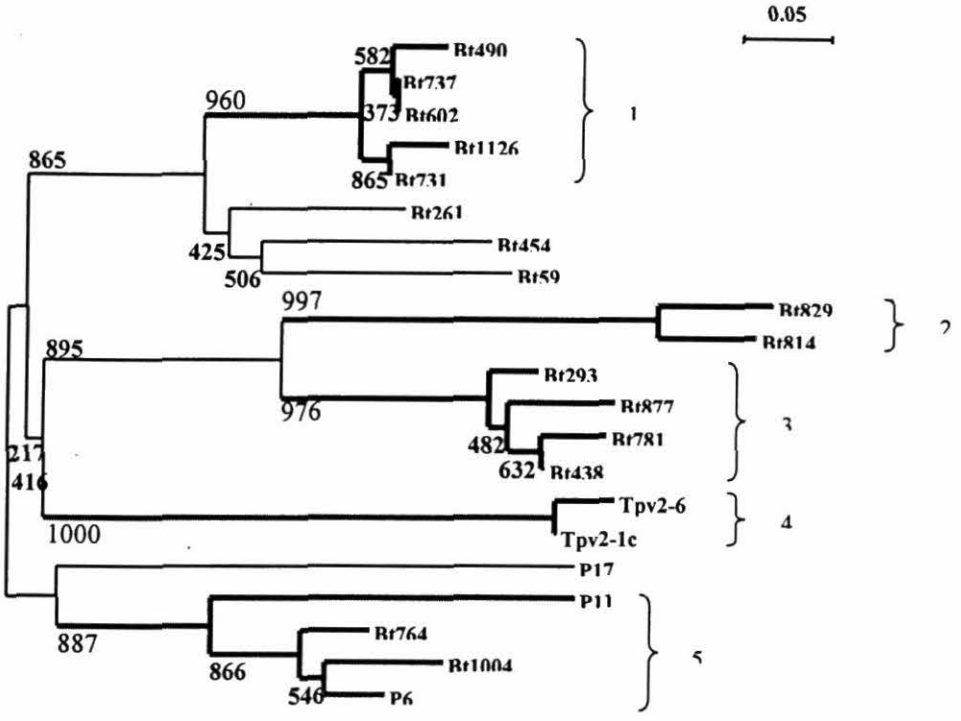


**Figure 3. Possible families of retroelements as determined by ribonuclease-isolated sections; Tpv elements were previously isolated (Garber et al., 1999).**

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## 1.3.8 Analysis of Ty1-copia retrotransposon LTR Sequences and Their Use for genome organization studies

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### Introduction

Long terminal repeats (LTRs) are a distinguishing feature of Ty1-copia retrotransposons. Short sequences in the LTR act as promoters and enhancers of transcription, and the majority of such sequences are in the U3 region. Activity of the different motifs has been well documented through transient reporter gene assays (Casacuberta and Grandbastien, 1993; Suonemi et al., 1996; Takeda et al., 1999). However, as functionality lies only through small stretches, the rest of the LTR is subjected to the high mutation rates of retroelements (Gojobori et al., 1990, cited in Flavell et al., 1992), generating polymorphisms at high levels. Differences in LTR length and sequence can be used to study the evolution of specific retrotransposon families in individuals or in different species; furthermore insertional polymorphism of retroelements can be easily assessed when information about LTRs is available.

In other research (see "Isolation and Characterization of Ty1-Copia Group Retrotransposon LTR Sequences in *Phaseolus vulgaris*," pp. xx of this report) we used the technique developed by Pearce et al. (1999) to isolate 24 different sequences corresponding to RNAse-PPT-LTR (ribonuclease- polypurine tract-long terminal repeat) sections of Ty1-copia retrotransposons. Here we performed an analysis of sequences derived from amplifications using polypurine tract primers (designed from the aforementioned isolated sequences) and *MseI* primers. We also initiated a study using primers derived from LTR sections. Primers were also useful in tests for Sequence-Specific Amplification Polymorphisms (SSAPs) and Inter-Retrotransposon Amplified Polymorphisms (IRAPs) on accessions G19833 and DOR364. Both varieties possess many agronomic traits of interest.

## Materials and Methods

A modification of the technique developed by Pearce et al. (1999) was developed to isolate polypurine-tract downstream sections using seven primers designed from polypurine tracts and their flanking regions. RNAse-PPT-LTR sections necessary for the primer design were previously isolated and characterized (Galindo, 2001).

Sequence analysis was carried out using the programs Sequencher 3.0, Clustal W 1.8, Clustal X 1.62, Blast n 2.0, Blast x 2.0 and Matinspector 2.2.

PCR products from amplifications using PPT and MseI primers were used for SSAP reactions with PPT primers and 9 MseI primers containing selective nucleotides. Amplification was carried out by a conventional AFLP touchdown protocol, modifying the annealing temperature according to primers. IRAP assays were modified from the methodology developed by Kalendar et al. (1999).

## Results and Discussion

Of the seven PPT primers, only five provided enough colonies for library construction, with a total of 1920 clones. Of the 72 fragments chosen for sequencing reactions, only 37 showed the expected pair of primers (PPT-MseI) and were used for further analysis with Blast, Clustal and Matinspector . SSAPs were carried out with all the seven primers, and IRAPs with two of them.

*BLAST search.* Several sequences downstream from the polypurine tracts (presumed LTRs) showed high similarity with previously isolated sequences in *Phaseolus vulgaris.* Analysis of the sequences led to the conclusion that these sections did not correspond to insertion sites of retroelements, but rather to upstream and downstream conserved regions of basal genome reading frames. Residual retrotransposon sections are a common feature of the flanking regions of normal plant genes (White et al., 1994) and play an important role in genome evolution, apparently concerning promoter regions.

*Clustal alignment.* Comparison of sequences resulted in a dendrogram of the LTR relations (result not shown). Related sequences (derived from a specific primer) exhibit a tendency to group, but some sequences are part of a mixed population. Unspecific grouping is possibly due to high levels of recombination between LTRs or inter-retroelement transposition (Pearce et al., 2000), mutations generated by retrotranscriptase enzyme, causing the loss of overall sequence similarity (Blusch et al., 1997) and unspecific amplification.

*Matinspector analysis.* Conserved regions required for transcription are usually found in LTRs. Isolated LTR sequence L814-90 presented high levels of similarity (3 out of 4 had 100% similarity) in all the basic motifs (CAAT and TATA boxes, polyadenilation signal, polyadenilation downstream signal) when compared to the consensus sequences of the program's database. The rest of the sequences had only a few motifs with conservation. High conservation levels in transcription factor-binding sites have proved useful, but not definitive for retrotransposon transcriptional competence (Poteau et al., 1991, cited in Manninen & Schulman, 1993).

*Retrotransposon molecular markers.* Preliminary SSAP tests with two PPT primers (PPT438 and PPT814) resulted in polymorphism of 22.56 and 12.56%, respectively, between accessions G19833 and DOR364, confirming the utility of this molecular marker for mapping purposes.

Polymorphic bands corresponded to both SSAP and AFLP markers as the retrotransposon-specific primers were not labeled.

The IRAPs also showed differential patterns between accessions, giving evidence to recent transpositional activity since the divergence of varieties (Kalendar et al., 1999).

**Ongoing Activities**

Standardization of a technique to use polymorphic bands as probes for microarray-based mapping. A modification of AFLP (Vos et al., 1995) is being used to evaluate the level of polymorphism between accessions G19833 and DOR364. PCR reactions include a pre-amplification step with EcoRI and MseI primers, a selective amplification step with specific LTR primers and MseI primer, and a +3 amplification step with LTR-specific primers and MseI primers with selective nucleotides.

Polymorphic bands are eluted from gels, re-suspended in water and re-amplified. After band identity confirmation in agarose gels, the PCR product is ligated to a PGEMT-easy vector and transformed into *Escherichia coli* DH5α cells. Several clones corresponding to each eluted band are re-amplified and electrophoresed on acrylamide gels to select the exact clone corresponding to the original eluted band. Selected clones are sequenced to confirm their retrotransposon origin and are used as probes for microarray tests.

IRAPs are being tested with the protocol of Kalendar et al. (1999) with some modifications.

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## 1.3.9 Application of the Diversity Array Technology (DarT) in beans for mapping and germplasm characterization

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### Introduction

The DNA microarray technology has been applied to several model organisms including *Escherichi coli*, yeast and *D. melanogaster* (Chu et al., 1998; Richmond et al., 1999; White et al., 1999). Currently, two complementary techniques are available: fragment-based DNA microarrays and oligonucleotide-based chips, also referred to as Affymetrix chips. Briefly, DNA microarrays allow the simultaneous hybridization of two fluorescent-labeled probes to an array of immobilized DNA fragments (usually PCR-amplified DNA sequences), each corresponding to a specific gene or genotype. After scanning the microarray with a laser scanner, the signal for each DNA fragment reflects the abundance of the corresponding messenger RNA in the sample. Recently a new methodology has been adapted using DNA microarrays: Diversity Array Technology (DarT) at Cambia-Australia (Jaccoud et al, 2001). DarT, which is not reliant on DNA sequence information, has several potential applications including germplasm characterization, genetic marker-assisted breeding and tracking genome methylation changes (Jaccoud et al., 2001).

A project was initiated to obtain molecular markers using DarT methodology and demonstrate their potential as markers in germplasm characterization, mapping and tagging of resistance genes.

### Methodology

Two different libraries were constructed to generate different representations: Diversity panels and a mapping panel for beans. The methodology used here followed Jaccoud et al. (2001).

*Generating diversity panels.* DNA from 8 accessions that included mapping parentals, wild and cultivated beans from Mesoamerican and Andean pools was used to generate diversity panels. One restriction endonuclease (EcoRI) was used to generate representations. About 6 ng of DNA from each genotype were bulked and digested with 2 units of EcoRI. After digestion, the EcoRI adapter was ligated to the fragments using T4 DNA ligase, diluted and amplified using a combination of 4 primers with 3 selective nucleotides in a final volume of 50 µl. Amplicons from representation were ligated to the pGEM T-easy vector, and the competent *E. coli* strain DH5α

was transformed by electrophoresis. White colonies were transferred onto a freezing medium and grown overnight at 37°C. Dilutions were made using 5 µl of bacterial and 45 µl of water. A 5-µl dilution was used to amplify by PCR reaction, using T7 and SP6 universal primers. After amplification, the PCR products were precipitated with 1 vol of isopropanol in ice. The DNA was re-suspended in 10 µl of TE+glycerol at about 20 ng/µl. The purified fragments were arrayed with two replicates per fragment onto poly-lysine microscope slides (homemade) using the SPBio microarrayer from Hitachi After PCR amplifications, representations were column purified, and fluorescent dye (Cy3 or Cy5) was incorporated using a Megaprimer Labeling Kit from Amersham. Probes were purified before hybridization. Then 5 µl of Cy3- and Cy5-labeled representations were mixed with 2 µl of 20 µg/µl salmon sperm DNA dissolved in ExpressHyb hybridization solution (Clontech, USA) and denatured at 96°C for 3 min. The denatured probes were then mixed with 50 µl of ExpressHyb hybridization solution, pipetted directly onto the microarray surface, and covered with a glass cover slip. Slides were placed in a 60°C water bath in hybridization chambers (Clontech) and left overnight. After hybridization the cover slips were removed, and the slides were rinsed at room temperature. Slides were dried quickly by centrifugation at 1000 rpm in a slide rack for 1 min. Slides were scanned using a Virtek Chipreader. Spot signal intensities were analyzed by ArrayPro.

*Generating a mapping panel.* The mapping panel was generated using two mapping parentals: DOR 364 and G19833. These two genotypes were digested using MseI to generate the representation. The remaining procedures were carried out as for the diversity panel methodology.

**Results**

*Diversity panel.* A total of 250 clones were spotted onto poly-lysine slides. Eight DNA fragments were identified between DOR364 and G19833 as being potentially polymorphic (with the red/green ratio >3.0, Figure 1). Several DNA fragments were identified among the other 6 accessions as being potentially polymorphic for diversity studies (red/green ratio >2.5). A total of 47 potentially polymorphic DNA fragment were identified.

*Mapping panel:* A total of 2000 clones were spotted onto poly-lysine slides and hybridized with DOR364 and G19833, labeled with Cy3 and Cy5. Three hybridizations were made to reconfirm the polymorphic clones. A total of 180 polymorphic clones (ratio >3.0) were obtained. Of these 180 clones, only 30 of them are contrastive between both parentals. Progeny have been processed for digestion/ligation, and they will be amplified to hybridize with contrastive clones.

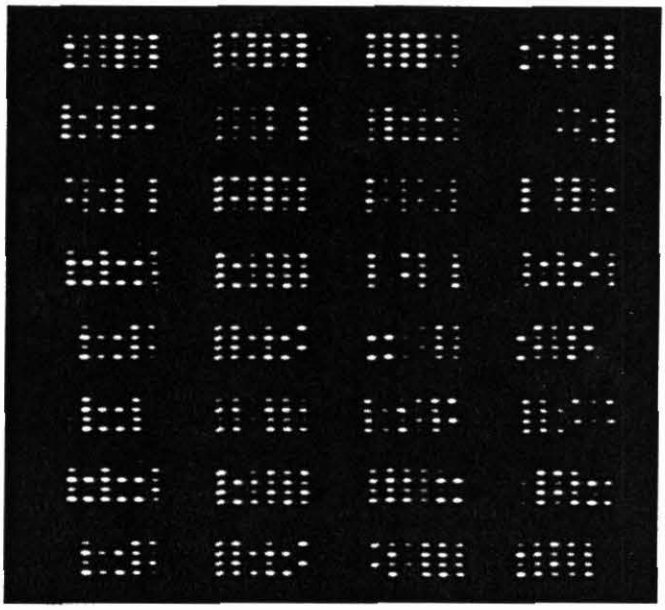

Fig 1. Hibridization pattern on a set of clones using DOR364 and G19833 parentals Labelled with Cy3 and Cy5.

## Ongoing Activities

- Hybridize polymorphic clones with a diversity panel
- Hybridize contratingclones with the progeny of DOR364*G19833
- Sequence the 30 contrasting clones to determine the level of duplicates
- Construct new libraries using different enzymes and two selective bases to increase the level of complexity

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## 1.3.10 Application of the Diversity Array Technology (DarT) in genepool characterization and marker assisted selection for Cassava improvement

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### Introduction

Recognizing that the length of time required to develop new cassava varieties through conventional plant breeding methods (up to 10 years) is a major bottleneck in the development and delivery of new varieties of this food security crop to farmers, CIAT has over the years been developing and disseminating molecular genetic tools to add markers assisted selection (MAS). The RFLP-based molecular genetic framework map of cassava (Fregene *et al.*, 1997) marked a major step in making cassava varietal development more efficient. However since most of the cassava breeding in the tropics is carried out by resource poor NARS in Asia, Africa and South America, the application of this map was limited on account of the prohibitive cost of the facilities required to gainfully and safely apply such a potentially hazardous radioactivity based protocol. In recognition of this, CIAT set out to provide Cassava Scientists with a fast and robust method to assess genetic diversity and conduct large scale mapping experiments with a significant reduction in costs and time through the development of efficient, cheap and easy to use PCR-based markers. A measure of success has been recorded in this with the development and deployment of about 500 microsatellite or simple sequence repeat (SSR) markers Chavarriaga *et al.*, 1998; Mba *et al.*, 2001; Fregene *et al.*, unpublished data; Mba *et al.*, unpublished data). Germplasm screening using these SSR markers is essentially by way of silver staining the amplification products on polyacrylamide gels or visualizing the PCR products on ethidium bromide stained agarose gels. While this will undoubtedly for a long time remain the marker of choice for these NARS on account of the ease of adoption it fails to address the need for high throughput.

In furtherance of CIAT's commitment to the development of cheap and reliable molecular tools for use in high throughput genetic fingerprinting of cassava germplasm collections, genetic fine mapping, gene tagging and molecular marker assisted (MAS) breeding, our Unit has begun applying the Diversity Array Technology (DArT) to eliminate this identified inherent shortcoming of lack of throughput protocols in the use of SSR markers. DArT is one of several applications of the novel DNA microarray technology platform that employs the solid state, open platform method for detecting DNA polymorphisms such as SNPs. In addition, this method is sequence-independent and requires only a minimal amount of DNA. The simplicity and low-cost of the technology strongly recommends it for use in tropical orphan crops like cassava where comprehensive genome sequences are definitely lacking and may not be available any time soon. The low-cost high-throughput technology of DArT, in addition to its obvious use in germplasm characterization, genetic mapping and gene tagging and MAS, can also be used in tracking genome methylation changes while the composite variant of the diversity panel permits the resolution of complex genomic samples into individual components. In our laboratory, we have employed the DArT technique to identify whole genomic DNA fragments that discriminate between cassava genotypes, including parents of each of our 2 mapping populations (that used for the framework map and for genetic mapping of cassava white fly resistance). This report describes a proof of concept pilot application of DArT to develop robes in the form of

polymorphic DNA fragments for use in the development of dense maps, cassava germplasm characterization, MAS and in assessing genetic diversity germplasm accessions.

**Methodology**

*Generation of panels*
Whole genomic DNA was extracted from the 4 parents of 2 mapping populations, NGA-2 or TMS 30572 and CM2177, for the $F_1$ mapping population; and ECU72 and MCOL2246, for the whitefly resistance gene mapping population, respectively. From this, 9ng of DNA from each of these 4 genotypes was bulked and digested with EcoRI. The digested bulk DNA was ligated, PCR amplified and purified using QIAGEN PCR cleaning kit. The cleaned PCR products were then used to transform competent bacterial cells. The transformed bacterial cells were incubated and plated out on LB+agar. Positively transformed cells (white colored colonies) were picked and cultured overnight in 384-well plates containing freezing media. These were then PCR amplified and the extension products alcohol precipitated, completely dried down and re-suspended in water. Equal volumes of each of these and spotting buffer (TE 20% glycerol) were introduced into fresh 384-well plates. These were then arrayed in duplicates onto 3 glass slides for each array using MiraiBio's spotter, SPBIO and the slides processed following Jacoud *et al.*, 2001.

*Generation of representations*
In each case, purified cloned amplicons of 2 representations i.e. the 2 parents of the respective mapping populations that had been bulked to generate the panel were labeled with Cy3 and Cy5 dyes, respectively, following usual procedures. The two labeling reactions were subsequently bulked and cleaned using a QIAGEN PCR purification kit.

**Hybridization**

The Cy3 and Cy5 labeled probes were hybridized onto the Diversity Panel overnight in Corning Hybridization chambers using Expresshyb hybridization solution from CLONTECH. These slides were post processed by serially washing in SSC and SDS followed by washes in decreasing concentrations of SSC. The slides were gotten ready for scanning by spin-drying in a tabletop SOIRVALL centrifuge at room temperature

**Scanning and image analyses**

Processed slides were scanned using the Cy3 and Cy5 channels of ChipReader (VIRTEK, Canada) and the images analyzed using ArrayPro4 from Cybernetics, USA. Positive clones, i.e. those with significant Cy3 or Cy5 fluorescence based on the ratio of their median intensities were identified. These values were obtained after normalization of the signals. Figure 1 shows a sample scan image with the Cy3 and Cy5 dyes having the green and red pseudo-colors, respectively. The yellow color represents a situation where both dyes hybridize positively to a clone. Clones were considered positive for Cy3 and Cy5 if they had Cy3/Cy5 ratios of more than 2.0 and less than 0.5, respectively.

**Results and Discussion**

A total of 350 putative polymorphic fragments have been identified as discriminating between the 2 parents of the Cassava $F_1$ mapping population. As a confirmation, a few of these would be used as probes to screen the 2 parents. An expected positive result would then lead to using these as probes on the 150 progenies that make up the mapping population.

**Future activities**

- Confirmation of the polymorphism of the isolated fragments
- Producing panels made up of the mapping population and screening these with the identified fragments as probes.
- Repeating same for the whitefly resistance mapping population
- Using the data for placing the fragments on the map
- Use of the probes for germplasm characterization
- Sharing of the information

**Reference**

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## 1.3.11 Genome location of SSR markers from a cassava cDNA library

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**Introduction**

There has been a concerted effort at the Biotechnology Research Unit (BRU) of CIAT to develop and disseminate molecular genetic tools that would make the development of new cassava varieties and germplasm characterization easier, cheaper and adoptable for cassava scientists especially in the National Agricultural Research Systems (NARS) where most of the cassava improvement work is concentrated. The first step in this direction was the RFLP based molecular genetic framework map of cassava (Fregene et al., 1997). Recognizing the inherent bottlenecks in the use of RFLP as molecular markers, especially by the NARS, a major thrust of the efforts has been directed towards saturating this map with PCR-based molecular genetic markers. RFLP based techniques are expensive, require the use of hazardous radioactive probes that are not available to many resource-poor developing country research programs, and these probes must be physically transferred from one site to another under strict safety protocols. In contrast, PCR-based markers are robust, inexpensive to assay, easily shared among researchers and readily accessible in public and private domains, making this a much more appropriate approach in these countries. With access to a simple text file containing the sequences of the oligonucleotide primers for the PCR-based markers of interest, a breeder can rapidly and efficiently evaluate the germplasm under study.

In this regard, our Unit has developed and made publicly available over 500 simple sequence repeat (SSR) or microsatellite markers (Chavarriaga et al., 19998; Mba et al., 2001; Mba et al., unpublished data; Fregene et al., unpublished data). Of these, a total of 157 were sourced from a cassava root and leaf cDNA library while the rest were from whole genomic libraries. From the latter, 85 have been located on the cassava genome. The present report describes the characterization and initial efforts at the mapping of the 157 SSR-containing cDNA fragments.

## Methodology

*Development of SSR's from a cassava cDNA library*
A cassava cDNA library was constructed commercially by Life Technologies, MD, USA from RNA extracted from leaves and roots tissue (see SB-2 Annual Report, 2000). The sequencing and initial primer designs were also as described for the whole genomic SSR clones using Perkin Elmer's ABI Prism 377 automated sequencer (Mba *et al.*, 2001).

*Parental Survey using the SSR markers*
Each of the SSR primer pairs were used to amplify the relevant loci of the cassava genome in the two parents of the F1 mapping population, TMS 30572(female) and CM2177 (male), respectively. Those that had at least one unique allele in at least one of the parents were used to screen the 150 members of the F1 mapping progeny.

*Mapping of SSR markers*
SSR markers that had a unique allele in either or both parents of the mapping population were used to screen the 150 progenies making up the $F_1$ mapping population. The segregation data of the markers that fitted the expected ratio of 1:1, presence: absence of the unique parental allele were used to place the markers on the framework map using the linkage analysis computer package MAPMAKER 2.0. (Lander *et al.*, 1987). The data analyses followed the same procedures as described for the mapping of the initial 36 SSR markers (Mba *et al.*, 2001).

## Results and Discussion

*Characterization of SSR loci types*
From the approximately 400 putative SSR-containing cDNA clones sequenced, a total of 157 clones contained SSR loci in good enough positions for primer design. Less than 10% redundancy has been observed. Many of these clones contained more than one repeat motif at times in different loci. However, a great majority of the repeat loci were the CT/GA repeat which accounted for over 81% of the SSR-containing clones for PCR primer design. A breakdown of the loci type is given in the table below.

Breakdown of loci types for SSR primers from the cDNA library

| Type | Percentage |
|---|---|
| CT/GA | 79 |
| CA/GT | 12 |
| TA/AT | 8 |
| Others | 5 |

*SSR Parental Survey*
All primer pairs successfully amplified the corresponding SSR loci in the parents of the cassava mapping progeny; even though with different $MgCl_2$ concentrations, and 2 annealing

temperatures, 55°C and 45°C respectively. In all, about 45 % of all SSR markers tested in the parents, revealed a unique allele in at least one of the parents while less than 20% showed a unique allele for both parents. A pattern of SSR polymorphism between the two parental loci is shown in Figure below.

*Genome location of SSR markers*

Figure 2 shows the map positions of 92 SSR loci from the SSR markers analyzed to date on the male- and female-derived molecular genetic map. The segregation data on the 150 members of the $F_1$ mapping population of another 47 SSR markers sourced from the cDNA library are currently being placed on the cassava map. Linkage group nomenclature is as described for the molecular genetic map of cassava by Fregene *et al.*(1997) except for groups L, O, and P that have now been merged with other groups. The SSR markers reveal a complete spread over the cassava genome – at least one marker being placed on all but one of the eighteen linkage groups. The existence of markers with unique alleles in both of the parents or "allelic bridges" (Ritter *et al.* 1991) will assist in the construction of a consensus map of analogous male- and female- derived linkage groups for the cassava genome.

**On-going activities**

Completion of the mapping of the rest of the SSR markers from the cDNA library.
Further dissemination of the SSR marker technology to international and national programs and other collaborators.
The use of the microarray Diversity Array Technology (DArT) to develop polymorphic DNA fragments to further saturate the map and for use in high throughput cassava genome characterization.

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## 1.3.12 Annotation of SAGE tags (Transcripts) differentially expressed in CMD resistant and susceptible genotypes

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### Introduction

The objective of the serial analysis of gene expression (SAGE) of CMD resistance in cassava is to identify candidate genes that are expressed in the *CMD2* – mediated response to the cassava mosaic virus (CMV). These candidate genes may be the novel dominant gene or genes expressed down stream that maybe the molecular basis of resistance. SAGE of CMD resistant and susceptible progeny from an $F_1$ mapping progeny yielded 12,700 15bp tags (representing expressed genes), divided into 5733 and 7053 tags for the resistant and susceptible genotypes respectively (CIAT 2000; Fregene et. al. 2001 in preparation). One hundred and seventy five transcripts were expressed 3 to12 times more in the resistant bulk compared 94 transcripts found 3-5 times in the susceptible bulk.

The next step of the SAGE experiment is to identify the transcripts represented by the 15bp tags, a task complicated by the scanty EST data available for cassava. A PCR approach was therefore employed to amplify longer fragments using the 15bp tags as primers and a cDNA library from resistant genotypes as template. At the same time an EST project was initiated in collaboration with the Iwate Biotech Research Center, Kitakami, Japan.

### Methodology

A cDNA library had earlier been constructed in pYES (Invitrogen Inc.) using mRNA from the CMD resistant bulk (Fregene and Terauchi 2000 unpublished data). The 13 or 15 bp SAGE tags served as forward primers and pYES vector sequences as reverse primer in PCR of a dilution of the cDNA library. PCR primers were synthesized for 28 tags expressed four times or more in the resistant bulk compared to the susceptible. PCR reaction conditions was 10 mM Tris-HCl, 50 mM KCl, 2.5mM $Mgcl_2$, 200µM each dNTPs,10pmol of the forward and reverse primer, and 2.5U of "hot star" Taq polymerase (Qiagen Gmbh) in a 50µl volume. DNA template was 1µl of a 10X dilutions of the cDNA library from the CMD resistant bulk. Thermal cycling conditions were 35 cycles of 95°C 4min, 94°C 30sec, 45°C 1min, 70°C 1min, and a final extension cycle of 70°C for 5min. PCR product was cloned into the PGEMT vector (Promega Inc.) transformed into *E.Coli* by electroporation and sequencing was off the purified plasmid template using the T7 forward primer.

To identify associations between differentially expressed genes and the dominant CMD resistance gene, cloned fragments from the tag PCR amplification were screened as RFLP probes in Southern blots of DNA of the parents and bulks of the CMD mapping progeny using 4 restriction enzymes namely: *Eco*RI, *Eco*RV, *Hind*III, and *Hae*III. DNA isolation, filter preparation and Southern hybridization were as described by Fregene et. al. (1997). Transcripts found to be polymorphic in the parental survey were analyzed in the 80 individuals of the resistant and susceptible bulks and if found polymorphic, will be analyzed in a large mapping progeny of about 2500 individuals to identify the precise position relative to the CMD resistance gene.

For EST generation, 2ul of the cDNA library was electroporated into 40ul of *E.Coli* HB101 cells (Gibco BRL) and plated on LB agar plates + ampicillin (100ug/ml). A total of 5,000 colonies were picked into 70ul of LB media + ampicillin (100ug/ml) in 384 well plates. Plasmid isolation was using the MONTAGE 96-well plate system (Millipore Inc), 4 96-well plates or 384 clones were processed at a time. PCR sequence reaction was with the 3' end primer designed from pYES (Invitrogen Inc.) and the big dye terminator kit (Applied Biosystems) on a 9600 Perkin Elmer Machine or an MJ Research DNA engine (Tetrad). The sequence reaction was cleaned using the multi screen 96-well plate format (Millipore Inc.) and analyzed on a Shimadzu RISA 384-capillary sequencing machine. A target of 3000 ESTs have been set for tag annotation.

**Results**

Of the 28 tag PCR, 24 gave good PCR products that could be cloned and sequenced, and 18 of these gave good and long enough sequence for BLAST (Atschul 1990) sequence similarity searches. Most of the tags were about 150-300bp long and the tag primer was found for 16 of the 18 sequenced tags, about 40bp from the 5' end. The putative identities of these transcripts and their tag primers are shown in Table 1. Parental and bulk filters were screened with all 24 tag PCR products. Results revealed polymorphism in 2 of the tags, 25 and 11. Tag 25 is a transcript showing homology to WPKY transcription factor, while tag 11 shows similarity to a bHLH transcription factor GBOF-1. These tags are being analyzed further in a larger population.

So far tag annotation has identified genes known to be involved in systemic acquired resistance (SAR) response to disease in plants. They include a WRKY transcription factor, catalases, a pectin-esterase and reductases. Other genes were also found implicated in plant response to disease but are part of the cell mechanism known to aid virus replication including elongation factor alpha-1. Elongation factor 1-alpha (EF1alpha), is an essential component of the translation machinery that delivers aminoacyl-tRNA to ribosomes. Virus proteins such as HIV-1 Gag polyprotein that play key functions at almost all stages of the viral and the conserved 3'-terminal stem-loop (3' SL) of the West Nile virus can bind to EF1alpha and incorporate it into the virus replication machinery (Blackwell and Brinton 1997; Cimarelli and Luban J 1999).

The 3' end sequencing of cDNA clones is ongoing and is expected to provide 3000 ESTs for tag annotation. Homology with known genes and proteins deposited in public data bases are being sought for as sequences are produced. Preliminary results reveal that that transcripts known to be abundant in cells such as ribosomal and chloroplast sequences constitute only about 10% of all sequences suggesting the cDNA library provides a good representation of genes expressed. Sequence data generated will be also be submitted to the Gene Bank.

**Future Plan**

- Annotate many more tags using 3' end ESTs
- Determine the function of some differentially expressed genes by over-expressing them in susceptible cassava plants and challenging them with infectious ACMV clones.

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**Table 1. BLAST sequence homology of 28 differentially expressed transcripts in bulks of CMD resistant and susceptible cassava genotypes.**

| Tag No. | Sequence | No. of transripts in resistant bulk | No. of transcripts in resistant bulk | BLAST homology of amplified cDNA fragment |
|---|---|---|---|---|
| 1 | CTAGAATGACCTTGT | 12 | 1 | Cytoskeleton related protein |
| 2 | CTAGCGCCAGACAGT | 11 | 3 | Elongation factor 1-alpha |
| 3 | CTAGCTCTGTGTATC | 8 | 2 | N/A |
| 4 | CTAGCAAAGCAGCGC | 7 | 0 | Pectin-esterase |
| 5 | CTAGGAAACAATCCT | 7 | 1 | Photosystem I chain II precursor |
| 6 | CTAGTACACAATGTA | 7 | 1 | N/A |
| 7 | CTAGCTCGCCGTAAG | 6 | 0 | Histone |
| 8 | CTAGTTAATATGGTA | 6 | 1 | N/A |
| 9 | CTAGTTCAAAGGAAG | 6 | 1 | Ribosomal protein |
| 10 | CTAGTTAAAATGTGA | 5 | 0 | Catalase |
| 11 | CTAGAGCTTTTCACT | 5 | 1 | bHLH transcription factor GBOF-1 |
| 12 | CTAGCCGGATCTCCT | 5 | 1 | N/A |
| 13 | CTAGCGATTAAAAAA | 5 | 1 | Rubredoxin |
| 14 | CTAGTGGAGCAATAC | 5 | 1 | DNA binding regulatory protein |
| 15 | CTAGTTGCTTTGCAC | 5 | 1 | Initiation factor 3k (A. thaliana) |
| 16 | CTAGAAGTGGTGCTT | 4 | 0 | Nuclear import protein |
| 17 | CTAGACTGAAGTCAG | 4 | 0 | Hypothetical protein (A. thaliana) |
| 18 | CTAGAGCACGAGT | 4 | 0 | Ubiquinol--cytochrome c reductase |
| 19 | CTAGATAATAAAAGG | 4 | 0 | N/A |
| 20 | CTAGATCCTTGCCTT | 4 | 0 | No significant similarity |
| 21 | CTAGGCAGGATCAAG | 4 | 0 | N/A |
| 22 | CTAGCTGAATTATAG | 4 | 0 | N/A |
| 23 | CTAGGCAGCCGCCGC | 4 | 0 | Catalase |
| 24 | CTAGGGATTATTCAT | 4 | 0 | N/A |
| 25 | CTAGGTGGACGAGAC | 4 | 0 | Transcription factor WRKY |
| 26 | CTAGTAATCGCTCAG | 4 | 0 | 40S ribosomal protein |
| 27 | CTAGTTGGATCTT | 4 | 0 | N/A |
| 28 | CTAGTTGGATTCTTT | 4 | 0 | N/A |

N/A cDNA sequence data not yet available

## 1.3.13 A comparison of marker assisted selection (MAS) and conventional selection for the rapid deployment of the novel CMD resistance gene (*CMD2*) in cassava gene pools.

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### Introduction

The principal objective of the project "Molecular Mapping of Genes Conferring Resistance to the Cassava Mosaic Disease (CMD) in African Cassava Germplasm" funded by the Rockefeller foundation is to identify markers tightly linked to different sources of CMD resistance for efficient and cost-effective deployment of resistance genes. Molecular markers linked to a novel and high level of resistance, designated *CMD2* (Akano et. al. 2001), have been identified. The effectiveness of *CMD2* against a wide spectrum of strains of the virus in sub Saharan Africa, including the aggressive Ugandan variant (UgV) (see section 1.4, this report), makes its deployment very appealing in protecting cassava production against the ravages of CMD both in Africa and Latin America. Of urgent importance is containing the rapidly advancing front of UgV that has now reached the Democratic Republic of Congo, Kenya, and Tanzania.

Conventional breeding for CMD resistance involves at least 4 cycles of selection for resistance at the seedling, clonal and preliminary and advanced yield trial stages. Disease pressure, which may vary from year to year, may lead to escapes that are carried along, in certain cases, up to the third cycle. Genetic markers for CMD resistance enables the elimination of susceptible genotypes at the seedling stage and reduces significantly, 50% in the case of *CMD2*, the materials to be evaluated in the field at the crucial single row (clonal) trial stage, where more than 95% of genotypes are eliminated. Markers are even more important when two or more genes/traits are involved, the reduction in progenies to be evaluated becomes even higher. In many cassava production scenarios, CMD resistance has to go hand-in-hand with cassava bacterial blight (CBB) resistance.

A MAS project for CMD resistance was therefore initiated with IITA to enable us to test the fidelity of CMD markers developed at CIAT, a very convenient approach considering that phenotypic and molecular data can be obtained at the same time and compared. The MAS project is also necessary to work out the details for routinely using these markers in cassava breeding.

### Methodology

The original group of CMD resistant land races for the study consists of TME3, TME4, TME28, and TME9. TME28 was dropped out due to very poor flowering, while seeds obtained from crosses with TME4 are not considered here due to its very close genetic similarity with TME3. Only crosses to TME3 and TME9, crossed to elite IITA parental lines, TMS30572, TMS91934, and TME117, a land race favored for his good cooking roots, are reported here. TMS30572 is moderately resistant to CMD, while the other two are susceptible. TMS91934 has a very high level of resistance to the all known strains of CBB (Verdier 1999, pers communication) and can serve as a source of CBB resistance in progenies of the highly CBB susceptible land races TME3 and TME9. The seedling nursery and field establishment were at the IITA sub-station in Mokwa located in the Guinea Savannah agro-ecology of Nigeria. This site is characterized by low CMD disease pressure and ideal for the multiplication of cuttings from CMD susceptible lines.

Before transplanting, 2 young leaves were harvested from each genotypes for DNA isolation and SSR analysis. The leaves were bagged in small plastic sample bags and carried on ice to IITA head quarters, Ibadan for DNA isolation. DNA isolation was by a miniprep isolation procedure of the Dellaporta et. al. (1983) protocol using 100-200mg of fresh leaf tissue and a twenty fold reduction in volumes of the isolation buffers and reagents. DNA isolated was shipped to CIAT head quarters for SSR marker analysis. DNA was not quantified for marker analysis, 5ul of a 10X dilution was used in PCR reactions. All the genotypes were analyzed with SSR markers tightly linked to *CMD2*, SSR118 and NS158. SSR marker analysis were as described by Akano et. al. (2001).

**Results**

Sexual seeds obtained from crosses to the CMD resistant land races and plantlets, transplanted to the field are summarized in Table1, a total of 2490 genotypes are currently growing in the field. Harvesting of two young leaves from plantlets just before transplanting was initially thought to be stressful to the young plants, however more than 99% of transplanted genotypes survived and only 2 were lost. This is an important observation as it suggests that molecular assisted selection can be done even while the plants are in the seedling nursery.

DNA was successfully isolated from all 2488 genotypes and the parental lines. The SSR markers, SSRY28 and NS158 have been analyzed in close to half of the genotypes and marker analysis is still ongoing. The large number of plants obtained from crosses from both TME3 and TME9 makes them ideal not only for making comparisons between MAS and conventional selection, and for marker-fidelity studies, but also for fine mapping markers linked to CMD resistance. Great care was therefore exercised in relating plants in the field to raw SSR data, a special template was set up in microsoft excel for this purpose.

Once molecular marker data becomes available for all genotypes, those with marker alleles linked to *CMD2* will be selected and five 20cm long woody stakes harvested for field establishment in a high CMD pressure location, in this case, IITA Ibadan. CMD resistance will be evaluated at 3 and 6 months after planting to confirm the results of marker analysis and for comparison with an unselected population of all genotypes also to be established at IITA, Ibadan.

**Future Plan**

- Evaluate all 2490 genotypes for CMD resistance under heavy disease pressure.
- Compare selection efficiency of marker-aided selection against phenotypic selection

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**Table 1. Summary of seeds and plants obtained from reciprocal crosses of TME3 and TME 9**

| Family name | Female | Male | Seeds harvested | Plants in field | Total plants in field |
|---|---|---|---|---|---|
| M1 | TME 3 | TME 117 | 36 | 18 | |
| M2 | TME 117 | TME 3 | 220 | 95 | 113 |
| M5 | TME 3 | 91934 | 103 | 49 | |
| M6 | 91934 | TME 3 | 60 | 12 | 61 |
| M7 | TME 3 | 30572 | 70 | 49 | |
| M8 | 30572 | TME 3 | 846 | 791 | 840 |
| M17 | TME 9 | TME 117 | 368 | 309 | |
| M18 | TME 117 | TME 9 | 174 | 107 | 416 |
| M21 | TME 9 | 91934 | 370 | 282 | |
| M22 | 91934 | TME 9 | 27 | 12 | 294 |
| M23 | TME 9 | 30572 | 264 | 214 | |
| M24 | 30572 | TME 9 | 700 | 552 | 766 |
| | | | | **Grand Total** | 2490 |

## 1.3.14 cDNA-AFLP analysis of differential gene expression in the Cassava - *Xanthomonasaxonopodis* pv. *manihotis* interaction

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### Introduction

The cassava bacterial blight (CBB) is a major disease, endemic in Latin America and Africa causing serious damage to cassava and resulting in severe yield losses. The causal agent is *Xanthomonas axonopodis* pv. *manihotis* (*Xam*). The most appropriate and realistic approach for controlling CBB is through host resistance. Resistance to CBB operates from the vascular system, and seems to be polygenic and additively inherited (Hahn, 1978). However, no resistance genes have been identified.

Plants in general, have a wide spectrum of cellular and molecular defenses including cell wall fortification, phytoalexins production and development of a hypersensitive response (HR), characterized by a necrotic reaction surrounding the invasion area to restrict pathogen expansion (Baker *et al.*, 1997, Hammond-Kosack & Jones, 1996, Culver & Dawson, 1991). These defense reactions are directly or indirectly activated by resistance genes. Understanding how these genes are involved in the recognition and response against pathogen attack will allow us get into the manipulation of the resistance against a wide range of pathogens.

The objectives of the study are to implement the cDNA-AFLP technique to identify differentially expressed bands between two different cassava cultivars, one resistant and one susceptible to CBB (MBRA 685 and MCOL 1522, respectively). We analyzed the pattern of cDNA-AFLP at different times post inoculation with a *Xam* strain and identified putative molecular disease resistance markers.

**Material and Methods**

*Sample preparation and cDNA synthesis*
We compared a resistant (MBRA 685) and a susceptible (MCOL 1522) variety for differential gene expressions over time after inoculation with *Xam* isolate CIO 151. Young plants were inoculated by stem puncture. Stem tissues were collected at 24 and 72 hours post inoculation (pi), 7, 15 and 30 days pi. The controls were healthy non-inoculated plants and plants inoculated with sterile water. The tissue was grounded in liquid nitrogen and total RNA was isolated using the Proteinase K method (Hall *et al.*, in Rocha, 1995). Poly (A) RNA was isolated using oligo (dT) coupled to DynaDeads (DYNAL). cDNA was synthesized using oligo (dT) primer and SuperScript II reverse transcriptase (GIBCO BRL) from 400-500 ng of mRNA, as starting material.

*cDNA-AFLP profiling analysis*
The template for cDNA-AFLP was prepared according to Bachem *et al.* (1996) using *EcoRI* and *MseI* restriction enzymes and adapters. Preamplification was carried out with one *EcoRI* and one *MseI* single chain adaptor with no (0) or one (1) selective bases. This product was checked on agarose gels and a 1/30 dilution was used for subsequent amplifications. These were done with a pair of primers with 2 or 3 selective bases (GIBCO primers and Plant AFLP Kit, GIBCO, respectively). Selective amplification products were separated on a 6% polyacrylamide gel run at 100W, 50°C for 2 and a half hours. It was then processed with the silver staining technique (Promega).

*Isolation, cloning and sequencing of target cDNA bands*
Bands of interest were marked, cut and eluted in ddHO. AFLP fragments were reamplified by PCR, ligated to pGEM®-Teasy (Promega) and sequenced using the automated sequencer (ABI Prism 377). The sequences were edited using Sequencher 3.0 (Gene Codes Corporation) and compared with the GenBank databases using BLASTx and BLASTn.

**Results and Discussion**

The cDNA-AFLP technique was successfully implemented using RNA isolated from stem tissue from cassava plants. We evaluated 32 and 40 combinations of AFLP primers with two (2) and three (3) selective bases, respectively. cDNA-AFLP profiles showed 40 to 70 bands per primer combination, ranging between 100 and 1500 base pair (bp), with ~3600 fragments screened (Figure 1). Differential expression was observed for 353 fragments putatively induced by the pathogen in the resistant variety, with an average of ~5 bands per combination. These fragments ranged between 130 to 650 bp. The more informative primer combinations were E-AA/M-AG, E-AA/M-CT, E-ACG/M-CTT and E-ACT/M-CTT with 11, 10, 14 and 16 differential bands, respectively.

We sequenced 201 bands and compared their homology with GenBank databases. Significant homologies with known genes or putative genes have been found for 149 sequences. 37 of these showed homology with plant resistance or defense related proteins or other type of plant proteins (Table 1). Sequences similar to the resistance genes Cf-2 and I2 from tomato were found, and

others with homology to putative resistance proteins. These fragments are expressed at 24 hpi in the resistant variety, indicating that they are induced by the pathogen or that their expression increased in the presence of the bacteria. This is in contrast with the hypothesis that resistance genes are constitutively expressed in plant cells (Hammond-Kosack & Jones, 1996, Staskawicz *et al.*, 1995, Lamb, 1994, Keen, 1990, Gabriel & Rolfe, 1990). Some of these sequences showed expression in the susceptible variety also, but later after the infection (15 dpi, bands E6, E18 and E45, Table 1), suggesting that these putative genes are present in both genotypes but expressed early in the resistant variety.

Figure 1.
AFLP Polyacrylamide gel electrophoresis stained with silver nitrate. Primer combination E-ACA/M-CAG.

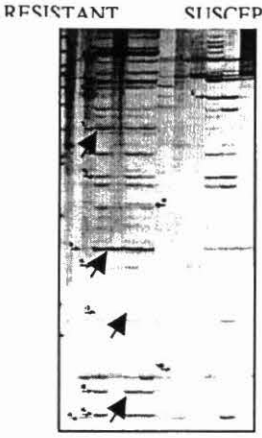


Table 1.
Nucleotide homology and probabilities from BLASTx search in GenBank of the differentially expressed cDNA-AFLP bands.

| Band | Bp. | Best Match | E Value | Expression |
|---|---|---|---|---|
| *Defense Related Proteins* | | | | |
| E6 | 213 | Disease resistance Cf-2 like protein (*L. esculentum*) | 2e-05 | 24 hpi |
| E45 | 260 | Resistance protein I2 (*L. esculentum*) | 2e-08 | 24 hpi |
| M62 | 830 | Putative resistance protein (*A. Thaliana*) | 3e-24 | 24 hpi |
| E18 | 171 | Resistance gene analog protein (*L. sativa*) | 1e-05 | 24 hpi |
| M56 | 350 | Putative receptor protein (*A. thaliana*) | 2e-14 | 24 hpi |
| M63 | 360 | Putative translation initiation factor IF-2 | 7e-24 | 24 hpi |
| E1 | 218 | Kinase protein SNF1 (*Cucumis sativus*) | | |

Fragments E6 and E45 showed significant homology with leucine rich repeats (LRR) from Cf2 and I2 genes (type LRR and NBS-LRR, respectively). The LRR motif in resistance genes is involved in protein-protein interactions and acts in the specific recognition of avirulence genes from pathogens (Staskawicz *et al.*, 1995). The Cf gene family recognizes the fungus *Cladosporium fulvum* and activates the defense response in several tomato species (Dixon *et al.*, 1996). The I2 gene family confers resistance to several species of *Fusarium sp.* and shares structural characteristics with NBS-LRR resistance genes (Ori *et al.*, 1997).

The fragment E18 showed homology with a putative resistance protein in lettuce. This putative gene has structural elements characteristic from NBS (kinase V motive and P-loop) and LRR (Shen *et al.*, 1998). Other fragments showed similarity with Serine/Threonine or receptor protein kinases that belong to a different type of resistance genes that modulate the phosphorylation of other proteins. They are involved in the signal transduction cascades that activate defense responses in plants. These results indicate that through the cDNA-AFLP technique we have isolated resistance and defense related fragments induced by *Xam*, corresponding to three different types of resistance genes in plants.

We also found significant homology with transcription factors that act in the last part of the signal transduction pathway that leads to the activation of defense related genes (Dixon *et al.*, 1994). Several fragments showed homology to senescence, apoptosis and dormancy associated proteins suggesting that they might be involved in the programmed cell death mechanism included on the hypersensitive response. This defense reaction, very common in plants, creates a toxic media for

the establishment and expansion of the invading pathogen (Hammond-Kosack & Jones, 1996, Staskawicz *et al.*, 1995).

Another 52 fragments did not show significant homology to known sequences in the databases. These were differentially expressed since 24 hpi indicating that were also induced by the pathogen, and might represent novel sequences in cassava putatively associated with resistance to *Xam*.

**Future Plans**

We are focusing our attention on those fragments that showed significant homology with known genes and those that are strongly induced to confirm their differential expression by Northern blot analysis.

These fragments will be used as probes to hybridize a cDNA library to isolate full-length clones. This information can be used to develop markers associated to resistance.

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## 1.3.15 Study of gene expression during pathogenesis of *Xanthomonas axonopodis* pv. *manihotis* using an AFLP-based microarray

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SB-2 Project

### Introduction

Systematic profiling of genes that are specifically expressed by a pathogenic bacterium in its host plant would assist in understanding basic virulence mechanisms (Okinaka et al., 2001). *Xanthomonas axonopodis* pv. *manihotis* is a plant pathogenic bacterium, causal agent of cassava bacterial blight. A plasmidic sequence containing a pathogenicity gene, *pth*B, has been described previously (Verdier *et al.*, 1996). However, others sequences should be involved into the pathogenesis process. By now, no new sequences have been reported. The elucidation of those bacterial expressed genes in infection process is a hard task when the entire genome sequence is not available.

Recent advances in functional genomic technologies such as DNA microarrays have provided a unique way to monitor gene expression on a genomic scale and under different conditions. The majority of microarrays have been constructed from organisms for which the whole genome sequence is known or from organism that have an important collection of ESTs.

We are constructing a *Xam* microarray based on the AFLP amplification of the *Xam* genome in order to identify genes implicated in pathogenesis and to study the global changes in gene expression associated with the process of infection of *Xam* during its interaction with cassava plants.

### Methodology

*AFLP libraries*
AFLP was carried out as described previously (Restrepo et al., 1999), using 250ng of *Xam* genomic DNA from strain Cio-46. The PCR was performed using the *Eco*RI + C and *Mse*I + A primer combination and the PCR product was cloned into pGEMT-Easy. Another library using AFLP adaptors as primers for the PCR amplification was constructed but only the fragments above 300 bp were eluted from the acrylamide gel and reamplified. The reamplified products were cloned. Plasmids obtained from the two libraries were introduced to *E. coli* by electroporation. Bacteria were grown overnight in freezing medium and 5µl of a 1:10 dilution was used for the insert amplification using T7 and SP6 primers.

PCR products were precipitated with isopropanol and resuspended in TE (10mM Tris; 1mM EDTA) and 50% DMSO. An aliquot of 2µl was used to confirm the amplification in a 1% agarose gel. Twenty µl of the PCR resuspended in the TE-DMSO mix were transferred into 384-well plates for slide printing.

*cDNA-AFLP library*
In another experiment, *Xam* strain Cio-46 was used to inoculate the susceptible cassava variety MCOL1522. Inoculations were performed as previously described (Restrepo *et al.*, 1997) by stem puncture with one bacterial colony. Stems (2 cm around the inoculation site) were collected 24,

48 hours and 5, 7 and 15 days after inoculation. Tissue was cut into small slices and resulting fragments were placed in DEPC-treated water. Sample was vigorously vortexed, supernatant was recovered and centrifuged for 5 min at 8000g. Bacterial pellet was resuspended in RLT buffer and RNA was extracted using the RNeasy plant mini kit (Qiagen, Valencia, CA). cDNA synthesis was performed using random primers and AFLP was conducted using the AFLP analysis system fro microorganisms (GIBCO). cDNA was also synthesized from ARN extracted from bacteria grown *in-vitro* in liquid medium. Nine primer combinations were assayed for the second AFLP amplification.
After the second AFLP amplification, the product was separated in a polyacrylamide gel (6%). Differential bands present in the inoculated bacteria and absent in the bacteria grown *in-vitro* were eluted from the gel, re-amplified and cloned as described above. A total of 118 clones were sequenced using the automated sequencer (ABI Prism 377). The sequences were edited using Sequencher 3.0 (Gene Codes Corporation) and compared with the GenBank databases using BLASTx and BLASTn.

*Slide printing and blocking*
Each DNA fragment was arrayed (arrayer SPBIO ver1.54, MiraiBIO, Inc) with four replicates onto glass slides coated with aminopropyltriethoxisilane (Sigma). The spacing between spots was 0.3mm. Slides were placed on a dessicant chamber wrapped into aluminum foil until use.
A set of control genes was printed in the slide. Controls were cassava housekeeping genes, *Xam* ribosomal genes, *pth*B, a *Xam* pathogenicity gene, N52, an internal sequence of the *pth*B gene and a repetitive sequence present in the *Xam* chromosome.
Two days after arraying, slides were processed. Slides were baked at 96C on hot plate for 1min. They were then crosslinked using a UV stratalinker at 650µJ. Slides were treated with amine blocking solution (1.5g of succinic anhydre in 250ml 1-methyl-2-pyrrolidinone and 250ml of 0.2M boric acid pH8) for 15 min, gently shaking at room temperature. Then, slides were placed in boiling distilled water for 2 min to denature DNA, soaked in 100% ethanol and dried by centrifugation (1 min at 1000rpm).

**Results**

From the AFLP-derived libraries, 768 clones were obtained (384 clones from each library). These clones were all printed in the array.
From the cDNA-AFLP-derived library, fragments varied in size from 50 to 800bp. For each primer combination, 20 to 80 bands were obtained. From 6 to 10 clones were picked for each differential band eluted from the gel. A total of 118 clones were sequenced, most of them showed homology to ribosomal genes. The 17 remaining (not showing a homology to ribosomal genes) did not presented an homoloy when compared with sequences in the Genbank We selected all clones from these 17 sequences to be arrayed in the *Xam* microarray.

**Future plans**

To hybridize *Xam* microarrays with ARN or cDNA obtained from *Xam* collected at different time points after inoculation and ARN from bacteria grown *in-vitro.*

The clones showing differential expression will be sequenced to identify genes involved in pathogenesis.

The clones, identified and sequenced, will be used as probes against northern blots to confirm their differential expression.

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## 1.3.16 Gene expression in cassava stems in response to infection by *Xanthomonas axonopodis* pv. *manihotis*

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### Introduction

The factors controlling the outcome of host-pathogen interactions where there is no obvious hypersensitive response are yet not well understood. This is the case for cassava bacterial blight, caused by *Xanthomonas axonopodis* pv. *manihotis* (*Xam*). *Xam* is a foliar and a vascular pathogen. No mechanism has been observed to limit the multiplication and development of the bacteria in the mesophyll of resistant cultivars during the foliar phase and intercellular multiplication in the mesophyll (Boher and Verdier, 1995). However defense mechanisms against the pathogen have been shown in the vascular system of infected cassava plants (Kpémoua *et al.*, 1996).

In order to establish which genes are involved in the expression of resistance of cassava (*Manihot esculenta* Crantz) to *Xam* infection, we used DNA microarrays containing 3872 *Arabidopsis thaliana* stress-related ESTs.

### Methodology

*Plant inoculations, RNA isolation and cDNA synthesis*

Four weeks old plants (variety MBRA 685) were inoculated by stem puncture with *Xam* isolate CIO 151. Stem tissues were collected at 24 and 72 hours post inoculation (pi), 7, 15 and 30 days pi. The controls were healthy non-inoculated plants. Tissue was grounded in liquid nitrogen and total RNA was isolated using the Proteinase K method (Hall *et al.*, in Rocha, 1995). Poly (A) RNA was isolated using oligo (dT) coupled to DynaDeads (DYNAL). cDNA was synthesized using oligo (dT) primer and SuperScript II reverse transcriptase (GIBCO BRL) from 400-500 ng of mRNA, as starting material.

*Preparation of labeled probes*

Fluorescence-labeled probes were prepared from cDNA. Each reaction (6µl) consisted of 2-3µg of cDNA, 3µg Random primers, 2mM each of dATP, dCTP, and dGTP, 0.65 mM dTTP, 2nmol of either Cy3-dUTP or Cy5-dUTP, and 10U of Klenow fragment in 1X reaction buffer. cDNA from healthy plants was labeled with Cy3 and a pool of cDNA from all time points after inoculation was labeled with Cy5. The labeling reaction proceeded for 1 hour at 37°C. After incubation, reactions of the two samples were combined and purified using the Qiaquick kit (Qiagen, Valencia, CA). The sample was then dried until 5µl were left and resuspended in 26µl of CLONTECH ExpressHyb buffer (with 40ng of salmon sperm DNA). The probe was denatured at 95°C for 5 min and applied to the microarray.

*Microarray hibridization and data analysis*

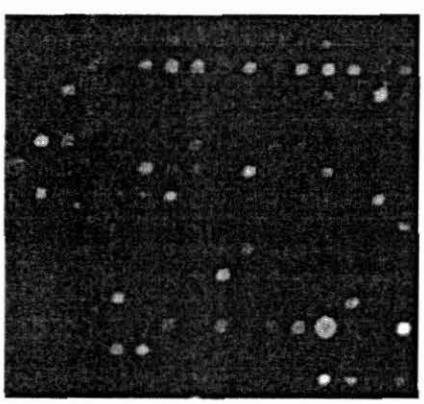
Hybridizations were performed overnight at 60°C in humidified chambers. The slides were sequentially washed in the following solutions: 1 X SSC and 0.1% SDS for 10 min, 1X SSC twice for 1min, 0.2X SSC twice for 2 min and 0.02 X SSC for 5 to 10 sec. Slides were dried by centrifugation.

After hybridization and washing, microarrays were scanned with Virtek chipreader®. Spots representing the arrayed genes were identified, and distinguished and analyzed using the Spotfinder and Arrayviewer softwares from TIGR (The Institute for Genomic Research). The average (integral) fluorescence intensity for each fluor and each gene was determined and background fluorescence was calculated as the median fluorescence signal of non-target pixels around each gene spot. Missing spots, spots with low signal intensity, and spots in high background areas were flagged and excluded from the analysis. Normalization between the Cy3 and Cy5 fluorescent dye emission channels was performed using the total intensity for each channel, based on the assumption that under the conditions being tested, most genes will not change in expression. In this experiment, we defined induction or repression of a gene as a minimum 2.5-fold change in its transcript level.

**Results**

Microarrays were used to study gene expression quantitatively after infection of cassava stems with *Xam.* Figure 1 shows an image of the microarray after hybridization.

**Figure 1: Results of gene expression experiments using *Arabidopsis* microarrays and cDNA obtained from cassava stems, healthy (Cy3) and inoculated (Cy5) as probes.**



Analysis of data revealed that 20 spots were not flagged as bad spots by the Spotfinder software and of these, 15 ESTs on the microarray showed significant differential expression in response to the infection of cassava stems with *Xam.* The position of these spots in the array, the Cy3 and Cy5 intensities after normalization and the Cy5/Cy3 ratio is shown in Table 1.

**Table 1. List of *A. thaliana* ESTs showing a differential expression after hybridization with labeled cDNA obtained from healthy (Cy 3) and inoculated (Cy5) cassava stems. Their position in the slide and total intensity obtained after scanning both fluors (Cy3 and Cy5) as well as the Cy5/Cy3 ratio are shown.**

| Row | Column | Metarow | Metacolumn | Subrow | Subcolumn | Cy3 | Cy5 | Cy5/Cy3 |
|---|---|---|---|---|---|---|---|---|
| 1 | 83 | 1 | 4 | 1 | 17 | 13468 | 45156 | 3.35 |
| 6 | 76 | 1 | 4 | 6 | 10 | 13183 | 43505 | 3.30 |
| 8 | 60 | 1 | 3 | 8 | 16 | 13565 | 63114 | 4.65 |
| 10 | 77 | 1 | 4 | 10 | 11 | 8538 | 1426286 | 167.05 |
| 10 | 84 | 1 | 4 | 10 | 18 | 14071 | 76568 | 5.44 |
| 28 | 65 | 2 | 3 | 6 | 21 | 112179 | 43910 | 0.39 |
| 34 | 61 | 2 | 3 | 12 | 17 | 14115 | 532074 | 37.70 |
| 34 | 63 | 2 | 3 | 12 | 19 | 81949 | 405565 | 4.95 |
| 40 | 21 | 2 | 1 | 18 | 21 | 24604 | 1833882 | 74.53 |
| 51 | 84 | 3 | 4 | 7 | 18 | 12098 | 1867275 | 154.35 |
| 64 | 80 | 3 | 4 | 20 | 14 | 26607 | 9477 | 0.36 |
| 70 | 24 | 4 | 2 | 4 | 2 | 37780 | 655197 | 17.34 |
| 78 | 4 | 4 | 1 | 12 | 4 | 21540 | 69820 | 3.24 |
| 79 | 54 | 4 | 3 | 13 | 10 | 192629 | 1311269 | 6.81 |
| 80 | 10 | 4 | 1 | 14 | 10 | 27473 | 9325 | 0.34 |

## Future plans

Identification of the ESTs showing a significant induction or repression pattern during the incompatible reaction between cassava and *Xam*.

Confirm the differential expression of the genes identified with new *Arabidopsis* microarray hybridizations and through northern blots analysis.

Develop microarrays containing cassava genes obtained from compatible and incompatible cassava-*Xam* interactions.

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## 1.3.17 Toward Fine-Mapping of Major Genes for Blast Resistance in Rice

Gerardo Gallego and Joe Tohme
SB-2 Project

### Introduction

We are making progress towards cloning a blast resistance gene using positional cloning. Tightly linked markers are required for marker-assisted breeding to ensure higher selection accuracy and efficiency. Our strategy for using positional cloning is based on:

The genome size of the rice is the smallest among the major crops (400 Mb),
The physical length of the genetic distance per unit is small, ca 200 Kb cM;
Rice has the most reliable transformation system among the monocots;
There are abundant restriction fragment length polymorphism (RFLP) markers and genetic information about rice blast resistance (Kawasaki et al 1996).
A large set of microsatellites distributed along the entire chromosome set, some of them linked to RFLPs next to Pi genes.
YACs and BACs contigs that contain Pi genes (Clemson University-USA and Rice Genome Program-Japan).
Updated ESTs database ( Wang et al 2001).

We have started fine mapping a region of chromosome 6 that contains genes Pi2, Pi13, and Pi9 for rice blast resistance, using as a source of resistance the variety Irat13.

### Methods

For mapping we used parental Fanny and Irat13, susceptible and resistant respectively for *Pyricularia grisea* isolates SRL-1 to SRL-6. The linkage map was constructed based on phenotypic segregation data from 104 doubled-haploid individuals. Plants were inoculated with blast SRL-1 isolates cica9-31-4 and cica9-6. MapMaker, with LOD 4,0, was used to analyze linkage of RFLP, RAPD, SCAR's, RGA, AFLP's and Microsatellites. For fine mapping, we selected a 6-7 cM interval between markers SCAR's B10 and microsatellite RM3 (easy to evaluate by PCR) in chromosome 6, which may involve the centromere. To search for recombinants within this interval, a 1500-2000 individuals, F2 population is being grown in the greenhouse. Evaluations with cica9-31-4 and cica9-6 isolates will be carried out with the F3.

The centromere is being mapped using probe N36 (donation of Dr. Q. Zhang from National Key Laboratory of Crop Genetic Improvement in China). Similarly, 29 RFLP-cDNA probes, mapped around the centromere and the interval for fine mapping (Harushima et al 1998), were donated by MAFF DNA BANK through RGP. Some ESTs close to RG648 (Z. wang et al 2001 ) and some mapped retrotransposons (S. Wang et al 1999) may be used for fine mapping theinterval. We also developed a cDNA expression library from Irat13, with 13824 clones that will be evaluated with microarrays.

**Results**

We obtained flanking markers for major resistance genes on chromosome 6 (figure 1) involved in resistance or susceptibility to SRL-1 isolates cica9-31-4 and cica9-6 in a 104, doubled-haploid population derived from the cross Fanny x Irat13.

Fifteen markers (1 SCAR's, 6 RAPD, 3 RFLP's, 2 AFLP's, 2 Microsatellites and 1 RGA), closely linked to resistance are within a 6-7 cM interval. This region may involve some Pi-2, Pi-13 or Pi-9 genes, possibly located in the centromere or within the pericentromeric region. Two PCR-based, flanking markers have been selected for fine mapping (B10 2+/-3cM y RM3 3.5+/-4.0 cM; (Figure 1).

Of the 15 restriction enzymes tested to search for polymorphisms for centromere mapping, ClaI and KpnI were selected in the parentals. Mapping the centromere and probes closely linked to it, will allow us to precisely define this region. For this purpose, we can use probes from other rice maps tightly linked to the centromere that map next to or within the interval B10-RM3.

Twenty nine RFLP-cDNA probes from the Japanese map, located within the centromeric region , were tested in parentals with XbaI, DraI, EcoRI, EcoRV and HindIII. No polymorphism was detected. The same probes are being tested with 15 more enzymes for future mapping to saturate the region.

SCAR's B10 developed at CIAT is a good starting point for fine mapping. It has shown perfect co-segregation with Pi-2 when tested in an Isolinea1 **x** Isolinea6 segregating population. (Fernando Correa, pers comm).

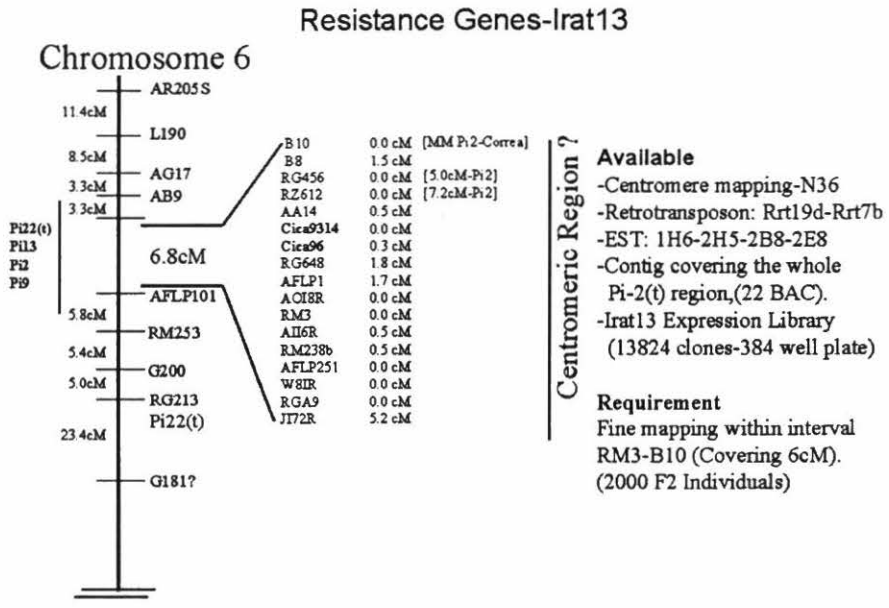


**Figure 1. Fine mapping of centromeric interval on chromosome 6 of Irat13. Tools required to achieve it and short term requisites**

**Ongoing Activities**

Fine mapping of chromosome 6 centromeric interval described above in an F2 population of 1500-2000 individuals to map tightly linked (0,1 cM) probes to genes of interest.
Evaluation of linked markers on rice BAC libraries.
Possible construction of a cosmid library from Irat13 to ensure markers closely linked to resistance in this parental genotype.
Analysis of cDNA libraries from Irat13 using microarrays.

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## 1.3.18 Constructing cDNA Expression Libraries for Oryzica Llanos 5 and Irat13

G. Gallego and J.Tohme
SB-2 Project

**Introduction**

Rice Blast, caused by *Pyricularia grisea* Sacc (Teleomorph Magnaporthe grisea Barr), is one of the most widespread and destructive diseases of rice (Ou 1985). Two varieties, Oryzica llanos 5 and Irat13, have shown resistance to isolates belonging to lineage SRL1 and SRL6 (Fernando Correa, pers. comm.). Dissecting and isolating resistance genes from these two varieties is crucial for breeding programs to produce durable resistance to blast. Linkage maps of Fanny **x** Irat13, and Fanny **x** O. Llanos 5, are available with different molecular markers. Fine mapping is being conducted in Fanny **x** Irat13, around genes Pi2, Pi13 y Pi9 next to the centromeric region of chromosome 6,. We have constructed a cDNA expression library for Irat13 and O. Llanos5 to speed up a Microarray-based search for genes expressed during blast infection.

**Methods**

To obtain representative amounts of mRNA of Oryzica llanos 5 and Irat13, plants were inoculated 20-25 days after planting in the greenhouse with strains cica 9-31-4 and cica 9-6, which belong to SRL1 (molecular markers linked to resistance to these two isolates have been previously

isolated). Leaves were collected in liquid Nitrogen after 6, 12 and 24 hours post-inoculation. Tissues were stored at -80 °C until needed.

To extract total RNA we made bulks of tissues collected at different times (100 mg from the bulk). Tissues were macerated in liquid Nitrogen, and RNA extracted using Rneasy® Plant Mini Kit protocol of QUIAGEN. mRNA purification involved several RNA mini-extractions, which were then run through Dynabeads® Oligo(dt)25 from Dynal. Once mRNA was ready, we proceeded to cDNA synthesis with the Kit Super Script Plasmid System® for cDNA Synthesis and Plasmid Cloning of GIBCO-BRL.

To fraction cDNA, we used columns from GIBCO-BRL and collected fractions 7,8 and 9 for cloning. Libraries were screened for insert size by endonuclease digestion and PCR amplification.

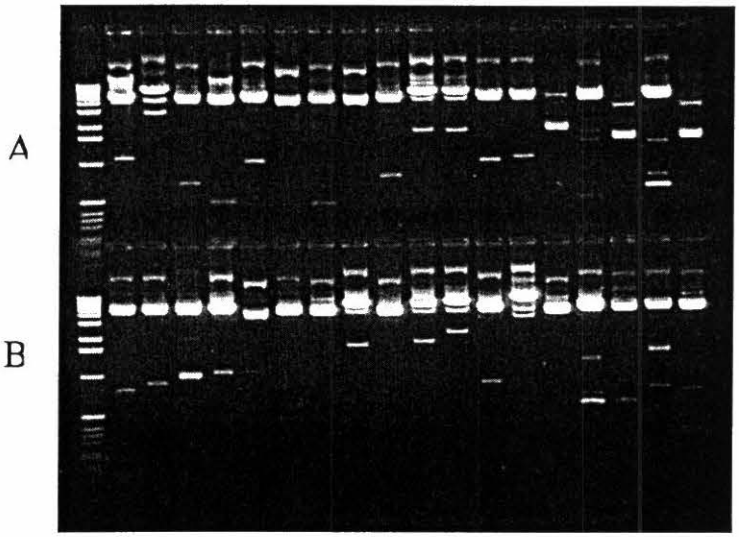


**Figure 1. Screening of cDNA expression libraries of O. Llanos 5 (A) and Irat13 (B) by digesting recombinant plasmids with NotI/Sal I.**

## Results

The two libraries showed an average size insert of 1,8 Kb, ranging from 0,5 to 2,0 Kb, which is optimum for cDNA Microarray (Xiang et al, 2000). The cDNA library of O.Llanos 5 was stored as ligations, at -80°C, for posterior electroporation into bacteria. Irat13 library was used to spot 13824 clones in 384-well plates, by duplicate, and stored at -80°C. Different clones from different plates were PCR-amplified directly from bacteria which guaranteed the presence of inserts in 90-95% of analyzed clones (Figure 1) .

Both cDNA libraries are expression libraries since inserts are directionally cloned respective to the transcriptional polarity of the mRNAs they derive from. Directional cloning thus facilitates the construction of subtraction libraries to search for diffrentially expressed genes.

**Ongoing Activities**

- Screening expression libraries with markers mapped and closely linked to blast resistance genes.
- Development of ESTs to saturate Fanny x Irat13 in regions of interest.
- Microarrays to monitor gene expression.
- Development of subtraction libraries to search for differential gene expression.

**References**

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## 1.3.19 Detection of Differentially Expressed Genes Related to Apomixis using cDNA Subtraction Coupled to Microarray Hybridization

Cortés Diego Fernando, Miles John & Joe Tohme
SB-2 Project

**Introduction**

The identification of differential gene expression between two organisms or cells types is a frequent goal in modern biological research. The possibility to determine mRNA expression differences between apomictic and sexual genotypes using the novel tools of functional genomics is a powerful tool to access the gene expression involve in this.

Several recent and rapid PCR-based method, including Subtractive Suppression Hybridization (SSH) and Representational Differences Analysis (RDA) have been for the cloning of genes that are differentially expressed between genotypes. Recently, cDNA microarrays have been developed and used to quantitative differential gene expression by hybridization a complex mRNA-derived prove onto an array of PCR products. Microarrays allow thousands of genes to be monitored simultaneously for expression level and compared between tissues.
Here we show the advancement in the merging of cDNA subtraction technique with microarray analysis as a potential method for detection of unique differential expressed genes related to apomixes in *Brachiaria.*

**Materials and Methods**

Based in the most common protocols for cDNA microarray different consensus protocols were tested in the implementation of this technique for the gene expression study with *Brachiaria* at CIAT.

The plasmids of 1920 selected cDNA clones were collected from the cDNA subtraction library, which was obtained from the subtractive suppressive hybridization (SSH) between the cDNA of *B. decumbes,* which contains specific transcrips and the reference cDNA of *B. ruzziciensis.*
The inserts of the cDNAs were amplified by PCR in 96-well plate format using T7 and SP6 primers pair specifics for the pGEMT-easy vector. PCR reaction of 50µl with 5µl of a dilution 1:20 directly from the grow bacterial as template. The PCR products were precipitated both by adding 50µl of ethanol and 5µl of 3M sodium acetate or 50µl of isopropanol. The precipitated samples were centrifuged at 3000rpm at 4°C for 30 minutes and washed with ethanol 70%. After dry down the samples it were resuspended in 10µl different spotting buffers (TE+glycerol 50%, SSC 3X, SSC 3X + SDS 0.1%, and TE+DMSO 50%). The yield and quality of the PCR products were analyzed by agarose gel electrophoresis.

The PCR samples were arrayed in duplicates from 384-well plates onto home made slides coated with poly-L-Lys as well as onto SigmaScreen™ coated slides. After spotting both types of slides were processed to avoid non-specific hybridization.

Two different were used for preparation of the labeled probe in the hybridization. In the first method we label cDNA directly by incorporating fluorescently labeled nucleotides during oligo-dT primed reverse transcription and in the second method the cDNA was prepared by a normal cDNA synthesis reaction and it was used as template for a random primer labeling reaction with exonuclease free klenow (USB). Following the hybridization the slides were submerged in the washing solutions and the fluorescent image was acquired for both fluorescent dyes used by scanning the slides with the Hitachi Genetic Systems.

**Results**

Experiments using cDNA microarrays, can encounter technical problems at any step. Different points, which are critical in the cDNA microarray implementation, has been solve, at least on the gene expression experiments with *Brachiaria.* The possibility provided by the microarray format, to include numerous internal controls facilitate the recognition and correction of many kinds of problems.

The different combination among the cleaning method of the PCR product, the spotting buffer, the type of slide and the probe labeling system allow us to establish a putative protocol for the gene expression analysis in *Brachiaria* with cDNA microarrays. We establish that the precipitation of the PCR product with isopropanol work better than the Ethanol and sodium acetate combination. Using isopropanol the amounts of PCR product recover is higher than with ethanol, and the steps in the precipitation and cleaning process is less time consuming. About the spotting buffers and the type of slide, the combinations between poly-L-Lysine home made slides and TE/Glycerol or TE/DMSO 50% give stronger signal than any other spotting buffer. Beside that poly-L-Lysine have more DNA affinity the background signal was also higher than when SigmaScreen slides were used. When the different spotting buffers were tested on SigmaScreen slides the only one which gives high affinity for the slide surface and regular spot form was TE/DMSO 50%, follow by 3x SSC, the rest of the spotting buffers spread the cDNA all over the slide joining each spot to the neighbors.

The hybridization results both incorporating fluorescently labeled nucleotides during oligo-dT primed reverse transcription of the total RNA or random primer labeling reaction with exonuclease free klenow (USB) using cDNA already synthesized give almost the same results without significant differences.

**Future Activities**

cDNA microarray spotting. 5,000 clones from each library will select after mass excision, PCR amplified and spotted onto replicate glass slides using the SPBIO spotting robot at CIAT.

Microarray hybridizations with Cy3 and Cy5 labeled cDNA derived from poly(A)+ of genotypes used in the cDNA subtraction library construction.

Microarray hybridizations with labeled PCR product of the SCARs markers includes in the *Brachiaria* genetic map, which are linked to the apomixes loci.

Generation of cDNA chips with clones derived from a full-length cDNA library both sexual and apomictic genotypes.

Identification of clones of interest. Computer analysis of the microarray hybridization output will be use to identify clones that change its expression patter between sexual and apomictic genotypes.

Microarray hybridization and sequencing of the full-length cDNA chips, using as prove cDNA clones identified as interesting in the cDNA sudstraction cDNA chip.

Interrogating of microarrays. Cy3 and Cy5 labeled cDNA derived from poly(A)+ of time points of interest will be used as probe to hybridize cDNA slides.

Identification of clones of interest. Computer analysis of the microarray hybridization output will be use to identify clones that change in abundance during the time course of PPD.

## 1.3.20 Isolation of Resistance Gene Analogs (RGAs) from *Brachiaria*

I.F. Acosta and J. Tohme
SB-2 Project

**Introduction**

The cloning of resistance genes in different species is providing a wealth of information about the structure, expression and function such genes. Recent genetic studies reporting that the *Mi* gene for resistance in tomato to the root knot nematode, *Meloidogyne incognita* is the same gene for resistance to specific isolates of the potato aphid, *Macrosiphum euphorbiae* (Rossi et al., 1998). *Mi* was the first example of a plant Resistance Gene (R-gene) active against two such distantly related organisms. Moreover, it was the first isolate-specific insect resistance gene to be cloned. The gene belongs to the nucleotide-binding (NBS), leucine-rich repeat (LRR) family which includes the majority of cloned R-genes.

Sequence similarity between cloned disease R-genes, especially those from the NBS class, allowed us to use degenerate primers in rice, cassava and common bean to isolate NBS-containing sequences that are potentially part of R-genes and are called Resistance

Gene Analogs (RGAs) (BRU Annual Report, 1998, 1999). RGAs have proved to be useful as "candidate genes" to map resistance loci, mainly in common bean (BRU Annual Report, 2000, 2001).

Spittlebug is the most harmful pest of *Brachiaria* in America. Different methods must be integrated to achieve effective control of this pest. An efficient method is the use of cultivars that are naturally resistant. However, the molecular mechanisms underlying resistance are not fully understood. The objective of this study is to initiate a candidate gene approach using degenerate primers to isolate RGAs the NBS-LRR class from *Brachiaria*.
Sequence similarity between cloned disease R-genes, especially those from the NBS class, allowed us the use of PCR with degenerate primers in rice, cassava and common bean to isolate NBS-containing sequences that are potentially part of R-genes. and are called Resistance Gene Analogs (RGAs) (BRU Annual Report, 1998, 1999). RGAs have proved to be useful as "candidate genes" to map resistance loci, mainly in common bean (BRU Annual Report, 2000, 2001).

Spittlebug is the most harmful pest of *Brachiaria* in America. Different methods must be integrated to achieve effective control of this pest. A low cost method is the use of cultivars that are naturally resistant. However, the molecular mechanisms underlying resistance are not fully understood which does not really allows to take advantage of it. The objective of this study is to initiate a candidate gene approach degenerate primers to isolate RGAs the NBS-LRR class from *Brachiaria*.

**Methods and results**

DNA templates were used from *B. brizantha* CIAT 6294 and *B. ruziziensis* BR4x44-02, which are resistant and susceptible to the spittlebug, respectively. A mapping population of 215 individuals has been derived from the interspecific cross of these species.

Degenerate primers based on the Nucleotide Binding Site (NBS) which is conserved in R-genes have been used successfully in rice, cassava and common bean to isolate RGAs of the NBS type (BRU Annual Report, 1998, 1999). For *Brachiaria*, we assayed degenerate primers designed by Leister et al. (1996, 1998), Lopez and Acosta (BRU Annual Report, 1999) and Silvia Peñuela (unpublished results). Amplifications were obtained for the primers indicated in Table 1. Primers targeting the TIR domain of R-genes or the TIR-type NBS were also tested but, as expected, no amplification was obtained because monocotyledonous does not have this type of R-genes (BRU, Annual Report, 1999; Meyers et al., 1999).

PCR products from *B. brizantha* CIAT 6294 were separated and bands of the expected size were purified, cloned and transformed into *E.coli* electrocompetent cells. A total of 144 clones (36 of each combination) were obtained and grouped by their restriction patterns with the 4-bp cutter enzyme *Alu*I. Twenty two groups were identified and one clone of each group was sequenced using the Dye Terminator Cycle Sequencing Kit and the Applied Biosystems Prism 377 DNA sequencer (Perkin-Elmer). Sequences of 19 clones corresponded to RGAs and were classified in 7 classes according to their similarity: BRGA1 to BRGA7.

## On-going work

The next step is locate these RGAs in the genetic map that is currently being constructed at the BRU (Olga X. Giraldo and Jaime Vargas, BRU, Annual Report, 2001) using the mapping population of the interspecific cross between *B. brizantha* CIAT 6294 and *B. ruziziensis* BR4x44-02. RGAs are usually mapped as RFLPs and we have already obtained good polymorphic band between the parental DNA. Screening of the mapping population will be conducted next year. We have also designed primers that specifically amplify each BRGA class to convert them in PCR-based markers that are easier to evaluate. BRGAs will be amplified from each parental and cut with *Alu*I. Digestion products will be separated on acrylamide gels and polymorphic bands between parents will be used for mapping. Resistance to one species of spittlebug has been evaluated quantitatively in each individual of the progeny. After location of BRGAs in the map, QTL analysis will be performed hoping to find one BRGA explaining some level of resistance to spittlebug.

**Table 1. Summary of the use of degenerate primers to isolate RGAs from *Brachiaria***

| Primer combination | Expected size (bp) | RGA class obtained |
|---|---|---|
| S2 + AS3 | 500 | BRGA1 |
| S2 + AS4 | 500 | - |
| S2 + GLPL3 | 500 | BRGA2 |
| S2 + PRS3 | 700 | BRGA2 to BRGA7 |

Primer sequences correspond to those in BRU Annual Report (1999)

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## 1.3.21 Construction of a molecular genetic map of Brachiaria and QTL analysis of spittlebug resistance

O. X. Giraldo[1], J. Vargas[1], E. Gaitán[1], M.C. Duque[1], J. Miles[1], C. Cardona[2], J. Tohme[1].
[1]SB-2 Project ; [2]IP-1 Project

### Introduction

The genus *Brachiaria* Griseb. belongs to the tribe Paniceae, comprises aproximately 100 species, mostly of African origin. Some of these have found commercial use as forage in tropical America, with approximately fourty million hectares of *Brachiaria* pastures in Brazil alone (Valle and Miles 1992). The commercial species of B. *brizantha* and B. *decumbens* are tetraploid apomitic (Valle 1986), The Construction of a *Brachiaria* molecular map was initiated (BRU annual report pp 123-127, 2000), using a population of 215 F1 individuals derived from a cross between an autotetraploid spittlebug susceptible individual *B. ruziziensis* and a tetraploid spittlebug resistant individual *B. brizantha*. The objective of the study is to increase the saturation of the map using SCARs and SSRs developed at CIAT, AFLPs, RFLPs probes from other grases species and tag the quantitative trait loci (QTLs) controlling spittlebug resistance in *Brachiaria*.

### Materials and Methods

*Plant Material:* A sexual tetraploid *B. ruziziensis* (Swenne et al., 1981), susceptible to spittlebug (Clone 44-3), was used as a female parent in a cross with natural and apomitic tetraploid genotype *B. brizantha* resistant to spittlebug (accession CIAT-6294).

*DNA Extraction:* DNA was extracted using the protocol described by Carlos Colombo (personal communication) with some modifications. 1g of tissue was dried at 48 °C for 20 hours and ground to fine power; 15 ml of extraction buffer (0.1M Tris-Hcl pH8.0, 0.05M EDTA pH8.0, 0.7 M NaCl, 4% CTAB and 1% ßMe) was added and incubated at 65 °C for 10 min; 15 ml of chloroform:isoamyl alcohol (24:1) was added and centrifuged at 3000 RPM for 30 min. The aqueous phase was transferred to a new tube and 8 ml of chloroform:isoamyl alcohol was added and centrifuged at 3000 RPM for 30 min, repeated twice. A volume of cold isopropanol was added to the supernatant and incubated over night at - 20 °C. The isopropanol mixture was centrifuged at 3000 RPM for 30 min at 4 °C. The DNA pellet was washed with cold 75% ethanol and dried at room temperature, and then resuspended in 300 ul of TE. Pancreatic RNAse was added to a final concentraction of 20 ug/ml. DNA was quantified on a DYNA QUANT 200 fluorometer (Hoffer Scientific Instruments, San Farancisco CA).

*Microsatellites:*The isolation of the microsatellites and the methodology for PCR amplification and evaluation of polymorphism have been described previously (BRU annual report pp 123-127, 2000). An additional set of 26 new SSRs was evaluated this year.

AFLP, RFLP RAPD: All 215 individuals were evaluated using the combination (E-ACG/M-CTA), The screening methodology was described in (BRU annual report pp 123-127, 2000). Protocols for RFLP and RAPD, markers in *Brachiaria* were described previously (BRU Annual report pp 105-110 1997).

*Linkage Analysis:* Segregation of markers as single dose restriction fragment (SDRF) markers according to the genetic model was determined by departure from the hypothesized 1:1 ratio by the Chi-square test. The data matrixes obtained for presence or absence of bands were analyzed

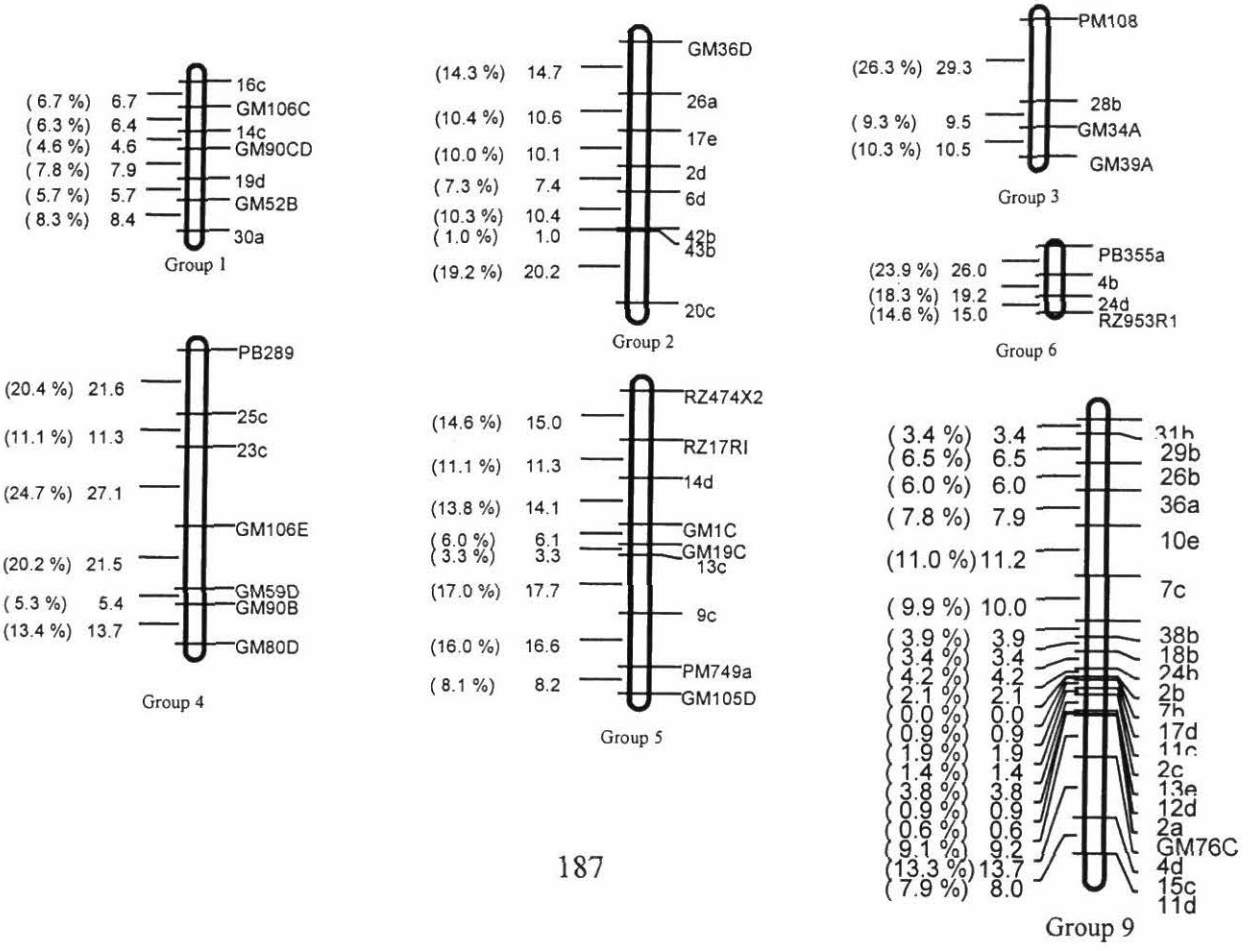
with MAPMAKER v 3.0b for PC (Lander et al. 1987), using LOD score of 6.0 and recombination fraction 0.3. Recombination was translated to genetic distances using the Kosambi map function.

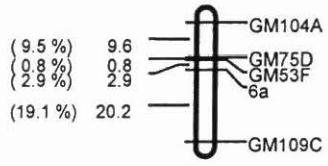
**Results and Discussion**

*Phenotypic screening:* The Tropical Forage Entomology section screened the population of the average damage of individual hybrid plant (C. Cardona et al., 1999). The results indicate that approximately 74.5% of the population can be classified as resistant or susceptible individuals to the spittlebug damage (table 1). The average damage values cover a continuous range from 1 to 5 suggesting a quantitative trait. Three different ranges were derived allowing the classification of the population as resistant, intermediate or susceptible individuals.

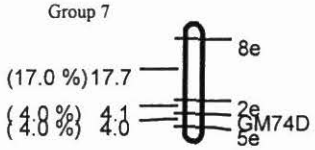
The Genetic Linkage Map: Sixty-eight SSRs, 5 combinations AFLPs (116 markers), and 35 RFLPs segregating in the male parent (CIAT-6294), were tested for linkage using MAPMAKER V.3.0b. Polymorphisms were scored for presence (H), and absence (A), and analyzed for dosage among F1 progeny using Chi-square tests (P<0,01). 45 SSRs, 67 AFLPs and 16 RFLPs markers, were found to define 22 linkage groups spanning 1079.031 cM, with an average marker density of 1 marker every 8.43 cM (Figure 1), map distance in centimorgans was calculated using the Kosambi mapping function. Linkage groups were organized according the number of markers presents in each group. The most densely populated linkage group 9 spanned 99.0 cM with 21 markers, followed by the linkage group 5 with 9 markers, spanned 92.306 cM, the groups 2 and 12 with 8 markers spanned 74.404 and 77.275 cM each one, while the least populated group was the linkage group 20 with 3 markers. The markers were grouped using a LOD = 6 and a recombination fraction of 0.3. Only the markers from linkage group 9 were grouped using a LOD=34 and recombination fraction of 0.3 (Figure 1).

**Fig 1** Preliminary *Brachiaria* framework map (LOD : 6 Tetha: 0.3)

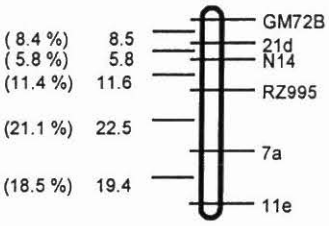




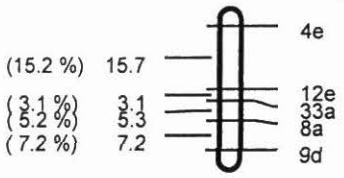

Group 7



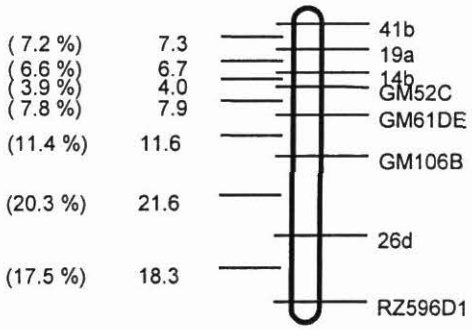

Group 14



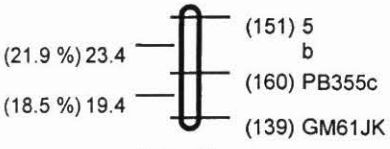

Group 8



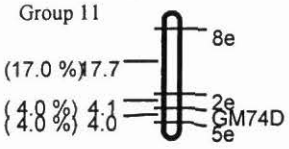

Group 10



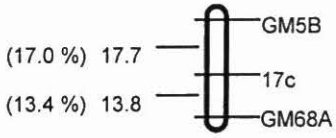

Group 12



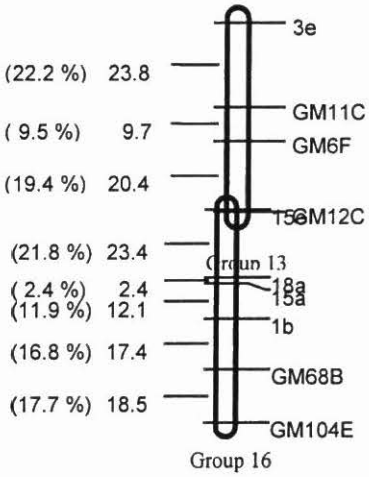

Group 11



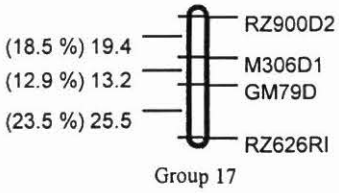

Group 14



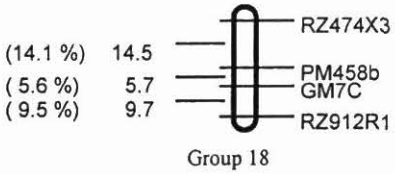

Group 15



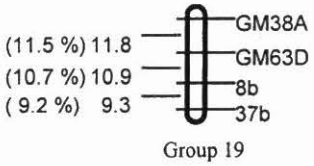

Group 16



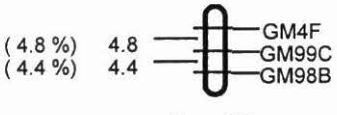

Group 17



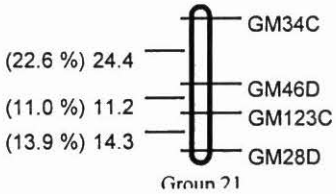

Group 18



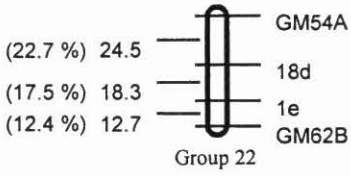

Group 19




Group 20




Group 21




Group 22

**Figure 1. Preliminary *Brachiaria* framework map (LOD : 6 Tetha: 0.3). Continuation.**

*Quantitative Trait Loci (QTL) analysis*

The average damage values from each F1 genotype were analyzed with QTL Cartographer software using the map generated by Mapmaker. First, a search test was conducted, to find the association of segregant markers and the trait of interest, through lineal regression for each marker in relation to the quantitative trait and using the Composite Interval mapping Method (CIM). Statistical significance levels of 0.01% were obtained by evaluation of F test in 6 markers of linkage group 2 (GM36D, 42b, 6d, 2d, 17e, 26a), and 3 markers of linkage group 16 (1b, 16a, 15a). These significance levels indicate a strong genetic linkage. Using the Composite Interval Mapping method, two major QTLs were found on linkage groups 2 and 16 with a LOD of 21 and 8 respectively (Figure 2a, 2 b) suggesting a strong evidence for the presence of QTLs for resistance to spittlebug. The most significant QTLs explained up to 37% and 15 % of the variance for QTL 1 and QTL2 respectively.

**On going activities**

More markers will be placed on the *Brachiaria* map as they become available. The QTLs data will be integrated with the work on the isolation of *Brachiaria* resistance gene analogs (RGAs). We plan to saturate the region of the different QTLs with additional markers using AFLP and Dart to fine map the region of QTL1 and QTL2. Such markers could eventually be used for a marker assisted selection program. However we will need to confirm the QTL1 and QTL2 in a different background.

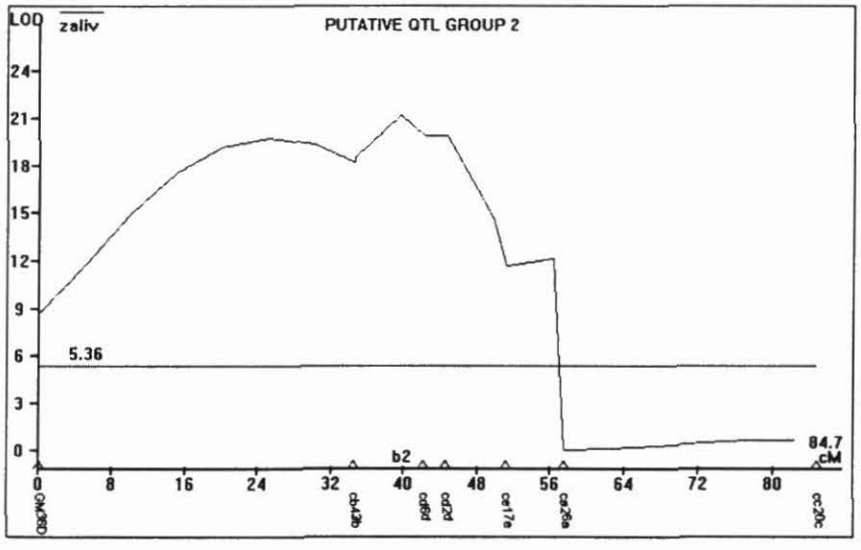



**Figure 2a Putative QTLs associate with susceptibility to spittlebug in the linkage group 2.**

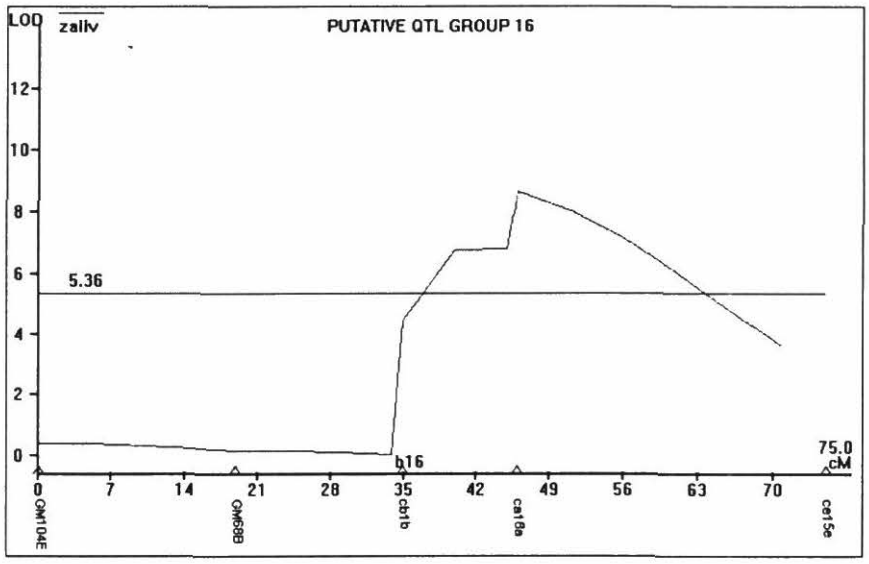



**Figure 2b. Putative QTLs associate with susceptibility to spittlebug in the linkage group 16.**

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**Table 1 level of spittlebug resistance F1 population of interspecific cross B. brizantha x B.ruziziensis**

| Accesion | Damage Average | Damage classification | Accesion | Damage Average | Damage classification | Accesion | Damage Average | Damage classification | Accesion | Damage Average | Damage classification | Accesion | Damage Average | Damage classification |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CIAT6294 | 2 | R | 1027-75 | 3.3 | S | 1078-0027 | 3.55 | S | 1082-0030 | 2.65 | I | 1094-0024 | 1.75 | R |
| clon 44-3 | 4.625 | S | 1027-79 | 3.2 | S | 1078-0031 | 1.6 | R | 1083-0001 | 1.9 | R | 1094-0025 | 3.3 | S |
| 1016-1 | 3.1 | S | 1027-82 | 3.2 | S | 1078-0032 | 1.6 | R | 1083-0004 | 3 | I | 1094-0026 | 3.65 | S |
| 1016-3 | 3.8 | S | 1027-84 | 4.5 | S | 1079-0002 | 2.133335 | I | 1083-0005 | 2.35 | I | 1094-0027 | 3.1 | S |
| 1016-8 | 2.4 | I | 1027-86 | 3.5 | S | 1079-0005 | 3.35 | S | 1084-0001 | 3.55 | S | 1094-0028 | 2.65 | I |
| 1016-9 | 3 | I | 1027-97 | 4.8 | S | 1079-0006 | 3.25 | S | 1084-0002 | 3.2 | S | 1094-0030 | 2.6 | I |
| 1016-10 | 4.2 | S | 1027-98 | 3.7 | S | 1079-0007 | 2.15 | I | 1084-0003 | 3.15 | S | 1094-0032 | 3.3 | S |
| 1016-11 | 3.7 | S | 1027-100 | 3.2 | S | 1079-0008 | 4.05 | S | 1084-0004 | 3.4 | S | 1094-0033 | 2.4 | I |
| 1016-12 | 3.9 | S | 1027-102 | 4.8 | S | 1079-0010 | 3.7 | S | 1084-0007 | 3.05 | S | 1094-0034 | | |
| 1016-17 | 4.5 | S | 1027-103 | 3.4 | S | 1079-0011 | 2.65 | I | 1084-0010 | 2.55 | I | 1094-0035 | 3.4 | S |
| 1016-19 | 4.1 | S | 1027-108 | 4.3 | S | 1079-0012 | 1.95 | R | 1092-0003 | 2.25 | I | 1094-0038 | 2.6 | I |
| 1016-21 | 4.8 | S | 1027-110 | 3.2 | S | 1079-0014 | 3.55 | S | 1092-0004 | 4.25 | S | 1094-0039 | 4.25 | S |
| 1016-22 | 4.7 | S | 1027-113 | 3.5 | S | 1079-0015 | 3.3125 | S | 1092-0005 | 3.1 | S | 1094-0042 | 1.85 | R |
| 1016-28 | 4.1 | S | 1027-116 | 3 | S | 1080-0001 | 2.05 | I | 1092-0008 | 3.8 | S | 1094-0045 | 1.8 | R |
| 1016-31 | 4.2 | S | 1027-117 | 4.2 | S | 1080-0007 | 4.3 | S | 1092-0010 | 1.7 | R | 1094-0048 | 2.8 | I |
| 1016-39 | 4.3 | S | 1027-118 | 2.6 | I | 1080-0010 | 2.95 | I | 1092-0011 | 2.7 | I | 1094-0049 | 1.5 | R |
| 1017-4 | 3.8 | S | 1027-119 | 3.9 | S | 1080-0013 | 1.35 | R | 1092-0014 | 3.85 | S | 1094-0050 | 3.85 | S |
| 1017-6 | 3.5 | S | 1027-120 | 4.8 | S | 1080-0014 | 3.4 | S | 1092-0015 | 3.75 | S | 1095-0001 | 3.55 | S |
| 1017-7 | 4.2 | S | 1027-122 | 3.9 | S | 1080-0015 | 2.5375 | I | 1092-0016 | 2.8 | I | 1095-0002 | 3.85 | S |
| 1017-9 | 3.6 | S | 1027-126 | 4.1 | S | 1080-0017 | 1.65 | R | 1092-0018 | 4.6 | S | 1095-0003 | 3.95 | S |
| 1017-12 | 4.3 | S | 1027-128 | 2.4 | R | 1080-0018 | 3.45 | S | 1092-0019 | 2.85 | I | 1095-0004 | 2.5 | I |
| 1017-16 | 4 | S | 1027-135 | 4.1 | S | 1080-0019 | 3.1 | S | 1092-0020 | 4.3875 | S | 1097-0003 | 2.15 | I |
| 1017-17 | 3.6 | S | 1027-136 | 3 | S | 1081-0003 | 4.45 | S | 1093-0002 | 3.5 | S | 1097-0004 | 4.625 | S |
| 1017-21 | 3.4 | S | 1028-2 | 4.2 | S | 1081-0004 | 4.5 | S | 1093-0005 | 4.55 | S | 1097-0005 | 3.3 | S |
| 1017-22 | 4.8 | S | 1028-3 | 4 | S | 1081-0005 | 2.75 | I | 1093-0006 | 1.65 | R | 1097-0006 | 1.7 | R |
| 1017-24 | 3.9 | S | 1028-7 | 4.2 | S | 1081-0006 | 1.95 | R | 1093-0010 | 3.8 | S | | | |
| 1017-25 | 4.1 | S | 1028-17 | 3 | S | 1081-0008 | 2.45 | I | 1093-0011 | 3.8 | S | | | |
| 1017-26 | 4.1 | S | 1028-18 | 4.2 | S | 1081-0010 | 2.95 | I | 1093-0012 | 1.7 | R | | | |
| 1017-28 | 4.6 | S | 1028-20 | 3.7 | S | 1081-0012 | 3 | I | 1093-0018 | 2.5 | I | | | |
| 1017-29 | 4.8 | S | 1028-22 | 2.8 | S | 1081-0017 | 2.675 | I | 1093-0019 | 2.15 | I | | | |
| 1017-30 | 3.8 | S | 1028-25 | 4.1 | S | 1081-0018 | 4.3 | S | 1093-0021 | 3.075 | S | | | |
| 1027-5 | 4.3 | S | 1028-27 | 2.3 | I | 1081-0019 | 2.15 | I | 1093-0024 | 2.4 | I | | | |
| 1027-6 | 4.6 | S | 1028-28 | 4.5 | S | 1081-0025 | 2.95 | I | 1093-0026 | 4.35 | S | | | |
| 1027-12 | 3.5 | S | 1028-29 | 3.5 | S | 1081-0027 | 1.85 | R | 1093-0028 | 3.95 | S | | | |
| 1027-24 | 4.5 | S | 1028-32 | 3.3 | S | 1081-0028 | 1.8 | R | 1093-0029 | 2.2 | I | | | |
| 1027-27 | 4.1 | S | 1028-36 | 3.8 | S | 1081-0030 | 2.35 | I | 1093-0031 | 2 | R | | | |
| 1027-29 | 3.7 | S | 1028-39 | 3.7 | S | 1082-0002 | 3.4 | S | 1094-0003 | 2.55 | I | | | |
| 1027-31 | 3.7 | S | 1078-0001 | 1.85 | R | 1082-0004 | 2.05 | I | 1094-0004 | 2.95 | I | | | |
| 1027-33 | 3.5 | S | 1078-0002 | 2.5 | I | 1082-0005 | 2.55 | I | 1094-0006 | 2.05 | I | | | |
| 1027-42 | 4 | S | 1078-0004 | 3.85 | S | 1082-0006 | 1.6875 | R | 1094-0007 | 3 | I | | | |
| 1027-43 | 3.5 | S | 1078-0007 | 1.45 | R | 1082-0011 | 3.25 | S | 1094-0008 | 3.8 | S | | | |
| 1027-49 | 4.5 | S | 1078-0009 | 2.8 | I | 1082-0013 | 3.35 | S | 1094-0010 | 2.9 | I | | | |
| 1027-59 | 4.1 | S | 1078-0010 | 2.5 | I | 1082-0018 | 2.65 | I | 1094-0011 | 3.95 | S | | | |
| 1027-61 | 4 | S | 1078-0013 | 2.45 | I | 1082-0020 | 4 | S | 1094-0012 | 1.7 | R | | | |
| 1027-62 | 4.3 | S | 1078-0014 | 3.15 | S | 1082-0021 | 1.7 | R | 1094-0016 | 3.35 | S | | | |
| 1027-65 | 4.2 | S | 1078-0015 | 3 | I | 1082-0024 | 3 | I | 1094-0018 | 4.25 | S | | | |
| 1027-68 | 4.3 | S | 1078-0016 | 3.7 | S | 1082-0028 | 3.65 | S | 1094-0021 | 2.8 | I | | | |
| 1027-69 | 3.9 | S | 1078-0022 | 2.45 | I | 1082-0029 | 4.175 | S | 1094-0023 | 2.35 | I | | | |

R: resistant, I: intermediate, S: susceptible

## 1.3.22 Isolation and characterization of microsatellite loci in *Bactris gasipaes*

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SB-2 Project

### Introduction

The peach palm *Bactris gasipaes* is the only palm domesticated in the Americas (Clement, 1992). It is very important economically because it yields two food crops with commercial potential: the fruit and the heart of the palm. Many peach palm populations have completely disappeared, and many more—both wild and cultivated—are in peril (Mora-Urpí et al., 1997). Knowledge of the genetic structure and diversity of populations, as well as characterization of *B. gasipaes* germplasm banks, is of great importance for conserving this species. Microsatellite loci are short tandem repeats, usually consisting of 2-6 nucleotides. These loci are very useful because they can be easily distributed between species and between individuals and populations within a species, providing a popular tool for learning about population structure (Chambers & MacAvoy, 2000). Moreover, in comparison with other markers utilized for germplasm analysis, microsatellites have the highest expected heterozygosity (Powell et al., 1996), which is very important for evaluating germplasm banks.

### Materials and Methods

*Construction of an enriched microsatellite library of Bactris gasipaes.* An enriched microsatellite library was constructed as described by Edwards et al. (1996). This involved the digestion of 200 ng of genomic DNA with *Rsa* I. An *Mlu*I adaptor (consisting of a 21-mer and a 25-mer primer) was ligated to the digested fragments. Filter-immobilized oligonucleotides representing the $CT_{20}$ and $GT_{20}$ SSR marker classes were used to select for the genomic fragments containing SSRs. Enriched fragments were amplified by PCR, using the 21-mer adaptor primer. PCR products were ligated in pGEM-T Easy vector (Promega). Clean ligation products were transformed into competent *Escherichia coli* DH5α cells and plated overnight on selective media (LB+ampicillin). Positive colonies were sequenced to characterize microsatellite loci using purified DNA template, T7 primer and Big-Dye terminator fluorescent chemistry of ABI- PRISM. Each sequence was aligned against all the other sequences, using the SEQUENCHER program in order to eliminate redundant clones. Primers were then designed for the unique clones using the PRIMER3.0 software program (available at http://waldo.wi.mit.edu/cgi-bin/primer/primer3).

*Microsatellite primer characterization.* DNA samples obtained from 14 peach palms in CORPOICA's germplasm bank (Caquetá, Colombia) were used to evaluate the primer pairs. In addition, 6 different species of *Bactris* (*B. glandulosa, B. setulosa, B. maraja, B. major, B. guineensis* and *B. gasipaes* var. *Chichagui*), the wild relatives of the peach palm, were analyzed to check cross-specific amplification, bringing the total number of common bean accessions used in the study to 20. PCR reaction (20 µL) consisted of 0.1 or 0.3 µM of each primer, 2.5 or 3.0 mM $MgCl_2$, 20 ng DNA, buffer 1x, 0.25 mM dNTPs, and 1 µ Taq polymerase. The initial denaturation was performed at 94°C for 3 min prior to 35 cycles of denaturation at 94°C for 15 s, annealing at 48-52°C for 15 s, and an extension at 72°C for 15 s, followed by a final extension at 72°C for 5 min. PCR products were resolved by electrophoresis on a standard 6% denaturing acrylamide sequencing gel and visualized by silver staining according to the manufacturer's guide (Promega, 1995). Alleles were sized relative to a 10-bp ladder, using the image analysis software 1D Kodak Digital Science™.

**Data analyses**

Gels containing PCR amplifications were scored for the presence or absence of alleles generated by each pair of primers for all 20 individuals. In this study the Discriminating Power (D) was used to compare the efficiency of the microsatellites in differentiating between genotypes. The D value represents the probability that two randomly chosen individuals show different allelic patterns with the microsatellite locus, thereby being distinguishable from one another. If *Pi* is the proportion of the population carrying $i^{th}$ banding pattern and is calculated for each microsatellite locus (Tessier et al., 1999), then $D=1-\Sigma p_i^2$. This is an extension of the Polymorphism Information Content or PIC (Anderson et al., 1993), available from the frequencies of the different banding patterns (or genotypes) generated by a primer.

**Results**

An enriched genomic library for *B. gasipaes* constructed and 18 polymorphic microsatellites markers developed. An average of 7 alleles per locus was found on the sample of the 14 individuals collected in a germplasm bank. Cross-species amplification was evaluated in 6 different species of *Bactris*. A total of 1920 resultant colonies were screened for $(GA)_n$ $(CA)_n$ repeats, using an oligonucleotide labeled with [$\gamma$ $^{32}$P]- dATP. A total of 62 (3.23%) were positive, and 39 of these clones had microsatellites; but locus-specific primer pairs were designed for only 27. Primers were synthesized by OPERON Technologies, Inc. Of these loci, only 89% appeared to be informative, with D values greater than D = 0.75, which make them very useful in population studies and germplasm characterization.

The 18 loci in *Bactris* evaluated in 6 different species of *Bactris* (*Bactris glandulosa, B. setulosa, B. maraja, B. major, B. guineensis* and *B. gasipaes* var. *Chichagui*), the wild relatives of the peach palm. Fifteen microsatellites markers produced amplification products in at least one species, with varying length between them. Bg17 was the only locus that had an amplification product in all species. In contrast, Bg1 and Bg21 had no amplification in the other *Bactris* spp., indicating that the flanking regions of these loci are not conserved in the *Bactris* genus. These results showed that markers identified in one species could be used for genetic analysis in related species.

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## Output 2 Genes and genes combinations made available for broadening the base of mandated and non mandated crops

### Activity 2.1 Transfer of gene and gene combinations using cellular and molecular techniques

#### Main Achievements

- Four lines of transgenic cassava plants, containing genes of interest for insect resistance, have been established in the biosafety greenhouse.
- Scaling up of transformation experiments with cassava has been achieved, using *Agrobacterium* and/or biolistics, by doing 2-3 transformations per month with at least four cultivars (TMS60444, SM1219-9, CM2306-4, MCol2215), for which FEC has been developed, with two genetic constructs containing genes of interest.
- The generation of transgenic rice with highest levels of resistance to RHBV (score 1) in the field, derived from a rustic variety Cica 8 grown by small farmers in Central and Northern South America, along being a good parental donor for rice breeding. Conferring protection to RHBV at 10 days after planting under high disease pressure, whereas the most common breeding resistance source is active after 20 days of planting.
- The development of a selection system for generating transgenic rice based on the use of mannose isomarase selection gene. The methodology allows the recovery of a high percentage of transgenic plants as indicated by Southern Analysis, although the plant regeneration frequency still needs to be increased. This system will allow moving away from using antibiotic resistance gene as a selection system for generating transgenic rice, in compliance with current food biosafety recommendations.
- The generation of a cDNA library from *Brachiaria* was accomplished and is being used to clone key genes involved in lignin biosynthesis for future modifications of the forage digestibility.

## 2.1.1 Genetic transformation of tepary beans and breeding of common bean genotypes amenable to *Agrobacterium* transformation

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### Introduction

Compared to direct gene transfer methodologies (particle bombardment, electroporation, etc.), *Agrobacterium* mediated transformation offers different advantages such as the possibility to transfer only one or few copies of DNA fragments carrying the genes of interest, and the transfer of very large DNA fragments of a size as large as 150kb. The most important advantage however is the possibility of producing transgenic plants with fragments of foreign DNA of the desired size, and free of marker genes. This has and will continue to have, enormous implications with regards to approval by regulatory agencies, public acceptance and marketability of transgenic crops.

Transgenic common bean plants have been produced only through particle bombardment of meristematic cells of mature seeds (Russell *et al.*, 1993; Aragao *et al.* 1996 and 1998). However, this technology is still far away from routine application, possibly because of its low efficiency and high labor costs. Plants produced by this way also seem to have problems in transgene expression, as was the case with the plants generated by Russell *et al.* (1993; Azzam *et al.*, 199?). Up to date, no transgenic common bean cultivars with genetically engineered agronomic traits have been developed .

*Agrobacterium* mediated transformation of a *Phaseolus* bean was first reported by Dillen *et al.* (1997). These authors reported the production of a single transgenic plant from a wild tepary bean (*P. acutifolius* G.) genotype NI576, after inoculating green nodular callus derived from pedicel explants. Between 1999 and 2000, we at CIAT in conjunction with our collaborators at the University of Hannover, developed a different transformation methodology through the direct inoculation of mature seed derived embryo explants with hypervirulent *Agrobacterium* strains. We tested this methodology in several genotypes and hybrids of tepary beans and several wild and cultivated genotypes of common bean. Transgenic hygromycin resistant GUS expressing tissues and two transgenic plants were obtained only from tepary bean hybrids involving the genotype NI576 as male parent: G40022 x NI576 and G40065 x NI576. Attempts to generate transgenic plants from the female parents of these crosses, G40022 and G40065 did not produce any transgenic tissues or plants. This would suggest that nuclear gene(s) of the genotype NI576 could be responsible for its competence to *Agrobacterium* transformation using the genetic transformation methodology developed.

In 2000, we started an interspecific common x tepary bean crossing program in order to transfer the gene(s) responsible for the *Agrobacterium* transformation competence from genotype NI576 to common bean. This crossing program continued through 2001. Fertile hybrid populations involving the genotype NI576 as parent has been produced, and these are currently being tested for competence to *Agrobacterium* mediated transformation.

### Methodology

Mature but not dry pods were used as sources of sterile explants (whole mature seeds without one cotyledon) for transformation experiments. *Agrobacterium* transformation was carried out with

the hypervirulent strain LBA4404 pTOK233 (Hiei *et al.*, 1994) following the protocols described in last year's (2000) annual report. As positive controls for the transformation conditions, leaf tissues of tobacco cv Xanthi were used, from which transgenic tissues were routinely obtained. Meristematic callus induction and plant regeneration were performed as described earlier (Mejía-Jiménez *et al.*, 1998). The crossing methodology for producing interspecific hybrids involving genotype NI576 was also described in the annual report of 2000 (Mejía Jiménez *et al.*, 2000)

**Results**

*Optimization of the Agrobacterium-mediated genetic transformation of tepary beans*
The CIAT-University of Hannover jointly developed protocol for genetic transformation of tepary beans through *Agrobacterium* called *Agrobacterium* mediated mature seed meristem transformation (AMMSM-transformation) consists of the following sequential steps:
(1) Sonication of the explants for wounding;
(2) inoculation with hypervirulent *Agrobacterium* strains;
(3) co-culture at 22°C for 3 days;
(4) induction of a meristematic callus for 1 month;
(5) selection of transformed meristematic callus tissue in a culture medium with hygromycin;
(6) induction of bud differentiation of the selected calli; and
(7) whole plant regeneration through micrografting.

In order to aid the discussion of the experiments, and justify the crossing program initiated, the results of the experiments carried out in the year 2000 are shown again (table 1).

From all the genotypes tested in this experiment, only the hybrids which involved the tepary bean NI576, G40022 x NI576 and G40065 x NI576 yielded transgenic callus clones and plants (table 1). However most of the hygromycin resistant, putatively transgenic calli that were obtained from these hybrids after selection died between the selection and plant regeneration stages. This may have been caused by the very small size reached by this calli after selection (between 0.5 and 2 mm in diameter), which made *in vitro* culture and regeneration difficult. This suggests that the transformation efficiency of the competent genotypes can be increased if the *in vitro* culture steps during transformation are optimized for obtaining larger resistant and easier to cultivate calli after selection.

For this purpose several modifications of the *in vitro* culture process of the transformed explants are being investigated, including the injection of 5000 ppm of $CO_2$ into the culture vessels to increase the efficiency of transformation.

Adaptation of the *Agrobacterium* mediated transformation methodology developed in tepary bean hybrids to common bean wild and cultivated genotypes
The AMMSM transformation methodology used to produce transgenic tepary bean plants of the hybrids G40022 x NI576 and G40065 x NI576 was applied to several common bean wild and cultivated genotypes (table 2).

Most of the genotypes tested showed low or no transient expression of the GUS-gene (fast histological method for the verification of the gene transfer process from the bacterium to the plant cell) after inoculation, in the tissues from which the transgenic buds develop (cotyledonary nodes and apical meristems). This indicates that low, or in most of the cases no gene transfer from *Agrobacterium* strain to these tissues occurred during coculture. After transformation and applying selective conditions *in vitro* (30 mg/l hygromycin), no transgenic plant or tissue could be obtained even from the genotypes that showed some levels of transient GUS expression (table 2).

Thus, not only is the gene transfer from *Agrobacterium* to the cells with regeneration competence necessary for the development of transgenic plants through the AMMSM transformation methodology, but additional cellular processes are needed after the gene transfer has occurred. May be an additional cellular rearrangement capacity not found in the common bean genotypes tested is needed for allowing the production of a whole stable transformed meristem from a single transformed meristematic cell.

**Table 1. Transgenic stable GUS expressing plants and meristematic callus tissue recovered after transformation of tepary bean mature seed meristems with the *Agrobacterium* strain LBA4404 pTOK233.**

| Genotype[1] | Number of inoculated explants | Transient GUS expression[2] | Selected GUS positive m-callus clones[3] | Regenerated transgenic GUS positive plants |
|---|---|---|---|---|
| G40025 | 80 | +++ | 0 | 0 |
| G40035 | 70 | +++ | 0 | 0 |
| G40022 x NI576 | 35 | +++ | 1 | 1 |
| G40065 x NI576 | 43 | +++ | 0 | 0 |
| G40022 x NI576 | 48 | +++ | 4 | 0 |
| G40025 | 34 | +++ | 0 | 0 |
| G40022 x NI576 | 28 | +++ | 0 | 0 |
| G40025 | 80 | +++ | 0 | 0 |
| G40022 | 365 | +++ | 0 | 0 |
| G40065 x NI576 | 164 | +++ | 0 | 0 |
| G40065 x NI576 | 63 | +++ | 1 | 1 |
| G40025 | 45 | +++ | 0 | 0 |
| G40022 x NI576 | 120 | +++ | 1 | 0 |
| G40065 | 88 | +++ | 0 | 0 |
| G40065 | 392 | +++ | 0 | 0 |
| G40065 | 198 | +++ | 0 | 0 |

All are cultivated tepary bean genotypes with the exception of NI576.
After three days of co-culture three explants were scored for GUS expression. A + or a – score was given for each of the explants expressing or not GUS in the regenerable regions of the explant, i.e. the cotyledonary nodes or apical meristem.

After a minimum of a six-weeks culture in the presence of 30 mg/l hygromycin
Development of novel strategies for interspecific common x tepary bean crossing to transfer transgenes or genes involved in conferring competence to *Agrobacterium* transformation from tepary to common bean As shown before, many attempts to adapt the AMMSM transformation methodology to common bean genotypes have failed. There are two alternatives for developing common bean cultivars or breeding lines through *Agrobacterium*:

By first transforming competent tepary bean genotypes and then transferring the transgenes to common bean through interspecific crosses

By transferring the genes responsible for AMMSM transformation competence, present in genotype NI576 of tepary bean to common bean or common x tepary beans hybrids and then using the de developed AMMSM competent genotypes to transfer the transgenes to common bean cultivars via intraspecific crosses.

Obtaining interspecific crosses between common and tepary beans and hence gene introgression have been difficult to achieve. However, we have been able to transfer many morphological, biochemical and even disease resistant genes from tepary to common bean using recurrent and congruity backcrosses (Mejía –Jiménez et al. 1994; Singh and Muñoz , 1999).

**Table 2 .Common bean (*P. vulgaris*) cultivars, breeding lines and wild genotypes tested for competence for AMMSM-transformation using the strain LBA4404 pTOK233 and the protocol developed for tepary bean.**

| Genotype | Number of inoculated explants | Transient expression[1] | Selected GUS+ m-callus clones | Transgenic GUS + plants regenerated |
|---|---|---|---|---|
| La Victorie | 110 | +++ | 0 | 0 |
| G3807 | 42 | ++- | 0 | 0 |
| G18253 | 51 | ++- | 0 | 0 |
| A295 | 48 | +-- | 0 | 0 |
| G04090 | 31 | +-- | 0 | 0 |
| G01853 | 35 | +-- | 0 | 0 |
| Tamazulapa | 29 | +-- | 0 | 0 |
| ICA Pijao | 95 | +-- | 0 | 0 |
| BAT 93 | 49 | +-- | 0 | 0 |
| G6413 | 33 | --- | 0 | 0 |
| G18255 | 41 | --- | 0 | 0 |
| G05706 | 25 | --- | 0 | 0 |
| G04449 | 46 | --- | 0 | 0 |
| G11071 | 47 | --- | 0 | 0 |
| G02997 | 38 | --- | 0 | 0 |
| Vax1 | 26 | --- | 0 | 0 |
| Vax2 | 68 | --- | 0 | 0 |
| Vax3 | 44 | --- | 0 | 0 |
| Vax4 | 37 | --- | 0 | 0 |
| Vax5 | 42 | --- | 0 | 0 |
| Vax6 | 48 | +-- | 0 | 0 |
| C20 | 53 | --- | 0 | 0 |
| Jalo EEP 558 | 31 | --- | 0 | 0 |
| Olathe Pinto | 40 | --- | 0 | 0 |
| Wild *P. vulgaris* genotypes | | | | |
| G10013 | 23 | --- | 0 | 0 |
| G10024 | 70 | +-- | 0 | 0 |
| G12875 | 10 | --- | 0 | 0 |
| G12922 | 27 | +-- | 0 | 0 |
| G12947 | 26 | --- | 0 | 0 |
| G23429 | 38 | +++ | 0 | 0 |
| G23490 | 39 | +-- | 0 | 0 |
| G23511A | 13 | --- | 0 | 0 |
| G23652 | 81 | ++- | 0 | 0 |
| G23653 | 83 | ++- | 0 | 0 |
| G27893 | 17 | --- | 0 | 0 |

1 After three days of co-culture three explants were scored for GUS expression. A + or a – score was
2 given for each of the explants expressing or not GUS in the regenerable regions of the explant, i.e. the cotyledonary nodes or apical meristem.

The possibility of transferring transgenes from genetically modified tepary bean plants to common bean has already been demonstrated last year, with the production of GUS positive

hybrids with the cytoplasm of common bean after a series of crosses with the GUS positive transgenic plant of tepary bean (see annual report 2000). At least three crosses to intermediate bridging hybrids are however needed in order to transfer a transgene to a common bean fertile plant. The successful transfer of a transgene will depend on the site of its insertion in the tepary genome, and a risk will always exist that it is linked to undesired characteristics of the tepary bean.
An easier and faster transfer of a transgene to a common bean cultivar, and fewer risks of this linkage to undesirable traits are expected if the second alternative is chosen, and a common bean genotype is developed which is competent to *Agrobacterium* transformation.

The crossing of common bean genotypes to the wild tepary bean genotype NI576, and the production of fertile progeny from the hybrids, has been especially difficult. However, the application of a novel crossing strategy (Mejia *et al.*, 2000), has led to the production of some populations of fertile hybrids.
These populations were tested for competence to *Agrobacterium* transformation, but up to date no competent line to *Agrobacterium* transformation has been identified (data not shown).

With the advancement in this progressive crossing program, more and more fertile populations involving the genotype NI576 in its pedigree are being developed and with less difficulty. Thus for the year 2002, several hybrid populations will be available to be screened for competence to *Agrobacterium* transformation, and transgenic plant production.

*Biolistic transformation of common bean*

Biolistics, that is the genetic transformation methodology that uses the particle gun to introduce the DNA into the plant cells, has been the only transformation methodology that has led to the recovery of transgenic common bean plants (Russell *et al.*, 1993; Aragao *et al.* 1996 and 1998). This methodology has been applied in bean transformation since 9 years in some labs (Smith *et al.*, 1992), but up to date no transgenic cultivars with engineered agronomic traits have been developed this way. One possible explanation for this failure could be the low efficiency of recovery of transgenic plants achieved. From the produced transgenic populations, the plants expressing the transgenes in the desired way have to be selected. High numbers of transgenic plants should be produced more efficiently in order to apply this technology to common bean breeding programs.

One way for increasing the efficiency of biolistic transformation could be the induction of meristematic calli (Mejía-Jiménez *et al.*, 1998) after particle bombardment of the meristems found in mature seeds. These regenerable callus masses that can be maintained in in vitro culture for several months could allow for a better selection of the transgenic cells and meristems after transformation. In the current year 2001, we attempted using this protocol of transformation followed by induction of meristematic calli to transform the common bean cultivar Bayo Madero, the best-known common bean genotype for developing meristematic calli. From these experiments we have not been able to select transgenic tissues expressing the transgenes. It seems therefore that biolistic transformation of plant meristems is not such a genotype-independent transformation methodology as has been proposed before.

In the coming year 2002, experiments will be carried out in biolistic transformation of different common bean cultivars, followed by meristematic callus induction. Cultivars that have yielded transgenic tissues or plants in experiments made before will be included.

## Conclusions

It seems that we would be able to solve the the "genetic transformation problem" of common bean by transferring the genes conferring competence to AMMSM-transformation from tepary beans via interspecific crosses. Also, the production of tepary x common bean hybrids (tepary bean cytoplasm) competent to AMMSM-transformation would be useful for a fast transfer of transgenes to common bean cultivars through sexual crosses.

## Future plans

To optimize the process of genetic transformation of competent genotypes to *Agrobacterium.* The objective is to increase the growth of the meristematic calli induced after transformation, in order to increase the survival rate of antibiotic resistant calli after selection

To further generate progenies of interspecific common and tepary beans involving the competent genotype of tepary bean to *Agrobacterium* transformation

To further screen common x tepary bean hybrid progenies which involve the genotype NI576 of tepary bean, for competence to *Agrobacterium*-mediated transformation of mature seed meristems

To perform biolistic transformation experiments using different cultivars of common bean

To initiate co-transformation experiments with binary *Agrobacterium* strains carrying marker genes and agronomic important genes in different plasmids, in order to produce marker gene free transgenic plants

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## 2.1.2 Transformation of cassava cultivars with a *cry*1Ab gene for insect resistance

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SB-2 Project

### Introduction

The control of Lepidopteran pests using transgenic plants that express *cry* genes is a reality for cash crops like maize, cotton and soybean (James 2000). Cassava also has pests of economic importance that belong to this family, the most important in Colombia being the stem borer *Chilomima clarkei*. Varietal resistance for the stem borer has not yet been identified. The rapid expansion of the stem imposes the pace to obtain resistant varieties. We are trying to deploy genetic resistance to *C. clarkei* by introducing a *cyr*1Ab gene from *Bacillus thuringiensis* () into commercial varieties. Two cassava cultivars of commercial interest (MCol 2215 or Venezolana , and CM-3306-4 or ICA Negrita),and one model cultivar (TMS60444) are the targets for transgenesis mediated by *Agrobacterium* and the particle gun. We describe here the latest advances in the production of transgenic cassava plants.

### Materials and Methods

Three cultivars are being used for transformation: TMS60444 (control), MCol2215 (Venezolana) and CM2177-3 (Ica Negrita). We are testing two plasmids containing the gene of interest, *cry*1Ab, the gus-intron gene for scoring early transformation events, and selectable marker genes. Plasmids are described in Annual Report 2000, Project SB-02. One plasmid (pSGManCry) contains the *pmi* gene (phospho mannose isomearse) for selection on media with mannose instead of sucrose. A second plasmid (pBIGCry) contains the *npt*II gene for selection on antibiotic-containing media. Both plasmids are being used with *Agrobacterium* and the Particle Gun for insertion into Friable Embryogenic Callus (FEC) of the three varieties. FEC is usually pretreated on medium with high sucrose concentration before shooting. It is desired to increase transformation efficiency.

### Results and Discussion

Stable transformation, mediated by *Agrobacterium*, has been achieved with TMS60444. There are four plant lines in vitro and/or the greenhouse, each one representing independent transformation events (Table 1; Figure 1).

**Table 1. Transgenic plant lines of cassava obtained through *Agrobacterium*-mediated transformation.**

| Plant lines of TMS60444 (date of transformation) | # of plants *in vitro* or greenhouse (GH) | *Agrobaterium* strain | Time under selection with antibiotics | Gus expression |
|---|---|---|---|---|
| 55 (May 5, 2000) | 28 *in vitro* | C58C1-pBIGCry | 4 months | Positive for FEC and Plants* |
| 80 (May 5, 2000) | 3 in GH >60 *in vitro* | Same | Same | Positive for FEC, not for plants |
| 92 (Oct 20, 2000) | > 20 *in vitro* | Same | Same | Positive for FEC and plants* |
| 270400 (Apr 27, 2000) | 1 in GH | LBA4404-pBIGCry | 7 months | Negative |

(*) Lines 55 and 92 (Figure 1) are high and fast expressors of the gus gene. Usually it takes less than an hour, at room temperature (25°C), for them to develop and homogeneous blue color in stems, shoots and leaves.

Molecular confirmation of the transgenic status of all four lines is currently done through PCR and RT-PCR for *cry* gene insertion and expression. Plants are still young for DNA extraction for Southern and Northern analysis. PCR has shown bands of the expected size for the *cry* gene. However, most probably due to the high GC content of primers, non-specific, amplified bands appeared in non-transgenic cassava lines as well. The identity of non-specific bands is being confirmed by Southern hybridization of PCR products. Further standardization of PCR conditions to amplify *cry* is required to report accurate data.

Transient expression of plasmid pBIGCry has been observed several times in MCol2215 after transformation with *Agrobacterium* strain C58C1. However, it has not been possible to recover FEC expressing *gus* (stable FEC transformants) on media with antibiotics as it was reported in the previous Annual Report (2000). We have found that besides the sensitivity to *Agrobacterium* infection, FEC from MCol2215 grows much slower than that of TMS60444 on non-selective media (Figure 2). This may partially explain the lack of success with the former cultivar. Rapid growth helps the cells to recover fast and survive on selective media after transformation. We are therefore intensifying our efforts with non-antibiotic, selectable markers like *pmi* for transformation with *Agrobacterium* and the particle gun. Although the gun may not be the most desirable method for transformation due to multiple and incomplete insertion events (which may speed up gene silencing), cells are less stressed after transformation, and do not undergo long treatments with antibiotics to eliminate supervirulent *Agrobacterium* strains.

The results with the gun show that pre-treating cells with high sucrose concentration before shooting them increases transient or stable expression of the *gus* gene after 2 to 6 weeks on selection with antibiotics or mannose. The expression of the *gus* gene from plasmid pBIGCry is usually stronger than that from pSGManCry, so more blue spots are observed with the former.

After shooting, cells from all three cultivars resume growth easily in liquid medium, maintaining their yellow color, indicating that shooting is not as stressful as infection with *Agrobacterium*.

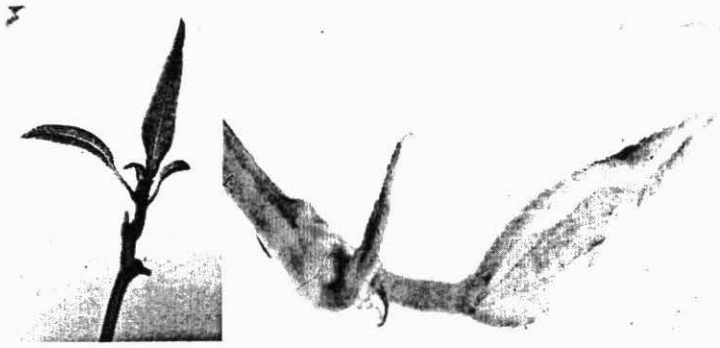

**Figure 1. Transgenic cassava plant lines 55 (left) and 92 from cultivar TMS6044, resistant to selection with geneticin and paramomycin, expressing gus and probably carrying the gen *cry*1Ab (inserted between the *gus*-intron and *npt*II genes in the same construct). Both lines were obtained through *Agrobacterium*-mediated transformation.**

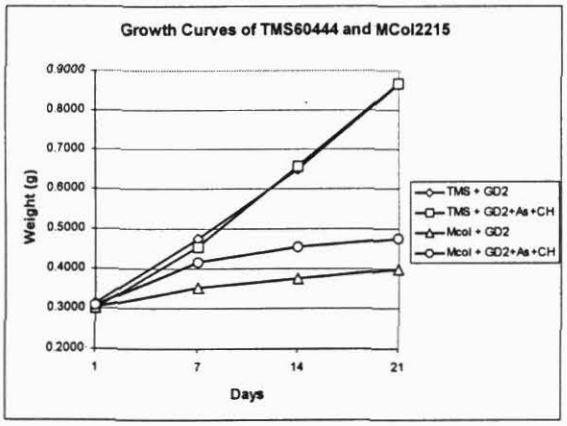


**Figure 2. *Growth curves of* FEC from cassava cultivars TMS60444 (TMS) and Mcol2215 (Mcol) on Gresshoff and Doy (1974) medium (GD2). Note that the doubling time for the former is less than 14 days, while for the latter it takes more than 21 days. (AS = asparagine; CH = *caseine hydrolyzate*)**

## Conclusions and Ongoing Work

Transformation of cassava cultivar TMS60444 using *Agrobacterium* has been achieved at BRU. Full transgenic plants, growing in the greenhouse and expressing inserted genes, can now be obtained in about 11 months. TMS60444 becomes then a model to fast-test genes of interest in cassava. We will perform molecular tests to confirm transgenesis of TMS lines and the expression of the *cry* gene.

These transgenic plants may now be used for bioassays to test if they can control Lepidopteran insect pests of cassava like the stem borer (*C. clarkei*).

Transformation experiments will continue using *Agrobacterium* and the gun with emphasis on commercial cultivars for which FEC has been established (i.e., for Mcol2215, CM3306-4 and SM1219-9). We will also emphasize on the use of non-antibotic markers (i.e., *pmi*) for selection of transgenic lines.

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## 2.1.3 Transformation and Regeneration of some CIAT Elite Cultivars: Towards Testing Candidate CMD Resistance Genes

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[1]SB-2 Project ; [2]IP-3 Project; [3]ILTAB

**Introduction**

Following the discovery of a single dominant gene(*CMD2*) that controls the novel form of resistance to CMD and efforts to identify candidate genes by the serial analysis of gene expression (SAGE), the stage is set for gene function analysis of *CMD2*. However genetic complementation of gene function is severely hampered by a lack of routine and robust transformation and regeneration methods for Latin American cultivars, the target group of CMD susceptible cultivars. A series of experiment was therefore set up in collaboration with the Danforth center to examine and refine the different steps for cassava regeneration and transformation

The first step in the development of tissue for transformation and regeneration experiments is the induction of embryogenesis. Cassava varieties are known to respond differently to embryogenesis. There is therefore a need to first screen for responsive lines and to identify optimal conditions for embryogenesis. A set of elite materials from CIAT were selected for the experiments based on their success with farmers and as parents for cassava breeding.

**Methodology**

A list of the seven CIAT cassava elite lines used in this study can be seen in Table 1. The lines were multiplied in-vitro in MS2 (Murashige and Skoog's basal medium supplemented with 2%sucrose) medium to generate large quantities of plantlets. This was to facilitate production of young leaf lobes for explanting to perform primary embryogenesis experiment. Unopened young leaf lobes were excised from these plantlets at 4weeks old and placed on Murashige and Skoog's media supplemented with 2% sucrose and 50Mm Picloram(MS2,50P). Alongside this work, explants from 4 week-old plantlets from model cultivar for cassava embryogenesis, TME 60444 were cultured on MS2, 50P and placed at different light intensity. This was to identify the optimal condition for embryogenesis, since light is known to be a crucial factor. Three light levels were tested-dark, low light (0.42 $\mu$mol $m^{-2}$ $s^{-2}$) and high light (7.2 $\mu$mol $m^{-2}$ $s^{-2}$). Globular embryogenic structures from model cultivar 60444 and the 4 responsive lines from CIAT were excised and placed on Gresshorf and Doy (1974) medium, supplemented with 2% sucrose, 50Mm Picloram. These cultures were in culture rooms and at 3-4 weeks, the new globular embryogenic structures produced were transferred to fresh GD2, 50P medium.

## Results

The result of the embryogenesis experiment in the CIAT lines are summarized in Table 1. Three CIAT lines, CM523-7, CM6740, CM2177-2 had a conversion rate to embryogenic structures from explant comparable to the control cultivar TMS60444. Pretreating *in vitro* plants in different light regimes did not have a significant influence on the induction of embryogenic tissues. However, when young unopened leaf lobes were explanted onto Murashige and Skoog basal media supplemented with 2% w/v sucrose and 50 µM picloram, low light was found to be significantly superior no light and high light treatments. Low light lead to an increase in the formation of embryogenic structures by a factor of two.

After two cycles FECs were produced in the model cultivar. These were then multiplied to generate more target tissues for transformation experiment. After 3 cycles FECs were generated in 3 out of the 4 lines from CIAT. Work is on going to increase the conversion rate among the CIAT lines and also produce more OES from explants in order to increase the generation of FEC

## Future Plan

Conduct transformation and regeneration efficiency experiments, with FEC from model cassava cultivar TME 60444 as target tissues, to compare particle gun bombardment and Agrobacterium mediated transformation

Translate the findings from above to elite lines from CIAT from which FEC have been generated to achieve a transgenic lines with genes of interest.

**Table 1. Conversion rate of explants from CIAT's elite cassava lines to embryogenic structures**

| Serial number | Genotype | Percent Putative Embryogenic Tissue | Percent Organized Embryogenic Tissue | No of explant used |
|---|---|---|---|---|
| 1 | CM523-7 | 20 | 70 | 80 |
| 2 | CM6740 | 20 | 50 | 90 |
| 3 | CM2177-2 | 20 | 75 | 80 |
| 4 | CM4574-7 | 1 | 1 | 80 |
| 5 | Tai1 | 1 | 1 | 80 |
| 6 | Tai8 | 0 | 60 | 80 |
| 7 | MBRA383 | 5 | 1 | 80 |
| 8 | Control TME60444 | 20 | 75 | |

## 2.1.4 Control of RHBV (Rice Hoja Blanca Virus) through nucleoprotein mediated cross protection in the greenhouse and in the field.

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### Introduction

Rice hoja blanca virus (RHBV) is a major virus disease of economic importance affecting rice in northern South America, Central America and the Caribbean. Rice transformed with the RHBV nucleocapsid protein (*N*) gene had a significant reduction in disease development. Reactions to inoculation with RHBV ranged from susceptible to completely resistant plants (immunity). The most frequent reaction was characterized by local necrotic lesions (hypersensitive reaction) followed by the production of new leaves without symptoms. Other plants developed chlorotic lesions in the inoculated leaves, but recovered producing tillers free of virus (recovery phenotype). These transgenic RHBV resistant rice lines expressed the *N* gene RNA at low levels that could be detected using RT-PRC but not by Northern blots analysis. The nucleocapsid protein could not be detected in any of the transgenic plants either by Western or ELISA tests. These results suggest that resistance conferred by the *N* gene is RNA mediated. Earlier reports indicated that besides the resistant phenotype when challenged with RHBV, the resistant transgenic lines showed significant increased performance for important agronomic traits including number of tillers, number of grains per plant, and yield as compared to the susceptible control. Upon inoculation some of the resistant transgenic plants showed agronomic traits similar to the uninoculated non-transgenic Cica 8 control. Using both agronomic traits and disease severity as criteria, several of the most resistant lines were followed through the R4 generation in the greenhouse and demonstrated that the *N* gene and RHBV resistance was inherited in a stable manner. Last year, results also suggested that the resistance conferred by the *N* transgene towards RHBV disease is expressed independently of the genotype background. The transgenic resistance could be used to complement the natural resistance source to the virus, when crossing selected transgenic lines with diverse genotypes carrying the breeding resistance gene(s). Results showed that the non-transgenic $F_1$s control plants were susceptible, whereas the transgenic $F_1$s were resistant even when inoculated at 10-day-old. These results suggested that the protection conferred by the RHBV-N transgene is inherited and expresses independently of the genotype background, and that the transgene could be used to complement the natural resistance source. This year report includes the characterization of mode expression of the transgenic resistance conferred by RHBV-N. The evaluation in the field for two semesters of transgenic lines representing various generations, and $F_2$ populations derived from crosses with Fedearroz 50, Oryzyca 1, Iniap 12, and Cica 8. Planting was coducted at CIAT headquarters upon approval from the Colombian Biosafety Committee on September 2000. It is also reported the progress generating transgenic rice containing the RHBV non-structural 4 ($NS_4$) gene from the RNA 4.

### Materials and Methods

#### RHBV Resistance Assays

For the greenhouse experiments, in order to characterize the RHBV-N resistance according to the plant age plants of 15 or 28 days old were inoculated with RHBV using four 2nd or 3rd instar *T.*

*orizicolus* nymphs per plant, from a colony of 80% virulence. Insects fed on the test plants for 5 days when insecticide is applied. Controls consisted of transgenic plants of Cica 8 carrying only the hygromycin resistance (*hpt*)gene, which was used as the selectable marker to generate the RHBV-N transgenic plants, or non-transgenic plants of Cica 8. Plants were scored for the development of RHBV disease symptoms every 3 days for 25 days and then evaluated once a week for 5 weeks. Plants were scored for the date of the first appearance of symptoms, and the percentage of leaf affected by RHBV was determined.

For the field evaluations, 280 transgenic lines and $F_2$ plants derived from crosses between resistant transgenic plants with Fedearroz 50, Oryzyca 1, Iniap 12, and Cica 8 were planted in November 2000, and 486 transgenic lines and $F_3$ plants derived from the same crosses described above were planted in July 2001. Lines were planted in a randomized plot design with 3 or 4 replications in the field, for years 2000 and 2001 respectively. Plants were inoculated at 15 days of age with two insects per plant from a colony of 80% virulence. Insects were feeding on the plants for 15 days in the first season, and for 10 days in the second season, upon when insecticide was applied as a biosafety control measurement. Plants were evaluated for the development of disease symptoms every two weeks until 45 days of age. Disease evaluations were conducted using an scale from 0 to 9, were 0 refers to no disease symptoms, and 9 indicates more than 90% leaf area is affected by the RHBV disease. Plants with rating from 1 to 3 = resistant, score 5 = intermediate, and 7-9 = susceptible.

**Results and Discussion**

*Comparative Level of RHBV-N Resistance in Transgenic Rice in the Greenhouse Respect to the Field.*

Last year we showed that line A3-49-60-12-3-3 showed the highest level of resistance throughout the whole life cycle. Between 74% to 81% of the plants did not show any disease symptoms when inoculated either at 15 days or 28 days of age, and only a 22% of the plants showed more than 25% of the leaf area affected when inoculated at 15day-old (Table 1). In contrast, Cica 8 control showed 100% of the plants with severe disease symptoms at 15-day-old (Table 1). Line A3-49-60-4-5-8 showed intermediate level of resistance at 14-day-old (Table 1) and 71% of the plants without symptoms at 28-day-old .About 70% of the plants of line A3-49-60-19 had less than 25% of leaf area affected at 15-day-old (Table 1). Sister lines A3-49-60-12-3-1 (susceptible) y A3-49-60-12-3-3 (resistant) showed different disease reaction indicating that the resistant phenotype was still segregating at the $T_4$ generation or gene silencing was affecting the expression of the RHBV-N gene in some of the plants. This year results indicated that there is a high correlation between level of resistance seen in the greenhouse with that in the field (Table 1).

*Field evaluation of RHBV resistance in transgenic rice containing the nucleoprotein gene*

Advanced generations of transgenic lines with stable RHBV resistance were selected first in the greenhouse until permit for the field test was granted by the Colombian Biosafety Committee on September 2000. Field was first planted on November 2000 to conduct evaluations for RHBV resistance and agronomic traits following International as well as the Colombian environmental biosafety regulations at the biosafety rice field located in CIAT experimental station Palmira.

**Table 1.- Comparative Disease Reaction in the Greenhouse and the Field in Plants Inoculated at 15 days old**

| | Greenhouse Leaf Area Affected (%) | | | Field[3] Disease reaction | |
|---|---|---|---|---|---|
| Line | 0 | >0-25 | >25-100 | 2000 | 2001 |
| A3-49-60-12-3-3 | 74 | 4 | 22 | 1 | 2 |
| A3-49-60-19 | 53 | 12 | 36 | 2 | 3 |
| A3-49-60-4-5-8 | 54 | 0 | 46 | 3 | |
| A3-49-60-13 | 25 | 4 | 70 | 3 | |
| A3-49-56-15 | 9 | 13 | 78 | 6 | 6 |
| A3-49-60-12-3-1 | 7 | 0 | 93 | 6 | 7 |
| A3-49-101-18-19-2 | 15 | 0 | 85 | 8 | 6 |
| A3-49-78[2] | 0 | 0 | 100 | ND | 7 |
| Cica 8 | 0 | 0 | 100 | 9 | 7 |

[2] Transgenic control with the *hpt* gene only. ND = not determined
[3] Mean values of three (in 2000) or four (in 2001) replicates

Replicated trials were conducted as described in materials and methods. Disease evaluations were conducted using an scale from 0 to 9, were 0 refers to no disease symptoms, and 9 indicates more than 90% leaf area is affected by the RHBV disease. Plants with rating from 1 to 3 = resistant, score 5 = intermediate, and 7-9 = susceptible. Besides the non-transgenic variety Cica 8, the transgenic Cica 8 line A3-49-78) carrying only the hygromycin resistance (*hpt*)gene, used as the selectable marker to generate the RHBV-N transgenic plants, and which does not contain the RHBV-N transgene , wasused herein as controls. Other 12 varieties were used as reference for differential disease reaction pattern. This variety differential included: Caribe 8, Capirona, Cimarrón, Colombia 1, Fedearroz 50, Fedearroz 2000, Fedearroz Victoria 1, Fundarroz PN1, Iniap 12, Linea 2, Oryzica 1, Oryzica Llanos 5, and Palmar.

Field evaluations corroborated results obtained previously in the greenhouse. Forty five entries derived from line A3-49-60-12-3-3 were highly resistant showing scores 1 to 3. A subset of these lines are shown in Table 1. Resistance in advanced transgenic lines ($T_6$ and $T_7$) was inherited stably (95% of derived progeny plants were reproducibly resistant) indicating that the RHBV-N transgene seems to be fixed in each of these transgenic lines (Table 1) . The most resistant lines were A3-49-60-12-3-3-79, A3-49-60-12-3-3-24, A3-49-60-12-3-3-28, A3-49-60-12-3-3-32, A3-49-60-12-3-3-68, A3-49-60-12-3-3-67, A3-49-60-12-3-3-72 (Figure 1). These lines were more resistant than Fedearroz 2000 in average over the two field evaluations (Figure 1).Other transgenic lines were intermediate giving ratings between 3 to 5, but several transgenic lines were as resistant as Fedearroz Victoria (Figure 1). Large number of lines were more resistant than commercial varieties, and the resistance genetic gain from Cica 8 to the transgenic lines was a reaction score change from 7-9 (Cica 8) to 1-3 (best transgenic lines) (Figure 1). Transgenic line A3-49-78 which only carries the hygromycin resistant gene and does not contain the RHBV-N gene was as susceptible as Cica 8 (Table 2) indicating that the resistance noted in the RHBV-N transgenic lines is due to the RHBV *N* viral gene. Fedearroz 50 , the main variety currently commercially grown in Colombia, was susceptible to the virus (score 7) and more susceptible than Fundarroz PN1 (score 5.5) which reacted as intermediate (Figure 1). Similar progress towards resistance was noted in the crosses between the highest resistant transgenic lines and the varieties. Crosses between non-transgenic Cica 8 and Fedearroz 50, Oryzica 1, and Iniap 12 gave an average of disease reaction of 8.0, 7.0, and 8.5 respectively corresponding to susceptible phenotype, in contrast the corresponding crosses generated with the transgenic resistant plants and the same varieties showed an average disease reaction of 4.5, 4.0, and 4.0 respectively . The crosses are at $F_3$ generation, thus the RHBV-N gene is still segregating in those lines. Resistant

plants (score 2-3) within those segregating $F_3$ lines had been identified. These field results corroborate earlier findings indicating that the protection conferred by the RHBV-N transgene is inherited and expresses independently of the genotype background, and that the transgene could betransferred to breeding populations by standar crossing. Sister lines of the resistant transgenic lines and transgenic crosses evaluated last season indicated that some of the plants showed a yield potential similar to varieties as Fedearroz 50, Fedearroz Victoria 1, and Oryzica 1. Currently progeny plants derived from resistant plants selected in the first field evaluation are currently being selected for yield potential and other agronomic traits.

**Table 2.- RHBV Resistance Evaluations of Transgenic RHBV-N Plants Over Two Consecutive Seasons in the Field**

| | Score[1] | |
|---|---|---|
| Line | 2000 | 2001 |
| A3-49-60-12-3-3-68 | 1 | 3 |
| A3-49-60-12-3-3-32 | 1 | 3 |
| A3-49-60-12-3-3-79 | 1 | 2 |
| Fedearroz 2000 | 2 | 3 |
| Victoria 1 | 2 | 4 |
| A3-49-60-12-3-3-24 | 2 | 2 |
| A3-49-60-12-3-3-28 | 2 | 3 |
| A3-49-60-4-5-8-79 | 2 | 4 |
| A3-49-60-12-3-3-72 | 2 | 3 |
| A3-49-60-13-2 | 2 | 2 |
| A3-49-60-12-3-3-19 | 3 | 3 |
| A3-49-60-12-3-3-18 | 3 | 4 |
| A3-49-60-12-3-3-78 | 3 | 3 |
| A3-49-60-13-8 | 3 | 4 |
| A3-49-60-13-1 | 3 | 3 |
| A3-49-60-12-3-3-77 | 3 | 4 |
| A3-49-60-12-3-3-12 | 3 | 3 |
| A3-49-60-12-3-3-59 | 3 | 4 |
| A3-49-60-12-3-3-3 | 3 | 3 |
| A3-49-60-12-3-3-14 | 3 | 3 |
| A3-49-60-12-3-3-67 | 3 | 2 |
| A3-49-60-19-8 | 4 | 4 |
| A3-49-60-12-3-3-23 | 5 | 4 |
| A3-49-101-18-19-2 | 5 | 6 |
| Fundarroz PN1 | 5 | 6 |
| Palmar | 6 | 6 |
| Iniap-12 | 8 | 8 |
| Colombia 1 | 9 | 7 |
| Oryzica 1 | 8 | 7 |
| Capirona | **8** | **7** |
| Caribe 8 | **8** | **8** |
| Cimarron | **8** | **8** |
| Fedearroz 50 | **7** | **7** |
| Linea 2 | **6** | **7** |
| Oryzica 1 | **7** | **7** |
| Oryzica Llanos 5 | **7** | **7** |
| A3-49-60-10-27 | **ND** | **1** |
| A3-49-78 * | **ND** | **7** |
| Cica 8 | **9** | **7** |

(*) Transgenic line carrying only *hygromycin resistance gene* .It does not contain the RHBV-N transgene.
[1]Mean values of three (in 2000) or four (in 2001) replicates

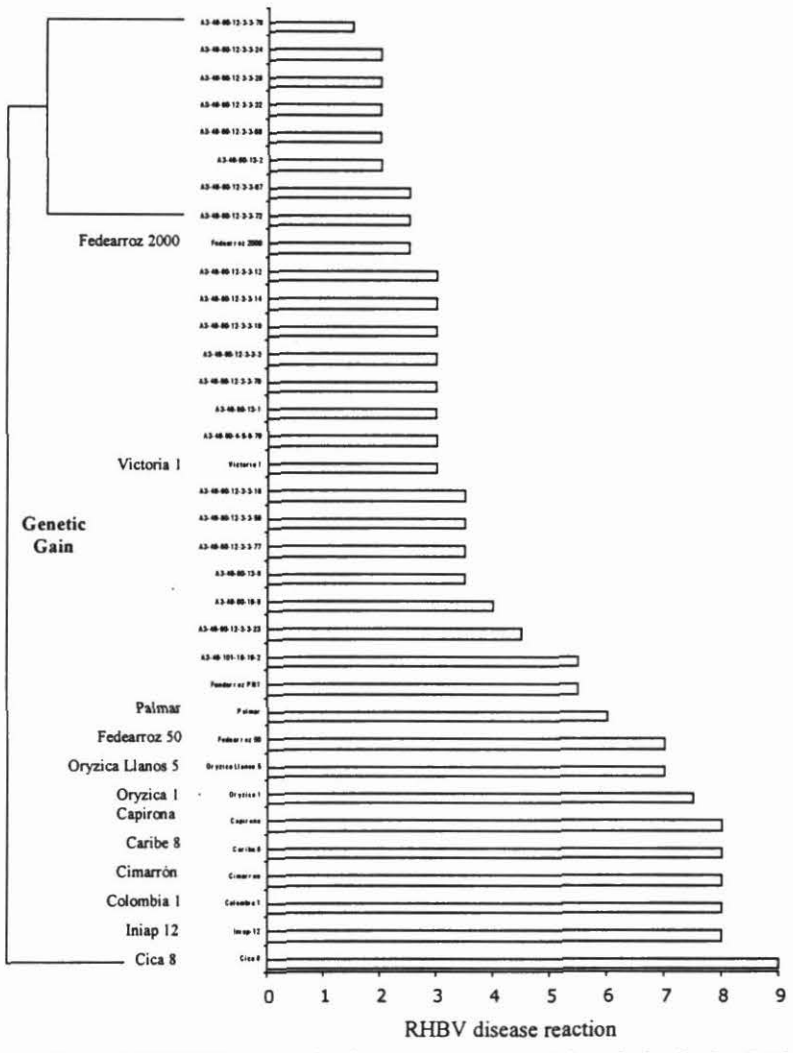


**Figure 1.- Disease reaction of RHBV-N transgenic plants and commercial varieties in the field. Mean values of two evaluations in November 2000 and July 2001.**

## 2.1.5 Characterization of Transgenic Rice Containing the RHBV Non-Structural 4 ($NS_4$) Gene from the RNA 4

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[1]SB-2 Project ; [2]IP-4 Project

### Introduction

The genome of RHBV consists of four species of ssRNA designated RNA 1, 2, 3 and 4. The RNA 4 consists of 1991 nucleotides with two open reading frames (ORFs). The most important ORFs is located in the 5′ proximal region of the viral RNA 4. The RNA 4 encodes a major non-structural protein ($NS_4$), which accumulates in the tissues of the infected plants with the RHBV. $NS_4$ protein is clearly distinguishable from the nucleoprotein (N) by specific antiserum. Another difference between $NS_4$ protein and N protein, is that the former only is expressed in the plant whereas the latter expresses both in the plant and the insect vector. It is inferred from the

differential expression of these proteins that the major $NS_4$ protein may has a function that is needed in the plant but not in the plant-hopper. The differential plant-insect $NS_4$ expression, and the similarity of $NS_4$ sequence with well characterized helper proteins described for other insect transmitted viruses, suggest that $NS_4$ might be involved in the RHBV transmission from the plant to the plant-hopper, or in the virus movement from cell to cell. The main goal for the expression of the RNA 4 in transgenic rice is to determine the function of the major $NS_4$ protein and study the potential for a novel and different method of producing viral resistant plants.

## Materials and Methods

### Rice Transformation

Mature embryos derived calli from *indica* varieties CICA 8, Palmar, Cimarrón and Fundarroz PN1, where used as targets. We used *Agrobacterium* Agl1 strain mediated transformation to introduce the $NS_4$ gene. Constructs *pIC002* and *pIC004* contain the RHBV $NS_4$ gene in sense and anti-sense orientations respectively, driven by the 35S CaMV promoter using as backbone the plasmid *pCAMBIA* 1301 which carries the *gus-intron* and hygromycin resistance genes. Constructs *pIC007*and *pIC009* contain the $NS_4$ sense gen, and *pIC008 the* $NS_4$ anti-sense gene driven by the ubiquitin or 35 S CaMV promoter. These genes were cloned into *pWBVec8* plasmid (from Peter Waterhouse's laboratory at CSIRO, Australia), which carries the hygromycin-cat 1 intron gene as selectable marker. Plants were regenerated after stepwise selection on 30 mg/l and 50 mg/l hygromycin, followed by 50 mg/l hygromycin throughout plant differentiation. Plants were grown to maturity in the biosafety glasshouse.

### Molecular Analysis of the Transgenic Rice Plants

*Southern Analysis.*15 µg DNA genomic were digested with Eco RI, fractionated in 1.0 % (W/V) agarose gels, and transferred to nylon membranes (N+ Amersham). The hybridization probe was a radioactively labeled 850 pb PCR fragment amplified using primers RHBV4 forward and reverse. DNA probes were random primer labeled and hybridization was carried out overnight at 55°C.

*RT-PCR and Northern Analysis.*Total RNA was extracted from 100 mg of fresh material using the RNAeasy ™ plant total RNA kit (Quiagen, Dorking, UK). The cDNA synthesis was done with the SUPERSCRIPT™ One Step RT-PCR system. Following the manufacturer's instructions and using primers RHBV4 forward and reverse. Northern analysis was carried out with 15 µg of total RNA per lane, using denature RNA gels with formaldehyde and formamide (Sambrook et al., 1989).

*Characterization of Sequence of* $NS_4$ *Gene.* Nucleotide sequence of the gene $NS_4$ in four $T_0$ generation transformed plants was carried out using the ABI PRISM. Dye terminator kit (Perkin-Elmer) with primers RHBV4 forward and reverse. These products of PCR were applied to Biosystems Prism 377 DNA sequencer (Perkin-Elmer) and edited with Sequencher (Genecodes, Ann Arbor, MI). The sequences were analyzed using the BLAST algorithm (Altschul et al., 1997).

*RHBV resistance assays.* Inoculations and evaluations were conducted in the greenhouse following the same procedure as described above for the RHBV N transgenic plants.

## Results and Discussion

Last year we reported the generation of 10 different constructs carrying the $NS_4$ gene in sense and anti-sense orientations. A total of 21 transgenic plants carrying the $NS_4$ sense orientation, and 70 plants carrying $NS_4$ anti-sense orientation were produced (Table 1). The plant regeneration effciency varied according to the genotype from 2% to 44%. Southern blot analyses using *Bam HI* or *Eco RI* which excise the complete $NS_4$ gene in sense or antisense orientation, or using *Sal I* which does not cut the gene cassette within the right and left borders indicated that between 50% to 100% of the regenerated plants analyzed contained the $NS_4$ gene (Table 1), and most cases the $NS_4$ gene is integrated as a single non-rearranged copy.

**Table 1. Transformation Efficiency of Three *indica* Varieties Using the RHBV $NS_4$ in Sense an Anti-Sense Genes**

| Genotype | Plasmid | $NS_4$ | Plants | RE % | Plants $S^+$ | % Plants $S^+$ | TE % |
|---|---|---|---|---|---|---|---|
| PALMAR | pICOO7 | Sense | 1 | 2 | ND | | |
| | pICOO9 | Sense | 1 | 3 | ND | | |
| | pICOO4 | @Sense | 13 | 28 | 13/13 | 100 | 28 |
| | pICOO8 | @Sense | 9 | 20 | 6/6 | 100 | 20 |
| CICA-8 | pICOO2 | Sense | 14 | 33 | 11/14 | 79 | 26 |
| | pICOO7 | Sense | 6 | 12 | 2/3 | 67 | 8 |
| | pICOO9 | Sense | 18 | 44 | 18/18 | 100 | 18 |
| | pICOO8 | @Sense | 10 | 18 | 5/10 | 50 | 9 |
| CIMARRON | pICOO8 | @Sense | 19 | 48 | ND | ND | ND |

@Sense = anti-sense. RE = plant regeneration efficiency. $S^+$ = Southern positive. TE = transformation efficiency . ND = not determined

The $NS_4$ gene was also amplified by PCR generating the expected gene size. The PCR product was sequenced, and in all cases the sequence corresponded to the entire $NS_4$ gene indicating that the transgene did not have rearrangements. PCR analysis for the *gus-intron*, *hpt*, and *$NS_4$* transgene; and Southern blot, RT-PCR and Northern analyses for the $NS_4$ of $T_1$ plants derived from $T_0$ identified as transgenic by Southern blot, indicated that not all $T_1$ plants inherited the three transgenes (i.e *gus-intron*, *hpt* and *$NS_4$* )(Table 2). Variation in the level of *gus* expression was also noted, and the expression of either *gus* or $NS_4$ was not always detected although plants contained the corresponding gene. These results suggestthe presence of gene silencing (Table 2). Most plants showed low levels of RNA expression from either the $NS_4$ sense or anti-sense genes. In these plants the RNA was detected by RT-PCR. $NS_4$ gene expression was detected by regular Northern in only one plant so far (Table 2).

**Table 2. Gus-intron, hpt, and $NS_4$ transgenes inheritance, and $NS_4$ expression in $T_1$ transgenic plants**

| Genotype | Plasmid | Plant $T_0$ | Plant $T_1$ | PCR[2] | | | | $NS_4$ | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | GUS[1] | *gus* | *hpt* | *NS4* | S[3] | RT-PCR | Northern |
| Cica 8 | PIC002 | 1 | 1 | - | - | - | - | - | ND | ND |
| | | 1 | 2 | - | - | - | - | - | - | - |
| | | 1 | 5 | + | - | - | - | - | ND | ND |
| | | 2 | 11 | - | - | - | - | - | ND | ND |
| | | 7 | 1 | +++ | + | + | + | - | ND | ND |
| | | 7 | 2 | +++ | + | + | + | - | ND | ND |
| | | 7 | 3 | +++ | + | + | + | + | ND | ND |
| | | 7 | 4 | - | + | + | + | + | - | - |
| | | 7 | 18 | ++ | + | + | + | + | + | - |
| | | 9 | 14 | +++ | + | + | + | + | - | - |
| | | 9 | 15 | +++ | + | + | - | - | ND | ND |
| | | 12 | 7 | +++ | + | + | + | + | - | + |
| | | 12 | 11 | ++ | + | + | - | - | ND | ND |
| | | 12 | 15 | +++ | + | + | - | - | ND | ND |
| Cica 8 | None | NT | NT | - | - | - | - | - | - | - |
| Palmar | PIC004 | 1 | 18 | + | - | - | - | - | - | - |
| | | 1 | 24 | - | - | - | - | - | - | - |
| | | 10 | 16 | ++ | + | + | - | - | + | - |
| | | 4 | 3 | +++ | + | + | + | + | + | - |
| | | 4 | 5 | +++ | + | + | + | - | + | - |
| | | 4 | 17 | +++ | + | + | + | + | + | - |
| | | 4 | 18 | - | - | - | - | - | - | - |
| | | 4 | 20 | +++ | + | + | + | + | + | - |
| | | 4 | 25 | +++ | + | + | + | + | + | - |
| | | 7 | 4 | - | + | + | - | - | - | - |
| | | 7 | 16 | - | + | + | + | - | + | - |
| | | 7 | 22 | + | + | + | - | - | + | - |
| | | 7 | 23 | - | - | - | - | - | ND | ND |
| Palmar | None | NT | NT | - | - | - | - | - | - | - |

[1] Test of Gus in leaves. The expression of GUS gene was scored based on the level of expression. (+) low; (++) intermidate; (+++ ) high. [2] PCR analysis to detect the transgenes: *gus* ;*hpt*, hygromycin; $NS_4$ . [3] S = + Positive to Southern blot analysis. ND= Not determined. $T_1$ plants derived from the same $T_0$ plant are sister lines. NT = Not transgenic

$T_1$ plants derived from self cross of eight $T_0$ plants, originally identified as transgenic based on Southern analysis, were selected to conduct the RHBV resistance evaluations in the greenhouse. Each $T_0$ line was represented by 13 $T_1$ plants and were inoculated at 18 days after germination with four insect vectors per plant derived from a colony with 80% virulence. Paralelly, 25 $T_1$ sister plants were used to determine the level of GUS expression. Results indicate that most $T_1$ plants from line 4 transformed with plasmid pIC004 showed the expected GUS expression indicating inheritance of the gus-intron gene. In this line, the number of plants with no o minor disease symptoms (<10% leaf area affected) was double respect to the non-transgenic control (Table 3). No difference in disease reaction were noted for the other transgenic lines and the

corresponding control (Table 3). However, for these lines there is indication that the transgene inheritance is significantly deviating from a Mendelian segregation and in 5 out of the 8 lines evaluated showed less than 25% $T_1$ plants with the transgene respect to the 75% expected. Because of this skewed segregation, commonly found in early generation of transgenic plants, it is necessary to advanced to the $T_2$ generation from those $T_1$ transgenic plants carrying and expressing the corresponding transgenes. Following, $T_2$ plants that inherited and expressed the $NS_4$ gene need to be dentified by molecular analyses, and challenged them with RHBV.

**Table 3. Disease resistance on $T_1$ transgenic plants derived from different $T_0$ plants carrying $NS_4$ sense (pIC002) or $NS_4$ anti-sense (pIC004) and inoculated with RHBV at 18 day old in the glasshouse**

| *Genotype* | Plasmid | $T_0$ Plant | % plants Gus[1] | Leaf Area Affected (% Plants) | |
|---|---|---|---|---|---|
| | | | | ≤ 10 | >10-100 |
| ***Cica 8*** | **pIC002** | **1** | **4** | **0** | **100** |
| | | 7 | 4 | 0 | 100 |
| | | 9 | 40 | 15 | 85 |
| | | 12 | 40 | 0 | 100 |
| | None | Control | 0 | 0 | 100 |
| Palmar | pIC004 | 1 | 12 | 31 | 69 |
| | | 4 | 84 | 62 | 38 |
| | | 7 | 12 | 46 | 54 |
| | | 10 | 24 | 46 | 54 |
| | None | control | 0 | 31 | 70 |

**Eighteen plants were evaluated per each $T_1$ line. [1] 25 plants per $T_1$ line were tested for GUS expression.**

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## 2.1.6 Foreign genes as novel sources of resistance for fungal resistance


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### Introduction

The fungal *Rhizoctonia solani* (sheath blight), *Helmithosporium, Rhincosporium,* and *Sarocladium* is already causing important rice yield losses in the Southern cone of South America

and increasing spreads had been reported in Colombia, Mexico and Venezuela. All rice varieties are susceptible and there are not known sources of stable genetic resistance for these diseases in rice. In the case of sheath blight, IRRI had placed a major effort in developing biological control strategies for this disease without success either. At present, the control of this complex mainly depends on use of fungicides (Dr. Fernando Correa, CIAT Rice Pathologist, Cali, Colombia, personal communication). Recently, FLAR suggested CIAT (Dr. Peter Jennings, personal communication), to develop molecular strategies for incorporating resistance to this fungal complex. However, very little is known about the interaction between the rice and these pathogens in order to designspecific resistance strategies for each of these fungi. Of the four, the plant-pathogen interaction with *Rhizoctonia solani* is the better known.

Work conducted by another principal investigator of this project (Dr. Nilgun Tumer, Biotechnology Center at Rutgers University, USA) showed that a pokeweed antiviral protein (PAP), a 29-kDa protein isolated from *Phytolacca americana* (a weed naturally found from USA to Argentina), has a ribosome-inactivating ability. Mutated versions of PAP gene has potent antifungal activity (Zoubenko et al., 1997). Homozygous progeny of transgenic tobacco plants expressing these PAP genes displayed resistance to the fungal pathogen *Rhizoctonia solani*. Transgenic PAP potato showed protection against *Phytophtora infestans*, and transgenic PAP turfgrass are resistant to various fungal pathogens. These results suggest the possibility of designing molecular strategies for incorporating fungal resistance by introgression of mutant PAP gene(s) in transgenic rice plants. Here we report the progress made during the second year of this project.

Last year we reported the generation of eight new PAP mutations directed to change the aminoacids composition in the PAP protein. These new mutated genes were placed into yeast vectors, and transformed into yeast to check for no toxicity. The non-toxic mutated genes are being transformed into tobacco first to check the gene expression and toxicity before using them for rice transformation. Two mutated versions of PAP (I deleted and II) already tested for no toxicity in turfgrass (another monocot species ) were used as the first approach to transform rice. These genes driven by the ubiquitin promoter were placed in the plasmid vectors pWBVec8, pWB10a, and pBGXiHGFP kindly supplied by Dr. Peter Waterhouse (CSIRO, Australia). These plasmids had been used successfully by Waterhouse to transform rice via *Agrobacterium*. They contain a hpt gene with a CAT-1 intron for increased expression of hygromycin resistance and selection in rice, a gus-intron-gene, or a gfp (green fish fluorescent) gene, respectively, to aid the recovery of transgenic plants. A total of 35 independent transgenic events carrying the PAPI deletion mutant gene, and 50 independent transgenic events carrying the PAPII gene were generated last year. A first set of plant tissue was sent to Rutgers this summer for analysis and plants with PAP gene expression were identified based on Western analysis. This year a new version of PAP gene (PAPY123) which include a deletion of 3 nucleotides was used to generate another set transgenic plants.

**Materials and Methods**

***Transformation and Plant Regeneration***

Mature embryos derived callus of *indica* varieties CICA 8, Palmar, Cimarrón and Fundarroz PN1, were used as targets. Palmar and Cimarrón showed high and moderate tolerance to sheath blight, whereas Fundarroz PN1 and Cica 8 are highly susceptible to sheath blight. The transformation experiments were conducted using *Agrobacterium tumefaciens* strain Agl1 (Wang et al.,1997) carrying one of the following plasmids, NT305, NT306 and NT446. Maize ubiquitin or 35SCaMV promoter drives these plasmids, which carry various mutant versions of the PAP gene (PAPI, PAPII and PAPY123). The hygromycin resistance conferred by the *hpt-cat intron gene*

was used as the selective marker. Plants were regenerated after stepwise selection on 30 mg/l and 50 mg/l, followed by other 30 mg/l selection throughout plant differentiation. Plants were grown to maturity in biosafety glasshouses.

***Molecular Analysis of the Transgenic Rice Plants***

Southern Blot and PCR analyses were used to detect the presence of the PAP and hygromycin genes. For this purpose, 15 µg of DNA were digested with different enzymes. The gels were denatured and neutralized by standard procedures. The DNA was transferred to nylon membranes (Hybond-N, Amersham). The filters were hybridized at 60°C. The presence of the kanamycin gen was determined throughout PCR.

**Results and Discussion**

A total of 59 transgenic plants carrying the PAPY123 gene were generated and confirmed by Southern blot (Table 1). More than 92% of the regenerated plants had integrated the PAPY123 gene in the genome as determined by Southern blot (Table 1). A similar total number of plants were generated for each of the PAP genes.

This year to confirm the integration of the PAP gene, 63 plants of the varieties Palmar and Cica 8 transformed with the plasmids NT 446 were evaluated by Southern Blot *and* PCR methods. The genomic DNA was digested with different enzymes ( Bam HI/EcoRI and Hind III) which excises the PAP gene. The results indicate the integration of at least one copy of the PAP gene in the rice genome. Moreover, a better analysis on the patterns of integration was obtained with the enzymes BamHI/EcoRI that split by half the PAPY123 gene into two fragments (Figure 1). This study showed that 67% of the plants analyzed revealed only one copy of the gene without rearrangements (Figure 1). The PCR analysis of some plants confirmed the presence of the PAPY123 gen. At present, the integration of the PAPY123 of varieties Palmar y Cica 8 varieties has been confirmed. The Southern blot analysis also revealed the presence of the hpt and nptII genes. Western analysis indicated that about 50% of the plants analyzed from PAPY123 showed gene expression of PAP protein, whereas 18% of the plants tested are expressing either PAPI or PAPII genes. PAP expressing plants will be evaluated for sheath blight resistance under greenhouse conditions, while detailed molecular analyses will be conducted to determine the number of gene copy.

**Tabla 1. Transformation efficiency of three *indica* varieties using the AgII (NT446)**

| Genotype | Plasmid | Callus | Plants | RE % | Plants Analyzed by Southern | Plants $S^+$ | % Plants S+ | % TE |
|---|---|---|---|---|---|---|---|---|
| PALMAR* | NT446 | 182 | 48 | 26.4 | 36 | 34 | 94.4 | 25 |
| CICA-8* | NT446 | 220 | 34 | 15.4 | 27 | 25 | 92.5 | 14.2 |
| CIMARRON** | NT446 | 16 | 5 | 31.2 | ND | ND | ND | ND |

* Five replicates. ** One replicate. RE = plant regeneration efficiency. $S^+$ = Southern positive. TE = transformation efficiency

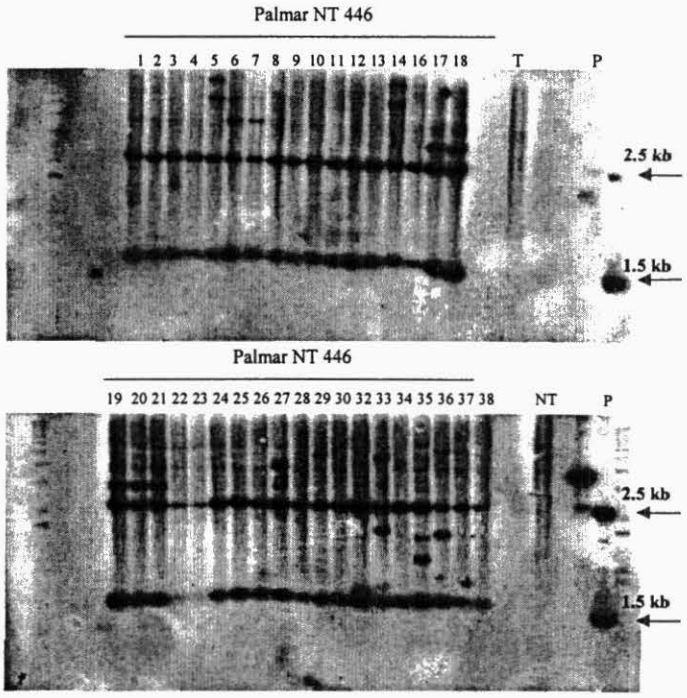


**Figure 1. Southern Blot Analysis of Genomic DNA of $T_0$ plants Transformed with *PAP* Y123 Gene. DNA was Cut with *Bam HI* and *Eco RI*. T = Control non Transgenic. P = Plasmid NT446 carrying PAPY123**

## 2.1.7 Development of genetic transformation of *Brachiaria* mediated by *Agrobacterium tumesfaciens*

C.P. Flores, Z. Lentini
SB-2 Project

### Introduction

*Brachiaria* grasses are the most widely grown pastures in subhumid and humid tropics. *B. decumbens* cv Basilisk (signalgrass) is important because of its high productivity under intensive use and its tolerance of low soil fertility and relative freedom from pests and diseases, apart from spittlebugs. Forage plant breeding has been largely based on phenotypic selection following sexual recombination of natural variation found between and within ecotypes. Advances in plant genetic manipulation over the last 15 years have provided convincing evidence that these powerful technologies can complement and enhance plant breeding programs. Molecular breeding based on transgenesis to overcome limitations in forage quality may be targeted to the individual subcharacters involved: dry matter digestibility, water-soluble carbohydrate content, secondary metabolites, alkaloids, etc. These molecular breeding approaches may include modification of lignin profile to increase dry matter digestibility and genetic manipulation of fructan metabolism to increase non-structural carbohydrate content (Spangenberg *et al* 2001). Most quality or anti-quality parameters are associated with specific metabolic pathways or production of specific proteins. This allows target enzymes or suitable foreign

proteins to be identified, corresponding genes isolated and their expression manipulated in transgenic forage plants. A protocol for genetic transformation of *Brachiaria* will be particularly useful to further improve the quality traits associated with the nutritional value of the pasture. Earlier work at CIAT's included the establishment of tissue culture methods for plant regeneration (Lenis, 1992), and genetic transformation mediated biolistic (Galindo, 1997) of *Brachiaria* species. Last year we reported the optimization of RITA system for an automated mass production of embryogenic calli of *Brachiaria* and the effects of medium composition on calli induction and plant regeneration in the same species. This year we report the progress made in the establishment of a protocol for *Agrobacterium*-mediated transformation of *Brachiaria* species and how the transformation is highly influenced by genotype.

## Materials and Methods

*Evaluation of different accessions of Brachiaria spp for in vitro tissue culture*
Genetic transformation mediated by *Agrobacterium* is highly influenced by the genotype. With the aim to increase the number transformation events, two accessions of *B. decumbens* (CIAT Nos. 606 and 16497), four accessions of *B. brizantha* (CIAT Nos. 16316, 25665, 6387 and 16467) and three hybrid materials (CIAT Nos. 36060, 36061 and 36062) were evaluated. Induction of embryogeneic callus from mature zygotic embryos of each of these genotypes was assayed.

*Genetic Transformation of B. decumbens mediated A. tumefaciens*
Scutellum-derived embryogenic callus of *B. decumbens* (CIAT Nos. 606 and 16497) and *B. brizantha* (CIAT 25665) were used as target explants. Two *A. tumefaciens* hypervirulent strains, AGL-1 and C58C1, both carrying the binary vector pCAMBIA 1305.2 (11,921 Kbp), kindly provided by Dr. Richard Jefferson, Australia were tested. The pCAMBIA1305.2 contains the hygromycin resistance (*hpt)* and *GUS-Plus-intron* genes. *GUS-Plus* is a new reporter gene isolated form *Staphylococcus* sp with some superior properties to *E. coli GUS* gene. The *GUS-Plus* gene contains the intron from the castor bean catalase gene to ensure detection of plant-specific-*GUS* expression and the glycine-rich protein signal peptide sequence. The protocol used was basically the same as reported by Florez and Lentini, 2000. An explant was considered GUS-positive when at least one blue spot was observed.

## Results and Discussion

Induction of embryogenic callus was highly dependant on the genotype (Table 1). Of the four *B. brizantha* accesions evaluated, accessions No. 16316 and No. 25665 showed the highest level of callus induction. *B. decumbens* accession 16497 showed the highest response of all the genotypes evaluated (Table 1).

**Table 1. Embryogenic callus induction on different *Brachiaria* species and accessions**

| Specie | CIAT accession | No. of isolated embryos | No. embryogenic callus | % Callus induction |
|---|---|---|---|---|
| *B. brizantha* | 6387 | 60 | 0 | 0 |
| | 16316 | 60 | 30 | 50 |
| | 25665 | 45 | 21 | 47 |
| | 16467 | 45 | 0 | 0 |
| *B. decumbens* | 606 | 75 | 40 | 53 |
| | 16497 | 66 | 50 | 76 |
| Hybrid | 36060 | 23 | 9 | 39 |
| Hybrid | 36061 | 33 | 3 | 9 |

Preliminary results indicate a higher response to *Agrobacterium* infection when using *B. decumbens* CIAT 16497. This accession showed an average of 26 % transient gus expression. Not difference were noted between the strains used (Table 2).

**Table 2. Gus transient expression of embryogenic callus co-cultivated *with Agrobacterium tumesfaciens* strains AGL-1 or C58C1**

| Specie | CIAT No. | Strain | Callus assayed | GUS $^{+}$ | % GUS $^{+}$ |
|---|---|---|---|---|---|
| *B. decumbens* | 16497 | C58C1 | 59 | 17 | 28.8 |
| | 16497 | AGL-1 | 77 | 18 | 23.4 |
| | 606 | C58C1 | 172 | 4 | 2.3 |
| | 606 | AGL-1 | 55 | 0 | 0.0 |
| *B. brizantha* | 25665 | C58C1 | 41 | 3 | 7.3 |
| | 25665 | AGL-1 | 37 | 3 | 8.1 |

### Future Plans

- To use *B. decumbens* CIAT 16497 as target for *Agrobacterium* infection
- To increase bacteria activity through utilization of new protocols for higher expression level
- To conduct assays until regeneration of putative transgenic plants is achieved

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## 2.1.8 Isolation of lignin biosynthetic genes from *Brachiaria decumbens*


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### Introduction

Lignin biosynthesis occurs through a series of reactions involving (1) the shikimate pathway which provides phenylalanine as a substrate, (2) the phenylpropanoid pathway which results in several cinnamoyl CoAs that act as precursors for a wide array of phenolic compounds, and (3)

the monolignol pathway which converts cinnamoyl-CoA moieties into monolignols and lignin. All enzymes in the phenylpropanoid and monolignol pathways, except one, have been cloned, usually from multiple plant species. Lignin concentration and composition both control herbage digestibility. Therefore, transgenic technology can be used to increase digestibility of forage crops by down regulation on enzymes in the phenylpropanoid or monolignol pathways. Except for a few highly unusual transgenics, the biggest difference between plant transformation and natural variation may be that the novel-lignin phenotypes occur at higher frequency within transgenic lines, making them easier to identify than novel-lignin phenotypes that occur relatively infrequently in natural populations (Casler and Kaeppler, 2001).

Relative small changes in quality of forage crops can lead to large changes in animal performance (Casler and Kaeppler, 2001). For these reason, CIAT jointly with the Plant Biotechnology Centre in Victoria, Australia are working to isolate genes from *B. decumbens* that are involved in lignin biosynthesis using *Lolium perenne OMT-1, 4CL-2, CCR-1* and *CAD-1* cDNA clones as probes. *OMT-1, 4CL-2, CCR-1* and *CAD-1* are the key gene in the lignin biosynthetic pathway.

**Materials and Methods**

*Northern Blot Hybridisation*
Total RNA from roots and shoots of ten days old *L. perenne* (ryegrass) and *B. decumbens* seedlings was isolated using the protocol described by Chang *et al* 1993. Fifteen to twenty μg of total RNA per lane was run. Ryegrass cDNA clones *OMT-1*, *4CL-2*, *CCR-1* and *CAD-1* were used as probes.

*cDNA Library Construction in ZAP*
Total RNA was cleaned using the Quiagen RNEasy kit. To yield mRNA from total RNA, Oligotex mRNA kit (Qiagen Company) was used. The Stratagene cDNA Synthesis kit was used for the construction of directionally cloned cDNA library. For the packing of the library, Stratagene GigaPack Gold III was chosen. For the screening of the library ryegrass cDNA clones *OMT-1*, *4CL-2*, *CCR-1* and *CAD-1* are being used as probes.

**Results**

The Northern hybridisation analysis was made to determine the homology between *L. perenne* and *B. decumbens* lignin biosynthetic genes. The results showed that *OMT-1, 4CL-2* and *CCR-1* transcripts are present in young roots and shoots of *B. decumbens* (Figure 1). These transcripts accumulate to higher levels in the roots than in the shoots. The presence of endogenous *OMT*, *4CL* and *CCR* genes in *B. decumbens* is confirmed.

**Future Plans**

To complete cDNA library screening for key lignin biosynthesic genes of *B. decumbens*
To isolate and characterize key lignin biosynthesic genes isolated from *Brachiaria* library
To produce transgenic germplasm with manipulated lignin metabolism
To obtain transgenic cultivars with enhanced forage quality

**Reference**

Casler, M.D. and Kaeppler, H.F. 2001. Molecular breeding for herbage quality in forage crops. In Spangenberg G. (Ed). Developments in Plant Breeding - Molecular Breeding of Forage Crops, 176-188.

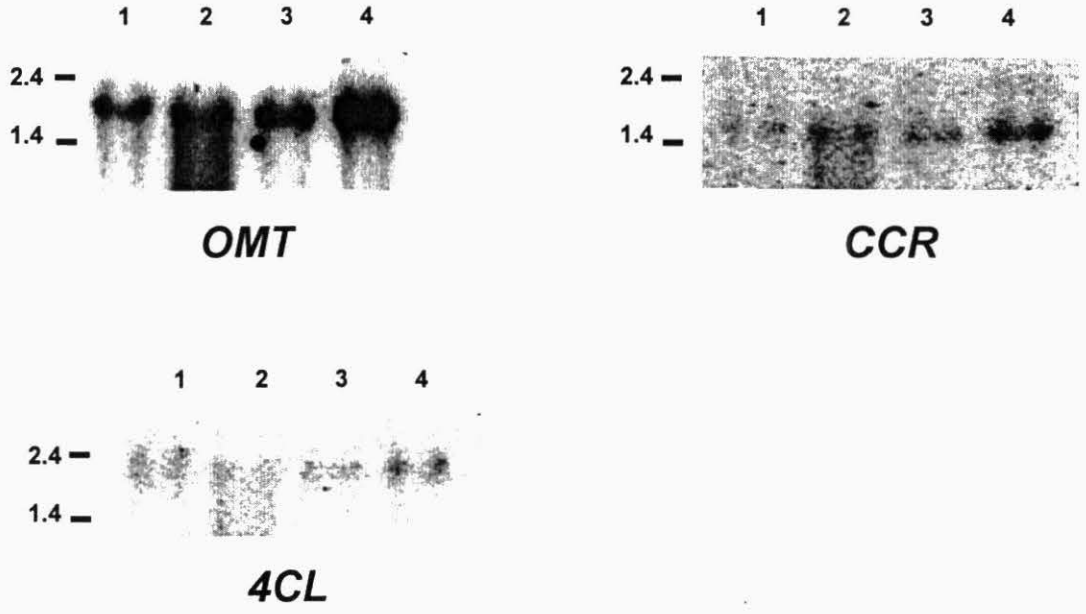


**Figure 1. Northern hybridisation analysis. *1* Shoots of *B. decumbens*. *2* Roots of *B. decumbens*. *3* Shoots of *L. perenne*. *4* Roots of *L. perenne*.**

## 2.1.9 Genetic transformation of tomato variety UNAPAL Arreboles for resistance to udworm (*Tuta absoluta*)

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### Introduction

Tomato (*Lycopersicon esculentum* Mill) is one of the most important crops in the fresh vegetable market as well as in the food processing industry (Rick and Yoder, 1988). Tomato is the major consumed vegetable crop in Colombia, with a planted area of 15,000 hectares yielding 450.000 tons per year (UNAL, 1997). In Colombia, this crop is highly affected by several pests and diseases, and abiotic stresses such as drought, high and low temperatures, and salinity. Since 1985, the vegetable breeding program at the Universidad Nacional de Colombia, Palmira Campus, has as main objective the development of varieties with resistance or tolerance to some of these traits. In 1997, this program released the tomato variety UNAPAL Arreboles, which has several traits attractive to tomato growers such as fruit firmness and good adaptability specially to the Valle del Cauca region. But this variety is susceptible to one of the major limitations to tomato production in this region: the budworm (*Tuta absoluta*), which affects the tomato buds and young leaves. It had been difficult to breed tomato resistant to this pest by standard breeding. The only sources of resistance genes is from wild tomato species which are incompatible with the cultivated tomato, and so far the attempts for an inter-specific breeding program has not been successful (Lourencao et al., 1985). The main objective of this work is to transform the tomato variety UNAPAL-Arreboles with the Bt gene cryIA(b), which had been used successfully to obtain resistance against Lepidoptera pests in various economical important crops (i.e. maize, cotton).

In previous reports it was described the evaluation of three protocols commonly used for tomato callus induction and plant regeneration (Fillatti et al., 1987, Narvaez, 1993, and Ultzen et al ., 1995). Results indicated that the highest response for callus induction and plant regeneration is noted on M3 medium sequence (Ultzen et al ., 1995). An increase in response of about 2-fold and 4-fold on callus induction and plant regeneration was noted on M3 media respect to the other media tested. The lowest response was obtained on M2 medium (Fillatti et al., 1987). This year it is reported the progress made on developing UNAPAL-Arreboles transgenic tomato containing the cryIA(b) gene.

**Materials and Methods**

Two *Agrobacterium* mediated transformation protocols commonly used for tomato (McCormick et al., 1986; Fillatti et al., 1987), were tested using the tomato variety UNAPAL-Arreboles. *Agrobacterium* strains C58C1, Agl1 and LBA4404 containing the pBIGCry construct (L.I. Mancilla at CIAT) were used. This gene construct contains the *cry1Ab* gene driven by the 35S CaMV promoter, the nptII gene for kanamycin resistance as selection markers, and the gus-intron as a reporter gene. Transgenic plants were identified by Southern blot analysis. Inheritance of gus expression and kanamyzin resistance was evaluated from T0 to T1 generation. Clonallys propagated plants of the original To plants were evaluated fro agronomic traits in the greenhouse.

**Results and Discusion**

Preliminary results indicated that the highest level of gus transient expression was attained with the *Agrobacterium* strain LBA4404. A total of four hundred tomato explants (cotyledonary leaves of 7-10day-old plantlets) were infected with LBA4404/pBIGCry weekly. After co-cultivation for 48 hour, about 10% of the explants were analyzed for gus transient expression. The rest of the explants from cultures showing transient expression were transferred to selection media containing kanamycin. After three weeks on selection media, regenerated plantlets were recovered. The number of regenerated plants recovered from kanamycin containing medium varied among the different experiments. From zero to ten plants were recovered per experiment.

A total of 51 putative transgenic plants were produced from 8 experiments (400 explants by experiment). This shows an efficiency of 1.6 % for recovering kanamycin resistant plants from the initial agro-infected explant. Of these plants 15 were transferred successfully to the greenhouse and 6 plants had shown stable gus expression throughout the vegetative and reproductive life cycle.

This year it was carried out the morphological and molecular characterization of kanamycin-resistant and gus-expression clones P28, P33 and P47 at the T0 and T1 generations. Self progeny derived from T0 plants (T1 clones) was evaluated for agronomic traits and resistance to kanamycin and Gus expression. No differences were noted neither between the T0 and T1 plants, nor between the transgenic plants and the tomato control plant for the various morphological traits evaluated (plant height, node lentgth, leaf type, presence of pubescence in stem, flower color and shape, fruit color and shape). These results indicated that no somaclonal variation is apparent in the transgenic plants. Preliminary results on the molecular characterization of clones P28,P33 and P47 by PCR and Southern analyses suggested that both genes are inserted in the genome of these plants. Molecular analysis for the patterns of insertions of the cryIA(b) in these plants is in progress. For the three clones evaluated, a 3:1 segregation ratio for gus expression and kanamycin resistance was noted. Indicating the insertion of an active locus for each of these genes. Variation was noted on the co-segregation of the two genes. Clones P28 and P33 showed a

95% and 82% co-segregation of both genes from T0 to T1, whereas P47 showed a cosegregation of 63%. Currently, inmunological and bioassays studies are being carried out to detect the Cry1Ab gene expression on the transformed tomato clones and to determine if these plants are insect resistant.

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## 2.1.10 Resistance to sugar cane yellow leaf virus (ScYLV): Genetic transformation an alternative aiding breeding of sugar cane


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### Introduction

Wide spread of yellow leaf syndrome disease caused by ScYLV was introduced in Colombia in1998 through the Brazilian variety SP 71-6163 (Victoria et al., 1999). The main source of the disease is the use of vegetative seeds from clonal propagated infected plants, and transmission by the aphid vector *Melanaphis sacchari* widely present in the sugar cane region of the country. The disease is associated with reduction in sucrose content and crop yield, although plants may be symptom-less. In Brazil, the variety SP 71-6163 infected with this virus showed yield losses of 60% to 80%. The molecular characterization and cloning of ScYLV was performed at Texas A&M (Mirkov et al., personal communication). Several constructs were generated, and one gene version containing part of the coat protein gene encoded high levels of protection in transformed sugar cane USA varieties. The main objective of this project is to transform sugar cane with the

truncated version of ScYLV coat protein gene via biolistics, since transgenic sugar cane has already being generated in other laboratories using this gene transfer method.

**Materials and Methods**

Genetic transformation of sugar cane was conducted using the PDS-1000 He particle accelerator device. A construct (pScYLV) containing the truncated version of the ScYLV coat protein gene driven by the ubiquitin promoter, and the nptII gene for genetycin resistance will be used. The protocol optimized at CIAT for rice transformation was tested and modifications introduced accordingly. In this regard, preliminary experiments were conducted using plasmids pGV1040, pCAMBIA 1201, and pCAMBIA 1301 all carrying the gus gene driven by the 35S CaMV promoter, to optimize bombardment conditions evaluating GUS transient expression. Simultaneously, callus induction and plant regeneration of the varieties CC 87-434, CC 85-63, CC 85-92, CC 85-96 and CC 84-75 was tested to select the most responsive genotype and use as target. Callus was induced in the dark from apical meristems of *in vitro* plantlets cultured on MS salts supplemented with 3 mg/l 2,4-D, 100 mg/l inositol, 18% coconut water, 3% sucrose, and 0.2% gelrite. Last year results indicated that the routine protocol used for clonal propagation of sugar in CENICAÑA although efficient for the generation of plants, it was not appropriate for the maintenance of embryogenic callus needed for bombardment since plant differentiation occurs as soon as callus induction is obtained. A modification of the standard tissue protocol was introduced by culturing the induced callus under dim light rather than direct light. This change in light culture conditions restrained callus differentiation by 85%, allowing the maintenance of embryogenic callus at a optimal for bombardment. Callus cultured under dim light conditions, showed about 80% of plant regeneration when transferred to direct light. These results suggest that changes of light conditions at early stages of callus induction, does not have deleterious effects on the plant differentiation process.

**Results and Discussion**

Last year results indicated that 51.3 % $\pm$ 27.8% of bombarded tissue showed gus transient expression 48 hr after bombardment. After a complete stepwise selection at genetycin 30 mg/l followed by genetycin 50 mg/l, a total of 68 petri plates with 20 to 25 explants each had been evaluated so far. About 50% $\pm$ 29 % of the bombarded callus were resistant to genetycin 50 mg/l. Of these resistant callus, about 4% $\pm$ 5% regenerated plants. Seventy regenerated plants were recovered after selection in genetycin 50 mg/l. Eleven plants have been tested by Southern blot so far. Six out of eleven tested plants were positive showing a 0.45 kb signal after digestion with *Hind* III indicating the excision of the complete truncated version of the ScYLV coat protein gene. Plants will be tested after digestion with *Pst* I and *BamHI* in order to determine number of copies and rearrangments present in transformed plants.

**References**

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## 2.1.11 Expression of recombinant CRYI(A)b Protein in *E. coli*

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[1]SB-2 Project ; [2]CIP-Peru

### Introduction

Bt genes of the *cry* family have been used to confer resistance to insects in different plants species (cotton, maize, canola, soybean). The toxic effect of the protein CRYI(A)b has been previously reported for several lepidopterans. To evaluate the toxic effect of this recombinant protein on cassava stem borer (*Chilomima clarkei*) larvae, the cry I(A)b gene was cloned into expression vectors and the protein purified. Different artificial diets were evaluated on larvae stages of stem borer from artificial colonies established at Nataima (Tolima) in an attempt to optimize conditions for toxicity tests.

### Methodology

The *cry*I(A)b gen from PLANTECK was cloned into a series of expression vectors (pGEX-5X-1, pGEX-5X-2 and pGEX-5X-3; Figure 1) to restore the open reading frame and to obtain the recombinant protein. The CRY recombinant protein was cloned attached to the 3'-end of a Gluatahione S-transferase gene sequence. The protein expression of several clones with these constructions were induced with IPTG in different concentrations, by four or six hours, at 30 °C or 37 °C. Positive clones with recombinant protein were identified by PAGE-SDS 10% (Figure 2). Induced bacterial cultures were concentrated and CRY protein was purified using Pharmacia chromatography column with Glutathione Sepharose 4B resine. After binding the fusion protein to Glutathion, the chromatography column was washed three times with PBS buffer before elution of proteins with reduced Glutathione 10mM pH 8.0. The concentration and molecular weight of purified protein was evaluated by electrophoresis.

Artificial diets were tested with stem borer larvae using different parts of the cassav plant. Flour made of lyophilized stems, leaves and rootw were ground and complemented with nutritive components like wheat germ, bacto-agar and beer yeast extract (Table 1).

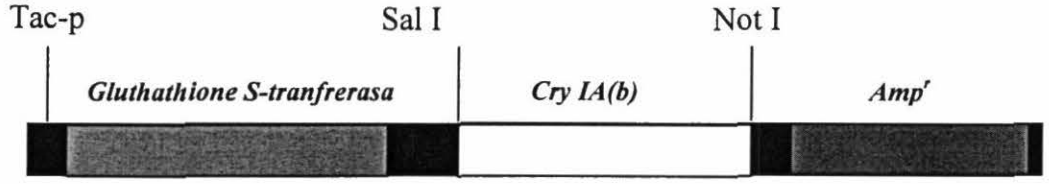
**Table 1. Contents of artificial diets (g/l) to rear Chilomima clarkei. (Reference: Lastra and Gómez, 1988)**

| *DIET Component* | *Carrot* | *Total Cassava stem* | *Cassava Xilem* | *Cassava Root* | *Cassava Leaves* |
|---|---|---|---|---|---|
| Wheat germ | 38.0 | | | | |
| Beer yeast extract | 40.0 | | | | |
| Carrot flour | 40.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Liophylized tissue | 0.0 | 40.0 | 40.0 | 40.0 | 40.0 |
| Hidrolizated casein | 8.0 | | | | |
| Powder Sucrose | 20.0 | | | | |
| Sugarcane leaf flour | 80.0 | | | | |
| Corn cob flour | 11.25 | | | | |
| Agar | 3.75 | | | | |
| Metil paraben | 3.5 | | | | |
| Stretopmicine | 1.5 | | | | |

## Results and Discussion

Recombinant Cry Protein Expression

Two of out three constructions with CRY in pGEX-5X vectors have been obtained. Expression of the protein has been detected on PAGE-SDS (Figure 2). pGEX1CRY and pGEX2CRY Recombinant CRY protein was detected as two fragments between 60 and 40 Kd.




**pGEXCry (7.72 Kbp)**

**Figure1. Diagram of constructs with *cry*1(A)b gene in series 1 and 2 of pGEX-5X expression vector.**

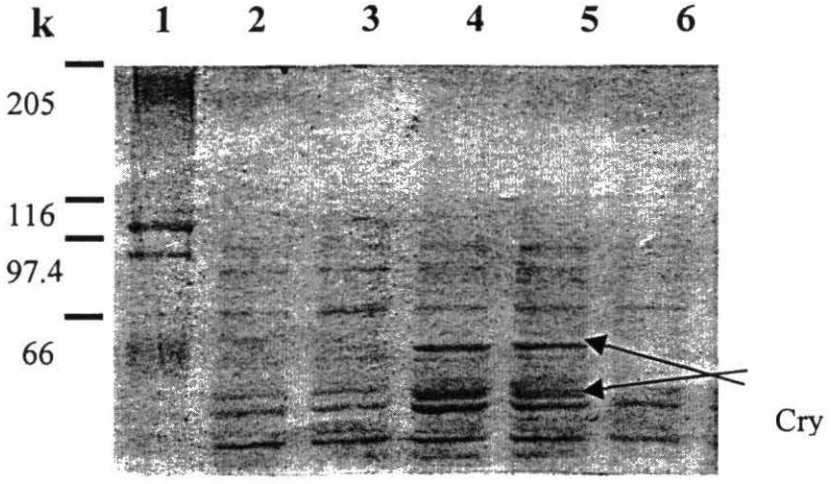



**Figure 2. Induction of expression of CRY recombinant protein in pGEX1CRY and pGEX2CRY clones with 0.2 mM of IPTG by 6 hours at 30°C. 1.Molecular marker; 2. pGEX-5X-1 (negative control); 3. pGE3 Cry; 4. pGEX2Cry; 5.pGEX1Cry; 6. pGEX-5X-1.**

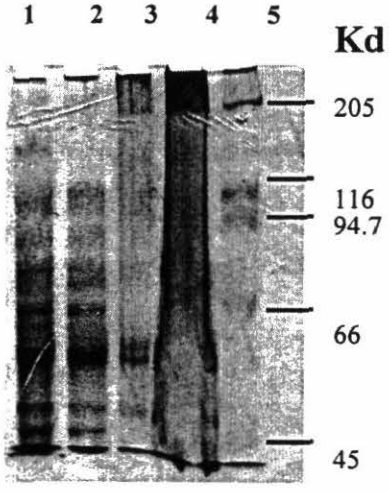



**Figure 3. PAGE-SDS with eluted fractions of purification of CRY recombinant protein from pGEXCRY clone. 1. Supernatant of bacterial lysate, 2. Elution with wash buffer, 3. Elution with reduced glutathione, 4. Bacterial pellet. 5. Molecular marker.**

**Artificial Diets to Rear C. clarkei**

Three different experiments with 5 repetitions were made with larvae of *C. clarkei* to evaluate different artificial diets. However, the viability of larval stages of *C. clarkei* was very low in these assays. *C. clarkei* larva showed the highest viability in diets with cassava stem flour and carrot flour and survived eighth days after inoculation. These results have to be duplicated. The time of larvae survival wasn't enough to evaluate protein toxicity.

**Conclusions and undergoing work**

The CRY IA(b) recombinant protein was obtained from expression vectors pGEX-5X-1 and pGEX-5X-2 and purified by Glutathione Sepharose 4B afinity cromatography. *C. clarkei* larvae have been difficult to rear on several artificial diets. An artificial colony has been established in CORPOICA-Nataima (Tolima). We will continue testing modifications on the diets to rear *Chilomima*. We will produce enough recombinant protein to test its toxicity on larvae artificially reared, or by sprying cassava cuttings with known concentrations of Cry1A(b).

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## Activity 2.2 Development of cellular and molecular techniques for the transfer of genes for broadening crop genetic base

### Main Achievements

- A novel backcross methodology for producing fertile common x tepary beans hybrids from otherwise incompatible genotypes was developed.
- Lines of Friable Embryogenic Callus of cassava commercial cultivars CM3306-4 (Ica Negrita, for the Northern Coast) and SM1219-9 (for Interandean Valleys) were established.
- The cryo-preservation protocol was tested with 43,5% of the cassava core collection. 82% of the accessions in the core have >30% recovery rates after freezing.
- A cryo-preservation protocol was adjusted for cassava wild relatives. Plants were recovered for *M. esculenta* subsp. *flabellifolia*, *M. esculenta* subsp. *peruviana* and *M. carthaginensis*.
- Friable Embryogeneic Callus from cassava cultivars TMS60444 and MCol2215 were recovered after freezing.
- Plants of the tropical fruit Tree tomato were recovered from frozen seeds.
- Propagation methods using RITA were validated with 16 commercial cassava clones reaching multiplication rates between 1:6 to 1:10.
- Cassava plants, produced in vitro by small farmers in Santa Ana (Cauca), were moved to the field for testing in Perico Negro (Cauca).
- Four cassava clones, selected for farmers from Santa Ana, have now been included in the propagation scheme.
- Simplification of RITA system and construction of cheap transfer hoods to reduce costs of implementing *in vitro* propagation systems for farmer communities and public schools.
- The use of bioreactors from increasing the response to rice anther culture was implemented.
- A methodology for the reproducible plant regeneration of naranjilla fruit (i.e.*lulo*) was developed.
- Selected soursop (*Annona muricata* L.) clones propagated *in vitro* through micrografting, planted three years ago in different locations, showed excelent agronomic behavior regarding general growth, tree architecture, flowering initiation and fruit quality, thus validating this technology as useful for producing planting material.

## 2.2.1 Regeneration of cassava plants from friable embryogenic callus (FEC) by combining conventional solid media and temporary immersion using RITA®

D Lopez[1], JE Montoya[1], R. Escobar[1], P Chavarriaga[1], J Tohme[1] WM Roca [1-2]
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### Introduction

Somatic embryogenesis has been the main regeneration method to obtain transgenic plants of cassava. However, the efficiency of embryo-to-plant conversion is still low with the few cultivars that have been transformed. We are investigating the efficiency of plant regeneration from FEC in three cultivars, two of which are of commercial importance in the Northern Coast of Colombia. Combinations of solid and liquid media (using temporary immersion in RITA®) are being tested to improving the rate of embryo-to-plant conversion. Improving this rate will have a positive impact on the efficiency of plant genetic transformation. It will also contribute towards developing synthetic seeds.

### Methods

Three cassava cultivars were used for the experiments: TMS60444, which is a model cultivar for regeneration and transformation, and Mcol2215 (Venezolana) and CM3306-4 (Ica Negrita) which are preferred cultivars among small-to-medium scale farmers. Approximately 0,17 g of FEC from each cultivar was explanted for embryo maturation on petri plates containing solid MS2 medium (López 2000) plus either hormone NAA or BAP. From this initial step, differentiating embryos in early torpedo and cotyledonary stages of TMS60444 were tested for further development on treatments A to D (*first test*). Based on the best results, a *second test* was carried out with TMS60444 plus CM3306-4 and Mcol2215 to check treatment E only (Figure 1). The treatments were as follows:

- liquid MS2 medium plus NAA, BAP and $GA_3$ in RITA®, with subsequent transfer to solid 4E propagation medium
- solid MS2 medium plus activated charcoal, transfer to solid MS2 plus BAP, and transfer to solid 4E
- liquid MS2 plus activated charcoal in RITA®, transfer to solid 4E
- solid MS2 plus activated charcoal, transfer to liquid MS2 plus BAP, and transfer to solid 4E
- like D, except that the liquid medium contained BAP plus $GA_3$

### Results and Discussion

For cultivar TMS60444 in the *first test*, embryos in the torpedo stage were observed eight days after the first maturation treatment on solid medium with NAA. Fourty days later we collected 160 embryos at the cotyledon stage (314 embryos per gram of tissue) from this initial treatment. They were equally split (60 each) among treatments A to D. Overall, two embryo harvests were made (30 and 90 days) after treatments were initiated, which produced 21 (52.5%), 13 (30.2%), 17 (42.5%) y 23 (53.4%) elongated embryos (plantlets) for each treatment respectively. Based on the quality of embryos, we determined that treatment D was the best from the *first test* to obtain the largest number of vigorous, elongated embryos.

The results reported here for the *second test* are partial since the experiment is still ongoing. So far the maturation on solid medium with BAP has produced larger amounts of mature embryos for CM3306-4 and Mcol2215, while medium with NAA seemed to be better for TMS60444 (Table 1). Embryos from MCol2215 were few, atypical and vitrified. Those from CM3306-4 were more normal-looking and vigorous (Figure 2). Currently embryos are being transferred to RITA with $GA_3$ as explained in Figure 1. $GA_3$ was added to liquid medium to improve shoot elongation of germinating embryos which, in general, from *test one*, did not seem to have well defined apical shoots.

**Table 1. Total number of mature embryos, from FEC, obtained after treatment with NAA or BAP on solid medium for three cassava cultivars.**

| Cultivar | Media | Total mature embryos | Embryos per gram of FEC |
|---|---|---|---|
| CM3306-4 | MS-NAA | 40 | 105 |
| | MS-BAP | 162 | 450 |
| MCOL 2215 | MS-NAA | 0 | - |
| | MS-BAP | 46 | 85 |
| TMS 60444 | MS-NAA | 247 | 1300 |
| | MS-BAP | 35 | 66 |

## Conclusions and Ongoing Work

Globular embryos from FEC differentiate asynchronously into torpedo and cotyledon-stage embryos at different rates and speeds depending upon genotype and hormones used. BAP seems to be more effective for embryo maturation with CM3306-4 and Mcol2215, while NAA produces a larger number of mature embryos in TMS60444.

Including RITA® in the process of somatic embryo maturation and germination seems to have a positive effect on the final embryo-to-plant conversion rate for at least cultivar TMS60444. Although the experiment is still ongoing with other cultivars, RITA® seem also to have a positive effect on the differentiation of root and shoot pole in somatic embryos of CM3306-4.

Future experiments should concentrate on achieving full plant regeneration from commercial cultivars for which FEC is already established, quantify and improve the "yield" of plants on a per-gram basis (of FEC) and try to synchronize embryo maturation using drying periods (in RITA®) and/or anti-auxin compounds.

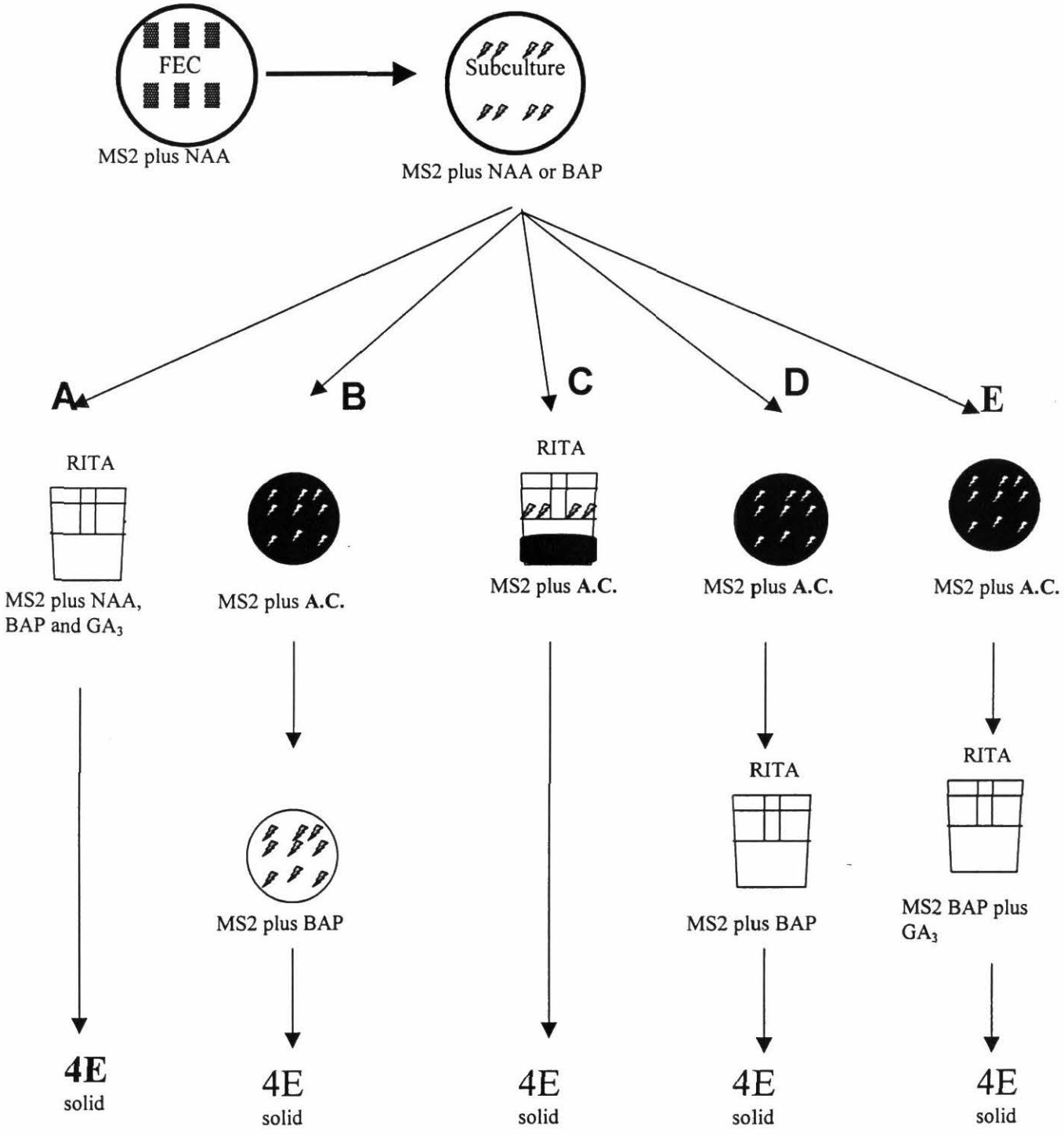


**Figure 1. Diagram of the methodology used for regeneration of plants from FEC of cassava cultivars TMS60444, Mcol2215 and CM3306-4. (A.C., Activated Charcoal).**

**Figure 2. Somatic embryos recovered from FEC of cassava cultivar CM3306-4. Note roots coming out of most embryos.**

### Reference

López D (2000) Inducción de callo embriogénico friable "CEF" y regeneracion de plantas de la variedad de yuca (*Manihot esculenta* Crantz) MCol 2215. Thesis, Universidad Nacional de Colombia,Palmira, Colombia, 85 pp

## 2.2.2 Induction of Friable Embryogenic Callus (FEC) in commercial cassava cultivars

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SB-2 Project

### Introduction

FEC is composed of several compact, embryogenic, cellular aggregates immersed in a liquid matrix of hyaline cells. FEC has been successfully obtained and used for genetic transformation of cassava with few cultivars of little-to-moderate commercial importance in Colombia. We report the production of FEC, and the establishment of cell lines, for cassava cultivars grown in different agro-ecological zones in Colombia.

### Materials and Methods

The following varieties were selected for induction of FEC: MTAI 8, CM 3306-4, CM 523-7, CM 4574-7, CM 6740-7, MBRA 383, SM 909-25 y SM 1219-9. The selection was based on their importance as cultivars in cassava-growing regions in Colombia. Young, immature leaves and axillary buds were used to induce somatic embryos (SE) on media MS-4 (Roca et al 1984) from which FEC was later induced on medium GD2-50Pi (Lopez 2000). To proliferate FEC, we used alternate growing cycles on solid GD-50Pi and liquid SH6-50Pi media (Lopez 2000).

## Results and Discussion

We observed a differential response of cultivars during the induction of somatic embryos and FEC. Besides the intrinsic embryogenic potential of each cultivar, the type of explant used to induce SE influenced the response of each variety. In five out of eight cultivars, young leaves seemed to produce more embryos than axillary buds (Table 1). Similarly, in the production of FEC cultivars responded differently: in about 50% of them FEC was obtained, although proliferation and maintenance was achieved with two (Table1). The time required to produce FEC also seemed to be variable among cultivars. Some showed FEC at 24 days after induction, while for others it took almost twice as much time. FEC appeared as groups of few clusters to ubiquitous masses of large cell aggregates. The regeneration of whole plants from FEC from the two cultivars reported here is underway using combinations of growing cycles on solid or liquid media in RITA® or Petri plates. Green, cotyledon-stage somatic embryos, with defined shoot and root poles are already germinating in both solid and liquid treatments for CM3306-4.

## Conclusions

Varieties that produce reasonable amounts of somatic embryos don't always produce detectable amounts of FEC.
There are genotype differences between Colombian cassava varieties to produce FEC.
FEC can be induced, although not always maintained as a cell line. It depends on the variety.

## Future Activities

Implementation of the FEC system with more cultivars of commercial interest
Improving methods to obtain FEC, and establish cell lines, for recalcitrant cultivars
Improving conditions for regeneration of plants from FEC
Introduce new FEC lines into the tranformation-regeneration scheme developed at CIAT

**Table 1. Average percentage (%) of explants generating SE and FEC, and time required to the appearance of FEC clusters.**

| | Cultivar | Average % of explants (L: leaves; B: Buds) producing SE | | Average % of SE generating FEC | Time required to obtain FEC (days) |
|---|---|---|---|---|---|
| Clones for Northern Coast | MTAI 8 | *L* | 70 | 43♠ | 24 |
| | | *B* | 32 | | |
| | CM3306-4 | *L* | 73 | 94♣♣ | 24 |
| | | *B* | 50 | | |
| Clones for Eastern Planes | CM 523-7 | *L* | 70 | 40♠ | 46 |
| | | *B* | 61 | | |
| | CM 4574-7 | *L* | 26 | 0 | - |
| | | *B* | 58 | | |
| | CM 6740-7 | *L* | 46 | 2.4♠ | 25 |
| | | *B* | 36 | | |
| Clones for Inter-Andean valleys | MBRA 383 | *L* | 2 | *0* | - |
| | | *B* | 13 | | |
| | SM 909-25 | *L* | 56 | 0 | - |
| | | *B* | 60 | | |
| | SM 1219-9 | *L* | 26 | 90♠ | 44 |
| | | *B* | 6 | | |

♠ FEC was induced but didn't proliferate.; ♣♣ FEC was induced, two lines established. ♣ FEC was induced, one line established.

**References**

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López D (2000) Inducción de callo embriogénico friable "CEF" y regeneracion de plantas de la variedad de yuca (*Manihot esculenta* Crantz) MCol 2215. Thesis, Universidad Nacional de Colombia, Palmira, Colombia, 85 pp

## 2.2.3 Development of a novel backcross methodology for producing fertile common x tepary beans hybrids from otherwise incompatible genotypes

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[1]SB-2 Project; [2]IP-1 Project

**Introduction**

The tepary beans (*P. acutifolius* A. Gray) possess several traits that are important for common bean improvement. Such incompatibility barriers as embryo abortion, hybrid lethality, hybrid weakness and hybrid sterility hinder crossing of common beans with tepary beans. Due to this, the tepary beans have been classified in the tertiary gene pool of the common bean (Debouck, 1991), the economically most important species of the genus.

Conventional crossing methodologies (such as recurrent backcrosses) have been used to transfer bacterial blight resistance from tepary to common beans (Mejía-Jiménez *et al.*, 1994; Singh and Muñoz, 1999). This crossing strategy however has not been successful for transferring other important agronomic traits.

Congruity backcrosses (CBC; the alternate backcross of the hybrids with genotypes of each species for several cycles; Haghighi and Ascher, 1988) promises to produce hybrids with higher genetic recombination and gene introgression rates. However CBC could only be applied to few facilitator genotypes of both species (Mejia-Jimenez *et al.*, 1994, and unpublished results).

As a result of the necessity to develop fertile common x tepary beans hybrids using the tepary bean genotype NI576 (genotype competent to *Agrobacterium* mediated genetic transformation, see Mejía-Jiménez *et al.* in this annual report), which could not be crossed using conventional or congruity backcrosses, a novel backcross methodology called Double Congruity Backcross (DCBC; Mejía-Jiménez et al. in preparation) has been developed. The DCBC methodology has allowed the production of fertile common x tepary beans hybrids involving the above-mentioned genotype. This methodology seems also to be generally applicable for the production of fertile hybrids from otherwise "incompatible" tepary and common beans genotypes.

In the year 2001, the DCBC-methodology was applied in crossing the common beans and tepary beans genotypes that have been also difficult to cross using recurrent or congruity backcrosses: G40023 and G40068 (drought), G40199 (bruchids, *A. obtectus and Z. subfaciatus*) and G40019 and G40036 (leaf hopper, *Empoasca kraemeri*).

## Methodology

Double congruity backcross hybrids with the cytoplasms of common or tepary bean (DCBCvulg and DCBCacut hybrids) were developed starting from advanced congruity backcross hybrids with cytoplasm of common bean developed previously (Mejía-Jiménez *et al.*, 1994), as described in the annual report 2000 (Mejía Jiménez et al. 2000).
Embryo rescue was applied when necessary to recover viable hybrids from aborting embryos (Mejía-Jiménez *et al.*, 1994).

## Results

Genotypes of tepary beans that were hitherto considered incompatible when crossed directly to common bean genotypes or even to advanced CBC hybrids, were crossed easily to advanced DCBC hybrids of the same cytoplasm (DCBCacut hybrids, table 1). Several fertile backcross hybrids were obtained from each parental combination. The produced plants were clearly recognizable as hybrids through morphological markers (hypocotyl and flower pigmentation, or primary leave size).

The generated hybrids are currently being used to "carry" the genes from the incompatible genotypes to DCBC hybrids with the cytoplasm of *P. vulgaris* (DCBCvulg hybrids, table 1). This cross is already generating vigorous hybrids, which are expected to be fertile or cross-fertile. Theoretically 25% of the alleles of the hybrids produced this way will be from the tepary bean genotype that hitherto had been incompatible with common beans when crossed using other methodologies.

**Table 1. Double congruity backcross (DCBC) strategy followed to produce fertile of cross-fertile hybrids between genotypes of *P. acutifolius* and *P. vulgaris*.**

| | *P. vulgaris* Cytoplasm | *P. acutifolius* Cytoplasm |
|---|---|---|
| (1) | | Incompatible *P. acutifolius* genotype (i.e. G40023) X Advanced DCBCacut[1] hybrids |
| | | ↓ |
| (2) | Advanced hybrids DCBCvulg[2] X | [G40023 x DCBCacut] hybrids (~50% of the genome of the incompatible genotype) Self-fertile |
| | ↓ | |
| (3) | [DCBCvulg x (G40023 x DCBCacut)] hybrids (25% of G40023) Fertile or cross-fertile | |

1 Double congruity backcross hybrid with *P. acutifolius* cytoplasm
2 Double congruity backcross hybrid with *P. vulgaris* cytoplasm
In the case of the genotype G40023, the G40023 x DCBCacut hybrids are being crossed to advanced congruity hybrid lines with common bean cytoplasm that were selected for several cycles as drought tolerant (S. Beebe, personal communication).

**Conclusions**

DCBC allows the production of viable and fertile or cross-fertile common x tepary hybrids from genotypes that were before incompatible using other crossing techniques.

**Future plans**

To produce fertile hybrid populations with common bean cytoplasm involving each of the selected tepary bean genotypes.
To measure the introgression of DNA fragments from the tepary bean genotypes in the fertile hybrid populations produced after DCBC, using AFLP techniques.

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## 2.2.4 Farmer's cassava seed production using *in vitro* low cost system

R. Escobar[1], C. Hernandez[2], J. Restrepo[3], G. Ospina[3], J. Tohme[1] and W.M Roca[1-4]
[1] SB1 Project, [2]Representative of Farmers community, [3]:FIDAR, [4]:CIP-Peru

**Introduction**

The use of cuttings of unknown phyto-sanitary conditions for cassava propagation reduces yield, farmer's income and seed quality and quantity for next crop cycle. An interdisciplinary group constituted by farmers, biotechnology researchers, and one NGO, sponsored by PRGA, modified and simplified *in vitro* propagation methods. Using a participatory scheme it was possible to establish a simple, low-cost cassava propagation system to produce certified, healthy and enough planting material (CIAT, BRU Annual Report 2000).

**Materials and methods**

A simple *in vitro* propagation system that minimized external inputs was implemented in a pilot experimental station located in Santa Ana, Cauca (Colombia). A representative person of the

community (Mr. C. Hernandez), selected by the community itself, trained a group of eleven women in tissue culture.

A propagation scheme was set up with MCol 1522 (Algodona), a traditional clone in the zone of which farmers say "it's very good but it gets sick easily".

**Results and discussions**

One hundred and thirty one different media have been tested during the last two years of the project. We selected three media with propagation rates (1:3-4) (BRU, Annual Report 2000) similar to that of conventional 4E medium (Roca 1984).

Other varieties were incorporated into the propagation system (CM 523-7, MBra 383, CM 6740-7).

We met with farmers to move in vitro plants to the field. Four blocks with 20 plants each were planted in Perico Negro, Puerto Tejada (Cauca, Colombia). One of the expected outcomes of this exercise is to help determine differences in root production between in vitro- and cutting-derived planting material. Furthermore, these plots could serve as the initial phase of a farmer's nursery system.

As an extra income source, we provided them with horticulture seeds to plant in the communal garden and in individual farms (seeds were donated by the Universidad Nacional de Colombia-Palmira).

Due to a long dry season in Cauca, and to contamination associated with animal feeding areas (swine and poultry) located too close to rural tissue culture labs, it was necessary to move plants back to CIAT's station and make adjustments to the rural plastic cabinets. Mr. Hernandez initiated a propagation scheme in CIAT to recover and increase the number of *in vitro* plants before returning them to Santa Ana.

We are currently testing options to replace MS medium. Explants (shoots and nodes) grown on media with Agrimins® show good response (root induction and growth). Tissue grown on media with Urea alone (46:0:0) died. A complete fertilizer (Coljap® desarrollo) induced callus proliferation with no root induction.

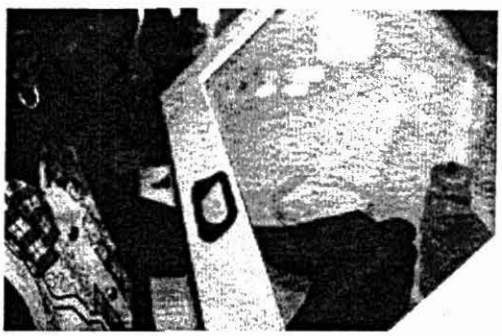
We built a prototype of a low-cost, sterile transfer cabinet, that reduced the purchasing price 12 times, from 3,000 to 250 US dollars/chamber.

CIAT maintains a pilot experimental area in its headquarters to optimize the system and include other crops to establish a farmer's seed platform. Multi-crop seed production systems could help farmers to establish a rural seed enterprise.

**Figure 1a: Farmers meeting to transplant *in vitro* plants to the field. (1b) Farmer using low-cost chamber**

1a

1b



**Conclusions**

Transplanting *in vitro* plants to the field was a success (99% recovery); it was the first time women from the farmers' group used in vitro-generated planting material.
Low-cost transfer cabinets reduce costs, maintaining an efficient propagation activity.
We replace all inputs for the conventional *in vitro* medium. This is an advantage if others farmer groups become interested in propagation activities.

**Future activities**

A propagation scheme needs to be established to integrate low-cost components with rapid propagation systems. This will help to initiate a cassava farm nursery system.
A cost analysis is critical for the process.
Include other crops in the propagation scheme.
A second phase to consolidate the propagation plan will be critical for the process.

**References**

Roca 1984

CIAT Annual report 2000

## 2.2.5 Estimation of the costs involved in a rapid multiplication scheme based on the use of micro-stakes

H. Ceballos [1-2]
[1]IP-3 Project ; [2]SB-2 Project

**Introduction**

The low multiplication rate of cassava has been listed as one of the most important constraints for cassava development. Several techniques have been developed for overcoming this problem,

including faster tissue culture or the use of micro-stakes alternatives. Although the protocols have been worked out, seldom these alternative multiplication rates have been applied at large scale. Therefore there is a need to scale up the process both to evaluate its viability when large volume of vegetative material is handled, and to estimate the costs of production. This information can be used by the private sector if the interest eventually develops for the creation of commercial seed production endeavors.

**Specific objectives**

- To set up a facility for a large production of vegetative material through the use of micro-stakes.
- To complete the system with an anti-white flies screenhouse.
- To produce clean vegetative propagules of elite clones.
- Estimate the costs of production.

**Materials and methods**

Several key elite clones were selected for multiplication using this system. Most of them are adapted to the mid-altitude valleys, where the common presence of white flies has resulted in a higher incidence of frog skin disease. The process starts with the indexation of "mother plants" to make sure they are free of frog skin and various virus that affect cassava.. The long stems obtained from these disease-free plants were cut into micro-stakes, about 5-cm long and with two buds, which were then put to sprout in moist chambers. Every two weeks the resulting shoots are "harvested" and put to produce roots in glass containers with plain water. Once they produce roots the plantlets are transferred to plastic bags with soil and eventually moved to the screenhouse. for hardening and further grow (about 1-2 months) before being transplanted to the field.

**Results**

The process has been used to multiply SM 909-25 (1355), SM 1219-9 (1213), SM 1460-1 (450), SM 1741-1 (272), CM 7514-7 (975), CM 7951-5 (490), MBRA 383 (998). The number of plants produced for each clone and already transplanted to the field is mentioned within parenthesis. These plants are certified to be disease free and constitute an important set of elite clones for this environment. It should be pointed out that the multiplication process begun while the indexation took place (frog skin disease indexation requires a grafting with the highly susceptible 'secondina' clone, which may take up to three months for results). As the results from the indexation came, several plants were found to be contaminated and, therefore, the plantlets obtained from them had to be discarded. Otherwise, the system proved to be operational and able to produce consistently large number of vegetative material with few problems to be solved now and then.

The system implemented allowed two people to produce about 10,000 plantlets in a period of three months. Costs have been estimated for a period of continuous production for two years (8 batches of 10,000 plants each). The total for infrastructure costs (mainly the moist chambers) was therefore divided by eight in the estimate of costs presented in Table 6. The cost of the special screen house was not included in this cost estimates because it is only necessary in areas where white flies are an important vector for cassava diseases.

**Table 6. Distribution of costs (US dollars[¶]) for the production of a batch of 10,000 plantlets using the micro-stakes multiplication system.**

| ITEM | COST |
|---|---|
| Agrochemicals, plastic bags, razor blades, detergent, fertilizers, etc. | 253.59 |
| Infrastructure (distributed in 8 batches) | 112.28 |
| Labor (two people) | 909.09 |
| **TOTAL** | **1274.97** |

[¶] 1 US$ -= .200 Colombian pesos

An important connection to of this activity for the SB2 project is that the later stages of the process described above apply equally to plantlets developed from tissue culture techniques. Particularly relevant in this case is the application that rapid temporary immersion systems could have in commercial multiplication of cassava propagules..

## 2.2.6 Rapid propagation of planting material by temporary immersion bioreactors.

[1]R. H. Escobar, [1]L. Muñoz, [1]J. Tohme and [1-2]W. Roca
[1]SB-2 Project, [2]CIP-Peru

### Introduction

Cassava has been considered the most important alternative crop since it is the only crop that yields acceptably under marginal conditions, with minimum inputs. Demand for cassava planting material is high and conventional methods of propagation don't satisfy the needs (Buitrago 1999). We are adapting RITA® to scale-up and produce enough cassava planting material of desired, indexed, commercial clones, using nodes as initial explants (Annual Report 2000).

### Methodology

Different immersion periods and growth regulators were tested. TDZ at low concentrations, combined with short immersion periods, resulted in better propagation rates than those obtained with BAP. The method is being adjusted with 16 commercial clones. In the last month we initiated RITA with clones MPer 183, HMC 1, CM3306-19 and MCol 1522.

### Results

We increased propagation rates up to 1:6 to 1:10 (Table 1), depending upon the genotype, which was higher if compared with rates (1:3-4) of normal propagation on 4E solid media (Roca 1984).

Plants produced with this system were transferred to the screenhouse and compared with plants produced on solid media. No morphological differences were observed.

Carbon dioxide was injected into RITA. Partial results indicate bud activation but not elongation. More time may be required for shoot elongation.

We are adapting bioreactors at low cost. RITA vessels are being replaced with recyclable soda glasses, and silicone tubing by aquarium flexible pipes. Hydrophobic, 0,22μm filters were

substituted by hand-made, cotton filters. We are setting up plans to run a low-cost, pilot bioreactor system, that can be transferred to small-scale farmer systems.

**Table 1: Propagation rates of commercial cassava clones using RITA system.**

| Cassava clone | Common name | Recovered tissue | | Propagation rate |
|---|---|---|---|---|
| | | Shoot | Nodes | |
| CM 3306-4 | Ica Negrita | 50 | 51 | 10.1 |
| CM 4574-7 | - | 40 | 25 | 6.5 |
| CM 523-7 | Catumare | 46 | 60 | 10.6 |
| MBra 383 | Brasileña | 43 | 25 | 6.8 |
| MBra 507 | Tucuma | 43 | 35 | 7.8 |
| MCol 2215 | Venezolana | 58 | 30 | 8.8 |
| MCol 1505 | Verdecita o P12 | 28 | 25 | 5.3 |
| Mcub 74 | Señorita | 31 | 42 | 7.3 |
| MEcu 72 | Injerta | 26 | 42 | 6.8 |
| Mtai 8 | Rayon 60 | 26 | 35 | 6.1 |
| SGB 765-2 | Caribeña | 37 | 20 | 5.7 |
| SBG 765-4 | Rojita | 27 | 32 | 5.9 |
| CM 6740-7 | Reina | 32 | 18 | 5.0 |
| CM 3555-6 | - | 46 | 40 | 8.6 |
| CG 1141-1 | Ica Costeña | 48 | 40 | 8.8 |
| Mven 25 | Querepa amarga | 40 | 40 | 8.0 |

A member of the PBA regional committee (Mr. Eduardo Erazo) discussed with us the importance of including in our propagation schemes some regional varieties. Farmers from the Atlantic Coast gave us stakes of local clones "Ramirana", "Yema de huevo" and "Por encima". A GRU staff isolated meristems and treated them with thermotherapy. We are now running RITAs with these materials.

We are also adapting RITAs to improve somatic embryo induction in *Brachiaria* and rice, and to improve rice anther culture. We are also using RITA for FEC regeneration to support cassava transformation activities (see transformation reports in this issue).

**Conclusions**

Propagation rates were improved form 1:3 in conventional solid media to 1:6-11 in RITA system.
Propagation rate is clone dependent.
RITA could be adapted to reduce implementation costs.
RITA could improve somatic embryogenesis and plant regeneration in other crops.

**Future activities**

Test RITA with local varieties (Ramirana, Yema de Huevo and Por Encima)
Transplant plants (80-100 each) of 10 commercial clones propagated with RITA and compare to conventional stakes.

A meeting with farmers, national programs and PBA-regional committee to exchange experiences.

Adjust low cost systems.

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## 2.2.7 Cryopreservation of cassava shoot tips using the encapsulation - dehydration technique.

R.H. Escobar[1, 2], N.C. Manrique[2], D.G. Debouck[1], J. Tohme[2], and W.M. Roca[2-3]
[1]SB1Project, [2]SB2 Project, [3]CIP-Peru

### Introduction

The encapsulation-dehydration technique allows the direct placing of cassava meristematic tissues into liquid nitrogen, avoiding thus the use of expensive equipment and opening the possibility for large-scale, long-term conservation at low cost (Escobar et al 2000). We are testing reproducibility between replications and conservation time in liquid nitrogen as steps to determine logistical aspects in the management of an *in vitro* base gene bank.

### Methodology

Methods have been previously described in Annual Report 2000, and Manrique 2000, and no modifications have been made since then.

### Results

We have currently tested 280 (43,5%) genotypes of the entire core collection (640 total accessions). With data obtain this year (from 127 genotypes) we could establish three groups based on percentage of shoot recovery after freezing:
a high-recovery group of 39 genotypes (up to 70%; Table 1a),
an intermediate group of 65 genotypes with 30-70% recovery (Table 1b)
23 genotypes with low-recovery (below 30%) (Table 1c).

Preliminary testing showed consistency among results (such as shoot recovery) in time (Table 2). We are planing to test the entire core for different conservation times in liquid nitrogen.

We propose a *Minimum Shoot Recovery Percentage* (MSRP) of 30% as the lower limit for the establishment of an *in vitro* base gene bank. This means that any cultivar to be included in the bank needs to have at least 30% of shoot recovery. A 30% MSRP has been constant among different experimental years (1999-2000) in experiments carried out so far. Of the clones tested, 82% comply with this minimum MSRP requirement.

To scale up cryopreservation of the collection, the limiting step is the initial tissue (shoots). It takes about 3-4 propagation cycles to increase the number of shoots to be frozen. As an average, one plant has one shoot and 3-4 nodes with buds. Nodes could also be used for cryopreservation. For this reason we checked axillary buds for cryopreservation. We found that in all clones it was possible to obtain shoots after freezing (Table 3). We plan to adopt buds as explants because it simplifies the work (reduces time, effort and propagation supplies).

**Future activities**

Conserve the entire core on cryotanks and establish a partial monitoring evaluation protocol for different conservation times
Optimized the use of buds as initial explants for cryopreservation
Establish a logistical aspect involved in management of cryo-banks

**Table 1: Response of 127 cassava clones from the core collection, cryopreserved in liquid nitrogen using encapsulation-dehydration technique: 1A. Clones grouping with highest shoot response after freezing (more than 70%)**

| Cassava clone | % Viability | % Shoot formation |
|---|---|---|
| CM 1999-5 | 100 | 83.3 |
| MARG 9 | 100 | 85 |
| MBOL 1 | 100 | 76.6 |
| MBRA 190 | 90.9 | 90.9 |
| MBRA 315 | 100 | 88.88 |
| MBRA 356 | 96.3 | 92.6 |
| MBRA 699 | 88.6 | 72 |
| MBRA 702 | 96.2 | 92.5 |
| MBRA 77 | 100 | 100 |
| MBRA 781 | 100 | 80 |
| MBRA 900 | 87.7 | 70.1 |
| MCOL 1178 | 94.4 | 88.8 |
| MCOL 1186-A | 96.6 | 96.6 |
| MCOL 1780 | 100 | 100 |
| MCOL 2032 | 100 | 90.45 |
| MCOL 2061 | 92.9 | 82.5 |
| MCOL 262 | 100 | 87.5 |
| MCOL 590 | 93.3 | 70 |
| MCOL 608 | 86.6 | 73.3 |
| MCOL 802 | 93.3 | 80 |

| Cassava clone | % Viability | % Shoot formation |
|---|---|---|
| MCOL 955 | 90.5 | 75.9 |
| MCUB 1 | 100 | 70 |
| MCUB 29 | 100 | 88.8 |
| MCUB 53 | 100 | 73.3 |
| MCUB 58 | 90.3 | 87.3 |
| MECU 166 | 100 | 100 |
| MMAL 24 | 85.9 | 75.5 |
| MMEX 17 | 95.75 | 76.93 |
| MMEX 49 | 100 | 100 |
| MMEX 59 | 90.6 | 77.4 |
| MPAN 131 | 92.9 | 79.2 |
| MPAR 135 | 100 | 95.83 |
| MPAR 51 | 90.6 | 77.57 |
| MPER 279 | 92.7 | 86.45 |
| MTAI 2 | 86.6 | 80 |
| MUSA 8 | 92.9 | 74.3 |
| MVEN 309 | 88.3 | 70.5 |
| MVEN 67-B | 100 | 87.87 |
| MVEN 82 | 93.3 | 72.9 |

**Table 1B: Intermediate response between 30-70%**

| Cassava clone | % Viability | % Shoot formation |
|---|---|---|
| CG 1372-5 | 100 | 35.45 |
| CG 915-1 | 100 | 65.8 |
| MARG 7 | 100 | 44.3 |
| MBRA 162 | 96.6 | 67.3 |
| MBRA 674 | 100 | 58.56 |
| MPER 243 | 100 | 65.6 |
| MPER 333 | 100 | 60 |
| MPER 431 | 96.6 | 46.6 |
| MPER 503 | 86.87 | 54.37 |
| MPER 518 | 67.42 | 49.7 |
| MPER 556 | 80.46 | 57.6 |
| MPER 569 | 94.4 | 53.85 |
| MVEN 173 | 86.3 | 42 |
| MVEN 174 | 97.2 | 55 |
| MVEN 208 | 59 | 52.3 |
| MVEN 217 | 81.8 | 48.6 |
| MVEN 297-A | 48.16 | 38.4 |
| MVEN 322 | 100 | 43.3 |
| MVEN 61 | 100 | 42.7 |
| SG 455-1 | 87.5 | 54.2 |
| MBRA 697 | 100 | 37.02 |
| MBRA 73 | 93.3 | 49.1 |

| Cassava clone | % Viability | % Shoot formation |
|---|---|---|
| MCUB 11 | 93.3 | 63.3 |
| MCUB 36 | 88.76 | 50.9 |
| MCUB 56 | 86.6 | 50 |
| MCUB 74 | 96.3 | 63.4 |
| MECU 10 | 74.4 | 43.3 |
| MECU 141-A | 66.6 | 46.6 |
| MECU 144 | 100 | 53 |
| MECU 166 | 90 | 50 |
| MECU 33 | 87.25 | 63.45 |
| MECU 68 | 95.2 | 43.8 |
| MGUA 44 | 91.9 | 55.2 |
| MGUA 7 | 84.4 | 36.6 |
| MIND 26 | 90.3 | 52.4 |
| MIND 3 | 96.6 | 60 |
| MIND 4 | 96.9 | 57.6 |
| MMAL 26 | 65.5 | 62.2 |
| MMEX 102 | 96.26 | 68.8 |
| MMEX 54 | 95.8 | 42.5 |
| MPAN 127 | 100 | 52.7 |
| MPAR 152 | 82.5 | 67.5 |
| MPAR 7 | 82.5 | 31.6 |
| MPER 184 | 100 | 58.1 |

| Cassava clone | % Viability | % Shoot formation |
|---|---|---|
| MBRA 730 | 94 | 46.1 |
| MBRA 792 | 92.9 | 44.8 |
| MBRA 897 | 100 | 65.1 |
| MBRA 916 | 100 | 46.6 |
| MCOL 112 | 88.8 | 41.6 |
| MCOL 1398 | 87.7 | 52 |
| MCOL 490 | 100 | 67 |
| MCOL 534-A | 87.7 | 58.86 |
| MCOL 590 | 100 | 57.7 |
| MCR 18 | 92.9 | 64 |

| Cassava clone | % Viability | % Shoot formation |
|---|---|---|
| MPER 241 | 90 | 51.8 |
| MCOL 1467 | 72.5 | 52.5 |
| MCOL 1736 | 76.38 | 47.2 |
| MCOL 2089 | 57.4 | 40.5 |
| MCOL 2157 | 85 | 53.85 |
| MCOL 2318 | 74.24 | 59 |
| MCOL 2361 | 63.3 | 49.9 |
| MCOL 2493 | 86.9 | 37.2 |
| MCOL 474 | 100 | 56.25 |
| MCR 84 | 100 | 48.6 |

**Table 1C: Clones with lowest shoot formation after freezing (less than 30%)**

| Cassava clone | % Viability | % Shoot formation |
|---|---|---|
| MBRA 589 | 63.3 | 23.3 |
| MBRA 891 | 76 | 14 |
| MBRA 915 | 91.8 | 21.8 |
| MCOL 2245 | 38.6 | 23.25 |
| MCOL 638 | 93.3 | 33.33 |
| MCUB 32 | 78.8 | 3.3 |
| MCUB 42 | 50 | 6.6 |
| MECU 141 | 35.5 | 21.1 |
| MECU 29 | 45.4 | 9 |
| MECU 71 | 70 | 21.8 |
| MECU 85 | 100 | 5 |
| MECU104 | 44.4 | 20 |
| MGUA 63 | 66.6 | 33.3 |
| MPAR 109 | 91.9 | 11.43 |
| MPAR 163 | 77.1 | 31.9 |
| MPAR 25 | 96.6 | 31.6 |
| MPAR 35 | 100 | 26 |
| MPAR 36 | 93.3 | 28.2 |
| MPER 584 | 31.8 | 22.12 |
| MVEN 200 | 96.26 | 20.7 |
| MVEN 210 | 90.09 | 21.21 |
| MVEN 219 | 32.8 | 14.7 |
| MVEN 329-A | 92.2 | 24.4 |

**Table 2:** Response after freezing of different explants (Escobar et al 2001).

| Cassava clone | Explant | Viability (%) | Shoot recovery (%) |
|---|---|---|---|
| MEcu 31 | Shoots | 35.0 | 25.0 |
| | Buds | 56.3 | 39.7 |
| MGua 14 | Shoots | 92.3 | 83.0 |
| | Buds | 78.3 | 36.6 |
| MCol 2215 | Shoots | 50.0 | 26.7 |
| | Buds | 58.3 | 33.3 |
| MCol 1505 | Shoots | 83.3 | 81.3 |
| | Buds | 37.7 | 22.2 |
| MCol 2016 | Shoots | 70.0 | 16.7 |
| | Buds | 96.3 | 79.6 |

**Table 3: Response of some clone cryopreserved across different conservation periods (Escobar et al 2001).**

| Cassava clone | Treatment | Viability (%) | Shoot recovery (%) |
|---|---|---|---|
| MCol 22 | Control Exp. | 88.1 | 88.1 |
| | 1 month | 100 | 100 |
| | 1 report | 95 | 85.45 |
| MPer 436 | Control Exp. | 100 | 87.5 |
| | 1 month | 91.65 | 78.75 |
| | 1 report | 94.1 | 79.7 |
| MVen 90 | Control Exp | 88.65 | 88.65 |
| | 1 month | 95 | 95 |
| | 1 report | 76.7 | 50 |

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## 2.2.8 Costing all expenses about the different methods to conserve cassava germplasm

P. Pardey[1]; B. Koo[1], G. Mafla[2] and D.G. Debouck [1-2]
[1]IFPRI; [2]SB-1 Project; [3]SB-2 Project

As part of a systemwide intiative in view of the establishment of an endowment fund for the germplasm collections held in-trust in the CGIAR, the GRU together with IFPRI has carried out a costing study of all its operations. The following table shows the costs per accession for the different conservation methods of cassava. It also shows how much would be needed to conserve the present collection in perpetuity. Another outstanding figure of this study is the amount of money saved through slow-growth *in vitro*, a piece of research initiated in 1997. If the GRU can extend the period between each subculturing from 12-14 months (an average between clones) up to 24 months, the saving could be of US$ 230.93 per accession, allowing a saving of US$ 1,404,079 for the entire collection of 6,080 accessions in perpetuity.

Costing studies of cassava germplasm conservation

*(cost per accession in US $; under 4%)*

| | *in vitro* | cryo | field | all |
|---|---|---|---|---|
| per year | 10.34/ 67.61 | 0.86/ 113.77 | 7.18 | N.A. |
| in perpetuity | 268.73 | 144.06 | 186.69 | 599.48 |

after Koo et al. 2001

## 2.2.9 Preliminary studies on the cryopreservation of meristems and seeds of wild *Manihot* species

R.H. Escobar[1,2], N.C. Manrique[2], F. Gil[1], J. Tohme[2], and D.G. Debouck[1]
[1]SB1 and [2]SB2

**Introduction**

CIAT maintains wild *Manihot* species as a field gene bank and as a small *in vitro* collection. Under field conditions, these species are exposed to pests and diseases, and their flowering and seed set are affected by environmental conditions. Twenty-nine species and 300 genotypes are maintained *in vitro* with subcultures every 6-12 month (personal communication, G. Mafla). Both options are seen as temporary given their costs, space and manpower requirements. Seeds in cold

storage have been kept up to seven years without loss of viability; if maintained at room temperature however, they lose viability after one year. Marin *et al* (1990) established a cryopreservation protocol with seeds and zygotic embryos. Later Escobar and Mafla modified the warming step after freezing, expediting the process (BRU, data not published). Two other approaches for the long-term conservation of *Manihot* diversity are being tested: cryopreservation of meristematic tissues (conservation of individual genotypes), and storage of botanical seeds (conservation of population genetic diversity)

**Methodology**

Modifications of the encapsulation-dehydration protocol, successfully used for shoot tips of cassava *(M. esculenta)* (Escobar *et al* 2000), were tested on two genotypes of *M. carthaginensis,* three genotypes of both *M. esculenta* subsp. *peruviana* and *M. esculenta* subsp. *flabellifolia.* These modifications consisted in different periods of dehydration on silica gel, and pre- and re-growth media components. The *in vitro* wild material selected by this study had completed passport data. Experiments have been undertaken with seeds of three *Manihot* species by freezing them in liquid nitrogen during different periods. Recovery of seeds will be monitored.

**Results and discussion**

According to Velasquez (1995), wild species had different behavior in *in vitro* conditions. It was necessary to adapt five propagation and conservation media (12A1, 12 A2, 12A3, WPM1 and WPM2) according to wild-group (genotype or species) response. When we tested the response after freezing using 12A3 medium in the process of regrowth, no growth was observed. We are currently using 12A3 only in the propagation scheme. At the beginning of this project we observed that wild frozen shoots responded with callus formation and tried to form shoots, but the *in vitro* growth conditions were not optimal. It was then necessary to increase Kinetin content, test 4E medium (Roca 1984) and MS with activated charcoal to obtain shoots after freezing.

The quality of cassava shoots is important for a good response after freezing (Escobar et al 2000). The morphology of wild shoot was different prior to freezing. *M. carthaginensis* showed no shoot growth on 4E medium before freezing (only differentiated leaves); *M. esculenta* subs. *peruviana* and *flabellifolia* showed stronger shoots after 3 days on 4E medium prior to freezing. Probably the lower response of *M. cathaginensis* was influenced by the previous conditions of donor tissues (Table 1). Different pre-growth periods (3,4 y 5 days) and media could be helpful to improve shoot quality before freezing.
We are introducing other wild species such as *M. orbicularis, M. cecropiaefolia , longipetiolata* to validate the method.

**Table 1: Response of wild *Manihot* species after freezing**

| Genotype | % Viability | % Shoot formation |
|---|---|---|
| *M. esculenta* subsp. *Flabellifolia* 439-003 | 51.35 | 39.55 |
| *M. esculenta* subsp. *Flabellifolia* 437-007 | 100 | 83.3 |
| *M. carthaginensis* 017E | 0 | 0 |

We compared different desiccation periods on silica gel (16h vs. 24 h) before freezing. We found that with *M. carthàginensis* 17f 417-001 it was possible to obtain plants after freezing (7.7%) with 16 h desiccation. With *M. esculenta* subsp. *flabellifolia* it was possible to obtain shoot formation in both conditions (Table 2).

**Table 2: Recovery of frozen wild *Manihot* species desiccation during different periods on silica gel.**

| | Rep | Viability % | % Shoot formation | Viability % | % Shoot formation |
|---|---|---|---|---|---|
| Desiccation duration | | 24 h | | 16 h | |
| Genotype | | | | | |
| *M. esculenta* subsp. *Peruviana* 413-003 | R3 | 35.7 | 21.4 | - | - |
| *M. esculenta* subsp. *Peruviana* 417-003 | R1 | 0 | 0 | 12.5 | 12.5 |
| *M. esculenta* subsp. *Peruviana* 417-003 | R3 | - | - | 60 | 30 |
| *M. esculenta* subsp. *Peruviana* 417-005 | R1 | 58.3 | 16.6 | 0 | 0 |
| *M. esculenta* subsp. *Peruviana* 417-005 | R2 | 0 | 0 | 0 | 0 |
| *M. esculenta* subsp. *Peruviana* 417-005 | R3 | 30 | 0 | 41.6 | 0 |
| *M. carthaginensis* 413-013 | R1 | 0 | 0 | - | - |
| *M. carthaginensis* 413-013 | R2 | 56.25 | 0 | 31.25 | 0 |
| *M. carthaginensis* 413-013 | R3 | 0 | 0 | 10 | 0 |
| *M. carthaginensis* 17f 417-001 | R1 | 0 | 0 | - | - |
| *M. carthaginensis* 17f 417-001 | R2 | - | - | 23 | 7.7 |
| *M. carthaginensis* 17f 417-001 | R3 | 14.28 | 0 | 7.14 | 0 |
| *M. esculenta* subsp. *flabellifolia*. 444-002 | R1 | - | - | 33.3 | 11.1 |
| *M. esculenta* subsp. *flabellifolia*. 444-002 | R2 | 100 | 60 | 100 | 100 |
| *M. esculenta* subsp. *Flabellifolia* 444-002 | R3 | 84.6 | 53.8 | 0 | 0 |
| *M. esculenta* subsp. *Flabellifolia* 433-002 | R1 | 76.9 | 61.5 | 53.8 | 46.1 |
| *M. esculenta* subsp. *Flabellifolia* 433-002 | R2 | 70 | 50 | 80 | 50 |
| *M. esculenta* subsp. *Flabellifolia* 433-002 | R3 | 50 | 10 | - | - |

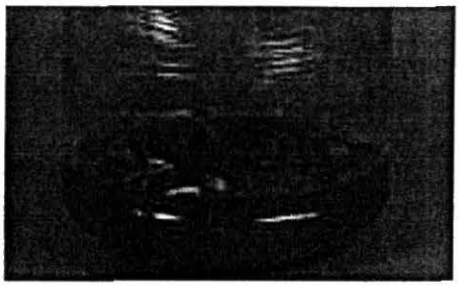

**Figure 1: Plants of *M. esculenta* subsp. *flabellifolia* 444-002 recovered form liquid nitrogen.**

## Conclusions

Preliminary results showed that plants from wild cassava species could be recovered after freezing with a 0-60% survival.

*In vitro* shoot tips of wild material had different aspect compared to cassava. It may be necessary to modify pre- and post-freezing conditions.

Simple MS with activated charcoal medium is a key factor to recover shoots after freezing.
Different dehydration periods could improve recovery rates after freezing of wild material.
*M. esculenta* subsp. *peruviana* and *flabellifolia* showed better response than *M. carthaginensis.*

## Future activities

The cryo-methodology needs to be validated with other wild material.
Keep fine-tuning conditions to improve recovery rates of wild material after freezing.
Compare cryopreservation method with conventional medium-term conservation (at 5°C or -20°C) helps to determine the best conservation alternatives.

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BRU Annual Reports
Manrique NC. 2000

## 2.2.10 Temporary Immersion System (RITA) for Anther Culture of Rice

E. Tabares[1], G. Delgado[2], R . Escobar[1], Z. Lentini[1]
[1]SB-2; [2]IP-4

### Introduction

Plant *in vitro* culture using temporary immersion offers all the advantages of a liquid medium system (automation, large scale production, easy changes of medium, filter sterilization, easy cleaning) without any of its drawbacks (reduced gas exchange, vitrification ). The RITA device has the additional advantages of low cost and automation. And it is suitable for both industrial production and research use. This system has proved its efficiency for somatic embryogenesis of banana (Alvarat *et al*, 1993; Escalant *et al*, 1994), coffee (Berthouly *et al*, 1995; Etienne *et al*, 1997), citrus (Cabasson *et al*, 1997), oil palm and rubber plant (Etienne *et al*, 1997), and at CIAT for cassava (Escobar and Roca , 1999); for clonal propagation through micro-cuttings of coffee, and sugar cane (Lorenzo *et al*, 1998); for proliferation of meristems of banana, and pineapple; and for micro-tuberization of potato (Teisson & Alavarad, 1998). RITA system improves plant cell nutrition and gas exchange, and enhances the quantity and quality of micro-propagated tissue. Last year we reported the use of RITA for the induction of embryogenic callus derived rice mature zygotic embryos. Callus induction was noted 8 to 15 days earlier in the RITA system respect to petri plates. A larger number of embryos showed callus formation, and between 2 to 4 fold increase in the number of embryogenic callus was seen in the automated system. One of the major bottlenecks for generating doubled haploids from rice anther culture (AC) is that the response is highly dependant on the genotype. Japonica types are generally highly responsive in contrast to indica rice, which shows low response and several genotypes are recalcitrant. The low response of indicas restrain the use of AC in breeding for the tropics. Following is reported preliminary experiments directed to induce embryogenic callus and plant regeneration from rice AC using the automated RITA system.

### Materials and Methods

For preliminary experiments, a japonica genotype (CT 6241-4-1-15-1) and an indica variety (Palmar) were used. Plants were grown in the field, panicles harvested, and anthers cultured according to Lentini et al., 1995. Anthers were either culture in liquid medium contained in baby food jars (control) or in RITA vessels. Seven hundred and fifty anthers were culture per RITA vessel. Immersion system was set up for cycles of 6 hr immersion. Induced callus from each culture system, was then transfer onto solid plant regeneration medium according to Lentini et al. 1995.

### Results and Discussion

Callus induction was noted 5 days earlier in the RITA system respect to the control. A significant larger number of embryogenic callus was noted in RITA for both genotypes, and between 3 to 5 fold increase in the number of regenerated plants was seen in the automated system (Figure 1). Experiments are in progress to determine the reproducibility of these results with larger number of *indica* genotypes, and to optimize the system for recalcitrant genotypes important for the breeding program.

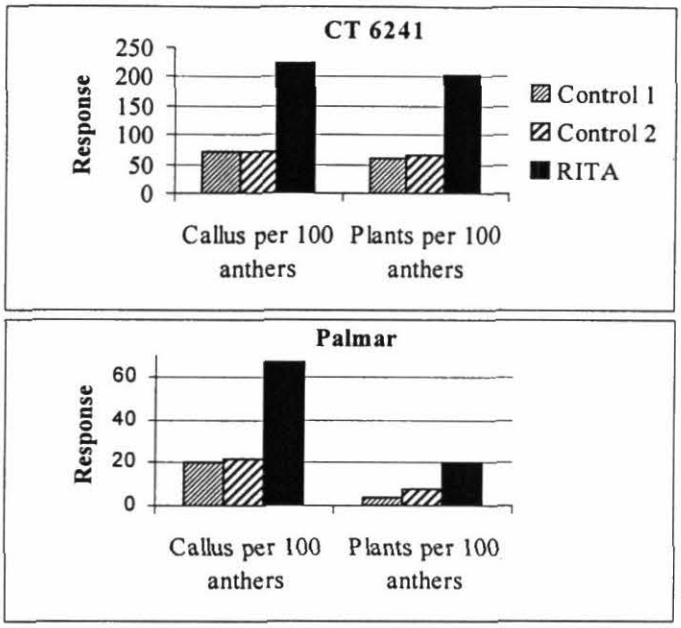


**Figure 1.- Callus induction and plant regeneration of japonica and indica rice in RITA system**

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## 2.2.11 Development of selection systems for the generation of transgenic rice according to current food biosafety requirements

E. Tabares[1], G. Delgado[2], Z. Lentini[1]
[1]SB-2 Project; [2]IP-4 Project

### Introduction

Most selection systems commonly used for the generation of transgenic rice using either biolistic or *Agrobacterium* mediated transformation, relies on the use of the *hpt* gene conferring antibiotic resistance to hygromycin. Although the selection using hygromycin resistance gene has been highly efficient for the production of transgenic rice, and *hpt* gene has been de-regulated for generating transgenic food for human consumption, it has not yet been approved for animal. As a response to public perception concerns, recent international food biosafety recommendations suggest to use non-antibiotic selection markers genes. From the year 2005 it is most likely that food or feed crop containing antibiotic resistance will not be approved for commercialization. Recent developments using selection systems different from either antibiotic or herbicide resistance suggest that the phosphomannose isomerase (*pmi*) is an efficient selection gene for production of transgenic plants from maize, sugar beet, and cassava (Wang, et al. 2000; Negrotto et al., 2000). When non-transgenic cell tissue are cultured on mannose, after uptake mannose is phosphorylated by a hexokinase, yielding mannose-6-phosphate which accumulates in plant cells and causes severe growth inhibition. Mannose isomerase gene converts mannose-6-phosphate to fructose-6-phosphate, thus giving plant cells the capacity of metabolizing mannose as a carbon source. Last year we reported the optimization of a positive selection protocol using mannose containing medium, for callus induction from mature zygotic embryos Agroinfected with a construct containing the *pmi* gene. Callus showed a significant reduction in growth when cultured on mannose containing medium respect to sucrose. An inhibition of 59%, 64%, 95% and 98% were noted when callus were cultured on mannose 1%, 2%, 3%, and 6%, respectively. The level of this inhibition was even higher than that observed on medium without carbon source which shows a 87% of growth inhibition respect to 3% sucrose. The inhibition of growth was due to the effect of mannose and not due to an increase of osmotic potential, since callus on 3% sucrose + 1.5% mannitol or 6% sucrose + 3% mannitol develop alike to those on 3% sucrose. Based on these results in the case of Cica 8 a concentration of 3% mannose is considered sub lethal, and 6% mannose a lethal dose. Following is described the progress made regenerating plants of rice from mannose resistant callus.

### Materials and Methods

Mature zygotic embryos of rice indica varieties Cica 8 and Palmar were co-cultivated with *Agrobacterium tumefaciens* strain LBA 4404 containing a constuct containing the phosphomannose isomerase (*pmi*) gene and the *uid-intron* gene encoding the β-glucoronidase for gus expression. After agroinfection , embryos were cultured on callus induction medium containing acetozyringone 100μM and cefotaxime 250 mg/l for bacteria elimination. A stepwise selection for callus induction was conducted first on medium containing 3% mannose (261 mOsmol/kg ), as the only carbon source, followed by a subcultured on induced callus on medium with 6% mannose. Mannose resistant callus were then transferred onto plant regeneration solid medium containing 2% mannose as based line complemented with either 0.5 %, 1.0%, 1.5% or 2.0% sucrose.

## Results and Discussion

For Palmar variety, preliminary experiments indicated that although plant differentiation was noted on medium containing only mannose without sucrose, shoots did not elongate when mannose was used as the only carbon source (data not shown). Therefore, various concentrations of sucrose were added besides the mannose to aid the plant regeneration process. Initially, a total of 16 different combinations were tested which included mannose from 0.5% to 6% in combination with sucrose from 0% to 3%. Results suggested that in order to have only transgenic plant regeneration in Palmar, a minimum of mannose 2% was needed in the plant regeneration medium (data not shown). Palmar showed about 50% plant regeneration on mannose 2% with either sucrose 1.5% or 2.0% (Table 1). All the regenerated plants showed gus expression (Table 1). In contrast, Cica 8 did not regenerate at all when mannose was included in the medium, although medium was complemented up to 3% sucrose (Table 1). About fifty percent plant regeneration was attained from Cica 8 mannose resistant callus only when 3% sucrose was used as the sole carbon source, however of these plants only 5% showed gus expression. Southern blot analysis of these plants is in progress. Results obtained so far, suggest that all gus expressing plants had simple insertions of both *pmi* and *iud-intron* genes in the genome.

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**Table 1. *Effect* fo mannose : sucrose ratio on plant regeneration of mannose resistant callus [1]**

| Genotype | Treatment | (%) Mannose | (%) Sucrose | Callus analyzed | Plants regenerated | (%) Regenerated plants | *iud-intron* gene expression | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Plants tested | Plants $Gus^+$ | (%) $Gus^+$ |
| Palmar | 1 | 2.0 | 0.5 | 4 | 0 | 0 | | | |
| | 2 | 2.0 | 1.0 | 20 | 0 | 0 | | | |
| | 3 | 2.0 | 1.5 | 9 | 5 | 56 | 5 | 5 | 100 |
| | 4 | 2.0 | 2.0 | 4 | 2 | 50 | 1 | 1 | 100 |
| | Control | 2.0 | 2.0 | 20 | 0 | 0 | | | |
| | | | | | | | | | |
| Cica 8 | 1 | 0.0 | 3.0 | 35 | 20 | 57 | 20 | 1 | 5 |
| | 2 | 2.0 | 0.5 | 6 | 0 | 0 | | | |
| | 3 | 2.0 | 1.0 | 5 | 0 | 0 | | | |
| | 4 | 2.0 | 1.5 | 6 | 0 | 0 | | | |
| | Control | 2.0 | 2.0 | 5 | 0 | 0 | | | |

[1] Putative transgenic callus derived on selection medium containing 3% mannose (sub-lethal) followed by 6% mannose (lethal)

## 2.2.12 A first initiative to support cassava seed production for the industry

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### Introduction

In the Northern Coast of Colombia, cassava has been considered the most important alternative crop since it is the only that yields acceptable under marginal conditions and with minimal inputs. There is an international starch industry, Corn Products, that has a branch located in Malambo (Barranquilla, Colombia), formerly known as *Inyucal*. It is considered one of the largest cassava consumers for industrial purposes in Colombia. However, Corn Products has encountered a chronic problem in the supply of raw material, fresh cassava roots for milling.

To continually operate the cassava milling plant, it would be desirable to provide up to 100 ton per day of fresh roots. Under the actual conditions, only 0.5 ton/day are ground, and in some days no material is available to take to the mill (M.C. Arzuza personal communication). In vitro propagation systems could be a quick and safe manner to put enough, healthy planting material in farmer's fields to support Corn Products' demand. This project is the first initiative to set up cassava seed production programs aimed to satisfy industrial needs.

### Methodology

Based on CIAT's experiences on handling cassava *in vitro* (Roca 1984; Escobar et al 2000, 2001; Annual Report 2000), we performed a feasibility analysis based on the final number of plants needed per hectare. This allowed the estimation of propagation rates, lab requirements, manpower, screenhouse facilities among other inputs to start the project. After establishing the scale and lab requirements, we trained staff from Corn Products. During the initial steps we formed a discussion group to diagram the lab to be established in Malambo. The lab included four essential areas: sterilization, propagation, washing and growth areas. Additionally, a screenhouse was also established. CIAT supported the technical and logistical scheme, while Corn Products provided financial support. Corn Products signed an MTA to access cassava germplasm MTai 8 and MVen 25. They received plants that were given to us later to initiate propagation schemes at CIAT while they were building the laboratory facilities in Barranquilla.

### Results and discussion

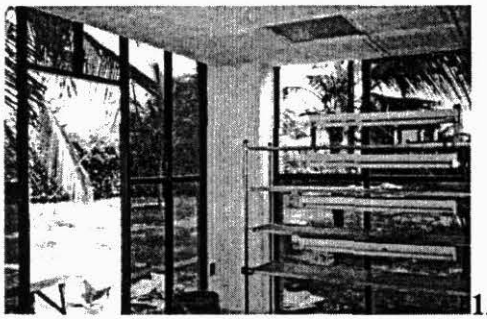
A 75,2 $m^2$ laboratory (including the four critical areas and a small office), and a 72 $m^2$ screenhouse were built (Figure 1). One staff from Corn Products (Maria C. Arzuza) was trained in tissue culture during one week. She then trained other partners in Malambo. They signed the MTA #028 to receive in vitro plants from the Genetic Resources Unit (5 plants per clone; April 23-1999). At BRU, using conventional propagation schemes, we increased the planting material and divided it in two sets:

1) a first set of 1336 *in vitro* plants (682 plants of MTai8 and 654 plants of MVen25; July 16-2001), was sent to Malambo to initiate propagation activities,
2) a second set was maintained at BRU as a back up under minimal growing conditions (35 Magenta boxes of MTai 8, and 12 Magenta boxes for MVen25; each magenta contained 20 plants).

Corn Product staff initiated propagation with material (1,336 plants) received from BRU. We estimated, based on a 1:3 propagation rate, that Corn Products would produce around 4,000 plants. They are currently propagating 25-40 containers/day/person, and maintaining 346 containers of MTai 8 (1384 plants) and 534 of MVen 25 (2136 plants), for a total of 3,400 plants (88% of the expected number of plants).

We maintain communication through e-mail, fax, phone and technical field visits to support improvements in staff skills and lab and screenhouse conditions in an attempt to keep losses below 3%. This will allow a more accurate planning of propagation schemes.

Although Mven25 was one of the clones selected initially for propagation, its poor adaptation to soil conditions in the area, and the early stake sprouting makes it unattractive to keep it in the propagation scheme. For this reason Corn Products is selecting other clones with better response and high dry matter content to propagate them.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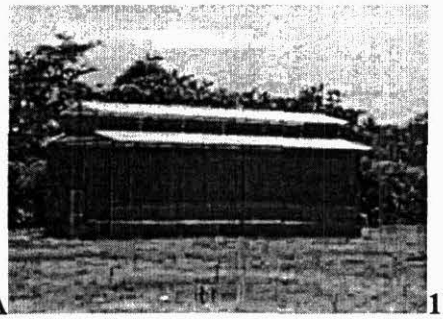
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**Figures: (1A) *In vitro* growth area in the laboratory and (1B) Screenhouse facilities in Malambo-Barranquilla.**

## Future activities

- Adjust screenhouse conditions to recover higher percentages of transplanted plants.
- Design propagation plans to maintain back ups in vitro to feed production lines, improve propagation rates and reduce costs.
- Introduce other clones into propagation schemes.

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## 2.2.13 Development of methodologies for *in vitro* multiplication, plant regeneration, and genetic transformation of naranjilla (lulo)

V. Segovia; Z. Lentini
SB-2 Project

### Introduction

A large number of fruits of Andean origin have great potential to become premium products for local and export markets with a high economic return for the farmers. Naranjilla (*Solanum quitoense*) is among these fruits. This species is native from Colombia and Ecuador, and it is normally cultivated between 700 and 2000 meters above sea level. Some of the main attributes of this fruit includes its high level of vitamin C, and the sub-shrubby perennial growth amenable for cultivation in hillsides and inter-cropping, aiding soil conservation practices. Recently in Colombia, naranjilla changed from being a fruit of local fresh consumption to become an important industrial fruit for juice and yogurt products, increasing its market value. A major constraint for the rapid adoption of naranjilla by the local farmers is the limited availability of elite germplasm free of pathogens. The high level of trait segregation restrains its multiplication through seeds. Rapid multiplication of quality planting materials is of paramount importance. One of the main objectives of this project is to develop a protocol for *in vitro* propagation of naranjilla with application for conservation and rapid multiplication of clones free of pathogens. The expected results include the mass multiplication of elite clones that then can be distributed to farmers. Since breeding for this species is almost non-existing, paralelly to the *in vitro* propagation effort, it will be important to develop plant regeneration and transformation systems to aid the development of germplasm. Last year it was reported preliminary results on developing a plant regeneration protocol. This year it is presented the advancement in establishing a system for maintenance of the *in vitro* germplasm collection, and the progress made identifying factors to increase the plant regeneration efficiency from elite naranjilla materials.

### Materials and Methods

High quality and elite clones provided by the Andean Fruit Center (Centro Frutícola Andino – CEFA) and Corpoica La Selva are used. This collection includes naranjilla with or without thorns commonly grown by farmers. Various media were tested for *in vitro* maintenance of the clones. Statistical experimental design was used to determine the optimal plant tissue and medium for an increase efficiency in plant regeneration. Regenerated plants were taken to the greenhouse and the field to evaluate plant growth and development to maturity. Plants initially grown *in vitro* and then in soil, were taken back to the *in vitro* system to establish a protocol for renewing the *in vitro* collection.

### Results and Discussion

*In Vitro* Propagation
Last year it was reported that plant develops healthier and faster *in vitro* when cultured onto ½ MS medium supplemented with ANA 0.02 mg/l, BAP 0.04 mg/l, and $GA_3$ 0.05 mg/l, and agar 4.5 g/l, in contrast to the regular micro-propagation medium used by CEFA or Corpoica La Selva (SB2 Annual Report 2000). However, after 10 months of sub-culturing naranjilla *in vitro* plants in this medium, most of the plants showed white stripes on leaves, thick stems, and slow development. Reason why other media were tested again. For this purpose, it was evaluated media commonly used for long term maintenance of *in vitro* germplasm of other *Solaneace*

species. Of the medium tested, a medium containing MS basal salts and vitamins, and supplemented with calcium pantothenic acid 2.5 mg/l and gelrite 3.5 g/l (Hussey and Stacey, 1981) used for potato, also showed to be the optimal for naranjilla (medium A). On this medium, *in vitro* shoots start showing root proliferation two weeks earlier respect to medium ½ MS medium. Fully expanded new leaves were present at one month after subculture on medium A, whereas it took about 6 weeks on ½ MS medium (Figure 1). Moreover, new leaves developed on medium A were completely green although the starting materials had leaves with white stripes. The number of leaves with white stripes increased as plants were sub-cultured on ½ MS medium. By sub-culturing plants on medium A contained in jars rather than in test tubes, it is possible to obtain a large number of explants to conduct series of experiments for optimizing plant regeneration with various replicates every month. Medium A also seems to be a more appropriate for a long term *in vitro* maintenance of the germplasm collection since plants are healthier, develop faster, and differentiate normal green looking leaves.

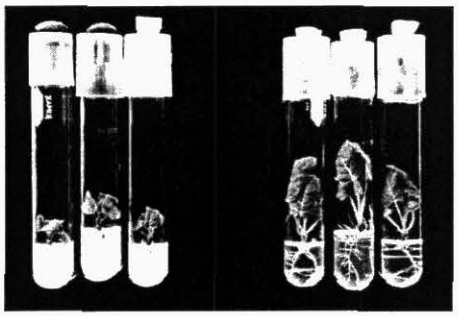

**Figure 1**. *In vitro* development of naranjilla plants in ½ MS medium (left) and medium A (right) one mothe after subculture

**Plant Regeneration**

A randomized block design of four replicates each of 15 experimental units was used to determine the best medium composition and explant to induce a direct plant regeneration in naranjilla. A non-parametric chi-square analysis indicated that petioles from the first and second node showed 17 times and about four times more plant regeneration that the corresponding leaves from thorny and non-thorny clones respectively. (Figure 2 and 3). A significant higher response was also noted on medium originally develop for plant regeneration on tomato (Ultzen et al, 1995), consisting on MS salts, B5 vitamins, supplemented with sucrose 10 g/l, glucose 10 g/l, gelrite 1.5 g/l, zeatine 2 mg/l and IAA 0.02 mg/l (Figure 2A). On this medium petioles from thorny genotypes showed three fold increase in plant regeneration respect to a medium reported for naranjila ( Hendrix et al., 1987) composed by MS salts and vitamins and supplemented with sucrose 30 g/l, agar 7 g/l, IAA 0.01 mg/l, kinetin 5 mg/l, or with a modification consisting on gelrite 2 g/l and BAP 2 mg/l (modification suggessted by Dr Richard Litz, Univeristy of Florida, laboratory which Hendrix work was conducted)(Figure 2B). Non-thorny genotypes did not regenerated any plant on medium developed by Hendrix (Figure 2B).

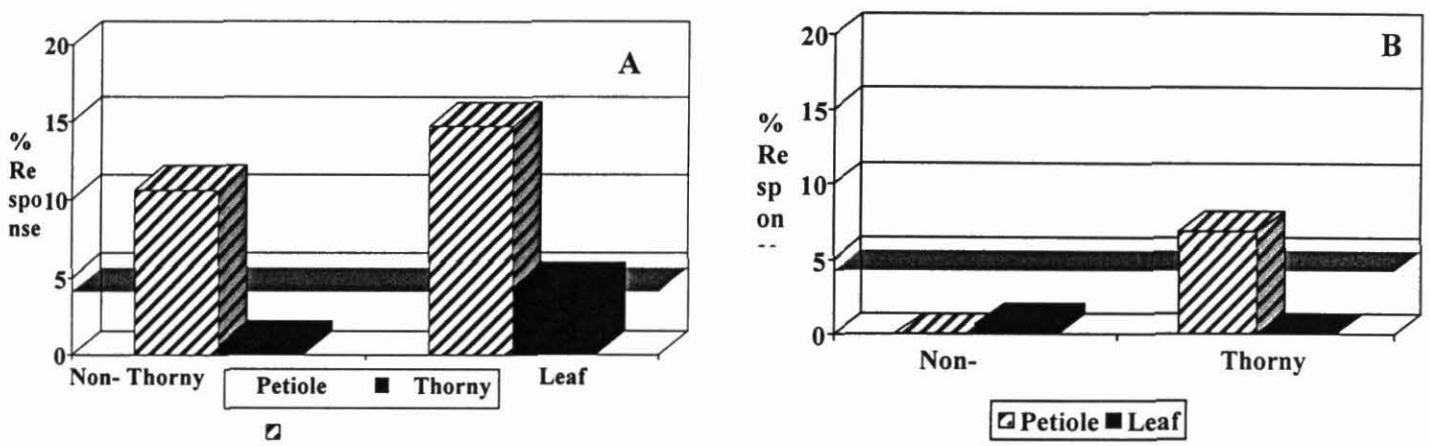


**Figure 2.- Plant regeneration from petiole or leaf explants of genotypes with or without thorns using medium previously develop for tomato (A) or reported by Hendrix for naranjilla (B).**

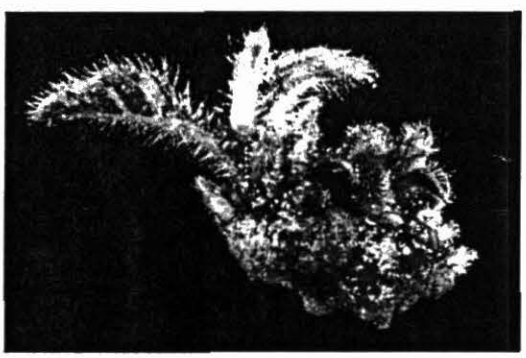

**Figure 3.- Multiple shoot formation from petiole of naranjilla**

**Plant Evaluations in the Greenhouse and the Field**

A methodology was establish to transfer *in vitro* material (from the *in vitro* germplasm collection and regenerated plants) to the greenhouse and there after, to the field. The first regenerated plants evaluated shown a normal plant growth and development to maturity in the greenhouse and in the field. Fruit formation is being evaluated in the field only, since temperatures in the greenhouse at CIAT headquarters is to high for naranjilla fructification. The field selected is located at 1,700 m.s.n.m. and with a mean temperature of 22 C, ideal for induction of fruit formation of naranjilla. The field plot is located in a farm (La Casona) at Dapa about 20 min from Cali, where there is a naranjilla production by farmers.

**Future plans**

Establish a cyclic culture from greenhouse to *in vitro*, to completely renew the existing germplasm *in vitro* collection and propagate it in medium A
Evaluate other factors affecting plant regeneration response to increase it at about 50%

Develop a genetic transformation protocol
Complete evaluation of regenerated plants in the field and compare the growth and development with *in vitro* propagated and seed derived materials

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## 2.2.14 Cryopreservation of Friable Embryogenic Callus (FEC) lines

LG Santos[1], R Escobar[1], P Chavarriaga[1], and WM Roca [1-2]
[1]SB-2 Project ;[2]CIP-Peru

**Introduction**

Friable Embryonic Callus lines were established at CIAT for some commercial Latin American cassava cultivars (Lopez 2000). In vitro-maintained cell suspensions are genetically unstable, besides, keeping them is labor intensive and costly (Reinhoud et al 2000). Cryopreservation of FEC may provide a means of ensuring genetic stability of cell lines, and could be a source of "fresh" tissue useful for genetic transformation.

**Methodology**

To establish a cryopreservation protocol we are using cultivars MCol 2215, CM 3306-4, and keeping the cultivar TMS60444 as a control. Different cryopreservation methods were tested: Classic, vitrification, encapsulation-dehydration, encapsulation-vitrification, desiccation and desiccation-vitrification. Different temperatures of loading, PVS3 dilutions, and pretreatment of FEC were also tested. A programmed freezing protocols (1°C/min) using a container with isopropanol was tested.

**Results and discussion**

Desiccation of FEC before freezing seemed to give tissues a good chance to recover. FEC grown on media with high agar content (6-9%) and growth regulators, desiccated during different times (10-30 days), are showing promising results after freezing. Frozen tissue was recovered on GD2-50Pi solid medium, grown under low light intensity and transferred to liquid medium (Figure 1). Fine tuning re-growth conditions (temperature, light intensity and humidity) will probably increase the recover of tissues. We are testing GRU's growth room conditions to compare with those we have at BRU. Initial observations indicate that there may be strong differences between both growth rooms, with the one from GRU giving better results.

- Protocols involving vitrification did not seem to work well for the recovery of FEC after freezing. Toxic effects of cryo-protectant solutions like PVS2 (Sakay et al 2000) were observed even with no frozen tissues. PVS3, another vitrification solution showed better results than PVS2. We will however continue testing protocols that mix vitrification and dessication to find out which one gives better recovery rates.

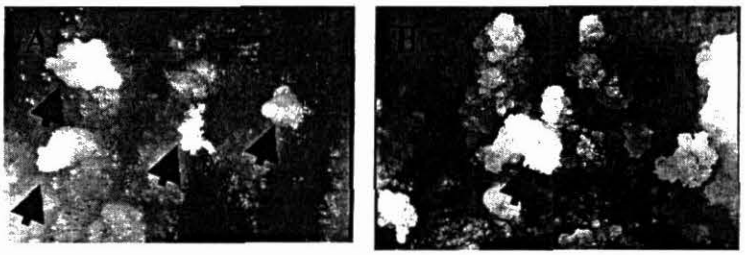

**Figure 1. Regrowth (arrows) of FEC after freezing (A) and control without freezing (B) for cultivar TMS60444.**

## Conclusions

- It is possible to recover cell growth after freezing of FEC.
- Modification of growth conditions could improve post-freezing response.
- Desiccation and desiccation-vitrification could be used as potential cryopreservation methods. Media with high agar content could be used as a desiccation treatment.
- PVS2 shows a detrimental effect on cassava cell suspensions. PVS3 has shown beneficial effect on tissues.

## Futures activities

- Adjust conditions to improve cell recovery after freezing.
- Test best conditions on other FEC lines from Latin-American cultivars.
- Recover plants from frozen cells.

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## 2.2.15 *In vitro* propagation through micrografting of selected clones of soursop (*Annona muricata* L.): Optimization of the technique and field evaluation of the agronomic performance of propagated trees

Juan Jairo Ruiz[3-8], Adriana Alzate[4], Nelson Royero[2-8], Silvio Cadena[3-8], Adriana Nuñez[3], Francisco Arboleda[3-5], Jorge Cabra[1], William Roca[7], Alvaro Mejía-Jiménez[1]
[1]SB-2 Project; [2]Corporación BIOTEC; [3]Project supported by Pronatta; [4]Project supported by Fundación Banco de la República; [5]Independent consultant; [7]Centro Internacional de la Papa, CIP, Perú; [8]Universidad Nacional de Palmira

### Introduction

The soursop (or guanábano in Spanish, *Annona muricata* L.) is a fruit tree native to the tropical Americas. Its white pulp is used for the production of juices, yogurts, ice creams and desserts.
One of the most important problems facing the soursop growers is the lack of disease-free planting materials from elite selections. Between 1997 and 1999, we developed a novel methodology for the *in vitro* clonal propagation of elite trees through micrografting (Royero *et al.*, 1998). This methodology allows a rapid clonal multiplication of elite varieties and the production of disease-free planting materials. The first trees derived through in vitro propagation were planted at CIAT and in farms belonging to experienced soursop growers located in Huila and Valle for field-testing between January 1999 and January 2000.
In 2001,our efforts were concentrated on the:

Evaluation of the agronomic performance of the micropropagated trees in the field;
Optimization of the propagation methodology;
Adaptation of the developed *in vitro* propagation methodology to new promising soursop clones;
Initiation of investigations on the use of rootstocks of different soursop selections or related annonaceus species; and
Initiation on investigations on the management of the micropropagated plants in the greenhouse.

### Methodology

The *in vitro* propagation methodology of soursop through micrografting has been described in previous reports (Royero *et al.* 1998 and 1999)

### Results

Optimization and adaptation of the micrografting methodology to new selected genotypes
The *in vitro* propagation methodology of soursop developed jointly by Corporación BIOTEC and CIAT, consists of the *in vitro* propagation of buds isolated from selected soursop trees or varieties, and their micrografting onto rootstocks produced from seedlings germinated *in vitro*. Buds for micrografting can be induced from *in vitro* cultured stem fragments, isolated from greenhouse growing plants or from other previously produced micrografts cultured *in vitro* (cyclic micrografting). Overall efficiency of propagation depends on (1) the multiplication rate of buds *in vitro*, (2) the efficiency of seed germination *in vitro*, (3) the efficiency of scion/rootstock union, and (4) further development *in vitro* and in the greenhouse of the micrografted plants. Each of these steps is being optimized independently in order to improve the overall propagation efficiency.

In general, the same methodology of propagation, developed initially with the commercial cultivar "Elita" (Rios Castaño and Reyes, 1996), could be adapted with few modifications to other three clones (named "Cristina", "Rosa" and "Francia") selected from large commercial plantations located in the Huila region of Colombia.

High efficiencies of scion/rootstock union are already being achieved with the actual methodology (between 100 and 65% depending on genotype combinations and source of buds, figure 1). However with some combinations, further development of the micrografted bud is still low (between 33 and 74%). The improvement of bud development *in vitro* can thus be identified as the most critical step for increasing the overall efficiency of the propagation methodology of soursop through micrografting. The highest levels of bud development *in vitro* are being achieved when rootstocks of the genotype Cristina, and buds coming from previously produced micrografted plants, cultured *in vitro,* are used.

Already new combinations of selected clones micrografted over different rootstocks have been planted at CIAT and sent to farms at Huila, for agronomic evaluation:

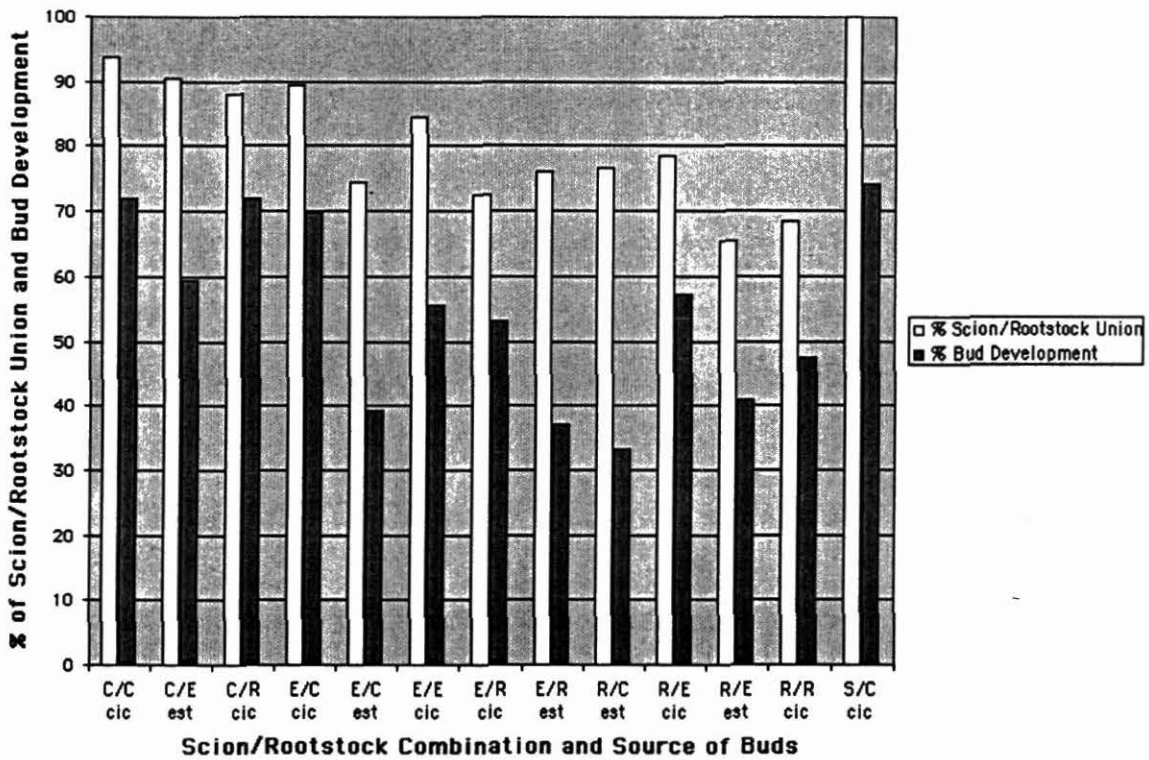


**Fig 1. Micrografting success (scion-rootstock union) and further development *in vitro* of micrografted plants of different combinations of soursop selections (E= Elita, C= Cristina, R= Rosa, F= Francia) and rootstocks. The buds used for producing the micrografts in the combinations marked with "cic" or "est" were isolated from micrografted plants produced previously and cultured *in vitro*, or from axillary buds induced *in vitro* from isolated stem sections of greenhouse growing plants, respectively.**

*Evaluation of the agronomic performance of soursop plants propagated through in vitro micrografting.*

The soursop plants produced through the *in vitro* micrografting methodology are being tested in the field in order to assess the usefulness of the technique for the production of planting materials of this tropical fruit species.

As a part of a M.Sc. thesis (Juan Ruiz, Universidad Nacional de Colombia), the agronomic performance of Elita/Elita micrografted plants is being evaluated in five different locations of the Valle and Huila states. Micrografted plants were planted in the field between January 1999 and January 2000, after 10 months of hardening in the greenhouse. In CIAT (where the highest growth rates are being achieved), the trees at 13 months after planting in the field had become vigorous and healthy with an average height of 2 m. Since the trees have to be pruned after reaching this height in order to initiate tree formation, other parameters in addition to tree height should be taken into account, in order to have a reliable estimate of tree growth. Perimeter of the stem 10 cm over micrografting point was measured in replacement (fig. 2). After 13 months in the field, an average of 5 reproductive structures (flower buds, flowers and developing fruits) were counted in each tree. First fruits were harvested 17 months after planting at CIAT. This is a rather fast development, and a first confirmation of the usefulness of the *in vitro* micrografting technique as a method for producing healthy and vigorous growing soursop planting material.

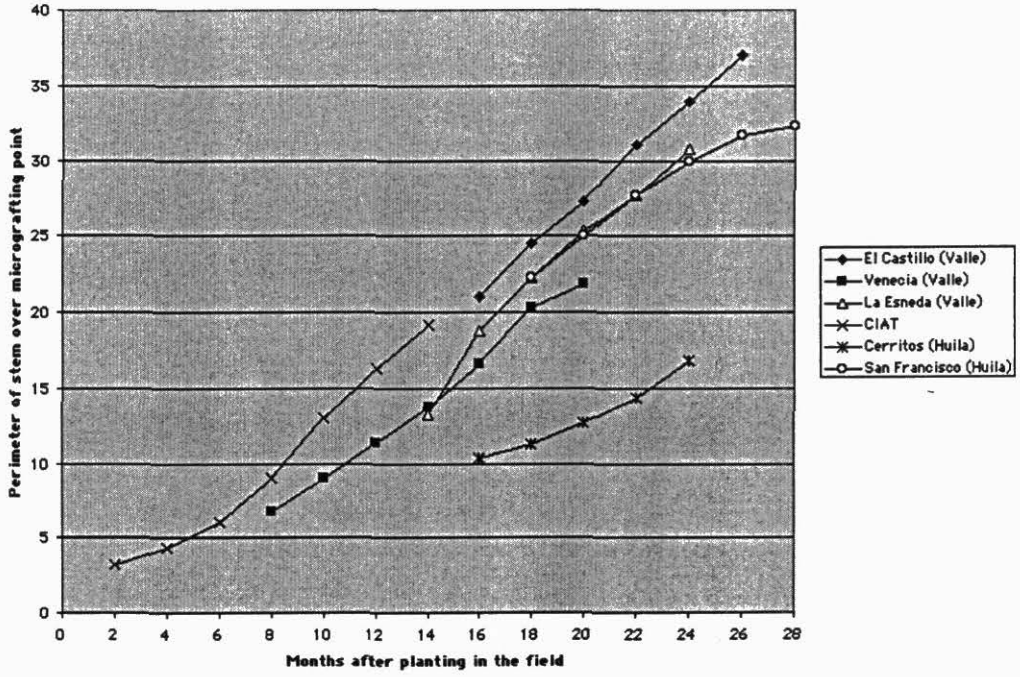


**Fig. 2 Growth of micrografted trees Elita/Elita in five locations of the Colombian states Valle and Huila measured as the perimeter of the stem 10 cm over the micrografting point.**

*Use of rootstocks of other Annona species for the production of more vigorous, disease resistant and widely adapted soursop trees.*

The use of rootstocks of different genotypes or species from that of the scion for the production of vigorous, widely adapted or disease resistant plants is a common practice in fruit tree propagation. In soursop this avenue has been largely under-exploited.

We are testing the compatibility and agronomic performance of combinations of scions of selected soursop clones Elita, Rosa and Cristina micrografted *in vitro* over rootstocks *of A. montana* and *A. glabra* L., two annonaceous species originally from the Chocó rainforest of Colombia. These two species do not show symptoms of anthracnose infections, the most important disease of soursop, even under conditions favorable for the development of this disease (high temperatures and humidity). Grafting soursop over *A. glabra* or *A. montana* may also improve resistance to drought, high humidity or soils with bad drainage (Escobar and Sánchez, 1992).

Compared to the *A. muricata*/*A. muricata* genotype combinations (for example, Elita/Cristina, fig. 3), micrografted plants produced over *A. montana* as rootstocks showed similar efficiencies of scion/rootstock union. However, further development efficiencies *in vitro* and later in the greenhouse, were much lower (13% greenhouse development for Cristina/*A. montana* micrografted plants; fig. 3) for some combinations. It is note worthy though that viable plants were obtained in all genotype combinations, indicating that the micrografted tissues of these two species are compatible. The produced plants were planted at CIAT or sent to farms at Huila State for agronomic evaluation.

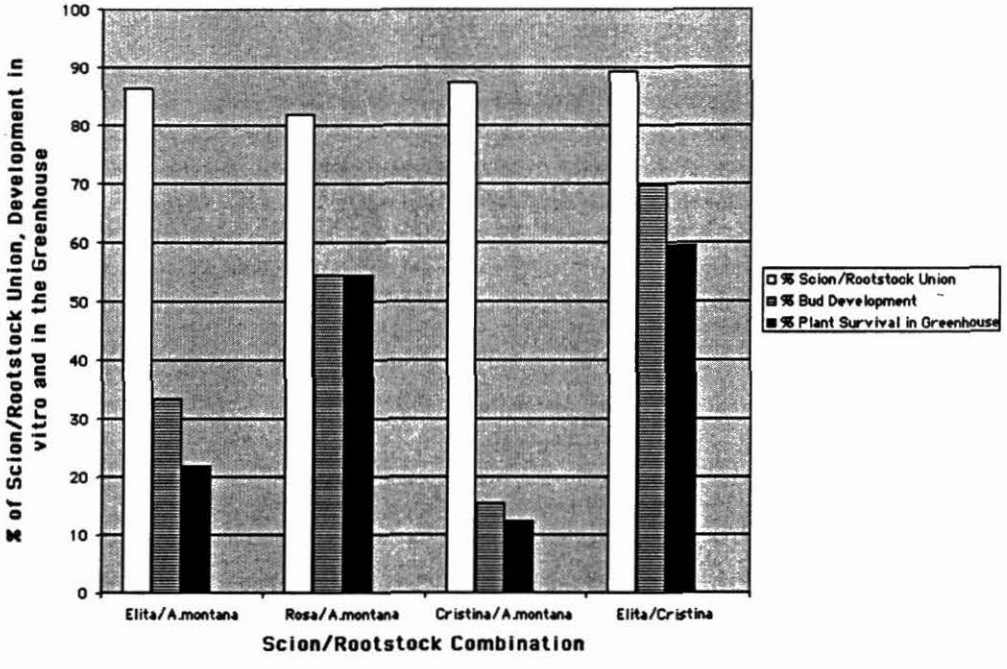


**Fig. 3. Efficiency of scion/rootstock union, further development *in vitro* and plant survival in the greenhouse of different soursop clones (Cristina, Elita and Rosa) micrografted over rootstocks of *A. montana*. For comparison the data obtained from the combination Elita/Cristina (*A. muricata*/*A. muricata*) are presented.**

*Greenhouse management of in vitro propagated soursop plants*

One important advantage of the *in vitro* propagated plants over plants propagated through traditional methods in nurseries, is the possibility to produce plants free of viral and other infectious diseases. This healthy state should be maintained during the greenhouse stage in order to prevent the recontamination of the plants and the spread of diseases through the planting material.

As a part of a M. Sc. thesis (Silvio Cadena, Universidad Nacional de Palmira), the management of the micrografted plants under greenhouse conditions is being standardized. The effect of four substrate combinations (mixtures of "cahaza", "carbonilla" , both by-products of the sugar industry- *Pinus* bark chips and CIAT-soil), and three strains of mycorrhizal endosymbiontic fungi: *Glomus deserticola, Gigaspora margarita and Gigaspora rosae* on the development of the micrografted plants is being investigated.

**Conclusions**

The *in vitro* micrografting propagation methodology developed initially with the clone "Elita" could be applied to other different soursop clones. The plants produced by this way develop healthy and vigorous growing trees, which initiate flowering after 13 months in the field under CIAT conditions. Efficiency of development of micrografted plantlets *in vitro* should be improved in order to apply the technology to the massive propagation of soursop clones.

**Future plans**

To continue with the evaluation in the field of the propagated soursop plants
To further optimize the *in vitro* growing conditions of the micrografted plants, in order to increase development efficiency of micrografted plants
To scale up the production of micrografted plants. The objective for 2002 is the production of at least 3000 plants
To standardize the methodologies of acclimatization and handling of the micrografted plants in the greenhouse
To continue with the evaluation of the compatibility between clones of soursop and rootstocks of different Annonaceous species, micrografted *in vitro*
To initiate field evaluation of novel selected soursop clones and include more farmers in this evaluation

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## 2.2.16 Developing cryopreservation alternatives for tropical fruits useful in National Programs: The case of Tree Tomato (*Cyphomandra betacea*).

Montoya, J.E.[1], Escobar, R.H[1]., and Debouck D [1-2].
[1]SB-2 Project ; [2]SB-1 Project

### Introduction

Based on our experience in cryopreservation of cassava (Escobar et al 1997, 2000), and keeping in mind that CIAT is adopting tropical fruits as part of its mandate crops, we selected Tree Tomato as a case study for cryopreservation. Our objective is design long-term conservation alternatives, comparable or better than standard conservation methods, which can be transferred to National Programs in the region.

Tree tomato is important for small-scale farmers of cold, tropical zones (average annual temperature: 16°C and 22°C; 1600-3000 m.a.s.l., and annual precipitation between 1300-1600 mm), representing an alternative income source.

### Methods

We collected ripen fruits of three materials (*yellow*, *tamarillo* and *comun*) available in local markets of Palmira and Cali. They were selected on the basis of color, appearance, brightness, phytosanitary state among other characteristics. Seeds were extracted, disinfected and dried on open petri dishes in a flow chamber for one hour. Then seeds were cryopreserved at least for one hour and thawed by warming them at 37°C for one minute. Seeds were then grown on germination medium for 40-50 days, and shoots transferred to rooting media before taken to the greenhouse.

We run five replicas with 20 seeds for each of two treatments (frozen and not frozen). Treatments were then evaluated based on germination percentages.

### Results and Discussion

No significant differences were observed between frozen and non-frozen seeds for *tamarillo* and *comun*, while for *yellow* we found differences at 40-day evaluation (Table 1.) .

**Table 1. Seed germination (%) after freezing Tree Tomato materials. (***, significant differences; ns = non-significant)**

| Species | Amarillo | | Tamarillo | | Común | |
|---|---|---|---|---|---|---|
| Treatments | Frozen | 37.4 % | Frozen | 86 % | Frozen | 94 % |
| | Non-frozen | 97.5 % | Non-frozen | 90 % | Non-frozen | 95 % |
| | Probability (0,001) | *** | | ns | | ns |

We evaluated seed viability after freezing for *Amarillo* using the Tetrazolium Chloride test (TZ) (viable tissues stain red). The results are summarized in Table 2.

**Table2:** TZ test with non-germinated seed after freezing.

| Repetition | total seeds | Viable seeds | Viability (%) |
|---|---|---|---|
| R1 | 18 | 15 | 83.33 % |
| R2 | 17 | 15 | 88.24 % |
| R3 | 16 | 11 | 68.75 % |
| R4 | 18 | 14 | 77.77 % |
| R5 | 18 | 15 | 83.33 % |

(a) (b) (c)

**Figure 1: Plants recovered after freezen steps growing in the greenhouse. (a) *Tamarillo*. (b) *Amarillo* and (c) *Comun*.**

## Conclusions

There are not significant differences in germination of frozen and non-frozen seeds for *Tamarillo* and *Común*.

*Amarillo* showed germination differences between treatments seeds, possibly due to fast desiccation rather than loss of embryo viability.

## Future Activities

Test slow desiccation methods for *Amarillo*
Compare cryopreservation with short-to-medium term conservation (5 to –20°C)
Transfer to National Germplasm Conservation Systems

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## Activity 2.3 Identification of points of genetic intervention and mechanisms of plant-stress interaction

### Main Achievements

- Characterizing genes of the carotene pathway in cassava was initiated using consensus sequences to amplify the genes -Lycopene Cyclase (BLyc), -Carotene desaturase (CDes), Phytoene synthase (PSyn) and Phytoene desaturase (PDes).

- The genetic variation in minerals concentrations of 411 cassava genotypes was evaluated. A substantial genetic variation that can be exploited to improve cassava micronutrient density was determined. Genotypes with higher concentrations of Fe, Zn and pro-vitamin A were identified

- The genetic variation of carotene content in leaves and roots of 682 cassava accessions was determined. A significant correlation (0.84) was observed for carotene content and color intensity of the roots. Carotene concentration in roots ranged from 0.13 to 0.92 mg/100 g FW, with a mean of 0.23 and a standard deviation of 0.10. Carotenes concentrate much more in the leaves than in the roots, illustrating, once again, the excellent nutritive value of cassava leaves. There was no correlation (0.02) between carotene concentration in the leaves and roots.

- The Identification of target points for the control of post-harvest physiological deterioration in cassava was pursued. Two classes of secondary metabolites, hydroxycoumarins and flavan-3-ols, were identified in deteriorated cassava root extracts. Those metabolites proved to be biologically active, as antioxidants and antimicrobials (4, 5). The dominant compounds were scopolin, scopoletin and (+)-gallocatechin.

## 2.3.1 Characterizing genes of the carotene pathway in cassava

L.I. Mancilla, A.F. Salcedo, J. Beeching, L. Chavez and J. Tohme
CIAT

### Introduction

Vitamin A deficiency is an important public health problem, especially in tropical countries. Cassava is one of the most important sources of energy in tropical countries, although it lacks the carotene content necessary, in the roots, to supply minimal carotene intake requirements. It would be desirable to breed cassava for increased carotene content in roots, taking advantage of the great genetic diversity that exists for this trait within cassava germplasm. One way to do it is by conventional breeding, which is the longest way. A second, faster approach is through genetic transformation, introducing and expressing genes to elevate carotene content in roots. Characterizing cassava genes of the -carotene pathway to understand their regulation in roots will help in the identification of root specific promoters that could be coupled to bacterial- or plant-derived genes known to enhance carotene content in other species (Ye et. al., 2000). Thus, the main objective of this work is to characterize cassava genes of the carotene pathway.

### Methodology

We used consensus sequences from GeneBank to design primers for PCR amplification of the genes -Lycopene Cyclase (BLyc), -Carotene desaturase (CDes), Phytoene synthase (PSyn) and Phytoene desaturase (PDes). Genomic DNA of high (Mper297) and low (CM523-7) carotene content varieties was extracted and used for PCR amplification. Amplified bands were cloned into pGEM-T Easy vector. Insert sizes were confirmed with M13 primers. Additionally, RT-PCR was also made from mRNA of roots from both varieties.

### Results and Discussion

Consensus primers were successful at amplifying all targeted genes. With the exception of Psyn, it was impossible to obtain a unique band. The optimal annealing conditions for PCR amplification were 52 $^{0}$C for genes BLyc, PDes, CDes and 50 $^{0}$C for Psyn (Figures 1,2). PCR product sizes from DNA and mRNA agreed with expect sizes based on consensus sequences.

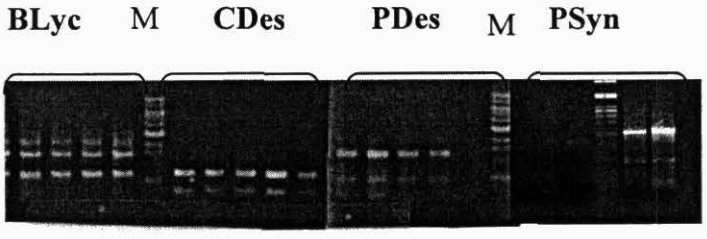


**Figure 1. PCR amplification of carotene genes from genomic DNA of cassava.**

mRNA samples from CM523-7 and MPer 297 genotypes were RT-PCR amplified. Several bands appeared. Genes BLyc, PDes and CDes showed expect size fragments. PSyn gene did not amplify. Bands were observed in both high and low carotene content genotypes. Different RT-PCR programs with other primer combinations, and annealing temperatures, will be performed for PSyn amplification.

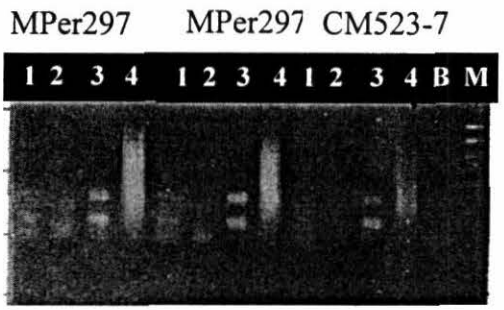


**Figure 2. RT-PCR of cassava roots mRNA with specific primers for carotene genes. 1. BLyc , 2. Pdes, 3. Cdes, 4. Psyn, B. Blank, M. /Pst I.**

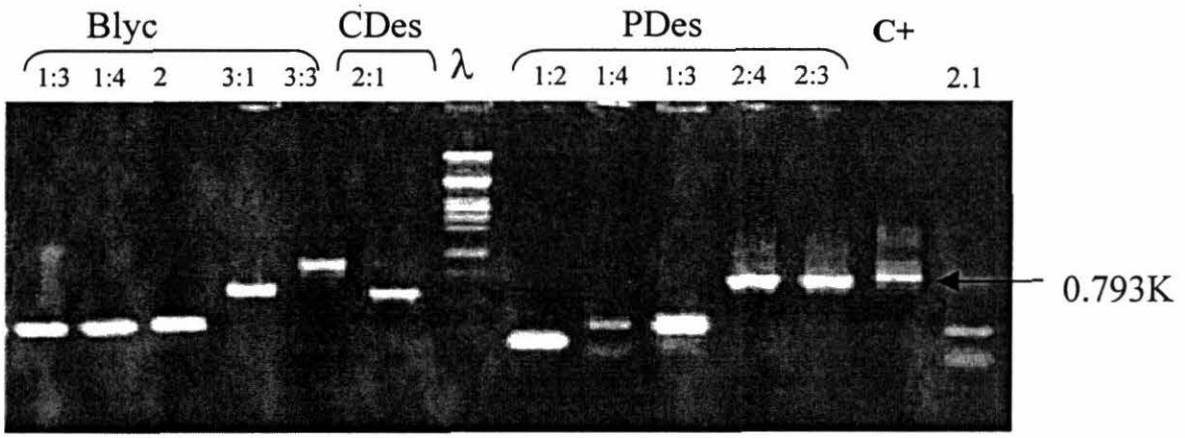


**Figure 3. Evaluation of carotene genes amplified by PCR with primers of pUC13/M13. The control clone (C+) has an insert size of 542 bp.**

Currently we have cloned 50 percent of the bands into pGEM-T vectors. Inserts from carotene clones have been confirmed by PCR using M13 and gene-specific primers. Results are shown in Figure 3.

**Conclusions and ongoing Work.**

Several fragments were amplified by PCR with primers designed from consensus sequences of carotenoid metabolism genes from cassava genomic DNA of two varieties having contrasting carotene levels in roots. Some of the amplified fragments have been cloned into pGEM T easy vector for sequencing and data base comparisons. RT-PCR of mRNA from roots of these cassava genotypes was also performed with successful amplification of bands. We will clone bands from

RT-PCR into the same vector for sequencing. Additionally, genomic and cDNA libraries will be constructed in GEM11 and pSPORT-1 vectors, respectively, to fish out genomic and cDNA clones.

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## 2.3.2 Genetic variation in total carotenes and minerals of cassava genotypes

L. Chávez,[1] ;H.Ceballos,[2] A. Bolaños, [2] F. Calle,[2] T. Sánchez,[2] M.C. Duque[1] and J. Tohme[1]
[1]SB-02 Project [2]IP-3 Project;

**Introduction**

Deficiencies of vitamin A, iron and zinc are widespread in sub-Saharan Africa and in many tropical areas where the diets of poor human populations are mainly plant-based and the intake of animal-derived products are low. As cassava is a staple food in regions where there are severe micronutrient deficiencies, the crop could be used as a vehicle to deliver vitamins and minerals in higher concentrations.

In the field of human and animal nutrition there is an increasing amount of evidence of a synergistic effect between vitamins and certain minerals. Preliminary results suggest that Fe and Zn contents in the diet increase vitamin A absorption and vice versa. Thus in the study of micronutrient availability from cassava roots and leaves, it is also important to measure mineral contents.

**Objectives**

The overall objective of this project is to improve the nutritional status of people living in marginal environments of the tropics, by selecting and promoting cassava genotypes with high bioavailability of micronutrients and vitamins. The objective of this research was to evaluate the extent of genetic variation of mineral concentrations in 411 genotypes from the world cassava germplasm bank held at CIAT.

## Methods

Mineral concentration measurement. Leaves and roots were collected, dried, ground to powder and sent to the Analytical Laboratory of the University of Adelaide (sampling was the same as that for evaluating carotene content; see "Evaluation of genetic diversity for total carotenes content in cassava leaves and roots". There the samples were analyzed by inductively coupled plasma atomic emission spectrometry. Care was taken in the processing to avoid contamination from soil, which has higher mineral concentrations than those of plant tissues.

Carotene concentration measurements. The extraction protocol for leaves and roots is described in "Evaluation of genetic diversity for total carotenes content in cassava leaves and roots".

Postharvest Physiological Deterioration (PPD) measurements. PPD was measured 7 days after harvest on genotypes whose concentration of carotenes in roots was measured simultaneously (see "Evaluation of genetic diversity for total carotenes content in cassava leaves and roots".

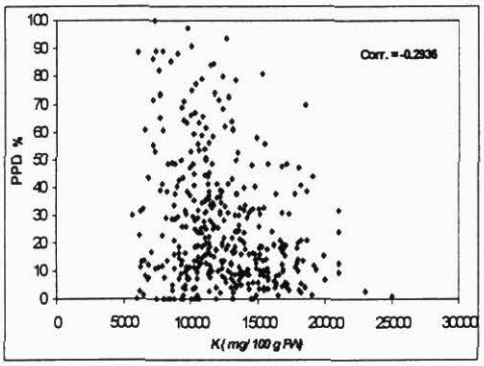
## Results

Trace mineral concentrations (mg/kg) Roots averaged 14.88 of Fe, 8.15 of Zn and 803.11 of Ca on a dry matter basis (Table 1). Mineral concentrations in the leaves were much higher, averaging 281.61 of Fe, 47.53 of Zn and 13760 of Ca. These leaf concentration figures are much higher than those in most staple foods. Although the leaves, which can be eaten as a vegetable, have a high water content and low mineral density, they supply high levels of minerals per calorie.

**Table 1. Simple descriptive statistics for mineral concentrations (mg/kg) of 411 genotypes of cassava**

| Mineral | Leaves | | | Roots | | |
|---|---|---|---|---|---|---|
| | Min. | Max. | Mean | Min. | Max. | Mean |
| Iron | 120.63 | 950.00 | 281.61 | 5.99 | 75.92 | 14.88 |
| Manganese | 18.67 | 200.00 | 61.39 | 0.47 | 5.00 | 1.47 |
| Boron | 4.02 | 31.16 | 14.04 | 1.14 | 3.44 | 1.95 |
| Copper | 2.81 | 12.36 | 7.39 | 0.49 | 40.31 | 6.78 |
| Zinc | 15.14 | 150.47 | 47.53 | 2.63 | 37.52 | 8.15 |
| Calcium | 6300 | 32000 | 13760 | 303 | 2500 | 803.11 |
| Magnesium | 2600 | 11300 | 4710 | 520 | 2400 | 1015.11 |
| Sodium | 10.27 | 113 | 29.47 | 18.56 | 1230 | 135.5 |
| Potassium | 8700 | 23000 | 15148 | 5700 | 25000 | 12381 |
| Phosphorus | 2300 | 7600 | 3901 | 980 | 3200 | 1720.07 |
| Sulfur | 2400 | 5200 | 3319 | 123 | 550 | 280.7 |
| Aluminum | 59.5 | 880 | 211 | 4.42 | 43.95 | 8.77 |

Correlations among minerals in roots and leaves. There was a high positive correlation between the mineral content in the roots and leaves for Mn (0.508) and K (0.407). The accumulation of the other minerals in roots and leaves seemed to be independent.



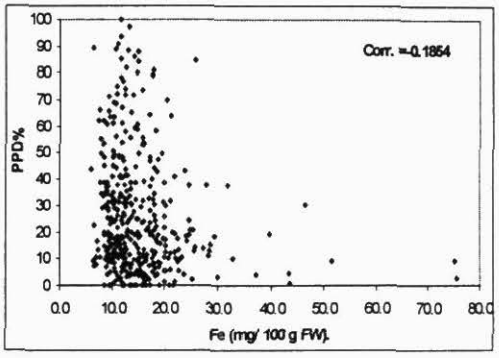




**Figure 1. Relationship between PPD and K concentration in cassava roots.**
**Figure 2. Relationship between PPD and Fe concentration in cassava roots.**

Correlations among minerals and carotene contents. There was a very weak relationship between total carotene content and Zn and Ca in the roots (correlation coefficients of 0.005 and 0.1792, respectively). There was no correlation for Fe. With respect to the leaves, the correlations were significant for Mn (0.150), Na (-0.148) and Ca (0.146). Correlation among minerals in roots and PPD. In general the correlations between PPD and mineral concentrations in the roots were low. The higher correlation coefficients found were negative for K (-0.2936, Fig. 1) and Fe (-0.1854, Fig. 2). Thus it would seem that there may be an inverse relationship between these minerals and the PPD process.

Cluster analysis. In order to identifying elite genotypes for breeding programs, this analysis constructs groups (clusters) that have interesting characteristics in common. Of the 15 clusters that were determined, the genotypes that had the highest concentrations of minerals among them were M Bra 11 and CM 6068-3. SM 722-13 had a low K concentration and a high PPD. M Col 2436, M Col 2439, M Col 2459 and M Bra 206 had a low PPD and a higher carotene content. Table 2 summarizes these data.

These results demonstrate that there is a substantial genetic variation that can be exploited to improve cassava micronutrient density. New varieties with higher concentrations of Fe, Zn and pro-vitamin A can be identified to exploit the synergy that may operate in absorption, internal transport and function among these micronutrients.

**Future Activities**

Search for a group of cassava cultivars with a higher micronutrient content to help in the breeding approach to improve their micronutrient content

Evaluate the expression and stability of minerals in elite genotypes under different environmental conditions

**Table 2. Genotypes with common characteristics identified from the cluster analysis for cassava roots.**

| Genotype | Characteristic |
|---|---|
| M Bra 11<br>CM 6068-3 | High Fe<br>High Zn<br>High Na, K, Cu, P, S and Al |
| SM 722-13 | Low K<br>High PPD |
| M Col 2436<br>M Col 2439<br>M Col 2459<br>M Bra 206 | High carotene<br>High K<br>High B<br>Low PPD |
| CM 5460-5, M Bra 49<br>M Bra 88, M Bra 89<br>M Bra 90, M Bra 103<br>M Bra 104, M Bra 194<br>M Col 480, M Col 847<br>M Col 2456, M Ecu 6<br>M Col 2389, M Cub 35 | Low K<br>Low B<br>High PPD |
| M Bra 122, M Bra 465<br>M Bra 468, M Col 1995<br>M Col 2272, M Col 2363<br>M Col 2412, M Col 2528<br>M Bra 214, M Col 2289 | High carotene<br>High Ca<br>High Mg |

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## 2.3.3 Evaluation of genetic diversity for total carotene content cassava leaves and roots

L. Chavez,[1] H. Ceballos[2], A. Bolaños, [2] F. Calle,[2] T. Sanchez[2] and J. Tohme[1]
[1]SB-02 Project; [2]IP-3 Project;

### Introduction

Cassava provides a large proportion of the daily intake of energy and other nutrients including vitamins for poor populations in many areas of sub-Saharan Africa. The cassava crop—given its flexibility with respect to planting and harvesting times and its tolerance to poor soils, pest and disease problems—plays an important role in food security as a stable food base in areas prone to drought.

Root crops are usually considered primarily sources of low-cost energy, but not important sources of other nutrients. In the case of cassava, however, CIAT has found a wide range in the content of total carotenoids after screening its germplasm collection (CIAT 1999, 2000). Developing

cassava cultivars with high available carotene content could significantly improve the health and nutritional status of the poor, especially women and children.

Postharvest physiological deterioration (PPD) is a constraint that limits the marketability of the fresh cassava roots and increases the costs of postharvest handling. Consequently, the roots must either be consumed or processed shortly after harvesting. In this study we evaluate the hypothesis that a high carotene content could help reduce PPD through its antioxidant capacity.

**Objectives**

The overall objective of this project is to improve the nutritional status of people living in marginal environments of the tropics, by selecting and promoting cassava genotypes with high and good bioavailability of micronutrients and vitamins.The specific objective of this research is to determine the extent of genetic variation of carotene content in 682 accessions from the cassava world germplasm bank held at CIAT.

**Methods**

Harvesting and sampling. A selection was made of 682 cassava genotypes to represent the wide variability in carotene content and PPD susceptibility from the CIAT germplasm collection (6000 genotypes). Three plants per cultivar were harvested at 9-12 months of maturity.

Carotene concentration measurements. The extraction procedure outlined by Safo-Katanga et al. (1984) was modified by extracting root parenchyma with petroleum ether. The extraction protocol for leaves had to be modified due to the presence of tannins and chlorophylls. The modified protocol included several extractions with petroleum ether at 35-65°C and washing with methanol in order to minimize interference from the other pigments. A 5-g sample was taken from randomly selected root or leaves, 10-11 months after planting. The quantification was done by ultraviolet spectrophotometry, using a Shimadzu UV-VIS 160A recording spectrophotometer. UV detection was done at l = 455 nm for root extracts and l = 490 nm for leaf extracts.

PPD measurements. Five commercially sized roots (minimum length 18 cm) were randomly chosen and analyzed using the method of Wheatley et al. (1985) with one modification: The prepared roots were stored under ambient conditions for 7 instead of 3 days. The proximal and distal root ends were cut off, and the distal end was covered with Clingfilm. After 7 days, seven 2-cm thick transversal slices were cut along the root, starting from the proximal end. A score of 1-10 was assigned to each slice, corresponding to the percent of the cut surface showing discoloration (1=10%, 2=20%, etc). The mean PPD score for each root was calculated.

**Results**

Analysis of carotene content. Carotene concentration in leaf tissue ranged from 18.71 to 96.2 mg/100 g FW, with a mean of 50.32 mg/100 g FW and a standard deviation of 10.43 (Table 1). These values are similar to those found in a group of 500 accessions evaluated the previous year (CIAT, 2000). The carotene distribution showed a symmetrical tendency (skewness = 0.2). Carotene concentration in roots ranged from 0.13 to 0.92 mg/100 g FW, with a mean of 0.23 and a standard deviation of 0.10 (Table 1). This distribution showed a strong skewness with long right tails (skewness = 2.83). A significant correlation (0.84) was observed for carotene content and color intensity of the roots. Carotenes concentrate much more in the leaves than in the roots, illustrating, once again, the excellent nutritive value of cassava leaves. There was no correlation (0.02) between carotene concentration in the leaves and roots.

**Table 1. Carotene concentration in leaves and roots of 682 cassava accessions from CIAT's germplasm bank collection.**

| Data from Leaves | | Data from Roots | |
|---|---|---|---|
| Range (mg/100 FW) | Frequency | Range (mg/100 FW) | Frequency |
| 18.71-26.46 | 6 | 0.13-0.21 | 437 |
| 26.47-34.22 | 38 | 0.22-0.30 | 166 |
| 34.23-41.98 | 94 | 0.31-0.39 | 29 |
| 41.99- 49.74 | 197 | 0.40-0.48 | 19 |
| 49.75-57.5 | 184 | 0.49-0.57 | 18 |
| 57.6-65.35 | 115 | 0.58-0.66 | 9 |
| 65.36-73.11 | 35 | 0.67-0.75 | 2 |
| 73.12-80.87 | 10 | 0.76-0.84 | 2 |
| 80.88-88.63 | 2 | 0.85-0.93 | 1 |
| >88.64 | 1 | >0.93 | 0 |
| Minimum | 18.71 | Minimum | 0.13 |
| Maximum | 96.2 | Maximum | 0.92 |
| Median | 57.45 | Median | 0.525 |
| Skewness | 0.2 | Skewness | 2.83 |
| Mean | 50.32 | Mean | 0.23 |
| SD | 10.43 | SD | 0.10 |

Correlations among vitamin content and PPD. The correlation between PPD and carotene content in cassava roots was -0.07 (Fig. 1). This relationship is similar to the one reported previously (-0.13). Although a high correlation between carotene content and PPD was not observed, it was noted previously (Chávez el al., 2000) that at carotene concentrations >50 mg carotene/100 FW in the roots, the PPD did not exceed 20%, suggesting a threshold effect (Table 2). Nevertheless, the results illustrated in Figure 1 clearly contradict previous findings. It has been founthat PPD studies are affected by large experimental errors arising from environmental variations at the time the evaluations are carried out. Because of logistical limitations, germplasm bank accessions must be harvested gradually over time; therefore the PPD evaluations are carried out at different times and environmental factors (particularly temperature). Because of this situation CIAT built a large chamber where temperature and relative humidity can be controlled. In the future all PPD evaluations will be performed under uniform environmental conditions.

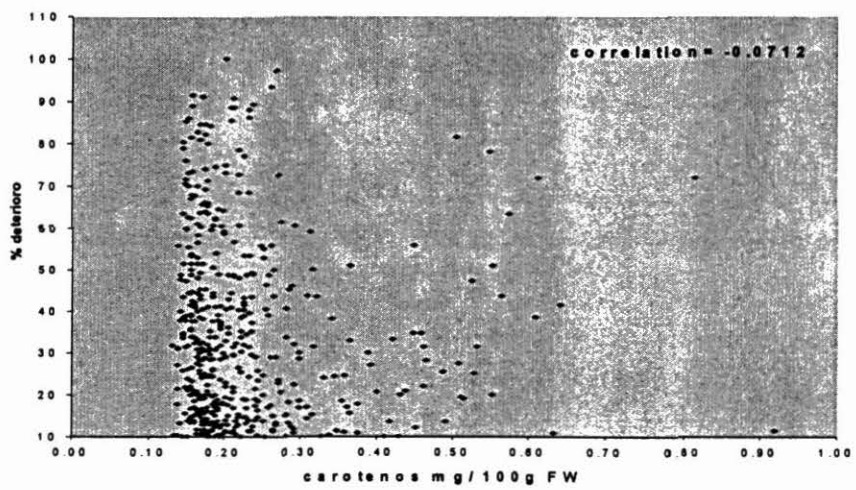


**Figure 1. Relationship between PPD and carotene content in cassava roots.**

**Table 2. Cassava genotypes with high carotene content and low PPD.**

| Genotype | PPD (%) | Carotene mg/ 100g FW |
|---|---|---|
| M Col 2086 | 5.7 | 0.51 |
| CM 3199 - 1 | 19.5 | 0.51 |
| M Col 676 | 19.0 | 0.51 |
| M Col 2068 | 5.7 | 0.53 |
| M Bra 487 | 20.0 | 0.55 |
| M Col 2498 | 4.3 | 0.57 |
| M Col 2099 | 9.5 | 0.57 |
| CM 5655 - 1 | 4.3 | 0.58 |
| M Bra 465 | 10.9 | 0.63 |
| M Col 2410 | 8.1 | 0.65 |
| M Col 2459 | 4.3 | 0.66 |
| M Col 2412 | 7.9 | 0.70 |
| M Bra 206 | 6.7 | 0.72 |
| M Col 2436 | 8.3 | 0.83 |
| M Col 2439 | 11.4 | 0.92 |

## Future Activities

- Continue screening the cassava landraces (only those with nonwhite roots) from CIAT's germplasm collection, exploring the genotypes with higher carotene contents.
- Identify a group of clones with the highest carotene content to help in the plant breeding approach for improving the micronutrient content of cassava.
- Evaluate the expression and stability of high concentrations of carotene in elite genotypes under different environmental conditions.
- Determine the proportion of carotenoids (given that only total carotenoids have been identified) with pro-vitamin A activity present in cassava roots and leaves

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## 2.3.4 Enabling Genomics Tools for Understanding and Exploiting the Genetic Potential of Cassava Starch

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### Introduction

Expressed sequence tags (EST's) as candidate loci as quantitative traits, has been suggested as a way of increasing the accuracy of mapping complex traits. The EST's simplifies and directs genomics sequencing and isolation and cloning genes of agronomical interest genes. Normally the generation of EST's has been by sequencing of random cDNA clones from libraries obtained from different tissues at various stages of development. Constructing cDNA libraries from tissues and developmental stages are keys for study certain traits.
Starch is certainly the principal carbohydrate storage form of CG mandate crops. Some of the key steps of the starch biosynthesis and deposition are well understood from the work in grain crops and root crop, such as cassava. A genome-wide gene expression approach will provide insights in the metabolism of starch in different crops. As one step to achieve this goal, a cDNA library from high and low starch content cultivars has been constructed.

### Materials and Methods

Source of plant tissue for the construction of the cDNA library were storage roots from plants 10 months of age. MPer 183 (low starch contend) and CM523-7 (high starch contend) were the cassava cultivars used in this study.
Fresh roots from each cultivars were harvested and immediately storage at –80°C. Root tissue was ground to fine powder using liquid nitrogen and 3 g used for RNA extraction with lithium chloride and cleaning steps with phenol:chloroform. Only total RNA samples with high purity (A260/A280 ratio 1.8 –2.0) and good concentration were selected for mRNA isolation using magnetic poly A DYNAbeads according to the manufacture. The cDNA synthesis and cloning was done using the Stratagene cDNA Synthesis Kit, ZAP-cDNA® Synthesis Kit and ZAP-cDNA® Gigapack® III Gold Cloning Kit according to the manufacture.

### Results

Prior to cloning, low molecular weight (less than 500 bp) cDNA was removed by size fractionation and the remaining fraction of cDNA was divided in two portions for each genotype, the firs portion contained fragments between 500bp and 1500bp and a second portion with fragments longer than 1500bp. The cDNA synthesis was primed with oligo (dt) primer which contains a Not I restriction site and an adaptor on the other site which contains the apropiate EcoR I site, allowing that the libraries were directionally cloned.
All libraries were obtained with a minimum titer of $10^8$ pfu/ml. The amplified libraries have titer of $10^8$ to $10^{10}$ pfu/ml. This quantity is sufficient for several thousands screening. An insert size screening after cloning and the average obtained was 1000 bp. Both cDNA libraries were transferred to IRD (Francia) for the differential subtraction and sequencing the clones differentially expressed, besides a copy of these libraries are still in CIAT for future activities

**Future Activities**

- Identification of clones differentially expressed between the cultivars with high and low starch content.
- Generation of thousands of EST's sequences.
- Establishment of starch related sequences obtained from public domain and EST's generated from the project.
- Comparative analysis between cassava EST and with available dbEST's from public accessible databases.

## 2.3.5 Functional Genomics Tools of Post-Harvest Physiological Deterioration in Cassava

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**Introduction**

The developing of genomics and bioinformatics tools is increasing our knowledge of plant genome structure, organization and gene function. Novel technologies such an Expressed Sequences Tags (EST's) and cDNA micro arrays are proving rapid ways to identify genes and to link sequence information to biological function.

The post-harvest physiological deterioration (PPD) is a major constrain to the development of cassava for producers, processors and costumers alike. Extending the shelf-life of cassava to one or two weeks is perceived as a goal that would benefit many, particularly the sustainable livelihoods of small-scale rural farmers and would contribute towards poverty alleviation.

The objective of this project is to identify the full set of major genes involved in the response by exploiting the powerful high throughput analysis of cDNA microarrays. This will enhance understanding of the problem and also provide the tools (clones) that could serve as components of gene constructs to modulate PPD. On this report we present an important step to make this goal achievable, like is the development of a cDNA library from different time-points during the PPD process in cassava roots.

**Materials and Methods**

Cassava roots from the cultivar CM2177-2, the male parent of the F1 cross used in generation the molecular genetic map of cassava, was used as source of plant tissue for the RNA extraction in the cDNA library construction.

Roots from the cultivar CM2177-2 were harvested from plants with 10 months of age. The RNA was isolated form the fooling time-points after harvest: 0, 3, 6, 12, 24, 48 and 96 hours. Poly (A)+ RNA was purified using magnetic poly A DYNAbeads according to the manufacture.

The cDNA synthesis and cloning was done using the Stratagene cDNA Synthesis Kit, ZAP-cDNA® Synthesis Kit and ZAP-cDNA® Gigapack® III Gold Cloning Kit according to the manufacture.

**Results**

Two directionally cloned cDNA libraries in Lambda ZAP II were obtained: "Early" from pooled 0, 3, 6 & 12 hour time points, and "Late", from 24, 48 & 96 hours.

Based on size fractionation, low molecular weight (less than 500 bp) cDNA was removed prior to cloning and the remaining fraction of cDNA was divided in two portions according the fragment's size, the firs portion contained fragments between 500bp and 1500bp and a second portion with fragments longer than 1500bp. The two "Early and Late" original libraries were obtained with a titer of $10^8$ pfu/ml. The amplified libraries have titer of $1.7 \times 10^9$ and $7 \times 10^8$ pfu/ml respectively. This quantity is sufficient for several thousands screaning. Both cDNA libraries were transferred to Dr. John Beeching's Laboratory in Bath University (United Kindong) for a specific gene screening and random clones sequencing A copy of these libraries are still in CIAT for future activities

Previous research showed that the key signalling events that trigger post-harvest physiological deterioration (PPD) in cassava occur during the earliest stages and that the lack of adequate wound repair in the detached root permits the spread of the deterioration response. The experimental work was to address these aspects.

The libraries were screened with PCR-generated subtraction probes enriched for PPD-related sequences. In addition we randomly selected clones from both libraries. The subtraction probes hybridised to 30 different clones, which were sequenced and characterised. Surprisingly 20 of these were for members of an extensin gene family, which fell into four classes. Extensins are proteins that are insolubilised in the plant cell wall by $H_2O_2$ during normal development and in response to wounding; they play a role in cell wall strengthening. This predominance of extensins could reflect their PPD-specificity or be an artefact of the PCR method used to produce the subtraction probes. Nonetheless, these results do demonstrate the activity of a large gene family of extensins during the early stages of PPD. Approximately 70 random clones from both libraries were also sequenced and characterized. The total of 100 clones sequenced included members of the following classes: cell wall strengthening, signal transduction, stress responses, senescence, defence, metabolism, transcription, translation, and the first evidence of the activity of a *Mutator*-like transposable element in cassava.

This and previous work had suggested that some aspects of wound repair (cell wall strengthening) occurred during PPD. Therefore, we used immunological methods to detect extensin accumulation during PPD. These data confirmed our supposition, showing an accumulation of extensin in the root, particularly associated with the vascular tissue; an accumulation that paralleled the development of PPD symptoms. However, the response occurred too late to heal the wound sites and thereby inhibit PPD.

**Future Activities**

cDNA microarray spotting. 5,000 clones from each library will select after mass excision, PCR amplified and spotted onto replicate glass slides using the SPBIO spotting robot at CIAT.
Interrogating of microarrays. Cy3 and Cy5 labeled cDNA derived from poly(A)+ of time points of interest will be used as probe to hybridize cDNA slides.

Identification of clones of interest. Computer analysis of the microarray hybridization output will be use to identify clones that change in abundance during the time course of PPD.

## 2.3.6 Identifying target points for the control of post-harvest physiological deterioration in cassava

M. X. Rodríguez[1], H. Buschmann[2], E. Okogbenin[1], J. Tohme[1] and J. R. Beeching[2]
[1]SB-02 Project; [2]University of Bath, U.K.

### Introduction

The major limitation for the development of cassava from a marginal farmers crop to an urban important crop is the rapid post-harvest deterioration starting 48 hours after harvesting, which renders the roots unpalatable and unmarketable for consumption and industrial utilization (6). The post-harvest deterioration process in cassava commences with a physiological deterioration (PPD), characterized by vascular streaking (blue-black discoloration of the xylem parenchyma followed by a general discoloration of the storage parenchyma). Subsequently, a microbial deterioration occurs. This secondary deterioration is a decay caused by the invasion of micro-organisms throughout wounds caused during root harvesting, handling and transport (2, 6).

PPD is an active process involving the increase and *de novo* synthesis of proteins and changes in gene expression, which resembles wound responses in other more studied plants. Nevertheless, in cassava the wound repair and the resultant down-modulation of the signals are inadequate, which leads to continuous cascades of wound responses that spread throughout the cassava root (observed as PPD) (1, 2).

The aim of this project is the identification of biochemical markers for post harvest physiological deterioration, studying the occurrence of secondary metabolites and enzymatic activities that may play a determinant role during PPD process. The identification of these PPD markers will generate the context necessary for the development of screening methods to exploit the existing genetic diversity with respect to PPD. These screening methods will also help to identify cultivars that combine agronomic performance and reduced post-harvest deterioration.

### Methodology

As previously reported, the strategy is based on the evaluation of cassava cultivars with contrasting responses to PPD, but following a deterioration time course of four days in order to avoid possible overlapping of defense responses caused by microbial deterioration. The biochemical tests are the quantification of coumarins (scopolin, scopoletin and esculin) and enzyme activities for Polyphenol oxydase (PPO), Peroxidase (POX), and Scopoletin-peroxidase. Taking advantage CIAT is generating a cassava genetic map and the parentals mapping cross have different responses to PPD, a representative percentage of the population was sampled to try to determine the role of major genes influencing the PPD response. Comparing the distribution of the PPD response presented by the family K between the two different ecosystems, CIAT Palmira and CIAT Quilichao (evaluation performed in 1998), CIAT-Palmira was chosen as sampling site because the deterioration score presented a normal distribution. Genotypes from the extremes (15 individuals) and the middle (five individuals) of the normal distribution were selected. From the three different groups the genotypes showing the minimal standard deviation between replications were chosen.

## Results

As reported before, two classes of secondary metabolites, hydroxycoumarins and flavan-3-ols, were identified in deteriorated cassava root extracts. Those metabolites proved to be biologically active, as antioxidants and antimicrobials (4, 5). The dominant compounds were scopolin, scopoletin and (+)-gallocatechin. Scopolin and scopoletin accumulate two to three days after harvest, whilst (+)-gallocatechin accumulates after four to six days. Based on this accumulation time, it was assumed that the coumarins are related to PPD while the flavan-3-ols are more related to microbial deterioration (3, 5).

The analysis of all root extracts chromatographic profiles showed two peaks, in the area of the non -polar compounds, which occurs with the onset of PPD. The structure elucidation of those candidates for PPD markers has not been successful. Besides that, the more prominent peak was quantified in terms of scopoletin. Figure 1 shows a major accumulation of the PPD peak in the cultivars with high response towards PPD, which may suggest this metabolite a good candidate for biochemical marker.

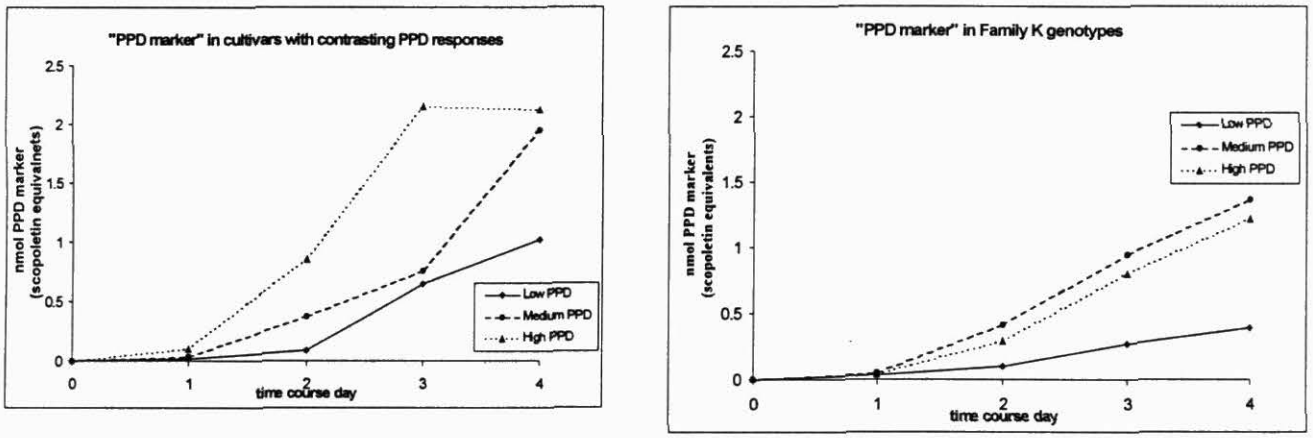


**Figure 1. "PPD marker" quantification in Scopoletin equivalents of different cassava cultivars and a percentage of Familiy K population with contrasting responses to PPD.**

Enzyme activities increase from the initial stage of the deterioration. Only POX presents a small decrease in activity by the end of the time course period, and the differences between cultivars with contrasting PPD responses are not as clear as with the other enzymes.

Previously was mentioned that peroxidases, in cassava roots, catalyse a reaction between scopoletin and $H_2O_2$ resulting in a black precipitate. The localisation of POX activity around the vascular parenchyma suggests a correlation between PPD and oxidation of hydroxicoumarins. As well, the increase of scopoletin-peroxidase could clarify the decrease of scopoletin at the lasts time course days (Figure 2).

The Family K was tested in detail for PPD damage. The entire population was grown, at the same time, in two different agro-ecological sites, Palmira and Quilichao, and during two following years, 1998 and 1999. Figure 3 shows the PPD response frequency for both planting locations and periods. Besides conclude PPD response is hereditable, is extensively affected by the environment. Even considerable differences in climate and soil factors exists between Palmira and Quilichao, it was not determined a specific environmental factor that could explain the genotype by environment (GxE) interaction.

The PPD scoring method used at CIAT, for more than 20 years, has been considered subjective and non reliable, therefore this project is looking for a biochemical analytical method which grants an objective and accurate measure of the PPD response. All tests are subject to large variability between roots of the same plant and between those of genetically identical plants. For that reason, it was assumed that the enormous variation, which indeed complicates the collection of statistically significant data, is more related to environmental factors than the specific analytical method used. While a rapid and economic biochemical assay can be developed, the traditional visual method should be used.

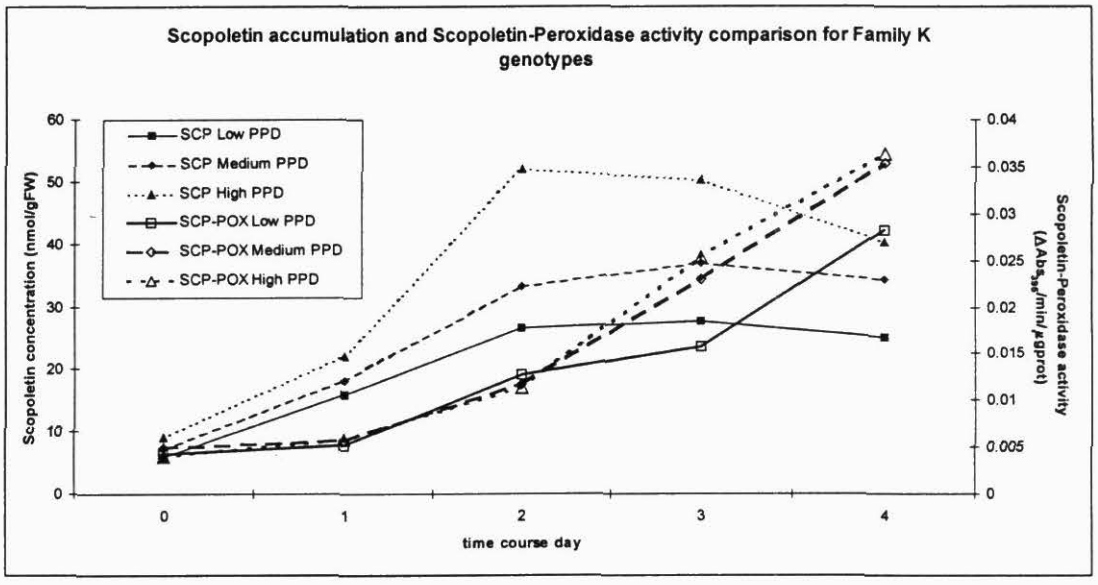


**Figure 2. Comparison between scopoletin (SCP) accumulation and scopoletin-peroxidase (SCP-POX) activity during a time course of four days, between a selected percentage of Family K population *with different responses* to PPD.**

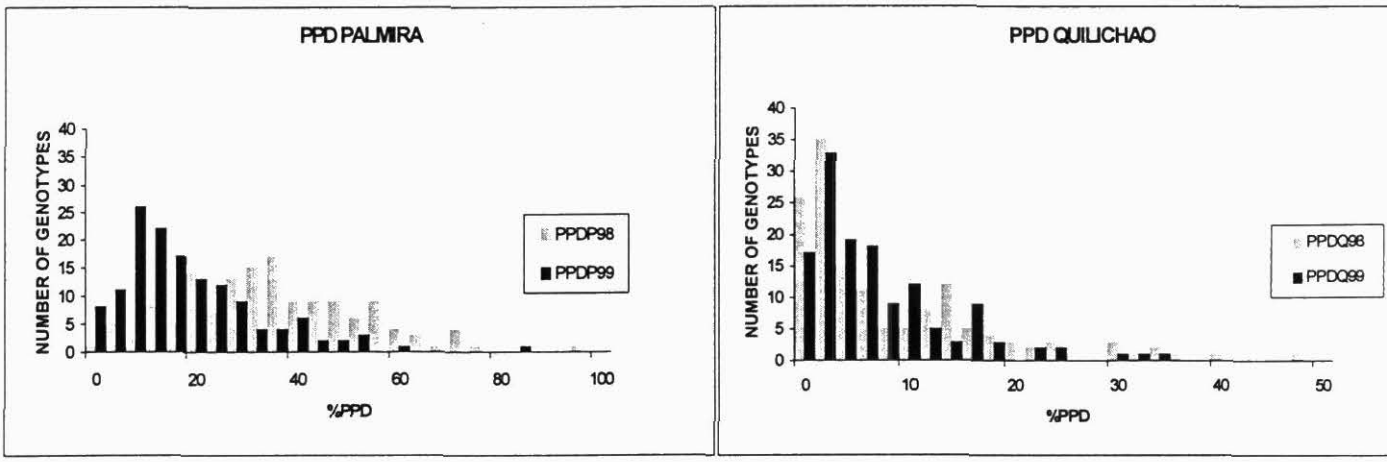


**Figure 3. Comparison of distributions of the PPD response amongst the Family K mapping population in two contrasting agro-ecologies in Colombia in 1998 and 1999.**

## On going activities

Present and future activities are the detailed analysis of all data compiled during the three years of research

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during post-harvest deterioration of tuberous roots of cassava (*Manihot esculenta* Crantz). Annals of Botany 86:1153-1160.

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# Output 3. Collaboration with public and private partners enhanced
# Activity 3.1 New collaborative arrangements and organization of workshops and training courses

## Main Achievements

- During the period of Oct 2000-2001 a total of more than 70 people (researchers, journalists, visitors and others) received training with SB-2 Project staff.
- A second workshop on Biotechnology and GMOs biosafety was given by CIAT to Colombian journalists.
- *Two training courses were held* at CIAT to train and upgrade SB-2 staff knowledge. One training was on molecular approaches for disease resistance, and modulating gene expression in transgenic plants was conducted at CIAT in collaboration with Eric Lam and Nilgun Tumer from the Biotechnology Center for Agriculture and the Environment Rutgers University, New Jersey, USA. Total of 43 participants including 37 CIAT Support Staff and 6 Principal Staff attended the course.
- The second course was on the use of micro array and the use of a novel technology. Diversity Array technology (DaRT). The course was given by Damian Jacouq from CAMB IA Australia and was attended by some 20 assistants from SB-2.
- CIAT obtained approval of a project from BMZ, Germany, on " *An Integrated Approach for Genetic Improvement of Aluminum Resistance of Crops on Low-fertility Acid Soils*" This proposal addresses a major strategic research issue through research partnerships linking CIAT, a regional network in Africa, two systemwide programs and an advanced research organization in Germany. The proposal will use a multidisciplinary approach to integrate the scientific capacity of both CIAT and the University of Hannover to alleviate a major soil constraint to agricultural productivity on low-fertility acid soils of Africa and Latin America.
- A collaborative project between Yale University and CIAT on insertion mutagenisis in rice using Ac-DS system was approved for funded by the USDA, opening the way to strengthen CIAT capacity in functional genomics.
- A collaborative project between CIAT and the University of Costa Rica on Breeding, biosafety and deployment of RHBV resistant transgenic rice was approved by the Rockefeller Foundation.
- With funding from the Rockefeller Foundation, SB-2 organized a legume genomics meeting between the CG working on legumes and US Universities resulting in the preparation of a common research agenda and a collaborative proposal.
- Staff of SB-2 organized the CG planning workshop for the biofortification proposal.
- A database for bean microsatellites was established.
- An updated version of FloraMap was released in 2001. Some 200 registered users from several countries have obtained a copy.
- Two assistants from the genome lab received full fellowships to attend visit a technology lab in Canada and to get trained in the development of full length cDNA libraries.
- In the period of Oct 2000-Sept 2001, SB-02 projects members published 40 Scientific papers in referred journal and books; abstracts and posters in conference. At he same time staff members gave keynotes and plenary presentations at International and regional meetings.
- In the period of Oct 2000-Sept 2001, projects members increased the contacts with private sector at the regional and international level to establish collaborative projects on technology transfer and to obtain freedom to operate for key technologies. Same kind of activities were established with regional NGOs in order to transfer CIAT technologies.
- The Cassava Biotech Network was reestablished with funding from DGIS and IDRC and with a focus for Latin America.
- In the same period 5 proposals were approved and 11 more submitted
- A total of 24 organizations contributed to funding projects in SB-2.

## 3.1.1 Highlights From The Cassava Biotechnology Network's Activities For 2001

Chusa Ginés
SB-2 Project

**Introduction**

The CBN initiated activities under the project *The Cassava Biotechnology Network in Latin America: Strategies for Integrating Small-Scale End-Users in Research Agenda-Setting, Testing and Evaluation,* jointly funded by DGIS and IDRC. The Coordination Office is based in Quito, Ecuador, a step taken in the process of decentralization of the Network to be eventually hosted with a Latin American organization.

The Coordinating Unit - now composed of Dr. Chusa Ginés, the Coordinator, and Verónica Mera, Social Scientist - established contact and built the collaborative basis with the potential pilot sites' organizations in Brazil, Colombia, Cuba, and Ecuador. The development of proposals and funding of small projects took place. The pilot sites "reconnect" with activities funded by the previous phase of the CBN. As well, the projects in Brazil and Colombia build on the collaboration with the PRGA, to take advantage of the synergistic opportunities for development of participatory methodologies. This usually involves capacity building on participatory and gender-sensitive methodologies and better integration of multidisciplinary teams. In the case of Brazil, the use of biotechnology will support the successful Participatory Plant Breeding Program of EMBRAPA Mandioca e Fruticultura. The focus is to stimulate local research on anchorage and adaptation of PRGA–linked biotechnology tools in key pilot sites.

The situation in Ecuador presents both a challenge and an opportunity. There is no organization with the specific mandate of doing R&D in cassava, and activities are fragmented. Therefore the CBN initiated a multi-stakeholder analysis, lead discussions with a variety of organizations involved with cassava activities in different regions and constituted a working group to carry out a diagnostic study on the Status of Production and Use of Cassava. This work has started in some depth in the province of Manabí. The results will be integrated into a GIS platform to improve targeting of activities and to increase the accuracy of analysis of crop performance. The outputs of GIS-based approach could include targeting and spatial analysis of potential / limitations of cassava production; analysis to identify possible genotype x environment interactions; definition of spatially variable risks, especially associated with future climate change. As well, socio-economic variables will be integrated. This study will form the basis for developing and integrated user-needs analysis, and ultimately, a more in/depth analysis on stakeholder involvement in priority setting and evaluation.

Presentations on the project were made at the following international meetings:

Programa de Biotecnología Agrícola, in Cartagena, Colombia, Feb. 2001. Possibilities for collaboration were explored with this DGIS-funded program with the intention to develop a joint research proposal to seek other funding and conduct a comparative analysis of the different methods of microprogation

RedBio meeting in Goiania, Brazil, June 2001– CBN co-organized a session on Cassava Biotechnology with presentations by CBN partners.

CBN V meeting in St. Louis, Missouri, 4-9 November 2001. This meeting served to invigorate the network and initiate the creation of a new vision for CBN. The program included sessions on participatory research and technology development.

International Society for Tropical Root Crops-Africa Branch (ISTRC-AB). 8th Symposium held at IITA, Ibadan, Nigeria, 12-16 November 2001. This meeting served to establish an exchange with other cassava researchers in Africa and start the formulation of a collaborative program for the CBN Network in Africa.

Sociedad Latinoamericana de Raíces y Tubérculos (SLART) meeting in Lima, Perú 28-30 November 2001. The objective was to strengthen the links with liked-minded organizations and projects in Latin America.

Overall, the first year of the project was focused on establishing a solid base for a decentralized regional network, and stimulating interdisciplinary collaborations for the development of a conceptual framework on participatory biotechnology.

### 3.1.2 Collaboration with Public and private sectors

- A CG planning meeting at CIAT was organized by SB-2 to prepare a global proposal on: "Harnessing agricultural technology to improve the health of the poor: "biofortified" crops to combat micronutrient malnutrition. The proposed project seeks to bring the full potential of agricultural science to bear on the persistent problem of micronutrient malnutrition by integrating breeding, nutrition, nutritional genomics, participatory and communities level research. CIAT and the International Food Policy Research Institute (IFPRI) will co-coordinate this inter-disciplinary effort among plant scientists, human nutritionists, and social scientists. CIAT will coordinate the breeding and biotechnology components while IFPRI will coordinate the nutrition and policy parts.

- To reinforce this global project SB-2 staff made a series of visit to Michigan State University, Cornell, IFRPI, Yale University, USAID and University of Frieburg to discuss the science and collaboration and establish the framework for the implementation of the project.

- SB-2 staff attended the Cereal genomics meeting organized at CIMMYT by Dr. Bob Zeigler from Kansas State University. The meeting was between CG and US researchers and resulted in the formulation of a research proposal.

- An intensive course on molecular approaches for disease resistance, and modulating gene expression in transgenic plants was conducted at CIAT in collaboration with Eric Lam and Nilgun Tumer from the Biotechnology Center for Agriculture and the Environment Rutgers University, New Jersey, USA.

- With funding from the Rockefeller Foundation, SB-2 staff organized a legume genomics meeting between the CG working on legumes and US Universities resulting in the preparation of a common research agenda and a collaborative proposal. Some 30 researchers attended from four CG centers (CIAT, ICARDA, IRCISAT, IITA) and several US universities. Washington, D.C., USA, Aug 19 - 24, 2001.

- SB-2 Staff prepared with researchers from the Danforth Center a framework proposal for a Global Cassava biotechnology initiative. The initiative also involved IITA, EMBRAPA and FAO. SB-2 staff visited the Danforth Center, Washington University sequencing centers, and FAO to prepare the proposal.

Other meetings and interactions included:

- SB-2 staff visited CIRAD and IRD to discuss a joint T-DNA project, Sept , 2001
- SB-2 staff visited University of Freiburg to explore possibilities of strategic alliance with CIAT. Cassava breeding through Biotechnology. Germany. September 9-11, 2001
- Clemson, South Carolina, USA, Aug 26-30, 2001, to visit collaborators at Clemson Unversity Genomics Institute and work on bioinformatics related to bean microsatellite development
- SB-2 staff Visited FEDEARROZ- Saldaña with Paola Ruíz and Juan José Vásquez July 17 - 20, 2001
- SB-2 staff visited CIAT whiteflies field at Ibagué - Tolima with Dra. Jeanne Jacobs, New Zeland. July 18, 2001
- SB-2 staff trip to Venezuela. Visited National Programs to link Projects of Investigation between both countries. July 7-9, 2001.
- SB-2 staff visited the Colombian Maize's Industry as part of a technology transfer agreement. June 16-17, 2001.
- SB-2 staff visited COLCIENCIAS- Bogotá. Genomic's Project. June 3-6, 2001
- SB-2 staff PBA Technical Meeting. Santa Marta. May 3-6, 2001
- SB-2 staff visited NOVARTIS, San Diego to discuss collaboration on rice genomics. April 14-22, 2001.
- SB-2 staff visited INIFAP, Mexico. April 22-29, 2001.
- Davis, California, USA, to visit collaborators at UC-Davis in the lab of P. Gepts. March 8-12, 2001
- Santa Fe, New Mexico, USA, to design software for CG bioinformatics program. Jan 14-17, 2001
- SB-2 staff nominated as a member of National Biotechnology Council of Colciencias, Colombia.
- Collaborators in ESALQ-Piracicaba and EMBRAPA / Arroz e Feijao - Goiania

- SB-2 staff member of Laboratory for training Latin American Trainees in Biosafety and related technology. Nominated as a member of the United Nations University

- SB-2 staff member of a review Panel to analyze the current Biosafety Regulation of GMOs in Colombia. (sponsors: Ministry of Environment and Agriculture; Colombian National Biosafety Council).

- SB-2 Staff aided the Colombian Association of Scientific Journalist with the public dissemination of a study funded by Colciencias on news cover of GMOs in Colombia

- SB-2 staff assisted the University of Costa Rica (UCR) on logistic to set up evaluations of RHBV resistant transgenic rice plants in Costa Rica according to biosafety recommendations

- SB-2 staff assisted the Colombian National University (UNAL) in Palmira, Colombia, and the Institute of Advanced Studies (IDEA) in Caracas, Venezuela on file application and construction of biosafety greenhouse facility complying with National biosafety regulations

- SB-2 Staff visited the Ministry of Science and Technology in Caracas, Venezuela, as a follow up initiative of Venezuela becoming a CIAT donor to fund research on rice biotechnology

- SB-2 staff and staff from University of Costa Rica initiated contacts with Val Giddings , Bio (USA), to get advice on how to proceed to get a non-interference approval from the private sector holding corresponding patents for the deployment to Latin American farmers of the RHBV transgenic resistant rice invention

- SB-2 staff initiated contact with the International Food Biotechnology Committee of ILSI to get advice on how to proceed to initiate food biosafety analysis of RHBV transgenic resistant rice

### 3.1.3 International Scientific Meetings

- SB-2 staff participated in several international meetings and gave keynote and plenary presentations at:

- Tohme, J. Opening presentation at the Euro conference on "Molecular genetics of Model legumes: impact for legume biology and breeding", organized by the Mx Planck Institute of Molecular Plant Physiology, Golm, Germany. Sept 15-19, 2001

- Tohme, J. Plenary presentation at the German Colombian workshop on Biotechnology research, University of Hanover, Sept 12-14, 2001

- Chavarriaga P., speaker International Symposium on Biotechnology. Plant Biotechnology at CIAT: from genomics, through tissue culture to farmers. Nairobi, Kenia. September 2-5, 2001

- Z. Lentini. Invited speaker: GMO derived food less safe than others?. IX Congress of the Colombian Association of Nutritionists. November 1- 2, 2001. Bogotá, Colombia.

- Lentini, Z. Invited speaker: Role of Biotechnology Developing Improved Germplasm for The Tropics. First International Congress on Challenges and Opportunities of GMOs for Agriculture and Agroindustry. Cartagena, Colombia. October 11-12, 2001.

- Z. Lentini, Invited speaker: Gene Flow Analysis for Assessing the Safety of GMOs in the Neo-Tropics: The case of beans and rice. International Congress on GMOs: Real risks or chimeras? Brasilia, Brazil. September 3-6, 2001

- SB-2 staff participated in ng livelihoods of the resource poor through biotechnology. London. August 30 - September 10, 2001

- Beebe, S Annual meeting of the Costa Rican national bean program (PITTA). Oral Presentation: Razas de frijol común y sus implicaciones para mejoramiento genético. August 8-9, 2001.

- Beebe, S. Annual meeting of the Costa Rican national bean program (PITTA). Oral Presentation: Reflexiones sobres retos y oportunidades en el mejoramiento de frijol en los próximos años August 8-9, 2001.

- Beebe, S. Jones, P Applications of GIS to tropical agriculture in CIAT. Oral presentation at the Conference on GIS and Biotechnology, VPI, Blacksburg, VA. 2001.

- Beebe, S.; Terán, H.; Quintero, C.; Pedraza, F.; Tohme, J Annual meeting of the Costa Rican national bean program (PITTA). Oral Presentation: Selección asistida por marcadores: consideraciones para su aplicación práctica. August 8-9, 2001.

- Beeching JR. Strategies for the Modulation of Post-harvest Physiological Deterioration in Cassava for the Benefit of Poor People. Report commissioned by DFID's Crop Post-Harvest Programme. 2001

- Tohme J. Moderator and presentation at the panel on biotechnology at the V Encuentro Nacional para la Productividad y la Competitividad organized by the ministerio de comercio exterior, Medellin, July 9-11, 2001

- Tabares E., L.Fory, L.Duque, F.Angel, G .Delgado, and Z. Lentini REDBIO. Rice Genetic Transformation efficiency using particle bombardment or mediated by *Agrobacterium tumesfaciens*. Goiania, Brazil. June 4-8, 2001

- Blair, M.W. Tohme, J. Beebe, S REDBIO. Bean genetic resources and genomics research at CIAT. IV Latin American meeting on Plant Biotechnology, Goianía, Brazil. June 4-8, 2001

- Florez C., R. Escobar, M.Duque and Z. Lentini REDBIO Optimization of RITA system for un automated mass production of embryogeni callus of *Brachiaria* species. Goiania, Brazil. June 4-8, 2001.

- Lentini Z. -key note speaker REDBIO. Gene Technology: Expanding Genetic Diversity and Adding Value to Rice. Brazil. June, 2001

- Lentini Z. Key note speaker. REDBIO. Biotechnology for Farmers and Consumers: Role of Biotechnology in Research and Delivering Improved Germplasm for Latin America. Goiania, Brazil. June 4-8, 2001

- Martinez César P.and J.Tohme. *2001.Progress in* the genetic improvement supported by molecular markers.Paper presented in :REDBIO 2001.IV Latin-American Meeting on Plant Biotechnology. June 4-8, 2001.Goiania,Goias.Brazil.

- Mora A., L. Fory, Iván Lozano, E. Tabares, L. Calvert, and Z. Lentini REDBIO. Transgenic Rice with Hypersensitive Resistance to Rice (*O. sativa*) Hoja Blanca Virus (RHBV) in the Field. Goiania, Brazil, June 4-8, 2001

- Lentini Z. - Plenary speaker Biosafety in Field Trials with Plants Modified with Gene Technology. VII Congress of the Colombian Society of Plant Breeding and Crop Production. Ibagué, Colombia. May 23-25, 2001.

- Beebe, S.Virginia Polytechnic Institute, Blacksburg, VA, May 17-19, 2001, to attend conference on GIS and biotechnology.

- Beeching JR. Molecular analysis of post-harvest physiological deterioration in cassava. BioVeg 2001: International Workshop on Plant Biotechnology. Ciego de Avila, Cuba, April 16-20, 2001

- Beeching JR. Post-harvest physiological deterioration in cassava: molecular and biochemical insights. INIVIT, Santa Clara, Cuba, April, 2001

- Gomez-Vasquez R. Defence responses in cassava suspension cells treated with elicitors. BioVeg: International Workshop on Plant Biotechnology. Ciego de Avila, Cuba, April, 16-20, 2001.

- Martínez, C. Symposium on Quantitative Genetics and Plant breeding in the 21st Century. Lousiana State University, March 26-28.2001.

- Tohme, J. Pleanry presentation on Biotechnology and agriculture productivity IV Encuentro de Competitividad y Productividad. Pereira, February 2001

- Tohme, J. Plenary presentation at a meeting organized by El Espectador and Ministerio de comercio for the sector industrial on biotechnology, 2001

- Beeching JR. Deterioracion fisiologica post-cosecha en yuca. Colombian Biotechnology Programme in Agriculture – International Seminar. Cartagena, Colombia. February 21 – 23,2001

- Beeching, J.; Tohme, J.; Escobar, R. Chavarriaga, P.; Verdier, V- Invited speakers International Program of Agricultural Biotechnology. Cartagena. January 20-24, 2001

- Chavarriaga P. invited speaker Conference on Genetic Engineering and Food for the World. USA, January 19-21, 2001

- Beeching JR. Post-harvest physiological deterioration in cassava: molecular and biochemical insights. IITA, Ibadan, Nigeria, November, 2000

- Buschmann H, Tohme J, Beeching JR. Biochemistry of post-harvest deterioration in cassava root tubers. German Society for Tropical Sciences. University of Hohenheim, Stuttgart. October 11-12, 2000

- Beeching JR. Post-harvest physiological deterioration in cassava: molecular and biochemical insights. Central Food Technological Research Institute, Mysore, India, October, 2000

- Beeching JR. Post-harvest physiological deterioration in cassava: molecular and biochemical insights. Central Tuber Crops Research Institute, Trivandrum, India, September, 2000

- Beeching JR. Post-harvest physiological deterioration in cassava. International Society for Tropical Root Crops Symposium. Tsukuba, Japan. September 11-15, 2000

- Tohme, J. Plenary presentation on the VIII congreso Colombiano de farmacologia y terapeutica, first Simposium International sobre biodiversidad como fuente de nuevos medicamentos., 19 August, 2001, Cali.

- Blair, Tohme, J. and Beebe, S PCCMCA. Bean genetic resources and genomics research at CIAT. Costa Rica April 1-6, 2000

- Muñoz, C. ; Blair, MW.; Roca, W. and Tohme, J. PCCMCA. Introgresión de genes de frijol tepari a frijol común por retrocruzas congruentes. Costa Rica April 1-6, 2000.

### 3.1.4 Workshops, training and Conferences

- SB-2 staff trained in construction of cDNA Libraries. Yale University. New Haven, Connecticut, USA. October 21 to November 18, 2001

- SB-2 staff trained in Métodos para cuantificar la variación genética y las relaciones filogenéticas en Bancos de Germoplasma. Costa Rica. November 5-7, 2001

- SB-2 staff attended: Conocimiento, construcción y análisis de técnicas de DNA Microarrays, October 13, 2001. North Carolina State University

- Biosafety in Developing and Using Insect Resistant Transgenic Plants Enthomology Forum. National University of Colombia. Palmira. October 11, 2001. (Z.Lentini).

- Workshop on Agriculture Biosafety for Colombian Journalists. CIAT. September 29-30, 2001

- Propagación a bajo costo en Santa Ana, Cauca." Perico Negro, Puerto Tejada, Septiembre 20 de 2001.

- SB-2 staff invited speaker in Fundamentos de Biología molecular para Fitopatologos". ASCOLFI. September 7, 2001

- SB-2 staff invited speaker in Analysis of Molecular Data. CIAT August 8-10 y 17, 2001
- SB-2 staff assistance to Seminar de Cryo' intercentre.Leuven, Amsterdam. June 30 - July 8, 2001
- SB-2 staff trained in VIRTEK. Toronto, July 7-16, 2001
- SB-2 Staff attended Workshop on Cryopreservation of vegetatively propagated tropical crops. INIBAP, 2-6 july 2001
- SB-2 staff attended Course on Desarrollo de metodologías participativas en Biotecnología. In: Curso sobremétodos y técnicas de participación de productores en la Investigación en CIAT. IPRA. June 26-29, 2001
- SB-2 staff attended Statistic Course, North Carolina State University, 29 de May 29 - June 19, 2001
- SB-2 staff visited IDIAF(Instituto Dominicano de Investigaciones Agricolas y Forestales).One month training in Plant Breeding.
- SB-2 staff trained in Summer Institute in Statistical Genetics NCSU May 30-June 16, 2001
- SB-2 staff participated in Microarrays Course. CIAT May 20-27, 2001
- SB-2 staff attended a workshop on *Phaseolus* genomics. Mexico, March 2-7, 2001,
- SB-2 staff participated in IV. Encuentro sobre Competitividad y Producción. Pereira, Colombia. February 15-16, 2001. (J.Tohme invited speaker)
- SB-2 staff trained a research scientist from Cenicaña on tissue and genetic transformation of sugar cane. 2001
- Course on the use of micro array and the use of a novel technology. Diversity Array technology (DaRT). The course was given by Damian Jacouq from CAMBIA Australia and was attended by some 20 assistants from SB-2. Mayo 20-27, 2001
- Course on molecular approaches for disease resistance, and modulating gene expression in transgenic plants was conducted at CIAT in collaboration with Eric Lam and Nilgun Tumer from the Biotechnology Center for Agriculture and the Environment Rutgers University, New Jersey, USA. Total of 43 participants including 37 CIAT Support Staff and 6 Principal Staff attended the course.

**Graduate students (current)**

- Ivan Ochoa – Pennsylvania State University, USA – conducting laboratory and field studies to understand the inheritance and mechanisms of low phosphorous tolerance in common bean and the role of adventitious rooting in adaptation to low phosphorous stress (collaboration with J. Lynch)

- Andrea Frei – ETH, Switzerland – studying the quantitative trait loci involved in resistance to the leaf-feeding insect, *Thrips palmi* in common bean (collaboration C. Cardona, S. Dorn, H. Gu)

- Oscar Checa – Universidad Nacional – Palmira, Colombia – studying the inheritance of climbing ability in common bean and the importance of genotype x environment interaction in this trait.

- Juan F. Fernandez; Molecular markers and population genetics of *Quercus* –PhD Program, Tropical Ecology, University of Missouri in Saint Louis –UMSL, USA.

- Hernando Ramírez; Tomato transformation for insect resistance –PhD program, Agronomic Sciences, Universidad Nacional de Colombia.

- Gerardo Gallego; Gene cloning of rice disease resistance genes - PhD program, Agronomic Sciences, Universidad Nacional de Colombia, Palmira, Colombia.

- Eliana Gaitán; Molecular markers and diversity of palm trees - PhD program, Agronomic Sciences, Universidad Nacional de Colombia, Palmira, Colombia.

- Roosevelt Escobar; Genotypic stability of cryopreserved cassava plants- MSc Program, Agronomic Sciences, Universidad Nacional de Colombia, Palmira, Colombia.

- Nelson Royero; Molecular markers and diversity of *Anonna* spp - MSc Program, Agronomic Sciences, Universidad Nacional de Colombia, Palmira, Colombia.

- Fabio Escobar; Molecular markers to certify seeds of rice - MSc Program, Agronomic Sciences, Universidad Nacional de Colombia, Palmira, Colombia.

- Edgar Barrera; Molecular markers for ACMD resistance- MSc Program, Agronomic Sciences, Universidad Nacional de Colombia, Palmira, Colombia.

- Juan J. Ruiz; Field evaluation of *in vitro* propagated *Annona* - MSc Program, Agronomic Sciences, Universidad Nacional de Colombia, Palmira, Colombia.

- Eyvar Andrés Bolaños Vidal. Caracterización de la diversidad genética en cuanto a contenidos de caroteno de raíces y hojas de 682 genotipos de yuca.

- Galindo, L.M. 2001. Aislamiento y caracterización de las secuencias LTR de retrotransposones del grupo TY1-copia en *Phaseolus vulgaris*. Dpto. de Biología, Universidad Nacional de Colombia, Bogotá. (Laureada).

- Martínez A.K. 2.001. Obtención de microsatélites en la palma de chontaduro *Bactris gasipaes* (Palmae). Dpto. de Biología, Universidad Nacional. Bogotá.

- Olga Ximena Giraldo. 2001. Universidad del Valle. Construcción del mapa genético de *Brachiaria* utilizando microsatélites y AFLP's. Dpto. de Biología. Fac. De Ciencias.

- Eliana González. 2001. Univalle. Diversidad genética de 3 poblaciones de *colombo balanus* excelsa (fagacia) especie endémica de los Andes Colombianos

- Claudia Patricia Florez. Ph.D. Thesis. Universidad Nacional. Sede Palmira.. Development of *Brachiaria* Genetic Transformation mediated by *Agrobacterium tumefacien*. Sponsor: Colciencias. Currently on leave at Dr. German Spangerberg's Laboratory, Plant Biotechnology Centre, Agriculture Victoria, La Trobe University, Bundoora, Victoria, Australia , establishing a CIAT- La Trobe Univeristy collaboration

**Undergraduate students (current)**

- Sergio Prieto – Universidad Nacional
- Juan José Vásquez. Universidad de los Andes, Bogotá
- Paola Ruíz. Universidad Javeriana, Bogotá
- Gloria Iriarte, Universidad de Tolima.
- Carolina Castaño, Universidad de los Andes, Bogotá
- Andrés Felipe Salcedo. Universidad del Valle
- Hector Fabio Buendía, Universidad de Tolima.
- Carolina Ramirez Rodríguez, Universidad del Tolima.
- Carolina Astudillo, Universidad del Valle.
- Wilfredo Pantoja, Universidad del Valle.
- Luis Guillermo Santos, Universidad Nacional -Palmira.
- Andrés Bolaños, Universidad Nacional –Palmira.
- Juan Esteban Montoya, Universidad Nacional - Medellín
- Morgan Echeverry, Universidad del Valle

### 3.1.5 Visiting Research

- Peter Wenzl, Center for the Application of Molecular Biology to International Agriculture, Cambia. (Oct 2001 - Dec. 2001)

- Rocío Gómez. Universidad de Bath (Oct. 2000-Aug.2001)

- Adebola Raji - Nigeria (Sept 1- Nov. 30, 2001)

- Maritza Berti, Universidad Santiago de Chile, Chile (Sept - Nov, 2001)

- Martha Isabel Moreno, UNIVALLE, Colombia. (Sept, 2001 - Dec. 2001)

- Alexandra Narváez, IRD, Ecuador ( August - Sept, 2001)

- Erika Alexandra Arnao, CONICIT - DANAC, Venezuela. (August, 2001 - Sep. 2001)

- Enmanuel Okogbenin, University of Ibadan, Nigeria ((July 1998 - Nov.2001)

- Juan Diego Palacio. Instituto von Humboldt, Colombia (July 1998 - Dec 2001)

- María Paola Rangel, Centro Nacional de Investigación de Caña de Azucar, Colombia (May 99 - Dec. 2001)
- Yvonne Lokko - Ghana (April 4, - Sept 5, 2001)
- Alba de las Mercedes Alvarez, OIEA, Cuba (Jan 2001 - Jun.2001)
- María Ximena Rodríguez, University of Bath (Jan. 1999 - Dec. 2001)
- Carlos Mario Hernández, Fundación para la Investigación y el Desarrollo Agrícola - FIDAR. Colombia ( Feb. 1999- Dec 2001)
- Inés Sánchez Mosquera, Corporación Colombiana de Investigación Agropecuaria, Colombia (Feb.1995 - Dec. 2001)

A total of more than 70 people (research, journalist, visitors) received trainning with SB-2 Project staff in different areas and /or for courses and workshops. Their backgrounds were diverse, having from BS to pos-doctoral degrees.

## Activity 3.2 Assembling databases, genetic stocks, maps probes and related information

### 3.2.1 Molecular genetics database constructed for a microsatellite parental survey of common bean germplasm

M.W. Blair[1], A.F. Guerrero[2], F. Rojas[3]
[1]SB-2; [2]IP-1 and [3]Unidad de Sistemas de Información

**Introduction**

We are developing two databases to store the information about microsatellites tested on common beans. The first, uses Oracle (Developer 2000) software that we initially tested for storing, handling and presenting images within a relational database about RAPDs that we constructed last year (Annual report 2000). The software has a more user-friendly interface and the capacity to be loaded onto the web. As a relational database, Oracle has the advantage of being an efficient program for organizing and managing data that has multiple layers of relational structure and which is based on a series of data tables. Oracle is also the standard program for databasing the information from the breeding programs at CIAT. The second database we are using to store microsatellite data is Beangenes (http://beangenes.cws.ndsu.nodak.edu/), which is the AceDB genome database for beans which was established by the USDA - Plant Genome program to specialize in the genetic information relating to the crop. We hope that these databases will be the basis for collecting genotypic information on common bean and a dynamic analysis tool allowing researchers to ask such questions as: how many polymorphisms can I expect when comparing two varieties that might be potential parents? ; and which polymorphisms distinguish one variety from another?

**Results and Discussion**

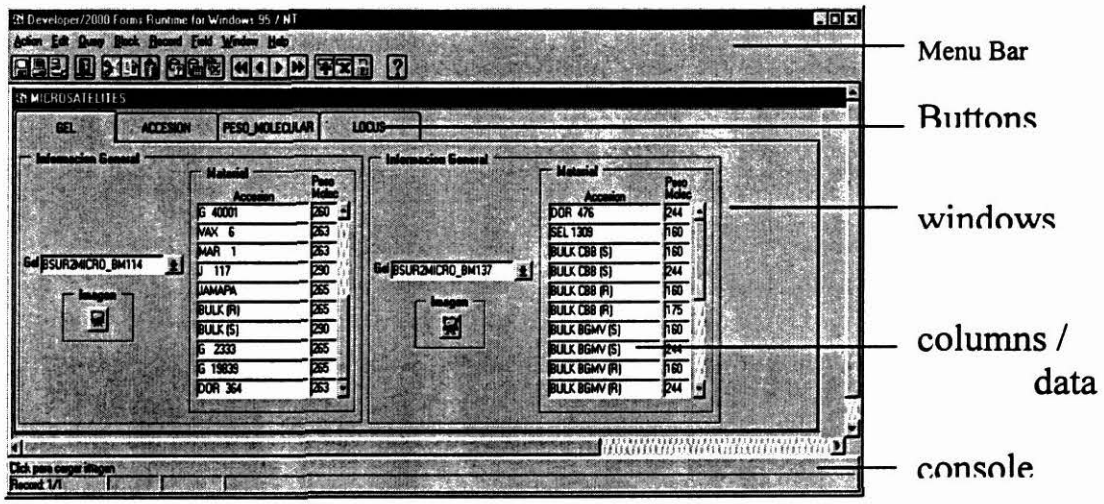
A total of 186 photographs of microsatellites surveys were scanned, annotated and loaded into both databases. Size estimates based on the molecular weight of each allele were also databased. This represents the diversity data for a total of 95 microsatellite loci.

*Oracle Database:* The gel images were loaded with the Oracle graphics development tool and the estimated band sizes were loaded using the Oracle worksheet development tool. The database provides a set of tools for asking new questions. The three principal components are tools to generate worksheets, reports and graphic images. The program has a web-compatible format that uses windows and buttons to allow for interactive searches and queries. The finished database has three main windows for "gel", "accession", "molecular weight" and "locus" as shown in Figure 1. Each of these has a datasheet format with columns and entries. The "gel" and "locus" windows allow the user to call up parental surveys for individual microsatellites and to compare the molecular weight of the alleles found in all the genotypes in that survey. These window can be used to compare two microsatellites at a time. Embedded windows are used to call additional items such as the gel images as shown in Figure 2. The "accession" window can be used to compare the markers present in two genotypes. The "molecular weight" window allows a user to search for microsatellites of a certain size. Activities are realized either through the menu bar or action buttons. A console line indicates the status and location of the user. The first version of this database was written in Spanish.

*AceDB Database:* A new class called "microsatellites" was created within Beangenes to contain the information about these markers. This was a central point for linking the images of the microsatellite parental surveys, which were added to an existing class, called "image". Beangenes is no longer being updated regularly at North Dakota Sate University, so it will be important for CIAT to incorporate and curate its own data for it to be included in the database. All the AceDB genome databases are being mirrored by the Demeter site at Cornell – USDA bioinformatics facility. These genome databases as well as Beangenes are written and curated in English.

Future plans: are to load additional molecular marker data into the present databases. The database could accommodate data from other studies of genetic diversity using the microsatellites, as well as any additional parental surveys that will be conducted. All of the microsatellties used in the parental survey are also being placed on the bean genetic map at CIAT and therefore the chromosomal location of the markers will be the next important data to put in the database. It will be very important to link the map information with the genetic diversity data accumulated for all microsatellites. A comparative mapping tool would allow researchers to compare the position of the markers in the CIAT population to mapping results from other laboratories (notably with the University of Florida and the University of California - Davis). Web access will be a priority, once the databases are released. AceDB is already internet compatible, through the program Web-Ace developed at the Sanger center. For the Oracle database, we hope to place the database on the internet using Microsoft Interdev or Web-DB. With either system, the database can be accessed from any type of computer, via common web-browsers such as Netscape or Internet Explorer. The information stored in these databases could be amenable to linkages with ICIS (International Crop Information System http://www.cgiar.org/icis), which is the database system for managing and integrating genetic resource, crop improvement and crop management information of the CG-system. IPHIS (International Phaseolus Information System - http://www.ciat.cgiar.org/icis/) is the version of the ICIS database that has been developed at CIAT to hold bean-breeding data. Molecular data is foreseen to be an important part of these databases in the future. To realize the maximum potential of a molecular marker database, it should also be linked with other existing databases that contain germplasm data on *Phaseolus* including SINGER (http://singer.cgiar.org), the

principal database on genetic resources held in the CGIAR system and GRIN (http://www.ars-grin.gov/npgs/), a comparable germplasm database of the USDA.



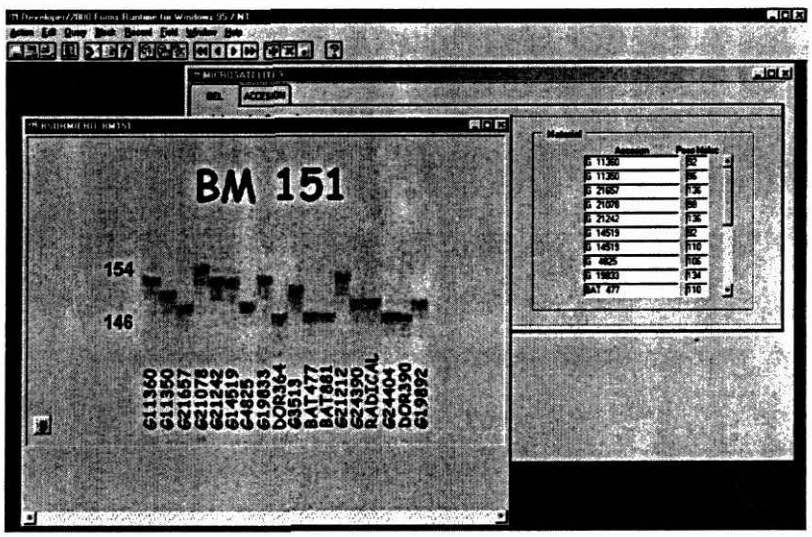




**Figure 1. Components of the main window**
**Figure 2. el-image window linked to accession and band data.**

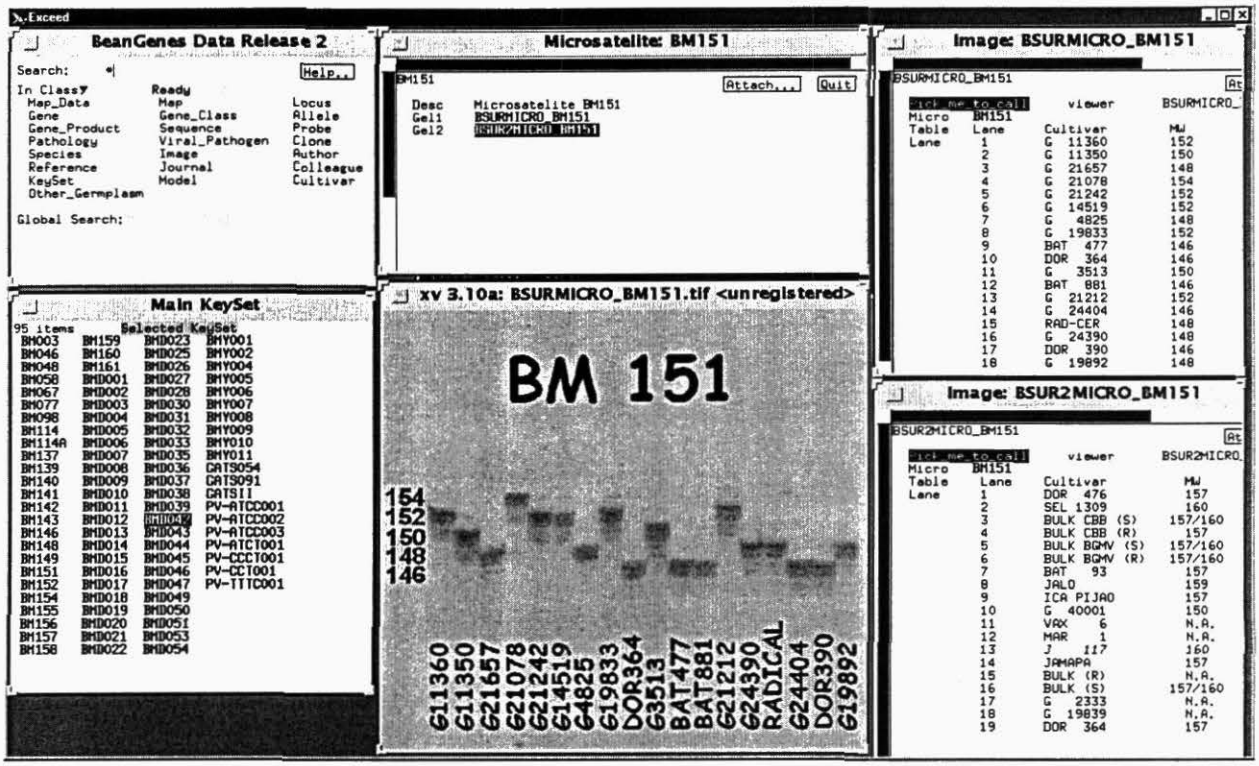



**Figure 3. Gel-image with microsatellite information in the AceDB database, Beangenes**

## 3.2.2 Flora Map: Release 1.01 with new climate grids

**P. Jones**
PE-4 Project

FloraMap was used as an example for the Intellectual property Audit. All together we exchanged almost 80 emails, many of them long and detailed. I learned a lot and the report clarified many aspects of our software protection, copyright and licensing. I modified our copyright statements on the manual and software and included overlooked acknowledgements to ESRI and Borland for the use of their software in FloraMap development. The auditors offered, free of charge, to register the FloraMap logo drawing as a trademark as well as just the name, something I did not consider in the original application for trademark.

We finished the release 1.01 of FloraMap incorporating some corrections and minor modifications and prepared a set of release notes. These are now available for download on the FloraMap web site. I have created the European MetGrid from files kindly provided by IWMI from their Land and Water Atlas of the World. These are 10 arc minute climate grids and, as such are at the lower limit of resolution for FloraMap, but are useful to extend the coverage. I also constructed the 2.5 arc minute grid for the Continental USA from data kindly made available by Dr. Chris Daly of the University o Oregon.

In the tropics, almost all climates are seasonally defined by the rainfall record. The annual march of temperature being marginally important. As we approach the temperate regions rainfall variability becomes less important and the seasons become much more markedly defined by

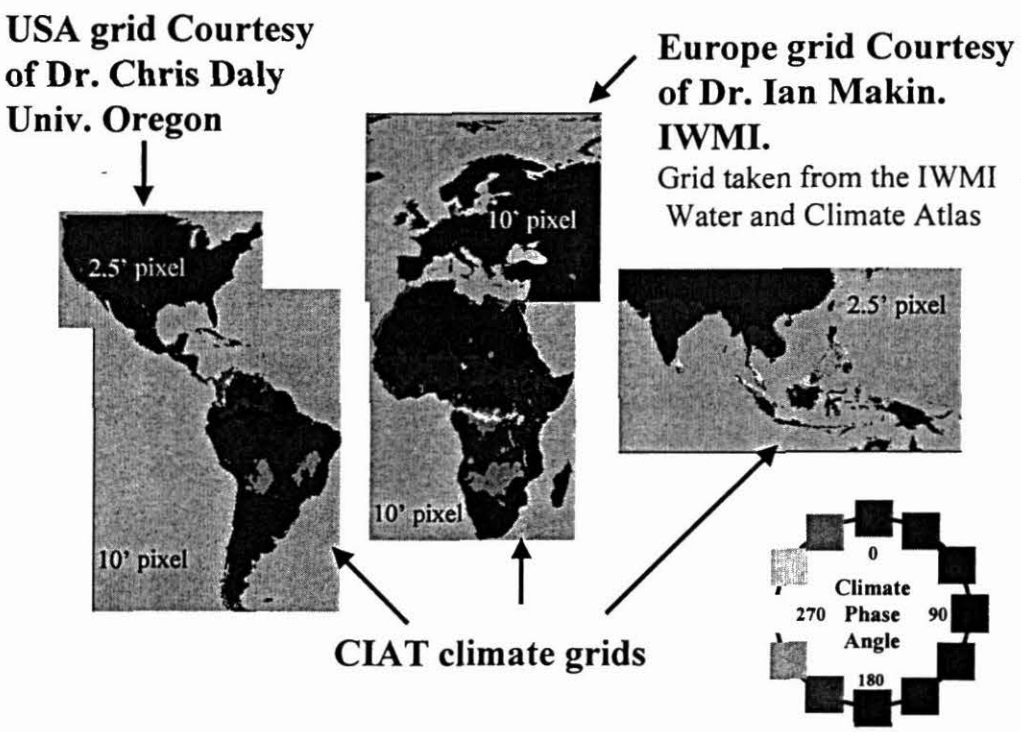
temperature. The original climate grids were rotated on rainfall alone. In the climate grids released to date this has not been a major problem, apart from some very limited areas of Mediterranean climates and deserts. I have now developed an alternative system using a weighted

$$s = \arctan \frac{|\mathbf{p} - \mathbf{t}|}{|\mathbf{p} + \mathbf{t}|}$$

rotation that favours rainfall seasonality in the tropics and temperature elsewhere. It proved to be a tricky balance of weightings and required repeated mappings of various indicators until I found the right combination. This proved to be reassuringly simple once I had found it. I use the vector sum of the rainfall first frequency vector p in mm, with vector t, the first frequency temperature vector multiplied by 2 times the latitude. The resultant rotation vector r gives the rotation phase angle. An index of rotational stability was formed as

This was mapped for all available MetGrids and inspected carefully for areas of instability. Some areas in the tropics show the values of s approaching instability in some bimodal rainfall areas where the first frequency vector amplitudes are low. However in all cases except three high altitude pixels in Colombia the rotation angle was stable. This aspect of bimodality now shows under the more sophisticated rotation algorithm and will need further work in the future before I implement high precision grids. However the present arrangement is adequate for the moment. It now grades evenly from one system to the other and will allow a unified approach throughout all climates.

We also created a grid for Honduras at 30 arc seconds and 5 arc minute and 10 arc minute grids for Asia for users whose computers would not handle the excess load of the 2.5 arc minute grids. All these new grids are now available for download from the web site.



During 13th and 14th Nov we assisted with the course on FloraMap, DIVA and SID given by IPGRI and CIP representative Robert Hijmans to IPGRI regional office personnel. We gave a detailed demonstration of FloraMap and was present during the tutorial session to answer questions, solve problems and give advice. William Diaz had distributed a feedback questionnaire for the participants and we got some useful comments.

**Measurable/ Expected Impact**

Although the exact number of copies actually in use is difficult to estimate, We believe that the number of active users is approaching 200 world wide. We would like to recognize the invaluable collaboration of my colleagues in IPGRI who have helped to widen the distribution and have given a remarkable service in putting on courses for training in FloraMap in many parts of the world.


**Floramap users registered with the FloraMap listserver**

## 3.2.3 Genetic constructs, stocks and databases

- Total of 280 *RHBV-N* transgenic lines and derived F3 progeny from crosses to Iniap 12, Oryzica 1, and Fedearroz 50.
- Forty five transgenic lines derived from *RHBV-N* transgenic Cica 8 with level of resistance to RHBV between 1 and 3.

- Seven lines *RHBV-N* transgenic Cica 8 lines more resistant than the highest commercial resistant variety Fedearroz 2000

- Total of 21 NS4 sense and 70 NS4 anti-sense transgenic Cica 8, Palmar, Cimarrón, and Fundarroz PN1 plants for novel RHBV resistance.

- Total of 59 PAPY123 transgenic Cica 8, Palmar, Cimarrón, and Fundarroz PN1 plants for novel RHBV resistance

- Total of 7,542 lines were generated from rice anther cultured for the various breeding efforts stationed at CIAT. Two hundred and sixty R2 , R3, and somaclone lines were distributed this year to national program in Latin America..

- A cDNA library from 10-day-old seedlings of *Brachiaria decumbens* was generated. The library is being used to clone key lignin biosynthetic genes from *Brachiaria.*

- At least two transgenic plant lines of cassava cultivars TMS60444 expressing GUS from construct containing a Cry1Ab gene.

- Construct carrrying a new version of pokeweed antiviral protein (PAP), gene isolated from Phytolacca americana, with a ribosome-inactivating ability, potent antiviral and antifungal activities driven, by the 35S promoter, and hygromycin as selection gene. E. coli and *Agrobacterium tumesfaciens* EHA101, EHA 105, and Ag11 strains.

## Activity 3.3 Project proposals and publications

### 3.3.1 Projects approved or on going

- Rice functional genomics consortium. USDA project on rice genomics with Yale University. Approved 2001.

- Delivery of transgenic rice cultivars to seed producers and farmers in tropical America: Following a multi-step approach involving biosafety assessment, nutritional testing and negotiations on intellectual property . The Rockefeller Foundation. Approved January 2001-2004 (Partnertship: CIAT and Univ. of Costa Rica).

- "Candidate Genes for Tolerance of Symbiotic Nitrogen Fixation (SNF) to Phosphorus Deficiency in Common Bean (*Phaseolus vulgaris L.*)". Approved by *Plate-forme de recherches avancées Agropolis – 2ème appel d'offre (2001 – 2003).* (partnership INRA, CIAT and INIFAP, Mexico)

- Gene Flow Analysis for Assessing the Safety of Bio-Engineered Crops in the Tropics. BMZ. 2000-2003. (Partnership: CIAT, Univ. of Costa Rica, Hannover University, and Federal Biology Institute-Germany).

- Genetic transformation of rice from fungal resistance. 1999-2001. Centro Tecnológico Polar (Partnership Rutgers University, IDEA-Venezuela, and CIAT.

## 3.3.2 Projects submitted, in preparation and concept notes

- Post-harvest physiological deterioration, starch & protein sections of CIAT / IITA / EMBRAPA / Danforth / et al. "Global Cassava Programme".Concept note submitted to DIFD for a CIAT-Bath

- Collaborative project "Enhancing Cassava's Potential for *Combating Vitamin A Deficiency*".

- Core genomics facilities for CIAT, Cenicafe, Cenicana, Corpoica. Submitted to the bilateral aid programbetween Colombia and Germany. Colociencias

- Concept note submitted to DFID's CPHP for a Bath-CIAT collaborative project "Knowledge and tools for the modulation of post-harvest physiological deterioration in cassava".

- Concept note submitted to the Novartis Foundation for a Bath-CIAT collaborative project "Enhancing the Protein Quality and Quantity of Cassava".

- "Bioavailability and clinical response to the consumption of high mineral beans and quality protein maize" proposal presented to the Micronutrient Initiative for funding of nutrition research in Colombia (submitted by Universidad del Valle with CIAT).

- "Manejo de germoplasma local y aumento de la agrobiodiversidad de frijol y maíz con variedades biofortificadas para mejorar la nutrición en comunidades rurales y urbanas de Nariño" proposal presented to Cuenta de las Americas (submitted by FIDAR with CIAT).

- "Mejoramiento de la nutricion humana en comuncidades pobres de America Latina utilizando maíz (QPM) y frijol común biofortificado con micronutrientes" proposal presented to Fontagro (IDB), to improve nutrition in rural and urban communitities in Colombia and Guatemala with NGO partners (submitted by FIDAR with CIAT).

- "Breeding staple crops for improved micronutrient value", a proposal submitted to a consortium convoked by the Gates Foundation, to improve the nutritional status of bean

- "Bean genomics for improved drought tolerance in Africa and Latin America", concept note prepared for BMZ-Germany.

- Regional distribution of Neotropical crops and their wild relatives: *gene flow risk assessment* for the management of transgenic plants. Submitted to USAID Biotecnology and Biodiversity Interface.

- Gene Flow Analysis for Assessing the Safety of Bio-Engineered Crops in the Tropics.

## 3.3.3 Publications

### 3.3.3.1 Refereed Journals, Books and Theses

- Mba, R.E.C., Stephenson, P., Edwards, K., Melzer, S., Mkumbira, J., Gullberg, U., Apel, K., Gale, M., Tohme, J. and Fregene, M. 2001. Simple Sequence Repeat (SSR) Markers Survey

of the Cassava (*Manihot esculenta* Crantz) Genome: Towards an SSR-Based Molecular Genetic Map of Cassava. Theoretical and Applied Genetics. 102:21-31.

- Moncada ,P., Martinez, CP, Borrero, J. Chatel, M.; Gauch H. Jr, Guimaraes, E. ; Tohme, J. and McCouch, SR. 2001. Quantitative trait loci for yield and yield components in an *Oryza sativa* x *Oryza rufipogon* BC2F2 population evaluated in an upland environment. Theor. Appl.Genet. 102:41-52

- Beebe, S., Rengifo, J. Gaitan, E. Duque,MC and Tohme, J. 2001. Diversity and Origin of Andean Landraces of Common Bean. Crop Sci. 41:854-862.

- Thomson, MJ. Tai, TH; McClung, AC; Hinga, MH; Lobos, KB; Xu, Y; Martinez, C. and McCouch, SR. 2001. Mapping quantitaive trait loci for yield, yield components, and Morphological traits in an advanced backcross population between Oryza rufipogon and the *Oryza sativa* cultivar Jefferson (To be submitted to Theor. Appl. Genet).

- Chavarriaga-Aguirre P, and Roca WM. (2001) Trangenic plants of cassava obtained through *Agrobacterium*-mediated transformation. *In*: Plant Genetic Engineering Vol. 2. Chapter 9 (pp xxx-xxx) Jaiwal PK and Singh RP Eds. Sci-Tech Publishing Company, Houston, USA (In press)

- Blair MW, Hedetale V, McCouch SR.2001. Fluorescent-labeled microsatellite panels useful for detecting allelic diversity in cultivated rice (*Oryza sativa* L.). Theor Appl Genet (accepted)

- Reilly K, Han Y, Tohme J, Beeching J R.2001. Isolation and characterisation of a cassava catalase expressed during post-harvest physiological deterioration. *Biochimica et Biophysica Acta*. 1518: 317-323.

- Tovar, E. 2001. Caracterización de la diversidad genética de la colección núcleo *de Phaseolus coccineus* L. y *Phaseolus polyanthus* Greenman. Mediante el uso de AFLPs. Dpto. de Biología, Universidad Nacional de Colombia, Bogotá.

- Wenzl, P. Patiño, G.M..;Chaves, A.L..Mayer,J.; Edgard.; Rao, I.; Madhusudana. 2001. High level of aluminum resistance in signalgrass is not associated with known mechanism of external aluminum detoxification in root apices. En: Plant Physiology (USA)Vol. 125, no. 3, p. 1473-1484

- Fregene M., Okogbenin, E.; Mba,C.; Angel,F. ; SuarezMC.; Guitiérrez, J.P.; Chavarriaga, W. Roca, Bonierbale, M and Tohme. J.2001.Genome mapping in cassava improvement: Challenges, achievements and opportunities. Euphytica 120(1): 159-165

- Han Y, Gómez-Vásquez R, Reilly K, Li H, Tohme J, Cooper RM, Beeching JR. 2001 Hydroxyproline-rich glycoproteins expressed during stress responses in cassava. Euphytica. 120: 59-70.

- Hguyen, VT, Bay D. Nguyen, Surapong Sarkarung, Martinez, M. Paterson, AH.; Henry T Nguyen. Maping genes controlling Al tolerance in rice :Comparing different genetic backgrounds.( To be submitted to MGG).

- Islam, F.M.A., K.E. Basford, Jara, C.; Redden,RJ and Beebe,S. 2001. Agronomic and seed compositional differences among gene pools in cultivated common bean. Genetic Resources and Crop Evolution (Accepted).

- Islam, F.M.A., Basford, KE.; Redden , RJ.; Jara, C and Beebe, S. 2001. Patterns of resistance to angular leaf spot, anthracnose and common bacterial blight in common bean germplasm. Australian Journal of Experimental Agriculture. (Accepted)

- Islam, F.M.A., Basford, KE.; Redden, RJ. ; Gonzalez,AV. ; Kroonenberg, PM and Beebe, S. 2001. Genetic variability in cultivated common bean beyond the two major gene pools. Genetic Resources and Crop Evolution (Accepted).

- Chavez, AL.; Bedoya, JM; Sanchez, T. Iglesias, C.; Ceballos, H. and Roca, W. 2000. Iron, carotene, and ascorbic acid in cassava roots and leaves. 2000. Food and nutrition Bulletin, vol. 21 # 4, 410-413.

- Beebe, S., Gonzalez, AV.; and Rengifo, J. 2000. Research on trace minerals in the common bean. Food Nutr. Bull. 21: 387-391.

- Buschmann, H., Reilly, K. Rodriguez, M.X.; Tohme, J. and Beeching, J.R. 2000. Hydrogen Peroxide and Flavan-3-ols in Sotrage Roots of *Cassava (Manihot esculenta* Crantz) during Postharvest Deterioration. J. Agric. Food Chem. 48, 5522-5529

- Buschmann, H.; Rodriguez, M.X., Tohme, J. and Beeching. J.R. 2000. Accumulation of Hydroxicoumarins during Post-Harvest Deterioration of Tuberous Roots of Cassava *(Manihot* esculenta Crantz). Annals of Botany 86: 1153-1160

- Fregene M, Okogbenin, E.; Angel,F. ; Suarez, MC; Guitierrez, J.; Chavarriaga, P. ; Roca,W. Bonierbale, M. and Tohme, J. (2000) Genome mapping in cassava improvement: Challenges, achievements and opportunities. *In*: Cassava Biotechnology, IV International Scientific Meeting. Carvalho, Thro and Vilarinhos Eds. Brasilia. (ISBN 85-87697-05-6; p 192-201).

- Jorge, V.; Fregene, M. A.; Duque, M. C.; Bonierbale, M. W.; Tohme, J.; Verdier, V., 2000. Genetic mapping of resistance to bacterial blight disease in cassava (*Manihot esculenta* Crantz). Theoretical and Applied Genetics. Vol. 101 Oct. p.865-872

- Mahuku, G.M., Jara, C. Cajiao, C and Beebe,S. 2001. Sources of resistance to angular leaf spot (*Phaeoisariopsis griseola*) in common bean core collection, wild *Phaseolus vulgaris* and secondary gene pool. Euphytica. (Accepted)

- Ojeda, S. Verdier, V.2000. Detecting *Xanthomonas axonopodis* pv. *manihotis* in cassava true seeds by nested polymerase chain reaction assay. En: Canadian Journal of Plant Pathology Vol. 22, no. 3, p. 241-247

- Chavarriaga, P.; Mancilla,LI.; Ladino, JJ.; Segovia, V. and Roca,WM. 2000. Developing transgenic strategies against cassava stem borers. *In*: Cassava Biotechnology, IV International Scientific Meeting. Carvalho, Thro and Vilarinhos Eds. Brasilia, (ISBN 85-87697-05-6; p 244-249).

- Chavarriaga-Aguirre,P.; Maya, MM; Duque, MC; Iglesias, C; Fregene, M.;Tohme, J. Bonierbale, M.; Kresovich, S. and Kochert, G. 2000. Microsatellites in cassava: Discovery, inheritance, variability and uses for germplasm characterization. *In*: Cassava Biotechnology, IV International Scientific Meeting. Carvalho, Thro and Vilarinhos Eds. Brasilia, (ISBN 85-87697-05-6; p 147-191).

- Restrepo, S.; Duque, MC; Verdier, V. 2000. Characterization of pathotypes among isolates of *Xanthomonas axonopodis* pv. *manihotis* in Colombia. En: Plant pathology (United Kingdom) Vol. 49, no. 6. p.680-687

- Restrepo, S.Vélez, C.M..Verdier, V. 2000. Measuring the genetic diversity of *Xanthomonas axonopodis* pv. *manihotis* within different fields in Colombia.En: Phytopathology (USA)Vol. 90, no.7, p. 683-690

- Roa, A.C.. Duque, MC.; Maya, MM.; Bonierbale, M.; Iglesias, C.; Tohme, J.; Chavarriaga Aguirre, P. 2000. Cross- species amplification of cassava (*Manihot esculenta*) (*Euphorbiaceae*) microsatellites : allelic polymorphism and degree of relationship. En: American Journal of Botany (USA)Vol. 87, no.11, p. 1647-1655

- Wenzl, P.; Chavez A.L, Mayer J, Rao, I, Nair M.G. 2000. Roots of Nutrient-deprived *Brachiaria* species accumulate 1,3-di-O-trans-feruloylquinic acid. Phytochemistry 55.pp.389-395.

**3.3.3.2 Proceedings, abstracts and others**

- Nguyen, Vinh T, Bay D Nguyen, Surapong S.; Martinez, C. Paterson, AH.; and Nguyen, HT. Mapping genes controlling Al tolerance in rice: Comaparing different genetic backgrounds (submitted for publication)

- Thomson, MJ., Tai, TH; McClung, AM.; Hinga,ME.; Lobos,KB.; Xu, Y.; Martinez, C. and McCouch, SR..Mapping quantitative trait loci for yield, yield components, and morphological traits in an advanced backcross population between *Oryza rufipogon* and the *Oryza sativa* cultivar Jefferson. ( To be submitted to Theor.Appl.Genet).

- Blair M., Hoyos A, Cajiao C (2001) "Desarrollo de frijol Andino voluble para la zona del Caribe" XLVI Annual Meeting PCCMCA. San Jose, Costa Rica. Arpil 2-6, 2001. Proceedings.

- Blair, M.; Hoyos, A.; Cajiao, C. 2001. Desarrollo de fríjol Andino voluble para la zona del Caribe"XLVI Annual Meeting PCCMCA. San José, Costa Rica. April 2-6, 2001.proceedings

- Manual de Inducción "Bienvenidos al Proyecto de Biotecnología. 2001

- Muñoz C., Blair MW, Roca, W and Tohme, J.2001 "Introgresión de genes de fríjol tepari a frijol común por retrocruzas congruentes" XLVI Annual Meeting PCCMCA. San José, Costa Rica. April 2-6, 2001. Proceedings.

- Muñoz, C. Blair, MW.; Roca, W. and Tohme, J.2001. Introgression de genes de fríjol tepari a frijol común por retrocruzas congruentes: XLVI Annual Meeting PCCMCA. San José, Costa Rica, April 2-6, 2001. Proceedings

- Chavarriaga P., Escobar RH., Tohme J, Roca. W. 2000. W.M. Biotechnology State of the Art: Plant Biotechnology in Colombia. *In*: Colombia-Germany a Scientific Alliance Endowed with Tradition and Future. Chapter 1. Working Document, COLCIENCIAS, Bogotá, Colombia, pp:11-34.

- Lentini, Z. 2001. Gene Technology: Expanding Genetic Diversity and Adding Value to Rice. IV International Congress of the Latin American Association on Plant Biotechnology. June 4-8, 2001. Goiania, Brazil.

- Lentini, Z. 2001.Biotechnology for Farmers and Consumers: Role of Biotechnology in Research and Delivering Improved Germplasm for Latin America. IV International Congress of the Latin American Association on Plant Biotechnology. Invited Key Note Speaker. June 4-8, 2001. Goiania, Brazil.

- Tabares E., Fory, L. ; Duque, L. ; Angel, F. ; Delgado, G. and Lentini, Z. 2001. Rice Genetic Transformation efficiency using particle bombardment or mediated by *Agrobacterium tumesfaciens*. IV International Congress of the Latin American Association on Plant Biotechnology. Poster. June 4-8, 2001. Goiania, Brazil.

- Florez C., Escobar, R. ; Duque, MC and Lentini, Z. 2001. Optimization of RITA system for un automated mass production of embryogeni callus of *Brachiaria* species. IV International Congress of the Latin American Association on Plant Biotechnology. Poster. June 4-8, 2001. Goiania, Brazil.

- Mora A., Fory, L. Lozano, I.; Tabares, E. Calvert, L. and Lentini, Z. 2001. Trangenic Rice with Hypersensitive Resistance to Rice (*O. sativa*) Hoja Blanca Virus (RHBV) in the Field. IV International Congress of the Latin American Association on Plant Biotechnology. Poster. June 4-8, 2001. Goiania, Brazil.

- Lentini, Z. 2001. Biosafety in Field Trials with Plants Modified with Gene Technology. VII Congress of the Colombian Society of Plant Breeding and Crop Production. May 23-25, 2001. Ibagué, Colombia. Plenary Invited Speaker. May 24, 2001.

## Activity 3.4 Donors contributing to Project SB-2 in the period Oct, 2000 Sept. 2001

- NEW ZELAND-Sustainable Integrated Management of Whiteflies through Host Plant Resistance

- GTZ-Project title: An Integrated Approach for Genetic Improvement of Aluminium Resistance of Crops on Low-Fertility Acid Soils

- MADR - Capacidad de regeneración y potencial de transformación genética de variedades de yuca de importancia comercia en Colombia-

- RF - Legume Genomics Meeting between US and CGIAR research
- RF - Development of a molecular map of cassava (Manihot Esculenta)
- BIOTEC-Caracterización molecular y agromorfológica de la variabilidad genética nativa de guanábana y especies anonáceas relacionadas
- WGF (Wallace Genetic Foundation) - The Development of Molecular Markets for the Breeding of Sustainable Pest Resistance in Common Beans: a Novel Strategy
- AGROPOLIS-Developing and exploiting expressed sequence tags for cassava starch and bacterial blight resistance - D203
- RF-Delivery of Transgenic Rice Cultivars to Seed Producers and Farmers in Tropical America, following a Multi-Step Approach Involving Biosafety Assessment, Nutritional Testing and Negotiations on Intellectual Property Rights.
- WARDA-Interspecific Hybridization Project
- COLCIENCIAS-Caracterización de la resistencia de la yuca a la bacteriosis vascular y su uso en el mejoramiento.
- Belgica-AGCD/BADC - Genetic Improvement of Common Beans Using Exotic Germplasm and Biotechnology
- The Netherlands - Cassava Biotechnology Network III – CBN
- RF-Molecular marker - aided analysis of traits of Agronomic importance in Cassava.
- RF - Rice biotechnology research
- CEGA-Control del Barrenador del Tallo de la Yuca en la Costa Norte de Colombia
- CENTRO TECNOLOGICO POLAR- Ensuring Stable and Durable Resistance of Rice to Pathogens and Pests: Rice Hoja Blanca Virus, Rhizoctonia solani and Sogota.
- CEGA-Producción y manejo de semillas de alta calidad para el desarrollo socioeconómico de pequeños y medianos productores de yuca de la Costa Atlántica
- Alexander von Humboldt-Use of morphological and molecular tecniques to study the diversity and conservation of endangered Colombian palm trees
- NRI - Identifying target points for the control of post-harvest physicological deterioration in cassava
- MADR-Propagación y Certificación de Yuca Libre de la Enfermedad de Cuero de Sapo-CONVENIO 128
- DfID - R7028(C)-Reviving the agricultural base of a region: use of genetic transformation and interactive testing to restore predominant locally - Adapte Cassava Varietes

- GTZ - Gene Flow Analysis for Assessing the Safety of Bio-Engineered Crops in the Tropics - No. 81038492

- IDRC-Strategies for Integrating Small-Scale End-Users in Cassava Biotechnology Research (Latin America)-File: 100386-001

## Activity 3.5 Project SB-02 Staff (2001)

### 3.5.1 SB2 Investigators: Discipline, position and time fraction

| Name | Discipline | Position | Time dedication% |
|---|---|---|---|
| Beebe Steve | Bean Breeding | Senior Staff | 30 |
| Beeching John | Plant Molecular Biology | Bath University. Visiting Scientist CIAT from Oct. 17/2000 - Oct 16/2001 | 100 |
| Bellotti Anthony | Cassava Entomology | Senior Staff | 20 |
| Blair Mathew | Bean Genetics and breeding | Senior Staff | 70 |
| Ceballos Hernan | Cassava Breeding | Senior Staff | 40 |
| Debouck Daniel | Botany | Senior Staff | 20 |
| Fregene Martin | Cassava Genetics and breeding | Senior Staff | 60 |
| Lentini Zaida | Biology/Genetics | Senior Staff | 80 |
| Martínez César | Breeding | Senior Staff | 49 |
| Mba Chikelu | Cassava genomics | Visiting Research Fellow | 100 |
| Mejía Alvaro | Cell Biology | Consultant | 20 |
| Restrepo Silvia | Molecular Plant Pathology | Research fellow from July 27/2001 | 100 |
| Roca William | Cellualr Physiology | CIP. Part time CIAT - Dec, 22/2001 | 10 |
| Tohme Joe | Genomics | Project Manager | 100 |
| Verdier Valerie | Molecular plant Pathology | IRD. CIAT Staff till - July 5/2001 | 100 |

**Tissue Culture/Cryopreservation/Transformation**

| | |
|---|---|
| Chavarriaga, P. | Research Associate |
| Escobar, R. | Research Assistant |
| Galindo, L.F. | Research Assistant |
| González,E. | Research Assistant |
| Ladino. J.J. | Research Assistant |
| Mancilla, L.I. | Research Assistant |
| Manrique, N.C. | Research Assistant |
| Muñoz, L. | Research Assistant |
| Segovia, V | Research Assistant |
| Tabares, E | Research Assistant |
| López,D. | Research Assistant |
| Fory, L. | Research Coordinator |
| Hernández,C.M. | Visiting Research |
| Bolaños,E | Technician |
| Dorado,C | Technician |
| Herrera,P. | Technician |
| Ríos, A. | Technician |
| Tigreros, H. | Technician |

**Genome Diversity**

| | |
|---|---|
| Gallego,G | Research Coordinator |
| Almeida,A. | Research Assistant |
| Gaitan, E. | Research Assistant |

| | |
|---|---|
| Barrera, E. | Research Assistant |
| Gutiérrez, J.P. | Research Assistant |
| Bohorquez, A. | Research Assistant |
| Vargas, J. | Research Assistant |
| Quintero | Research Assistant |
| Giraldo,M | Research Assistant |
| Pedraza,F. | Research Assistant |
| Cortés,DF. | Research Assistant |
| Giraldo,OX. | Research Assistant |
| Acosta, I. | Research Assistant |
| Galindo,LM. | Research Assistant |
| Oogbening, E. | Visiting Researcher |
| Reyes, N. | Technician |

**Plant-Stress interactions**

| | |
|---|---|
| Chaves,A.L. | Associated Research |
| Santaella,M. | Research Assistant |
| Mosquera,G. | Research Assitant |
| Rodriguez, M.X. | Visiting Research |

**Administrative**

| | |
|---|---|
| Cruz,O.L. | Bilingual Secretary |
| Zuñiga,C.S. | Bilingual Secretary |
| M.C. Duque | Statistical Consultant |

**A.V. Humboldt**

| | |
|---|---|
| Palacio, J.D | Visiting Researcher |
| Torres, J. | Visiting Researcher |
| García, V.H. | Visiting Researcher |

**Corporación BIOTEC**

| | |
|---|---|
| Royero,N. | Visiting Researcher |
| Alzate,A. | Visiting Researcher |
| Nuñez, A. | Technician |
| Ruíz,J.J. | Visiting Researcher |
| Cadena,S. | Visiting Researcher |

**CORPOICA**

| | |
|---|---|
| Sanchez,I. | Visiting Researcher |

**CENICAÑA**

| | |
|---|---|
| Rangel, P. | Visiting Researcher |