# PROJECT SB-2

# Enhancing the Understanding and Use of Agrobiodiversity Through Biotechnological Methods

# ANNUAL REPORT 1996

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# PROJECT SB-2: Enhancing the Understanding and Use of Agrobiodiversity Through Biotechnological Methods

## EXECUTIVE SUMMARY

Project SB-2 was organized in the second part of 1996, and resulted from the merging of former Projects #14 (Understanding Species Genetic Diversity) and #15 (Broadening the Genetic Base of Gene Pools). In retrospective, the phylosophy and strategy on agrobiodiversity research at CIAT in general, and about project 14 resulted from earlier activities within the Scientific Resources Group (SRG) on Genetic Diversity, between 1994-95. The major highlights of project SB-2 are summarized by outputs.

**Output 1.1: Knowledge on gene pool structure at intra and inter specific level enhanced**

(i) In 1996, the AFLP technology has been used for assessing changes in genetic variability of common bean landraces resulting from social-economic forces in Rwanda on the one hand; and provided more information on the taxonomic relationships of *P. lunatus* and three related *Phaseolus* spp. On the other hand, unique identification of 105 *Manihot* accessions suggests that AFLP can help to develop species-specific markers for studies of introgression.

(ii) Assessment of genetic relationships of Cuban rice varieties, and *Passiflora* genetic resources have been conducted using RAPDs, AFLPs and chloroplast DNA markers. The latter is a collaborative effort with CORPOICA and IPGRI with Andean countries for the conservation and use of passifloras.

(iii) Molecular markers have also been used for studying the population structure of cassava bacterial blight pathogen *(Xanthomonas axonopodis, pv. manihotis)*. A PCR-based assay, using a dot-blot technique has been developed for diagnostics of this pathogen within the ORSTOM-CIAT project.

**Output 1.2: Agroecological, agronomic and genomic information assembled and integrated**

(i) Generation of trait-specific genetic stocks is key to the use of molecular marker technology for gene tagging and marker-assisted selection in germplasm development. RILs of common bean for tagging important traits, e.g. beanfly, root length, low P efficiency have been generated. Similarly, cassava stocks are being constructed, under common genetic background, for key traits such as cyanogenic potential, photosynthetic rate, resistance to bacteriosis, post harvest deterioration, dry matter content and morphological traits. Genetic stocks for resistance to specific rice blast lineages and for

apomixis in *Brachiaria* have also been generated at CIAT.

(ii) In 1996, molecular markers were used for gene tagging of following traits:

- One SCAR was designed for a gene tag for resistance to common bacterial blight (CBB) of beans, explaining up to 23% of variability in CBB reaction. SCARs are useful for more reliable gene identification using PCR technology.

- An AFLP cloned fragment was linked to a resistance gene for rice blast pathogen lineage SRL-1. And a RFLP marker linked to the cv Fanny resistance gene was mapped to rice chromosome 11. The approach known as Genomic RFLP Substraction is being implemented to fine map rice blast resistance genes of the durable resistant cv. O. Llanos 5.

- A SCAR was designed from a RAPD marker linked to the apomictic phenotype in *Brachiaria ruzienzis* x *B. brizantha* population. Mapping distance was estimated at 4 cM in the *B. ruzienzis* x *B. decumbens* population and at 13 cM in the *B. ruzienzis* x *B. brizantha* population. A RFLP probe from the Cornell rice map was also linked to the SCAR and with apomixis.

(iii) In the last few years, GIS have been applied to the exploration of biodiversity in the wild relatives of *Phaseolus vulgaris*, *Stylosanthes* spp and *Manihot* spp. Bean "favoring" climates were identified where wild beans have not previously been collected; similarly, new collecting areas were suggested for *Stylosanthes* sp. off. *scabra* and information to clarify *S. guianensis* taxonomy was also provided by GIS analysis of genetic resources. Further to the generation of probability maps for five *Manihot* spp, GIS was recently also applied to the mapping the potential presence of pests.

**Outputs 1.3: Techniques for assessing genetic diversity developed**

(i) Construction of the cassava molecular genetic map continued in the BRU in 1996. Linkage maps for the female and male parents have been constructed involving 168 and 159 markers (RFLP, RAPD, microsatellite and isozyme loci), with 20 and 24 linkage groups, respectively. Further work will seek to merge both maps and increase the mapping density. A project has been initiated to map ACMV resistance genes in collaboration with IITA.

(ii) Refinement of AFLP and microsatellite marker technologies for characterization and analysis of bean and cassava diversity has been carried out in 1996. It was determined that the number of primers for sound germplasm classification using AFLPs can be one; on the other hand, a procedure was carried out to speed up the isolation of genomic fragments containing GA sequence repeats for use as microsatellite markers in *P. vulgaris*. In collaboration with the USDA Plant Conservation Unit, Georgia and the

University of Georgia, 8 cassava microsatellites have been generated and three tested at CIAT, with large genetic diversity detected in 7 *Manihot* Taxa.

(iii) Using rubber plant DNA probes from CIRAD, 60% homology was detected by hybridization with DNA of the cassava mapping parents, opening the way for comparative genomic studies. Through collaboration with the Univ. of California, Davis, cassava sequences were identified homologous to a rice resistant gene against *Xanthomonas* bacterial blight. Work is underway to test if the cassava clone, homologous to the rice gene, has a role in the reaction of cassava to CBB.

**Output 2.1: Exotic or novel genes and gene combinations made accessible for broadening the genetic base of cultivated gene pools.**

(i) Identification, transfer and utilization of useful genes and gene combinations from wild germplasm and related species in rice and common bean continued in 1996. Exploitation of transgressive segregation in rice through crosses with *Oryza rufipogon*, *O. barthii* and *O. glaberrima* has moved forward with generation of second backcross F2 families, and agronomic and molecular characterization of these is underway in order to identify QTLs on yield and other important traits.

(ii) Earlier successful transfer of useful traits from *Phaseolus acutifolius* to *P. vulgaris* through interespecific hybridization aided by embryo rescue continued in 1996 with congruity backcrossing for transferring genes(s) for leafhopper and bruchid resistance. From the initial recurrent backcrosses, two lines with comparatively high levels of resistance to bacterial blight were developed after 6 years of intensive field evaluation and selection.

(iii) Research on rice genetic transformation as a means of efficiently transferring otherwise non-accessible genes, has continued with RHBV. This year, transgenic rice plants generated at CIAT have been challenged with the virus, and plants selected with very low or no disease symptoms vis-a-vis the non-transformed control plants.

**Output 2.2:Knowledge generated on mechanisms of genetic variability of plant response to biotic/abiotic stress.**

(i) Identification of points for genetic intervention is a pre-requisite to the implementation of efficient germplasm screening and the control/management of constraints. One biotic and one abiotic constraint have been pursued in 1996. The strategy for searching genes involved in resistance to the bean weevil included the isolation of specific cDNA clones, from resistant genotypes using a differential display approach. On the other hand, using *Brachiaria* as a model insights into mechanisms of acid soil tolerance were obtained as

they relate to the activities of key enzymes involved in nutrient uptake, the mapping of nutrients in root tips, and the characterization of plant organic acids.

(ii) Work on the potential of cassava as a carotene source continued with the evaluation of high carotene genotypes in different environments. A yellow root gene pool is being formed using selected parental genotypes to develop a selection program for increased carotene content.

(iii) Fifteen cassava clones with low cyanide levels were selected as promising resistance to the burrowing bug, *C. bergi*. Previously resistance to *C. bergi* had only been detected in high cyanide (above 200 ppm) clones.

**Output 2.3: Improved gene transfer methodologies developed for broadening the genetic base of cultivated gene pools.**

(i) Two critical developments occurred this year at CIAT in genetic transformation. On the one hand, the generation of transgenic cassava through an *Agrobacterium* mediated methodology was confirmed by molecular and gene expression assays; on the other hand, generation of common bean transgenic plants was achieved using particle bombardment and a regeneration technique with three well known varieties. Transformation cassettes have been constructed harboring transgenes for transformation resistance against the cassava stem borer.

(ii) Transformation efficiency of indica rice vars. has been improved, and development of a methodology for *Brachiaria* transgenesis has further advanced using the particle bombardment in both cases. Study and management of apomixis will require an efficient transformation system in *Brachiaria*.

**Output 3.1: Availability of genes, genetic stocks, genomic maps, probes, cell cultures/lines.**

Development of a cassava genome data base is underway with CBN cooperation. Other material such as plant genetic stocks for genome mapping, maps and probes, vectors and tissue cultures for transformation are being assembled and documented.

**Output 3.2: Collaboration with CIAT partners on methods and techniques for understanding genetic diversity and broadening the genetic base of gene pools.**

In 1996, two Workshops and one Training Course were organized at CIAT on topics dealing with agrobiodiversity evaluation/assessment, conservation, and use of modern molecular and cellular technologies. In addition, in 1996, 32 scientists from NARS and AROS attended specialized training on selected advanced biotechnologies. Participants from : Colombia,

Argentina, Cuba, Peru, Costa Rica, Ecuador, Belgium, USA. CIAT Regional Cooperation contributed with partial support for LDCs participants.

## 1996 PUBLICATIONS

In 1996, staff contributing to Project SB-2 published: 16 papers in Refereed Journals, 15 as Conference Proceedings and Posters, and 5 as research Theses.

### CIAT AND PARTNERS' PRINCIPAL STAFF CONTRIBUTING TO PROJECT SB-2

In 1996, 28 principal staff from CIAT and 26 from partner organizations in developing and developed countries contributed to Project SB-2

### CONTRIBUTING DONORS TO PROJECT SB-2

In 1996, eleven donor agencies from developed (8) and developing (3) countries contributed to Project SB-2.

CONTENTS PAGE


| | | |
|---|---|---|
| **PROJECT OBJECTIVE 1.** | | 1 |
| OUTPUT 1.1 | Knowledge on gene pool structure at intra and interespecific level enhanced | 1 |
| 1.1.1 | Genetic structure of *P. vulgaris* | 1 |
| 1.1.2 | Studies of *P. lunatus* and related spp of South America using AFLPs | 2 |
| 1.1.3 | Assessment of *P. vulgaris* genetic diversity in Rwanda | 4 |
| 1.1.4 | Evaluation of *Manihot* genetic diversity with molecular markers: AFLP analysis of inter-and intraspecific diversity | 5 |
| 1.1.5 | Molecular characterization of cassava bacterial blight | 8 |
| 1.1.6 | Analysis of genetic relationships among Cuban rice vars using RAPD and AFLP markers | 12 |
| 1.1.7 | Characterization and genetic variability of *Passiflora* spp from the Andean region | 13 |
| 1.1.8 | A PCR method to identify *Fusarium*, *Thielaviopsis* and *Pythium* species: Characterization of strain | 15 |
| OUTPUT 1.2 | Agroecological, agronomic and genomic information assembled and integrated | 16 |
| 1.2.1 | Integration of agroecological, agronomic and molecular data | 16 |
| 1.2.2 | A new variant of Arcelin from Southern Mexico | 16 |
| 1.2.3 | Gene tagging of agronomic traits in bean | 18 |
| 1.2.4 | Tagging of rice blast resistance genes | 19 |
| 1.2.5 | Identifying markers linked to the apomixis gene in *Brachiaria* | 20 |
| 1.2.6 | Evaluation of useful variability in cassava using traits-specific genetic stocks | 21 |
| 1.2.7 | GIS mapping for germplasms distribution | 22 |
| OUTPUT 1.3 | Techniques for assessing genetic diversity developed | 29 |
| 1.3.1 | Construction of a molecular genetic map of cassava | 29 |
| 1..3.2 | Determination of adequate number of AFLP primers for germplasm classification in beans | 31 |
| 1.3.3 | Enrichment of a *P. vulgaris* genomic library for GA sequence repeats | 33 |
| 1.3.4 | Microsatellite analysis in cassava | 34 |
| 1.3.5 | Evaluation of cassava diversity with heterologous probes (comparative genome mapping) | 35 |
| **PROJECT OBJECTIVE 2** | | 37 |
| OUTPUT 2.1 | Exotic or novel genes and gene combinations made accessible for broadening the genetic base of cultivated gene pools | 37 |
| 2.1.1 | A novel approach for the identification and utilization of genes from wild *Oryza* germplasm | 37 |

CONTENTS PAGE


| | | |
|---|---|---|
| 2.1.2 | Making useful traits available to *P. vulgaris* through interespecific hybridization with *P. acutifolius* | 39 |
| 2.1.3 | Control of RHBV through coat protein mediated cross protection and anti-sense RNA strategies | 42 |
| OUTPUT 2.2 | Knowledge generated on mechanism of genetic variability of plant response to biotic/abiotic stress. | 46 |
| 2.2.1 | Molecular basis of resistance to the bean weevil *Acanthoscelides obtectus* | 46 |
| 2.2.2 | Mecanisms of acid soil tolerance in *Brachiaria* | 47 |
| 2.2.3 | Resistance of cassava to *Cyrtomenus bergi* | 49 |
| 2.24 | Exploring the genetic potential and stability of carotene content in cassava roots | 54 |
| 2.2.5 | The effects of pre-harvest pruning of cassava upon post-harvest physiological deterioration (PPD) potential, scopoletin and dry matter contents | 56 |
| 2.2.6 | Molecular mechanisms of cassava physiology under stress: The role of glycine decarboxylase | 57 |
| OUTPUT 2.3 | Improved gene transfer methodologies developed for broadening the genetic base of cultivated gene pools | 58 |
| 2.3.1 | Comparative study of two methodologies for improving genetic transformation of rice | 58 |
| 2.3.2 | Genetic transformation of cassava | 61 |
| 2.3.3 | Development of an efficient genetic transformation protocol for common bean | 65 |
| 2.3.4 | Development of *Brachiaria* genetic transformation | 67 |
| 2.3.5 | Cryopreservation of cassava shoot tips in liquid nitrogen | 69 |
| **PROJECT OBJECTIVE 3** | | |
| OUTPUT 3.1 | Availability of genes, genetic stocks, genomic maps, probes, cell cultures/lines. | 74 |
| 3.1.1 | Development of the cassava genome database | 74 |
| 3.1.2 | Development of collections of genetic-molecular-cell material at CIAT | 74 |
| OUTPUT 3.2 | Cooperation with CIAT partners on methods and techniques for understanding genetic diversity and broadening the genetic base of gene pools | 75 |
| 3.2.1 | Workshops and Training Courses | 75 |
| 3.2.2 | Individual Training | 76 |
| 1996 PUBLICATIONS | | 77 |
| CIAT AND PARTNER'S PRINCIPAL STAFF CONTRIBUTION TO PROJECT SB-2 IN 1996 | | 81 |
| CONTRIBUTING DONORS TO PROJECT SB-2 IN 1996 | | 82 |

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## REPORT PROJECT OBJECTIVE 1.

To improve the understanding of the genetic diversity of wild and cultivated species for improved conservation and broadening the genetic base of gene pools.

### OUTPUT 1.1: KNOWLEDGE ON GENE POOL STRUCTURE AT INTRA AND INTER SPECIFIC LEVEL ENHANCED

#### *1.1.1 Genetic structure of P. vulgaris*

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Progress was registered in the goal of elucidating the genetic structure of cultivated landraces of *P. vulgaris*. The statistical analysis of 269 Mesoamerican landraces which was reported on a preliminary basis in 1995 was completed and a manuscript prepared for submission. This has now passed the CIAT internal review process and will be submitted as a companion paper to one on a comparison of the core and reserve collections based on RAPD band frequencies. Both studies utilized the same data on Mexican accessions, with the study of genetic structure including an additional 89 accessions from Central America and other regions. Following the analysis of the 269 accessions, a subset of 8 primers was identified among the 39 used. These 8 primers reproduced the groups formed by the 39 with about 88% accuracy (ie, with 12% errors) at the level of races and groups within races. Plans were formulated to screen the remainder of the Mesoamerican accessions in the core to complete a molecular classification of these. DNA has been extracted and at time of this writing, RAPD analysis is ready to begin.

A subset of the Andean accessions in the core was identified to represent in a total of 200 landraces the range of growth habits, agroecological environments and geographical distribution. A large sample of nuna types was included. These 200 accessions have been analyzed at the University of Wisconsin using RAPD and an analysis by AFLP in CIAT is nearly complete. These analyses will permit a comparison of RAPD and AFLP as tools for study of genetic diversity of bean, and will provide a broad database of molecular markers with which to visualize the structure of cultivated Andean germplasm.

### 1.1.2 Studies of *Phaseolus lunatus* L. and related species of South America using Amplified Fragment Length Polymorphism (AFLPs)

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The genus *Phaseolus* may have about fifty species all of neotropical origin. While the Mesoamerica region, may harbor at least some forty species (Debouck, 1991; Delgado Salinas, 1985), South America may only harbor six to eight species as wild plants. Most South American *Phaseolus* sensu stricto species have their range of distribution apparently limited to the Andean region.

Harms (Harms, 1921) has described two new species *P. augusti* Harms *and P. pachyrrhizoides* Harms, from Huancavelica and Junín, Peru, respectively, the latter having larger peduncles and bracts. Macbride, 1943 noted the little differences between these two taxa, observing that *P. pachyrrhizoides* sometimes displays unsymmetrical, lobed lateral leaflets. On the other hand, Piper, 1926 named *P. bolivianus* Piper, a new species from Cochabamba, Bolivia. He mentioned *P. pachyrrhizoides*, but curiously not *P. augusti*, and did not cross-refer to any of them. Macbride, 1943 considered *P. bolivianus* as not distinct enough from *P. augusti* and put it in synonymy.

The questions we are trying to address are: i) are *P. augusti* and *P. pachyrrhizoides* distinct species?, ii) can *P. bolivianus* be merged with P. augusti?, iii) is *P. rosei* different from the Andean wild Lima bean. Some new insights on these questions seem possible thanks to new germplasm made available by explorations carried out in original Andean habitats in 1985-1996, and to the use of AFLP technique to measure and understand genetic diversity (Vos *et al.*, 1995; Tohme et al. 1996) that has been successfully applied to *Phaseolus* beans.

Progress Report. One-hundred-and-twenty accessions, belonging to a total of 71 populations of wild South American *Phaseolus* species, were included in this study. *P. lunatus*: Eighteen accessions from Colombia (Magdalena, Cundinamarca, and Boyaca), 14 from Ecuador (Chimborazo, El Oro, and Loja), 15 from Peru (Cajamarca and Junin), and one accession from Argentina was used. *P. augusti*: Four accessions from Ecuador (Azuay and Loja), 18 from Peru (Cuzco and Piura), ten from Bolivia (Cochabamba and Chuquisaca), and seven accessions from Argentina (Salta, Tucuman, and Jujuy) were included. *P. pachyrrhizoides*: Thirty accessions, exclusively from Peru (Amazonas, Cajamarca, Junin, and Apurimac), were studied. *P. bolivianus*: Only three accessions were obtained, one from Peru (Cuzco) and two from Bolivia (Cochabamba). In addition, two Central American *P. lunatus* accessions, from Guatemala and Costa Rica, were analyzed to determine their relationship with the South American material. Five accessions of South American *P. vulgaris* and one *P. costaricensis* were also included as outgroups.

Amplified fragment length polymorphisms were generated using the technique described by Vos et al. (1995) and modified by the Biotechnology Research Unit at CIAT (Gonzalez et al., 1995). Primers complementary to adapter sequences, having one additional nucleotide on their 3' end, were used to carry out a selective pre-amplification of the DNA template. The primer sequence complementary to the EcoRI end was: 5'-GACTGCGTACCAATTCA (E + A). The MseI primer

sequence was: GATGAGTCCTGAGTAA**G** (M + G). Letters in bold correspond to the first selective nucleotide. The primer complementary to the EcoRI end of the template DNA, E + AAC (5'-GACTGCGTACCA ATTC**AAC**), was used in combination with the MseI primer M + GTA (5'-GATGAGTCCTGAGTAA**GTA**). Another EcoRI primer, E + AGT (5'-GACTGCGTACCAATTC**AGT**) was used in combination with the MseI primer, M + GAC (5'-GATGAGTCCTGAGTAA**GAC**).

Data was scored as presence/absence of band, where 1 corresponded to presence of band and 0 to its absence. Only intense bands were scored; faint and ladderlike sequences of bands were ignored. The Nei-Li coefficient of similarity (1979) was calculated for each pair of accessions with each primer combination. Dendogram results were supported by multiple correspondence analyses were carried out with "Corresp" procedure of the SAS statistics package (SAS Institute, Inc., 1989).

Heterogeneity or gene diversity for each observed gene pool was calculated according to formulas presented by Nei (1987). Heterogeneity was calculated for each band level and then averaged out for the total measure.

The results elicit the following points for discussion. First, in comparison to species such as *P. vulgaris* and *P. costaricensis*, *P. lunatus* and related taxa are relatively distant (Table 2). Although our analysis includes only a few *Phaseolus* species, these results are consistent with those obtained by (Maréchal *et al.*, 1978), showing an organization of this genus into gene pools with the common bean and the Lima bean being at the extremes. They are also consistent with cpDNA analysis data (Llaca *et al.*, 1994; Schmit *et al.*, 1993) showing noticeable distance between *P. lunatus* and the group of species related to *P. vulgaris.* Again in spite of too few species being analyzed, *P. augusti* and *P. pachyrrizoides* form a continuum linked to *P. lunatus*, confirming early observations (Debouck, 1991).

Second, the existence of two major gene pools in wild Lima bean, as evidenced on polymorphisms in seed storage proteins (Gutiérrez Salgado *et al.*, 1995) and allozymes (Maquet *et al.*, 1994), seems to be confirmed with 3 groups. The wild form with slightly larger seeds in the western Andean range of Ecuador and northern Peru clearly separates at 0.58-0.71 with 95% confidence from the form distributed in the eastern lowland South American tropics. Interestingly, the small-seeded wild Lima beans from Central America separate from the ones of South America, but the sample is too small to raise a definitive conclusion. However, some Colombian materials, namely from the Andean departments of Boyacá and Cundinamarca, although close to the lowland neotropical wild Lima bean, separate to form another group.

Third, *P. augusti*, *P. bolivianus* and *P. pachyrrizoides* seem to form a continuum rather than three different clear-cut entities. Their low level of separation would question maintaining these three taxa as separate species; there would be instead considered as one but polymorphic species.

### 1.1.3 Assessment of *P. vulgaris* genetic diversity in Rwanda

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A project was initiated as part of the Seed of Hope initiative to assess the degree of genetic diversity change and/or loss as a result of the civil war in Rwanda. Initially, collections form 1984 and 1995 were compared by using seeds shape, color pattern, seeds coat brilliance, and seed size. These comparisons suggested that there has been a loss of variability in 1995 compared to what existed in 1984. We extended the morphological analysis to include AFLP fingerprinting data.

Progress Report. AFLP fingerprinting was performed as described by Vos et. al., 1995 with some modifications (Tohme et al. 1996). Five hundred ng of DNA form each sample were double digested with EcoRI and MseI enzymes and incubated at 37°C 1 h. and then adapters were added and this mixture.

Eighty one combinations for EcoRI and MseI adapters were designed by adding 1 and 3 randomly selected nucleotides to the 3' end. Based on the result of the survey two combinations of primers were selected to be used in this study. Two consecutive amplifications with one and three extra nucleotides per primer were carried out in a PTC-100 programmable thermal controller (MJ Research). 5µl of double digested and adapter ligated DNA were amplified using each primer with an extra nucleotide. A slight smear was observed when 20µl of the PCR product were loaded on a 1.5% agarose gel. For the second amplification 5 ng of primers for EcoRI with three extra nucleotides were end labeled by using gamma 32P-ATP, thirty ng of the non labeled for MseI were added and 10µl of a 1:20 dilution of the initial PCR product. This second amplification was carried out by programming a touchdown cycle profile (Don, et. Al., 1991). Selective amplified fragments were mixed with an equal volume of loading buffer, denatured at 95°C for 3 min and incubated in an ice bath for 5 min. Four µl of each sample were loaded into 6% polyacrylamide denaturing gel and running at 40 V/cm for 2 h at 50°C

Part of these two collections had been fingerprinted using AFLP technique. DNA was extracted from 593 accessions from the collection held at CIAT and 324 form 1995 collection. 462 accessions have been screened with two pair of AFLP primers combinations. PE1A/ PM1A combination gave a average of 92 well scorable bands and PE1C/PM1C combination gave 84 bands.

The data matrix obtained for presence or absence of bands was Nei-Li (1979) definition. The matrix of distances was analyzed using the UPMGA method of the software NTSYS. Preliminary dendrograms were generated with the TREE program of NTSYS. Maximum diversity among groups formed by NTSYS analysis was 0.48, as measured by Nei's distance. We observed greater heterogeneity in 1984 collection that 1995 corresponding 0.27 to 1985 collection and 0.23 to 1995 collection.

Classification of beans: Major groups or gene pools were recognized in Mesoamerica and Andes *P. vulgaris* germplasm, although the separation among communities was not clearly defined. In this

analysis we observed a mixture of genotypes between close and distant communities. We plan to continue the AFLP analysis using two primers combinations for both 1984 and 1995 collections and comparing these collections with a selected sub-set of Latin America germplams from the core collection.



### *1.1.4 Evaluation of Manihot Genetic Diversity with Molecular Markers:*

**AFLP analysis of inter- and intraspecific diversity**

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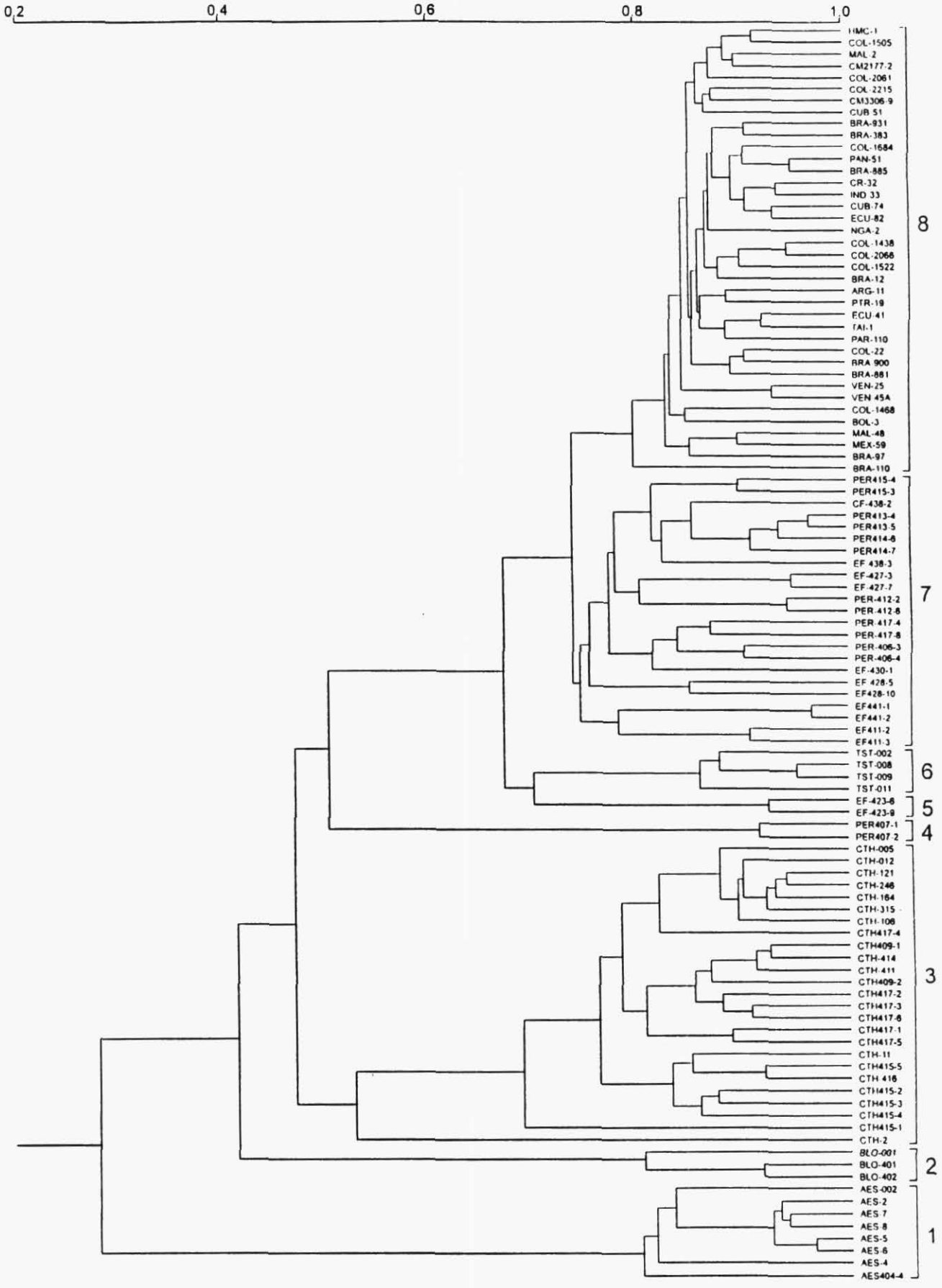
Advantages of AFLP as genetic markers for cassava include the large amount of data points generated per experiment and the lack of requirement for much previous information about the species under study. A set of 105 *Manihot* accessions has been evaluated with AFLPs at CIAT to estimate genetic relationships within the genus. The set included 35 cassava landraces, 3 improved hybrids, and 67 individuals from the following taxa: *M. aesculifolia*, *M. carthaginensis*, *M. brachyloba*, *M. tristis*, *M. esculenta* subsp. *flabellifolia* and *M. esculenta* subsp. *peruviana*. Selective amplification of *Eco*RI - *Mse*I restriction fragments was realized with two different +1/+3 AFLP primer combinations.

Progress Report. Fingerprints of each genotype consisted of from 17 to 51 bands, confirming the high information content obtainable with this type of marker. Data were registered as presence or absence of bands in each accession, and the results converted to a similarity matrix, based on AFLP products held in common between pairs of individuals. UPGMA analysis clustered the accessions into eight groups that correspond largely with prior taxonomic classification (fig. 1). Discrete groups were formed for the species *M. aesculifolia*, *M. brachyloba*, and *M. carthaginensis* (clusters 1, 2, and 3, respectively), and cassava (*M. esculenta*, cluster 8). Most accessions pertaining to *M. esculenta* subspp. *flabellifolia* and *peruviana* fell into a mixed cluster (7). Three smaller groups were formed by two pairs of individuals collected at higher altitudes, of the *M. esculenta* subspecies (4 and 5), and *M. tristis* (6). The accessions representing cassava appear as a compact group (8), with greater similarity among its members than in the next cluster (7). Because cluster 7 comprised a mixture of *M. esculenta* subspp. *flabellifolia* and *peruviana* accessions, these two subspecies were pooled into a single group for subsequent analyses. This group of accessions comprised of the wild subspecies of *M. esculenta* was the most closely related to cassava among the taxa analyzed. The analysis permitted the unique identification of each individual, indicating that the collection does not contain genetic duplicates, and facilitated analysis of the distribution of genetic diversity among accessions and species. Species-specific markers which may be useful in germplasm classification or introgression studies, were suggested by the unique presence and absence of AFLP products in samples of each of the three wild species.

Mean similarities between pairs of individuals within the six resulting groups were significantly different ($P<0.05$), according to Duncan's multiple range test (Table 1). As expected, intraspecific similarity (Table, 1 diagonal) was greater in all cases than interspecific (Table 1, upper triangle).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IND 33
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ECU-82
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COL-1438
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PTR-19
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PER-412-2
PER-412-6
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PER-417-8
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EF411-3
7
TST-002
TST-008
TST-009
TST-011
6
EF-423-6
EF-423-9
5
PER407-1
PER407-2
4
CTH-005
CTH-012
CTH-121
CTH-246
CTH-164
CTH-315
CTH-106
CTH417-4
CTH409-1
CTH-414
CTH-411
CTH409-2
CTH417-2
CTH417-3
CTH417-6
CTH417-1
CTH417-5
CTH-11
CTH415-5
CTH-416
CTH415-2
CTH415-3
CTH415-4
CTH415-1
CTH-2
3
BLO-001
BLO-401
BLO-402
2
AES-002
AES-2
AES-7
AES-8
AES-5
AES-6
AES-4
AES404-4
1

**Table 1.** Genetic similarity values (mean±SD) between (upper triangle) and within (diagonal) groups of*Manihot* germplasm, based on all pairwise similarities between individuals. Different letters on the diagonal indicate significant differences (P<0.05) by Duncan's multiple range test

| Group[a] | ESC (n=38) | AES (n=8) | BLO (n=3) | CTH (n=25) | TST (n=4) | ESC-FLA-PER (n=14) |
|---|---|---|---|---|---|---|
| ESC | 0.85±0.03ab | 0.31±0.03 | 0.41±0.02 | 0.47±0.03 | 0.65±0.03 | 0.70±0.11 |
| AES | | 0.86±0.06a | 0.26±0.02 | 0.26±0.02 | 0.27±0.02 | 0.27±0.05 |
| BLO | | | 0.84±0.07ab | 0.43±0.04 | 0.41±0.03 | 0.40±0.05 |
| CTH | | | | 0.77±0.09cb | 0.45±0.04 | 0.46±0.06 |
| TST | | | | | 0.88±0.04a | 0.67±0.11 |
| ESC FLA-PER | | | | | | 0.70±0.15c |

[a] ESC = *M. esculenta*; AES = *M. aesculifolia*; BLO = *M. brachyloba*; CTH = *M. carthaginensis*; TST = *M. tristis*; ESC FLA-PER = *M. esculenta* subspp. *flabellifolia* and *peruviana*

### 1.1.5 Molecular characterization of Cassava Bacterial Blight - Collaborative project ORSTOM- CIAT

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Cassava bacterial blight (CBB) is a major disease in Latin America causing serious crop losses which affects both yield and planting material. The causal agent is *Xanthomonas axonopodis* pv. manihotis (*Xam*). Since the beginning of the project (April 1995) surveys were conducted in Colombia in order to evaluate the impact of the disease, the genetic and pathogenic diversity of the causal agent and the level of resistance of the cassava core collection and the varieties cultivated. The principal objective of this project is to develop information on pathogen population structure, host pathogen interactions, genetics of resistance and detection methods. Results will permit to define a global strategy to control the disease.

#### 1.1.5.1 Analysis of pathogen population diversity

***Genetic characterization.*** Knowledge of pathogen population structure is essential for formulating long term disease management strategies. Information on pathogen diversity can be used in characterizing and deploying resistant germplasm. In Colombia, *Xam* strains were collected in different edaphoclimatic zones, in different sites and in various fields. A combination of virulence analysis and molecular genetic characterization is being used for the evaluation of existing biodiversity among strains of *Xam*. Different types of genetic methods are being applied for strain characterization based on restriction fragment length polymorphism analysis (RFLP) with *Xam* probes, either genomic or plasmidic as well as universal probes (ribotyping). In Colombia 250 isolates have been characterized with these tools, resulting in the description of 26 different genetic groups. Some of these groups were widely distributed in the different Colombian ecozones, suggesting patterns of pathogen migration. Cluster analysis among the groups tends to reflect their ecogeographic distribution, and areas of unique diversity have been identified. The highest level of genetic diversity was found among *Xam* strains collected in experimental fields where a high number of genotypes were cultivated (Villavicencio, Carimagua). The lowest level of genetic diversity was found in ECZ5 (Cajibio-Mondomo), a zone of high altitude isolated by the andean mountains. Few number of cassava varieties are cultivated in this zone which can explain the low level of genetic diversity observed within the pathogen population. Strains of this zone were also studied for plasmid content showing a great homogeneity. AFLP's studies are being developed to further characterize this population of *Xam*.

***Pathogenic characterization.*** Pathogenicity of all Colombian strains was assessed on a susceptible cultivar (MCol1522) by stem puncture and leaf inoculation in greenhouse conditions. Results have shown that most of the strains are highly pathogenic when inoculated by stem puncture.Variation among the reactions to foliar inoculation indicates variable aggressivity. A correlation between genetic groups and pathogenicity has not yet been demonstrated.

### 1.1.5.2 Characterization of strain - cultivar interactions.

Until now, no differentiation of races of *Xam* are known and no genes governing resistance in cassava have been described. Representative strains of *Xam* from each genetic group described (26) are being used to screen a set of 12 cassava germplasm representing a range of resistance and susceptibility. Differential reactions between strains and genotypes have been observed and the analysis are under study.

### 1.1.5.3 Genetics of the pathogen - Expression of a pathogenicity gene.

A *path*ogenicity gene (*pth*B located on a plasmid that is widespread within *Xam* strains, has been previously characterized. DNA sequencing revealed that this gene is a member of a gene family named *avr/pth* which is well described in other plant pathogenic *Xanthomonas*. The expression of the *pth*B gene and its role in the plant-pathogen interaction are being studied. The coding region of the *pth*B gene (fragment Bam-Bam) has been cloned in a pET vector in order to express the corresponding protein. Further studies on the purification, characterization and immunocytolocalization of this protein are planned.

### 1.1.5.4 Identification of source of resistance.

*Xam* is a vascular pathogen that enters vessels after a preliminary phase of intercellular development in the mesophyl. In susceptible plants, symptoms are characterized by leaf wilting and stem exudates, while resistant plant shows limited stem necrosis. The number of infected vessels is lower in resistant than in susceptible cultivars.

***Screening cassava core collection***. The main objective is to evaluate resistance to CBB in cassava cultivars both in field and in greenhouse conditions. The principal criterium used for the greenhouse evaluation is scoring symptom evolution over one month. Stem inoculations are done on one month old cassava plants.The reactions are evaluated at 8, 15, 30 days after inoculation according to a scale ranged from 0 to 5. Fifty one varieties were inoculated with different strains, each experiment was replicated at least 12 times. A group of resistant cultivars has been identified among which Mnga19 (=TMS91934), 6 cultivars MBRA, Mcol 22, CM523-7, CM6438-14, SM524-1, SM1053-12, SG107-35. A set of 12 cultivars showing different reactions were selected for further evaluation with a larger group of strains (see 1.2).

The 51 cultivars tested were multiplicated in the field (ICA Palmira) in order to obtain a good quality and quantity of stake materials. These cultivars have been transferred for the first evaluation in Los Llanos and are evaluated in field condition (4 evaluation during the cycle). Evaluation of this material is planned in different sites of the 3 ECZ where we have shown a clear differentiation of the pathogen population structure.

***Genetic characterization of resistance.*** Evaluation of the CIAT mapping progeny (TMS 30572 x CM 2177-2) with one reference strain of *Xam* revealed a wide range of transgressive genetic

segregation. This population is being evaluate with a representative set of strains as a mean of localizing resistance genes (see MB). We hope to identify combinations of alleles that provide greater resistance than the parental clones exhibit. The genetic map of cassava will be applied to the development of molecular markers to assist in breeding for resistance to CBB.

### 1.1.5.5 Development of detection methods for *Xam.*

*Xam* is transmitted in vegetative and sexual seed of cassava and is a target of international phytosanitary quarantine efforts. Diagnosis based on field inspection, has limitations especially since *Xam* can survive in tissues without causing symptoms. Different approaches have been developed based on molecular genetic information and monoclonal antibody technology (MABs) in order to improve the detection methods.

***Seed transmission.*** Fruits collected in infected fields have been treated in the laboratory in order to reveal the existence of natural *Xam* infection. The pathogen has been isolated both from fruits and seeds collected in different fields. Presence of the bacteria was revealed after seed washes and embryon maceration, dilution series and numeration on LPGA medium.

***Development of seed and seedlings inoculation methods.*** Artificial inoculation techniques have been developed on dry seeds (immersion and vacuum infiltration with a calibrated suspension of bacteria) and on germinated seeds (puncture of the embryon with a calibrated suspension). Both techniques revealed the apparition of CBB symptoms on seedlings 5 to 7 days after inoculation confirming that *Xam* can induce symptom into seedlings. Another inoculation technique has been developed on seedling (by stem puncture) which also allowed the apparition of disease symptoms in the plantlet.

***Survival of Xam in the seed.*** Studies on the survival of *Xam* into the seed have been investigated. After inoculation (immersion and vacuum infiltration) seeds were held at room temperature for several days. The number of bacteria present in the seed was estimated 8, 15, 22, 30 days after inoculation. Results obtained have shown the ability of *Xam* to survive in the seed.

***Development of Elisa.*** Two MABs were generated which react to all pathogenic *Xam* strains (ORSTOM), and their use in ELISA is being developed. First results have shown a positive reaction with bacterial culture and infected leaf samples with both the two MABs.

***Development of PCR and dot-blot assays.*** We have developed a PCR-based assay for *Xam*. Two primers *Xam*1V and *Xam*1K which are highly specific for *Xam* allow the amplification of a DNA fragment (900bp) only from pathogenic strains of *Xam*. PCR has been performed by using *Xam* DNA, inoculated leaf and stem tissues and field infected samples. The PCR reaction was sensitive as the minimum concentration detected was $3.10^2$ cfu per ml in infected samples.
A dot-blot test using the amplified DNA fragment (900bp) has been developed and the sensitivity of detection was in the range of 10ng of DNA per dot and as $1.3.10^3$ cfu/ml in plant extracts. Interference from other bacteria associated with *Manihot spp.* was not observed. The two techniques have given promising results allowing the detection of *Xam* in infected plant samples. Methods for the detection of *Xam* in cassava seeds are in progress.

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### *1.1.6 Analysis of genetic relationships among Cuban rice varieties* using RAPD and AFLP markers

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As part of a training program a comparison of RAPD and AFLP markers for germplasm classification and the assessment of the level of the genetic diversity within a group of Cuban rice varieties was carried out by Ing. Jorge Fuentes from the Institute for Applied Nuclear Research, Cuba.

Progress Report. Twenty-one rice varieties were selected from those used by major genetic improvement programs in Cuba. DNA samples were extracted according to the method described by Dellaporta et al. (1983). For RAPD analysis, 50 ng DNA of each variety were amplified, using 24 oligonucleotides. The samples were amplified in a buffer solution containing 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, 0.2 mM dNTPs, 0.01% BSA, and 80 nM primer, under the following conditions: 5 min at 95 °C, 1 min at 95 °C, 1 min at 36 °C, and 2 min at 72 °C for 35 cycles; then 5 min at 72 °C. The amplified DNA were separated in 1.5% agarose gel that contained 0.5 mcg/mL of ethidium bromide, and then photographed with POLAROID FOTODYNE equipment. AFLP fingerprinting was performed as described by Vos et. al., 1995 with some modifications (Tohme et al. 1996). Four AFLP combinations were used.

To study genetic similarity, data were obtained by reading the fingerprints of the different varieties studied on the basis of the presence (1) or absence (0) of each band. Genetic similarity between varieties was estimated by using the similarity indexes proposed by Nei and Li (1979) and Sokal and Michener (1958), and the statistical software NTSYS.PC (Rohlf 1988). Dendrograms were constructed, using cluster analysis (UPGMA), with the same statistical software. The cophenetic coefficients of correlation (Sokal & Rohlf 1962) for the matrixes with data on RAPD, AFLP, and both types of markers were calculated to verify the usefulness of the method used to explain genetic diversity among individuals. Also, the three matrixes of similarity were compared among themselves by Mantel's test (Mantel 1967), and the degree of congruence in estimating genetic similarity was determined for each type of marker.

Sixty RAPD oligonucleotides were analyzed in the study; of these, 24 showed consistent and reproducible bands, of which 87% (21) were polymorphic. In general, the different oligonucleotides detected polymorphisms among the 21 varieties studied. The average number of polymorphic bands per primer for RAPD data was 2.95, which does not differ from the overall average calculated from other authors (2.86). Results indicate that these primers may be very useful for rice germplasm classification.

The four AFLP combinations yielded an average number of 20.75 polymorphic bands per combination, which is twice that obtained by Mackill et al. (1996). This significant increase could be attributed to the low number of combinations tested by these authors.

The average value of genetic similarity among Japonica varieties was 0.48 for RAPD data and 0.55 for AFLP data, whereas corresponding values for Indica varieties were 0.87 (RAPDs) and 0.67 (AFLPs). AFLP markers detect 18% more variability than RAPD markers. When only Cuban varieties were included in the analysis, this value was 21%. AFLP polymorphisms was detected in the fingerprints of mutants or lines resulting from somaclonal variation data, indicating the capacity of this type of marker to detect differences, even in varieties that genetically are closely related.

Cluster analyzed was also conducted and dendrograms, constructed from matrixes of genetic distance with data from RAPD and AFLP markers, both alone and combined. In both cases, the different varieties were grouped into two well-defined groups belonging to the Indica and Japonica subspecies. Also, in both analysis, the rice variety Pokkali was grouped apart, apparently being more associated with the Indica group of varieties despite its Japonica origin. The Japonica germplasm has been further subdivided into Japonica Tropical (Oka 1958) or Javanica (Chang 1976) and Japonicum varieties from temperate countries (Mackill 1995). These findings are supported by the results of the study.

The high consistency of results with both types of markers should be highlighted. Only small differences were found in the position of the cultivars (varieties Oryzica Llanos 5, L-8554, IAC-16) within the dendrograms. Such consistency is supported by the correlation values obtained by Mentel's test (Mentel, 1967), which compared the matrixes of similarity for RAPD and AFLP data, both independently and combined. The correlation value obtained for RAPD and AFLP markers (0.71) also indicates that the estimates of genetic relationship per type of marker are adequately related.



### 1.1.7 Characterization and genetic variability of *Passiflora* spp. from the Andean region



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Passion fruits are very important as genetic resources and its characterization and evaluation of wild and cultivated population is a priority for Andean countries. Very little is known on the considerable genetic variability of *Passiflora* as their prospection, evaluation and study is just starting. *Passiflora* genus originates from the tropical rainforest regions of the new world (South America). These species are found from sea level to the high Andes generally as low density populations. The species are open pollinated and probably pollinated by large bees, other by hummingbirds. Passion flowers belong to the order *Violales* and the family *Passifloraceae* which has 18 genera. *Passiflora*, is by far the largest genus with 400 species. (Escobar, 1988)

*Passiflora* species are very difficult to classify acurately. Some of them are so widely varied and other so resemblant to each other. Many species and varieties of *Passiflora* have been carefully and acurately recorded, however it would be very difficult to obtain living specimens of most of these species. In order to extend our knowledge by keeping comprehensive collections of the genus such as the national collections organised by IPGRI in Colombia, Ecuador, Venezuela and Perú, many rare species will always be safe from extinction and available for investigation.

In this study genetic characterization, variability evaluation and mutational analysis of chloroplast DNA variation have been used to explore the evolution and the relationship of species complex in *Passiflora.*

The results by genomic and molecular characterization derived from *Passiflora* analysis will provide the bases to characterize different species, accesions, and origins; traits in intra and interspecific crosses for further improvement, with multiplication of selected genotypes and conservation of the *Passiflora* as a valious genetic resources. This work will contribute to the recent national efforts to collect, characterize and evaluate the *Passiflora* fruit germplasm, which is recognized as a regional priority.

Progress Report. *Passiflora* seeds or explants were germinated in the green house and transfered to the field at La Selva, Rio Negro (Antioquia). The youngest leaves were harvested from each plant and frozen in liquid nitrogen for the DNA extraction. Total DNA from thirty six (36) *Passiflora* sp. was isolated from 3.5 g. frozen leaves from a single plant per accession as described by Dellaporta et al. with some modifications. Approximately 150 μg of total DNA was obtained per gram of young fresh leaf tissue. RFLP and RAPD markers were carried out. For RFLP markers, total DNA (4 μg) was digested with four different restriction endonucleases. They are*Hind* III, *Eco* RI, *Ava* I and *Hae* III. DNA labelling and hibridization procedures were carried out as previously described (Angel et al, 1993)

Eight heterologous cpDNA probes (*Petunia* and mungbean) were hybridized sequentially to DNA, displaying 18 different chloroplast DNA molecules. Of the 32 successful hybridizations, 28 gave rise to polymorphic patterns. The large majority interspecific polymorphisms were observed in the inverted repeat region (IR) region homologous to the 16.2-Kb mungbean cpDNA fragment MBI and in the large single copy (LSC) region homologous to the 7.8-Kb mungbean chloroplast DNA fragment MB10. *Eco* RI and *Ava* I restriction digests showed the majority of the observed interspecific cpDNA variation followed by *Hae*III and *Hind*III. The most polymorphic combinations probe/restriction enzyme were MBI/*Ava* I and MB10/*Eco* RI showing each 9 different hybridization patterns in 36 accessions used. No polymorphism was found when *Hae* III restricction enzyme was combined with three (MB2, P14. P22) out of 8 probes tested, indicating no variation in G-C rich sequences in *Passiflora* present in regions homologous to these probes. Similarly, MB10 located in the large single copy region did not show polymorphism when was combined with *Hind* III.

In this work, 6 of the 8 probes evaluated were able to detect at least one intraspecific polymorphism. *Eco* RI restriction digests showed the majority of the observed intraspecific cpDNA variation. Intraspecific cpDNA variation was observed among individuals within *P. ligularis, P. edulis*, *P mixta* and *P. maliformis*. The RFLPs observed may have resulted from deletion and insertion phenomena. However, comparative restriction-site mapping of the cp genomes in different *Passiflora* species are necessary to determine the cause and the exact locations of the observed polymorphisms. The genetic distances and cluster analysis (UPGMA and WARD's method) expresed as genetic diferences between two *Passiflora* sp. accessions showed high level of polymorphism among the species studied. These results and the future studies with new accessions also could help us to elucidate the problems in the actual taxonomy.

The RAPD markers were carried out with 52 *Passiflora* accessions issues from 14 species. For these analysis 183 random primers were evaluated, 50 of them showed polymorphism, indicating high level of polymorphism intra species in *P. ligularis*, givin good bases for the improvement of this species. The RAPD markers also indicated a great genetic flow inter species specially between *P. mixta* y *P. mollissima.* This result is of high interest to carry out taxonomic classification studies. The Parsimony analysis shows that *P. coriacea* and *P. adenopoda* are the more ancient species. This analysis also showed *P. ligularis* and *P. maliformis* originate from the same branch as well as in *P. mollisima* and *P. mixta.* These analysis are in progress and will be carried out with new accessions. All these results will help us to start the genetic improvement of the most promising *Passiflora* species and their closest relatives.

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### 1.1.8 A PCR method to identify *Fusarium* , *Thielaviopsis* and *Pythium* species: Characterization of strains




Elizabeth Álvarez
Cassava Pathology


Isolation. A collection of 27 *Fusarium spp*, 13 *Thielaviopsis spp* and 9 *Pythium spp* strains was established from plantations at Villavicencio and Tumaco. In addition, monosporic cultures from these strains were obtained.

Pathogenicity. A pathogenicity test was performed at the greenhouse by cutting oil palm leaves with scissors previously deepen into an spore suspension (1x 10 spore/ml). The seedlings were observed for necrotic lesions developed along the leaves, 4 to 7 days after the incubation. Of the 49 strains tested only 13 *Thielaviopsis spp* induced necrotic leaf symptoms.Strains obtained from Tumaco were highly pathogenic, compared with the ones from Villavicencio.

Results of the two experiments measuring aggressiveness were very similar, those isolates considered to be most aggressive in the first remained the same in the second experiment and the

same was true for those strains that were the least aggressive. The greatest variability occurred with isolates of intermediate infection level, the non-pathogenic isolates, including 27 *Fusarium spp* and 9 *Pythium spp* strains, manifested the same negative response in the two experiments.

Molecular characterization. DNA was extracted from all strains following the protocol of Lee and Taylor (PCR protocols 1991) amplification of the I T S region was obtained with all strains. The P C R products generated with the primer I T S -4 and I T S -5 were digested with each of four restriction enzymes. Restriction of the rDNA yielded 2 or 3 bands depending on the enzyme and the strain. Different restriction pattern types were recorded using the restriction enzymes CfoI, AluI, MspI and the patterns corresponded to *Fusarium, Pythium* and *Thielaviopsis.*

RAPPDs we screened 11 random 10 mer primers and found four that generated polymorphism and consistently produced the same bands in replicate trails. RAPD primer OPA4,OPA10 detected genetic variation among *Thielaviopsis spp* strains.

## OUTPUT 1.2. AGROECOLOGICAL, AGRONOMIC AND GENOMIC INFORMATION ASSEMBLED AND INTEGRATED



### 1.2.1 Integration of Agroecological, agronomic and molecular data

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(i) Yield data was taken on two repetitions of the bean core collection in Popayán (1700 masl). Response to photoperiod was evaluated on the bush types (habits 1, 2 and 3) in Palmira, and those accessions which were evaluated as sensitive were evaluated as well in Popayán.
(ii) In order to use wild germplasm for improving yield in beans, three wild bean accessions were identified based on AFLP analysis as being especially diverse. These originated in Mexico, Colombia and Argentina. All have been crossed to three commercial cultivars of contrasting bean races: DOR390, Pinto Villa and ICA Cerinsa. The cross with Mexican and Colombian wilds have been advanced to the first backcross. These will be carried forward to a second backcross, selfed and evaluated for yield to identify QTLs for yield potential.



### 1.2.2 A new variant of arcelin from southern Mexico

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The wild form of *P. vulgaris* is widely distributed in Mexico, from the northern state of Chihuahua to the southern one of Chiapas, along the western Sierra Madre and in the volcanic axes in central Mexico. In its range of distribution *P. vulgaris* colonizes many and varied ecological niches with

secondary vegetation (disturbed habitats) for example it grows in the semiarid region in the state of Durango in areas that receive around 350 mm of yearly precipitation to around 2000 mm in the subtropical areas of Chiapas. *P. vulgaris* is mainly found at altitudes between 800 and 1800 masl (Miranda, 1987; Gentry, 1969; Delgado-Salinas et al., 1988). Although most of the traits related to adaptation are quantitative (Bennett, 1970), there are also important traits under the control of major genes. An example of the last traits is the resistance to bruchids, which is due to the presence of a specific seed protein, Arcelin, only found in the wild form of *P. vulgaris* (Shoonhoven et al., 1983).

Progress Report. A field exploration aimed to collect wild germplasm of *Phaseolus* spp was conducted in the fall of 1992 in the Southern states of Oaxaca and Chiapas, Mexico, area located in the middle of the region known as MesoAmerica. This exploration was part of an INIFAP-CIAT initiative to fill major collecting gaps in the representation of wild *P. vulgaris* in that region of Mexico (Acosta et al., 1993). The collected populations were assayed at CIAT for reactions to both bruchids and seed proteins (phaseolin and arcelin). Resistance to Zabrotes was detected. The resistant lines were then screened for the presence of arcelin and to determine the kind of variants present.

Total seed protein was extracted from 0.01 g of crude cotyledon tissue in 0.1 ml 0.5 M Nacl buffered at pH 2.4. After being shaken three times at 5 min intervals, the mixture was centrifuged at 12000 rpm for 15 min. Equal volume of supernatant and cracking buffer were mixed Brown *et. al.* (1981), boiled for 5 min and centrifuged at 14000 rpm for 15 min.

Proteins were separated according to their molecular weight, using one-dimensional SDS-PAGE electrophoresis in a vertical apparatus Laemmli U. K. (1970), as modified by Ma and Bliss (1978).The slab gels were 1.5 mm thick with 13.5% acrylamide in the running gel and 4% acrylamide in the stacking gel. An aliquot of 10 ul was then applied to the gel. Electrophoresis was performed for 5 h at 50 mA. Gels were stained overnight with Coomassie Blue and then destained with mixture of methanol, acetic acid and destilled water (in a ratio of 6:1:13).

Two-dimensional IEF/SDS-PAGE was done according to a method described by Anderson L. (1988), using 13.86% acrylamide slab gel for SDS-PAGE, except that no stacking gel was included. Electrophoresis was performed overnight at 40 mA. Arcelin was silver-stained according to a method described by Blum *et. al.* (1987).

The arcelin variant present in the lines from Chiapas was clearly distinct fromthe previously reported variants. The arcelin variants 1 2 3 and 4 which had been characterized previously Hartweck *et.al.* (1991), arcelin-5 Lioi L. and R. Bollini (1989), Goossens *et.al.* (1994) and arcelin-6 Santino *et. al.* (1991), all consists of several polypeptides. These polypeptides were also found for the novel arcelin-7 variant, which consists of two related polypeptides, like arcelin-5, with the exception that arcelin-7 does not have the polipeptide Arc 5c as described in Goossens *et.al.* (1994), for arcelin-5. The populations containing the new arcelin alleles were collected at the 'Depresion Central' of Chiapas, area located between the Sierra Madre del Sur and the Central Highlands. The area is of subtropical climate with a yearly precipitation ranging across locations from 1000 to 2000 mm. Most of the area is devoted to cattle raising with small portions of rainfed and irrigated agriculture. In addition to *P. vulgaris*, others wild species of *Phaseolus* also grow in the area (Acosta et al., 1993).

During the exploration conducted in Chiapas during 1992, only a small portion of the central part of the state was covered. In addition, in the sampled area new lands had been recently opened for cropping and cattle raising with a indiscriminated use of herbicides that according to interviewed farmers, were damaging the previously large populations of 'frijol de monte' (wild *P. vulgaris*) (Acosta et al., 1992). Furthermore, in the state there exists other wild and cultivated species of *Phaseolus*. However, due to an accelerated rate of erosion there is a real need for exploring and collecting in the rest of the state. The finding of this new variant is the first reported in the state of Chiapas, stressing the need for further exploration and characterization of *P. vulgaris*.



### 1.2.3 Gene tagging of agronomic traits in bean

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1. Bean Genetics; 2. Biotechnology Research Unit

Phenotypic data was obtained or is in the process for several important traits. All phenotypic data in beans is taken on Recombinant Inbred Lines (RILs), usually on F5 derived lines. One population segregating for bean fly resistance was sent to Tanzania for screening there with the insect. A very interesting population between an Andean low phosphorus tolerant landrace, G19833, and a Mesoamerican bred cultivar, DOR364, was evaluated for root traits. Broad differences were observed for total root length which appeared to be the trait that explained best the difference among the parental genotypes in response to low P. RFLP were utilized to analyze the two parents and 80% of probes proved to be polymorphic. A framework will be applied to the progeny to tag genes for root length. Meanwhile, data on yield performance with and without P stress have been obtained; yield QTL will also be sought. Other populations of RILs are being advanced for other traits.

Another population is being advanced which is segregating for traits relevant to P nutrition. G21212 is especially efficient in P uptake per gram root, BAT881 is inefficient. It is hoped to study this population with colleagues at Pennsylvania State University to tag relevant traits. Two other populations involving accessions with P use efficiency traits are also being advanced.

Sequence Characterized Amplified Regions (SCARs) are PCR fragments that have been converted to site specific primers. SCARs are an important part of a MAS strategy, since they permit more reliable PCR identification of desirable genes, with less variability than RAPDs among different laboratories and PCR procedures. Important progress in *Phaseolus* was recorded in the preparation of SCARs for two traits. One SCAR was obtained for a gene tag for resistance to Common Bacterial Blight (CBB). This gene was obtained originally from *P. acutifolius*, through XAN159 and several intermediate generations. The tag (as well as the SCAR amplified product) was present in the original *acutifolius* parental line, and of course in SEL1309 which was used as the CBB resistant parent in the marking population. This marker explained 23% of the variability in CBB reaction, and represented a difference of about 1.5 on the 1-9 CBB scale.

The second SCAR developed was based on a pair of codominant bands marking an important BGMV resistance gene derived from 'Garrapatos'. These bands were identified by colleagues in Puerto Rico (P. Miklas' team at USDA and J. Beaver at University of Mayaguez). In the process of

sequencing these bands we realized that unique sequences in the two bands were almost non-existent. Two sets of SCARs were developed but both sets continued to amplify both codominant bands, and it is still necessary to select on the basis of band weight. It was also possible to create a SCAR that amplfied the resistant band exclusively. We continue to compare notes with colleagues to identify other important BGMV gene tags to develop as SCARs.

### 1.2.4 Tagging of rice blast resistance genes



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1. Biotechnology Research Unit; 2. Rice Pathology

The mapping of resistant genes to Colombian rice blast in several crosses has been continued with the aid of diverse techniques such as RFLP, AFLP, microsatellites and lately with the construction of a highly polymorphic genomic library (genomic subtraction).

In the case of AFLPs, a marker linked to a resistant gene for lineage SRL-1 has been found in the Doubled Haploid cross between Fanny, a susceptible cultivar, and Irat13, resistant to many of the Colombian blast isolates. Only 6% recombination between marker and the resistance gene was detected. The band has been cloned and sequenced and SCAR primers are being designed. The AFLP cloned fragment has already been used as an RFLP probe showing a single band pattern.

The second mapping population, consisting of DH lines from the cross between Fanny, the standard susceptible line and Colombia 1 a cultivar resistant to many blast isolates-lineages, shows very little polymorphism when using RFLPs (79 polymorphic probes out of 274 tested), as well as a very high distortion in the segregation of alleles, usually towards Fanny's alleles. Nevertheless, it has been possible to associate a few RFLP markers to different clusters of resistant genes in this population. Linked markers belong either to chromosome 11 or 12 of the molecular map, though some inconsistencies have been found, probably due to the skewed segregation. A linkage to the Fanny resistant gene to a selected isolates form ALL-7 has been confirmed. The gene mapped to chromosome 11 near the Pi-1. The gene is either closely linked to Pi-1 or allelic to it. Fine mapping of the regions containing the resistant genes is underway. We plan to use microsatellites to complete the mapping in this population.

Dissection of resistance to blast in Llanos 5: Recombinant inbred lines were generated between the cultivar Fanny, (susceptible to most isolates), and Oryzica-Llanos-5, considered resistant to all common isolates-lineages in Colombia. Isolates from the main MGR lineage have been used to screen the whole RILs. Screening the RILs with RFLP probes from the maps of Cornell and Japan is also underway. 90 probes out of 140 tested so far, proved to be polymorphic. The mapping of these probes is proceeding as well as the identification of more polymorphic clones. We have also initiated the use of AFLP primers and microsatellites on the RILs.

A novel method called Genomic RFLP Subtraction is also being introduced to fine map the resistance genes in O. Llanos 5. The method implies the isolation of unique fragments of DNA present in a population or in a genotype (called Tracer) in this case present in O.Llanos 5 and absent

in the other population or genotype such as Fanny (Driver) . These fragments are then used as probes to screen both genotypes and their progeny in a present/absent allele pattern. The identification of such RFLP fragments could accelerate the fine mapping of resistance genes in O. Llanos 5. The first subtraction library is scheduled for testing in December.



### 1.2.5 Identifying markers linked to the apomixis gene in Brachiaria

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Several species of *Brachiaria* forage grasses are widely sown in tropical America. Two closely related commercial species, *B. decumbens* and *B. brizantha* are tetraploid and reproduce by aposporous apomixis. Reproductive mode is conditioned by a single gene, with apomixis dominant to sexuality. Our genome research seeks to: 1) tag and fine-map the apomixis locus with molecular markers; 2) exploit tightly linked PCR-based markers in an applied *Brachiaria* breeding program; 3) locate the apomixis locus on a complete *Brachiaria* genetic map constructed with heterologous probes from the rice and maize RFLP maps.

Progress Report. Two hybrid mapping populations were generated by crossing a *B. brizantha* and a *B. decumbens* clone each to the same tetraploidized, sexual *B. ruziziensis* biotype (*B. ruzizienzis* (CIAT 4403)X *B. brizantha* (CIAT 26646) [1013 cross] and *B. ruzizienzis* (CIAT 4403)X *B. decumbens* (CIAT 606) [1015 cross]) Both progenies were phenotyped with b esterase analysis for the selection of the hybrids. Individual hybrids in each population were phenotyped for reproductive mode by microscopic observation of embryo sac structure in a minimum of 20 ovaries with classifiable sacs. RAPDs, AFLP and RFLPs markers were used to screen the two mapping populations. The data was analyzed with MAPMAKER (LOD score 6.00 and recombination value of 25%).

The project made a significant progress in 1996. To date, 111 RFLP probes selected from the framework map of rice have been evaluated on parentals; 624 RAPD and 175 AFLP primers have been screened on bulk DNA. Linked primers were identified and a SCAR was designed from the RAPD primer linked to the apomictic phenotype in the *B. ruziziensis* x *B. brizantha* population. The SCAR was linked with reproductive mode phenotype in both populations, with mapping distance estimated at 4 cM in the *B. ruziziensis* x *B. decumbens* population and 13 cM in the *B. ruziziensis* x *B. brizantha* population. We have also identified a RFLP probe from the Cornell rice map which is linked with the SCAR and with apomixis. Current efforts are aimed at fine mapping and at comparative mapping between *Brachiaria*, maize, and rice.

### 1.2.6 Evaluation of useful variability in cassava using trait-specific genetic stocks

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1. Cassava Genetics; 2. Cassava Pathology; 3. Cassava Physiology

Cassava varieties are fixed hybrid genotypes that have been selected to posses a successful combination of genes controlling important adaptive and quality traits. Sexual progenies from crosses among clones, however, represent random combinations of the factors that have contributed to the varieties' success. and as such offer advantages in genetic studies. Recombinant progenies from controlled crosses between selected have been produce ideal genetic stocks for studies of parameters such as heritability, genetic linkage, and correlation among important cassava characteristics. Such genetic stocks have been constructed at CIAT in order to provide a wide range of genetic variability for each of several key characteristics in a common genetic background, or sets of backgrounds; and optimal linkage disequilibrium among genetic factors which contribute to a trait; as well as among different traits under simultaneous evaluation.

*Heritability of cyanogenic potential.*

Individuals from 10 sexual progenies from crosses between cassava clones with low and high cyanogenic potential and the parental clones were evaluated in two edaphoclimatic zones during 1995-6, to contribute to estimates of the heritability of cyanogenic potential. A subset of this material, representing the most variable families identified in 1994-5 were also grown in two additional sites to estimate the influence of environmental effects and genotype x environment interaction on variability for the same trait. Heritability of cyanogens in the root parenchyma was found to range between 0.6 and 0.9 (P= 0.0001), with correlations as high as r2= .9 in three years, by analysis of parent-offspring regression. Evaluation of the same genetic stocks in contrasting environments of Palmira (Valle), Quilichao (Cauca), Pivijay (Magdalena), and Aramasin (Guajira) will contribute to our knowledge of genotype x environmental interaction for cyanogenesis.

*Variation in photosynthetic rates*

A similar approach to the above is being taken to analyze the heritability of photosynthetic rates and the degree of their genetic correlation with productivity under drought stress. Photosynthetic rate is measured with a Licor meter in mmoles CO2/m2/sec, in replicated measurements taken throughout the growing season. Six recombinant families are under field evaluation in Quilichao during 1996, and early analysis shows moderate heritability; that is, progeny performance tends to correlate well with parental performance for this characteristic. Agronomic data including yield of above and below ground parts will be taken on these materials in early 1997 for the second consecutive year. These genetic stocks will be valuable for assessing the relationship between photosynthetic rate and biomass accumulation, as the wide range of variability presented in the unselected progenies is under evaluated in a single environment.

*Genetic mapping population*

The molecular map of cassava now provides direct measures of genetic distinction at discrete loci

throughout the genome. The application of the map to breeding objectives depends on the simultaneous monitoring of molecular (genotypic) and whole plant (phenotypic) variation in a the mapping progeny. Although the cassava map was drawn on segregation of molecular markers in 90 individuals of the progeny, an additional 60 individuals (total 150) from the same cross have been propagated vegetatively, to allow the analysis of an adequate stock for quantitative traits. Molecular markers from the map of 90 individuals are currently being scored on the remaining individuals which will add resolution to the segregation data.

The progeny on which the cassava map was developed was selected for both genetic polymorphism and important phenotypic differences presented by its parents. During the development of the map, 150 clones of mapping progeny have been propagated for field evaluation, and preliminary information has been gathered on the range of variability presented for root quality, resistance, and physiological parameters. Only non destructive assays, such as root quality and physiological characters, can be measured during the propagation phase; in addition to analyses such as for disease resistance that can be made on separate cuttings grown in the greenhouse. Data was taken in 1996 for the mapping progeny on 1) morphological descriptors, resistance to bacteriosis (using one strain under greenhouse conditions), cyanogenic potential (root parenchyma), post harvest deterioration, and dry matter; evaluation of photosynthetic rate is currently being conducted on the mapping progeny, together with the analysis of the trait-specific genetic stocks. A wide range of segregation has been observed for resistance to bacteriosis, photosynthetic rate, cyanogenesis, perishability and dry matter content in these preliminary trials, and replicated experiments are planned to gain increased precision on measurements of the genetic components of this variance. Our goals are to identify the important loci controlling these traits by their cosegregation with mapped molecular markers, and apply the resulting genetic information and gene tags to improving selection schemes. Once markers representing genetic loci that explain a significant proportion of the phenotypic variability are identified, they can be developed as gene tags, and used as correlative screens in selection programs.

### 1.2.7 GIS mapping for germplasms distribution

P. G. Jones

Land Management Unit



With the early collaboration of Dr. Nick Galwey, we have been investigating the possibility of mapping the distribution of wild *Phaseolus vulgaris*. We developed a number of useful techniques for relating a "calibration set" of germplasm collection or herbarium passport data to climate data from the CIAT climate database.

In 1996 the original paper delineating these methods (Jones et al., 1996) was accepted for publication in the journal "Biodiversity and Conservation". We have gone on to study the genera of *Stylosanthes* and *Manihot*. We have even applied the mapping techniques to a major pest of cassava and other crops.

Geographical Information Systems (GIS) have to date been applied mainly to the study and planning of land use, but we should be able to apply the technique to exploring biodiversity in the wild relatives of crop plants. Germplasm collections of these wild relatives contain in their passport data a wealth of information on the geographic distribution of the species. Herbarium collections are another important source of such data.

Here we present a method of objectively evaluating the criteria from the spatial distribution of germplasm bank accessions or Herbaria specimens. We envisage that we will incorporate in future studies further variables such as soil characteristics and disturbance by human agencies.

***Phaseolus Vulgaris***

The data available from the wild *P. vulgaris* collection indicate that it gives an incomplete coverage of the geographical distribution of the species and thus probably also of its genetic diversity. Further exploration and collection is needed. A method that can focus the exploration effort is invaluable in completing our picture of the genetic diversity of this species. We have developed a procedure to use the germplasm collection held in trust at CIAT, together with the CIAT climate data base for Latin America, as a basis for predicting the sites where wild *P. vulgaris* beans are likely to be found.

**Identifying "bean-favoring" climates**

The passport data for the location of origin of each wild bean accession consist of the latitude, longitude, and elevation of the collection site. We use this climatic data to estimate the climate of each location where wild beans have been obtained, using a two-stage process.

First, we estimate the mean monthly values for rainfall, temperature, and diurnal temperature range at the location in question by interpolation between the five closest stations for which records are available, using an inverse-square weighted distance. We adjust the temperature values according to the interpolation point's elevation.

Second, we address the problem of the timing of the major seasons throughout the year. We use the modified 12 point Fourier transform to rotate the climate data to a standard date. This version of the transform conserves the integral within each monthly division. The Fourier coefficients are then expressed as the phase angles and amplitudes of the six Fourier-function frequencies. The phase angle for the first component of the rainfall Fourier function often represents the date, relative to the 1st of January, on which the rainfall is at a maximum. We subtract this from all the phase angles for all climatic variables and then calculate the inverse of the Fourier transforms to produce a rigidly rotated set of climate data having the same form and integral as the original. This ensures that climates at different locations are not treated as dissimilar merely because their rainfall maxima and associated values of other variables occur at different times of the year.

We apply principal components analysis produced by the PCP command of Genstat (PCP) to the resulting 36 climatic variables to identify a smaller number of principal components, composite variables uncorrelated with each other, which account for most of the variation in climates among wild-bean locations. We fit a multivariate-Normal distribution to the principal components so that we can calculate a probability density for any climate.

Next we apply the climate estimation procedure to a continent-wide dataset derived by interpolation to the 10-minute (18 km at the equator) grid given by the National Oceanographic and Atmospheric Agency (NOAA) digital elevation model, using the same interpolation procedure as for the accession points.

The procedure described above depends on the assumption that the species occurs in a continuous range of climates that can be described by a single multivariate normal distribution. This might not be the case. To check this we apply hierarchical cluster analysis, using the group average method, to climates of the accession locations. We can then map the clusters using the Fortran program to construct an IDRISI image.

We thus identified a number of areas in Colombia that have "bean-favoring" climates but where wild beans have not previously been collected. We have confirmed that the natural vegetation in one of these areas corresponds closely to the type with which the wild bean is associated and we have recovered weedy types from this area, although the wild bean itself has not so far been found. In another promising area, farmer anecdotes mention wild beans growing along roadsides and field margins.

The most telling confirmation of the procedure's validity comes from area 3 (North Santander Department), which is clearly a continuation of a major potential area in the highlands of Venezuela. Evidence that the wild bean occurs in this area comes from published herbarium sources not included in the calibration set. Further field exploration of the distribution of *P. vulgaris*, in which the predictions made here will be both used and tested, is underway.

**Classification of climates where the wild common bean occurs**

There is evidence that we may be dealing with more than one population. The method was therefore extended to divide crop or species climates into several groups. The procedure identifies groups as Andean, Central American, and Mexican.

***Stylosanthes Species***

We selected five sets of accessions of *Stylosanthes* species from the germplasm banks of CIAT and CSIRO, then plotted the species distributions, as for *Phaseolus vulgaris*. The tropical legume genus

*Stylosanthes* has provided several species with potential for pasture and soil improvement that have been comprehensively investigated and improved over the last 20 years. As most species are native to the Americas, this is their center of diversity.

**Other factors may be important locally**

*Stylosanthes guianensis sensu lato* is a Pan-American species, most widely found throughout the continent. Many of the potential areas for collection have been explored. Yet, there would appear to be a broad area of potential exploration for *S. guianensis* through southern Peru and northern Bolivia. The apparent absence of *S. guianensis* from the interior lowlands of South America could be because *S. guianensis* is intolerant of periods of extended waterlogging, or because of its poor adaptation to the shady forest environment. However, *S. guianensis* has been collected in high rainfall areas (annual rainfall >7000 mm) of the humid tropics, such as the Colombian Chocó department where it was found relatively frequently at roadsides. Its apparent absence could also be because of minimal collection carried out in these regions. Thus it may be worthwhile to consider other environmental characteristics, such as chemical and physical soil factors, for potential distribution of the species. This would be in addition to plant exploration missions to areas where the species has potential but has not yet been collected.

**A validation of the method**

A serendipitous difficulty led us to an unplanned validation of the classification and mapping procedure. There are usually small errors of location in the files used for the analysis. In the case of *S. hamata*, over 20 accessions were collected in the small islands throughout the Caribbean, particularly some in the Bahamas. Slight errors in the passport data and minor systematic errors in the climate database made it difficult for us to align some accessions with the correct island. Since they then fell in the sea, we decided to eliminate them from the calibration set. We went ahead and fitted the model to the continental points. Once mapped, the unplanned validation became obvious. The islands onto which we had been trying to align the recalcitrant points lit up as highly probable climates for *S. hamata*.

**Potential for further collection**

The most striking indication of potential new collecting areas is the case of *Stylosanthes* sp. aff. *scabra*. There is now evidence that this species is distinct from *S. scabra*. Recently, much interest has been generated for this plant in Australia because of its frost tolerance and adaptation to heavy soils of central and southern Queensland.

Accession points are limited to an area in the states of Bahia and Minas Gerais in eastern Brazil. The climate probability density map shows major potential collection areas in southern Brazil, eastern Paraguay, and northern Argentina. New collection missions might explore these areas.

**Species consist of different populations**

*S. guianensis* has a wide natural distribution in tropical America, but the collection points do not all fall in high probability areas, whereas most of the points in Brazil do. Are there in fact two or more populations of the species with different climatic requirements?

Fitting a single multivariate normal distribution to the calibration set when the set is a composite population may cause spurious regions of high probability where no accessions exist, or accession sites in areas of low probability. We applied the climate clustering technique already used on *Phaseolus vulgaris* this case. Further work on the genetic characterization of samples of these populations is helping to prove this hypothesis.

In May, Mark Sawkins arrived to work with us for 3 months, financed by the Royal Botanic Gardens at Kew. He is studying at the University of Birmingham in England. We pushed on with the studies of *Stylosanthes* and mapped species probability models of *S. viscosa, S. humilis,* and *S. capitata.*

***Stylosanthes guianensis***

We obtained data of 561 characterized accessions of *S. guianensis.* Polyacrylamide gel electrophoresis (PAGE) had been used on three enzyme systems (∝-esterase, β-esterase, and ∝βACP) with a total of 40 bands of isoenzyme data.

We merged the data with location data for the species. From this we created a combined climates file of 449 records and undertook a PCP analysis. We then put the scores through a hierarchical cluster analysis. From scatterplots of the PCP scores on pairs of dimensions, we found that a small group of accessions had formed away from the main group within an ellipse of two standard deviations from the origin. Identifying the accessions in this group from passport data, we found that the group was catalogued as being *S. guianensis* var. *pauciflora.* After clustering the data, we identified and removed the accessions of this variety from the main *S. guianensis* file. This is another case of the power of the technique. Had we not known that these accessions clustered differently to the rest, we would not have noticed them as different taxonomically.

Since the presence or absence of an enzyme type is marked in the bands of an electrophoresis gel, it occurred to us that this presence or absence in binary form could be regarded as a simple binary sequence analogous to a chromosome for the purposes of a genetic algorithm. A genetic algorithm operates on a binary string to produce mutations and recombinations as if it were the DNA of a living organism. A separate subroutine evaluates the fitness of the "DNA" string. The selection process can often converge on a mathematical solution much faster than an exhaustive search for the total number of combinations.

Using the isoenzyme data as the chromosomes and the climate clusters as the phenotypic solution, we made many different analyses for *S. guianensis*. We wrote a number of different routines to evaluate the "fitness" of a "chromosome". The basis of all was that they should explain to a maximum the differentiation of isoenzyme data between the chosen climate cluster groups.

The exhaustive search for combinations of isoenzyme bands would have taken !40 (factorial 40 operations = $0.8 \times 10^{48}$). The most modern computer would be unable to finish this operation before the end of the universe as we know it. The genetic algorithm homed in on possible solutions within minutes.

It would be vain to expect that the raw isoenzyme data would produce a complete solution explaining the climate clusters. However, we have indications that some isoenzyme bands may be differentiating some climate clusters. Table 1 shows the frequencies of ∝-esterase bands 8 and 10 in selected climate groups as identified by the genetic algorithm. These data give us no insight into what is actually occurring but do give us hope for the future.

**Table 1.** Frequency of combinations of ∝-esterase bands 8 and 10 in selected climate groups.

| Climate group | Number of accessions in bands | | | | |
|---|---|---|---|---|---|
| | Neither | Only 8 | Only 10 | Both | Total |
| 1 | 3 | 23 | 2 | 6 | 34 |
| 2 | 1 | 3 | 0 | 10 | 14 |
| 5 | 20 | 37 | 14 | 81 | 152 |
| 6 | 9 | 63 | 6 | 70 | 147 |
| 7 | 5 | 65 | 2 | 16 | 88 |

## *Manihot* Species

We have also produced probability maps for five species of *Manihot*, the wild relatives of cassava *Manihot esculentum*. Of the five species mapped, many drew concerned attention from genetic experts in the field. *Manihot carthinagensis* has been collected mainly from the North Coast region of Colombia and from Venezuela. Our mapping shows that there may be potential collecting areas in Yucatan and Cuba but the main area of climatic similarity is in northeast Brazil. It would appear strange that a species could cross the Orinoco/Amazon region when it was adapted to severe drought stress in an area of the Caribbean north coast. However, our colleague Dr. Antonio Costa Allem, taxonomist at CENARGEN in Brazil, assured us that there did exist a *Manihot* species taxonomically indistinguishable from *Carthinagensis* in Brazil in the area in question.

An area where we are on solid ground is in the direct precursors of *Manihot esculentum*. *M. flabellifolia* and *M. peruviana* are known to be the closest living relatives of *M. esculentum* from genetic studies. The distribution maps of these species are based on a few accessions, but show a striking similarity. It is telling that the major modern plantings of cassava are geographically isolated from these areas. Crops transplanted from their areas of origin often escape their principal predators and diseases—note the case of the mealybug in Africa. It would seem prudent to study the plantings of cassava in the new colonist areas of Acre and Rondonia in Brazil to see if pathogens and pests new to the rest of the cassava planting world may come to the fore and be exported from these areas.

### *Cyrtomenus bergi*, a Pest of Cassava

The mapping techniques are not limited to plant species. They can also be used to track the presence of insects and the diseases they cause. We have been collaborating with the entomology unit of the Cassava Program in mapping the potential presence of the pest *Cyrtomenus bergi*.

We found once again that a single climate population did not describe the full range of the organism. We have now made a number of analyses seeking a rational clustering of the environments reported for the pest. Most cases (41 out of 66) can apparently be assigned to a common climate. However at least four more climate clusters are needed to explain the remaining 25 accessions.

### *Arachis* sp. the Ancestors of the Peanut and Promising Forage Species

We have begun analysis of accessions of the genus *Arachis*. Taken from herbarium and germplasm collection records, we are working up the geographic calibration sets for over 60 species.

### Conclusions

By applying the technique to many different cases we are learning both their power and their potential pitfalls for the uncritical user. The results must be used together with traditional knowledge of centers of origin and factors influencing dissemination.

The resolution of the climate files at 10 minutes of arc (about 18 km) leaves a lot to be desired, particularly in broken, mountainous terrain. This will be solved in the near future when we construct new interpolated climate files with a precision of 30 seconds of arc (about 1 km).

Apparently we can use the methods to ask three types of questions. First, where are other potential areas? Second, are we dealing with a single uniform population? Third, are species that have been

named as different occupying the same environmental niche and, if so, could they be closely related taxonomically and genetically? If we combine traditional germplasm collection and taxonomy with GIS and genetic marking techniques we should have a powerful tool to both pose and answer such questions. The technique seems to hold considerable promise in identifying key areas for in situ conservation.

For a full treatment of this research, see the 1996 detailed report of the Land Management Unit.

**Reference**

Jones, P. G.; Galwey, N. W.; Beebe, S. E.; and Tohme, J. 1996. The use of Geographic Information Systems in biodiversity exploration and conservation. Biodiversity and Conservation. Forthcoming.



## OUTPUT 1.3 TECHNIQUES FOR ASSESSING GENETIC DIVERSITY DEVELOPED



### 1.3.1 Construction of a molecular genetic map of cassava

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The genetics of important agronomic traits of cassava (*Manihot esculenta* Crantz) are less well understood contrasted to other major sources of calories that feed mankind. A genetic linkage map of cassava has been constructed with molecular markers as a first step towards marker-aided analyisis of quantitative traits in cassava. The map will also serve as a tool for cassava breeding via association of map-based markers with important traits in and the development of marker-aided selection schemes. Current status of the genetic map of cassava is summarised below and future perspectives briefly stated.

Progress Report. One hundred and thirty-two RFLP, 30 RAPD, 3 microsatellite, and 3 isoenyme loci segregating in the gametes of the female parent (TMS 30572) of the $F_1$ mapping population define 20 linkage groups spanning 931.6cM, with an average marker density of one marker every 8cM. Twenty six RFLP markers and one isoenzyme marker remain unlinked. Linkage groups are named alphabetically until they can be correlated to earlier named chromosome karyotypes (Magoon et al. 1969). Based on the segregation data reported here, the length of the cassava genome is estimated to be 1, 610cM. With a total map length of 932cM, this implies that the present framework map covers roughly 60% of the cassava genome. One hundred and seven RFLP, 50 RAPD, 1 microsatellite, and 1 isoenzyme single-dose markers, segregating from the gametes of the male parent (CM 2177-2) scored in the $F_1$ mapping populations, define 24 linkage groups (not shown) with

a total distance of 1, 220cM. A similar estimate of genome size based on mapping data from the male gametes suggests that the length of the cassava genome is 2,010cM.

The most salient difference observed between the male- and female-derived framework maps is the greater genetic distances on the male-derived map between markers common to both parents (allelic bridges). Intervals are larger in the male-derived map than in the female-derived map in eight instances and in only two instances does the female-derived map display larger genetic distances. A paired T-test of the 10 intervals showed significantly ($P<0.01$) greater distances in the male-derived map suggesting a reduced recombination rate in gametes of the female parent. This observation is reinforced by the greater overall length of the male-derived famework map and its larger estimate of genome size compared to the female.. We are currently working with the computer package Join Map 2.0 (Stam et al. 1993) to develop a consensus map based on male- and female-derived framework maps.

Thirty percent of all RFLP markers on the female-derived cassava genetic map were found to be linked in the repulsion phase. This figure is less than the 50% expected for linkage of single-dose markers in allopolyploids or diploids (Wu et al. 1992). On the other hand, it differs markedly from the complete absence of linkages in the repulsion phase expected for autopolyploids. The 30% figure of markers linked in the repulsion phase and presence of entire groups not presenting markers linked in repulsion implies that a significant amount of random pairing occurs in cassava. The karyology of the 18 haploid chromosomes of cassava reveals 6 identical pairs and 3 different pairs of homologous chromosomes (Magoon et al. 1969). Assuming random assortment between homoeologous chromosomes of the six pairs of identical chromosomes, a reduction of 66.7% would be expected of all markers linked in repulsion (16.7% as against 50%). The percentage of markers linked in the repulsion phase in the genetic map of cassava reported here, 30%, is significantly higher than this. It is not clear at this stage if the higher number of markers found in repulsion than expected from the karyology of cassava (Magoon et al. 1969), supports the predominance of disomic inheritance as suggested by pairing behaviour (Bai 1992) and inheritance of isoenzyme loci (Roca et al. 1992; Lefevre and Charrier 1993).

Six duplicated loci were detected from the segregation of single dose markers derived from 36 cDNA, and over 200 genomic sequences. Our results reveal a few randomly distributed duplicated loci, less than 5% of the total number of markers, a number corresponding roughly to that reported in many diploids (Causse et al. 1994). It remains to be determined whether the duplicated loci represent vestiges of an ancient allopolyploid or random genomic duplication events in a diploid or diploidized genome. We are currently placing more cDNA markers on the cassava map in an attempt to resolve the enigma of genome duplication in cassava, a putative allopolyploid. cDNA sequences are known to be relatively more conserved than genomic clones, and are expected to be very valuable additions to the cassava map.

Planned developments for the genetic map of cassava includes addition of more molecular markers, preferably microsatellite markers and cDNA clones, and joining the male/female derived maps. Microsatellite markers showed a higher level of polymorphism than any other marker in the intraspecific cross used in generating the cassava genetic map, while cDNAs will be most useful in defining the genome structure. Secondly the cDNA/AFLP technique (Bachem et al. 1996) would be employed in generating expressed sequence tags (ESTs) for the cassava map in an attempt to

place known gene sequences on the map. The 150 plants of the F1 mapping population have also been scored for several traits in the field, in two environments, and QTL analysis have been initiated using map-based markers (Bonierbale 1996, unpublished data). A backcross population (backcrossed to the female parent, TMS 30572) has become available for better resolution of identified QTLs and for the genetic mapping of sources of resistance to the African cassava mosaic disease (ACMD) (in collaboration with the International Institute of Tropical Agriculture IITA). The backcross population would be genotyped shortly, making available a "biologically" unified map for comparison with the compromise map using Join map.

### 1.3.2 Determination of adequate number of AFLP primers for germplasm classification in beans

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In order to estimate the number of primers necessary for a sound phylogenetic analysis and germplasm classification a subset of wild Phaseolus accessions were analyzed with a set of 6 different AFLP primers. Thirty-nine accessions of wild South American *Phaseolus* (*P. lunatus, P. augusti. P. pachyrrhizoides,* and *P. bolivianus*) species where chosen at random from a bigger collection consisting of 120 accessions. One cultivated accession from the common bean, *P. vulgaris*, was also included as a an outgroup.

Progress Report. The AFLP protocol used was that described by Vos et al. (1995), with modifications introduced by the Biotechnology Research Unit at CIAT (Gónzalez et al., 1995). The following primer combinations were used: (E + AAC/ M + GTA (A), E + AAC/ M + GCG (AB), E + AGT/ M + GAC (C), E + ACA/ M + GGT (D), E + ATC/ M + GCA (G), E + AGA/ M + GAG (H)).

All forty accessions were primed with six different primer combinations to carry out the second selective amplification. Data was scored as presence and absence of band, with a 1 corresponding to presence, and 0 corresponding to absence of band. Only well defined bands were included in the analysis. For each of the primers employed, Nei-Li coefficients of similarity (1979) were derived between all accessions. Dendograms were generated using the algorithm UPGMA (Sneath and Sokal, 1973), from the SAHN clustering procedure of NTSYS version 1.80 (Rohlf, 1994). Cophenetic correlations and correlations between similarity matrixes of each primer combination were obtained using the program MXCOMP of NTSYS.

Additionally, similarity matrixes were generated for each possible permutation of the six primers used. That is, first, data from each primer was combined with data from every other primer, to generate two-primer similarity matrixes; then, data from each of these primer-pairs was combined with data from every remaining primer, to generate three-primer similarity matrixes. This procedure was continued until all 63 possible primer combinations had been used to obtain similarity matrixes. The last matrix obtained was derived from data of all primer combinations pooled together. Correlation coeficients between similarity matrixes of each permutation and the last matrix obtained

were calculated. For each of the matrixes obtained, the average similarity value, the standard error, the variation coefficient, and the longitude of the confidence interval were also calculated.

Each of the primers employed in the analysis revealed different levels of polymorphism. All primer combinations had similar levels of citosines and guanines, varying between two and four. However, the primer with the lowest CG content (E + AAC/ M + GTA) did reveal the greatest amount of amplification products. The primer with the greatest CG content (E + AAC/ M + GCG), did not amplify the lowest number of bands, contrary to expectation.

Similarity matrixes obtained for the forty accessions evaluated with each of the six primer combinations, were highly correlated (Table), with $r \geq 0.75$ in most cases.There was one exception, however. Primer E + AAC/ M + GCG gave very low levels of matrix correlation with other primers ($0.42 \leq r \leq 0.64$), suggesting that the fragments amplified contained different information about the stucture of accesion relationships.

To observe if there was any relationship between the different characteristics which a primer may exhibit, two multiple correspondence analyses were carried out on data from all primer combinations (Table). One analysis included all forty accesions studied (complete), and the other excluded the only individual belonging to *P. vulgaris* (restricted). In the complete analysis, only bands which contributed to the formation of groups in the second dimension were taken into account. This was done because the great difference exhibited between *P. vulgaris* and the other individuals obscured all other variation in the first dimension; differences between the groups made up of *P. lunatus, P. augusti, P. pachyrrhizoides,* and *P. bolivianus* could only be observed from the second dimension. In the restricted multiple correspondence analysis, bands contributing to the first and second dimension were taken into account.

No clear relationship was observed between the number of polymorphic bands amplified by each primer combination and the number of bands with which each contributed to the formation of discrete groups in the multiple correspondence analysis. It should be pointed out, however, that the two primers with the lowest number of polymorphic bands (E + AGT/ M + GAC and E + ATC/ M + GCA) were the ones which contributed the least to group formation in the multiple correspondence analysis. Contrary to this evidence was the fact that the highly polymorphic primer, E + AAC/ M + GTA, also contributed very little information to the analysis. Correlation coefficients calculated between each similarity matrix and that of all primers combined, were, in general, very high and with little difference between them. It is noteworthy, that the primer E + AAC/ M + GCG was, once again, the one with the lowest value of r ($r = 0.71$).

The correlation analysis carried out on all possible primer permutations revealed that, as the number of primers used increased, the correlation between these similarity matrixes and that obtained from all six primers also increased. In addition, the range of values taken on by each correlation coefficient decreased with each primer added. The first tendency is highly predictable, as a proportion of a total is being increased with each primer addition, which leads to increasing similarity between the the part and the whole. However, the range of values which r took on was highly interesting: just changing from the use of one primer to two, diminished the range by 56% (from $r = 0.71 - 9.94$ to $r = 0.86 - 0.96$).

Average similarity values also showed a tendency to stabilize as primers were added to the analysis. Most of the primer permutations gave average values of similarity inside the 0.6 and 0.8 range, independent of the number of primers employed. Similarity values tended to stabilize around 0.7. Therefore, no permutation gave values radically different to the last one obtained.

The variation coefficient, measure which gives, in percentages, a relationship between the standard deviation and the average similarity value of each matrix, stabilized at a value close to 20%. Using one primer gave values between 15 and 35%. With three primers, these variation coefficient values diminished by 60%, to a range between 17 and 25%. This analysis suggests that analyses carried out with more than three primers would be unnecesary.

Confidence interval lengths obtained for each of the primer permutations evaluated were all very small and similar to each other (close to 0.02), even when only one primer was used (Fig.). The lengths of the confidence intervals were a very small percentage of the actual similarity values, never exceeding the values of 2-4% (data not shown). As the size of the confidence intervals did not change appreciably as the number of primers used was increased, the analysis suggests that the use of only one primer could be sufficient.

### 1.3.3 Enrichment of a P. vulgaris genomic library for GA sequence repeats

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Short tandem repeat (STR) loci, as know as microsatellite loci are widely used for genetic linkage and mapping studies due to their high levels of polymorphism, widespread genomic distribution and rapid analysis. Several methods have been developed to speed up the isolation of genomic fragments containing such simple repeats. Screening genomic short fragment libraries with simple repeat oligonucleotide probes has proven successful for isolation of large number of GA repeats but this approach requires a lot of time and needs several rounds of hybridization to obtain a pure clone.

50 ug of genomic DNA were digested using 500 units of Tsp 509 (AATT) at 65°C overnigth and ran on a in 1.5% low melting point (lmp) agarose gel. Fragments between 100 and 500bp were excised and purified from the low melting point agarose gel using Phenol-Chloroform methodology. About 5 ug size-fractionated genomic DNA were ligated with 50 uM of Tsp adapters containing EcoRI site.

A total of 500 pmol of biotinilated oligoprobe (GA)13 was attached to 2 mg of steptavidin-coated magnetic beads (previouly washed with PBS Buffer) in 200 µl of water at room temperature for 1 hour. Excess unbound oligo was removed with 400µl of1X BW Buffer. The beads were dissolved in 400µl 5XSSC-O.1%SDS and then dissolved in 300 µl 10XSSC+ 0.1%SDS prewarmed at 65°C. 5ug of DNA were denatured in a volumen of 150 µl by pleacing it at 95°C for 5 min. The DNA was hybridized with beads+oligonucleotide during 3 hours at 65°C.

Beads were repeatedly sedimented by application of a magnetic field and washed 2X 5min each at room temperature in 400µl of 2XSSC+0.1%SDS, followed by once at 65°C in 2XSSC+0.1%SDS

and a final wash in 200µl of TE + 50mM NaCl. Finally this mixture was resuspended in a final volumen of 200µl of TE.The last of each set of washes was retained for PCR amplification. Following these washes, the beads were resuspended in 50µl of water plus 3µl of 2M NaOH and incubated at room temperature for 20 min to release any bound sequence. The beads were then removed, and the supernatant was accurately neutralized by addition of 3µl 10XTE (10mM Tris-HCl, 10mM EDTA,pH 7.3), 2µl 1.25M actic acid. The supernatant was desalted by passage through Magic Minicolumns following manufacturer's intructions. Aliquots were amplified exponentially in 25µl PCR containing 2µl of desalted template,0.2mM total dNTP, 0.2µl Taq DNA Polymerase,0.8µM of both primers using a Hot-Start PCR amplification: once 5min at 94°C , once 3 min at 80°C (add the enzime mix) and 20 times 45 sec at 94°C, 45 sec at 56°C and 1.5 min at 72°C. A finally extension step at 72°C for 5 min.This PCR product was desalted again using Magic Minicolumns and resuspended in 40µl of water for future digestion. The PCR fragments enriched for (GA)13 sequences were digested with EcoRI and re-ligated into fresh EcoRI-pBluescript (Stratagene) digested and dephosphorilated using T4 DNA Ligase. These recombinants were transformed into competent MRF' Blue *E.coli* cells, and the resulting colonies were screened with the biotinylated probe (GA)13 using standard methods.

Several clones from this library were sequenced and different sizes of GA simple sequence repeat were identified. We are in the process of generated flanking primers for subsequent use in mapping and germplasm characterization.



### 1.3.4 Microsatellite analysis in Cassava

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Microsatellites are codominant genetic markers especially useful for mapping and diversity studies in heterozygous species such as cassava. They represent a type of DNA sequence that is 'hypervariable' and therefore usually detect a larger number of alleles per locus within populations or germplasm collections than other types of markers. Primer sequences for the amplification of 15 microsatellites cloned from cassava at the Univ. of Georgia were made available to CIAT in 1996. The majority of the cassava microsatellites identified so far correspond to short stretches of GA-repeats, despite a search for different types with a set of 7 oligonucleotides. Informativeness of the primers was assessed at the Univ. of Georgia on a set of 44 cassava genotypes, and the most informative are being used to characterize the core collection. This analysis was facilitated by a small grant from USAID, permitting training of a CIAT assistant in the USDA Plant Conservation Unit of Griffin Georgia.

At CIAT, three microsatellites have been applied to the collection of cultivated and wild germplasm described above. Table 1 presents an example of the genetic diversity detected in 7 Manihot taxa with the microsatellite marker GA12. A total of 18 alleles were detected in 105 genotypes, with between 2 and 10 alleles in a given taxon (Table 1). The frequencies of various alleles in different components of the gene pool can be used to describe the structure of genetic diversity. Additional microsatellites, GAGG5 and GA21 revealed 8 and 9 alleles, respectively in same germplasm set.

Segregation analysis of 8 microsatellites in the cassava mapping population (CM7857) indicate that 7 represent single loci and one corresponds to a duplicated locus in the cassava genome. The remaining microsatellites will be applied to the wild species collection during 1997 to contribute to the analysis of genetic relationships.

**Table 1.** Frequencies of alleles of microsatellite GA 12 recorded in different Manihot species

| | ESC | AES | BLO | CTH | ESC-FLA | ESC-PER | TST |
|---|---|---|---|---|---|---|---|
| Allele | Frequency ($p_i$) | | | | | | |
| 1 | | | 0.17 | | | 0.03 | |
| 2 | | | | | | 0.03 | 0.25 |
| 3 | | | | | | | 0.13 |
| 4 | | | | 0.08 | | | 0.50 |
| 5 | | | | 0.13 | | | |
| 6 | | | | | | 0.06 | 0.13 |
| 7 | | | | 0.02 | | 0.06 | |
| 8 | | | | 0.40 | 0.21 | | |
| 9 | | | | 0.02 | | | |
| 10 | 0.22 | | | 0.13 | 0.08 | 0.09 | |
| 11 | 0.37 | | | 0.10 | 0.33 | 0.25 | |
| 12 | | | | 0.06 | 0.08 | 0.06 | |
| 13 | | | | | 0.08 | 0.03 | |
| 14 | | | 0.83 | | 0.13 | 0.06 | |
| 15 | 0.41 | | | | 0.08 | 0.31 | |
| 16 | . | 0.06 | | | | | |
| 17 | | 0.94 | | 0.04 | | | |
| 18 | | | | 0.02 | | | |
| Number of Individuals (n) | 38 | 8 | 3 | 24 | 12 | 16 | 4 |

## *1.3.5 Evaluation of cassava diversity with heterologous probes*(comparative genome mapping)

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*Euphorbiaceae*

Conservation between related species at the DNA level often permits the use of DNA markers across members of the same plant family. This can provide complementary information on genome evolution and gene phylogeny, as well as increasing the number of markers available for map development. Hybridization of DNA of the cassava mapping parents (TMS30572 and CM2177-2) on Southern blots was performed using DNA probes from rubber (*Hevea brasiliensis*, Euphorbiaceae) from CIRAD, to determine the feasibility of future comparative mapping. Twenty five genomic probes from rubber have been evaluated for homology and polymorphism in cassava,

using five restriction enzymes and various hybridization stringencies. Fifteen (60%) of the probes tested showed strong homology with cassava DNA, and polymorphism which should permit their localization on the cassava genetic map. Two probes did not hybridize, and the remainder produced weak signals, indicating that various parts of the cassava and rubber genomes have diverged at different rates.

***Heterologous genes for disease resistance***

*Xanthomonas campestris* pv manihoti (*Xcm*) is the causal agent of the serious systemic disease, cassava bacterial blight (CBB). Homology among pathogenicity determinants (*avr*, *pth* and *hrp* genes) from various *Xanthomonas species* on hosts as diverse as rice, tomato, citrus and cassava was reported by ORSTOM in 1994. In the interest of testing the hypothesis that homologous systems of resistance to related pathogens, such as *Xanthomonas,* may function in distantly related plants, the CIAT-ORSTOM collaboration in Cassava Pathology and Genetics was extended to the Univ. of California, Davis, where a gene for resistance to *Xanthomonas* species (Xa-21) has been isolated from rice (Gramineae). The discovery of resistance genes in cassava via information from heterologous systems would be a significant advance both in plant pathology, and cassava genetics/ breeding. In Davis, the protein encoded by *Xa-21* was expressed and sequenced following PCR amplification of the resistance gene. Four cassava varieties with varying reactions to CBB were sent as DNA extracts to UC Davis, and amplification of homologous sequences from cassava with primer sequences from *Xa21* (rice) was successful with the resistant varieties only. An 846bp PCR product from cassava was cloned and sequenced, revealing high homology with the rice gene.

The new cassava clone (PCR250), homologous to *Xa21* in rice, was used at CIAT as an RFLP probe to investigate a possible role in host reaction to CBB. Upon hybridization to survey filters, the cassava genetic mapping population was polymorphic for PCR250 with the restriction enzyme *Hind*lll, and the probe was mapped to a minor (female parent-derived) linkage group of 2 RAPDs and 1 cassava cDNA marker. Simultaneously, molecular segregation data was tested for correlation with phenotypic data, generated by the greenhouse inoculation of the same progeny with a strain (X27) of *Xcm*. The cassava mapping population presented a full range of resistance- susceptible reactions to this strain of *Xcm*. However, no correlation was found between the population's genotype at the PCR250 locus and the greenhouse reaction to X27: *Xcm*. Two hypothesis were generated which will be tested in further collaboration: 1) the gene responsible for resistance in rice is present in cassava, but does not serve a resistance function; or 2) strain X27 of *Xcm* does not respond to the cloned gene, but additional strains of the pathogen affecting cassava may well be sensitive to it. Additional strains are now available through the CIAT-ORSTOM collaboration, and will be tested on this population, together with additional, related DNA sequences that have been isolated from rice. This and other related DNA sequences from rice will be also investigated for homology in a wider range of cassava germplasm which has been selected for their different reactions to CBB to expand on earlier results which showed differential reaction with resistant and susceptible varieties.

# REPORT PROJECT OBJECTIVE 2



**To identify and access exotic or novel genes and gene combinations for broadening the genetic base of gene pools.**

## OUTPUT 2.1: EXOTIC OR NOVEL GENES AND GENE COMBINATIONS MADE ACCESSIBLE FOR BROADENING THE GENETIC BASE OF CULTIVATED GENE POOLS

### 2.1.1 A novel approach for the identification and utilization of genes from wild *Oryza* germplasm.

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Much concern and high priority has been given to increasing the yield potential and broadening the genetic base of cultivated rice. Different approaches (hybrid rice,recurrent selection, new plant type, etc) are being used by several groups to address these issues. However, another alternative, already proven to work well in tomato, offers a great potential for rice. This breeding strategy makes use of wild germplasm, a backcrossing scheme, and molecular markers for the genetic improvement of cultivated rice. Molecular markers will allow the identification of "positive" alleles (QTLs) from the wild species in early segregating generations and the introgression of these positive alleles into selected improved cultivars via marker-aided selection. The main goal is to implement a breeding strategy for the systematic discovery and transfer of genes associated with yield and other agronomic traits of economic importance from wild germplasm into cultivated rice.

Progress Report. Starting in 1994, single crosses between seven improved irrigated cultivars (Bg-90-2, *Oryzica Llanos* 5, *Oryzica* 3; *Morelos* A-88, *Lemont, Cypres, and Jupiter),* five upland cultivars *(Caiapo, Progresso,* CT6196-33-11-1-3, *Oryzica* Sabana 6, and *Oryzica* Turipana 7), and three wild rice species (*O. rufipogon, O. barthii, and O. glaberrima*) were made (Table 1). $F_1$ plants were backcrossed to the improved rice cultivar and approx. 100-180 $F_1BC_1$ seeds were produced. Based on field performance and genetic potential three cross combinations, mainly BG90-2/*0 rufipogon, O. Llanos 5/ O. rufipogon,* and *Caiapo / O. rufipogon,* were chosen for the next backcrossing. Best F1BC1 plants (40-50) were identified using negative selection against obvious undesirable traits (spreading plant type, excessive shattering, long awn, dark color grains, high sterility) and used for the second backcross to the improved cultivar. Approx. 900-1000 $F_1BC_2$ seeds were produced per cross combination; the F1BC2 generation was evaluated under field conditions early in 1996 and again negative selection against undesirable agronomic traits was applied. Around 300 $F_1BC_2$ plants were selected per cross combination and harvested individually; $F_2$ seed was used to plant replicated yield trials is four locations (CIAT-Palmira and Santa Rosa, la Libertad Exp. Station in Villavicencio, and Saldaña. Tolima) including the parents, $F_1$ and commercial standard varieties as checks. These yield trials are under way; however, field observations suggest that in the case of Bg90-2/ *O. rufipogon* there are several

$F_2BC_2$ progenies that look superior to Bg90-2 in terms of yield potential and grain length. In all three cross combinations, all F2BC2 families are very similar to the recurrent parent and show very little segregation. Molecular characterization of the parents, $F_1$, and F1BC1 was already done while molecular characterization of the F2 BC2 families is underway. This molecular data together with data on 12 agronomic traits (including yield) will be used to identify F2BC2 families carrying positive alleles from the wild parent to continue the backcrossing scheme.

**Table 1**. Inter-specific crosses made between several improved irrigated and upland rice cultivars, and three wild species of rice.

| | |
|---|---|
| **O. rufipogon /** | BG90-2 // 2 * BG90-2 (3)<br>MORELOS A88 // 2* MORELOS A88 (3)<br>ORYZICA 3 // 2* ORYZICA 3 (3)<br>ORYZICA LLANOS 5 // 2* ORYZICA LLANOS 5 (3)<br>LEMONT // LEMONT (2)<br>RU94030006 // RU94030006 (2)<br>CYPRESS // CYPRESS (2)<br>ORYZICA SABANA 6 // 2 * ORYZICA SABANA 6 (3)<br>ORYZICA TURIPANA 7 (1)<br>PROGRESSO (1)<br>CT6196-33-11-1-3 (1)<br>CAIAPO // 2* CAIAPO (3) |
| **O. barthii /** | BG90-2 // BG90-2 (2)<br>MORELOS A88 // 2* MORELOS A88 (3)<br>ORYZICA 3 // 2 * ORYZICA 3 (3)<br>ORYICA LLANOS 5 // ORYZICA LLANOS 5 (2)<br>LEMONT // LEMONT (2)<br>RU94030006 // RU94030006 (2)<br>CYPRESS // CYPRESS (2)<br>ORYZICA SABANA 6 // ORYZICA SABANA 6 (2)<br>ORYZICA TURIPANA 7 // ORYZICA TURIPANA 7 (2)<br>PROGRESSO (1)<br>CT6196-33-11-1-3 // CT6196-33-11-1-3 (2)<br>CAIAPO (1) |
| **O. glaberrima /** | BG90-2 // BG90-2 (2)<br>MORELOS A88 (1)<br>ORYZICA 3 // ORYZICA 3 (2)<br>ORYZICA LLANOS 5 (1)<br>LEMONT (1)<br>RU94030006 // RU94030006 (2)<br>CYPRESS (1)<br>ORYZICA SABANA 6 // ORYZICA SABANA 6 (2)<br>ORYZICA TURIPANA 7 // ORYZICA SABANA 7 (2)<br>PROGRESSO (1)<br>CT6196-33-11-1-3 (1)<br>CAIAPO (1) |

Remarks :

(1) = SINGLE CROSS MADE

(2) = FIRST BACKCROSS MADE; IN SOME CASES SECOND BACKCROSS UNDERWAY

(3) = SECOND BACK CROSS MADE; IN SOME CASES REPLICATED YIELD TRIALS WITH F2BC2 PROGENIES UNDERWAY

## 2.1.2 Making useful traits available to Phaseolus vulgaris through interespecific hybridization with P. acutifolius

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This research started in 1989 and so far has resulted in the production of large numbers of fertile interespecific hybrids for field evaluation and selection of agronomically desirable lines;

The progress achieved so far is a consequence of solving the following barriers to gene transfer between *P. acutifolius* and *P. vulgaris*:

(i) Identification of non-carrier genotypes of incompatibility factors, as "facilitator" parentals particularly for the first interespecific crosses. The common bean cv Ica Pijao and the tepary cv G40001 were used as facilitators of interespecific hybridization.

(ii) Design and application of a two-step *in vitro* culture technique of rescued embryos, to avoid embryo abortion, especially after 18-20 days from pollination.

(iii) Identification of hybrid seedlings at an very early stage using Diaphorase iso-enzyme fingerprinting.

(iv) Increasing introgression of tepary bean traits through congruity backcrossing. (Haghigi and Ascher, 1988). Congruity backcrossing also allowed to increase fertility and reduce the requirement of embryo rescue in advanced hybrids (Mejia-Jimenez, et al, 1994).

### Progress in 1996

1. Interespecific congruity back crosses between *P. vulgaris* and cultivated and wild *P. acutifolius* genotypes continued along two avenues: (i) congruity backcrosses continued from the 8th to the 10th cycle using the tepary bean cv G40001 (resistant to bacterial blight); (ii) in order to improve the level of bacterial blight resistance on the one hand, and to add new sources of resistances, additional tepary genotypes were introduced into congruity backcrossing: cv G40022 (resistant to bacterial blight), cv G40035 (resistant to Empoasca and bacterial blight); wild genotypes G40102 (Empoasca, *Acanthoscelides*, obtectus), G40253 (resistant to A. obtectus and bacterial blight), G40054 (transformed with Arcelin 5 gene).

2. The aim of crossing common bean (*Phaseolus vulgaris*) with tepary bean (*P. acutifolius*) was expanded from that of transferring high levels of resistance to common bacterial blight to those of transferring genes(s) for leafhopper and bruchid resistance and transformation capacity. Tepary accessions G40035, G40036, G40054, G 40102, G40199, and G40253 were therefore hybridized, succesfully, with common bean cultivar ICA Pijao. A separate congruity backcrossing program between ICAPijao and each tepary accession was initiated. Progenies from other congruity backcrosses involving tepary accession G40001 were already advanced.

3. From the initial recurrent backcrosses of common bean with tepary beans, two lines -- VAX 1 and VAX 2 -- with comparatively high levels of resistance (Table 1) were developed after 6 years of intensive field evaluation and selection among several thousand progenies. When the common blight reaction of a resistant tepary bean (e.g., accession G40001) was compared with that of VAX 1 and VAX 2, it was clear that not all the resistance genes found in the tepary beans were transferred to the two lines. Pyramiding resistance from various species and sources seems to be the next most promising step for CBB resistance breeding (Table 1).

4. Congruity backcrossing with runner bean (*P. coccineus*) accessions G 35170 and G35171, both immune to the bean golden mosaic virus (BGMV), was also continued. The $F_2$ and $F_3$ progenies obtained from the initial crosses and congruity backcrosses were grown in the field at CIAT- Quilichao to advance generation and fixation of traits.

5. One accession of *P. costarisensis* was crossed with ICA Pijao without using embryo rescue. This accession did not flower for the few years it grew at CIAT-Popayan. But when Dr. Daniel Debouck took cuttings from Popayan to Tenerife, it flowered within less than a year. Thus, taking advantage of its flowering, crosses were made with ICA Pijao. But, so far, all $F_1$ hybrids have had problems. Seedlings and young plants have either been very weak or died. We do not yer understand why. Attempts are being made to grow the $F_1$ hybrids in a cooler environment and to induce rooting to make cuttings for multiplication. Neither do we know the complementary attributes of the *P. costaricensis* accession.

**Plans for 1997**

The common bean molecular maps generated at the Univs of California and Florida have been merged (Gepts, Univ. of Calif., Davis). Markers from the merged map can be used for monitoring the introgression of chromosomal segments from tepary into congruity generations.

This approach will provide a quick and secure way for the selection of introgressed lines and thus accelerate the production of hybrids.

*In situ* DNA hybridization can also contribute towards the same objective.

**Table 1**. Comparison of reaction to Xanthomonas campestris pv. phaseoli (Xcp) of common bean lines with pyramided resistance and those obtained from interespecific crosses of P. vulgaris x P. acutifolius, evaluated at CIAT-Quilichao in 1996

| | Xcp reaction[1] | |
|---|---|---|
| Identification | Razor blade | Aspersion |
| **From Pyramided resistance** | | |
| PVPA 9576-14-1 x XAN 309 | 1.4 | 2.2 |
| PVPA 9576-14-2 x XAN 263 | 1.5 | 2.0 |
| PVPA 9576-14-1 x XAN 310 | 1.8 | 2.0 |
| **From P. vulgaris x P. acutifolius** | | |
| VAX 1 | 3.0 | 2.3 |
| VAX 2 | 2.8 | 2.5 |
| Checks | | |
| ICA Pijao [2] | 8.4 | 7.7 |
| G 40001[3] | 1.3 | 2.7 |
| LSD (0.05) | 0.8 | 1.3 |

1. Mean of five evaluations in each of three replicates on a 1 to 9 scale where 1-3= resistant, 4-6 = intermediate, and 7-9 = susceptible.
2. Susceptible *P. vulgaris*
3. Resistant *P. acutifolius*

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## 2.1.3. Control of RHBV through Coat Protein Mediated Cross Protection and Anti-Sense RNA Strategies.

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Background. Rice hoja blanca virus (RHBV) is present throughout much of tropical America (Morales and Niessen, 1983). When epidemics occur, yield losses may reach 100% since there is limited distribution of the varieties that are resistant to both the vector and the virus. The planthopper insect Tagosodes oryzicola (Muir) is the vector of RHBV and also a serious pest of rice that causes direct damage. Some very popular varieties are resistant to the vector but are susceptible to the virus. The uncertainty of epidemics induces farmers to spray up to 5-6 times to control this planthopper vector of RHBV. There is a need to incorporate additional sources of resistance into improved germplasm to ensure stable and durable resistance, since the resistance is from a single resistant source. Moreover, the source of resistance does not confer immunity in most commercial varieties. Therefore, farmers start applying insecticides even when about 10% of the plants from resistant cultivars start showing RHBV symptoms.

The main goal of this project is to provide new source(s) of resistance to minimize the possibility of an outbreak of the disease by (i) transforming rice with novel gene(s) for RHBV resistance; and (ii) incorporating these genes into Latin American commercial varieties or into genotypes to be used as parents in breeding. RHBV is a member of the tenuivirus group. The molecular characterization of RHBV and the preparation of cDNA libraries (Ramirez et al., 1992 and 1993) has led to the design of novel virus-resistant strategies to genetically engineer commercially-grown rice cultivar. Two different strategies are being attempted: a) the nucleocapside (NC) cross protection and b) the antisense-gene down regulation of the major NS4 protein. The NC-mediated cross protection has been successful for the tenuivirus RStV (Hayakawa et al., 1992). The strategy for the expression of the RNA4 is to determine the function of the major NS4 protein and study the potential for a different method of producing viral resistant plants. The down regulation of this protein may be a novel method of producing virus-resistant plants by breaking the cycle of transmission.

Progress Report. The direct deliver of genes into immature embryos or immature panicle-derived calli is conducted using DNA-coated gold particles accelerated by the PDS-1000/He system. The tropical irrigated Latin American *indicas* varieties Oryzica 1, Cica 8, and Inti and the tropical upland japonica line CT 6241-17-1-5-1 are used as targets. Constructs containing the RHBV-NC or the antisense RHBV-NS4 genes driven by the 35S CaMV promoter are being used. The 35S CaMV - hph gene is used as the selective marker. The putative transgenic events are recovered using a step-wise selection on culture medium containing 30 mg/l hygromycin B (hyg B) followed by 50 mg/l hyg B throughout plant regeneration (Li et al., 1993). With this system one $Hyg^r$ plant line might be recovered from 2 to 33 explants initially bombarded depending on the genotype. Single or multiple copies of the transgenes are noted. Segregations of 3:1 among offspring of transgenic plants are recovered, indicating Mendelian inheritance from single genetic locus of a functional hph gene. But also some transgenic plants showing skewed segregation patterns are

obtained. Similar results had been reported in transgenic rice (Cooley et al., 1995; Christou, 1995). Possible interpretation of these results may include the linkage of the transgene with semidominant or dominant lethal mutations, inactivation of the transgene by methylation, and/or excision of the transgene from the genome (Hayakawa et al., 1992). The co-transformation rate for two unlinked transgenes is from 30% to 60%. Detailed data on the efficiency of recovering transgenic plants, and the stability of expression and inheritance of the transgenes introduced using this methodology can be found in the Annual Reports of 1995 and 1994.

After the complete step-wise selection process throughout plant regeneration on 50 mg/l hyg B, a total of 165 plants from the antisense RHBV-NS4 and 187 plants from the RHBV-NC bombardments had been recovered. Southern blot of genomic DNA and Northern blot of the plants recovered from the antisense RHBV-NS4 bombardments indicated that 2 of these plants (1.2 %) contain and express the antisense-RNA4 gene. The identification of transgenic plants that express the RHBV antisense may allow for the analysis of the affect of the major non-structural gene and to determine the down regulation of this viral gene confers resistance to RHBV.

Sixty of the 187 (32%) plants recovered from the RHBV-NC experiments contain the RHBV gene. In all cases, larger NC fragments than the expected length were visualized on the Southern blots suggesting the presence of rearrangements. Apparently, a variety of integration patterns had been obtained in other works specially when circular plasmid is used (Hayakawa et al., 1992). Therefore, currents experiments include the linearization of the expression vector before bombardment.
Nineteen T0 plants showing integration of the RHBV-NC gene as indicated by Southern blots, were analyzed for inheritance of the hph resistance and RHBV-NC genes by genetic and molecular analyses of the transgenic T1 progeny. Genetic analyses were conducted by evaluating the resistance to hygromycin of T1 seeds germinated *in vitro*. Five of the nineteen plants did not inherited hygromycin resistance (ratio 0:1 resistant: suceptible) in the T1 progeny (Table 1). About 58% of the To lines showed a skewed segregation of 1:1 (resistant: suceptible) and whereas 16% showed a segregation of 3:1 indicating the inheritance of a single active locus (Table 1). A sample of 9 plants including 1:1 or 3:1 segregations ratios were analyzed by Southern blots. The two lines that showed a 3:1 ratio for hygromycin resistance, also showed a 3:1 ratio for the presence of the hph and RHBV-NC genes in the T1 progeny, confirming the inheritance of a single active locus for the transgenes. However, those lines showing a 1:1 or 0:1 (resistant:susceptible) ratio for hygromycin resistance showed segretations of 1:0 (homozygous) or 3:1 (heterozygous single locus) for the integration of the transgenes in the genome of the plants suggesting that the skewed segregations noted for hygromycin resistance is probably due to inactivation of the hph gene expression
(Table 1). Based on the inheritance analyses, six plants were choosen (Table 1) and evaluated for RHBV resistance under biosafety greenhouse conditions. Twenty five day-old T1 plants of each T0 line choosen, were infected with proven viruliferous plant hoppers, two per plant. Plants were enclosed within a plastic tube and the insects were allowed to feed on the plants for 5 days to ensure a high pressure of infectation. Plants were scored for the presence of the RHBV disease symptoms every two days for 25 days. At 40 day-old plants were evaluated for the level of the disease reaction with a scale of 1 (dead plant), 2 (diseased, low vigor); 3 (diseased, intermediate vigor); 4 (vigor, some tillers free of disease symptoms); and 5 (healthy plant, no disease symptoms); and leaf tissue were analyzed by ELISA using an antibody specific for the RHBV-RNA4 to detect the presence of the virus particles.

Preliminary evaluations tests showed 8 T1 lines derived from the A3-49 T0 line with attenuated disease symptoms, and increased performance for various agronomic traits respect to the non-transgenic control infected with RHBV (Table 2). These results are promising, however to clearly determine if these plants are true resistant, selfed progeny seeds (T2 generation) of each of these T1 lines will be evaluated for RHBV resistance and for expression and integration of the RHBV-transgene to confirm the inheritance and stability of the RHBV transgenes. Currently, the generation of transgenic plants carrying various versions of the RHBV-NS4 and RHBV-NC sense and antisense to modulate different levels of the RHBV transgene expression is in progress. Future work includes the genetic and molecular characterizarion of these plants jointly with the RHBV resistance evaluations to determine the efficiency of the different strategies to confer protection to the virus.

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**Table 1**. Inheritance of the hph resistance and RHBV-NC genes by genetic and molecular analyses of the transgenic T1 progeny.

| T0 line | Hygromycin resistance[1] observed ratio R:S | $X^2$ | $p$ | Southern blot[2] observed ratio Present:absent | $X^2$ | $p$ |
|---|---|---|---|---|---|---|
| A3-48 | 1:1 | 0.40 | 0.53 | 3:1 | 0.05 | 0.83 |
| A3-49* | 1:1 | 0.34 | 0.56 | 1:0 | ------ | ------ |
| A3-50 | 1:1 | 0.69 | 0.41 | 1:0 | ------ | ------ |
| A3-57* | 3:1 | 0.13 | 0.72 | 3:1 | 0.01 | 0.90 |
| A3-58* | 1:1 | 0.29 | 0.59 | 3:1 | 0.05 | 0.83 |
| A3-59 | 0:1 | ------ | ------ | NE | | |
| A3-60 | 0:1 | ------ | ------ | NE | | |
| A3-61 | 0:1 | ------ | ------ | NE | | |
| A3-64 | 1:1 | 0.82 | 0.37 | NE | | |
| A3-72 | 0:1 | ------ | ------ | NE | | |
| A3-74 | 1:1 | 0.69 | 0.41 | NE | | |
| A3-75 | 0:1 | ------ | ------ | 3:1 | 0.00 | 1.00 |
| A3-76 | 1:1 | 0.82 | 0.37 | NE | | |
| A3-77* | 3:1 | 0.05 | 0.83 | NE | | |
| A3-78* | 1:1 | 0.53 | 0.47 | 1:0 | ------ | ------ |
| A3-81 | 1:1 | 0.09 | 0.76 | NE | | |
| A3-83 | 1:1 | 0.34 | 0.56 | 3:1 | 0.43 | 0.52 |
| A3-84 | 1:1 | 0.29 | 0.59 | NE | | |
| A3-85* | 3:1 | 0.67 | 0.41 | 3:1 | 0.11 | 0.74 |

[1] Twenty T1 seeds analyzed per T0 line. [2] Ten plants analyzed per T0 line, except for A3-57 where 23 plants were assayed. * Lines choosen for RHBV resistance tests. NE= not evaluated.

**Table 2.** Evaluations of T1 progeny plants from line A3-49 for RHBV resistance and agronomic traits under greenhouse conditions

| Line A3 | | Flowering (days) | Height (cm) | Tillers | Flowers/ panicle | Grains/ Plant | Fertility (%) | Disease reaction | ELISA (units) |
|---|---|---|---|---|---|---|---|---|---|
| 49-27 | | 121 | 55 | 8 | 67 | 110 | 23 | 4 | 0.568 |
| 49-34 | | 127 | 55 | 44 | 54 | 5 | 9 | 3 | 0.153 |
| 49-37 | | 124 | 62 | 23 | 56 | 40 | 3 | 5 | 0.071 |
| 49-39 | | 126 | 57 | 15 | 63 | 287 | 22 | 4 | 0.372 |
| 49-56 | | 126 | 86 | 12 | 83 | 696 | 70 | 4 | 0.558 |
| 49-60 | | 133 | 66 | 12 | 114 | 396 | 32 | 4 | 0.787 |
| 49-75 | | 125 | 78 | 15 | 67 | 147 | 16 | 3 | 0.152 |
| 49-101 | | 113 | 78 | 14 | 71 | 376 | 28 | 4 | 0.432 |
| Control | | | | | | | | | |
| Infected | mean | 141 | 66 | 5 | 53 | 0 | 0 | 2 | 0.440 |
| | Sd | 29 | 15 | 4 | 55 | 0 | 0 | 1 | 0.111 |
| Non-infected | mean | 117 | 83 | 15 | 106 | 1594 | 87 | 5 | 0.005 |
| | Sd | 11 | 3 | 8 | 18 | 454 | 8 | 0 | 0.001 |

## OUTPUT 2.2. KNOWLEDGE GENERATED ON MECHANISMS OF GENETIC VARIABILITY OF PLANT RESPONSE TO BIOTIC/ABIOTIC STRESS.

### 2.2.1 Molecular Basis of Resistance to the Bean Weevil Acanthoscelides obtectus

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The bean weevil (*Acanthoscelides obtectus*) is a major pest of stored beans in the Americas and in Africa. It has not been possible so far to transfer resistance genes, naturaly found in some wild Mexican *Phaseolus* spp, into commercial cvs. Inheritance seems to be complex.

The main objective of this project is to search for gene products that differ between resistant and susceptible genotypes using differential display. For this purpose, we are using a novel technique by which differential amplification of cDNA sequences is generated with a random PCR approach (Liang et al, 1992). Recently a non radioactive protocol for this technique in mammalian cells was described (Sokolov et al, 1994).

Progress Report. We have implemented the non-radioactive technique for *Phaseolus* during pod filling stage. Total RNA has been isolated from different genotypes resistant and susceptible to *Acanthoscelides obtectus* belonging to *P. acutifolius* and *P. lunatus*. Genotypes chosen for the assay were from Brazil in the case of *P. lunatus* and from Mexico in the case of *P. acutifolius*.

In order to minimize differences between transcripts from different genotypes, total RNAs were bulked into two groups: RNA from resistant and RNA from susceptible genotypes from each species. Poly A+ RNA was isolated by chromatography using oligodT cellulose columns and mRNA isolated was quantified and used in reverse transcription (RT)-PCR experiments. For RT-PCR assays, primers used for first strand synthesis were hexanucleotides as suggested previously (Sokolov et al, 1994). The second strand synthesis was carried out using random primers of 10 nucleotides in length. The PCR products were separated in agarose gels (2%) and bands present in resistant but absent in susceptible samples have been isolated from the gel, reamplified and are ready to be cloned in pCRII plasmid.

So far, 21 bands have been isolated and are being cloned in order to be used as probes in Northern hybridization experiments. Specific clones, only hybridizing with RNA isolated from resistant genotypes, will be subjected to more analysis as temporal expression occurs during pod-filling; subsequently these will be sequenced.

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## 2.2.2 Mechanisms of acid soil tolerance in Brachiaria

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The aim of the project is to get insights into mechanisms of acid soil tolerance of three *Brachiaria* species which are being used as parents in a breeding program at CIAT: *B. decumbens* cv. Basilisk (well adapted), *B. brizantha* cv. Marandú (less persistent), and *B. ruziziensis* cv. Common (poorly persistent).

The so-called "acid soil syndrome", a multiple edaphic stress situation consisting of Al toxicity and low availability of essential nutrients such as N, P, K, Ca, and Mg, was simulated by low ionic strength hydroponic solution cultures using GEOCHEM 2.0 model. Main treatments were: a control solution *(co)* that contained sufficient nutrients and a multiple stress solution *(st+Al)* that composed of low levels of N, P, K, Ca, and Mg in combination with a high Al concentration ($\{Al^{3+}\}$ = 44 mM). To separate the effects of low nutrient stress and Al-toxicity an Al-containing control solution *(co+Al)* and a nutrient stress solution without Al *(st)* were used as well. Relative growth in the *st+Al*treatment with respect to the *co* treatment was in the same order as acid soil persistence under field conditions (BRU Annual Report, 1995). Using these four different treatments, several experimental approaches are being pursued: (i) evaluation of plant growth responses to nutrient supply and Al toxicity with a special focus on root system architecture, (ii) measurement of activities of two key enzymes involved in nutrient uptake, (iii) mapping of nutrient distribution in root tips on a micro-scale, (iv) characterization of plant metabolites such as phenolic secondary compounds and organic acids, which could be relevant to nutrient acquisition and Al-detoxification, and (v) isolation of acid soil stress-induced genes.

Progress Report. (i) *Plant growth responses to nutrient supply:* Plants were grown in a series of solutions with decreasing levels of a single nutrient or increasing levels of Al. Several shoot and root growth characteristics including shoot biomass, leaf area, root biomass, total root length, root diameter distribution, root system topology (magnitude, total exterior path length, altitude), and root system fractal dimension were measured. Root parameters were evaluated on scanned images of root systems with the program "WinRHIZO" which helped to characterize more closely the interspecific differences in adaptive phenotypic plasticity of the root system as influenced by low levels of certain essential nutrients in culture medium.

(ii) *Key enzymes involved in nutrient uptake and utilization:* The $H^+$-translocating ATPase of the plasma membrane plays a key role in plant nutrition because it creates an electrochemical proton gradient that is used as driving force for nutrient uptake. Plants were grown under four treatment conditions described above and root plasma membranes were extracted and purified by means of a two-phase partition method. Studies with specific inhibitors of the $H^+$-ATPase as well as of other enzymes from other membranes revealed a high degree of purity. Measurements on enzyme kinetics

yielded the following results: (i) Al stress - under high and low nutrient supply *(co+Al, st+Al)* - increased the speed of proton transport across the plasmamembrane, as monitored by the $v_{max}$ value of ATP hydrolysis, with the notable exception of *B. ruziziensis* grown under *st+Al* conditions; (ii) the affinity of the enzyme towards ATP (described by its $K_m$ value) decreased under Al stress for both *B. decumbens* and *B. ruziziensis* with the exception of *B. ruziziensis* grown in the *st+Al* solution where it remained high; and (iii) the $H^+$-ATPase of *B. brizantha* was generally less responsive.

Root acid phosphatases play an important role in the intra- and extracellular mobilization of organically bound P reserves under P-deficient conditions. Acid phosphatase activity was measured in root extracts of hydroponically grown plants. Results indicated that: (i) Al stress could induce a 5 to 10-fold increase of specific activity of the enzyme in all three species; (ii) low nutrient stress could trigger a 5-fold increase of specific activity in *B. ruziziensis* but not in the other two species; and (iii) the root acid phosphatase of *B. decumbens* plants grown under Al stress became more Al tolerant than that of those that were grown without Al, a reaction that was not observed with the other two species (Table 1).

**Table 1**. Characteristics of the root acid phosphatase from three *Brachiaria* species.

| | ***B. ruziziensis*** | | ***B. decumbens*** | | ***B. brizantha*** | |
|---|---|---|---|---|---|---|
| Treatment | Specific activity[a] | Al inhibition[b] | Specific activity | Al inhibition | Specific activity | Al inhibition |
| **co** | 20±1 | 81±3 | 17±1 | 60±0 | 20±3 | 88±4 |
| **co+Al** | 74±2 | 88±5 | 183±2 | 20±2 | 96±2 | 84±1 |
| **st** | 105±1 | 52±2 | 26±2 | 74±3 | 29±5 | 79±2 |
| **st+Al** | 93±2 | 64±2 | 91±0 | 42±1 | 112±2 | 85±2 |

[a] in nmole substrate•mg protein$^{-1}$•h$^{-1}$
[b] in %, 1 mM $AlCl_3$

(iii) *Nutrient mapping in root tips:* Nutrient uptake is generally considered to be specially active in root tips. Differences in uptake mechanisms or preferences towards certain elements, e.g. in the context of the anion-cation balance, could result in different levels and/or distributions of nutrients in root tip tissue. In order to map these changes, seminal root tips of plants grown under four different treatment solutions were lyophilized and scanned with "Proton Induced X-ray Emission" (PIXE) with a spatial resolution at 5 $\mu$m. The resulting 2-dimensional maps of Al, P, Ca, K, Mg, Si, S, Cl, and Fe are being analyzed.

(iv) *Characterization of plant metabolites:* A series of phenolic, secondary compounds have been found to act as signaling molecules for the initiation and functioning of plant-microbe symbioses (symbioses with N-fixing bacteria and arbuscular-mycorrhizae)which play a key role in nutrient acquisition from infertile soils. Thus, phenolic compounds in roots of hydroponically grown plants were analyzed by reverse-phase HPLC. A dramatic accumulation (at least 10-fold) of two major compounds was observed with *B. ruziziensis* but not with either *B. brizantha* or *B. decumbens* when grown under low nutrient stress *(st, st+Al)*. This observation was consistent with previous finding that the concentration of these compounds in soil-grown plants of *B. ruziziensis* was about 5 to 10-fold greater than that of the other two species. Further research work is in progress to determine

whether lack of supply of which essential nutrient could induce this effect in *B. ruziziensis*. A purification procedure is being developed to determine the molecular structure of these two compounds. Organic acids have been shown to be involved in Al tolerance as well as in P stress response in plants. A technique for organic acid purification and HPLC analysis was optimized to permit the quantification of 11 physiologically important acids. Application of this technique to root extracts of plants grown under four different treatments clearly demonstrated that all three species accumulated citric acid under Al stress. Accumulation and/or exudation of citric acid was implicated to confer Al tolerance in maize and beans. Interestingly, citric acid was not induced by low levels of P, which suggests that the presence of Al rather than Al-induced P deficiency triggers this response.

(v) *Induction of genes under simulated acid soil stress.* Isolation of stress-induced genes provides a direct approach to characterize molecular mechanisms underlying abiotic stresses. This approach is not biased towards the supposition of certain hypotheses concerning adaptation mechanisms. Comparison of the DNA-sequences of isolated genes with known genes in databases and evaluation of their inducibility by different stress factors can yield valuable information with respect to their role in acid soil adaptation. Since the offspring of an interspecific cross between the best and the less-adapted species (*B. decumbens* x *B. ruziziensis*) shows a pronounced heterosis effect, it cannot be used to isolate genes involved in acid soil adaptation by means of a segregating population-based approach. Thus, a subtractive hybridization technique is being applied to isolate genes that are specifically induced in *B. decumbens* but not in *B. ruziziensis* under simulated acid soil stress *(st+Al)* when compared to control conditions *(co)*. In order to obtain full-length clones, a number of techniques including isolation of total RNA, mRNA purification, ds-cDNA synthesis on paramagnetic beads, and PCR amplification of cDNA have been currently optimized.

### 2.2.3 Resistance of cassava to Cyrtomenus bergi

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The screening of 175 low cyanide clones for resistance against the *C. bergi* was repeated during 1996. The clones had been selected from the CIAT Core Collection for their cyanide content of less than 50 ppm in fresh weight. The screening took place in Santágueda, in the province of Caldas, Colombia, an area heavily infested by *C. bergi*, on land leased from Universidad de Caldas. The clones were planted in a randomized block design of 6 replicates with each clone represented once in each replicate.

The damage level was generally higher this year compared to 1995. Fifteen common clones within the group of the 25 less damaged clones each year were selected as promising clones of resistance to *C. bergi*, (Table 1). Eleven of these fifteen clones are high yielding. The common most damaged clone both years was MCUB8, which will serve as a control in the future.

The resistance of these clones will have to be verified in a monoculture design with plots consisting of a similar clone. The mechanisms causing the apparent resistance is still a subject of speculation.

*Cyanide.*

*C. bergi* is a generalist with many host plants, which has not co-evolved with cassava. It feeds on the cassava roots, which is the part of cassava, that varies the most in terms of cyanide content. The susceptibility to *C. bergi* among 33 cassava clones (Euro Core Collection) representing a broad distribution of cyanide and starch content was tested in the field in a randomized block design with 4 replicates.

No relation was found between the level of damage caused by *C. bergi* and the starch content nor the dry matter content. When the damage level was plotted against the cyanide content the means fell within a triangle with no high damage scores in high cyanide containing clones and both high and low damage scores in low cyanide containing clones, (Figure 1). No statistical model could fit the data well. This data fit into a triangle has been seen before when testing clones with very different genetic background and too much 'noise' interfering.

**Table 1**. Fifteen low cyanide cassava clones of low damage level caused by *C. bergi* and a highly susceptible control

| Clone | Damage level* (0-6 scale) | Max. damage level | Yield CIAT (t/ha) | Cyanide content (ppm, dry weight) | |
|---|---|---|---|---|---|
| | | | | 1991 | 1993 |
| MECU21 | 0.55 ± 0.12 | 2.00 | 3.80 | 67 | 78 |
| MCOL317 | 0.66 ± 0.11 | 1.33 | 2.62 | 104 | 163 |
| MCOL1667 | 0.54 ± 0.14 | 1.07 | 16.56 | 218 | 87 |
| MBRA675 | 0.80 ± 0.11 | 1.75 | 23.93 | 59 | 175 |
| MPAN100 | 0.67 ± 0.16 | 2.00 | 2.40 | 67 | 49 |
| MCOL1389 | 0.95 ± 0.17 | 2.67 | 10.00 | 117 | 97 |
| MPER597 | 0.70 ± 0.11 | 1.69 | 10.38 | 90 | 148 |
| MPER569 | 0.90 ± 0.14 | 2.75 | 7.14 | 34 | 99 |
| MPER213 | 0.91 ± 0.11 | 3.13 | 9.34 | 92 | 131 |
| MBRA712 | 0.80 ± 0.13 | 2.78 | 3.68 | 133 | - |
| MCOL707 | 1.08 ± 0.14 | 2.60 | 14.30 | 76 | 60 |
| MPER458 | 0.80 ± 0.11 | 2.20 | 9.98 | 56 | 122 |
| CM489-1 | 1.01 ± 0.10 | 2.50 | 31.79 | 86 | 170 |
| MPER183 | 0.88 ± 0.15 | 2.40 | 23.94 | 102 | 148 |
| MCOL1185 | 1.02 ± 0.17 | 4.00 | 8.42 | 39 | 86 |
| MCUB8■ | 4.54 ± 0.12 | 5.67 | 12.44 | 105 | 139 |

*Values are means ± standard error

However, we aspired to test the effect of cyanogen in its real medium, i.e. in cassava roots, rather than an artificial medium without being able to incorporate the cyanide the same way as in the plant tissue.

Thirteen siblings from the AM264 family had appeared with varying cyanide content. Using these to test the effect of cyanogen to *C. bergi* with hope to be able to reduce the 'noise' due to more genetic similarity between the clones. The siblings were derived from a cross between a high and a low cyanide containing clone which had been selfed to segregate for cyanide levels in the roots.

Damage level caused by *C. bergi* in response to the cyanide content in the 13 sibling clones was tested in the field in a randomized block design of ten replicates. The mean damage score of commercial root (>250 gram) was plotted against the mean cyanide content of each clone and a sigmoid model was found to describe the relation, see figure 2. Despite the low damage level in general and the low cyanide level found in the clones at this field site, it was found that clones with a cyanide content less than 120 ppm was significantly higher than clones with a cyanide content above 200 ppm. Damage level of clones of a cyanide content between 120 and 200 ppm was very variable.

Reproduction of *C. bergi* in response to the cyanide content in the 13 sibling clones was tested in the laboratory using four replicates of each clone and 20 pars within each replicate. The trial lasted 80 days. Cassava roots were changed daily and the total and the free cyanide content was measured before the root parenchyma was exposed to the insects. The mean of the accumulated oviposition per female during 80 days was plotted against the mean cyanide content of each clone and a sigmoid model was found to describe the relation, (Figure 3). Oviposition on clones with a cyanide content less than 120 ppm was significantly higher than clones with a cyanide content above 200 ppm. Oviposition on clones of a cyanide content between 120 and 200 ppm was very variable.

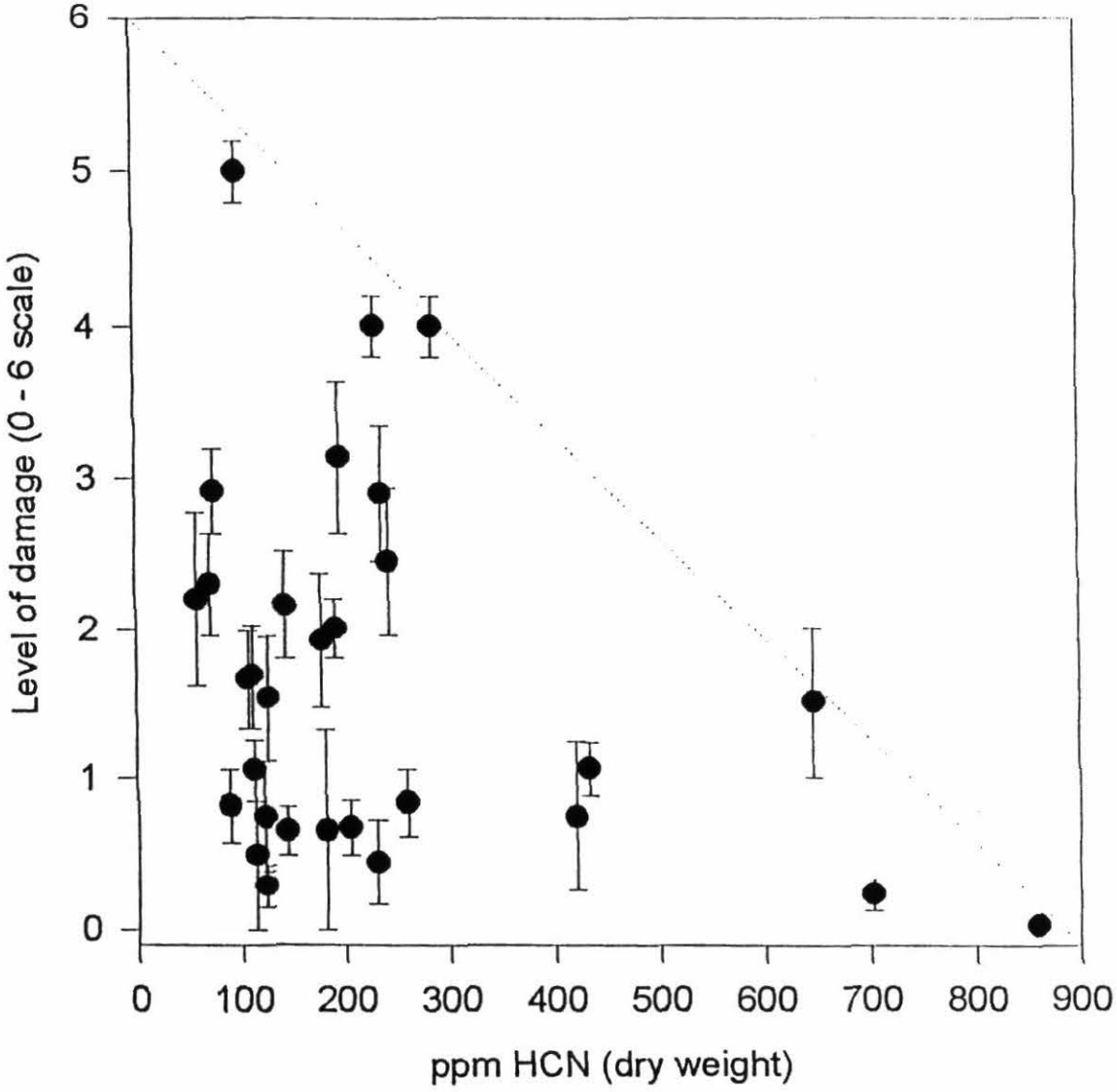



**Figure 1**. Damage level caused by *Cyrtomenus bergi* in commercial cassava roots (>250g) of 33 varieties (Euro Core Collection) in response to cyanide content (HCN) in the root parenchyma.

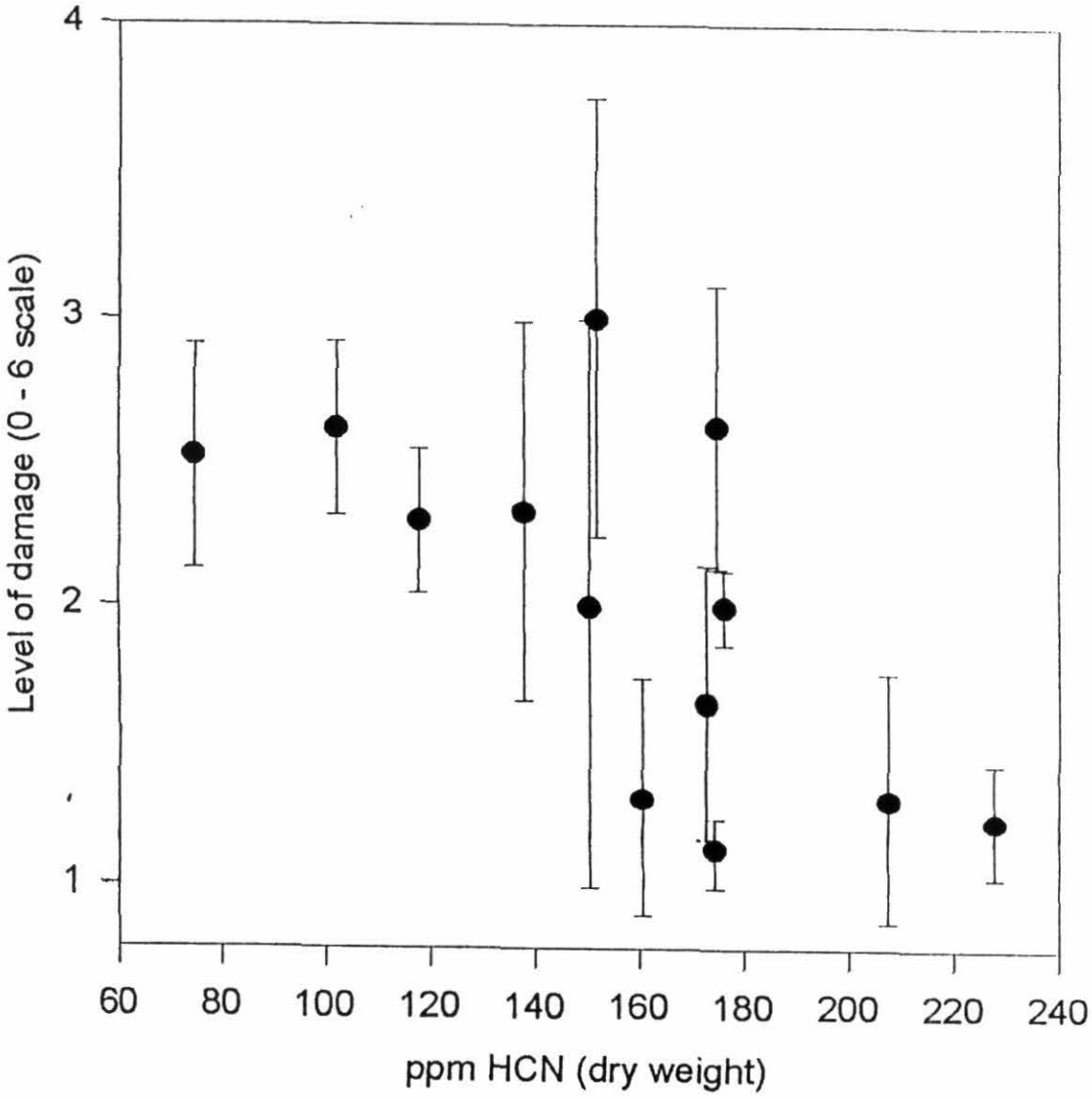



**Figure 2.** Damage level caused by Cirtomenug bergi in commercial cassava roots (>250 gram) of 13 sibling of the family AM264 as a function of cyanide content (HCN) in the root parenchyma.

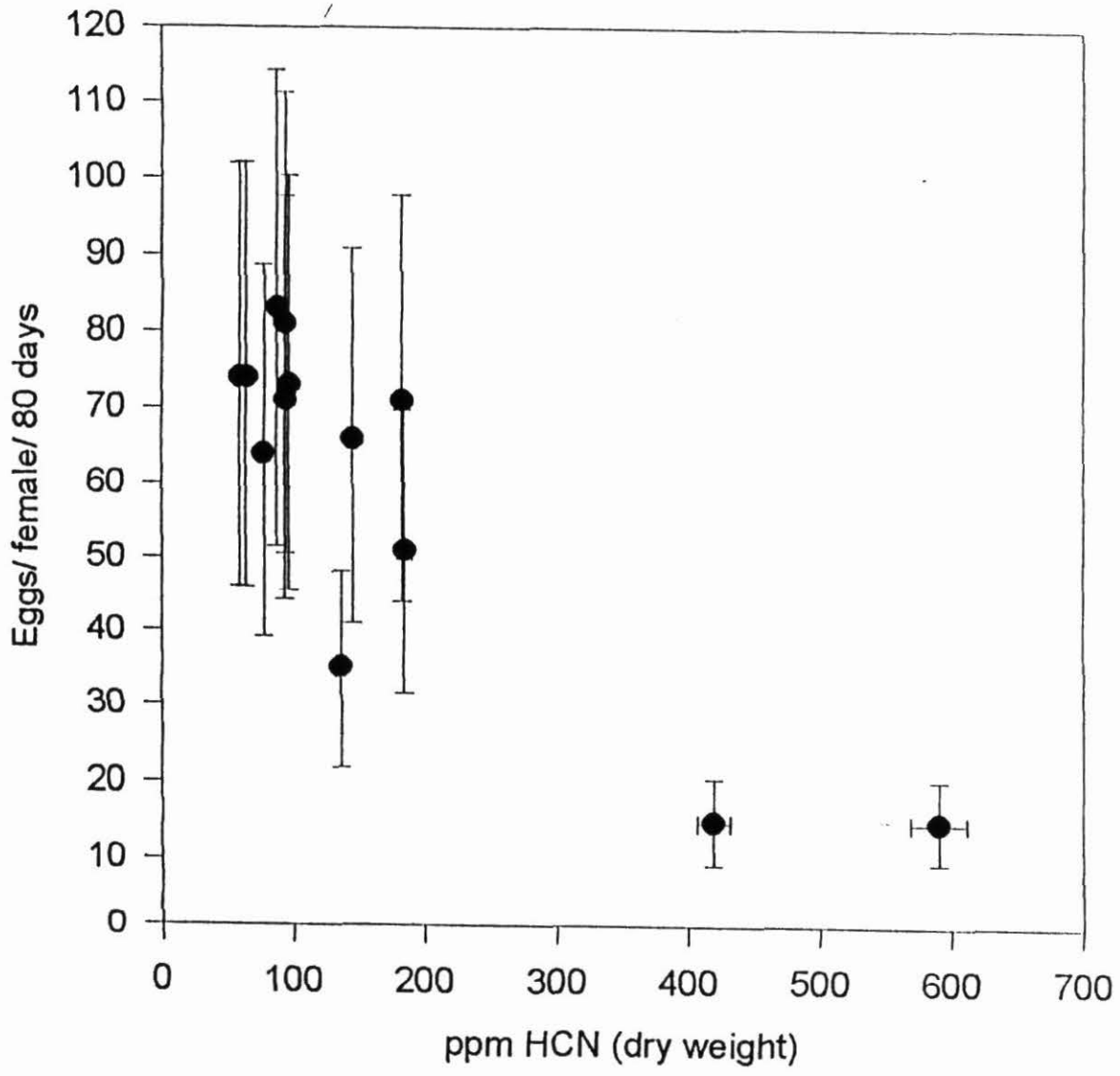



**Figure 3.** Oviposition per female during 80 days while exposed to cassava roots of 13 sibling of the AM264 family as a function of cyanide content (HCN) in the root parenchyma.

## 2.2.4 Exploring the genetic potential and stability of carotene content in cassava roots

C. Iglesias[1], A.L. Chaves[2] and F. Calle[2]
1Cassava Program; 2. Biotechnology Research Unit



Improving the efficiency with which cassava acquires micronutrients and accumulates them in the roots and leaves, can have an enormous potential not only in terms of human nutrition, but also in terms of crop production. An strategy to approach this problem is to enhance the micro-nutrient status of cassava through plant breeding.

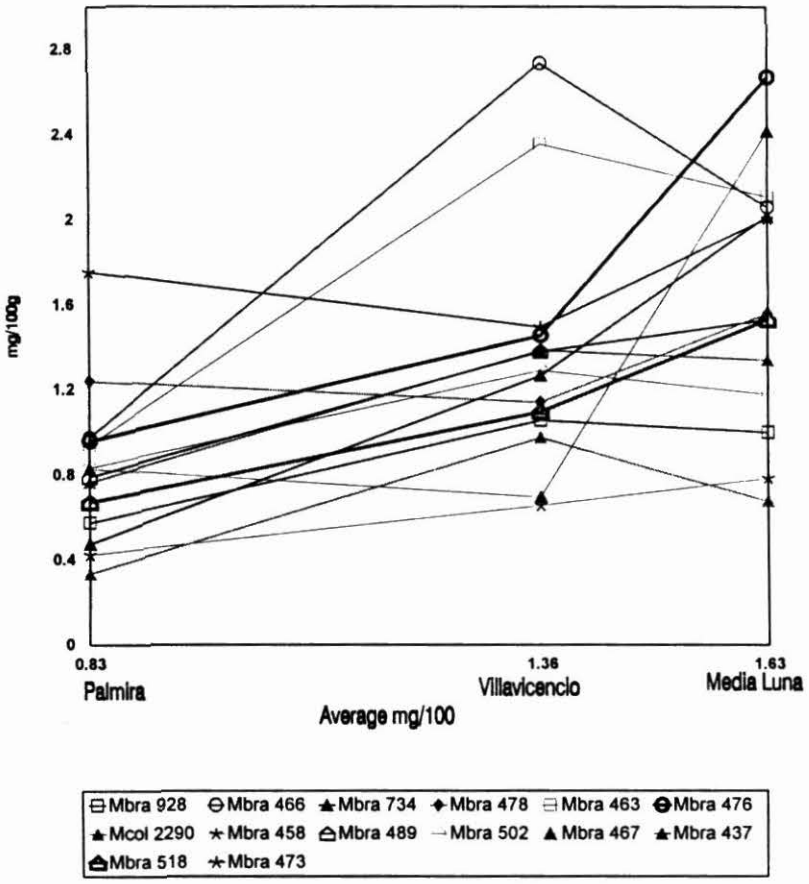
The activities carried out here included: (i) quantification of total carotenes content on fresh roots of 14 selected genotypes planted in three differents regions (Palmira, Villavicencio and Media Luna); (ii) evaluation of the effects of enviromental variation on carotenes content in the selected genotypes; (iii) determination of the environments with greater potential for expression of the trait.

Progress Report. Previous work demonstrated the feasibility of breeding cassava for high carotene content the roots and maintaining those high levels in the final products after processing ( Iglesias, et al., 1996; Euphytica, in press). A group of 14 selected genotypes was recently evaluated in three different ecosystems: sub-humid (CIAT, Palmira)), acid soil savanna (Villavicencio) and mid altitude (Media Luna) in order to study the stability of carotene concentration in front of environmental changes.

There were significant differences ($P<0.001$) among evaluation sites. Average carotene concentration for all the evaluates genotypes (See Figure 1) in the sub-humid ecosystem was double (1.63 mg/100g of roots) the one observed in the more fertile mid-altitude site (0.82 mg/100g). With the acid-soil savanna ecosystem falling in-between (1.35 mg/100g).. Although there were significant interactions with the site of evaluation (particularly for 5 of the evaluated genotypes), testing in the site with the greatest expression for the trait (sub-humid ecosystem) will result in a more precise screening, a broader expression range and a higher heritability than in other environments. Sub-humid tropics is the most important ecosystem for cassava production in Africa and Latin-America.

A yellow root gene pool is being formed by the recombination of selected parental genotypes, with the purpose of starting a recurrent selection program for increased carotene content. such populations will also be transferred to Africa, where adatative selection can lead to the identification of suitable genotypes in areas of high demand for yellow-root cassava (i.e. Ghana).

**Figure 1:** Interaction by genotype for root carotene content




Future Work. We are in the process of screening leaves of selected genotypes with high carotene content of CIAT's germplasm collection in order to correlate it with the results obtained with cassava roots.
We wil also characterize the genetic diversity within cultivated cassava with respect to vitamin C content.

## References

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Biacs, Peter A., and Daood Hussein G. J. Plant Physiol. 143 (1994) 520-525.

## 2.2.5 The effects of pre-harvest pruning of cassava upon post-harvest physiological deterioration (PPD) potential, scopoletin and dry matter contents.

G.M. O'Brien[1], Q. van Oirschot[1], O. Orozco[1], A.L. Chaves[2]
1. Cassava Quality; 2. Biotechnology Research

Plants of two cassava cultivars, one susceptible to PPD, and the other highly resistant, were harvested (i) without pruning, and (ii) at various intervals after pruning. Sample roots were taken for the evaluation of scopoletin content, dry matter content and PPD. The three parameters were observed in relation to time after pruning.

In the PPD-susceptible cultivar, a sharp downward trend in visible PPD during the post-pruning period was accompanied by a similar trend in the scopoletin contents in samples taken. A significant correlation between the two parameters was observed ($R = 0.81$), implying that pruning, as well as leading to reduced PPD-susceptibility, also led to reduced parenchymal scopoletin contents.
In the PPD-resistant cultivar, a notable overall reduction in PPD was observed, and scopoletin content also decreased generally. However, some individual results contradicted the general trend: around 3% of results matched relatively high scopoletin levels (> 4 mg/kg, fresh weight basis) with zero visible PPD.

With both cultivars it was noted that DMC, which underwent some minor fluctuations after pruning, did not correlate significantly with visible PPD ($R < 0.1$).
The evidence suggests that there is no simple, straightforward link between either DMC or scopoletin and PPD.

**Comments:**

Effect of pre-harvest pruning upon PPD. Substantial reductions in PPD-susceptibility occurred during the 39-day post-pruning period. Between days 0-4 and day 25, a major decrease in PPD-susceptibility was indicated in the PPD-susceptible cultivar, M Col 22. The PPD-resistant cultivar, M Bra 337, consistently had very low PPD results, at no point higher than 20%. Both cultivars reached a minimum value for PPD susceptibility between day 25 and day 39. The final results on day 39 remained well below the initial values. The effect of pruning upon PPD in the cultivar M Col 22, which produced a pre-pruning mean PPD result of 65% was remarkable: at 25 days after pruning, a mean PPD result of 4.3% was observed.

Parenchymal scopoletin in relation to PPD after pruning. With the cultivar M Col 22, notable correlations between scopoletin content and visible PPD were observed throughout the post-pruning period ($R = 0.81$, $p > 0.0001$ with PPD assayed 24 h. post-harvest, 176 observations; $R = 0.61$, $p > 0.05$ with PPD assayed 5 days post-harvest, 11 pooled observations, Figure 1). As PPD decreased after pruning, so did scopoletin content.

The cultivar M Bra 337, on the other hand, showed a much lower correlation between scopoletin content and visible PPD ($R = 0.26$, $p > 0.0005$ with PPD assayed 24 h. post-harvest, 176 observations; $R = 0.01$, $p > 0.97$ with PPD assayed 5 days post-harvest, 11 pooled observations, Figure 2). It should be stressed, however, that over 70% of the individual M Bra 337 observations involved scopoletin contents of less than 1 mg/kg (fresh basis), and that 48% of observations were within two times the magnitude of the detection limit (0.2 mg/kg, f.b.). With such low levels of scopoletin in the samples, it could be argued that any correlation between scopoletin and visible PPD might be distorted to a considerable degree. Even so, there occurred 5 individual observations with scopoletin values of over 4 mg/kg (f.b.), with PPD values of 0%. This appears to demonstrate conclusively that there cannot be a straightforward correlation between parenchymal scopoletin content and PPD.

Parenchymal DMC in relation to PPD after pruning. With the cultivar M Col 22, no significant correlation was observed between DMC and PPD ($R = 0.08$, $p > 0.3$ with DMC assayed 24 h. post-harvest, 176 observations; $R = 0.006$, $p > 0.98$ with DMC assayed 5 days post-harvest, 11 pooled observations, Figure 3).

With the cultivar M Bra 337, no significant correlation was observed between DMC and PPD when DMC was assayed at 24 h. post-harvest ($R = 0.03$, $p > 0.71$, 174 observations). Interestingly, the pooled and averaged DMC results taken at 5 days post-harvest produced a much better correlation ($R = 0.59$; $p > 0.06$, 11 pooled observations, Figure 4). However, any inferences of a true correlation here should be treated with caution, as the 24 h. results, when pooled and averaged, also improved markedly in terms of correlation between the two parameters ($R = 0.24$; $p > 0.48$).

Further research. The evidence suggests that there is no simple, straightforward link between either scopoletin or DMC and PPD.

Further research aimed at controlling PPD by non-conventional genetic means should therefore be focused upon other enzymes and/or metabolites. The enzyme chalcone synthase, of key importance in flavonoid biosynthesis, along with polyphenol oxidase and related enzymes, could be of prime importance and high specificity in causing the symptoms of PPD.

### 2.2.6 Molecular Mechanisms of cassava physiology under stress: The role of glycine decarboxylase



Faustina Giraldo[1], Fernando Angel[1], Mabrouk El Sharkawi[2], William Roca[1]
1. Biotechnology Research Unit; 2. Cassava Physiology.

Earlier work at CIAT has shown that cassava can adopt to stress situations such as drought by expressing a photosynthetic mechanism resembling intermediate C3:C4 plants. Particularly, certain enzymes involved in photosynthesis/photorespiration change their specific activities under stress on the one hand and the foliar anatomy of some cassava varieties and wild Manihot spp studied, also shows intermediate characteristics.

Our research aims at characterizing enzyme activities localized in particular foliar tissues and cell.

Glycine decarboxylase is a multienzyme complex of the photorespiratory pathway, made up by four subunit, proteins P, H, Y and L. Genes coding for T and H subunits have been cloned and sequenced in *Pisum sativum* (pea), *Solanum tuberosum* (potato) and *Flaveria pringlei*.

Progress Report. In order to design primers for isolation of the genes coding for T and H subunits in cassava, the three sequences belonging to the three species mentioned, were aligned and some conserved sequences were chosen to construct specific primers. Primers, 19 nucleotides in length, derived from sequences coding for T and H proteins to carry out PCR amplifications, using cassava DNA as template, were used.

An amplified fragment (0.4 Kb) obtained with primers derived from T sequence was isolated and cloned in pCRII plasmid. Cloned fragment was labeled and probed with DNA digests of cassava, pea and potato. Strong hybridization was observed with digests of cassava DNA and one band of 2.0 Kb was detected in Hind III digests. When Eco RI digests were probed a 1.6 Kb fragment was observed. In Pea and potato, one Eco RI fragment (1.4 Kb) and one Hind III fragment (1.7 Kb) were detected, but the hybridization signal was weaker than the one observed in cassava. The distribution of glycine decarboxylase has been recently detemined in C3, C4 and C3:C4 intermediate species (Reyes Fernandez et al, 1995). In C3 species, it was located in mithocondria of mesophyl cells, while in C4 and in intermediate species the enzyme was found exclusively in bundle sheat mitochondria. Cloned fragments here can be used to isolate cDNA clones from a cDNA library of cassava in order to be used as probe in *In situ* hybridization experiments. *In situ* hybridization could show in which tissue is expressed this enzyme. This approach can contribute to gain an understanding about in cassava, tissues, the compartmentation of this enzyme and hence to better understand the physiological mechanisms underlying the photosyntetic rates of cassava under drought stress and high temperatures.

**References**

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## OUTPUT 2.3 IMPROVED GENE TRANSFER METHODOLOGIES DEVELOPED FOR BROADENING THE GENETIC BASE OF CULTIVATED GENE POOLS.

### 2.3.1 Comparative Study of Two Methodologies for Improving Genetic Transformation of Rice.

Z. Lentini, E. Tabares, W. Roca.
Biotechnology Research Unit.



In order to apply genetic engineering successfully to crop improvement, it should be possible to generate transgenic plants with a high efficiency. An increased efficiency for the generation of

transgenic plants is relevant since there is a number of evidences indicating that generally a low percentage of transgenic lines show the level of gene expression needed to confer the trait of interest, and few trangenic lines will stably express and inherited the transgene introduce after several generations of sexual cycle. With the aim of improving the generation of transgenic rice from *indica* rice at CIAT, a comparative study was conducted to revise two most used methodologies for genetic transformation of rice by particle bombardment. Herein is reported the progress attained using the *indica* variety BR-IRGA 409, a popular Brazilian variety developed from CIAT germplasm, mainly grown in Southern part of the country which is the major rice producing area of Latin America.

Progress Report. The direct deliver of genes into mature embryos was conducted using DNA-coated gold particles accelerated by the PDS-1000/He system. The tropical irrigated Latin American *indica* variety BR-IRGA 409 was used as target. Co-transformation experiments were conducted using equal amounts of the pAct1D construct (McElroy et al., 1990; kindly provided by Dr. R. Wu, Cornell University) containing the GUS reporter gene under the control of the rice actin-1 promoter, and a construct containing the hph selective gene encoding for hygromycin resistance (Hyg $^r$) driven by the 35S CaMV promoter.

Mature embryos were pre-treated for 4 hours before bombardment, culturing them on NBA (Li et al., 1993) medium, standard medium used at CIAT, or CC (Christou, 1995) medium, both containing 0.4 M Mannitol. About 16 hr after bombardment, embryos were transferred onto medium without mannitol, and with or without hygromycin (hyg) for selection. Four selection systems for hygromycin resistance were used after bombardment: (1) Embryos bombarded on NBA were cultured on NBA without hygromycin for a week, and then transferred to 30 mg/l hyg for 3 weeks, resistant callus were subcultured on the same medium for a month, selected callus on this medium were then transferred onto 50 mg/l Hyg and then to regeneration medium containing 50 mg/l Hyg (standard selecction schemme used at CIAT according to Li et al., 1993); (2) The same procedure as before, but replacing NBA medium for CC medium; (3) Embryos bombarded on CC medium were transferred onto CC medium containing 30 mg/l Hyg, or (4) Onto CC with 50 mg/l one day after bombardment, according to Christou (1995); resistant callus recovered from 30 mg/l Hyg were transferred onto 50 mg/l , callus selected on 50 mg/l were cultured on regeneration medium containing 50 mg/l Hyg.

About twice as many embryos bombarded on medium CC showed GUS transient expression after 24 hr of bombardment respect to those on NBA medium. The number of blue spots per embryo bombarded was also higher (data not shown). Eighty four to 100% of bombarded embryos developed Hyg resistant (Hyg$^r$) callus when selection was initiated a day after bombardment on 50 mg/l or 30 mg/l hyg respectively, whereas 36% to 68% embryos developed Hyg$^r$ callus when selection started a week after bombardment (Table 1). The number Hyg$^r$ callus generated on CC medium was also higher than on NBA medium (Table 1).

**Table 1**. Hygromycin resistant callus obtained from mature embryos of BR-IRGA 409 after four different selection treatments.

| Treatment[a] | Explant Bombardment | Explants with Hyg$^r$ callus % | Hyg$^r$ callus | Hygr callus'/explant |
|---|---|---|---|---|
| 1 | 33 | 36(7) | 27 | 2.3(2.1) |
| 2 | 28 | 68(46) | 66 | 3.5(2.1) |
| 3 | 32 | 100(0) | 101 | 3.1(2.4) |
| 4 | 34 | 84(22) | 114 | 3.9(2.6) |

[a]Treatments for selection on hygromycin after bombardment: (1) and (2) embryos were cultured on NBA or CC for a week without hygromycin and then transferred onto 30 mg/l; (3) and (4) embryos cultured on CC and placed on selection medium with 30 mg/l or 50 mg/l Hyg one day after bombardment, respectively. Numbers in parentheses refer to the standard deviation.

The CC medium contains 3.5 higher level of calcium than NBA, and rice callus develops slower respect to the NBA medium (data not shown). The higher levels of calcium in the CC medium may aid the repairing of plama membrane and cell wall increasing the survival of the cells from the physical damage caused by the bombardment; and may also favored the division of the transgenic cells respect to the nontransgenic ones in the presence of the selective agent, since CC medium is suboptimal for callus formation. The formation of Hyg$^r$ callus was visible within 1-2 weeks when selection started one day after the bombardment, whereas it took 4-6 weeks in the other treatments. The possibility of initiating earlier the selection on 50 mg/l hyg (the lethal concentration for selection) will allow to reduce the process of generating transgenic rice from 4 months to two months since the regeneration onto the selection medium containing 50 mg/l hyg could be initiated one month after bombardment. Plant regeneration from these Hyg$^r$ callus is underway. Comparative evaluations of these treatments with other genotypes is also in progress.

## References

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Li L., Qu R. , de Kochko A. , Fauquet C. , Beachy R.N. 1993. An improved rice transformation system using the biolistic method. Plant Cell Rep. 12: 250-255.

## 2.3.2 Genetic transformation of cassava.



E. Torres, L. Mancilla, F. Angel , W.M. Roca
Biotechnology Research Unit.

Following our initial work of transforming cassava via *Agrobacterium tumefaciens*, this year our efforts focused on (i) confirmation of insertion and expression of transgenes in regenerated-transformed cassava plants using molecular and resistance assays; (ii) testing and choosing a selectable marker for transformation experiments; (iii) the construction of vector a transformation vector for resistance against the cassava stem borer; (iv) isolation and cloning of starch branching enzyme.

Progress Report.

### 1. Confirmation of cassava transformation

(i) Molecular assay: PCR and Southern analysis. Plant DNA was amplified using *npt* II specific primers transferred to Hybond N+ was hybridized with the PGV 1040 plasmid 6 kb fragment. At least one of the four DNAs was clearly positive, (plant 53-5.2); a weak signal was detected in plant 207 also indicating amplification of *npt* II specific sequence. Detected band was 1.2 Kb in size corresponding to the fragment previously amplified by PCR. DNA from two other (plants 137-1.1 and 57-1.2) were negative as well as the non transformed plants.

When total plant DNA was digested with control Bgl II and hybridized with *npt* II purified fragment obtained from T-DNA, three strong signals in plant 53-5.2 were evident, indicating three different T-DNA insertions into host DNA. Two of the three insertions lost *Bgl* II restriction sites, generating bands heavier than 6 kb . In order to confirm that the T-DNA was integrated into host DNA, a restriction enzyme *Sca* I having only restriction site into the T-DNA was used to digest total DNA. When the *npt* II gene was used as probe, three different bands were detected confirming that 3 different insertions are present in plant 53-5.2. When *Sca* I was used, the smallest band detected was 9.3 kb, indicating that the next *Sca* I restriction site is a least 3.3 kb into host DNA from *Bgl* II site, in the right border flaking the T-DNA.
As expected, no hybridizing band were observed in control DNA.

(ii) Basta Resistance assay. Analysis for expression of the *bar* gene was made only on the preselected plants with PCR. Plants were propageted in the green house and were sprayed with 40ml/$m^2$ of a 150mg/L PPT solution. Previous experiments with " Basta" where were carried out with differents concentrations (25-250 mg/l) in order to determine the maximum resistance level to PPT. The transformed plants expressing herbicide resistance were: 53-5.2 and 207, the same plants that gave positive signals with PCR.

In conclusion our results demonstrate the generation of non-chimeric transgenic cassava plants with rate efficiency transformation of 2% .

## 2. Choice of a selectable marker

In orden to determine the effectiveness of marker genes in selecting transformed embryogenic tissue, tissue growth inhibitor curves were established for cotyledonary leaves obtained from somatic embryos the antibiotic hygromycin (2-64 mg/l), the herbicide phosphinotricin (8-50mg/l). and the sugar mannose (1-20 g/l), were tested as selectim agents. The genes encoding these products are: *hph* (hygromycin phosphotransferase), *bar* (neomycin phosphotransferase) and mannose isomerase.
The parameters evaluated, at 8, 15 and 30 days after culture, were viability, callus and embryos.
(i). Hygromycin: after 15 days in the introduction medium of somatic embryos, the viability was reduced to 20% with 16 mg/l; higher concentrations, killed the tissue and induction of friable and embryogenic callus were arrested (Fig. 1).

(ii) Phosphinotricin: two sources of phophnotricin was used :
PPT (Sigma) and the commercial herbicide Basta (Hoechst ). The latter containis 200 g/l PPT. Explant viability was more affected with Basta than with PPT. Concentrations of Basta up to 8mg/l inhibited callus and embryos, while concentrationes higher than 40 mg/l killed the tissue.

(iii) Mannose: Concectrations between 5.0 to20 g/l reduced up to 60% of tissue viability. The induction of callus and embryos was inhibited at concentrations above 5 g/l.. (Fig. 2)

In conclusion, both hygromycin (15mg/l) and phosphinotricin (Basta:8 mg/l; PPT: 40mg/l) were efficient as selection agents. Mannose had no effect on viability of explants.

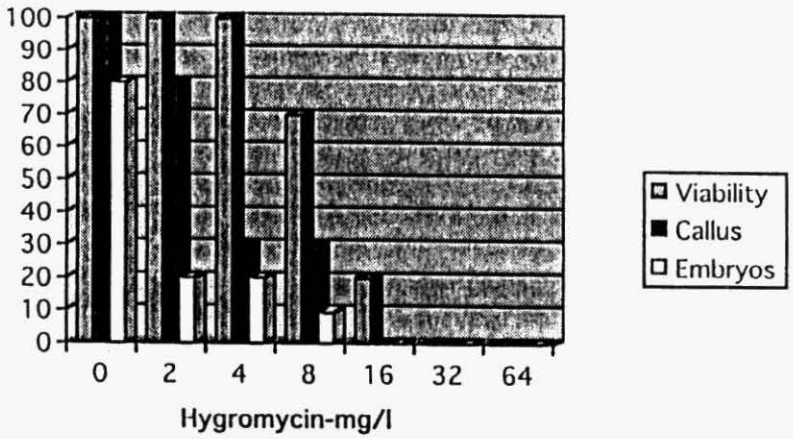



**Fig.1**. Effect of hygromycin on tissue viability, callus growht and embryos induction, after 20 day of culture.

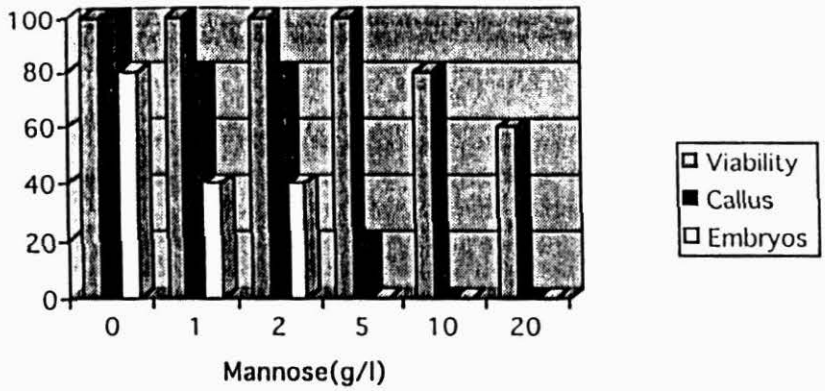
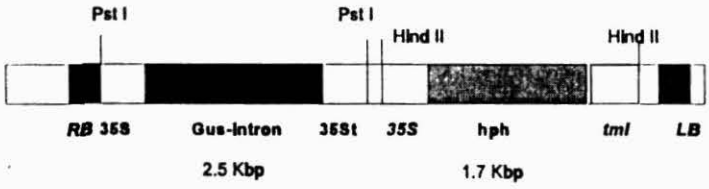



**Fig. 2.** Effect of mannose on tissue callus growth and embryos induction after 20 days of culture

### 3. Construction of transformation cassettes for cassava

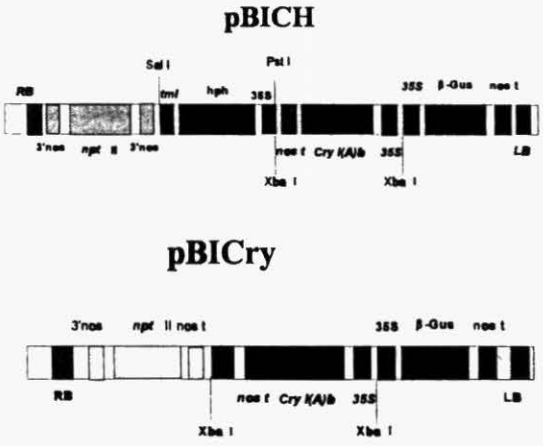
(i)Plasmid pSGh. The plasmid pSGh was derived from pSG-Man1.The mannose isomerase gene of pSG-Man1 was detected using Hind III enzyme. The hygromycin B (*hph*) gene, from plasmid pTRA 151, was cloned into this site. pSGh contains the coding region of *Streptomices hygroscopicus hph* gene betwen the enhanced 35S promotor of CaMV and the transcription terminator from a tumor morphology large gen (*tml*). The *hph* gene is 1.7 Kbp and can be isolated using Hind III digestion. In addition, pSGh contains the *gus*-intron gene and the spectinomicin/streptomicin resistence genes.The 2.5 Kbp Gus-intron gene is obtained from the plasmid digested with Pst I. Ttransformed bacteria with pSGh were selected by their resistantce in to LB- with hygromycin, up to 100 μg/ml.

**Fig. 1.** Organization of plasmid pSGh.



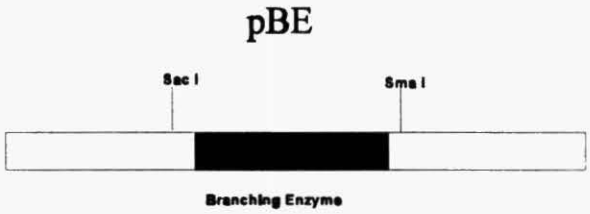

(ii) Plasmid PBI101. Adicionally, another gene was cloned in to pBI101 plasmid. The plasmids pBICH and PBICry were constructed by ligation of the 3.0 Kbp codin region of *Cry* IA(b) gene from *Bacillus thuringiensis* (Plantek Co.) in to the Xba I site of pB101. The pBICH contains the *hph* gene from pTRA 151 in the Sma I site of pB101. The product of Sma I digestion is a 4.7 Kbp fragment that consists of *Cry* and *hph* gene. The pBI101 derivative has a kanamycin resistance (*npt* II) and β *gus* genes. The molecular size of plasmid PBICry is 15.2 Kbp and 17 Kbp for pBICH. (Fig. 2).

**Fig. 2**. Plasmids pBICH and pBICry containing the *Cry* IA(b) and *hph* genes and




## 4. Cloning and sequencing of the starch branching enzyme

A 3.0 Kbp clone containing the starch branching enzyme gene (BE) was isolated from a cDNA library of *S. tuberosum* using the homology gene of pea. The BE of potatoes was amplified by PCR and cloned into Eco R I site of PCR-Script vector. In order to express the protein in *E. coli*, the Suma I/Sac I Be digested gene was cloned into Sma I site of pUC18 and pUC 19 vectors, and the transformed bacteria were induced with IPTG.




Future plans.

- Place into the pSGh construct the *Cry* IA(b) gen and for cassava transformation via *Agrobacterium* ,using the derived binary system.

- Evaluation of insecticidal activity of Bt toxin with larvaes of the lepidopter stemborer *Chilomina clarkei*.

- Test other strains of *Agrobacterium tumefaciens* for cassava transformation

## 2.3.3 Development of an efficient genetic transformation protocol in common bean

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The Genetic transformation of common bean has been largely difficult mainly because of the recalcitrance of this species to conventional *in vitro* tissue culture plant regeneration. To overcome problems arising from the lack of protocols for plant regeneration, Russell et al. (1993) applied the biolistic methodology to directly introduce DNA into of meristematic cells of mature seeds, and produced the first transgenic bean plants. This approach was based on the methodology developed by Christou et al. (1990) with soybean.

Although these works showed for the first time that transgenic bean plants can be obtained by DNA bombardment into meristems, the methodology used, however has limitations, mainly the low rate of meristem transformation, the difficulty in applying *in vitro* selection for the identification of transgenic shoot, and the low efficiency in regeneration of transgenic plants.

Using *Agrobacterium tumefaciens* as a vector, the genetic transformation of a wild genotype of the tepary bean (*Phaseolus acutifolius*) has been achieved recently (Dillen et al., in press). The transformation protocol however couldn't be applied to common bean, economically, the most important species of the genus.

Progress report. In 1994, as an outcomes of this project, we were able to induce an organogenic callus, named meristematic callus (m-callus), involving hundreds of meristems, with none or few unorganized growth. During 1995 we also developed a methodology for recovering fertile plants efficiently by micrografting shoots developed from m-calli. Use of m-calli for genetic transformation of common bean has the following advantages over the bombardment of mature seed meristems: a) many more meristematic, regenerable regions are exposed to the projectiles carrying DNA; b) an *in vitro* selection step of transformed tissue can be applied efficiently.

In 1995 we bombarded 238 petri-dishes with m-calli of 5 agronomic important cultivars of common bean in 29 sessions. Marker genes that confer resistance to hygromycin (hph gene) or phosphinothricin (pat and bar genes) and the gus-intron gene have been used for developing the transformation methodology. These marker genes were cotransformed in two separate plasmids.

From the bombarded tissue, the project team was able to select more than 500 clones which have shown resistance during at least 3 subcultures on selection media. From the selected clones, in total 107 plants have been regenerated during 1996, 99 of which have shown to be fertile and have produced progeny (Table 1).

**Table 1**. Fertile plants regenerated from transformed m-calli selected as hygromycin or phosphinothricin resistant.

| Cultivar | Bombarded Petri dishes | As phosphinothricin or hygromycin resistant selected clones | Fertile plants regenerated |
|---|---|---|---|
| Bayo Madero | 113 | 430 | 80 |
| C20 | 40 | 63 | 14 |
| A295 | 37 | 31 | 3 |
| ICA Pijao | 36 | 13 | 1 |
| Carioca | 12 | 2 | 1 |
| Total | 238 | 539 | 99 |

Genetic analysis using the polymerase chain reaction has been performed in some of the regenerated plants. Of 37 analyzed plants, regenerated from clones selected with phosphinothricin or hygromycin, 21 have shown the presence of fragments of one or both of these genes (Table 2).

**Table 2**. Results of the PCR-analysis of primary transformants and its progeny for the presence or absence of transferred marker genes.

| Cultivar | Positive plants for the hyg-gene/Analyzed plants | Positive plants for the pat-gene/Analyzed plants |
|---|---|---|
| Bayo Madero | 14/25 | 8/26 |
| C20 | 5/9 | 6/8 |
| A295 | 2/3 | 2/3 |
| Total | 21/37 | 16/37 |

As expected, a segregation of the presence of the hph and the pat gene has been found in the progeny of all transformed lines with positive progeny.

Furthermore, hygromycin resistance has been analyzed in progeny plants. The progeny of 5 plants from 3 different cultivars, that have resisted germination *in vitro* in a culture medium with hygromycin, have shown to be PCR-positive for the hph gene. These results show that this gene is expressed in some transformants at the seedling level.

Currently, we are working to obtain further molecular-genetic evidences for stable integration of the transferred genes in progeny plants, by analyzing the genomic DNA by Southern blot hybridization.

These results show that the methodology developed for the genetic transformation of common bean using the particle bombardment of meristematic calli can be used in the production of transgenic plants of several common bean cultivars of agronomic importance.

Future work. The project, that has a duration of three years and is financed by BMZ-GTZ, is now coming to an end (Dec. 1996). A request for additional support for a two year period has been presented to the funding agency. During this time we plan to perform further inheritance and gene expression analysis of the transferred genes and to develop transgenic stocks with tolerance to phosphinothricin, the active compound of the herbicide Basta™. This trait has been identified by

some breeders and bean producers as an important trait that could reduce costs of bean production.

Transformation with two genes that modify fatty acid metabolism (an antisense DNA of the sn-Glycerol-3-Phosphate Acyltransferase -GPAT- from common bean and oleate selective GPAT of spinach, see Wolter et al. 1993) isolated an analyzed in the laboratory of Prof. Dr. Heiz in Hamburg, Germany, will be initiated. Expression of the two genes in transgenic common bean should confer chilling tolerance.

Other genes of agronomic importance that could be tested in transgenic bean plants are genes that confer resistance to insects like the Bt-toxin, proteinase and alfa amylase inhibitors.

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### 2.3.4 Development of Brachiaria genetic transformation.

L.Galindo, Z. Lentini, W. Roca.

Biotechnology Research Unit



The development of genetic transformation of brachiaria is underway for its use in genetic studies with homologous traits (i.e. apomixis) and on germplasm development with heterologous traits (i.e. insect resistance). A methodology was previously developed at the Unit for plant regeneration from various *Brachiaria* species. Proliferation of embryogenic callus derived from the scutelum node of mature embryos, and plant differentiation through somatic embryogenesis were achieved (Lenis, 1994). Last year a preliminary report was presented on initial attempts for the development of transformation by particle bombardment. This year progress is presented on the evaluation of culturing parameters that may allow to increase the efficiency of transgene transfer into *Brachiaria*.

Progress Report. The effect of positioning scutellum explant when culturing isolated embryos for callus induction was evaluated. Table 1 shows an increase of about two -fold in the production of embryogenic callus when the scutellum is facing up, without direct contact with the culture medium, allowing a higher gas exchange. Current work includes the comparison of various media composition to optimize callus formation from isolated embryos, used as target for bombardment, and from complete seeds for the mass production of callus. Use individual embryogenic callus is sought as target tissue when the response of isolated embryos is low and highly heterogenous as it occurs with *B. ruziziensis* (data not shown) .

**Table 1**. Production of embryogenic callus from isolated mature embyos of *Brachiaria*.

| Species | Scutellum[1] position | Explants[2] No. | Embryogenic callus | | Embryogenic callus (%) | | Embryogenic callus/explant | |
|---|---|---|---|---|---|---|---|---|
| *B. brizantha* | Side up | 60 | 45 | (24) | 32 | (18) | 2.2 | (0.7) |
| | Side down | 60 | 24 | (14) | 23 | (14) | 1.1 | (0.4) |
| *B. decumbens* | Side up | 60 | 66 | (5) | 45 | (5) | 6.7 | (2.0) |
| | Side down | 60 | 24 | (2) | 30 | (10) | 2.4 | (1.1) |

[1] Position for callus induction; Side down, scutellum side in contact with the culture medium.[2]Three replicates per treatment of twenty embryos per replicate. Number is parentheses refer to Sderror.

The culture postion of embryos after the bombardment also affects the level of transient expression of the *gus* gene (Table 2). A substantial increase in *B-glucoronidase* expression was noted when embryos were cultured scutellum side up after bombardment . This level of expression was improved respect to last year results.

**Table 2.** Transient expression of gus gene in *Brachiaria* scutellum after 48hr of bombardment with pAct-1D plasmid using the PDS-1000 He biolistic system.

| Species | Scutellum[1] position | No. embryos | No. blue spots total | No. blue spots per embryo |
|---|---|---|---|---|
| *B. brizantha* | Side down | 280 | 306 | 1.1 |
| | Side up | 40 | 426 | 10.4(1.1) |
| *B. decumbens* | Side down | 300 | 240 | 0.8 |
| | Side up | 40 | 123 | 3.1 (0.6) |

At present , the effect of the scutellum position is being monitored on the stable expression of the *gus* gene several weeks after bombardment, and on the efficiency for recovering transgenic plants both expressing the *B-glucoroniadase* and resistant to hygromicin.

**Reference**

Lenis, S.J. 1991. Regeneración de plantas de la gramínea forragera *Brachiaria spp*, a partir de tejidos cultivados *in vitro*. CIAT, Cali-Colombia

**2.3.5 Cryopreservation of cassava shoot tips in liquid nitrogen.**

R. H. Escobar[1], W. M. Roca[1], M. Giraldo[1] G. Reyna[1], G. Mafla[2]

1. Biotechnology Research Unit, 2. Genetic Resources Unit

We have developed an improved technique, involving programmed freezing of pre-cultured shoot tips to increase success rate of cassava cryopreservation. Observed genotypic response to cryopreservation has prompted to adjust the basic protocol for obtaining increased response and accessibility of the technique to a wider range of cassava varieties.

Progress Report. 1. We evaluated the CIAT cryopreservation protocol (1992), under two freezing rates (rapid and programmed). It was observed that no differences exist in viability between the two freezing rates with cultivars tested (Table 1). Some, such as MMex 71, MVen 232, MEcu 117, MCol 1389 and MCol 297 showed low shoot growth response. MCol 1468 revealed high viability response but without shoot development. MCol 1468 is known for its low tissue culture response.

2. When the shoot tip size was increased a dramatically improved response with two of the most recalcitrant genotypes was obtained (Table 2). There seems to exist a strong relation ship between size and pre-treatment on 4E medium. This medium induces cells with meristematic behavior, able to support dehydration and show good response after cryopreservation.

3. Higher responses were obtained with a range of genotypes when larger shoot tips were used (Table 3). This shows a critical step in the reculture medium, making it necessary to modify the current to reculture medium. In this experiment we observed structures-like-shoots that stopped growing and were eventually covered with callus. Addition of 1mM asparagine and high citokinin, without auxins has improved the survival and shoot growth on reculture media, as callus formation stopped.

It is known that cassava shoot tips respond well to Kinetin and 2iP (0.3-1 mg/l), and to BAP (0.04-0.3 mg/l) ( BRU Annual Report 1995). We have tested asparagine with some cultivars that show high viability response, with the aim at improving shoot tips recovery (Table 4).

4. Use of large quantity of canes per freezing experiment, (eight varieties per experiment use 24 canes in the freezing chamber), causes changes in the freezing curve. At a different temperature (freezing point) this change could modify the response of cryopreserved tissue (Table 5). For this reason it is necessary to specify the quantity of canes per experiment in order to modify the temperature curve.

Future Work. 1. It has been shown that shoots from *in vitro* culture schow different response compared to greenhouse-derived. For this reason, a system will be developed to provide shoot apices rapidly and in a large quanting. Testing different citokinins for a short time, will also be pursued for increasing viability and frequency of shoot recovery after freezing.

2. As we haven't observed differences between freezing rates, it might be beneficial to implemente a shoot tip encapsulation technique.

3. Media with lower osmotic concentration will be tested on cultivars with recalcitrant behavior.

4. Preculture medium will be modified to improve shoot tips recovery.

5. Develop the logistical aspects for implementaring the cassava *in vitro* base collection in liquid nitrogen at CIAT.

**References**

R. H. Escobar, G. Mafla and W. M. Roca . 1996. A Methodology for recovering cassava plants from shoot tips maintained in liquid nitrogen. Plant Cell Reports, *in press*.

**Table 1**: Effect of slow (programmed) and rapid freezing rates on cryopreservation of different cassava genotypes.

| Variety | Freezing | Viability | Shoot growth |
|---|---|---|---|
| M Bra 12 | Rapid | 46.6 | 20,0 |
| | Programmed | 60,0 | 20,0 |
| M Bra 507 | Rapid | 86.6 | 13.3 |
| | Programmed | 66.6 | 20,0 |
| M Col 297 | Rapid | 9.1 | 0,0 |
| | Programmed | 20,0 | 0,0 |
| M Col 304 | Rapid | 26.6 | 6.6 |
| | Programmed | 6.6 | 0,0 |
| M Col 1389 | Rapid | 25,0 | 0,0 |
| | Programmed | 14.3 | 0,0 |
| M Col 1468 | Rapid | 66,0 | 6.6 |
| | Programmed | 7.7 | 0,0 |
| M Ecu 50 | Rapid | 7.7 | 0,0 |
| | Programmed | 20,0 | 6.6 |
| M Ecu 117 | Rapid | 18.2 | 0,0 |
| | Programmed | 8.3 | 0,0 |
| M Gua 14 | Rapid | 7.7 | 7.7 |
| | Programmed | 23,0 | 0,0 |
| M Mal 48 | Rapid | 33.3 | 33.3 |
| | Programmed | 50,0 | 18.7 |
| M Mex 71 | Rapid | 30,0 | 0,0 |
| | Programmed | 15.4 | 0,0 |
| M Par 71 | Rapid | 71.4 | 28.6 |
| | Programmed | 28.6 | 14.3 |
| M Ven 232 | Rapid | 0,0 | 0,0 |
| | Programmed | 7.7 | 0,0 |
| M Col 22 | Rapid | 94.4 | 55.5 |
| | Programmed | 88.8 | 66.6 |

**Table 2**: Effect of shoot tips size on cryopreservation of recalcitran cassava varieties.

| Variety | Size | Freezing | Viability | Shoot growth |
|---|---|---|---|---|
| M Col 1389 | Large | Rapid | 70,0 | 0,0 |
| | | Programm | 72.7 | 9.1 |
| M Col 1389 | Central | Rapid | 25,0 | 0,0 |
| | | Programm | 14.3 | 0,0 |
| M Ecu 117 | Large | Rapid | 100.0 | 85.7 |
| | | Programm | 100,0 | 14.3 |
| M Ecu 117 | Central | Rapid | 18.2 | 0,0 |
| | | Programm | 8.3 | 0,0 |

Large: apical dome + 4-5 primordia

Central: apical dome + 2-3 primordia

**Table 3:**Genotypic effect on cassava cryopreservation using large shoot tips with rapid and slow (programmed) freezing.

| Variety | Freezing | Viability | Shoot growth |
|---|---|---|---|
| M Bra 12 | Rapid | 64.7 | 29.4 |
| | Programmed | 78.6 | 28.6 |
| M Bra 507 | Rapid | 75,0 | 18.7 |
| | Programmed | 56.2 | 18.7 |
| M Bra 191 | Rapid | 80,0 | 53.3 |
| | Programmed | 100,0 | 68.7 |
| M Col 297 | Rapid | 70.6 | 17.6 |
| | Programmed | 40,0 | 33.3 |
| M Col 304 | Rapid | 64.7 | 0,0 |
| | Programmed | 46.6 | 0,0 |
| M Col 1389 | Rapid | 13.3 | 6.6 |
| | Programmed | 30.7 | 7.7 |
| M Col 1468 | Rapid | 25,0 | 0,0 |
| | Programmed | 28.6 | 0,0 |
| M Col 1734 | Rapid | 25,0 | 8.3 |
| | Programmed | 11.7 | 5.8 |
| M Col 1736 | Rapid | 16.6 | 0,0 |
| | Programmed | 0,0 | 0,0 |
| M CR 113 | Rapid | 53.3 | 6.6 |
| | Programmed | 75,0 | 12.5 |
| M Dom 2 | Rapid | 52.6 | 5.3 |
| | Programmed | 38.8 | 22.2 |
| M Ecu 50 | Rapid | 56.2 | 18.7 |
| | Programmed | 42.8 | 28.5 |
| M Ecu 117 | Rapid | 52,0 | 21.4 |
| | Programmed | 70.6 | 17.6 |
| M Gua 14 | Rapid | 64.3 | 35.7 |
| | Programmed | 47.1 | 17.6 |
| M Mal 48 | Rapid | 86.6 | 53.3 |
| | Programmed | 44.4 | 0,0 |
| M Mex 71 | Rapid | 78.5 | 7.2 |
| | Programmed | 0,0 | 0,0 |
| M Par 71 | Rapid | 100,0 | 82.3 |
| | Programmed | 66.6 | 0,0 |
| M Per 303 | Rapid | 53.3 | 26.6 |
| | Programmed | 71.4 | 14.3 |
| M Ven 232 | Rapid | 11.1 | 0,0 |
| | Programmed | 6.2 | 0,0 |
| M Col 22 | Rapid | 87.5 | 50,0 |
| | Programmed | 46.6 | 0,0 |

**Table 4:**Effect of rapid and slow (programmed) freezing on cassava cryopreserved shoot tips recovered on a modified re-culture medium.

| Variety | Freezing | Viability |
|---|---|---|
| M Bra 191 | Rapid | 73.3 |
| | Programmed | 84.2 |
| M Col 1734 | Rapid | 91.6 |
| | Programmed | 71.4 |
| M Col 1736 | Rapid | 53.3 |
| | Programmed | 21.4 |
| M CR 113 | Rapid | 45,0 |
| | Programmed | 56.2 |

**Table 5:** Effect of rapid and slow (programmed) freezing using larger numbers of varieties per freezing experiment .

| Varieties | Freezing | Viability | Shoot growth |
|---|---|---|---|
| M Col 304 | Rapid | 63.6 | 18.1 |
| | Programmed | 100,0 | 54.5 |
| M Col 1389 | Rapid | 90.9 | 9.1 |
| | Programmed | 90,0 | 10,0 |
| M Col 1468 | Rapid | 53.8 | 0,0 |
| | Programmed | 84.6 | 0,0 |
| M Par 71 | Rapid | 84.6 | 76.9 |
| | Programmed | 92.8 | 71.4 |

(ii) Project members participated in the organization and conduction of the Third scientific meeting of the Cassava Biotechnology Network (CBN), held in Kampala, Uganda, August, 1996.

(iii) Planning Workshop between Inst. A. von Humboldt (AvH) and CIAT (Sept. 1996). Scientists of the two institutions discusseed and agreed on potential areas of collaboration, including inventory/prospecting, conservation (*in-situ, ex-situ*), and value adding. Working groups, comprising scientists of AvH and CIAT were set per research area, and charged with follow up activities.

(iv) Course on Applications of PCR technology, organized by CIAT BRU in collaboration with Universidad Nacional, Colombia ( Dec 1996).

### 3.2.2 Individual Training

In 1996, nearly 32 scientists from NARS and AROS attended specialized training on selected advanced biotechnologies. Participants from: Colombia, Argentina, Cuba, Peru, Costa Rica, Ecuador, Belgium, USA, France, India, Mexico, Nigeria, Austria.

Funding: CIAT Regional Cooperation contributed with support for LDCs participants.

## 1996 PUBLICATIONS

### A. REFEREED JOURNALS

1. Tohme, J.; Gonzalez, DO.; Beebe, S.; and Duque, MC. 1996. AFLP Analysis of genepools of a wild bean core collection. Crop Science. 36(5): 1375-1384.

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**1996 THESIS**

1. Caracterización y análisis de la variabilidad genetica de Passifloraceas Juss mediante el uso de marcadores Moleculares (RAPD). **Diego Alberto Fajardo Ramirez**.

2. Optimización de una metodología para la inducción y proliferación de embriones somáticos de yuca (*Manihot esculenta* Crantz). **Juan Guillermo Cobo Borrero.**

3. AFLP characterization of wild South American *Phaseolus* species. **Ana Lucia Caicedo**

4. Marcaje de genes que confieren resistencia al arroz a linajes colombianos de *Pyricularia grisea*. **Mauricio La Rota,**

5. Aislamiento y Caracterización de cDNAs específicos de *Phaseolus* resistentes a *Acanthoscelides obtectus*. **Luisa Fernanda Fory.**

## CIAT AND PARTNERS' PRINCIPAL STAFF CONTRIBUTING TO PROJECT SB-2 IN 1996

### 1. CIAT

1.1 Biotechnology Research Unit: W. Roca, J. Tohme, Z. Lentini, F. Angel, A. Mejia, M. Fregene, P. Wenzl

1.2 Genetic Resources Unit: D. Debouck

1.3 Bean Program: S. Beebe, S.Singh, C. Cardona

1.4 Cassava Program: M. Bonierbale, V. Verdier, C. Iglesias, M.El-Sharkawy, G. O'Brien, A. Bellotti, A.M. Thro.

1.5 Rice Program: C. Martinez, F. Correa

1.6 Tropical Forrages Program: J. Miles, I. Rao

1.7 Land Use Unit: P. Jones, R. Uribe

1.8 Biometry: M.C. Duque

1.9 Virology: L. Calvert

1.10 Regional Cooperation: R. Posada, A. Caldas

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