ANNUAL REPORT 1998
PROJECT SB-02

ASSESSING AND UTILIZING AGROBIODIVERSITY THROUGH BIOTECHNOLOGY

CIAT

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CIAT
Centro Internacional de Agricultura Tropical
International Center for Tropical Agriculture

PROJECT SB-2: ASSESSING AND UTILIZING AGROBIODIVERSITY THROUGH BIOTECHNOLOGY

PROJECT OVERVIEW

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

Objective: To apply modern biotechnology to identify and utilize genetic diversity for broadening the genetic base and increasing the productivity of mandated and non-mandated crops.

Outputs: Improved characterization of genetic diversity of wild and cultivated species and associated organisms. Genes and gene combinations accessed and utilized. Collaboration with public and private sector partners enhanced.

Initiation: 1997

Duration: Five years

Milestones:

1998. Molecular linkage maps and DNA-based markers available for assessing diversity and tagging useful traits of *Phaseolus, Manihot*, and associated organisms. Transgenic resistant plants generated with rice; populations generated with useful traits from wild *Oryza* spp. Collaborative activities with CIAT partners implemented.

1999.DNA-based markers available for other species in CIAT mandated agroecosystems. Modern methods developed for the rapid propagation of cassava and other species in CIAT mandated agroecosystems. Trangenic resistance to insect pests available in cassava. Cryopreservation technique for cassava.

2000.QTL, from wild germplasm identified and mapped involved in yield and quality of rice and beans. Collaborative activities with CIAT partners, including privated sector implemented.

2001. Gene transfer utilized for broadening the genetic base and germplasm enhancement of rice, cassava, beans, and *Brachiaria*. Collaborative activities with CIAT partners implemented.

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

Collaborators: IARCs (IPGRI: systemwide program, CIP and IITA: root-tuber crops research; ISNAR: training, policies. NARS (CORPOICA, ICA, EMBRAPA, INIAs,). AROS of DCs and LDCs. Biodiversity institutions (A. von Humboldt, INBIO, SINCHI, Smithsonian). Corporations and private organizations (Corp. BIOTEC, BRL, Novartis).

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

CIAT project linkages: Inputs to SB-2: Germplasm accessions from gene bank project. Segregant populations from crop productivity projects. Characterized insect and pathogen strains and populations from crop protection projects. GIS services from land use project. Outputs from SB-2: Genetic and molecular information on gene pools, and populations for gene bank, and crop productivity projects. Information and material on identified genes and gene combinations for productivity and crop protection projects. Methods and techniques of propagation and conservation for gene bank and productivity projects. Interspecific hybrids and transgenic stocks for crop productivity and crop protection (IPM) projects.

WORK BREAKDOWN STRUCTURE

PROJECT SB-02: ASSESSING AND UTILIZING AGROBIODIVERSITY THROUGH BIOTECHNOLOGY

PROJECT GOAL

To contribute to increased productivity and to the conservation of agrobiodiversity in tropical countries

PROJECT PURPOSE

To apply modern biotechnology to identify and utilize genetic diversity for broadening the genetic base and increasing the productivity of mandated and non-mandated crops

OUTPUT 1. Genomes of wild and cultivated species and associated organisms characterized.

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

OUTPUT 2. Genes and gene combinations made available for broadening crop genetic bases.

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

OUTPUT3. Collaboration with public and private sector partners enhanced.

Organization of conferences, networks, workshops and training courses.

- Assembling of data bases, genetic stocks, maps and probes, and related information.
- Publications, project proposal development and contribution to IPR and biosafety management.

PROJECT LOG-FRAME (1998)

NARRATIVE SUMMARY	MEASURABLE INDICATORS	MEANS OF VERIFICATION	IMPORTANT ASSUMPTIONS
GOAL To contribute to increased productivity and to the conservation of agrobiodiversity in tropical countries.	CIAT partners using improved germplasm and more effective breeding and conservation methods	Center's NARS and other publications, statistics.	
PURPOSE To apply modern biotechnology to identify and utilize genetic diversity for broadening the genetic base and increasing productivity of mandated and non-mandated crops.	Characterized gene pools, improved genotypes and useful genes are available for crop improvement.	More efficient breeding techniques, publications.	Continuation of donor support; successful partnership with private sector; good regulatory framework.
OUTPUT 1. Genomes of wild and cultivated spp and associated organisms characterized.	Conservation strategies based on molecular knowledge of diversity; integration of genome analysis with agroecological data; projects using molecular markers; incorporation of new molecular and bioinformatic techniques.	Reports, publications, databases	Availability of phenotypic data for CIAT crops; establishment of effective partnerships; up-to-date equipment available at CIAT.
OUTPUT 2. Genes and gene combinations made available for broadening crop genetic bases	Availability of rice lines with transgenic and wide crossing, improved traits through, respectively; cassava and *Brachiaria* tissue cultures expressing transgenes; engineered transformation cassettes available;	Reports, publications, germplasm.	Adequate support continues; private sector involvement.
OUTPUT 3. Collaboration with public and private sector partners enhanced	NARS scientist participates in training courses and adapt some biotechnologies; partners use CIAT assembled strains and constructs, databases and maps; new partners become involved in CIAT work.	Courses organized; material distributed to partners; new work agreements; additional project proposals submitted; additional funding; reports, publications.	Adequate support; equipment and operational support.

PROJECT RESEARCH HIGHLIGHTS 1998

The research highlights for the period Oct. 1997- Sept 98 are summarized by outputs:

1. **Genetic diversity characterized**. Microsatellites (SSRs) and expressed sequenced tags (ESTs) were added to CIAT's repertoir of molecular genetic markers. We have used these and other markers to describe diversity at molecular level, determine population structure and genetic distances and phylogenetic relationships, in mandated crop germplasm and extended to non-mandated crops in cooperation with CORPOICA, A.V. Humboldt and the SINCHI Institutes. Genetic diversity of the cassava bacterial blight pathogen in Colombia was linked to the ecoregion and microgeographic origin of germplasm. Biological control organisms against the cassava green mite were also subject of genetic characterization this year. Assessment of diversity at DNA level provides information on potential new sources of variability for broadening crop genetic base, and for linking diversity *in-situ* with *ex-situ* collections. This year we have also used molecular markers for mapping crop genomic regions associated with resistance genes against cassava bacterial blight, for apomixis in *Brachiaria*, and for BGMV and P uptake in common beans. DNA sequencing allowed us to identify a putative Antracnose resistance gene in beans and an R-gene for rice blast. Finally, through cooperation with ARIs we have constructed the first bacterial artificial chromosome (BAC) gene library for cassava. This is a step to the cloning of large genomic segments for future manipulation and usse in crop improvement.

2. **Crop plant genomes modified.** We have obtained transgressive segregation for higher yield (15-25%) in interspecific backcross progenies of rice with wild *Oryza spp*. Screening these progenies with molecular markers, allowed to identify and map chromosomal regions (QTLs) from *O. barthii* and *O. rufipogon* associated to yield and yield components. Evaluation of RHBV resistant transgenic rice resulted in up to 65% higher yield than non-transgenic control; furthermore, we have obtained indications that the transgene can be transferred by crossing to other rice vars. In preparing for the genetic engineering of cassava, we have screened a range of Latin American cassava cvs for their response to somatic embryogenesis. This year we have also made genetic constructs harboring a Bt gene agains the cassava stem borer. As a step towards an understanding of pathogen-host interactions, a CBB pathogenicity gene has been cloned and its protein characterized, and a highly sensitive and specific, PCR-based, detection method was developed. Due to their unique tolerance to Al toxicity, low N and low *P, Brachiaria* spp constitute an important source of stress-related genes. In collaboration with an ARI we have isolated two secondary metabolites which are biosynthesized in *Brachiaria* roots under stress; its anti-fungal role will be investigated. Long-term preservation of tissue and cells is basic to integral genome modification strategies; we have moved forward in the developing of a simplified, low cost cryoconservation technique for cassava shoot tips by an encapsulation-dehydration method. As above, CIAT technological capacities have been extended to micropropagation/micrografting of a fruit tree and genetic transformation of a horticultural crop in collaboration with NARS.

3. **Collaboration with CIAT partners enhanced.** In the period covered by this report, project SB-02 staff has organized 6 workshops and courses dealing with biotechnology and biodiversity topics and planned a second phase of CBN. The BRU has assembled a collection of near 100 *E. coli* strains, containing plasmid constructs for genetic transformation and over 100 *A. tumefaciens* strains. In the period Oct 1997 – Sept 1998, the project staff has produced 19 refereed publications, 14 non-refereed and 13 project concept notes and proposals. Ten donor organizations have contributed to project SB-02 complementary outputs/activities in 1998.

PROJEC T SB-02: ANNUAL REPORT 1998

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OUTPUT 1. GENOMES OF WILD AND CULTIVATED SPECIES AND ASSOCIATED ORGANISMS CHARACTERIZED

ACTIVITY 1.1 Molecular characterization of genetic diversity.

SUMMARY OF ACHIEVEMENTS

- In describing the phylologeny of the genus *Phaseolus*, 20 species have been grouped using internal regions of ribosomal DNA.
- As a collaboration with the ESALQ, Univ. of São Paulo, on the structure of Brazilian cassava landraces using AFLPs, alleles that are unique to Brazilian germplasm have identified.
- In collaboration with CORPOICA, 52 *Passiflora* spp have been characterized and patterns of genetic distances described. Genome grouping, was carried out with the Colombian *Musas* collection for duplicate identification.
- AFLPs identified genetic groups within the cassava bacterial blight pathogenic population of Colombia, at ecoregional and microgeographic level. Further, AFLPs showed great discriminatory power to distinguish genetic differences at even the intrapathova level.
- AFLPs where also used to characterize biological control agents and entomopathogens of the cassava mite.

1.1.1. Phylogenetic analysis in *Phaseolus.*

Background

Molecular data plays an essential role in the reconstruction of evolutionary relationships among many organisms. For assessing molecular phylogenetic relationships among plants, the nuclear genes coding for the 18S and 25S ribosomal RNA components of the cytosolic ribosomes have been used extensively at family and higher taxa levels (Hamby and Zimmer, 1991). To study the phylogeny among closely related genera or species, however, a more rapidly evolving gene is needed for the analysis. Internal transcribed spacer (ITS) regions have been shown to be relatively conservative in length and can be used as a source of nuclear DNA characters for phylogenetic reconstruction in plants (Baldwin, 1992). Both ITS regions (ITS1 and ITS2) are part of the rRNA genes (rDNA), which are organized in a tandemly repeated manner. Each rDNA repeat unit consists of the genes coding for the 18S, 5.8S, and 25S rRNA, which are separated from the next repeat unit by the intergenic spacer region (IGS). The coding regions are interrupted by two internal transcribed spacers: ITS1 and ITS2.

We have initiated a molecular phylogenetic study of the different section of the *Phaseolus* genus starting with the group of species related to the common bean (*Phaseolus vulgaris*). The second group to be analyzed is of the Lima bean. A third target for this project is a total phylogeny for the genus including all species available at CIAT.

Methodology

Total DNAs were isolated from fresh or frozen leaves following the protocol of Afanador et al. (Afanador, L. pers comunication). PCR amplifications of the ITS sequences were done using a total volume of 50µl, containing 2.5mM $MgCl_2$, 0.1uM each primer, 1mM dNTPs mixture and 10X *Taq* polymerase buffer and 1 unit *Taq* DNA polymerase was added to each reaction. PCRs were performed in a MJ research thermocycler. PCR products were purified using Wizard PCR Preps (Promega Corp.) or by elution in a low melting point agarose. Fragments were sequenced on a ABI Prism 377 (Perkin-Elmer) using dye terminators and AmpliTaq FS (Perkin-Elmer). The sequences obtained were aligned using the Sel-Al program and phylogenetic analysis will carried using PAUP version 4.

Results

Twenty different species of the genus *Phaseolus* have been amplified using primers from the ITS regions and complete sequences were obtained. A preliminary phylogenetic tree was constrcuted using *macroptilium* sp as an outgroup. *P. lunatus* and *P. vulgaris* complex were found to be in separated groups, as expected. *P. vulgaris* was closer to *P. coccineus*. The *P. lunatus* complex was found to be closer to *P. augusti* and *P. pachyrrhyzoides* than to *P. vulgaris* . These results are in agreement with previous analysis conducted at CIAT using the AFLP methodology (A.L. Caicedo, BRU annual report 1997)

On going activities

- Analysis of the data for the *P. vulgaris* complex and *P. lunatus* complex and associated wild *Phaseolus* species
- Comparison with different species.
- Sequencing of ITS from a wide range of *Phaseolus* species
- Construction of phylogenetic tree for all species of the genus *Phaseolus* .

References

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Collaborators: E. Gaitán, O. Toro, D. Debouck and J. Tohme

1.1.2. Microsatellite variability in Brazilian cassava landraces.

Introduction

Cassava is one of the most important cultivated species among traditional farmers in Brazil, especially those from local indigenous communities in Amazonian and sub-Amazonian regions. Cassava flour constitutes the basis of these communities' diets. In these regions, farmers grow in small gardens dozens of cassava landraces (Cury, 1998). This type of agricultural system conserves and maintains a substantial genetic variability *in situ,* in a way that is still not well understood. The expansion of modern commercial agriculture that affects directly and indirectly these traditional systems, often results in the genetic erosion of existing landraces (folk varieties, ethnovarieties).

To understand better the genetic structure of local landraces a project was initiated by the Genetic Department, ESALQ-USP, Piracicaba-SP, Brazil. One component of the project involves the analysis at CIAT of cassava microsatellites to evaluate the genetic variability of 55 cassava landraces from 13 'gardens', obtained from 8 communities in 3 Brazilian regions: Solimoes river, Negro river (amazonia), and Ribeira valley (Atlantic jungle, southern cost of Sao Paulo State).

Material and methods

DNA from lyophilized leafs of 55 Brazilian folk varieties was extracted by the method described in Dellaporta (1983). The tissue was obtained from the cassava germplasm collection of the Genetic Department, ESALQ-USP, Piracicaba-SP, Brazil. Twelve cassava microsatellites (Chavarriaga-aguirre, in press) were amplified with fluorescent primers purchased from Perkin-Elmer or Research Genetic. The fragments were separated in an ABI PRISM ^{TM} 377 DNA Sequencer. The protocols used to amplify and separate the microsatellites were described in CHAVARRIAGA-AGUIRRE (in press).

DNA from the accession MCol-22 from the CIAT core collection was used as a standard in all gels to facilitate the comparison of results. Microsatellite data from the core of the core collection on CIAT were obtained by Roa, A.C. (BRU, personal communication). The results were analyzed with the GeneScan^{TM} and Genopyper software.

Results

The data of this study were also compared to 38 accessions that constitute the core of the cassava core collection present at CIAT, using the same set of microsatellites. The number of alleles found is displayed in the Table 1. We found 14 alleles that are not shared by the 2 groups of plants. Seven are exclusives of the Brazilian landraces and the other ones are exclusive of the core of the core collection of CIAT.

On going activities

1. Statistical analysis of the microsatellite data to determine the genetic structure of the Brazilian collection
2. Compare the diversity of the Brazilian collection wit h the core collection
3. Determine the change if any on the core collection

Table 1. Microsatellites primers analyzed in cassava and wild *Manihot* species.

Microsatellite name	Forward primer/ reverse primer[a]	Repeat sequence[ab]	Size range (bp)	No. of alleles[c]	% of polymorphism
GAGG 5	TAATGTCATCGTCGGCTTCG GCTGATAGCACAGAACACAG	$(GGGA)_2(GCGA)$ $(GGGA)(GA)_9$	113-127	8	86
GA 12	GATTCCTCTAGCAGTTAAGC CGATGATGCTCTTCGGAGGG	$(GA)_{13}$	133-171	17	100
GA 13	TTCCCTCGCTAGAACTTGTC CTATTTGACCGTCTTCGCCG	$(GA)_2GC(GA)_8$	134-140	4	57
GA 16	GTACATCACCACCAACGGGC AGAGCGGTGGGGCGAAGAGC	$(GA)_{11}$	98-134	15	71
GA 21	GGCTTCATCATGGAAAAACC CAATGCTTTACGGAAGAGCC	$(GA)_3$ $AA(GA)_{13}$	104-118	8	86
GA 126	AGTGGAAATAAGCCATGTGATG CCCATAATTGATGCCAGGTT	$(GA)_8(GT)_7G(GT)_4$	178-403	20	86
GA 131	TTCCAGAAAGACTTCCGTTCA CTCAACTACTGCACTGCACTC	$(GA)_{19}$	75-128	21	100
GA 134	ACAATGTCCCAATTGGAGGA ACCATGGATAGAGCTCACCG	$(GA)_{12}GTA(GA)_2$	309-338	10	71
GA 136	CGTTGATAAAGTGGAAAGAGCA ACTCCACTCCCGATGCTCGC	$(GA)_{15}$	148-171	11	100
GA 140	TTCAAGGAAGCCTTCAGCTC GAGCCACATCTACTCGACACC	$(GA)_{14}$	148-172	10	57

[a] Chavarriaga et al., 1998.
[b] Sequence of the SSR loci isolated from the cassava cultivar M COL 22.
[c] Null alleles are not included.

References

Chavarriaga-Aguirre,P; Maya, M.M.; Bonierbale,M.W.; Kresovich,S.; Fregene, M.A.; Tohme,J.; Kochert,G. 1998. Microsatellites in cassava (*Manihot esculenta* Crantz): discovery, inheritance and variability. *Trends in Genetics* (in press).

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Dellaporta, S.L.; Wood, J.; Hicks, J.B. A plant DNA minipreparation: version II. *Plant Molecular Biology Reporter* 14: 19-21 .

Collaborators: Muhlen, G.S[1].; Roa, A.C.; Tohme, J.
1. ESALQ, Universidade de São Paulo, Brazil

1.1.3 Measurement of the genetic diversity in *Xanthomonas axonopodis* pv. *Manihotis* within different fields in Colombia.

Introduction

Cassava Bacterial Blight (CBB) caused by *Xanthomonas axonopodis pv. manihotis (Xam),* is a major constraint to cassava cultivation worldwide. The movement of *Xam* by the exchange of contaminated planting material has important implications for the control of the disease. Thus it is important to understand pathogen migration and how it influences population genetic structure. A study of the distribution of pathogen diversity among ecological zones and sites in Colombia was conducted in 1995-1996. This study revealed the genetic variability of *Xam* in Colombia and population sub-structuring at the level of ecological zones (Restrepo and Verdier, 1997).

The objective of the present study was to continue the analysis of *Xam* population structure in Colombia by examining the variation and microgeographic distribution of the pathogen in different fields located in four ecological zones.

Materials and Methods

A *Xam* collection was sampled in 1997 from eight experimental fields and analyzed by RFLP, rep-PCR and by inoculation on a cassava cultivar. The genetic diversity for all *Xam* isolates was estimated by Nei's diversity index (H_T). A genetic differentiation coefficient (G_{ST}) was calculated as $G_{ST} = (H_T - Hs)/H_T$ where Ht is the total genetic diversity of *Xam* in Colombia and Hs is the average genetic diversity in each ECZ or each field.

Results and Conclusions

In Colombia, the *Xam* population shows a high degree of genetic diversity (table 1). 19 distinct haplotypes were found among the 244 Colombian isolates collected in 1997. Ten new haplotypes, that were not detected in a previous study, were characterized. This may reflect the potential of change of the *Xam* populations. Our results allowed us to assess the role of host selection in structuring pathogen population. Pathogen diversity was highly correlated to the number of cultivars planted.

Seven haplotypes out of the 8 described in the ECZ2 were detected at site E. All the haplotypes present in ECZ1 and ECZ5 could be detected in one site, site G and B respectively. For future studies to evaluate pathogen diversity in Colombia, we suggest to collect extensively within one field representative of each ecological zone. Further studies in single fields (e.g, site F) will give insights into the effects of cultivation of a host genotype on pathogen diversity. Based on the multiple correspondence analysis, the 244 isolates were grouped into six clusters. Three clusters contained isolates collected in only one ecozone, clusters 1, 2 and 6 (ECZ 5, 2 and 1 respectively).

ERIC and REP primers yielded two and three fingerprint types respectively. The rep-PCR was less discriminative than RFLP, showing less that 1% of polymorphism among the analyzed strains.

It was possible to detect pathogen migration with the use of the RFLP analyses. While the haplotypic distribution is specific to each ecological zone (ECZ), within an ECZ the same haplotype strains were present in different sites. This supports the evidence that movement of the pathogen within ECZ results from the transmission of the strains through contaminated germplasm.

References

Restrepo, S. & V. Verdier. 1997. *Appl Environ Microbiol* 63, 4427-4434.

Table 1: Haplotypic diversity (Hs) of *Xam* population within each field, each ecozone and for the *Xam* collection in Colombia (H_T).

ECZ [a]	Locality	Field	Cassava cultivar planted	Genetic diversity index	
				Hs (field)	Hs (ECZ)
5	Cajibio	A	Traditional	0	0.53
	Mondomo	B	Improved	0.66	
2	Matazul	C	Improved	0.62	
	Carimagua	D	Improved	0.64	0.68
	Villavicencio	E	Improved	0.68	
2-5	Santander Quilichao	P	Improved	0.46	0.46
1	Caracoli	F	CG1141-1	0	
					0.52
	Pivijay	G	Improved	0.61	

[a] ECZ = edaphoclimatic zone, ECZ 1: subhumid tropics, ECZ 2: acid-soil savannas, ECZ 5: high-altitud tropics and ECZ 2-5: which shares ECZ 2 and ECZ 5 characteristics

Collaborators: C. Vélez, S. Restrepo and V. Verdier[1]
1. ORSTORM-CIAT

1.1.4 AFLP fingerprinting: an efficient technique for detecting genetic variation of *Xanthomonas axonopodis* pv *manihotis*

Introduction

Xanthomonas axonopodis pv. *manihotis (Xam)* is the causal agent of cassava bacterial blight (CBB), a particularly destructive disease of cassava in South America and Africa.genetic diversity of *Xam* has been mostly characterized by RFLP analyses. The analysis of a Colombian population of *Xam*, collected from different ecological zones, showed the existence of at least one clonal population, which was found in the high-altitude tropics (ecozone 5) (Restrepo and Verdier, 1997). To verify the genetic homogeneity of this population, we developed a molecular technique that can detect smaller sequence variations than can the RFLP technique.

Amplified restriction fragment length polymorphism (AFLP) is a recently developed technique for the fingerprinting of DNAs of any origin or complexity. The high resolution power of AFLP was demonstrated in characterizing bacterial strains at the subgeneric level. Until now, AFLP has not been applied to population studies of plant-pathogenic bacteria at the pathovar level. We previously established the conditions for applying the technique to the study of *Xam*. Here, we report the use of the AFLP technique in assessing the genetic diversity of *Xam* at the infrapathovar level. The efficiency of RFLP and AFLP analysis for measuring genetic diversity in *Xam* was compared.

Methods

Bacterial strains, DNA isolation and RFLP analysis. *Xam* isolates were collected in different sites in three ecological zones. Forty-six field isolates collected in 1995 and 1996 and one reference strain, CIAT 1121 were analysed. The RFLP-*pth*B haplotype of 37 of the 47 *Xam* strains were previously characterized (Restrepo and Verdier, 1997).

AFLP reactions. AFLP markers were assayed as previously described with modifications (Vos et al., 1995). The second PCR reaction was performed with the touch-down PCR thermal profile, with the initial annealing temperature of 60°C and subsequent reduction by 1° to 55°C over five cycles (Fig.1). The primers used were those selected in a previous study (CIAT, annual report, 97). The amplified products were separated on a 6% polyacrylamide denaturing gel.

Data analysis. RFLP or AFLP markers from eight primer combinations were scored as either present (1) or absent (0). We used NTSYS-PC to calculate a similarity matrix, to do the cluster analysis and to construct the dendrogram. A correlation index was calculated between the similarity matrices resulting from AFLP and RFLP to determine the complementarity or redundancy of results.

Results and Conclusion

AFLP is an extremely useful and reliable technique for detecting polymorphisms in bacterial populations. Analysis of 47 *Xam* strains presented a total of 322 AFLP bands for the eight primer combinations. Between 28 and 64 bands per strain were obtained, ranging from 40 bp to 350 bp. 173 bands were polymorphic and considered for the cluster analysis. AFLP allowed the discrimination of closely related strains collected in ECZ5. Ten clusters were obtained and ECZ5 strains were grouped in two separate clusters. The cluster analysis performed for the RFLP markers resulted in 8 clusters and ECZ5 strains were grouped in one cluster.

The results obtained by AFLP and RFLP are correlated but not redundant. Matrices of genetic similarity estimates, based on both methods were correlated (r = 0.79). The AFLP data not only supported but also extended the RFLP/*pth*B analysis by revealing the existence of subgroups among the ECZ5 *Xam* population. By having a superior discriminative power in differentiating highly related strains belonging to the same pathovar, AFLP analysis is a valuable alternative in *Xam* population studies.

AFLP results support the hypothesis that strains from ECZ5 form a genetically and evolutively separate group. Strong evidence exists to suggest that the pathogen migrates between and within ECZs in Colombia, but because of the low similarity observed between ECZ5 strains and strains collected in other ECZs, the origin of the ECZ5 strains remains unclear.

AFLP markers can also be used to identify particular races or pathotypes and for monitoring the dynamics of AFLP haplotypes within each region. In addition to population studies, AFLP DNA fingerprinting may facilitate the identification of polymorphisms linked to virulence factors and contribute to the understanding of plant-bacteria interactions at the molecular level.

References

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Collaborators:
Silvia Restrepo, Myriam Duque, Joe Tohme and Valérie Verdier [1]

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1.1.5 Virulence variability of a *Xanthomonas axonopodis* pv *manihotis* Colombian populations.

Introduction

Cassava bacterial blight (CBB) caused by *Xanthomonas axonopodis* pv. *manihotis (Xam)* is a particularly destructive disease in Colombia. The most appropriate and realistic approach for controlling the disease is to identify and deploy host-plant resistance. However, effective

breeding for resistance depends on information about the pathogen diversity and the spatial distribution of the diversity. Virulence variation of *Xam* has been reported by different authors and their results suggest that besides variation in virulence, some pathogenic specialization exists. Our main objectives are to determine the possible existence of pathotypes among the *Xam* population and to evaluate the usefulness of cassava cultivars as host differentials.

Materials and Methods

Cassava cultivars and bacterial strains. Based on a previous study of the germplasm diversity (Sánchez *et al.*, 1998), we selected a set of 17 cultivars as potential CBB differential hosts. A set of 26 *Xam* isolates representing the 26 haplotypes described in Colombia in 1996 was used for inoculation. Plants were arranged in the greenhouse according to a randomized block design with five replications per isolate x cultivar combination. The experiment was conducted twice. Inoculation techniques and disease rating were as previously described (Restrepo and Verdier, 1997; Verdier *et al.*, 1994).

Data analysis. Inoculation datas were analyzed by a non-parametric analysis of variance, the Kruskal-Wallis analysis. The area under the disease progress curve (AUDPC) was calculated for each interaction and data transformed to log (AUDPC) and then analyzed. Data from the AUDPC analysis and the leaf inoculations were analyzed by an analysis of variance using the General Linear Models procedure (SAS). A Duncan's multiple range test was used to compare the entry means (significance was declared for $p < 0.05$). The leaf reaction was assigned to one of five pathogenicity classes (A to E), which were determined by a descriptive method (univariate procedure of SAS) (SAS). The analysis was done for each cultivar separately.

Results

Leaf and stem inoculations for detecting strain X genotype interactions.. Lesion sizes obtained after the leaf inoculation varied widely among cultivars. By the stem and the leaf inoculation methods, it was possible to detect a variation in the reaction of the isolates when inoculated on the cultivars. However, using the leaf inoculation method the genotype X isolate interactions were not statistically significant ($p = 0.1884$). Data of the stem puncture of the 26 isolates on the same 4 cultivars showed that the interactions were highly significant ($p < 0.001$).

Isolate X cultivar interactions. The interactions between *Xam* isolates and cassava genotypes were highly significant ($p<0.01$). No significant differences were found between the two repetitions of the experiment. Each *Xam* strain showed different reaction after the inoculation with the 17 cultivars. Reactions between isolates and cultivars adapted to the ECZ of origin of the isolates were studied and specific pathotypes were defined for each ECZ. Among ECZ2 isolates, 8 different pathotypes were defined. Six pathotypes were found among ECZ1 isolates. Cultivars adapted to ECZ5 were all susceptible to ECZ5 isolates therefore only one pathotype was described. The ANOVA analysis of the areas under the disease progress curve demonstrated that significant differences between cultivars, isolates and among the interactions (isolate x cultivar) ($p<0.001$) exist.

Discussion

Two inoculation methods were assessed for their usefulness in detecting cultivar x isolate interactions. Multiplication and spread of *Xam* were monitored in cassava leaves in susceptible and resistant cultivars as expressed by the leaf area lesion. Our results confirmed previous results that have shown that bacterial spread in the mesophyll is identical in resistant or susceptible cultivars. Cassava bacterial blight is a foliar and vascular disease and plant resistance mechanisms occurred at a vascular level. Stem inoculation is the most appropriate method for detecting isolate x genotype interactions and must be recommended for such studies. The lesion sizes obtained after the leaf inoculation may be correlated with the leaf structure rather than with the resistance as expressed in the vascular stem.

The results of the Kruskal-Wallis analysis of the stem inoculations and results of the ANOVA analysis of the areas under the disease progress curve showed the possibility of some varietal-specificity. Resistant cultivars react differently to different highly virulent isolates, indicating that different resistant factors may be involved in the expression of resistance. Isolates were grouped into pathotypes based on their reactions on the cultivars adapted to the ECZ where they were collected.

Our results provide a basis for CBB control strategy in specific ECZ utilizing a selective deployment of ECZ-adapted cassava resistant cultivars. In the case of *Xam* in Colombia, some genotypes with good level of resistance could be proposed for further breeding. MBRA 685 and MNGA-2 present a good level of resistance to the majority of ECZ2 isolates, CM6438-14 and CM523-7 are resistant to ECZ1 isolates. Resistance to ECZ5 isolates has to be found. The durability of these sources of resistance is likely to be affected by the capacity of the bacterium to undergo mutation for pathotype change in the field and by the changes in the pathogen population due to pathogen's migrations.

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1. ORSTOM- CIAT

1.1.6 Molecular Characterization of Selected Species of Phytoseiidae

The complex of phytoseiidae found on cassava in the Neotropics has its greater genetic diversity in Colombia where 40 species have been sent to Africa for the biological control of *M. tanajoa.* Three of these have become established, *Neoseiulus idaeus, Typholodromalus manihoti* and *T. aripo.* It has been found that isolates of *N. idaeus* do not show any variability at the isoenzymatic level, perhaps oving to its wide distribution in different ecosystems. *T. manihoti* is the predominant species in most of the cassava growing areas of South America. In isoenzymatic studies done at CIAT show that *T.*

manihoti has considerable diversity in its banding pattern. Because of this hybridization studies have been carried out between populations of this species to determine if reproductive differences exist or if there is an isolation of this group.

Materials and Methods

In the present research molecular studies are using AFLP's is being done in populations of the different species found in hot semiarid and high altitude regions of the neotropics that correspond to similar regions of Africa (the Sahara and the East African plateau. The objective of this work is to identify their phylogenic relationships and contribute to the selection and release of the most promising species for biological control of *M. tanajoa* in Africa.

Results and Discussion

Eight phytoseiid species, including numerous populations collected in several countries were analyzed with AFLP's **(Table 1).** Due to the problems that have occurred in the reproducibility of banding patterns of AFLP's with arthropods in general, special extraction techniques have been developed, which have given good results. The methodology of Vos et al, 1996, with Operon Technologiest adaptors and Primers was used. Non degenerated DNA, at good concentrations, between 10 and 25ng/ul, was obtained.

Of the species indicated in **(Table 1)** diverse combinations have been analyzed and at this time results have been obtained for the combinations PEIC/PMIC for *N. idaeus* and *T. manihoti.* Using a NTSYS program, with UPGMA methods of classification realized with a matrix of similarity calculated with the DICE index, the following dendograms were obtained **(Fig. 1 and 2).**

Populations of *N. idaeus* showed a very high similarity index of 0.96 in the case of populations coming from different geographic zones such as Brazil and Venezuela and up to 1.00 in the case of populations form the same geographic zone. These similarity indexes, being so high, show no significant difference and we can say they are monomorphic populations.

Populations of *T. manihoti* display indexes of 0.2 to 0.7, tending to be more similar when from the same geographic zones, as in the case of Villanueva (Colombia) and Yaracuy (Venezuela) of 0.7. These populations come from the north coast of both countries, geographically close and with similar climates. It can also be concluded that the populations of *T. manihoti* analyzed show a high polymorphism. The analysis of the remaining populations is continuing.

Collaborators: A.Bohorquez, J. Tohme, A. Bellotti

Table 1. Phytoseiidae species analyzed with AFLP's.

Species	Country	Locality
T. manihoti	Colombia	Cajibío, Cauca
		Chinchiná, Caldas
		Barbosa, Antioquia
		Armenia, Quindío
		Copacabana, Antioquia
		Sta. Rosa Cabal, Risaralda
		Bijagual, Santander
		Bucaramanga, Santander
		Villanueva, Guajira
		Pivijay, Magdalena
	Venezuela	Marin, Yaracuy
	Ecuador	.Calderon, Manabí
	Brazil	Cruz das Almas, Bahia
T. aripo	Colombia	Palmira, Valle
		Pivijay, Magdalena
	Brazil	Cruz das Almas, Bahia
	Benin	Ab-Calavi, Benin Station
T. tenuiscutus	Colombia	Los Córdobas, Córdoba
	Ecuador	Chone, Manabí
		Cantagallo, Portocayo, Manabí
		Portoviejo, Manabí
		Guayaquil, Guayas
N. ideus	Colombia	Armenia, Quindío
		Fonseca, Guajira
		Carretalito, Guajira
		La Paz, Cesar
	Ecuador	Danzarin, Manabí
	Brazil	Petrolina, Pernambuco
		Crató, Ceará
		Capim Grosso, Bahia
		Piritiba, Bahia
	Venezuela	Carora, Lara
G. annectens	Ecuador	Crucita, Portoviejo, Manabí
		Calderón, Manabí
		Charapoto, Manabí
		Rocafuerte, Danzarín, Manabí
	Colombia	Fonseca, Guajira
G. helveolus	Ecuador	Santa Ana, Manabí
		El Rodeo, Portoviejo, Manabí
		Portocayo, Cantagallo, Manabí
Euseius ho	Ecuador	Santa Ana, La Teodomira, Manabí
		Santa Ana, Santa Ana, Manabí
		Rocafuerte, Danazarín, Manabí
		Puertocayo,Cantagallo, Manabí
N. californicus	Ecuador	Portoviejo, Manabí
		Chone, Manabí
		Machala

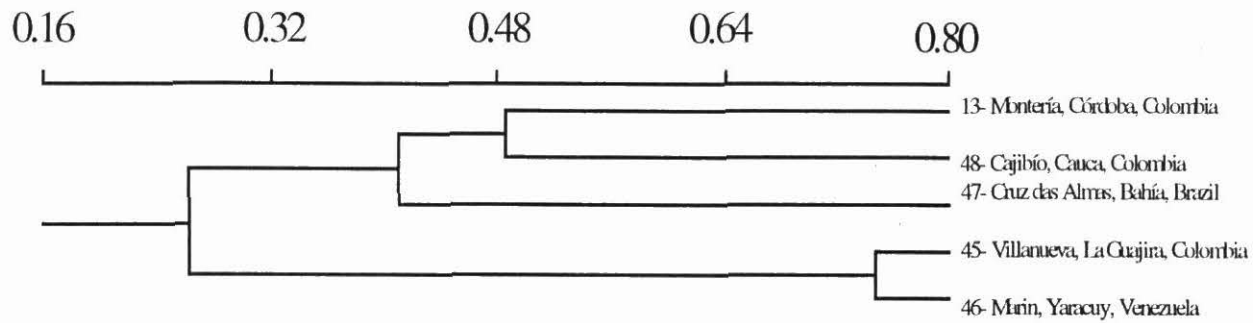

Figure 1. **Similarity index of five populations of *T. manihoti* with the PE1C combination.**

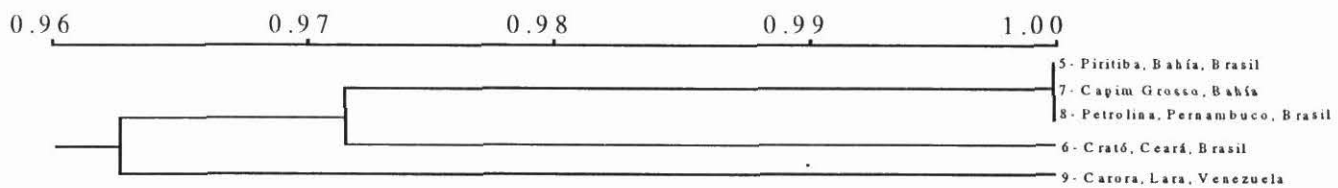

Figure 2. **Similarity index of five populations of *N. idaeus* with the PE1C/PM1C combination.**

1.1.7 Molecular characterization of the entomopathogenic fungus, *Neozygites* sp, pathogen of phytophagous mites.

Neozygites cf. *floridana*, a fungal pathogen (Zygomycetes: Entomophthorales), cause irregular or periodic mortalities on mite populations in Colombia and the NE Brazil. The pathogen has been found on cassava mites throughout many cassava growing regions of the neotropics. This fungus shows considerable promise for biological control of the CGM and is being studied in Brazil and Africa, as well as at CIAT.

Due to taxonomic problems with this type of fungus, it has been difficult to determine exact species identification. There is interest to introduce the neotropical strain of the fungus into Africa to control CGM, but before this can be done, it is necessary to determine certain characteristics of the fungus, such as the existence of different strains or species.

Material and Methods

Molecular techniques are presently being used to determine taxonomic identification of entomopathogenic fungi, such as AFLP's. However due to the difficulties in rearing this fungus *in vitro*, it is difficult to obtain sufficient quantities of DNA. We have improved on the method developed by Luis Leite and Donald Roberts (Boyce Thompson Institute, Ithaca, New York) (see 1997 Annual Report). Work by Delalibera (CNPMF/EMBRAPA, Cruz das Almas, Brazil) has also contributed significantly to the in vitro culture of this fungus.

DNA extraction was done by modifying the methodology of DNA MiniCTAB (developed by Zeller at Purdue University) developed for the *Pyricularia* fungus. The extraction of DNA is made from fungal isolates that have been growing in a liquid media, TNMFH, for 23 days. This is then lyophilized to reduce humidity and facilitate maceration. Fu AFLP's, the methodology developed by Vos et. al. (1995), with some modifications was used. Two kits were tried, the Small Genome of Gibco BRL Technologists, and the kit that contains the primers and adaptors of Operon Technologists.

Results and Discussion

It was possible to obtain (Zeller technique) DNA of good quality and concentrations of 5 to 150 ng, depending on the quantity of tissue obtained from the in vitro culture. Strains from several different sources were used in the analysis **(Table 1).** In some cases (Mt Benin) we were unable to obtain sufficient DNA to observe bands in the gels, perhaps due to the insufficient amount of hifal bodies produced in the in vitro culture.

Table 1. Strains of Neozygites used for DNA analysis collected from several sources.

Mite Host	Locality where collected	Name
Tetranychus urticae	CIAT, Palmira, Valle (Colombia)	TuCIAT 1
T. urticae	CIAT, Palmira, Valle (Colombia)	TuCIAT 2
T. urticae	Avakpa, Benin	TuBenin
Mononychellus tanajoa	CIAT, Palmira, Valle (Colombia)	MtCIAT 1
M. tanajoa	CIAT, Palmira, Valle (Colombia)	MtCIAT 2
M. tanajoa	CIAT, Palmira, Valle (Colombia)	MtCIAT 3
M. tanajoa	Media Luna, Magdalena (Colombia)	MtML
M. tanajoa	Santander de Quilichao, Cauca (Colombia)	MtSQ
M. tanajoa	Avakpa, Benin	MtBenin
M. tanajoa	Cruz das Almas, Bahia (Brazil)	MtCDA 1
M. tanajoa	Cruz das Almas, Bahia (Brazil)	MtCDA 2
M. tanajoa	Caruaru, Pernambuco (Brazil)	MtCar
M. tanajoa	Piritiba, Bahia (Brazil)	MtPir

With the small Genome kit it was possible to obtain good banding patterns, although the numbers are small (10 to 20) and insufficient to make a true characterization. With the Operon kit, it was possible to evaluate more primer combinations **(Table 2).** The combinations with the greatest numbers of bands were +1/+3 and the analysis has focused on these types of combinations. At this time we only have partial characterization that indicate marked differences between the fungal strains. However these analysis need to be repeated to confirm these results and the statistical analysis is being done.

Table 2. Strains of Neozygites and primer combination for AFLP analysis.

No. Strains of *Neozygites* sp.	Type of Combination	Combination	No. of Bands
6	+2 / +3	PE1AA/PM1A	32
8	+2 / +3	PE1AA/PM1B	44
8	+2 / +3	PE1AA/PM1C	39
5	+2 / +3	PE1AA/PM1E	24
5	+2 / +3	PE1AA/PM1F	40
3	+ 1 / + 3	PE1A/PM1B	44
3	+ 1 / + 3	PE1A/PM1C	40
3	+ 1 / + 3	PE1A/PM1D	34
3	+ 1 / + 3	PE1A/PM1F	57
3	+ 1 / + 3	PE1A/PM1G	70
3	+ 1 / + 3	PE1A/PM1H	64
3	+ 1 / + 3	PE1A/PM1I	67

Collaborators: A.Bohorquez, J. Tohme, A. Bellotti

1.1.8 Studies on the Viral Pathogens found in *Mononychellus tanajoa* and *M. caribbeanae.*

Previously we have reported at CIAT, epizootics causing high mortality in field and greenhouse populations of *M. tanajoa* and *M. caribbeanae*. The pathogen, analyzed under the election microscope appears to be of viral origin. The particles are

approximately 60 nm in size. Extractions of DNA have been made from the infected mite populations for molecular analysis. In agarose gels that displayed complete DNA, the presence of six different sized molecular bands was observed. This confirmed the presence viral particles. It has also been observed that these viral epizootics occur normally during wet or humid periods.

Reports in the literature on viral diseases of mites are few and little is known about its biology, mode of infection, symptoms, transmission, mite stages attacked, etc. In addition it is not known if it will attack beneficial mite predators. The present study is aimed at understanding and describing the signs and symptoms, oviposition of diseased females, mortality, viral presence all mite stages and transoval transmission.

Material and Methods

Four cassava leaves containing diseased *M. tanajoa* mites, and four leaves with diseased *M. caribbeanae* mites were used. From each leaf ten repetitions of each mite stage (eggs, larvae, nimphs, and adults), that showed symptoms of viral infection were removed. Infested leaves with healthy mites were used as a control. Observations with the election microscope were used to insure that mites were infected with the virus.

The biology, mortality and oviposition of mite stages were observed for 12 days. Each infected development stage was placed on a clean cassava leaf disc in a petri dish with moist tissue and in a 23 to 25°C chamber.

Viral disease symptoms are expressed by a clear amber discoloration of mite eggs, larvae and nymphs, and dark amber to brown colored adults. Upon death, hemocyle can be observed coming out of the anal pore resulting in a completely dry cadaver. Mortality is probably produced by the virus as virus particles have been found in the cadavers, of all stages of both species of mites. Mortality is highest in eggs and adults which could be due to the delicacy of the eggs and the age of the adults and the time of infection of the virus **(Fig. 1).** After 12 days, individuals in the control treatments showed no evidence of viral infection and those examined under the electron microscope showed no virus particles.

Oviposition by diseased females was greatly reduced when compared to healthy females **(Fig. 2).** When the F2 generation was evaluated mortality for all the stages of *M. caribbeanae* was 25.3% and for *M. tanajoa* 29.8% due to the virus. These results indicate that the virus is tranovil transmission of the virus. The F2 generation of the control mites did not show these symptoms.

Preliminary results with this virus disease indicate a good potential for biological control of cassava mites. Considerable research, however, still needs to be accomplished before this disease can be of practical use in an IPM program for control of cassava green mite.

Collaborators: A.Bohorquez, J. Tohme, A. Bellotti

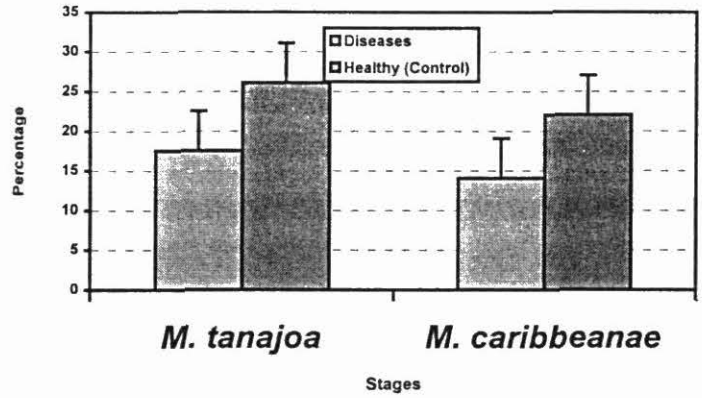

Figure 1. Percent Mortality of for Stages of *Mononychellus tanajoa* and *M. caribbeanae* due to Virus Infection.

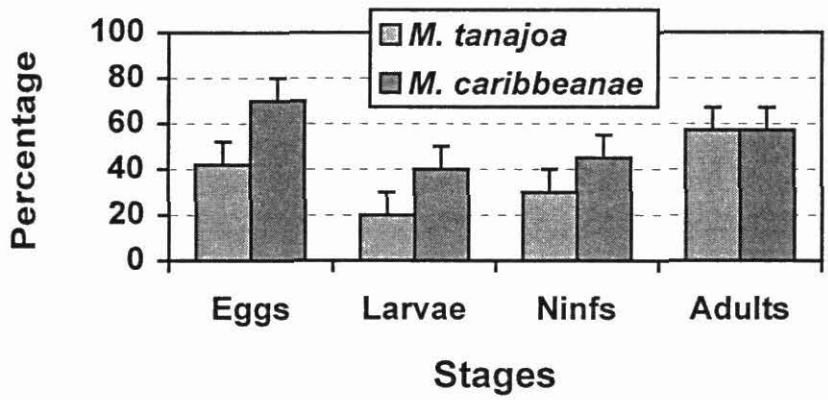

Figure 2. Oviposition of *M. tanajoa* and *M. caribbeanae* females infected with a virus disease.

1.1.9 Characterization and genetic variability analysis of *Passiflora* L. sp. from the Andean region, using molecular markers.

Introduction

The characterization and evaluation of wild and cultivated population of *Passiflora* is a priority for Andean countries.

In this study genetic characterization of variability evaluation with RAPD markers 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 valuable genetic resource. This work will contribute to the recent national efforts to collect, characterize and evaluate the *Passiflora* fruit germplasm.

Material and Methods

The collections of *Passiflora* a were established at the Experimental Station La Selva, Rionegro (Antioquia) of CORPOICA. DNA Extraction was described by Dellaporta et al. (1983). The RAPD markers were carried out as described by Williams et al. (1990). For the RFLP, five cloned fragments of cpDNA from Mung bean (4) were used as probes for detecting variation in the *Passiflora* chloroplast genome. DNA labeling and hybridization procedures were carried out as described by Angel et al. (1993). Three *Pst*I chloroplast probes from *Petunia hybrida* were also used. RFLP and RAPD band patterns were scored as a 1/0 data matrix. The dendrograms were constructed by the clustering method of UPGMA.

Results and Discussion

The resuls with RAPD markers carried out on 52 accessions representing 14 species of the genus *Passiflora* L. showed polymorphism, indicating high level of polymorphism intra species in *P. ligularis* given good bases for the improvement of this species. The RAPD markers also indicated a great genetic flow inter species specially between *P. india* and *P. mollissima.* This result of a great value for carring out taxonomic classification studies, shows similarity coefficients ranged from 0.929 to 0.075 showing a diverse genepool in the genus.

The studies with 71 more accessions shows a large intraespecific variation in *P. ligularis* confirming previous studies published in Euphytica 101:341-347. 1998. Further estudies will be carried out with new accessions. The results with RFLP markers demostrated profound cpDNA diversity among the species evaluated. Intraspecific variatiation was observed in four of seven species.

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Collaborators: I. Sánchez[1], F. Angel[2], D. Fajardo, María Fernanda Castillo, J. Tohme, and W. Roca.
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1.1.10 Biochemical and molecular characterization of the *Musa* L. Colombian Collection.

Introduction

The Colombian *Musa* Collection has 130 accesions but very few of them have been characterized at a morphologic and agronomic level. The genetic studies which we propose, based on a molecular characterization, will allow a better understanding of *Musa*'s genome and genetic diversity of the CCM.

This proyect also allow the identification of duplicated accessions of Colombian *Musa* Collection (CCM), thus contributing to reduce the costs of mantaining this collection *in vitro* and in field, by allowing its appropriate management.

Material and Methods

The agronomic characterization will be done to the field collection which is kept at the Agrado, Armenia - Quindío, 1310 m.o.s.l.

DNA was isolated, using the method of Doyle, 1990.

The molecular biology methods that will be used are: AFLP (Amplified fragment length plymorphism) described by VOS, P. et al. 1995. and Random Amplified Polymorphic DNA (RAPD), described by Williams, et al.1990.

Result and Discussion

Pattern differences between the accessions were evident in eight of the 52 tested RAPD primers. The dendrogram with RAPD markers diferentiated the plantain groups (AAB and ABB) from the *acuminata.*(AA and AAA).

From 32 primer combinations of AFLP tested, the combinations E-AAG+M-CAT and E-AAG+M-CTT were selected . The number of bands scored per gel was of 166 which an average of 30% were polymorphic. similarity matrix based on the similarity coefficient of DICE (1945), was produced. The greatest similarity (100%) was found between of the cavendish, gros michel genotypes, larger differences were found between *poupoulou* genotypes. A dendrogram based on average linkage cluster analysis was generated from the AFLP data The dendrogram shows clustering of the genotypes according to their genome origin .

AFLP analysis was found to be higly effective in distinguish genotypes from genome groupe. The percent similarity values between the 128 *Musa* genotypes, using one primer combination, ranged from 0,4 to 100%. In the previous study isozyme electrophoresis has been shown to have potential for discriminating between cultivars of *Musa* L. and RAPD variation was also found to be effective in distinguishing among the groups *acuminata* and *balbisiana*. On the other hand AFLP markers reveal differences within each group. Mientras que los AFLP muestran las diferencias existestes al interior de cada grupo. The AFLP markers data show that, genetically, the plantain genotypes are more closely related to those of the bananas. In the present study, with a few exceptions, the 128 genotypes clustered together according to genome type.

Future Activities

Characterization of Musa clones using microsatelites primers, given by the CIRAD -labs.

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Collaborators:

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ACTIVITY 1.2 Identification and mapping of useful genes and gene pools

SUMMARY OF ACHIEVEMENTS

- QTLs for P uptake by roots has been mapped; and major and minor resistance genes to BGMV, bean anthracnose and bean angular leaf spot have also been mapped; and QTL for BNF in presence of low P was tagged.
- Using the strategy of degenerate primers, we have identified sequences of a putative anthracnose resistance gene in beans and of a *Pyricularia* R-gene in rice.
- A genomic region involved in general defense against cassava bacterial blight was localized using the cassava molecular map. But initial work found no QTLs associated with marker genes involved in cassava post harvest deterioration.
- A region of the *Brachiaria* genome was associated to the apomixis gene and linked to an RFLP marker at 3 cM from the gene, to an AFLP at 11cM and to a SCAR marker at 3 cM from the gene. All these markers correspond to the rice chromosome 1.

1.2.1 Mapping of important agronomic traits in bean

Introduction

Last year we reported on the tagging and mapping of genes in a population of RILs derived from the cross of DOR 364 x G19833. QTL for two traits were mapped previously: root architectural differences that contribute to phosphorus (P) uptake in bean; and resistance to BGMV. Since that time we have obtained additional data for BGMV reaction, for other traits related to P absorption, and for other diseases.

Materials and Methods

In addition to data on root growth and P uptake in the field in Darién, Colombia, this year we obtained from colleagues at Penn State University, data on basal (or crown) root development, on root hair development, on leaf acid phosphatase activity, and on H+ ion exudation. At CIAT the RILs were evaluated for resistance to three different isolates of the anthracnose pathogen, and one isolate of the angular leaf spot (ALS) pathogen. All these isolates were characterized previously as Mesoamerican in reaction, as were used to test for the presence of resistance genes derived from the Andean parent, G19833. Days to flower, seed size and grain yield were also registered. Phenotypic data were analyzed with the QGene program to identify likely regions in the genome for QTL.

Results

It was possible to identify QTL for all the traits for which the analysis was performed. Thus, this is perhaps the most agronomic traits of common bean for which genes have been mapped on any single map to date. The mapping of QTL for the several traits related to P uptake permitted a far better understanding of the mechanisms that contribute to P acquisition in bean. Similarly, the mapping of resistance genes for anthracnose and ALS revealed aspects of the genetics of resistance. Both major and minor genes were identified. The biological dimensions of these results are discussed in sections 1.1 and 3.2 of Project IP-1. For purposes of the present report, we shall refer to an overview of the bean map that is emerging from these activities.

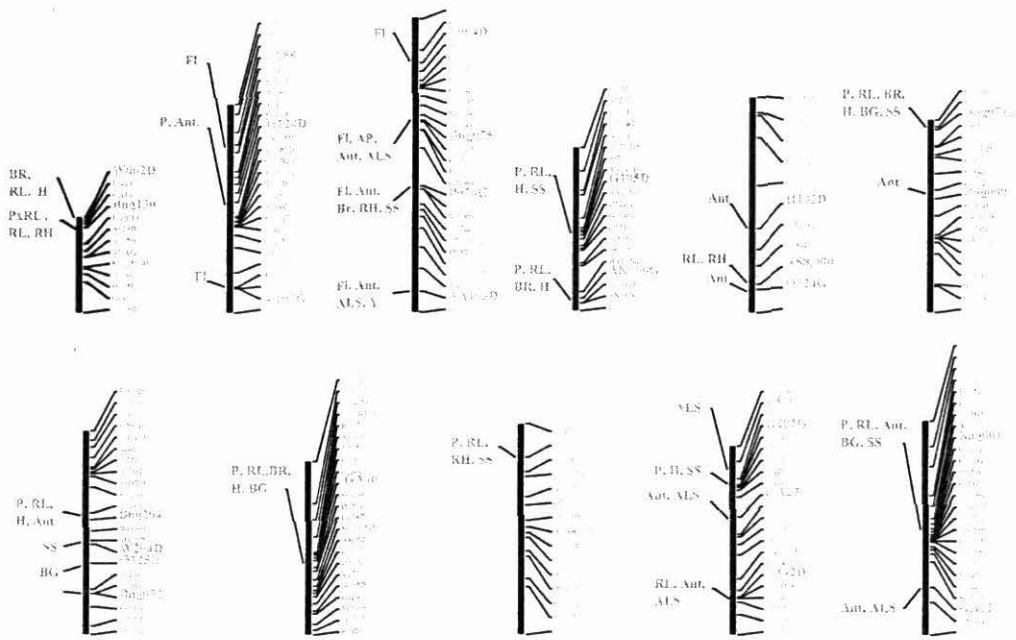
For the traits analyzed, at least 28 important regions of the genome were identified. These were distributed across all eleven linkage groups of the common bean genome, and are represented in **Fig.1** was observed that QTL for traits related to P absorption tended to cluster together. This was observed in almost all linkage groups. Some of these traits may in fact have a common physiological basis, although not necessarily. Likewise, some coincidence of resistance genes for anthracnose and ALS was observed (eg, around the region marked by the RAPD AA193D in linkage group B03). This could occur if these genes have a common evolutionary origin.

However, clustering of genes for apparently distinct traits was also observed. For example, in linkage group B03, the RAPD P076G was associated with P uptake and root architecture, with anthracnose resistance, with flowering date and with seed size. In linkage group B11, the RFLP Bng001 was associated with P uptake and root architecture,

with anthracnose and BGMV resistance, and with seed size. Many other examples were observed. The significance of this for the evolution of the crop genome is not clear. However, apart from the genetics of any single trait or possible applications in marker-assisted selection - the clustering of genes for very different types of traits is significant from the standpoint of breeding. If useful genes are linked in repulsion, it will be difficult to recombine such genes in breeding lines, in which case breeders may need to prioritize among closely linked genes and choose those that are indispensable for their objectives. Breeders may wish to develop strategies to utilize those genes that are not linked when different genetic strategies are available (eg, alternative genes for a disease resistance), or to make a special effort to detect genetic recombinants. This issue is especially important when breeding objectives call for the improvement of a larger number of traits. In any case, a systematic strategy requires the continued mapping of genes.

Collaborators: S. Beebe, F. Pedraza, J. Tohme

MAPPING OF QTL FOR AGRONOMIC TRAITS



1.2.2 GeneTagging with PCR Markers for Bean Breeding: Adaptation to Low Fertility Conditions

Introduction

Phosphorus (P) deficiency is thought to be a primary limitation on Biological Nitrogen Fixation (BNF) of common bean in tropical soils. In 1997 it was reported in Project IP-1 that bean accessions had been identified for the tolerance of BNF to low soil P. A breeding line, BAT 477 was found to be relatively tolerant to low P with regards to SNF. It has also demonstrated excellent N fixation capacity in unstressed conditions in two contrasting soil types and in drought conditions. A germplasm accession , G3513 that was previously identified as tolerant to low P in grain yield was also found to be relatively tolerant in BNF. In contrast, DOR 364 was found to be sensitive to low P with regards to BNF. The work with the sources of low P tolerant BNF was extended to the search for the genes responsible through the use of molecular markers.

Materials and Methods

RILs were developed of crosses of DOR 364 x BAT 477 (DxB)and DOR 364 x G3513 (DxG). These were evaluated in the F5 and F6 generations in the case of DxB, and F4 and F5 generations for DxG. A greenhouse sand culture system was used employing 3 treatments: 1) Low P+BNF, in which plants were inoculated with *Rhizobium tropici* strain CIAT 899; 2) Low P without Rhizobium, to estimate the contribution of seed N to plant growth; 3) High P with Rhizobium, to estimate BNF capacity in the presence of adequate P supply. Data taken on all treatments included aerial, root and total DW. %N and %P analyses were performed on the Low P+BNF treatment in both the F5 and F6 generations, using a bulk sample of ground tissue from the four repetitions. Shoot/root ratio, Total N and Total P were calculated from primary data.

After an initial screening of more than 300 primers to identify those that produced polymorphisms among the parents, primers were tested on genotypes in each population that presented extreme values in biomass accumulation and/or total N. Eventually 42 and 32 primers were evaluated on all RILs of DxB and DxG, producing 82 and 49 RAPD, respectively. A total of 12 linkage groups were thus formed in the population DxB, identified as DB1 to DB12, with 9 RAPD unlinked. These represented about 615 cM which is approximately half of the total length of the bean genome (Vallejos et al, 1991: Nodari et al, 1993; Beebe et al, 1998). Nine linkage groups were formed in the population DxG covering 563 cM.

Results: Several genomic regions in both populations were identified as containing QTL for different traits. In the population DxB, the most important linkage group, DB5, was composed of only three RAPD covering 5.1 cM. Within this group the marker N201 explained 16% of DW and 10% of Total N variability in the Low P + BNF treatment in the F5 generation. In the F6 generation the magnitude of its effect dropped to about 5%, but was significant nonetheless. This linkage group was also the only one in the DxB cross that presented a consistent effect for %P. In linkage group DB10 the RAPD U1403 had a significant effect on DW (13%) and also Total N (9%) in F5, while expressing a

much reduced effect in F6. Markers in groups DB13 and an unlinked marker G1002 also presented relatively stable effects. The possible agronomic significance of these QTL is discussed in the IP-1 report. Field trials are underway to determine the contribution of the QTL to field performance.

In the DxG population, QTL were identified in the Low P + BNF treatment that were expressed in both generations for: root DW; shoot DW; root/shoot ratio; %N; %P; and N/P ratio. Subsequently the RILs of DxG were planted in the low P field in Darién and were evaluated for yield. Three promising QTL were identified for yield at low P, but only one of these corresponded to a QTL recognized in the greenhouse system, where it expressed an effect on root/shoot ratio. While it is hopeful that yield QTL can be identified, these yield QTL remain to be confirmed with additional yield trials. It remains to be seen what significance exists in the field for the QTL that were marked in the greenhouse in the DxG cross. G3513 expresses other unique traits related to N transport, therefore the RILs will be used for studying those traits and the partial genetic map that has been developed will possibly be useful for those traits as well.

			R^2																			
Linkage Group		Size (bp)	DW				%N				%P				Total N				N/P				DW high P	
			F5		F6		F5		F6		F5		F6		F5		F6		F5		F6		F6	
	Marker			RSq		RSq		RSq		RSq		RSq		RSq		RSq		RSq		RSq		RSq		RSq
DB1	O1601		B	6.1	B	0.8	D	0.3	D	0.2	B	0.4	D	0.9	B	6.02	B	0.79	D	1.1	B	0.6	B	0.6
	H1201		B	7.9	B	1.9	D	0.2	D	0.3	B	0.1	D	1.5	B	8.33	B	1.77	D	0.4	B	1	B	0.2
	P701		**B**	**8.8**	**B**	**3.7**	D	2.8	D	0.4	D	0	D	2.1	**B**	**5.74**	**B**	**3.58**	D	0.6	B	0.7	B	0.9
	X303		B	3.1	B	0.5	B	0.1	B	1.9	B	1.5	B	0	B	4	B	3.47	D	1	B	2.5	D	0
DB5	N201		**B**	**16**	**B**	**4.7**	D	5.3	D	0.1	**D**	**5.8**	**D**	**3.7**	**B**	**10.4**	**B**	**3.28**	B	2.3	B	3.8	**B**	**6.8**
	U1801		**B**	**12**	**B**	**5.2**	D	3.5	B	0	**D**	**6.3**	**D**	**4**	**B**	**8.93**	**B**	**4.89**	**B**	**3.9**	**B**	**7.3**	**B**	**4.5**
	O2001		**B**	**12**	**B**	**5.1**	D	3.4	D	0	**D**	**6.3**	**D**	**5**	**B**	**9.2**	**B**	**4.39**	**B**	**4**	**B**	**7.8**	**B**	**3.4**
DB10	M901		B	6.3	B	0	D	6.5	D	5	D	0.1	D	3	B	2.29	D	0.98	D	0.6	D	0.1	B	3.7
	U1304		B	6.6	B	0.6	D	5.3	D	6	D	0.1	D	6.7	B	2.84	D	0.26	D	0.6	B	0.2	B	2.5
	U1303		B	7.5	B	1.1	D	5.3	D	5.2	D	0.4	D	6.3	B	3.52	D	0.01	D	0.1	B	0.3	B	3
	X903		**B**	**10**	**B**	**3.4**	D	2.2	D	3.5	D	0.3	D	5.4	B	8.03	B	0.85	B	0.2	B	0.6	**B**	**6.2**
	U1403		**B**	**13**	**B**	**3.1**	D	3.7	D	3.2	D	0.3	D	6.3	B	9.08	B	0.77	D	0	B	1	**B**	**5.3**
	U1402		**B**	**13**	**B**	**3.1**	D	3.7	D	3.2	D	0.3	D	6.3	B	9.08	B	0.77	D	0	B	1	**B**	**5.3**
	M902		B	12	B	0.8	D	3.6	D	2.6	D	0.3	D	2.8	B	8.21	D	0	D	0	D	0	B	5.9
DB13	Q1701		D	1.4	D	5.2	B	0.4	B	0.1	D	0.2	B	1	D	1.14	D	4.22	B	0.9	D	0.8	D	0.8
	Q1001		**D**	**5.6**	**D**	**5.3**	B	0	B	0	B	0.2	B	0.6	**D**	**6.33**	**D**	**5.08**	D	0.1	D	0.3	**D**	**6.2**
	X901		D	6	D	3.6	D	0.3	D	1.3	D	0	B	0.3	D	10.5	D	7.97	D	0.7	D	4	D	3.8
UL	G1002		**D**	**3.7**	**D**	**8.9**	D	0	D	0	B	0.7	B	3.4	**D**	**4.95**	**D**	**11.9**	D	0.7	D	5.1	B	0.5
Adj R^2																								

Conclusions

Several QTL were identified that were expressed in two generations in a greenhouse test of SNF under low P, although the level of expression varied widely. Some of these were also expressed at high P. At least one should be considered for conversion to a SCAR for use in marker assisted selection.

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1.2.3 Identification of Disease Resistance Gene Analogs (RGA): Identification of NBS type putative resistance gene in common bean.

Introduction

In the past few years several resistance genes have been isolated and cloned different plant species, using either map-based cloning or transposon-tagging. Molecular characterization of these genes uncovered common sequence motifs, even though they confer resistance to a wide spectrum of pathogens, i.e., viruses, bacteria or fungi. The majority of cloned R-genes are characterized by the presence of an N-terminal nucleotide binding sites (NBS) and a C-terminal stretch of leucine-rich repeats (LRR). The presence of these conserved domains allowed the grouping of these genes into several classes, and established their possible function in the defense response as part of the signal transduction pathway (Baker et al., 1997). Proteins similar to serine-threonine kinases are another group which has been suggested to interfere in protein phosphorylation, one of the common mechanisms of protein control (Bent, 1996). Other families include transmembrane receptors either with long extracytoplasmic LRR domains, like Cf-2 and Cf-9 in tomato, or with a LRR transmembrane region as seen in the product of $HS1^{pro-1}$ from sugar beet. There are also transmembrane receptors with extracellular LRR domains and also an intracellular serine/threonine kinase, like the one encoded by Xa21 in rice (Baker et al., 1997).

PCR-based cloning with degenerate primers made possible the identification of Resistance Gene Analogues (RGAs) in rice, *A. thaliana*, and lettuce (Leister et al., 1998; Aarts et al., 1998; Shen et al., 1998). Their sequences have shown high homology with R-genes previously reported, and they mapped within or near to disease resistance loci.

We have initiated a project to use this strategy to identify potential R-genes in rice and *Phaseolus vulgaris* L.

Common bean (*Phaseolus vulgaris L.*) production is severely affected by several diseases such as anthracnose, angular leaf spot and Bean Golden Mosaic Virus (BGMV). Anthracnose is caused by the fungi *Colletotricum lindemuthianum* (Sacc et Magn) and is the principal pest in Africa and Latin America. Genetic analyses of the disease, and race-cultivar specificity, strongly suggest a gene-for-gene interaction (Adam-Blondon et al., 1994), where the resistance is determined by the interaction of dominant resistant genes (R) in the plant and the corresponding avirulence genes (Avr) in the pathogen.

Materials and Methods

DNA was extracted from leaf tissues of two genotypes, G19833 and DOR364. G19833 is highly resistant to anthracnose and some strains of angular leaf spot but susceptible to BGMV, whereas DOR364 is susceptible to angular leaf spot and anthracnose, but is resistant to BGMV. Data on 87 RIL-F_9 plants from the cross of G19833 by DOR364 screened with several Andean and mesoamerican isolates of anthracnose, angular leaf spot and BGMV are available. A RAPD, RFLP and AFLP map is also available (BRU annual report, 1997).

PCR reactions were performed using degenerate primer combination I (s1 y as1) and II (s2 y as2) (Table 1). These primers were designed based on Leister et al. (1996). The PCR products were separated by electrophoresis in a 1.2% low melting point agarose (LMP –GIBCO-BRL). Each band was eluted and purified with the PCR-Preps kit (Promega). PCR-purified products cloned into the pGEM-T vector system (Promega) and transformed into *E.coli* DH5α cells by electroporation following GIBCO-BRL instructions. One hundred clones from each band were randomly picked and grouped based on restriction digestion patterns using 4-base-pair cutting enzymes. At least three clones from each group were used as probes. They were hybridized to blots containing parental DNA digested with a set of five restriction enzymes (EcoRI, EcoRV, XbaI, HindIII y DraI). RFLP segregation was evaluated on the 87 RIL-F_9 plants. Polymorphic probes and at least one clone from each group were sequenced using the Dye Terminator Cycle Sequencing Kit and an Applied Biosystems Prism 377 DNA sequencer (Perkin-Elmer).

Table 1. Primers used for PCR based on Leister et al. (1996).

	N-terminal						
Peptide sequence	**G**	**G**	**V**	**G**	**K**	**T**	**T**
Primer s1	GGT	GGG	GTT	GGG	AAG	ACA	ACG
Primer s2	GGI	GGI	GTI	GGI	AAI	ACI	AC
	C-terminal						
Peptide sequence	**G**	**L**	**P**	**L**	**A**	**L**	
Primer as1	CAA	CGC	TAG	TGG	CAA	TCC	
Primer as2	IAA	IGC	IAG	IGG	IAA	ICC	

Results

With primer combination I it was possible to amplify a single band, RGA1.1, of about 1200 bp. By using primer combination II we detected three bands: RGA2.1 (1200bp), RGA2.2 (800bp) and RGA2.3 (500bp). The restriction pattern of the RGA1.1 was the same for 100 clones, indicating homogeneity of PCR products (i.e., a single group was formed). The restriction pattern of bands RGA2.1, RGA2.2 and RGA2.3 was heterogeneous allowing the creation of groups 9, 10 and 7 respectively.

We have sequenced 35 clones from different groups of each band. A GenBank search, using the BLASTX algorithm, of sequences from bands RGA2.1, RGA2.2 and RGA1.1 did not show any significant homology with R-genes or RGAs reported in this database.

Eighteen clones of band RGA2.3 were sequenced and grouped, according to sequence homology, into three classes: 1) Class I contained five clones identical to each other (98% sequence identity); 2) Class II included clone RGA2.3.33.alone, although it had 73% sequence identity to Class I; and 3) Class III formed by clone RGA2.3.78, with 47% sequence identity to Class I. A fourth class grouped clones that did not have homology to the aforementioned classes, nor homology to any R-gene or RGAs. The majority of them

did not have continuous open reading frames and their hybridization patterns suggested multiple-copy sequences.

Clones of Classes I and II showed significant sequence similarity not only to resistance genes but also to like-NBS RGAs. However, clone RGA2.3.33 did not have a full-length open reading frame, suggesting that it is a pseudogene. A GeneBank search of clone RGA2.3.78 revealed high homology to RGAs of the kind NBS-LRR.

Several clones of the first class, and the clone RGA2.3.33, were mapped and were placed at the distal region of linkage group B11 of the integrated new bean map developed by Gepts's lab a t UC Davis. A highly significant QTL for anthracnose resistance (LR140) is mapped at this region (Pedraza, F. Personal communication). These clones may be part of the anthracnose R-gene of common bean.

Sequence of bean NBS gene fragment have been deposited in the GenBank database (accesion no. AF084026) (Figure 1).

On going activities will concentrate on:

4. Mapping RGA2.3.78r
5. Using different degenerate primers to amplify new RGAs
6. Sequencing more clones,
7. Generating cDNA libraries to isolate the candidate R-gene.

Figure 1. Sequence of bean NBS gene fragment *GenBank* database accession no. AF084026

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   1 acgactctcg ctcaacatgt attcaatgac ctgagggtgg atgaggctaa atttgatgtt
  61 aaagtttggg tttgtgtttc agatgaattt gatgttttca agatatctag agcaattctt
 121 gaggcagtta ctaaatcagc cgatgatagt agagatctgg agatggtcca tagaggaatg
 181 aaagaagaat tgacgggaaa gaaatttctt cttgttttgg atgacgtttg gaacgaaaac
 241 caacctaaat gggaggaagt gcagaagccc cttgttttag gagtccaagg gagtaagatt
 301 cttgtgacca cacgtagtaa ggaagttgct tctaccatgc gttcagaaga atactcccta
 361 caacaattac aagaagatga ttgttggaag ttgtttgcta aacatgcatt tcgaggtgat
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KKCK"
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Reference:

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1.2.4. Gene Tagging of Resistance to the African Cassava Mosaic Disease (ACMD).

Introduction

The Ugandan epidemic of the African cassava mosaic disease, caused by a recombinant strain of the African cassava mosaic virus (ACMV), reinforces the need for new approaches to resistance breeding as a preventive measure for new situations which may arise elsewhere in East Africa and sub-Saharan Africa, as the virus continues to evolve. Identifying and pyramiding disease resistance genes will provide stable resistance against a broad spectrum of the viral strains. Pyramiding genes, known only by indistinguishable phenotypes, requires molecular marker-aided genetic analysis. Map-based cloning of resistance genes, using the existing BAC library and tagged genes, will facilitate even more efficient movement of genes around cassava genepools, and is the ultimate goal. Strategies for gene tagging of disease resistance genes in out breeding crops have been

described and the require a population segregating for disease resistance and a molecular genetic map.

A half-sib backcross mapping population, generated at CIAT by crossing, in both directions, a set of 5 F_1 plants, chosen for their profuse flowering, to their maternal parent, TMS30572, an ACMD resistant improved cassava variety from West Africa, is an ideal segregating population. The paternal parent of the F_1 progeny, CM2177-2, an improved cassava variety adapted to the Colombian Caribbean coast, is susceptible. The back-cross population was established *in vitro* from immature seeds, 40 days after pollination, to considerably reduce the long period required for establishing cassava mapping populations. An existing embryo culture protocol for immature cassava seeds was modified for *in vitro* germination of the immature seeds. A total of 276 plants were transferred to to the International Institute of Tropical Agriculture (IITA), Ibadan for post-flask management, field establishment and disease resistance scoring. Gene tagging of ACMD resistance required that this half-sib BC1 population is genotyped with frame work markers from the genetic map of cassava and phenotyped for disease resistance in mutli-locational, replicated trials.

Materials and Methods

Two hundred and thirty plants showing sufficient growth were micropropagated at IITA, from the initial *in-vitro* cultures, by explanting and transferred to the green house in preparation for transfer to the field Ocober 1997. The green house material was established in the field in Abuja, Nigeria, a disease- free area for multiplication of vegetative material from the half-sib family January 1998. An ACMD disease resistance trial, with 3 replicates, 6 genotypes per replicate, in 3 high disease pressure sites, in a randomized complete block design was established early September 1998. Disease resistance will be scored at 3 and 5 months after planting.

Controlled screening, in the green house, of ACMD disease resistance is also desired, to maximise virus infection efficiency, and thus obtain true disease resistance phenotypes from each genotype of the BC1 cross. A full-length, infectious DNA clone of the West African strain from Nigeria was recently obtained by abutting primer PCR (AbP-PCR) at the John Innes center (JIC) (Briddon et al. 1993). The infectious clone was used to infect cassava plants in the green house without the whitefly vector, using a hand held particle gun. Twenty four stakes each of the parental genotypes of the original F1 mapping population were shipped to the JIC and bombarded with the infectious viral clone.

Transferring the genetic map of cassava to the half-sib backcross population has also begun at CIAT. One hundred and fifty RFLPs, 5 microsatellites, and 3 isoenzyme frame work markers, from the female- and male-derived genetic map of cassava will be used to genotype the population, and a genetic map constructed using the MAPMAKER linkage analysis program.

Results and Discussion

Preliminary screening of ACMD resistance with a virus DNA clone from ACMV-Nigeria, conducted at the John Innes center, on twelve plants established from stakes in

the green house, from each of the parents of the F_1 Map population reveal the female, parent, TMS30572, is indeed resistant, while the male parent is susceptible. TMS30572 showed no symptoms after bombardment with the infectious viral particle after several weeks after inoculation, while symptoms developed in 10 plants out of twelve in the susceptible, male, parent, CM2177-2, after two weeks. This results shows the appropriateness of these populations for gene tagging studies of diesease resistance. The half sib backcross population is expected to provide increased variation in the phenotypic expression of disease resistance thereby maximixing the chance that underlying segregating QTLs can be detected.

DNA was extracted from 240 genotypes from the BC1 half-sib cross, they correspond to genotypes in the field in Nigeria and also in the field here at CIAT. Southern blots for survey of the BC1 parental genotypes, with five restriction enzymes are being prepared, to screen the selected markers for polymorphisms. Progeny filters with the relevant filters will then be prepared.

Further activities

- Phenotype the BC1 half-sib cross for disease resistance both in the field in Nigerian, and the screen house at JIC.

- Genotype the BC1 with frame work markers from the genetic map of cassava.

References

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1.2.5 Marker-Assisted Genetic Analysis of Earliness and Root quality traits in Cassava

Introduction

A quantitative trait loci (QTL) trial to identify region of the cassava genome controlling time of initiation and rate of storage root thickening, post-harvest deterioration (PHD), starch content, culinary quality, and salient morphological traits in cassava with molecular markers from the cassava map, was established earlier in the year. Genetic analysis of genes controlling these important traits is expected to lead to more efficient schemes for simultaneous development of early, better keeping, and high starch cassava varieties. In order to take powerful technologies for marker-assisted cassava breeding out of International centers to national programs in regions where the most pressing needs for cassava improvement exists, a young African scientist was involved in the QTL experiments through the award of a Ph.D. studentship, funded by the Rockefeller foundation.

Materials and Methods

The F1 mapping population, from which the cassava map was developed, was also used as the QTL mapping population; the F1 cross has been genotyped with over 200 markers, including known starch biosynthesis genes, cyanogenesis genes, and expressed sequence tags. The field experiment was a triple partially balanced lattice design with 20 plant per genotype/plot per replication, with three replications, in two sites, CIAT, Palmira and CIAT, Quilichao. The Quilichao experiment was planted on the 15th of January 1998, while the CIAT plot was established the 29th of January, both plots were irrigated prior to planting. To test for early genotypes, storage roots from three plants from the border row of genotype plots, were harvested at 7 months, or early August, and dry matter content of the roots determined. Leaf morphology was also be measured at 6 months. The rest of the traits wil be harvested at maturity. Heritability of the different traits will be calculated from environmental, genotypic and total variance derived from the ANOVA of results. Single point by regression and t-test with conditioning will be carried out on the mean of phenotypic scores from the three replication by the QTL analysis software package Q-gene and PGRI.

Results and Discussion

Storage roots was harvested from three border plants from each plot at 7 months. Fresh roots from all three plants were weighed in air and water and the specific gravity of roots determined, which was later used to calculate dry matter. Phenotype values were averaged over replication for each site and analyzed by single point analysis using regression, with markers from the female-derived map being the independent variable, with the Q-gene package. Table 1 shows significant QTLs ($P<0.01$) found only on chomosome D for weight of storage roots at 7 months for the data from the Quilichao trial. Two regions on Chromosome D explains more than 60% of phenotypic variance. Heritability estimates, calculated from the ANOVA, (SAS Institute) remains to be completed, and should yield estimates of genotypic variance explained by the markers. No significant QTLs were found with the CIAT, Palmira data. It was however noted that the CIAT Palmira trial suffered from multiple problems, including: flooding in March, extensive pesticide damage late March, residual maize pesticide "atrazin" effect in May, and sporadic pest and disease problems. The Quilichao trial remained relatively clean. The experiment will be repeated the following year and in controlled trials in the green house to confirm the identified. The leaf morphology data is currently being analyzed.

Further activities

- Scoring the QTL trial in November for dry matter, starch content, post-harvest deterioration, culinary quality and important plant morphological characteristics such as plant height, height of first branching, stem color etc.
- Analyze earliness data with an aim of setting up controlled green house experiments with 40 genotyopes each from the extremes to measure components of earliness such as, increase in girth of secondary roots, time of first starch accumulation, or differentiation of cambia, rate of starch accumulation, etc.

Table 1. Markers of linkage group D of the genetic map of cassava showing significant linkage to a QTL effect for storage root dry matter weight at 7 months. QTL analysis used only data from the Quilichao trial.

Marker	Chrom	F	RSq	P	AA	SE	N	Aa	SE
Ai18b	NgD	15.72	0.1733	0.0002	521.72	32.46	39	695.5	29.35
GY219	NgD	14.45	0.1598	0.0003	530.49	33.07	41	696.76	27.86
GY42	NgD	14.43	0.1632	0.0003	530.8	33.25	41	701.23	28.97
rGY167	NgD	14.37	0.1608	0.0003	536.93	31.7	44	706.36	29.55
rGY180	NgD	14.24	0.1578	0.0003	532.95	32.6	42	698.5	28.23
GY181	NgD	14.01	0.1556	0.0004	535.42	31.93	43	700.2	29
GY50	NgD	13.96	0.1569	0.0004	533.93	32.57	42	700.29	29.14
rGOT-2	NgD	13.81	0.1521	0.0004	537.47	30.49	47	704.25	30.81
GY125	NgD	13.8	0.1554	0.0004	536.26	32.11	43	702.24	29.55
GY222	NgD	12.18	0.1447	0.0008	532.27	33.4	41	692.3	29.82
GY179	NgD	10.51	0.1229	0.0018	539.05	34.14	40	685.76	29.16
O19	NgD	8.97	0.1068	0.0037	554.42	30.85	40	684.22	30.26
AD4e	NgD	8.89	0.1086	0.0039	550.79	31.97	38	682.32	30.34
K11b	NgD	7.52	0.097	0.0077	536.29	35.21	38	669.35	32.88

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1.2.6 Mapping of genetic resistance of Cassava to bacterial blight disease (*Xanthomonas axonopodis* pv *manihotis*)

Introduction

The most common biotic constraint to cassava (*Manihot esculenta* Crantz) production worldwide, cassava bacterial blight (CBB), is invoked by a vascular bacterial pathogen *Xanthomonas axonopodis* pv. *manihotis* (*Xam*). Resistance has been introgressed in improved varieties through intra and interspecific crosses (*M esculenta* × *M. glaziovii*). However, genetic of resistance to CBB have never been extensively studied until now. Although *Xam* populations genetic studies have been recently extended, there is no gene for gene relation clearly demonstrated. The difference between resistant and susceptible varieties invoked a variation in the speed of vascular reaction to *Xam* colonization, and suggest that resistance is quantitative. The objective of the present study was to locate cassava genomic regions responsible of resistance to *Xam* in order to facilitate their manipulation in breeding programs.

Material and Methods

Material. The plant material used in this study was the F1 (MNGA 2x CM2177-2) composed of 150 individuals including 90 individuals described previously (Fregene *et al.*, 1997). Five strains of *Xam* belonging to different haplotypes and collected from different ecozones have been selected.

Evaluation of resistance. In greenhouse, nine plants per genotype and per strain were evaluated at seven, 15 and 30 days after inoculation by puncture following a scale previously described. Bacterial population densities in the stem were scored at seven and 15 days. The average of the $\log_{10}$ value of *Xam* population was used in QTL mapping. In the field, the 150 F1 individuals were evaluated for resistance to CBB during 2 cyles (1997 and 1998) in completely randomized experiment at CIAT center 'La Libertad' in Llanos. Each plant was evaluated at four and 7 months using a scale from 1 (no symptom) to 5 (dieback of the whole plant). The disease rating of each plant was used separately for the QTL analysis.

Mapping of QTL. The framework linkage map used is the female map and contains 142 markers including RFLP, RAPD, isoenzymes and microsatellites as described previously (Fregene et al., 1997). Molecular data for each individual were used to QTL single marker analysis. Statistical analysis of qualitative data was performed with SAS for resistance measured on an ordinal scale (greenhouse and field), and with QTLcartographer computer package for bacterial population densities in stem

Results and Conclusions.

For greenhouse evaluations, the χ^2 test for homogeneity showed significant differences between resistance rating to the five different strains. For bacterial quantification in stem, the χ^2 test showed that high levels of population are associated with higher notes and low levels with lower notes at 7 and 15 .da.i.. Test for association between each of these populations and symptoms at 30 d.a.i. showed significant association only in few cases. Spearman correlation analysis showed that there is no significant correlation between field disease rating at four and 7 month. There was no correlation between greenhouse and field evaluation. Among possible explanations, one can be that strains inoculated in greenhouse are very different from those present in field, and another is that some individuals may have escaped the disease in field. A second cycle of evaluation is under progress.

A total of five linkage groups and 26 markers were involved in resistance based on greenhouse evaluation to the five strains of *Xam*. Three linkage groups were common to almost two strains with several common markers (C, D, and L linkage groups), 16 markers being in common for resistance to CIO-84 and ORSTX-27. For the other strains, regions of cassava genome involved in resistance to *Xam* are different. This result indicates that there is probably an interaction between pathogen and plant i. e. factors of resistance are specific for each strain. We know that the difference between each strain used in this study at molecular level is based on a probe corresponding to pathogenicity

gene. These factors of pathogenitcity may interact with resistance factors in a quantitative mode. Five markers on five different linkage groups are significantly associated with $\log_{10}$ of bacterial population in stem at 7 and 15 d.a.i. Nine linkage groups and 17 markers were involved in field resistance at 7 month and none at 4 month. Three linkage groups (D, K and X) and seven markers were common to greenhouse and field evaluation based in disease rating at 30 d.a.i.. Despite the lack of correlation between field and greenhouse disease rating, linkage analysis showed that linkage group D was tightly linked to resistance in both cases. Resistance factors of this linkage group must be involved to general defense mechanisms against CBB. These resistance factors may have come from *M. glaziovii*. Two evidences for this hypothesis are (i) the numerous markers present on group D reveals low levels of recombination like found in interspecific crosses; (ii) an hypothesis is that resistance to CBB has been inherited from *M. glaziovii*; it will be interesting to identify and map markers specific of *M. glaziovii* that are present in TMS 30572.

A second population of 270 individuals was developed in CIAT backcrossing five F1 individuals with female parent TMS 30372. Increased size of this population will allow us to map resistance to CBB more precisely. Obtention of homozygous individuals for some genome segments will give us the possibility to identify resistance loci with recessive effects.

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Fregene MA, Angel F, Gómez R, Rodriguez F, Roca W, Tohme J, Bonierbale M (1997). Theor Appl Genet 95 : 431-441

Collaborators: V. Jorge[1] , M. Fregene, M.C. Duque, M. Bonierbale[2], J. Tohme, and V. Verdier[1]

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1.2.7. Identification of genomic regions responsible for the determination of whitefly resistance in cassava

Introduction

Whiteflies are a major pest of many agricultural crops in most areas of the world. Yield losses are estimated in the hundreds of millions of dollars. Most whitefly species cause crop losses through direct feeding, while some are very efficient vectors of several economically-important plant viruses. There are almost 1200 species with a wide range of hosts like legumes, fruit trees and ornamentals where this insect causes big economic losses (Butler and Henneberry 1994, Brown et al. 1995).

A single whitefly species *Bemisia tabaci* (this is actually a complex of biotypes and may include the species *B. argentifolia*), is the vector of Africa Cassava Mosaic, Bean Golden Mosaic, Bean Dwarf Mosaic and at least 30 other geminiviruses of important food crops. The species *Aleurothrachellus socialis* and *Trialeurodes vaporariorum* cause considerable yield loss on cassava due to their direct feeding damage. The main symptoms in the plant are: total chlorosis, the apical leaves turn curly, the basal leaves

turn yellow and dry, and it stops the developing of the plant. The adult insects are found preferentially in the apical zones of the plant, where they extracting large quantities the sap of the conductive vessels, causing a considerable damage by loss of vigor, with low yield in the production. The honeydew which excrete, as a result of the copious sap intake, serves as a substrate for sooty mold fungus, which can also damage hosts by blocking photosynthesis.

The entomology section has reported the existence of different sources of resistance to whitefly (CIAT, 1995). The most resistant genotypes are: Mbra-12 and MEcu-72. We have initiated a project to map the resistance genes of MEcu-72 using RFLP markers from the Cassava map, RAPDs and AFLP.

Materials and methods

Two mapping populations were generated from the crosses of CG489-34, a resistant clone and two susceptible genotypes Mcol-2026 and Mcol-1505. CG489-34 is resistant clones that combine the both source of resistance Mbra-12 and MEcu-72. A third mapping population was generated from the cross of MBra-12 by Mcol-2026. The population size ranges from 108 to 135

Based on field testing, contrasting individuals (highly resistant and susceptible clones) were selected for each population. DNA was extracted from the different individuals and susceptible and resistant groups were formed and bulked with the respective parental genotypes. Bulk segregant analysis was conducted using RAPD primers.

Results

Sixty two RAPDs markers have been screened with the resistant and susceptible bulks of the three populations (table 1). One primer, OP.P3, showed a clear polymorphism between the susceptible and resistant group; presence of a strong band in the resistant bulk and absence in the susceptible bulk. This marker is being evaluated on the whole population of each cross to confirm its association with the resistance. Parental genotypes from each family were also evaluated with RFLP markers from the cassava map (Fregene et al, 1997). Seventy-two probes were found to be polymorphic

On going activities

1. Isolation and sequencing of the polymorphic band from OP.P3 and development of a Scar for efficient and rapid screening.

2. Screening of the bulk DNA AFLP markers

3. Development of a framework map using RFLP, microsatellites from the CIAT cassava map, RAPDs and AFLP markers for the linkage analysis.

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Collaborators: E.Barrera Sabogal, A.Bohórquez Chaux, C. Iglesias, T. Bellotti and J. Tohme.

1.2.8 molecular markers of genes involved in physiological post-harvest deterioration (ppd) of cassava roots.

Introduction

The short period of storage of the roots of cassava affects the marketing of the product. The process of deterioration begins 2 or 3 days after the harvest, followed by a microbial deterioration between the 5 to 7 next days. The physiologic deterioration is observed as a discoloration of the vascular tissues and the stored parenquima, accompanied by changes typical in the response to lesions in other plants systems (Beeching et al., 1994a; Beeching et al., 1994b).

The mechanism response of plants to lesions and attacks of pathogens include multiple biochemical responses, which are common to a wide range of plants. This include: increase in the synthesis of some proteins as phenylalanine ammonia-lyase (PAL), an associate enzyme with the synthesis of phenol, peroxidases and phenoloxidases, key enzymes in the biosynthesis of phenols and poliphenoles; changes in the composition of the membranes lipids and production of etilen lithic enzymes like b, 1-3, glucanase and Chitinase (Beeching et al. 1994a; Beeching et al. 1994b; Rickard, J.E. 1985).

The correlation among phenotypic values of deterioration and specific neutrals molecular markers will allow indirect selection in breeding programs forthese traits. Recently a molecular linkage map of cassava was developed at CIAT on a F1 population using RFLP's, RAPD's and isoenzimes markers. The mapping population is the result of a cross between two lines elite of cassava, MNga 2 (TMS 30572) and CM 2177-2 (ICA Cebucan) (Fregene et al., 1994).

The objectives of this investigation, are to transfer the existent knowledge and the molecular tools developed in other plants systems to cassava, and the mapping of the genes invovled in the physiologic post-harvest deterioration.

Methodology

Plant material. The mapping population of cassava consists 150 F1plants, from a cross between "TMS 30572" "CM2177-2" (the male parent). This population was developed to construct a genetic map of cassava (*Manihot esculenta* Crantz) by Fregene et al, (1997).

DNA Extraction. DNA was obtained from young leaves (3-4 gr. from fresh weight) The tissue was collected from the plants maintained at CIAT at -80°C. The obtained DNA using Dellaporta et al. 1983 protocol was dissolved in $T_{10}E_1$ for incubation to 4°C over night and then quantified by fluorometry (TKO 100 Hoefer).

RFLP's. Digestion with restriction enzymes (EcoRI, EcoRV, HaeIII, HindIII and PstI) was made using 10 ng of DNA,. The digestions were carried out following standard protocols provided by the manufacturer (Biolabs New England). The digestion products was runned into a gel agar 0.8%, using TBE 0.5X buffer solution for 16-18 hours. They were transferred to a membrane of nylon Hybond N+ (Amersham) following the method of Southern blotting alkaline (Southern, E. M. 1975). Then the filter were prehybridized for four hours and later hybridized over night to 65°C with the probes marked with α32P dNT.

Evaluation PPD. The phenotypic evaluation of the population to PPD susceptibility was carried out in two different environments: 1. CIAT Palmira and 2. CIAT Station at Villavicencio, during the first semester of 1998. In addition an evaluation carried out by Fregene in 1997 at CIAT, was also included as a different environment. These evaluations are based on a subjective method, based on the works of Wheatley, C. (1982).

Analysis of Information. The analysis of variance was carried out in order to determine the range of the components of variance for PPD, genotypic [G], environmental [E] and interaction genotype- environment [GxE].

The phenotypic correlation of PPD was analyzed employing the statistical programs of analysis linkage: Q-Gene, MapMarker and QTL Cartographer (Lander, et al., 1989; Nelson, J.C., 1995; Basten, C.J., 1997).

Generation of the Map Linkage. Four of the seven clones have been included in the map previously developed by Fregene (1997), GLU1 and MEPX segregating for the female parent (NgA-2), PAL1 and MEPAL segregating for the male parent (CM2177-2). The linkage map was generated using the program MAPMAKER/ EXP 3.0 (Lander et al., 1987; Lincoln et al., 1992). The RFLP's markers were grouped using a LOD 4.0 (probability of error is of 1 in 10^4).

Mapping QTLs. The QGENE program (Nelson, 1994) was used to obtained the linear regression of the markers from the map over the trait (PPD) and to identify through simple interval the regions or QTLs associate markers and the variability of the characteristic explained (R2). The mapping for compound interval was carried out using QTL Cartographer (Basten, C.J., 1997).
Results

Obtaining the Genes. The genes of Pheylalanine ammonia-lyase (PAL1), Catalase (CAT1), Acc Oxidise (ACCO1), Hydroxyproline- Rich Glycoproteins (HRGP1), and b-glucanase (GLU1) were given by J.R. Beeching (University of Bath, United Kingdom). This were cloned in the site of restriction for Eco RI of plasmid pUC18. L.F. Pereira (University of Guelph, Canada) gave the genes of Peroxidase (MEPX) and another Phenylalanine ammonia-lyase (MEPAL1). This cloned in the site of restriction for Eco RI of the plasmid pGEM 10. These were amplified by reaction PCR using primers M13, obtaining amplified fragments of the expect sizes.

RFLP's markers. With the availability of seven candidates genes in order to include in the genetic map of cassava, was carried out hybridization in order to determine polymorphism generated restriction enzymes of between the parents, which have a high probability of segregating in the individuals of the progeny.

For each one of the genes polymorphism was detected with the following restriction enzymes: Eco RI for GLU1, Taq I and Hae III for PAL1 and ACCO1, Eco RI and Taq I for MEPX, Taq I and Hind III for HRGP1, Hind III and Taq I for MEPAL. CAT1, is the only gene that was monomorphic with the tested enzymes. Only the 23% of the enzymes were polymorphic. The 46% of the polymorphism founded were with Taq I and 18% for Eco RI, Hae III and Hind III respectively.

The evaluation of the clones in the progeny was carried out with the enzymes that generated polymorphism in the parents. Except GLU1 and MEPX, the clones were multiple bands, that could correspond to several loci of the same gene or several sequences of the same gene family. In the progeny not all of the polymorphism was as evident as which how it in the figure 4, and the difficulty genotyping the individuals concerning several markers was something frequent, running the risk of identifying the genotype of the individuals concerning to one or several clones of erroneous way.

Phenotypic Evaluation. The phenotypic evaluation was carried out in the two planned environments. It was found that the values for PPD, doesn't have a normal distribution. The observed distribution is asymmetric on the left, presenting a high frequency low values of PPD between 0- 5%. A similar distribution was obtained by M. Fregene. The frequencies of individuals by any interval of values of PPD were different among the two environments. The highest grade of asymmetry was in environment 1 (Villavicencio), prevailed the low value deterioration. The data were adjusted to a normal distribution through statistical transformation (ln PPD).

The data already in a normal distribution was used for the analysis of variance in Q-GENE and QTL-Cartographer.

PD's QTL. Table 1 summarizes the QTLs found in the analysis of the segregates marker in the map of the female parent only.

Table 1. Summary of QTLs associated with markets, R2 and value additive. Analysis for parent female (NGA-2).

		Martin			**Villavicencio**			**CIAT**		
	Grupo de Ligamiento	QTL	R^2	a	QTL	R^2	a	QTL	R^2	a
P	**A**	**RGY208**	**0.25**	**0.94**	**RGY208**	**0.25**	**-1.143**	**RGY208**	**0.25**	**-1.006**
P		GY213	0.14	0.70						
D	**B**	RGY191	0.185	-0.786						
		GY197	0.14	-0.673						
					rGY147	0.14	0.82			
								GY197	0.17	-0.81
	C							RGY174	0.23	0.915
								GY196	0.17	0.769
	F				GY203	0.156	-0.852			
					GY122	0.160	-0.849			
					GY186	0.214	-1.000			
	G							rAM18	0.18	0.783
	J				AD1C	0.149	-0.859			
					GY34	0.246	-1.079			
	K				GY119	0.180	0.921			
					rCDY106	0.332	1.059			
	N	GY148	0.189	0.746						
					rGY74	0.207	1.042			
	Q							F19A	0.23	0.883
								GY183	0.19	0.832
	X							CBB1	0.10	0.649

Discusión

The apparent low level of polymorphism (23%) between the parent lines of the mapped population could be attributed the high percentage of relationship between the cassava genotypes.

The linkage map is of great use in the development of markers for indirect selection, the management of germplasm and the genetic study of complex traits. The inclusion of markers in the genetic map of cassava in order to select either resistant or susceptible genotype to the PPD has been identified as an important objective. The effort for the development of these markers involves the study of the inheritance genes implied in the defense process of the plant, which is supposed of quantitative inheritance because the high contribution of the environment in the variability expression of the characteristic. The next step includes the evaluation of the PPD in several environments in order to

reduce the as much as possible the component of environmental variance and elucidate the genetic component. The RFLP's markers are useful for this analysis.

The restriction enzymes Taq I, EcoRI , Hae III, and Hind III generated polymorphism for genes involved in the PPD, that it is segregates in the progeny.

The results of the phenotypic evaluations have been confusing. At CIAT, like at Villavicencio, a high frequency of values of very low PPD was observed, even 10 days after the harvest. This was not expected for a progeny, in which the parents have intermediate values of PPD. However the mapping and QTLs analysis were carried out.

The markers of PAL1, MEPX, GLU1 and MEPAL present high distances in centimorgans are from the nearest markers. This indicates either that markers are wrongly mapped or that saturation of the map of cassava is low.

No QTL showed association with the markers of the genes involved in PPD. The QTLs were associated with effect between environments suggesting that the environmental variation is high. Only the group of linkage was a QTL that maintained its effect and association to a marker between the ambient.

In order to resolve this molecular and of field problems, a news analysis is being carried. In addition, all the genes will be mapped again using RFLP-based PCR and make other phenotypic evaluation will be made with three repetitions in every environment.

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1.2.9 Identification of NBS and Protein Kinase type putative resistance genes to Rice Blast

Introduction

In rice we are specifically interested in finding the resistance genes against *Pyricularia grisea*, one of the most devastating and variable fungus that affect up to 85 countries where rice is cultivated. Breakdown of resistance is quite common and most varieties One major exception is Oryzica Llanos 5, developed at CIAT. This cultivar has been grown experimentally and commercially for more than 8 years without any symptoms of a breakdown. Resistance remains stable because several resistance sources are combined in the lines, which were selected through a long and complex breeding scheme. Such characteristics make it ideal l to study blast resistance and to design breeding strategies incorporating source of resistance to the different blast lineages. We are working 1) on the dissection of resistance of Llanos 5 using gene tagging and 2) on the identification of RGA linked to resistance genes.

Methods

The same strategy described for the identification of RGA in bean is been followed for rice. Based on a consensus sequence in the protein domain, degenerate primers were designed (see Table No.1) and were used to amplify fragments analog to resistance genes. DNA templates were used from the varieties Fanny and Oryzica Llanos 5, susceptible and resistant to blast, respectively.

After the PCR reaction the different bands were separated and cloned into *E. coli.* Groups of sequences for the different bands were established using restriction enzyme patterns

following the methods used by Leister et al. (1998). Then five to ten clones of each group were tested by RFLPs in the parental lines; and the polymorphic ones were hybridized to DNA from the progeny of the cross O. Llanos 5 x Fanny. Using the MapMaker program (Lander et al., 1987), these probes were placed on the RFLP map generated for the F7 RILs population. The fragments linked to known resistance genes were sequenced using the automatic ABIPrism 377 DNA sequencer (Perkin-Elmer) and compared with the database of proteins (blastx) in GenBank.

Results

Sa far, we have sequenced 9 fragments from the 776 clones of the NBS library and 3 from the 140 clones in the protein kinase library. All of them showed high percentage of homology with known resistance proteins like *Oryza longistaminata* receptor kinase, *Oryza sativa* receptor kinase-like protein or NBS-LRR type resistance protein [*Oryza sativa*].

We have placed 11 of the identified resistance gene analogs (RGA) as new markers on the rice map derived from the cross of Llanos 5 and Fanny. Many of them (6) map to chromosome 11 where Xa 21 as well as Pi-1 and Pi-7 are located, suggesting that we might be getting close to resistance genes to *Pyricularia* in rice. Another one map to chromosome 6 and one more map to chromosome 7.

On going activities:

- fine map rice blast resistance genes in the O.Llanos x Fanny cross;
- Sequence additional fragments and map them on the RIL populations
- Initiate the isolation of the genes responsible for that resistance by screening rice BAC libraries with the different RGA obtained from degenerate primers.

Table No.1

Primer	Name	Nucleotide Sequence	Reference
NBS	AS1	5′-CAACGCTAGTGGCAATCC-3′	Leister et al., 1996
NBS	AS2	IAAIGCIAGIGGIAAICC	"
NBS	S1	GGTGGGGTTGGGAAGACAAGG	"
NBS	S2	GGIGGIGTIGGIAAIACIAC	"
NBS	AS3	IAGIGCIAGIGGIAGICC	"
LRR		TCAAGCAACAATTTGTCAGGICAIATICC	Ronald, P. *pers.com.*
PK	PK1	TAACAGCACATTGCTTGATTTIAIITCICGITG	"
PK	PK2	TAACAGCACATTGCTTGATTTIAIITCICAITG	"
PK	PK3	TAACAGCACATTGCTTGATTTIAIITCICTITG	"

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1.2.10 Genetic mapping and fine mapping of the apomixis gene in *Brachiaria*

Introduction

The gene tagging of apomixis and construction of a *Brachiaria* map was pursued using the crosses *B. ruziziensis* x *B. decumbens* and*B. ruziziensis* x *B brizantha* . Heterologous RFLP probes from the rice (Cornell University), maize (Missouri University) and sorghum (Texas University) map were used. Other molecular markers used in this study were RAPDs, SCARs and AFLPs. A modified protocol for DNA extraction was also implemented in order to improve the efficiency of the mapping project.

Materials And Methods

A sexual tetraploid *B. ruziziensis* clone (CIAT 44-3) was used as a female parent in a cross with natural and apomictic tetraploid genotypes *B decumbens* and *B ruziziesis B brizantha.*

DNA Extraction DNA was extracted using the protocol described by Carlos Colombo (personal communication) with modifications. 1g of tissue was dried at 48 °C for 20 hours and ground to fine power using a modified piston constructed for grinding bean seeds; 15 ml of extraction buffer (0.1M Tris-Hcl pH8.0, 0.05M EDTA pH8.0, 0.7 M NaCl, 4% CTAB and 1% ßMe) was added and incubated at 65 °C for 10 min; 15 ml of chloroform:isoamyl alcohol (24:1) was added and centrifuged at 3000 RPM for 30 min. The aqueous phase was transferred to a new tube and 8 ml of chloroform:isoamyl alcohol was added and centrifuged at 3000 RPM for 30 min, repeated twice. A volume of cold isopropanol was added to the supernatant and incubated over night at - 20 °C. The isopropanol mixture was centrifuged at 3000 RPM for 30 min at 4 °C. The DNA pellet was washed with cold 75% ethanol and dried at room temperature, and then resuspended in 300 ul of TE. Pancreatic RNAse was added to a final concentration of 20 ug/ml. DNA was quantified on a DYNA QUANT 200 fluorometer (Hoffer Scientific Instruments, San Farancisco CA). Protocols for RFLP, RAPD, and AFLP markers in *Brachiaria* have been described previously (BRU Annual report pp 105-110 1997).

Segregation of markers in the genetic model for single dose restriction fragment (SDRR) markers was determined by departure from the hypothesized 1:1 ratio by the Chi-square test. The data matrixes obtained for presence or absence of the bands were analyzed for

linkage to the apomixis gene with Mapmaker Macintosh using the Kosambi function and LOD score of 6.0

Results

Four sets of heterologous probes were screened on the parental genotypes and the mapping populations. Table 1 shows percentage of hybridization, polymorphism and mapped probes). Linkage analysis revealed a region of *Brachiaria* genome, corresponding to chromosome 1 in rice, bearing the apomixis gene linked to RFLP, AFLP and SCAR markers. The apomixis gene is linked to three rice RFLP markers (RZ 276, RZ 995 and RZ 413), at a distance of approx. 3 cM from the apomixis gene. All three makers map near the centromere region in the rice map. The AFLP marker EeMf28 was located at 11 cM and SCAR marker (SN14) at 6 cM. Attempts to fine map the apomixis gene failed so far.

A second activity involves the screening of selected *Brachiaria* genotpyes with the SCAR SN14 (MW = c.a. 1100 bp) to determine the efficiency of the marker as a tool for indirect selection of the reproductive mode of F_1 plants or *Brachiaria* accessions.

Table 1 Percentage hybridization, polymorphism and mapped probes

Source	Hybridized probes	%Polimorphic probes	%Mapped probes
Rice probes from the Japan map	6	83	50
Rice cDna	83	82	41
Oats cDNA from the rice map	18	100	72
Sorghum probes	13	23	8
Maize probes	4	100	75

On going activities

- Implementation of a modified bulk segregant analysis for fine mapping of the apomixis gene
- Mapping of *Brachiaria* microsatellites obtained
- Development of a framework map using RFLP, RAPDs, AFLP and microsatellites markers
- Screening *Brachiaria* accessions and hybrids plants with the Scar SN14 and compare with embryo sac analysis.

Collaborators: J. Vargas, C. Roa, J. Miles and J. Tohme

ACTIVITY 1.3 Development of molecular genetic techniques for assessing genetic diversity

SUMMARY OF ACHIEVEMENTS

- We have embarked in an effort to generate microsatellite (SSR) markers for cassava and *Brachiaria*. Cassava SSR were highly polymorphic and have more allelic diversity with wild *Manihot* than cassava. Through a collaboration with the Swiss and Sweedish, the aim is to generate 500 SSRs to saturate the cassava molecular map.
- For the first time we have constructed a Bacterial Artificial Chromosome (BAC) library for cassava. The BAC library contains over 55,000 clones and has 5 x coverage of the genome; and thus 95% probability of funding a desider DNA clone in a gene cloning effort.

1.3.1 Development of cassava microsatellite markers and its use for assessing genetic diversity at genus level

Introduction

Microsatellites are very polymorphic simple-sequence repeats, abundantly present in eukaryotic genomes. Their amenability for PCR amplification and semiautomated genotyping makes them suitable markers for typing large germplasm collections. Due to conservation of priming sites, microsatellites may be amplified across species and genera. We characterized 14 microsatellites in cassava [1]. To evaluate the genetic diversity in *Manihot*, and the ability of cassava microsatellite primers to amplify (homologous) loci at the genus level, we typed a genetically representative sample of cassava, as well as six wild *Manihot* species with ten-primer pairs [2]. The degree of relationship between cassava and its wild relatives was also assessed. Furthermore, 521 accessions of the cassava core collection were typed using four primer pairs [3] to characterize diversity and to identify redundancies.

Materials and Methods

Microsatellites were isolated from accession MCol22 using standard cloning protocols. Typing of accessions was performed using radioactive- and fluorescent-labeled primers. Multiplex PCR reactions were performed combining 2 up to 4 different fluorescent primers. Electrophoresis and allele detection was carried out on automatic DNA sequencers (models ABI Prism 373 and 377). Statistical analysis included heterozygosity estimation, allele number and frequency, segregation analysis, similarity indexes calculation and dendrogram construction employing the unweighted pair group method (UPGMA).

Results and Discussion

Heterozygosity values in the cassava core collection ranged from 0.00 to 0.88 and the number of alleles detected varied from 1 to 15. Chi-Square tests (X^2) showed that most microsatellite loci segregated following Mendelian ratios. Linkage was detected among few loci, and most loci showed two alleles per genotype. Typing the core collection with four microsatellite loci revealed between-country allele number and frequency variation, which agreed with between-country allele size variation at the same loci. Unique alleles were present in countries such as Brazil, Colombia and Guatemala. A low percentage (1,34%) of redundancy was found in the core collection through microsatellite, isozyme and AFLP analysis. Based on these findings we recommend 1) to use more microsatellites for typing cassava collections (i.e., the entire core), and 2) to employ DNA-based markers for future selections of cassava germplasm to ensure genetically representative, non-redundant samples.

Cassava-derived microsatellite primers produced PCR-amplified products across the genus demonstrating their utility for cross-species amplification. However, a decline of amplification success was observed with increase of genetic distance. For example, in *M. aesculifolia*, *M. carthaginensis* and *M. brachyloba* no PCR product was detected for two loci. The presence of null alleles in these species also affected the level of observed heterozygosity. Previous work with AFLP markers showed that these three species are the most distant relatives of cassava, whereas *M. esculenta* susbsp. *flabellifolia* and *M. esculenta* subsp. *peruviana* are the wild forms most closely related to the crop. Such findings were confirmed with microsatellite analysis.

Overall, a high level of polymorphism was observed (83 %) in the *Manihot* genus. The number of alleles varied greatly (from 4 to 21) among the loci evaluated, but in all cases more alleles (79 out of 124) were found in the wild taxa than in cassava. Considering only cassava and the wild subspecies, 53 additional alleles were present in the second group, suggesting a larger pool of alleles for the *M. esculenta* subspecies than for cassava. This result pointed out the primary gene pool of cassava as a potentially source of diversity that could be useful for the improvement of the crop.

References

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and AFLPs to evaluate genetic diversity and redundancy in the cassava core collection and to assess the usefulness of DNA-based markers to maintain germplasm collections. Submitted to Molecular Breeding (Sept-1998)

Collaborators: P. Chavarriaga-Aguirre, A.C. Roa, M.M. Maya[1], M.C. Duque, M. Bonierbale[2], C. Iglesias and J. Tohme.
1. Purdue University; 2. CIP, Lima

1.3.2. Construction of a Cassava Bacterial Artificial Chromosome (BAC) Library: Towards Cloning of Disease and Pest Resistance Genes.

Introduction

Disease and pest epidemics arises from time to time in major cassava growing regions with the attendant loss of income for resource poor farmers and erosion of valuable germplasm. Resistance breeding is the most effective way of combating intermittent, widespread disease and pest attacks. But, cassava's long growth cycle, heterozygous state, and vegetatively propagated nature considerably slows down resistance breeding. The advent of cloning resistance genes in plants, together with reproducible transformation protocols in cassava makes genetic transformation with homologous disease and pest resistance genes, a faster and more efficient way of taking on disease epidemics.

Cloning genes known only by their phenotypes and position relative to molecular markers on a genetic map, or map-based gene cloning, depends on the ability to generate large DNA fragments and ordering them into contigs, spanning the genomic region carrying the gene(s) of interest. Two important criteria therefore are markers closely linked to genes of interest, and large DNA fragment libraries. The cassava genetic mapping project at CIAT has identified regions of the cassava genome controlling resistance to several strains of the cassava bacterial blight (CBB). Similar experiments are ongoing to identify genes controlling resistance to the cassava mosaic disease (CMD).

Materials And Methods

Megabase size DNA was isolated from the cassava variety TMS30001 and embedded in agarose plugs as described by Zhang *et al.* (1995). TMS30001, developed at the International Institute of Tropical Agriculture (IITA), shows extreme resistance to the cassava mosaic disease (CMD) and resistance to the cassava bacterial blight (CBB). Large genomic DNA, in one-third of an agarose plug, was partially digested with Hind III, 1.5U for 20 min at 37°C, and DNA fragments of sizes 100-300kb size selected by pulse field gel electrophoresis (CHEF MAPPER, Bio-Rad Corp). Size selected DNA was ligated into the Hind III cloning site of pBeloBAC11 in a vector:insert ratio of 10:1, using 14U of ligase, in a final volume of 100µl. Twenty microliters of DH10B competent cells (GIBCO BRL) were transformed, with 2µl of the ligation reaction, by electroporation, and white colonies picked for DNA insert sizing. Colonies were grown for 14hr in LB+ 30mg/ml Chlorampenicol and plasmid DNA isolated by the Autogen automatic plasmid

isolation robot (Kurabo Inc.). Plasmid DNA was digested with 10U of *Not* I to liberate inserts and separated on a 1% agarose gel by pulse field gel electrophoresis. The rest of the ligation was transformed, plated out and picked with the Q-bot robot (Genetix PLC).

Results And Discussions

Twenty colonies carrying recombinant plasmids were randomly picked and inserts were sized. DNA insert sizes ranged from 40 to 110kb, with an average of 80kb. Using the Q-bot robot (Genetix PLC), 55296 clones were picked and transferred to 384-well plates containing culture media suitable for storage of bacteria colonies at -80°C. A total of 144 plates were required to store the library. The BAC library was also gridded onto 3 high density filters, each containing 18,432 clones per filter, for rapid screening of the library. The cassava genome has a size of 760 million base pairs. The cassava BAC library therefore has a 5X coverage of the genome and a 95% probability of finding any desired DNA clone. Screening the BAC library with cassava genomic DNA clones has so far revealed a satisfactory representation, up to 30 hits. The BAC library was constructed with the objective of map-based cloning of disease and pest resistance genes in cassava. A good commencement point of cloning resistance genes will be already identified markers tightly linked to CBB resistance genes. Fine mapping of these genomic regions has also been initiated, to be followed by assembling a contig of BAC clones traversing the region.

Further activities

- The cassava BAC library has 55,296 clones, of average size 80kb available for gene cloning, the next stage of this project will be to use the BAC library to construct contigs of regions already identified in cassava carrying resistance genes to CBB. The library is publicly available through CIAT.

References

Zhang H., Zhao X., Ding X., Paterson A., Wing R. 1995. Preparation of megabase-size DNA from plant nuclei. The Plant Journal 7(1): 175-184.

Collaborators: M. Fregene, G. Gallego, J. Lopez, R. Wing[1], J. Tohme
1. Clemson University, USA

1.3.3 Expressed Sequence Tags (ESTs) for the Genetic Map of Cassava.

Introduction

A project was initiated last year to saturate the molecular genetic map of cassava with EST markers. ESTs markers have been widely used in the human, mouse and several plant genome projects for the candidate loci approach to mapping polygenic traits, to organize YACs, BACs, or other large-insert DNA clones into physical maps, and for identification of DNA regulatory sequences responsible for gene expression. The EST

data base division of Gene Bank contains over 1.2 million sequences, derived from a diverse collection of organisms. Cassava EST markers, obtained from sequencing transcipt derived fragment (TDF) polymorphic between the parents of the F_1 mapping population for cassava, is expected to provide more informative markers for the map. Putative identities of ESTs so far identified confirms the usefulness of this approach in saturating the molecular genetic map of cassava, and is also giving added insight into the genome organization of a putative allotetraploid.

Materials and Methods

Total RNA was isolated from young leaves and roots of the two genotypes of the parents of the cassava mapping population TMS30572 y CM2177-2 at 4 weeks after planting. Messenger RNA was isolated from total RNA by Dyna beads and cDNA was syntesized from the mRNA templates as descibed by Bachem et al. (1996). Using the modified AFLP method for cDNA templates (Bachem et al, 1996), a preamplification PCR without selective bases primers and a subsequent amplification with two selective nucleotides primers, using all the possible 256 combinations, was done on the cDNA library. PCR reaction was electrophoresed on a 5% sequencing gel. Polymorphic AFLP bands correspond to TDFs differentially expressed in the parents excised from the dried gels, was eluted and reamplified.

The eluted cDNA fragment was cloned into pGEMT (Promega Inc) and transformed into *E.Coli* strain, DH5 alpha . Plasmid DNA was isolated from overnight miniprep cultures and TDFs were PCR labelled for use as probes in an RFLP screen of parental filters. Filters contained genomic DNA from the parental genotypes digested with five enzymes: EcoRI, HindIII, HaeII, AseI, TaqI. TDFs found polymorphic in the parental survey were hybridized to progeny filters prepared with the corresponding enzyme. Raw marker data was combined with previous segregation data from the frame work map of cassava and linkage analysis was by the MAPMAKER program. New markers are added to the existing framework map with a LOD thresh hold score of 3.0. TDFs were also sequenced, regardless of if they have been mapped or not using the automated ABI 377 sequencer in the Biotech Research Unit, CIAT. The Basic Alignment Search Tool (BLAST) version 2.0, of the National Center for Biotechnology Information found at http://www.ncbi.nlm.nih.gov/gorf/wblast2.cgi was used to search for homology to known genes published sequences of Gene Bank.

Results and Discussion

More than 500 TDF polymorphisms were obtained from the AFLP screening using 256 2-nucleotide selective primer combinations. Two hundred of these were eluted from the gel and PCR amplified. Forty eight of the TDFs were randomly chosen for mapping, and cloned. Forty four were used in the parental survey. Level of RFLP polymorphism in the parents was 55, and six of the polymorphic TDFs were mapped to the existing frame work of cassava, four to the female-derived map and two to the male-derived map. The others have to be confirmed in the hibridization with the progeny. Forty four of the TDFs were sequenced and homology to known genes in revealed mostly housekeeping genes. They include Chlorophyll binding proteins, in *Lycopersicum esculentum* and *Brassica napus,* a transcription factor from *Nicotiana tabaccum* PRL-2 genes from *Arabidopsis*

thaliana , and an AFLP fragment from *Dioscorea tocoro*. These sequences, in the process of submission to the EST data base of the GenBank.

Complementary DNA derived from cDNA/AFLPs show much higher RFLP polymorphism compared to random full length cDNAs found during genetic mappin gof cassava, 55% to 20%. The technique is therefore a powerful way to map many more cDNAs, using the highly informative RFLP technique, towards a transcript map of cassava. Preliminary QTL mapping with mapped TDFs reveals the power of mapped cDNAs to uncover genes involved in complex trait, a chlorophyl binding protein mapped close to a QTL for dry matter content and explained 10% of phenotypic variance. However, the short length of the TDFs, 200-400bp considerably reduces sensitivity of Southern hybridization resulting in rather weak signals. Other marker techniques, such as single nucleotide polymorphisms (SNP) might provide a way around the RFLP bottle neck encountered in mapping TDFs. Results thus far have shown that it is possible to develop a transcrip map of cassava, that might simplify genetic analysis of agronomic traits of interest.

Further activities

- Primers are being designed for sequences TDFs to implement the SNP mapping approach.
- More TDFs arwill be cloned, sequenced and mapped and their putative identities determined

Collaborators: M.C. Suarez, M. Fregene, A. Bernal, J. Guitierrez and J. Tohme

1.3.4 Lowering the Cost of Reliable Biotechnologies for National Cassava Programs: Simple Sequence Repeat (SSR) to Saturate and Facilitate Use of the Cassava Molecular Genetic Map.

Introduction

The applicability of the majority of molecular markers, currently RFLP markers, available on the molecular genetic map of cassava, in marker-assisted studies of genetic diversity, complex traits and marker-assisted improvement of cassava has been questioned by collaborators in the NARs. Considering the low technology status of most cassava breeding programs, RFLP markers, though highly informative, are beyond the capacity of all centers in Africa and Latin America and most centers in Asia. Recent advances in the identification and development of simple sequence repeat (SSR) markers, combined with its speed and high levels of allelic diversity, PCR-based assay, and codominant nature make them the markers of choice for saturation of the cassava genetic map and for transfer of marker technology to collaborators in the National Programs. PCR technology requires a relatively small capital out-lay for its integration into day-to-day plant breeding and germplasm management operations. More SSR markers can be assayed for a large number of plants, in a given time, for a given lab attendant, for a given budget, compared

to RFLP markers. Besides SSR markers can be shared "online" with collaborators, since only information as to primer sequences are required.

A project to generate a few hundred SSR markers for the molecular genetic map of cassava was developed, with support from the Swiss Development Cooperation (SDC) and the Swedish International Development Agency (SIDA). A cassava breeder from the National Root and Tuber Program in Nigeria was invited to participate in the entire process of SSR marker identification, development and mapping.

Materials and Methods

A DNA library from variety TMS30572, was enriched for SSR markers, using the affinity capture method for the following di-, tri, and tetra- nucleotides: $(CT)_8$, $(GT)_8$, $(CAA)_6$, $(CAG)_6$, $(AAT)_6$, $(ACG)_6$, $(GATA)_4$, and $(CAGA)_4$ and cloned into pUC18 plasmid as described by Edwards et al. (1996) . The library was transformed into DH10B competent cells and total of 18,000 colonies from the enriched library was screened for SSR markers by hybridization with $(CT)_{20}$, $(GT)_{20}$, $(CAA)_{14}$, $(CAG)_{14}$, $(AAT)_{14}$, $(ACG)_{14}$, $(GATA)_{10}$, and $(CAGA)_4$ oligonucleotides and by anchored primer PCR. A second enriched library from variety CMC40 enriched for $(CT)_8$, $(GT)_8$, developed by Keith Edwards, Bristol University, UK, and cloned in pJV1, was screened with the oligos $(CT)_{20}$, and $(GT)_{20}$ by dot blot screening. Plasmid DNA was isolated from putative clones by the QIAprep plasmid isolation kit, and 1-3ul of plasmid preparation was sequenced on an ABI377 automated sequencer, using the Universal and reverse M13 primers. Forward and reverse sequence strands were aligned using, either the GCG software, or the Basic Alignment Search Tool (BLAST) version 2.0 site of the National Centerfor Biotechnology Information found at http://www.ncbi.nlm.nih.gov/gorf/wblast2.cgi. Primer design was by the web based software Primer 3.0 found at http://waldo.wi.mit.edu/cgi-bin/primer/primer3.cgi.

Results and Discussions

A total of 120 putative clones were identified in the first library and sequenced. Of this, 64 clones contained unique SSR markers; primers, 20-mers long, could be designed for 40 clones from regions flanking the di-or tri—nucleotide repeats. Other clones had the SSR too close to the end of the fragment, or had very short, <4 di- or tri-nucleotide repeats. Results reveal a low efficiency of enrichment <1% enrichment. More than 1100 putative SSR markers were identified from the second library, corresponding to an enrichment of 50%. Plasmid DNA was isolated from 550 clones and so far 50 of these clones have been sequenced, all clones contained the corresponding di-nucleotide repeat.

Further activities

- Sequencing of putative SSR clones from the second enrichment, and primer design is continuing; the target by the end of the year is 500 sequenced clones with corresponding primer pair.
- Genetic mapping of the SSR markers is expected to begin at the beginning of next year.

Reference.

Edwards KJ, Barker JHA, Daly A, Jones C., and Karp A 1996. Microsatellite libraries enriched for several microsatellite sequences in plants. Biotechniques vol 20, No. 5:758-760

Collaborators: M. Fregene, C. Mba[1], J. Guitierrez, E. Gaitan, K. Edwards[2] , J. Tohme
1. National Root Crops Research Institute, Nigeria.
2. Bristol University, U.K.

1.3.5 Isolation and characterization of microsatellites in *Brachiaria* sp.

Background

Microsatellites are short DNA sequences of repeated nucleotide motifs between two and six bases, flanked by conserved sequences . They are codominant inherited genetic markers and have proven to be highly polymorphic. They can be analysed using PCR with specific primers flanking the repeat region, resulting in simple sequence length polymorphism (SSLPs). DNA microsatellites have been used for mapping, gene tagging, germplasm characterisation and evolutionary studies. Several methods have been developed to speed up the isolation of genomics fragments containing such simple repeats (J.M.H. Kijas, 1994, K.J. Edwards, 1995). We have initiated a project to assess the presence of microsatellites in *Brachiaria decumbens* and their potential as markers for fine mapping the apomixis gene and spitellbug resistance..

Methodology

50 ug of genomic DNA were digested using 500 units of Tsp509-I (AATT) at 5°C during 3 hours and ran on a 1.0% low melting point agarose gel. Fragments between 100 and 600 bp were excised and purified using Phenol- Chloroform methodology. About 5ug size- fractionated genomic DNA were ligated with 50uM of Tsp adaptors containing EcoRI and Tsp509-1 sites. Enrichment for microsatellites was carried out using 100ng of the ligated, denatured DNA in 500ul of hybridisation buffer using filters with GA and CA,AT,GC oligonucleotides for 24 h at 37°C. After hybridisation, filters were washed during 10 minutes in 1xSSC at 65°C. Bound DNA was eluted in 200 ul sterile distilled water by boiling fo 5 min. Two microlites from the eluted DNA were amplified by PCR in 25 ul using 1 unit *Taq* DNA Polymerase. After the PCR enrichment step, amplification were repeated once more using all of DNA generated from the previous step.

Approximately 250 ng from enriched and cleaned DNA were taken directly from the final amplification and ligated to 50 ng of pGem-T easy vector (Promega) during 24 hours at 15°C. Plasmids were cleaned and electroporated into DH5α cells and plated on LB-agar plates containing ampicilin, IPTG and X-Gal. Following incubation overnight at 37°C, colonies were transferred to nitrocellulose filters and hybridised with each oligonucletide.

Positive colonies were prepared using the Promega Wizard Kit and sequenced in a 377 Perkin- Elmer sequencer with dye terminators.

Results

1771 colonies were hybridised with GA oligonucleotide, 759 colonies with GC oligonucleotide, 1012 colonies with CA oligonucleotide and 1518 colonies with AT oligonucleotide. 92 positive colonies were obtained of which 68 were GA, 6 were GC and 18 were CA. We not found positive colonies using AT oligonucleotide. Primers for the relevant SSR are being designed and will be used to screened the different mapping populations available.

On-going activities

1. Using enrichment methodologies for other motifs.
2. Sequencing of additional GA and CA clones.
3. Design of primers
4. Estimation of the number alleles in *Brachiaria* sp
5. Testing the usefulness of the microsatellites in gene tagging and genome mapping in *Brachiaria* sp.

References

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K.J. Edwards, J.H.A Barker, A. Daly , C. Jones and A. Karp, 1995. Microsatellite Libraries Enriched fo several microsatellite sequences in Plants. Biotechniques 20(5):758-760.

Collaborators: R. Pineda, E.Gaitán, J. Miles and J. Tohme

OUTPUT 2. GENES AND GENE COMBINATIONS MADE AVAILABLE FOR BROADENING CROP GENETIC BASES

ACTIVITY 2.1 Utilization of novel genes and gene combinations by means of cellular and molecular gene transfer techniques

SUMMARY OF ACHIEVEMENTS

- Resistant interspecific progenies involving *Phaseolus coccineus* and *P. polyanthus* have been sent to Africa for field trials against the Bean Fly, a major pest of common bean in that continent.
- We have screened 23 Latin American Cassava vars for response to somatic embryogenesis. A gene construct harboring the cry 1A(b) Bt gene has been made in the BRU, and a highly virulent *A. tumefaciens* strain identified, for use in a transgenic approach agains the cassava stem borer.
- A pathogenicity gene (Pth B) of cassava bacterial blight (CBB) pathogen has been cloned. Characterization of the PthB protein has began to allow interaction studies with the host plant. We have also developed a PCR-based highly sensitive, specific, and simple technique for the detection of the CBB pathogen in sexual seed.
- A simpler, lower cost, cryopreservation technique through encapsulation-dehydration of shoot tips has been tested with several cassava genotypes.
- QTLs from *O. rufipogon* and *O. barthii* associated with yield increase (up to 25%) have been localized using microsatellite markers from the rice molecular map.
- Transgenic rice resistant to RHBV displayed 45-65% higher yield potential than non-transgenic CICA 8 control. Inheritance and expression of the transgen indicates that it can be utilized to complement the natural resistance source in different rice varieties.
- Work has been initiated to adapt *Agrobacterium*- mediated transformation of rice and *Brachiaria*, with promising results. Common bean transformation appears more promissing with the biolistic method.
- Extension of CIAT biotech capacities has included collaboration with the Biotec Corporation, Cali through the developing of a micrografting-micropropagation technique for the Soursop fruit tree. Similarly, in collaboration with the Agronomy Faculty, Univ. Nac. Palmira, a highly efficient plant regeneration protocol for tomato has been developed as a step towards a transgenic approach to the tomato fruitworm and budworm.

2.1.1 Gene transfer between *Phaseolus* spp through interspecific hybridization

Background

Useful traits such as resistance to somepests (ej. Bruchids and leaf hoppers) and diseases (e.g. *Ascochyta* blight, bean golden mosaic, common bacterial blight, web blight, and white mold) are not found, or are inadequately expressed or the genetic base is very narrow in common bean.

There is need of introgression of useful genes from related genepools, and wild populations of common bean (primary gene pool) and from related spp (of the secondary and tertiary gene pools).

Common bean breeding and selection methods using genes from distantly related races, gene pools, wild populations, and species often results intermediary products or parental stocks. (Singh, et al 1998). The stocks can be used as parents in subsequent crossing with elite lines to develop commercial cvs. Backcrossing is often used to transfer the necessary traits, like recurrent and congruity backcrossing. Introgression of useful genes from distant sources often requires plant-the-lant hybridizations, sometimes bridging-parents, or embryo rescue (Mejía-Jimenez, et al, 1994).

Material and Methods

In the period late 1997 mid 1998, interspecific crosses were performed between the wild *P. acutifolius P. parvifolius, P. parvifolius* is highly, tolerant to CBB and presses good levels of resistance to leafhopper and bruchids (Singh, et al, 1998).

Results and Discussion

(i) Congruity backcrosses carried between a *P. parvifolius* genotype with ICA Pijao, required embryo rescue. Progenies were selfed for 2 and 3 generations to develop inbred-backcross lines. Seed of these material, will be multiplied for extensive evaluation.

(ii) Hybrid plants also were generated (15-50%) from *P. vulgaris x P. acutifolius* crosses. Around 70-75% of the plantlets had hybrid constitution, as evidence through Dehydrogenase isozyme profiles. More congruity backcross are underway in order to allow increased recombination.

(iii) From the original between common and tepary beans carried out since 1995, several hybrid lines, highly resistant to bacterial blight, have been produced. These lines have moved further to regional trials, and others used as donor parents of CBB resistance.

Use molecular markers, molecular linkage maps, and *in situ* DNA hybridization will be implemented to facilitate genes introgression. The availability of routine transformation protocols for *P. vulgaris* will make gene transfer from alien source possible.

P. acutifolius can be genetically transformed since wild it can be used as a bridge for alien gene introgression into common bean through wide crossing with the transgenic tepary.

References

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and efficienty of hibridization through recurrent and congruity backcrossing. Theor. Appl. Genet. 88: 324-331.

Collaborators: S. Singh, D. Debouck, P.V. Herrera, W. Roca

2.1.2. Use of *P coccineus* and *P polyanthus* to improve Common Bean

Phaseolus coccineus and *Phaseolus polyanthus* express traits that are largely or totally absent from the primary gene pool of *P. vulgaris*, including resistance to Bean Stem Maggot (BSM) and ascochyta blight. *P. coccineus* also has a significant level of resistance to BGMV which could be of utility for common bean.

Material and Methods

Under a Belgian funded project, we are investigating the potential of these species through the use of a core collection of 140 accessions drawn from CIAT's collection of some 1,000 entries. To select the core we employed a GIS model that involved a clustering of environments to assure sampling a range of ecological origins. For seed increase we have made an effort to utilize seed that is as close to original seed as possible, that is to say, seed that has not been increased under open pollenation, although such seed is not always available. We have performed seed increases under controlled conditions and now have seed available of about 60% of the core to initiate evaluations.

We have also given continuity to the former Belgian-sponsored project and evaluated the interspecific progeny that were generated previously.

Results and Discussions

Seed Production. It has proven more difficult to produce seed of (*P. coccineus*) and (*P. polyanthus*) than we had expected. Seed production by different accessions varies from more than 2,000 seed to none at all at the time of writing. However, about 80% of the accessions have produced enough seed to initiate evaluations in the coming. Accessions from the north and central parts of Mexico produced seed without problems in most cases. However, accessions from further south (starting in the state of Chiapas in Mexico and continuing through to Peru) were variable in seed production and often produced little seed (<200 seeds) as of yet. This pattern implies a problem of adaptation related to latitude. The seed production as per agroecological cluster will be investigated further. Although the difference in daylength in Colombia is small as seasons change, there still might be some slight response as days become slightly shorter in September.

A nursery for the vegetative propagation of the accessions has been established, from which to draw cuttings and thereby to conserve sexual seed for the traits for which it is absolutely necessary. It is hoped to use vegetative cuttings for evaluations of foliar pathogens. Accessions have likewise displayed wide differences in the ability to reproduce from cuttings.

Inheritance studies. For this purpose we have made the necessary crosses for BGMV, for BSM and for ascochyta. Populations are in different stages of development.

In the case of BGMV, we studied the reaction of a number of accessions in the glasshouse to mechanical (hand) inoculation with the virus. The PC accessions previously identified as resistant maintained their expected reaction, while PP accessions expressed a high degree of susceptibility. However, contrary to expectations, most of the PC accessions inoculated in the greenhouse expressed high resistance, although in the past we had observed that many PC are susceptible under field conditions. Two cross combinations are in F2 generation and another four are in F1. Families in the F3 generation must now be produced for replicated field trials.

In the case of Bean Fly, crosses have been successful. Two populations are in F2 , and another two are in F1 (Table 1). We are waiting for feedback from Africa on the reaction of the parents which are putatively resistant and susceptible, based on past data.

In the case of ascochyta blight, we could confirm the reaction of several selections with a PP or PC plant morphology but expressing susceptibility to ascochyta blight. Twelve such selections were programmed in crosses with resistant PP and PC. The PP crosses have proven difficult but the crosses with resistant PC have produced a good number of F1 seed. In the next year we will advance these populations and initiate inheritance studies.

Interspecific crosses. Within the families selected for ascochyta resistance, 344 individual plant selections were taken and planted for reinoculation in the second semester. Another 44 selections were made among progeny with a PC or PP morphology but presenting a susceptible reaction, to be used in the inheritance studies. All selections were inoculated three times in disease plots, while a separate lot was protected with fungicides to produce pathogen-free seed for export. In the disease plots, ascochyta pressure was even more severe than in the first semester, and 10 lines were identified as most resistant although their resistance did not equal that of resistant PP or PC.

One of the objectives is to take advantage of the genetic variability in PC and PP in the practical improvement of bean varieties. Four of the resistant lines were programmed in 8 simple crosses for crossing in multiple combinations with common bean cultivars (Table2). Crosses will involve cultivar parents of both dry bean and snap bean types. Previous experience with small-scale producers of snap beans in the Andean zone had demonstrated that pesticide abuse is now a common phenomenon, and that most producers apply fungicides as often as once a week. Furthermore, ascochyta is the first disease to appear, and therefore is the key to setting off a vicious cycle of pesticide application. Therefore, the deployment of ascochyta resistance as posed in the present project would have a doubly beneficial effect in snap beans by improving yields and by forestalling pesticide applications.

Other crosses have been planned to combine sources of traits with other common bean cultivars.

Among the progeny evaluated in the field in 1997, a great number contained PP or PC parents that reputedly possess resistance or tolerance to Bean Fly. These progeny were selected for agronomic value, and seed of the most promising were produced for shipment to Tanzania for testing there with the insect (Table 3).

Some accessions of PC have been reported to be resistant to the white mold fungus (*Sclerotinia sclerotiorum*). Therefore, it is of interest to evaluate interspecific progeny for this disease also.

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Table 1: Hybrid seeds for inheritance study of BGMV resistance.

Obs.	Female	Species	X	Male	Species	Generation	No. seeds
1	G35171	COCC	x	G35337	PLAN	F1	59
2	G35171	COCC	x	G35347	PLAN	F1	101
3	G35172	COCC	x	G35337	PLAN	F1	41
4	G35172	COCC	x	G35347	PLAN	F1	35
5	G35171	COCC	x	G35337	PLAN	F2	964
6	G35172	COCC	X	G35337	PLAN	F2	1047

Table 2: Crosses involving the best interspecific selections for ascochyta resistance, to create multiple crosses. Popayán, 1998a.

Obs	Female (*)	Species	x	Male (*)	Species	Purpose	F1 Seed
1	239-(8)A-F11	VULG	x	346-F4	VULG	ASC	27
2	239-(8)A-F11	VULG	x	ICTA Hunapu	VULG	ASC	53
3	249-(8)A-F11	VULG	x	346-F4	VULG	ASC	40
4	249-(8)A-F11	VULG	x	ICTA Hunapu	VULG	ASC	15
5	282-(2)A-F12	VULG	x	346-F4	VULG	ASC	22
6	282-(2)A-F12	VULG	x	ICTA Hunapu	VULG	ASC	39
7	283-(6)A-F12	VULG	x	346-F4	VULG	ASC	9
8	283-(6)A-F12	VULG	x	ICTA Hunapu	VULG	ASC	45

Table 3: Interspecific crosses involving *P. vulgaris* and *P. coccineus* or *P. polyanthus*.

Obs	Female	Spp.	x	Male	Spp.	Purpose	F1 Seeds
1	G 4090	VULG	x	G35171	COCC	BGMV	50
2	G 4090	VULG	x	G35172	COCC	BGMV	27
3	RAB 50	VULG	x	G35171	COCC	BGMV	35
4	RAB 50	VULG	x	G35172	COCC	BGMV	38
5	TALAMANCA	VULG	x	G35171	COCC	BGMV	43
6	TALAMANCA	VULG	x	G35172	COCC	BGMV	177
7	G 2333	VULG	x	G35345	PLAN	BSM	6
8	G 2333	VULG	x	G35348	PLAN	BSM	61
9	G 2333	VULG	x	G35350	PLAN	BSM	56
10	G 685	VULG	x	G35345	PLAN	BSM	7
11	G 685	VULG	x	G35348	PLAN	BSM	4
12	G 685	VULG	x	G35350	PLAN	BSM	5
13	FEB 192	VULG	x	G35348	PLAN	BSM	38
14	FEB 192	VULG	x	G35350	PLAN	BSM	18
15	CAL 143	VULG	x	G35345	PLAN	BSM	42
16	CAL 143	VULG	x	G35348	PLAN	BSM	19
17	CAL 143	VULG	x	G35350	PLAN	BSM	1

2.1.3 Development of genetic transformation methods for common bean

Introduction

The genetic transformation of common bean has been reported by Russell et al. (1993) and Aragao et al. (1996) by particle bombardment of mature seed meristems. This methodology however has not yielded until now transgenic plants expressing agronomic important genes, possibly because of the low efficiency of transformation achieved and/or transgene expression problems. Using *Agrobacterium* as a vector the genetic transformation of a wild genotype of tepary bean have been possible (Dillen et al. 1997). Due to the difficulties in common bean transformation, the latter authors suggested the use of tepary bean as bridge to introgress transgenes to the common bean, by means of interspecific hybridization.

The main obstacle impeding the development of efficient genetic transformation in common bean, has been the lack of protocols for efficient *in vitro* plant regeneration from somatic cells or tissues.

In the past years, we have been able to develop a methodology for *in vitro* plant regeneration of common bean (Mejía-Jiménez et al. 1998), based in: The induction of an undifferentiated, long

term maintainable, highly morphogenic tissue composed solely of meristems (meristematic calli or m-calli) in agronomic important cultivars of common bean and other *Phaseolus* species. The regeneration of fertile plants through micrografting of differentiated buds from the m-calli.

During 1996-1997 we have attempted to transform m-calli through particle bombardment, but problems possibly related to gene integration, germline transformation or gene silencing prevented the recovery of transgenic plants expressing correctly the transformed genes.

During 1998 our efforts were concentrated in increasing the transformation efficiencies of m-calli, and in the achievement of the genetic transformation of m-calli through *Agrobacterium*.

Materials and Methods

A meristematic callus line of the cultivar Bayo Madero has been used in the particle bombardment experiments. For *Agrobacterium* transformation, this and other genotypes of common and tepary bean have been used.

Before bombardment or *Agrobacterium* inoculation the tissue were sonified in medium containing 2,4-D, in order to induce at the meristems wounding, cellular reorganization, activate cell divisions and expose cell layers below the epidermis to the transforming treatment. The bombarded plasmid was the pCambia 1304, which contains the GUS gene fused to the GFP gene and a Hygromycin resistance gene both under the control of the p35S promoter. The plasmid was bombarded in four different configurations: Closed circular, linearized, single circular DNA strand + (coding strand for the GUS gene) and single circular DNA strand - (complementary strand). Single strand DNA was produced by cloning the whole plasmid pCambia 1304 into the plasmid pBluescript SK + or -. GUS expression of the bombarded m-calli was evaluated 2 and 15 days after bombardment.

For the transformation with *Agrobacterium* sonified m-calli were cocultivated with overnight cultures (OD 0.5) of binary (EHA101 pIB GusIntron and A281 pIB GusIntron) and cointegrate LBA4404 pTOK233 hypervirulent *Agrobacterium* strains, which carry the GUS-Intron gene and the Basta™ or hygromycin resistance genes. Transient GUS expression were assayed after 3 days of coculture. *Agrobacterium* growth was controlled using ticarcillin, clavulonic acid, combined with vancomycin

Two weeks after transformation, the m-calli were transferred to selective medium containing antibiotics (hygromycin or phosphinothricin).

Results

Meristematic calli induction and long term maintenance.

- The number of genotypes responding to the methodology of induction of m-calli could be expanded. This has been possible in six agronomic important genotypes of common bean, 5 of tepary bean and one of *P. polyanthus*.
- Meristematic calli lines of Bayo Madero older than three years retain its regeneration capacity. This shows a high genetic and physiological stability of this kind of cultures.
- Efficient plant regeneration from m-calli is performed now routinely

Genetic transformation through particle bombardment

- The highest levels of GUS-expression 15 days after bombardment were obtained by bombarding with a single strand + DNA plasmid (the coding strand for the GUS gene). No expression 2 or 15 days after bombardment was obtained by bombarding with its complementary single DNA strand! This shows a totally elimination of the transient reporter gene expression by bombarding with the – strand.
- From these experiments more than 50 putatively transgenic clones that are resistant to concentrations of up to 30 mg/l hygromycin are still under selection

Agrobacterium transformation

- Methodologies for large scale wounding and inoculation of m-calli with *Agrobacterium* and have been developed.
- High levels of transient expression of the GUS Intron gene (up to 20 GUS expression spots/100mg tissue) in m-calli inoculated with *Agrobacterium* is routinely obtained. Factors important for achieving this were the inoculation medium, coculture temperature, the *Phaseolus* genotype, and the use of hypervirulent *Agrobacterium* strains.
- The m-calli of common bean are usually very susceptible to media changes and antibiotics presence in the media, making it difficult the controll of *Agrobacterium* growth after inoculation. A treatment for controlling *Agrobacterium* growth without killing the m-calli with the high doses of antibiotics required has been developed.

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Improved regeneration method may speed common bean transformation (communicated by Timothy Pratt) In ISB News report from July 1998 http://www.nbiap.vt.edu/news/news.rpt.html

Collaborators: A. Mejía, F. Giraldo, L. F. Galindo, A. Hassa[1], H. J. Jacobsen[1] and W. M. Roca

[1] University of Hannover

2.1.4 Improving somatic embryogenesis of a range of Latin American cassava cultivars.

Introduction

Agrobacterium-mediated transformation offers a powerful tool to introduce genes of agronomic interest and thus contribute to expand the genetic base of cassava. To use this technology, efficient transformation-regeneration systems are required for cultivars of economic importance. We tested 23 cultivars from nine Latin American cassava-growing countries for their ability to produce somatic embryos. An economically important cultivar in northern Colombia and Venezuela (MCol2215), was further tested for its ability to produce *in vitro*-derived plantlets from somatic embryos (somatic embryo maturation and elongation).

For somatic embryo induction, the CIAT's standard medium (CIAT,1995) was tested. Similarly, for somatic embryo elongation-maturation several media and growth conditions were tested. Treatments to evaluate embryo germination included the effects of light, desiccation and the presence of activated charcoal in different media. Criteria for evaluation of embryogenesis included embryo developmental stage and growth, and percentage of friable, non-embryogenic callus produced.

Results and Discussion

Table 1 show six cassava genotypes, including MCol2215 and the advanced line CM 2177-2, which had high-to-very-high embryogenic response. It was comparable to the embryogenic response shown by the model cultivars MCol22 and MCol1505. The other cultivars tested had medium-to-low embryogenic response, although they produced friable, potentially-embryogenic calli.

Regarding induction of somatic embryos and their development into plants with MCol2215, in vitro-derived, immature apical leaves, and axillary buds were the best explants for embryo induction. The best gelling-agent/auxin combination for embryo induction was Gel rite and 2,4-D. Somatic embryo proliferation responded better on medium containing different concentrations of 2,4-D and a mixture of Gel rite-Agar.

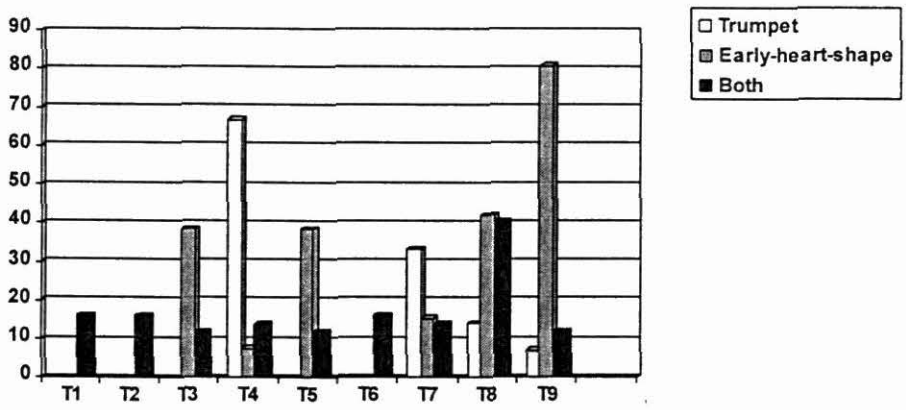
Treatments did not show significant differences for the production of friable, non-embryogenic calli, neither for embryo growth. For embryo development the best treatment included MS medium plus NAA, and MS plus ABA and cytokinin (BAP) (Figure 1). Embryos in the stages of early heart-shape and trumpet developed better under light conditions (Figure 2). Desiccation favored embryo germination (Figure 3). Currently we are evaluating elongation conditions.

Table 1. Somatic embryogenic response of Latin Americans cassava cultivars

	CULTIVAR	RESPONSE		CULTIVAR	RESPONSE		CULTIVAR	RESPONSE
1	MCOL 1505	++++	**9**	MBRA 893	-	**17**	MDOM 2	++
2	MCOL 2215	++++	**10**	MBRA 888	-	**18**	MPAR 110	-
3	MCOL 22	++++	**11**	MPAN 139	+	**19**	CM 523-7	+
4	MBRA 12	++++	**12**	MMEX 59	-	**20**	CM 2177-2	++++
5	MCOL 1468	++++	**13**	MPAR 104	+	**21**	CM 3306-4	-
6	MPER 183	++++	**14**	MCOL1522	++	**22**	CG 1141-1	-
7	MECU 72	++	**15**	MBRA 894	-	**23**	*MC RG3*	+
8	MCUB 74	+++	**16**	MBRA 900	-			

(-) Absence; (+) Low; (++) Medium; (+++) High; (++++) Very High

Collaborators: Ladino,Y.J; Segovia, V; Chavarriaga, P; Roca, W.



- **T1:** MS - No hormone.
- **T2:** MS + cytokinin.
- **T3:** MS + ABA.
- **T4:** MS + ABA - cytokinin.
- **T5 & T6:** MS + cytokinin.
- **T7:** MS + activated charcoal.
- **T8:** MS + auxin.
- **T9:** MS + auxin - mannitol.

Fig. 1 Effect of medium composition on growth and Development of cassava (cv. Mcol 2215) somatic embryos

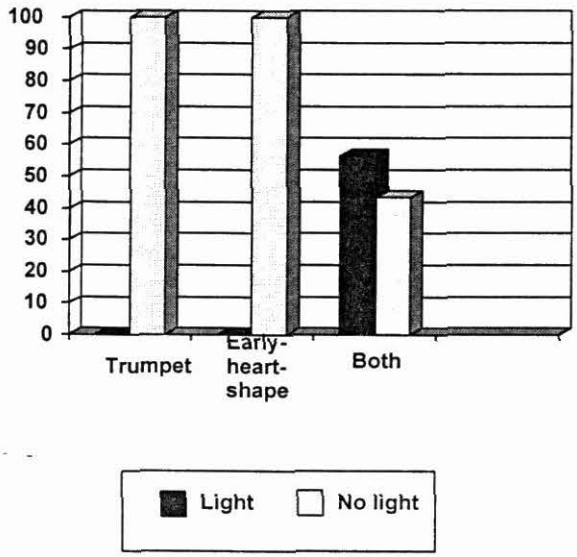


Fig 2. Effect of light on cassava (cv. MCol 215) embryogenesis.

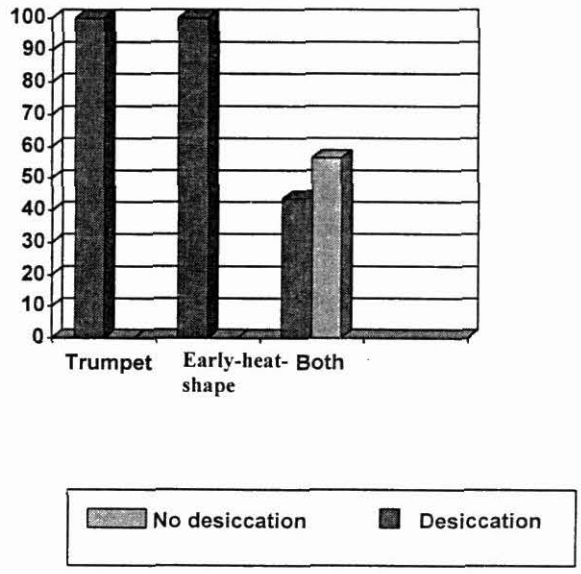


Fig 3. Effect desiccation on cassava (cv. MCol 2215) embryogenesis.

2.1.5 Construction and testing of plasmids containing a *Bt*-gene for cassava transformation

Introduction

The cassava stem borer (*Chilomina clarkei*, Lepidoptera) causes important economic losses in cassava growing area of Northern Colombia and in Venezuela. The introduction of insect resistant genes (Bt-genes) through *Agrobacterium*-mediated transformation offers a powerful component of integrated control of the pest. We constructed several plasmids containing a Bt gene (*cry*IA(b); Figures 1 & 2) plus several selectable and scorable markers employing standard cloning techniques. These plasmids were introduced into different *Agrobacterium* strains and the bacteria tested for their ability to transfer the T-DNA into cassava embryogenic tissues. A supervirulent *Agrobacterium* strain (LBA4404-pTOK233) was also tested to study the effect of containing extra *vir* genes on the efficiency of transformation of cassava tissues. Cassava has been transformed with this strain [1]. Potential expression of the *cry*IA(b) gene, and the effect of extra *vir* genes on transformation efficiency were indirectly measured by the transient expression of the GUS-intron gene.

Results and Discussion

Initial results indicated that C58C1-pBIGCry was very efficient at transferring T-DNA and expressing the GUS-intron gene in cassava explants (Table 1). Between 23 and 50% of explants showed multiple infections (blue foci) and whole blue tissues after 3-4 days of coculture and 15 to 30 min. of incubation in GUS buffer. With the strain LBA4404-pTOK233 the percentage of explants showing multiple infections increased to 66-87%. The overall efficiency of transformation was comparable between these two strains although the presence of extra *vir* genes enhanced significantly T-DNA transfer. Non-supervirulent strains, carrying no extra *vir* genes (i.e., LBA4404-pBIGCry) produced few-to-none multiple infections. Since somatic embryogenesis in cassava is of multicellular origin [2], regeneration of non-chimeral transgenic plants requires multiple infections, i.e., multiple, adjacent cells must be transformed simultaneously on the same explant. As we show here, supervirulent strains like C58C1, or strains carrying extra *vir* genes like LBA4404-pTOK233 produced multiple infections, which, with proper selection methods, can potentially developed into whole transgenic plants. We are currently in the process of selecting transgenic cassava tissues and constructing plasmids that carry extra *vir* genes to enhance T-DNA transfer efficiency.

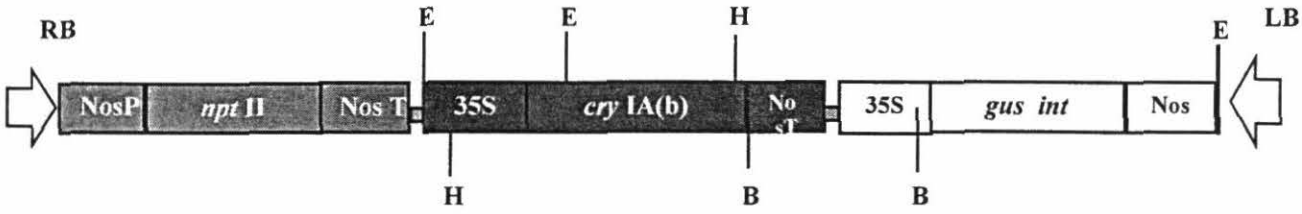
Table 1. Efficiency of transient expression of the GUS-intron gene in cassava embryogenic tissues with different strain-plasmid combinations

Plasmids:	LBA4404	C58C1
pBIGCry	++	++++
pKC2301	+	n.d.
pTOK233*	++++	n.d.

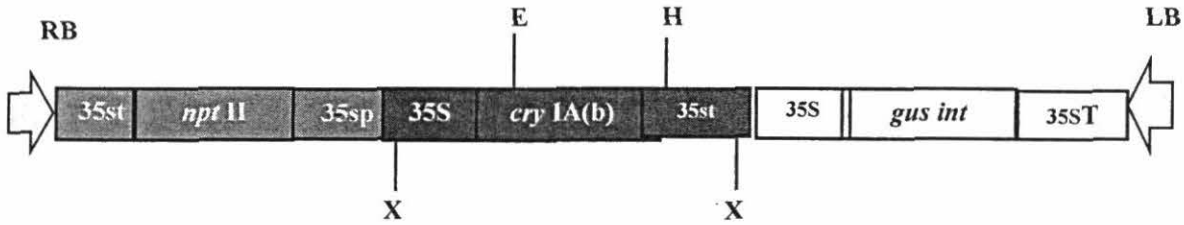
(++++) Between 20 and more than 80% multiple infections produced
(+), (++) Below 10% multiple infections
(n.d.) Not done

Figures 1 and 2. Plasmids pBIGCry and pKC2301 containing the cry1A(b) gene.

pBIGCry(15.4 kb)



pKC2301 (14.62 kb)



H : Hind III; **E**: Eco R I; **B**:Bam H I;**X**:Xba I

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Collaborators: P. Chavarriaga, V. Segovia and L.I.Mancilla, W. Roca

2.1.6 Characterization of the PthB protein of *Xanthomonas axonopodis pv. manihotis.*

Introduction

Phytopathogenic bacteria generally have limited host ranges, often to members of a single plant species or genus. Microbial genes involved in plant-microbe interactions may be functionally classified into four broad categories: those involved in parasitism, pathogenicity, host range, and avirulence (1). A family of avirulence (*avr*) and pathogenicity (*pth*) genes has been identified in the genus *Xanthomonas*, including *X. campestris* pvs. *vesicatoria (avrBs3)* and *malvacearum*, *X. oryzae* pv. *oryzae* , *X. citri* (reviewed by Leach and White, 1996) and recently *Xanthomonas axonopodis* pv. *manihotis* named *pth*B (Verdier *et al.*, 1996). The *avrBs3*-like avirulence genes sequenced to date are 94 to 98% identical with each other. Avirulence genes which are present in bacterial pathogen interact with matching resistance genes in the host to triggering a cascade of responses in the plant. The major plant defense mechanisms include production of anti-microbial compounds and enzymes, cell-wall fortification, production of reactive oxigen species, and the hypersensitive reaction (HR), characterized by the rapid necrosis of the plant at the site of infection and the accumulation of phytoalexins.

Recently, it has been shown that bacterial avirulence proteins functions as elicitors that are perceived within the plant cell. Our purpose was to express and purify the *Pth*B protein for producing polyclonal antibodies. Interaction of the PthB protein with the host plant will be further studied by immunoelectronmicroscopy.

Methods.

Bacterial strains and plasmids. Strains and plasmids used in this study were *Escherichia coli* DH5 α , *X. axonopodis* pv. *manihotis* CIO46 and CFBP1851 and vectors pGEX series 5X-1, 5X-2 and 5X-3 (Pharmacia). The *pth*B gene of *Xam* was previously cloned and purified using standart methods (*Bam*HI fragment ≅ 2.9kb). The *Bam*HI fragment of *pth*B was cloned in pGEX vectors previously digested with *Bam*HI and dephosphorylated. Strain A1-28, A3-15 (pGEX 1 and 3 respectively with the insert pthB) were grown on LB supplemented with ampicillin at 100ng/ml.

Expression of the pthB gene and purification of the protein PthB/GST

Strain A3-15 was grown in LB+Amp and induced with IPTG 1.0mM. Bacterial suspension were sonificated and run in a SDS-PAGE gel, stained with comassie blue R-250 for detecting the PthB protein. The protein was ecised from the gel and purified by an amonium carbonate 0.5%, SDS 0.1% and mercaptoetanol 2% treatment, then dyalised for 48h to eliminate salts residues. Quantification of total protein was performed using the Bradfor method and using bovine serum albumine (0.5mg/ml) as standard protein. For immunization of white rabbit, the protein (0.3mg) was injected intramuscularly three times at 10 days interval. Specificity of the serum was evaluated by ELISA and western blot. Antibodies for immunoelectron microscopy were purified from the serum by an amonium sulfate and dyalisis procedures.

To determine the activity of protein, leaves of the susceptible cassava cultivar MCOL 1522 were inoculated with 0.03mg of protein; bacterial suspensions of *X. axonopodis* (CIO 46 and CIO 1851). IPTG induced strain of *E.Coli* A3-15 was used as positive control. Sterile water, polyacrylamide gel and non induced A3-15 strain were used as negative control.

IPTG induced strain of *E.Coli* A3-15 was used as positive control. Sterile water, polyacrylamide gel and non induced A3-15 strain were used as negative control.

Results and discussion

Cloning the *pth*B gene in pGEX vectors was successful with the pGEX 5X-1, 5X-2 and 5X-3 series, the insert orientation was then evaluated by restriction digests with *Eco*RI and *Xho*I respectively. Strain A3-15 (pGEX5-3 - *Bam*HI fragment) was selected for further studies. The protein fusion PthB/GST was induced by adding IPTG (100mM) to the cultured cells of strain A3-15 (O.D 600= 0.6-0.8). The protein was then separated by SDS-PAGE gels. SDS-polyacrylamide gels were stained and showed a band of 123KDa which corresponds to the protein fusion pthB/GST. Negative control (pGEX 5X-1 alone) showed one band between 30-26 kDa corresponding to the glutation transferase protein (GST). The protein was excised from SDS-polycarylamide gels and 1.3mg of purified protein was obtained.

Specificity of the serum was tested by ELISA. Using a dilution of 1/250 of the protein (0.1mg) Elisa tests were positive with a O.D 490 was > 2.0. Positive reactions were observed using dilutions of the serum (1/10.000). A rabbit serum inoculated with freund's adjuvant was used as negative control, (O.D = 0.2). Different samples were used as antigen control: strains of *Xam* CIO 46 and CIO 1851 (O.D > 1.5), strains A3-15induced (O.D >0.8) and not induced (O.D<0.5), extracts of cassava leaves MCOL 1522 (O.D <0.2). Western blots were used to confirm the reactivity of the serum (1/1000 dilution) versus protein fusion PthB/GST. Our results demonstrated the high specifity of the *Pth*B serum against the recombinant protein. Reactivity of the polyclonal antibody to PthB protein will be further evaluated.

Cassava leaves inoculated with 0.03 mg of protein show a yellow/brown spot after 48 hours post-inoculation. Leaf angular spots appear 5 days after inoculation with the *Xam* strain CIO 46. Induced strain A3-15 caused light brown spot while inoculations with sterile water, acrylamide gels, and non induced strain A3-15 did not induce any symptoms. Serial dilutions of the protein extracts were made and 6 days after inoculation light leaf brown spots were visible.

We will futher investigate the location of the PthB protein from *Xam* in inoculated plant tissues and bacterial cultures. Knowledge of the location of pthB gene products in bacterial-plant interactions could provide new insight regarding AVR protein function in the elicitation of resistance.

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Collaborators: Valérie Verdier [1], Monica Chávez , Lida Mancilla.

1. ORSTOM-CIAT

2.1.7 Detection of *Xanthomonas axonopodis* pv. *manihotis* in cassava true seeds by Nested-Polymerase Chain Reaction assay(N-PCR)

Introduction

Cassava Bacterial Blight (CBB) caused by *Xanthomonas axonopodis* pv. *manihotis (Xam* is a particularly destructive disease in South America and Africa. The success of a cassava seed certification program will depend on the availability of reliable tests to detect the pathogen in true seeds. Current methods to identify and detect *Xam* rely on isolating the bacterium and conducting immunoassays of tissue. Enzyme-linked immunobsorbent assays to detect this pathogen are not entirely specific. Recently, a PCR assay has been developed for detection of CBB in cassava leaves and stems (Verdier *et al.*, 1998). In cassava seeds, the bacterium was not detected by current PCR assays.

The nested polymerase chain reaction (N- PCR), is a PCR based assay for which a second round of amplification is developed by using primers internal to the first amplification product. For the present study, we developed a pair of internal primers and established the conditions of a N-PCR for seed detection. Our objective is to develop a rapid and more sensitive method for detecting *Xanthomonas axonopodis* pv. *manihotis* in true cassava seeds.

Materials and Methods

PCR Conditions. PCR reactions were as previously described (Verdier et al., 1998). 20ng of DNA were used for the amplification. For the experiments performed with cultured cells, the bacterium was suspended and serially diluted in distilled sterile water; plated on LPG and the colonies developed were counted. Each dilution was boiled for 10 min prior to PCR and 10 µl of each dilution was used as source of DNA template for the PCR reaction. Negative control reactions (no DNA or bacterium) were run in all the experiments. 10 µl of each PCR product were then transferred to the N-PCR reaction mix with the internal primers which were designed with the software Cprimer. Amplification profile was as previously described. The amplification products were analyzed by agarose electrophoresis, stained with etidium bromide.

Detection of *Xanthomonas axonopodis* pv. *manihotis* in artificially infected seeds. Cassava healthy seeds were inoculated as follows: an inoculum of 10^8 cfu/ml (optical density at 600nm= 0.1) was prepared from a fresh culture. Three groups of ten seeds each were washed and scarified. The seeds were then inoculated by absorption in vacuum for 45 min. The inoculated seeds were washed twice by shaking in distilled sterile water, then each seed was macerated individually. Each sample was serially diluted in distilled water, and plated on LPG, the colonies that developed were counted. 10µl of each dilution were used as the source of DNA template and incubated at 95 °C for 10 min. One µl proteinase K (10 µg/ml) was added and samples were incubated at 55 °C for 12 min. Finally, the samples were incubated at 20 °C for 3 min during the addition of *Taq* polymerase. PCR profile was as described above. For the N-PCR, 10 µl of each PCR product were used as the source of DNA template.

Detection of *Xanthomonas axonopodis* pv. *manihotis* in seed samples collected in infected fields. Cassava fruits collected in infected fields were dried and the seeds were extracted. Samples of ten seeds were washed in distilled sterile water, serially diluted and plated on LPG. 10 µl of each dilution were used as DNA template for PCR after being treated as indicated above. 10µl of each PCR product were used for the N-PCR.

Results and Conclusions

We have developed a N-PCR based assay for the detection of Xanthomonas axonopodis pv. manihotis, in true sexual seeds. The primers X-V2 and X-K2 directed the amplification of an 509bp nucleotide product internal to the 898bp product amplified with the first round PCR. The N-PCR assay improves the sensitivity of our previous single stage PCR assay. The N-PCR method allowed us to detect 1.4 cfu/reaction in cultured cells, 1 to 2 cfu/reaction in artificially infected seeds and 1.2. 10^2 cfu/reaction in naturally infected seeds. The PCR and N-PCR procedures are simple and fast methods to perform. With its specificity and sensitivity the combined PCR assays described here have a potential as a reliable procedure for detecting and identifying the CBB pathogen in seeds.

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Collaborators: S. Ojeda and V. Verdier[1]

1. ORSTOM - CIAT

2.1.8. Cryopreservation of cassava shoot tips: Encapsulation-dehydration technique.

Background

Cassava shoot tips were successfully cryopreserved using an encapsulation/dehydration technique. The technique (Palacio, 1997) allows direct placing of shoots into liquid nitrogen, preventing the use of expensive programmable freezing equipment and opening the possibility of large-scale conservation methodology at CIAT (Escobar *et al.* 1998).

Materials and Methods

To optimize the factors controlling the rates of plant recovery after freezing, cytokinins, gelling agents mixtures and supplementation of beads were tested.

Results and Discussion

Results obtained in 1997-98 are shown in Table 1, 2 and 3.

Table 1: Effect of growth regulators on viability and shoot recovery after freezing of cassava encapsulated-dehydrated shoot tips.

Cultivar	Growthregulator (mg/l)	% Viability	% Shoot recovery
M Col 1468	BKZ (0.33 each)	90	25
	K1	71.4	4.8
	2iPKZ (0.17 each)	90	45
	2iPKZ (0.33 each)	80	20
	2iPKZ (0.5 each)	90	20
M Bra 507	BKZ (0.33 each)	100	35
	K1	80	40
	2iPKZ (0.17 each)	95	65
	2iPKZ (0.33 each)	95	50
	2iPKZ (0.5 each)	90	10
M Ven 232	BKZ (0.33 each)	84.2	0
	K1	90	65
	2iPKZ (0.17 each)	88.8	5.6
	2iPKZ (0.33 each)	89.5	10.5
	2iPKZ (0.5 each)	89.5	0
M Col 22	BKZ (0.33 each)	100	45
	K1	100	47.4
	2iPKZ (0.17 each)	95.4	72.8
	2iPKZ (0.33 each)	93.7	68
	2iPKZ (0.5 each)	100	52.6

B, K, Z and 2iP are all growth regulators

Plant regeneration rates could be increased through the inclusion of Benzyladenine (B), Kinetine (K), Zeatin (Z) and 2iP. We previously found that the type and concentration of cytokinin could improve shoot response after freezing (BRU-Annual Report 1995). K1 and 2iPKZ (at 0.17-0.33 mg/l each) had the best effect on shoot recovery after freezing (Table 1). Two recalcitrant cvs, MVen 232 and MCol 1468, improved their response with this treatment.

Viability after freezing is more consistent with the encapsulation-dehydration than with programmed freezing; the average viability value per cv. was more than 80%. This gives us the opportunity to recover more shoots per treatment. We have observed that when using beads with growth regulator response of frozen shoot improved.

Table 2. Effect of agar brand and consistency of recovery medium on viability and plant recovery from frozen shoot tips.

Cultivar	Consistency of medium	Agar relation ®Duchefa:®Phytagel	% Viability	% Shoot recovery
M Col 1468	Solid (0.45%)	3:1	89.4	58
	Semisolid (0.35%)	3:1	88.9	50
	Control (0.35%)	1:0	100	56.2
M Bra 507	Solid (0.45%)	3:1	100	66.7
	Semisolid (0.35%)	3:1	100	38.9
	Control (0.35%)	1:0	100	52.6

Some agars contain inhibitory substances which may prevent morphogenesis in certain cultures, rate of growth can be slow, toxic exudates from explants do not diffuse away quickly. Hyperhydracity could be avoided using mixtures of Gel-rite and Agar. We have found that a relation 3:1 with the K1 medium shows improves shoot recovery from frozen cassava shoot tips (Table 2).

Table 3. Effect of bead supplementation on the response of cassava shoot tips after freezing in L.N.

Cultivar	Bead supplementation	% Viability	% Shoot recovery
MCol 1468	4E	83.3	50
	K1	80	70
	BKZ (0.33 each)	100	90
	Without	81.8	63.6
MBra 507	4E	90	50
	K1	63.6	45.4
	BKZ (0.33 each)	54.5	9.1
	Without	66.6	41.7
MVen 232	4E	100	81.8
	K1	90.9	90.9
	BKZ (0.33 each)	91.6	83.3
	Without	66.7	66.7
MCol 22	4E	91.7	75
	K1	75	66.7
	BKZ (0.33 each)	75	50
	Without	66.7	50

It seems that beads are not so permeable to media components at the beginning of culture; tissue could starve. Supplementation of beads with media components could support the initial growth till shoot emerges. We have found that medium K1 is more effective especially after freezing MVen 232 and MCol 1468 (table 3).

Conclusion and future plans

We found that adjusting the shoot recovery steps (media and conditions) the percentage of shoot recovery after freezing can be increased. Encapsulation/dehydration could be a simple way to introduce cassava collection liquid nitrogen. We will test this improved methodology on a sub set of the cassava core collection.

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Collaborators: R. H. Escobar, M. P. Rangel, W. M. Roca

2.1.9 Genes from Wild Rice Contribute to Yield Increase in Cultivated Rice

Introduction

Twenty one wild species and two cultivated species (*O.sativa* and *O. glaberrima*) represent ample genetic variability for rice breeding programs. It has been suggested that the *Oryza* wild species represent a potential source of new alleles for improving yield, quality, and stress resistance of cultivated rice. However, limited use of this variability has taken place. Barriers still exist in effectively utilizing genes from wild species and molecular mapping techniques are needed to readily detect these new alleles in segregating populations.

CIAT started in 1994 a collaborative project aimed at characterizing and utilizing wild rice species for the improvement of cultivated rice. We here report on progress made in the identification of QTLs associated with yield increase in *O. rufipogon* and *O. barthii*.

Materials and Methods

Two improved rice cultivars (BG90-2 and Lemont) were crossed *to O. rufipogon* and *O. barthii*, respectively (Table 1). Few plants (2-3) in each of the wild species were hybridized to several plants of each of the improved cultivars (recurrent parents). Single crosses were obtained and grown in the greenhouse at CIAT in 1994. Three F_1 hybrid plants were backcrossed to the improved cultivar; 153-198 BC_1F_1 seeds were obtained per cross combination. The resulting BC_1F_1 plants were transplanted (30x50 cm) and evaluated based on phenotype; negative phenotypic selection for undesirable agronomic traits (spreading plant type, excessive shattering, long awns, dark-color grains, high sterility, etc.) was used to narrow the selection down to the best (30-50) individuals. Each selected BC_1 individual was back crossed again to the recurrent parent and approx. 30 BC_2F_1 seed were sown in wooden trays in the screenhouse and later on transplanted (30x40 cm) under irrigated conditions. A negative phenotypic selection was applied again and best individuals per cross were selected and harvested individually to generate BC_2F_2 seed; 300 BC_2F_1 plants were selected in the BG90-2/ *O. rufipogon* cross for field testing, whilst high sterility was found in the Lemont / *O. barthii* cross (Fig 2). Therefore, another BC to Lemont was done and 326 BC_3F_1 plants were selected for field testing as BC_3F_2 *families*.

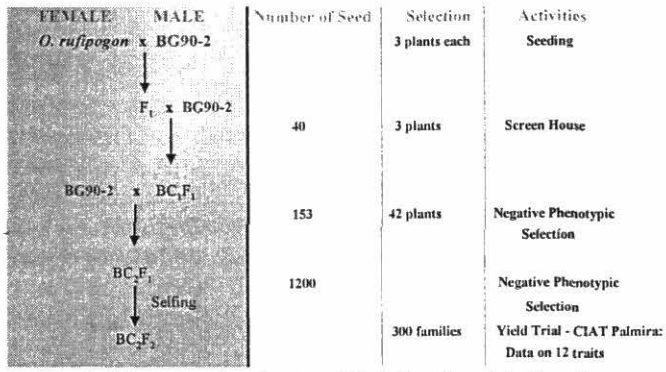


Fig. 1. Scheme used to develop BC_2F_2 families with *O. rufipogon*

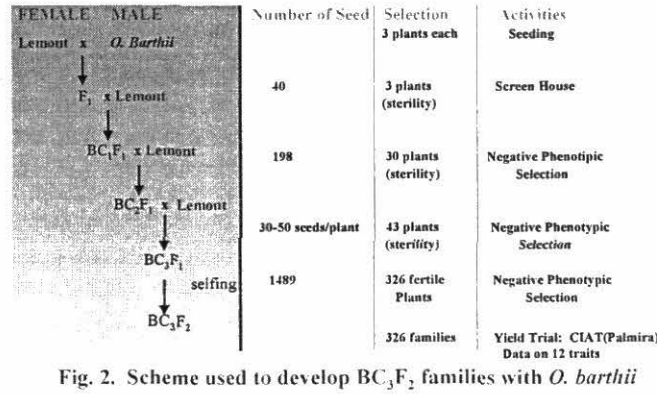
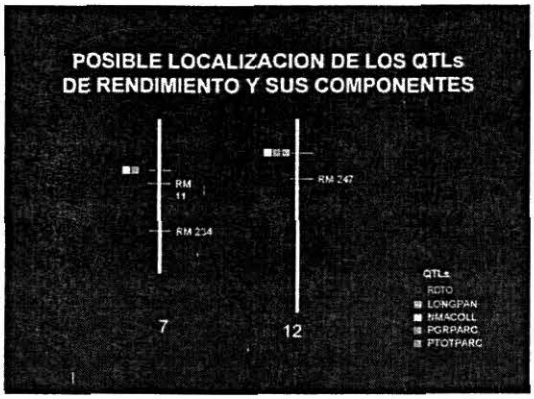
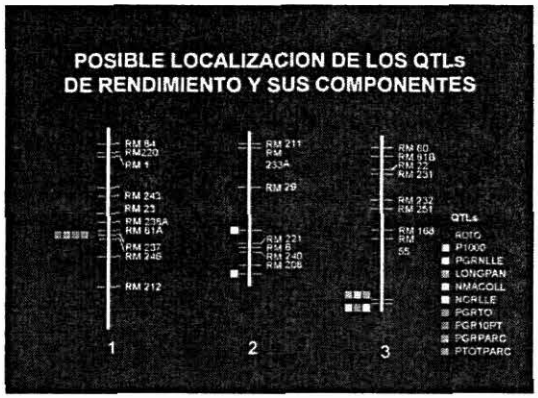


Fig. 2. Scheme used to develop BC_3F_2 families with *O. barthii*

Field trials

The 300 BC_2F_2 families derived from the cross BG90-2/ *O. rufipogon*, and the 326 BC_3F_2 families from the cross Lemont / *O.barthii* were planted in replicated yield trials in CIAT-Palmira. Transplanting (20x30) was done and a completely randomized design with two reps., 2 row-plot, 5 m. long was used. Data on 12 agronomic data, including plot yield/family were taken on 10 randomly selected plants/plot.





Based on yield potential and good agronomic traits, 38 BC_2F_2 families from the cross BG90-2 / *O. rufipogon* were selected and further evaluated for grain yield; a completely randomized design with four reps, 4 row-plot, and 5 m. long was used.

Molecular characterization

DNA of young leaves from the parentales genotypes, the BC_2F_2 and BC_3F_2 families was extract by the Dellaporta Method (McCouch et al. 1988) modified for PCR assay by CIAT Biotechnology Research Unit (unpublished data). Parental surveys filters containing *O. rufipogon*, *O. barthii*, BG90-2, and Lemont were prepared using five restriction enzymes (EcoRI, EcoRV, HindIII, XbaI and DraI). Approximately 140 markers from the rice molecular framework linkage map were selected at 10-20 cM intervals throughout the genome. A set of 78 mapped rice microsatellite markers, developed at Cornell University, was also used to complement the RFLPs in QTL analysis.

Conclusions

The distribution of grain yield (kg/ha) of 300 BC_2F_2 families (BG90-2/*O. rufipogon*) derived from plot yields of 40 plants (20 plants/row x 2 row) averaged over two replications.

Transgressive segregation can be observed, with several lines (11%) having between 5 and 25% higher yield than the recurrent parent BG90-2. Transgressive segregation for other yield components was also observed. Grain yield data taken on 38 BC_2F_3 families (Table 1A) confirmed results obtained in the BC_2F_2 generation.

Based on the 88 RFLP and 14 microsatellites from the RF- Cornell framework map screened on 300 BC_2F_2 families, putative linkages were identified with yield, and yield components from replicated data available for the whole mapping population. Preliminary results using one way anova and t- test indicate associations between markers and yield on chromosome 2 similar to J.Xiao and S. McCouch results (Fig. 2). No linkage for yield was detected on chromosome 1 as reported by J. Xiao and S. McCouch. Other associations were also identified for yield and the various yield components (Fig. 3-4). These data from different groups working with diverse recurrent parents suggest that DNA introgressed from *O. rufipogon* can contribute positively to yield in elite rice cultivars.

The distribution of grain yield (kg/ha.) of 326 BC_3F_2 families (Lemont / *O. barthii*) derived from plot yields of 40 plants (20 plants/row x 2 row) averaged over two replications. Transgressive segregation can be observed, with several lines having up to 30% higher yield than Lemont. Based on the 54 microsatellites from RF-

Cornell framework map screened on 326 BC_3F_2 families derived from the cross Lemont / *O. barthii* putative linkages were identified. Preliminary results using one way anova and t-test indicate association between markers and yield on chromosome 2 similar to Xiao et al. and on chromosome 7.

Table 1. Grain Yield (Kg/ha) of some BC_2F_3 families from BG90-2/*O. rufipogon* cross at CIAT. 1997

Line/pedigree	Yield	(%) of BG90-2
CT13941-11-M	7880	21
CT13958-12-M	7746	19
CT131946-1-M	7535	16
CT13976-7-M	7519	16
Ct13946-26-M	7359	13
BG90-2	6496	0
O. rufipogon	4998	-23

- Minimum signif difference. Dunnett's – 1582

Grain Yield (Kg/ha) of some BC_3F_2 families from Lemont/*O. Barthii* cross at CIAT. 1997.

Line/pedigree	Yield	(%) de rendimiento
CT14949-28	6193.5	136.05
CT14937-24	5899.24	129.59
CT14964-3	5629.59	123.66
CT14938-5	5620.73	123.47
CT14937-10	5526.44	121.4
CT14955-15	5514.25	121.13
CT14966-9	5513.34	121.11
CT14937-5	5498.15	120.78
CT14946-69	5496.81	120.75
CT14937-26	5450.14	119.72
Lemont	4552.39	100
O. barthii	1065.72	23.41

ON-GOING ACTIVITIES

1. Complete the characterization of agronomic and molecular data, and QTL analysis to determine the number of QTLs associated with yield increase across environments for both crosses.

2. Determination of contribution for positive alleles of each of the parents.

3. Development of Nils based on QTL analysis carrying specific QTLs for use in breeding programs.

4. Start agronomic and molecular characterization of several other populations involving crosses with *O. glaberrima* to determine the presence of QTLs for yield increase.

Other Activities

Agronomic evaluation of 346BC2F2 families from the cross Oryzica3/*O.rufipogon* under irrigated conditions at CIAT.Palmira. Grain yield data showed that several lines outyielded Oryzica 3. Molecular characterization needs to be done.

Seed multiplication and agronomic evaluation of 350 doubled haploid lines from the cross Lemont/ *O.barthii.* These lines were developed from BC3F1 plants and evaluated under irrigated conditions at CIAT.Palmira. Bulked seed from each line was harvested for further evaluation in a replicated yield trial.

Two hundred ninety six BC2F2 families from the cross Bg90-2/ *O.barthii* are being evaluated in a replicated yield trial under irrigated conditions at CIAT. Palmira. Data on main agronomic traits are taken.

The BC2F1 population from the cross Progresso/*O.barthii* was planted and evaluated at CIAT. Palmira. Very high sterility was observed and 320 fertile plants were harvested for further evaluation under upland conditions in a replicated yield trial. This population was also run through anther culture and the response in terms of callus production has been very good.

The BC2 cross between BG90-2 and *O.glaberrima* was completed and the BC1 population is under evaluation at CIAT.Palmira. The F2 seed will be shared with WARDA for evaluation in West Africa in 1999.

One hundred advanced lines were received from WARDA's rice interspecific hybridization project (*O.sativa/O.glaberrima*) and evaluated under upland conditions at La Libertad Experiment Station, Villavicencio in collaboration with M.Chatel and Yolima Ospina. Data on main agronomic traits, disease reaction, tolerance to acidic soil conditions, and grain yield were taken;15 lines were selected for further evaluation in 1999.

Seed of aprox.600 F3 lines from several interspecific crosses (BG90-2/*O.rufipogon*, Oryzica 3/*O.rufipogon*, and Lemont/*O.barthii*) was provided to C.Bruzzone for field evaluation in Santa Rosa and Palmira. On the other hand, seed of 305 BC2F2 families from the cross BG90-2/*O.rufipogon* was sent to WARDA and evaluated in an observational nursery in Ivory Coast .Many lines were selected by WARDA's breeders for further evaluation in several ecologies next year .

Funding proposals and concept notes for Colciencias ,the European Community ,CIDA, and South Korea were developed and presented to potencial donors .

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Collaborators: C. P. Martínez , J. López, A. Almeida, G. Gallego, J. Borrero, W. Roca, M. C. Duque and J. Tohme.

2.1.10. Control of RHBV (rice hoja blanca virus) through nuclear protein mediated cross protection in transgenic rice.

Rice hoja blanca virus (RHBV) is one of the major diseases afecting rice in tropical Americas. RHBV disease was first reported in 1935 and since then, mayor outbreaks of the disease had caused up to 80% of yield loss. Most popular varieties are resistant to the vector but are

susceptible to the virus. The breeding resistance is conferred by one or two genes, but plants carrying this source of resistance are susceptible at younger ages than 25-day-old. The uncertainty of epidemics induces farmers to spray insecticides to control sogata, the 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 present in most varieties is from a single resistant source. 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. Previous reports described the particle bombardment genetic transformation protocol optimized for indica Latinamerican genotypes, the preparation of gene constructs containing the RHBV nuclear (N) protein gene and the RHBV NS4 nonstructural protein antisense gene. Last year we reported the generation and selection of RHBV-N transgenic lines from the Colombian rice commercial variety Cica 8, showing stable RHBV resistance on T2 progeny plants. The RHBV-N transgenic lines A3-49-56, -60, and –101 showed a significant delay in the development of the disease and reduced severity of the symptoms (disease reaction from 0.1 to 0.3) in contrast to the non-transgenic control Cica 8 (disease reaction 0.8, when using a rating scale of 0 to 1) which was highly susceptible at 23 days after the RHBV infection. These transgenic lines showed a yield potential of 46% to 64% higher than the non-transgenic Cica 8 control.

Following is reported the study in progress directed to determine the inheritance and expression of the N-protection in other genetic backgrounds of interest for breeding. This study aims to elucidate if the N transgene could be used to complement the breeding resistance source already available to fully protect plants younger than 25 days of age.

Materials and Methods

Inheritance and expression of the RHBV nucleoprotein cross protection in different genetic backgrounds.

In this work, T3 progeny plants were selected based on the resistance level and agronomic traits from the corresponding T2 progeny line (Table 1). Individual T3 progeny plants from the selected Cica 8 N-transgenic lines were first analyzed by nested-PCR to detect the plants carrying the N-transgene. Those plants were chosen as female parents and crossed with: 1) the breeding fixed line CT8008-3-12-3P-M-1P highly susceptible to RHBV; 2) the variety Oryzica 1 with moderate resistance; and 3) the variety Fedearroz 50 (FD50), highly resistant to RHBV. Controls consisted of F1 crosses between the non-transgenic Cica 8 and CT8008, Oryzica 1, and FD50, respectively. Plants 10-day-old and 20-day-old of each F1 cross were infected with RHBV under greenhouse conditions. Viruliferous nimphs from a vector colony with at least 85% of virulence were used. Five nimphs per plant were placed onto each plant contained within a plastic tube. Nimphs were allowed to feed on the plant for 5 days. Plants were evaluated weekly and up to 54 days after infection for the development of RHBV disease and plant vigor. Evaluations for performance of agronomic traits is still in progress.

Results

Results showed that the non-transgenic F1s were significantly more susceptible than the resistant parent, suggessting that the natural resistance source is encoded by non-dominant gene(s). Crosses with the transgenic lines A3-49-60-4-5, A3-49-60-4-13, A3-49-60-12-3, and A3-49-101-18-19 were significantly more resistant (about 40%) to RHBV than the corresponding F1 non-transgenic cross when using 10-day-old plants (Figure 1). A similar trend was obtained when plants were infected at 20-day-old. The higher level of resistance of the transgenic F1s was noted on the crosses with the susceptible, the intermediate resistant, and the highly resistant genotypes, and in some cases the resistance level was similar to the resistant parent (Figure 1). These results suggest that the protection conferred by the RHBV-N transgene is expressed independently of the genotype background, and that the transgene could be used to complement the natural resistance source. These crosses are currently being evaluated for its performance for agronomic traits. Future work will include the evaluation of the resistance segregation in F2 population to determine the inheritance and stability of this trait through selfing, and the initiation of a marker assisted selection backcross breeding scheme for the introgression of the RHBV-N transgene into CT8008-3-12-3P-M-1P, Oryzica 1, Fedearroz 50, and Cica 8 parental genotypes. The selection process will be aided by MAS using the presence of the RHBV-N transgene as the trace marker. In order to implement this type of molecular selection, the correlation between the level of resistance and the presence of the transgene as well as the effect of the plant age on the level of the resistance conferred by the RHBV-N gene are currently being analyzed.

Table 1.- Disease reaction and yield potential of T2 progeny plants from RHBV-N Cica 8 transgenic lines selected as female parents.

Line A3-49	Disease reaction	Grains/ plant
56-17	0.04	1332
60-4	0.09	728
60-12	0.04	582
101-5	0.00	1240
101-18	0.03	67
Cica 8 infected	0.51 (0.10)	70 (48)
Cica 8 non-infected	0.00	1218 (343)

Numbers in parentheses refer to the standard error.

Collaborators: Z. Lentini, L. Calvert (IP4), C.Gamboa[1] , L.F. Fory (IP4), I. Lozano (IP4), E. Tabares.

1. Danac, Venezuela/FLAR.

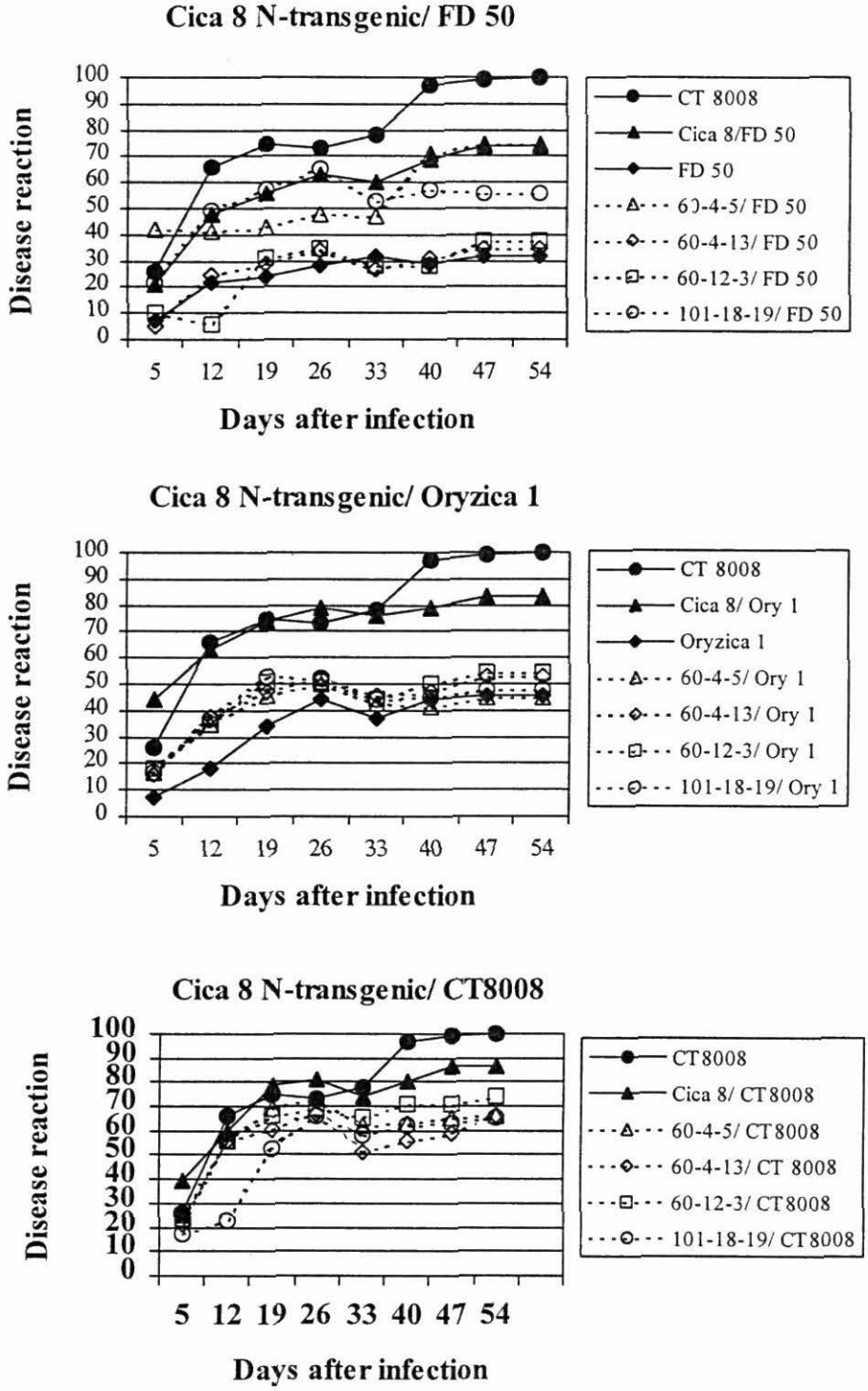

Figure 1.- Disease development on 10-day-old progeny plants from crosses between Cica 8 RHBV N-transgenic lines and three genotypes with different levels of RHBV resistance. Fedearroz 50 (FD 50), highly resistant; Oryzica 1, intermediate resistant; and CT 8008 highly susceptible.

2.1.11. *Agrobacterium* mediated genetic transformation of rice genotypes adapted to irrigated, upland, and hillside agroecosystems.

Until recent years it was thought that monocots, such as rice, were recalcitrant to *Agrobacterium*-mediated transformation. Recent reports have shown high transformation efficiency and demonstrate the potential of agroinfection in economically important cereals, such as maize, wheat, barley and rice. Transformation based on the use of *Agrobacterium tumesfaciens* is being preferred in a larger number of laboratories nowadays, because it appears to result in high transformation efficiencies and in more predictable segregation patterns of the transgenes when compared to the particle delivery system or electroporation. At CIAT there is interest to adopt this technology for transformation of local rice varieties such as *indica* rices (CICA 8, IR-72, INIAP 12) adapted to irrigated (flooded) conditions, and *japonica* rices (CT 6241, CT 10069, and Lastisday-Fofifa) adapted to acid soils (savanna) and highland (hillside) environments. Research was initiated this year to review the protocols established for rice (Hiei *et al.*, 1994; Aldemita,1996; and Toki , 1997) and introduced the necessary modifications for optimization using the selected breeding genotypes.

Material and Methods

Optimization of *Agrobacterium* mediated transformation six genotypes adapted to Latin American rice habitats

The three protocols developed for rice (Hiei *et al.*, 1994 and 1997; Aldemita,1996; and Toki , 1997) were tested. Preliminary results showed differences between the three protocols established for rice transformation. Following are described the modifications introduced into Aldemita and Hodges (1996)'s protocol, which gave the highest response for the genotypes tested so far. Instead of using N6 as recommended by Aldemita and Hodges (1996), scutellum derived callus was induced and sub-cultured for 0, 3 and 7 days on NBA medium (Li te al., 1993) containing proline and NAA prior the co-cultivation (preculture) with the bacteria. The the callus was co-cultivated with *A.tumefaciens* strain LBA4404 (pTOK233) in NBA-AS medium containing 100µM acetosyringone for 3 days. Casamino acids and kinetin were omitted in the co-cultivation and selection media, and 20µl 100µM acetosyringone were added 2 hours prior the co-cultivation and onto the co-cultivated callus to reactivate further the *vir* genes. After co-cultivation, the agro-infected callus were washed with N6 salts (Chu et al., 1975) containing carbenicillin (250 mg/L), cefotaxime (100 mg/L), and hygromycin (50 mg/L) to kill the bacteria. The callus were then transferred onto the selection medium A [NBA containing carbenicillin (250 mg/L), cefotaxime (100 mg/L), and hygromycin (30 mg/L)] for three weeks. The healthy looking callus were sub-cultivated onto medium B [NBA containing carbenicillin (250 mg/L), cefotaxime (100 mg/L), and hygromycin (50 mg/L)] for other three weeks. Following the transgenic calli were first transferred onto a proliferation medium (LS with 0.5 mg/l 2,4-D and 50 mg/L hygromycin) for 3 weeks and then to a regeneration medium (MS with NAA 1 mg/l, kinetin 4 mg/l).

Results and Discussion

Preliminary observations indicated that embryogenic scutellum derived callus showed higher transient GUS expression than immature embryos (data not shown). The effect of the callus preculture time on GUS expression was evaluated. Embryogenic scutellum derived callus from Cica 8, CT10069, CT6241, INIAP 12, IR-72 and Latsihday-Fofifa were preculture for 0, 3 and 7 days on NBA medium prior co-cultivation, then co-cultivated with the bacteria for 3 days, and transient GUS expression was determined. Results indicated higher GUS transient expression on callus precultured for 0 or 3 days prior co-cultivation for *indica* rice (Cica 8 and INIAP 12), whereas no effect of preculture time was noted for *japonica* type (CT6241, CT10069, and Latsihday-Fofifa) (Table 1). Higher number of hygromycin resistant callus (Hyg^r) was recovered from Cica 8 (irrigated, *indica*) with three days of preculture, and from CT6241 (lowland-upland, *japonica*) and CT10069 (highland-upland, *japonica*) with 0 days of preculture after selection with 30 mg/l and 50 mg/l of hygromycin, respectively (Table 2). Between 60% and 100% of these Hyg^r callus showed stable GUS expresion (GUS^+) at 60 days (2 months) and 90 days (3 months) after the agro-infection (Table 2). Plant regeneration from these Hyg^r and GUS^+ callus is in progress. The studies conducted so far suggest that the selection of the explant source and preculture of the target tissue are majorkey factors for *Agrobacterium* mediated transformation of rice.

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Table 1.- Transient GUS expression on scutellum-derived callus after cocultivation with *A. tumefaciens* strain LBA4404 (*p*TOK233).

Rice Genotype	Pre-culture (days)	Callus Co-cultivated	Callus GUS +	(%)	Mean (Serror)
IR – 72	0	20	5	25	1.7 (0.7)
	0	39	0	0	
	0	34	1	2.9	
	3	33	4	12.1	ND
INIAP 12	0	6	5	83.3	48.8
	0	28	4	14.2	(24.4)
	3	28	22	78.5	
	3	7	1	14.2	35.6
	3	7	1	14.2	(17.5)
	7	18	1	5	
	7	6	0	0	
					2.5 (1.8)
CICA 8	0	18	17	94.4	71.3
	0	42	23	54.7	(13.4)
	0	25	9	36	
	0	32	32	100	
	3	67	62	92.5	
	3	20	20	100	78.9
	3	22	8	36.3	(10.0
	3	8	3	37.5	
	3	37	12/14	85.7	
	3	14	14	100	
	3	24	24	100	
	7	12	0	0	
	7	8	3	37.5	
	7	9	4	44.4	37.9
	7	34	7/10	70	(12.5)
CT6241	0	19	8	50	65.1
	0	40	11	27.5	(13.2)
	0	42	20	47.6	
	0	24	24	100	
	0	27	27	100	
	3	41	39	95.1	
	3	25	24	96	74.9 (9.1)
	3	12	9	75	
	3	18	10	55.5	
	3	22	8	36.3	
	3	12	11	91.6	
	7	7	3	42.8	
	7	18	10	55.5	63.3
	7	12	11	91.6	(11.9)
CT10069	0	30	18	60	67.1
	0	67	21	31.3	(12.7)
	0	36	16	44.4	
	0	27	27	100	
	0	24	24	100	
	3	41	39	95.1	
	3	26	25	96.1	55.3
	3	14	8	57.1	(16.4)
	3	12	1	8.3	
	3	25	2/10	20	
	7	14	12	85.7	
					ND
Latsihday-Fofifa	3	10	2	20.0	23.8 (3.9)
	3	11	2	18.1	
	3	15	5	33.3	
	7	10	7	70.0	ND)

Table 2.- Production of Hygromycin-resistant (HygR) calli derived from scutellum inoculated with *A. tumefaciens* strain LBA4404 (*p*TOK233).

Rice genotype	Pre-culture (days)	Callus Analyzed	Selective medium							GUS expression			
			A			Callus Analyzed	B			Callus GUS$^+$/ evaluated			
			Hyg^r callus		%Hyg^r callus		Hyg^r callus	%Hyg^r callus	%Hyg^r in A and B	60 days	(%) GUS^+	90 days	(%) GUS^+
CICA 8	3	23	15		65	15	10	67	43	12/12	100	8/12	67
	7	24	8		33	8	6	75	25	0/15	0	0/12	0
CT6241	0	18	18		100	18	16	89	89	ND	ND	17/17	100
	3	25	15		60	15	9	60	36	ND	ND	ND	ND
	7	20	10		50	10	6	60	30	ND	ND	ND	ND
CT10069	0	24	24		100	24	22	92	92	ND	ND	14/14	100
	3	13	9		69	9	4	44	31	ND	ND	ND	ND
	7	8	3		37	3	1	33	12	ND	ND	ND	ND

2.1.12. Advances in *Brachiaria* genetic transformation mediated by *Agrobacterium tumefaciens.*

Brachiaria species are important components of the pastures grown in the tropical lowlands of America, Asia, Africa, and Australia. *B. decumbens* cv *Basilisk* is one of the most extensively cultivated species, which has adaptation to acid soils, rapid growth, good soil coverage, and high nutrional value as a feed pasture. This species however, is highly susceptible to spittle bug (homoptera). Resistance to this pest is present in *B. brizantha* which does not outcross with *B. decumbens. B. ruziziensis* is used as a bridge between *decumbens* and *brizantha* species, thus recurrent backcross is needed to recover the agronomic characteristics from *decumbens*. Plant genetic transformation offers an expedite alternative to transfer genes between unrelated species. A protocol for genetic transformation of *Brachiaria* will be particularly useful to introduce resistance gene(s) for this homoptera pest, and to improve further the quality traits associated with the nutritional value of the pasture.

Earlier work at CIAT's included the establishment of tissue culture methods for plant regeneration (Lenis, 1993), and genetic transformation by direct methods using particle bombardment (Galindo, 1997) of *Brachiaria* species. *Agrobacterium tumefaciens* is a high efficient vector for the transfer of allien genes into dicotyledonous. Recent advances in the development of protocols for monocotyledoneous species is allowing to use this technology for transforming various cereal species such as rice (Hiei et al., 1994; Rashid et al., 1996; Dong et al., 1996; Toki, 1997), maize (Ishida et al., 1996; Escudero et al., 1996), and barley (Tingay et al., 1997). Genetic transformation mediated by *Agrobacterium* seems to have certain advantages respect to direct methods, which include a higher transformation effficiency, the transfer of large segments of DNA with minimal rearrangement, and the integration of fewer numbers of copies of the transgene(s) into the plant genome.

Materials and Methods

Improvement and adaptation of callus culture and plant regeneration for genetic transformation of *Brachiaria*

The present work is in its initial phase and is aimed to establish a transformation protocol mediated by *A. tumesfaciens* of *B. decumbens*. In this research, matured embryos and embryogenic scutellum derived callus of *B. decumbens* cv Basilisk accession 606 is used as target explants. The bacteria strain LBA 4404 carrying the hypervirulent plasmid *p*TOK 233 (50.35 Kb, kindly provided by Dr. Toshihiko Komari, Japan Tobacco Inc., Japan) or other constructs provided by CAMBIA (Dr. Richard Jefferson, Australia) will be tested. The *p*TOK 233 plasmid is a cointegrated system with three chimeric genes for expression in plants, including hygromycin resistance (*hph)*, *gus-intron* (*uid-intron*) and neomycin resistance (*npt II)* genes driven by the 35S promoter. This plasmid has been used successfully in *Agrobacterium* mediated transformation of various monocots species. As a first step, the transformation protocols already established for rice, maize, barley and other grasses will be tested and modify accordinghly to optimize agroinfection for *B. decumbens*. Following is reported the preliminary work in progress..

Results and Discussion

The success of plant genetic transformation highly depends on the type and physiological status of the explant used. Work suggest that in monocots, the target explant should be in an active cellular division stage to be amenable for allien gene introduction. Embryogenic callus derived from the scutellum has shown to be the most appropriate explant for transformation of cereals.

The protocols for callus induction and plant regeneration of *Brachiaria* developed at CIAT (Lenis, 1992, and Galindo, 1997) include a very short callus phase followed by plant differentiation as soon as the embryogenic callus is being formed. This rapid plant formation difficults using this tissue as target for agroinfection. At present, different basal medium compositions are being tested to define the most appropriate medium sequence for callus induction, maintenance of embryogenic callus, and plant regeneration. Preliminary results suggest that matured embryos cultured on a modified N6 medium develop embryogenic callus that can be maintained in the callus phase by subculture on the same medium. The embryogenic structures differentiate into plants when transferred onto a modified MS medium. Different medium sequences are being tested to define the best protocol to maintain the target tissue in the optimal stage for agroinfection, to select the putative transgenic tissues, and regenerate the transgenic plants.

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2.1.13 Incorporation of resistance to fruitworm (*Neoleucinodes elegantalis*) and budworm (*Scrobipalpuloides absoluta*) in the tomato variety UNAPAL Arreboles by genetic transformation

Tomato (*Lycopersicon esculentum* Mill) is one of the most important crops in the fresh vegetable market as well as in the food processing industry (Rick and Yoder, 1988). Tomato is the major consumed vegetable crop in Colombia, with a planted area of 15.000 hectares yielding 450.000 tons per year (UNAL, 1997). In Colombia, this crop is highly affected by several pests and diseases, and abiotic stresses such as drought, high and low temperatures, and salinity. Since 1985, the vegetable breeding program at the Universidad Nacional de Colombia, Palmira

Campus, has as main objective the development of varieties with resistance or tolerance to some of these traits. In 1997, this program released the tomato variety UNAPAL Arreboles, which has several traits atractive to tomato growers such as fruit firmness and good adaptability specially to the Valle del Cauca region. But this variety is susceptible to the two major limitations to tomato in this region: the fruitworm (*Neoleucinodes elegantalis*) which damage the fruit even at early stages of development, and the budworm (*Scrobipalpuloides absoluta*), which eats the tomato buds and young leaves. It had been dificult to breed tomato resistant to these two pests by standard breeding. The only sources of resistance genes is from wild tomato species which are incompatible with the cultivated tomato, and so far the attempts for an interspecific breeding program has not been successful (Lourencao et al., 1985).

An attractive alternative to introduce resistance in tomato against these two pests is by genetic transformation. Nowadays, it is well known the progress attained in the incorporation by genetic transformation of crystal proteins genes from *Bacillus thuringiensis* (Bt) for resistance to Lepidoptera pests. At present, other novel insecticidal proteins genes for insect resistance, such as vegetative insecticidal proteins (ViPs), proteinase inhibitors, chitinases, peroxidases, cholesterol oxidases, among others have been reported. The main objective of this work is to transform the tomato variety Arreboles with the Bt gene cryIA(b), which had been used susscessfully to obtain resistance against Lepidoptera pests in various economical important crops (i.e. maize, cotton).

A prerequisite for transfering genes into plants is the availability of efficient transformation and plant regeneration systems. Although the transfer of genes into tomato using *Agrobacterium* mediated transformation has been reported (McCormick et al., 1986; Fillatti et al., 1987; Ultzen et al.,1995 etc.), the efficiency of plant regeneration and transformation is genotype dependent. Following is reported the results from the first phase of this project on the adoption and adaptation of an efficient plant regeneration system of tomato Arreboles.

Material and Methods

Comparison of three media sequence for callus induction and plant regeneration from the tomato variety Arreboles.

Three protocols commonly used for tomato were compared. These protocols included the media M1 (Narvaez, 1993), M2 (Fillatti et al., 1987), and M3 (Ultzen et al ., 1995). The components of M1, M2 and M3 callus induction media are shown in Table 1. M1 is the most simple while M3 is most complex of the three media. M3 consists of MS salts, B5 vitamins, with the addition of 0.05 mg/l biotin, 0.5 mg/l folic acid and 2 mg/l glycine. Sucrose is reduced from 30g/l to 10g/l and 10g/l of glucose is added. The response following the three protocol medium sequence were compared by evaluating callus induction and plant regeneration using the following criteria: No. of callus per explant, No. of callus with shoots, No. of shoots per explant, and No. of plantlets per explant. Three replications with a total of 108 explants were evaluated per each medium. Data was analyzed using a randomized complete block design (SAS,1988).

Results and Discussion

Cotyledonary leaves from 7-10 day-old seedlings were used as starting materials. Callus formation from the cotiledonary leaf explants was noted two weeks after culture on the callus induction medium. One month later, callus were transferred onto the corresponding plant regeneration medium. Duncan's Multiple Range Test for each of the parameters analyzed indicated that the highest response for callus induction and plant regeneration is noted on M3 medium sequence (Table 2). An increase in response of about 2-fold and 4-fold on callus induction and plant regeneration was noted on M3 media respect to M1 and M3 medium, respectively. The lowest response was obtained on M2 medium. M2 contains 3 fold as much sucrose as M3, and does not contain glucose nor an auxin source. A total of 238 plantlets were regenerated from 278 initial explants on medium M3 giving a regeneration efficiency of 86%, whereas the plant regeneration efficiency for M1 and M2 were of 73% and 34%, respectively. Based on these results M3 medium has been selected to conduct the genetic transformation studies with the variety Arreboles. The review of key factors affecting the efficiency of *Agrobacterium* mediated genetic transformation of tomato Arreboles is in progress.

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Table 1. Medium composition for tomato callus induction

	Medium		
	M1	M2	M3
MS salts	4.5 g / l	4.5 g / l	4.5 g / l
Myo-Inositol	100 mg / l	100 mg / l	100 mg / l
Thiamine-HCl	10 mg / l	0.5 mg / l	0.5 mg / l
Nicotinic acid	1 mg / l	5 mg / l	5 mg / l
Pyridoxine	1 mg / l	0.5 mg / l	0.5 mg / l
Biotin	-	0.05 mg / l	0.05 mg / l
Folic acid	-	0.5 mg / l	0.5 mg / l
Glycine	-	2 mg / l	2 mg / l
Sucrose	30 g / l	30 g / l	10 g / l
Glucose	-	-	10 g / l
BAP	2.5 mg / l	-	-
IAA	1 mg / l	-	0.02 mg / l
Kinetin	-	4 mg / l	4 mg / l
PH	5.8	6.0	6.0
Phytagel	1.8 g /l	1.8 g /l	1.8 g /l

Table 2. *In vitro* response of tomato Arreboles on medium M1, M2, and M3 commonly used for tomato callus induction and plant regeneration.

	M1			M2			M3		
	Means	Total per 108 explants	% [5]	Means	Total per 108 explants	%	Means	Total per 108 explants	%
Callus per explant	1.90 b	206	32.1[1]	1.45 c	157	24.5	2.57 a	278	43.4
Callus w/ shoots/Expl	1.12 b	121	29.4[2]	0.62 c	67	16.3	2.07 a	223	54.3
Shoots / Explant	1.53 b	165	27.3[3]	0.78 c	84	13.9	3.30 a	356	58.8
Plantlets / Explant	1.40 b	151	34.1[4]	0.50 c	54	12.2	2.20 a	238	53.7

Means followed by the same letter are not significantly different at p: 0.01.

1/ Fraction from the total number of callus
2/ Fraction from the total number of callus with shoots
3/ Fraction from the total number of shoots
4/ Fraction from the total number of plantlets
5/ Response percentage relative to the other media

Table 3. Comparison of the three plant culture media on the base of the evaluation of four parameters

	Callus[1] Per Expl	Total Callus No.	Relative Response To others Media(%)	Callus With shoots	Total Callus w/shoots No.	Relative Response To others Media(%)	Shoots Per Explant	Total shoots No.	Relative Response To others Media(%)	Plantlets Per Explant	Total Plantlets No.	Relative Response To others Media(%)
M1	1.90 b	206	32.1	1.12 b	121	29.4	1.53 b	165	27.3	1.40 b	151	34.1
M2	1.45 c	157	24.5	0.62 c	67	16.3	0.78 c	84	13.9	0.50 c	54	12.2
M3	2.57 a*	278	43.4	2.07 a	223	54.3	3.30 a	356	58.8	2.20 a	238	53.7

- Means with the same letter are not significantly different

1/ Mean from total of 108 explants

2.1.14 development and standardization of an *in vitro* clonal propagation method of Soursop (*Annona muricata* l.)

Introduction

Annona muricata L. (soursop) is a tropical fruit tree originated from Colombia and/or Brazil (J. León, 1968). Its fruits have interesting properties for agroindustry (Toro, 1995) and as a source of antitumoral and insecticide metabolites (Cavé et al., 1993).
Soursop production in Colombia is very inefficient due to the use of sexual seed for propagation. Genome heterogeneity of soursop is very high and the development of seed-originated trees is therefore heterogeneous.

Asexual propagation methods like grafting are also used but they contribute to the dissemination of diseases like the anthracnose produced by *Colletotrichum gloesporioides*. Clonal propagation methods that use pathogen-free, elite clones will improve the production of soursop in Colombia.

Materials and Methods

We developed a micrografting method for micropropagation using elongated axillary shoots of "Elita" clon (Ríos Castaño et al., 1996) as scions and *in vitro* germinated "Elita" plantlets as rootstocks. The explants were surface sterilized in 1% NaOCl (v/v) and washed with sterile distilled water. Axillary shoots were elongated aseptically in Woody Plant Medium (WPM – Lloyd and McCown, 1980) containing low concentrations of benzylaminopurine (BAP). Seeds were germinated aseptically in B5 (Gamborg, 1968) medium containing low concentrations of gibberellic acid (GA_3).

Axyllary shoots and apexes were grafted onto hypocotyls of 10-15 days-old, *in vitro* germinated plantlets. Micrografted plants were grown in half strength WPM incubated at 29 °C (under white fluorescent tubes, 3600 lux, with a 12-h photoperiod). After 30 days, plantlets were transferred to the greenhouse under semi-shade conditions.

Results and Discussion.

Table 1 summarizes the steps required to obtain micrografted, pathogen-free soursop plants.

Table 1. Steps required to obtain micrografted, pathogen-free soursop plants

Steps	Time (days)	Average success
Mother plants* selection and preparation in the screen-house (trimming, fungicide and antibiotics sprying, hormone treatment); selection of best stems for *in vitro* planting.	**60**	8 stems / 2 year-old mother plant
Stem planting *in vitro* and elongation of axillary buds. @	**30**	47% of stems produce optimum scions
Rootstock production from sexual seed (seed sterilization, *in vitro* germination and growth) @	**30-45**	81% of seeds produce optimum rootstocks
In vitro micrografting.	**35-40**	61% successfully graft
Hardening in greenhouse conditions.	**180**	66% of successful micrograftings continue developing in the greenhouse
	Total: 355	

(*) A mother plant is a nursery-grown, vegetatively grafted soursop plant that serves as source of stems.
(@) These two steps are performed simultaneously to synchronize the process.

To reduce *in vitro* contamination below 10%, it was necessary to treat mother plants with fungicides and antibiotics in the screen-house.

To get an average of 81% *in vitro* germination of seeds it was necessary to 1) dry the seeds after sterilization using an oven (15 days at 37°C) or air-drying (21 days); 2) scarify completely the seeds; and 3) supplement the germination medium (B5) with GA_3 (0.5 mg/l).

"V shaped" grafting, which has been first used for bean *in vitro* grafting (A. Mejía, 1995), seemed to be an efficient method to guarantee grafting survival.

From each stem planted *in vitro* we could obtain up to 12 scions for *in vitro* grafting, which increases the multiplication rate and shortens the whole process to 10 months (one month shorter than the time required for standard commercial production of vegetatively-grafted plants).

After 35-40 days micrografts were transferred to the greenhouse to a non-sterile soil mixture containing soil/sand/ rice husk/perlita.(1:2:1:1). Survival index (66%) in the greenhouse was higher than that (35%) reported by Bejoy and Hariharan (1992) for micropropagated, non-micrografted *A. muricata* plantlets.

Currently we have 200 micrografted, two months old plants growing in the greenhouse that will be transferred to the field to begin agronomic evaluations.

The entire *in vitro* micropropagation process is being improved towards its transfer to soursop growers.

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Collaborators: N. Royero[1], L. Muñoz, A. Mejía, J. Cabra[1], W. Roca.
[1] Corporación BIOTEC, UNIVALLE, Cali

ACTIVITY 2.2 Identification of points for genetic intervention in plants/stress Interactions

SUMMARY OF ACHIEVEMENTS

- Al-tolerance of all *Brachiaria* spp is superior to that reported for Al-tolerant crop vars. *B. decumbes* is best adapted to low N- supply and has higher N-uptake efficiency. *Brachiaria* thus becomes an important source of genes for these traits.

- Two new secondary metabolities, biosynthesized via the shikimate pathway, have been isolated and chemically characterized from *Brachiaria decumbens* roots grown under N and P stress conditions. Inhibitory effects by the two compounds against fungal pathogens will be evaluated.
- CIAT work in exploring the potential of cassava germplasm for vitamins and anti-oxidants sources has been extended to the Colombian tropical oil palm 'mil pesos' as a collaborative extension with Univ. del Choco. Antioxidants Carotene and Tocopherol content are similar or higher than sunflower, but lower than soybean.

2.2.1 Exploring the Genetic Potential and Stability of Vitamin Content in Cassava

Introduction

Improving cassava's nutritional value can improve living conditions of poor farmers and urban consumers who use the crop as the main source of nutritional energy. Few have studied the concentration and variability of vitamins and minerals in cassava root parenchyma. This work intends to determine the potential improvement that can be reached through selection and recombination. The nutritional value also depends on the availability of nutrients to the human body once the product is processed and consumed.

The objectives of this work are to:

a) Characterize the genetic diversity within CIAT cassava core collection, (630 genotypes) selected to represent the genetic diversity in the global cassava germplasm collection (approximately 5,500 genotypes) with respect to ascorbic acid (vitamin C) and carotenes (vitamin A) content.
b) Screen elite genotypes for physiological post-harvest deterioration and stability of the vitamins after processing.
c) Evaluate the potential of cassava leaves as a source of vitamins for human nutrition.

Materials and Methods

Carotenes. The extraction procedure outlined by Safo-Katanga et al. (1984) was adjusted by extracting root parenchyma with petroleum ether. The extraction protocol for leaves had to be modified due to the presence of tanins and chlorophylls. A sample of 10 g was taken out of the root or leaves, at random 10 to 11 months after planting. The quantification was done by ultraviolet spectrophotometry using a Shimadzu UV-VIS 160A recording spectrophotometer. UV detection was done at λ = 455nm for root extracts and λ = 490 nm for leave extracts.

Ascorbic acid. The protocol for the determination of ascorbic acid by Fung and Luk (1985) was adjusted for cassava leaf and roots taking as base the procedure outlined and involved the following steps:

a) Homogenization of 1 g of fresh leaves or 6 g of fresh roots in a turrax with 20 ml of extraction buffer (3% phosphoric acid and 8% glacial acetic acid).
b) Centrifugation for 5 min at 10°C and 3000 rpm.
c) Separation of supernatant and vortex of 1 ml of the extract with 2 ml of 10% hydrochloric acid. Reading was taken immediately with an UV-VIS spectrophotometer. UV detection was done at λ =245 nm. Quantification was done using a previously decomposed extract with 1M sodium hydroxide solution as blank. During the whole process, samples were protected from air in order to avoid oxidation.

Results

Ascorbic acid concentration in leaf tissue ranged from 1.68 mg/100 g FW to 419.25 mg/100 g FW. Concentration of ascorbic acid in leaves is higher than in the roots. Average ascorbic acid concentration in the leaves was ten times higher than the one observed in the roots. The data about ascorbic acid concentration in root parenchyma showed a broad distribution of concentrations from less than 1.0 mg/100 g FW to 39.52 mg/100 g FW of fresh roots. Vitamin content in the leaves was not correlated to those in the roots.

Cassava is processed before comsumption using heat treatments which can affect the ascorbic acid and carotene content. At the moment we are measuring the effect of following treatments on the ascorbic acid content of 40 elite clones (genotypes with the highest concentration of ascorbic acid): solar drying of cassava flour, oven drying of cassava flour and cooking fresh roots 30 minutes. Results from this study are pending. Boiling reduced ascorbic acid concentration at least in 60-70% from fresh root content. In addition we are evaluating post-harvest root deterioration of selected genotypes in order to correlate this results with the carotene and ascorbic acid content.

Another target for 1998 is to complete the evaluation of the carotene content in the cassava core collection. So far screening of the leaves of the 600 accessions has revealed a wide range of variation in carotene content, 23.28 mg/100 g FW to 172.45 mg/100 g FW. This implies that 6 g of fresh leaves (2 g of dry leaf flour) of genotypes with the highest concentration of carotenes in the leaves will supply the daily requirement of vitamin A for an adult male (between 600-700 ug vitamin A/day).

Once screening of the roots is completed, we hope to be able to identify germplasm with particularly high or low carotene concentration.

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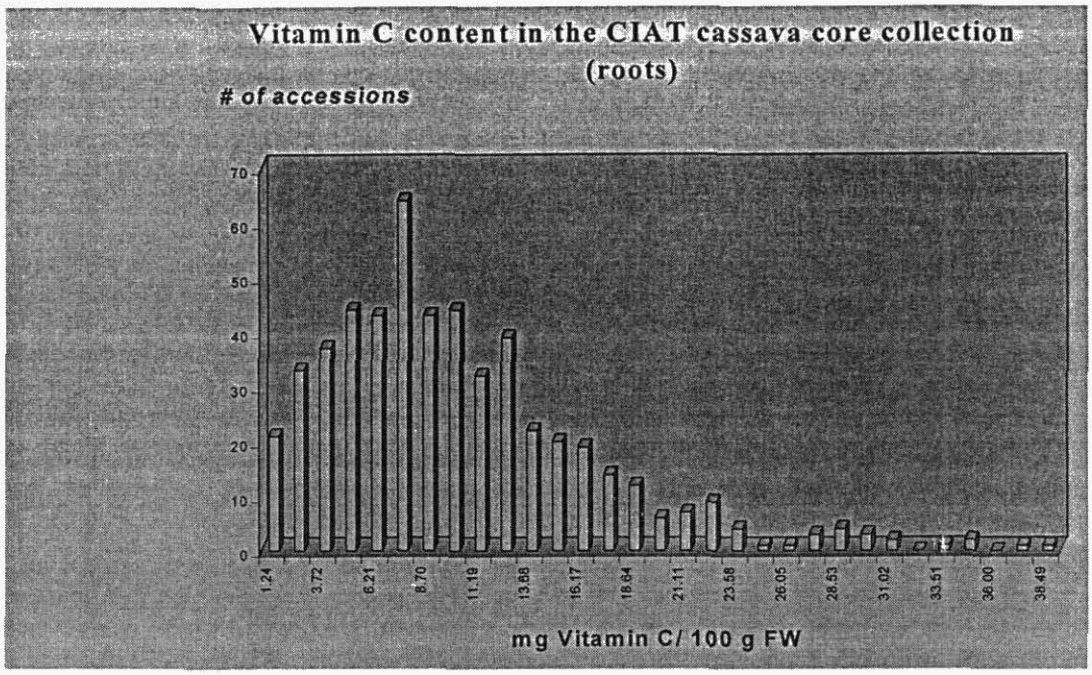
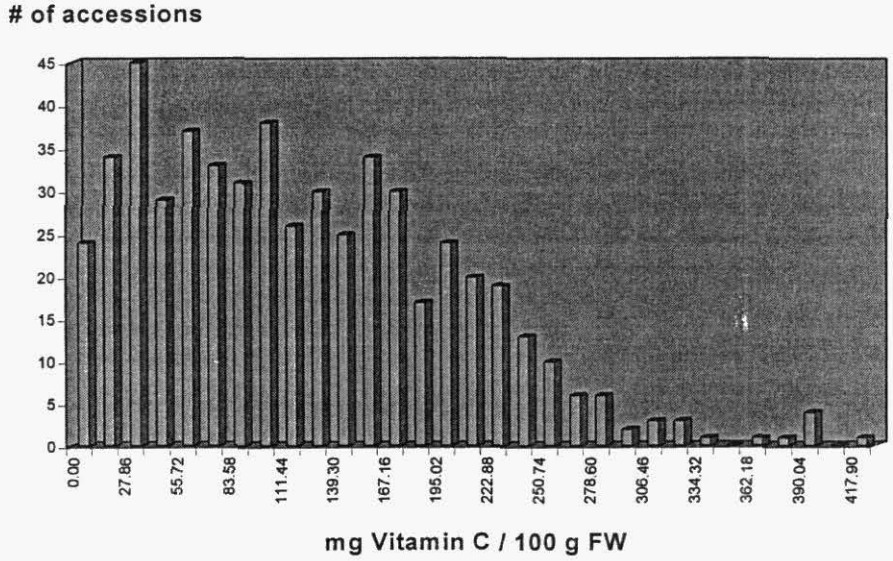
Collaborators: C.Iglesias, W. Roca, A.L. Chavez, J.M. Bedoya, F. Calle, T. Sanchez



Vitamin C content in the CIAT cassava core collection (leaves)



2.2.2 Mechanisms of Acid Soil Adaptation in *Brachiaria* Cultivars*

Background

Brachiaria cultivars are the most widely sown forage grasses used for livestock production in tropical savannas. An ongoing breeding program at CIAT seeks to combine favorable traits, such as adaptation to acid soils, resistance to spittlebug and forage quality, within new apomictic cultivars. Easy and quick methods are thus required to screen large progenies for acid soil adaptation. To develop physiologically based screening methods, we focused on the three major factors that probably contribute to the poor persistence of less adapted *Brachiaria* cultivars on low fertility acid soils: Al-toxicity, P-deficiency and N-deficiency.

Interspecific differences in adaptation to acid soil stress

Al-toxicity. Al toxicity has been long been identified as the major growth-limiting factor in acid soils. The elongation of the primary root of seedlings in solutions containing only Al^{3+}, Ca^{2+}, and Cl^- ions was measured after 3 days to evaluate whether interspecific differences in Al-tolerance exist among *Brachiaria* species. The results demonstrated that (i) Al-tolerance of all *Brachiaria* species is superior to that reported for Al-tolerant crop varieties, and that (ii) *B. ruziziensis* was clearly less Al-tolerant than *B. decumbens* and *B. brizantha* (Fig. 1; left half). At present, HPLC analyses of organic acids in roots and root exudates are underway. Preliminary results suggest that citric acid as well as other organic acids within roots might contribute to the high level of Al-tolerance in *Brachiaria* species. However, there seem to be no significant interspecific differences with respect to organic acid accumulation/exudation. This implies that B. *decumbens* must have additional Al-tolerance mechanisms, which are probably not related to chelation of Al^{3+} ions by organic acids.

Adaptation to P-deficiency. P in acid soils is extremely immobile because of chemical fixation by Al^{3+} and/or Fe^{3+} ions. The root systems of acid soil-adapted plants must thus explore large soil volumes to take up sufficient P. This can be accomplished by extensive root systems. Thin roots can reduce the amount of biomass needed to construct a root system of a given length. An experiment was set up to investigate aspects of root system morphology, such as root thickness, in relation to P-deficiency. Plants were grown in hydroponic culture with declining P-supply in the greenhouse. Upon harvest, root systems were stained and scanned with a flatbed scanner. The resulting images were then analyzed with WinRHIZO software.

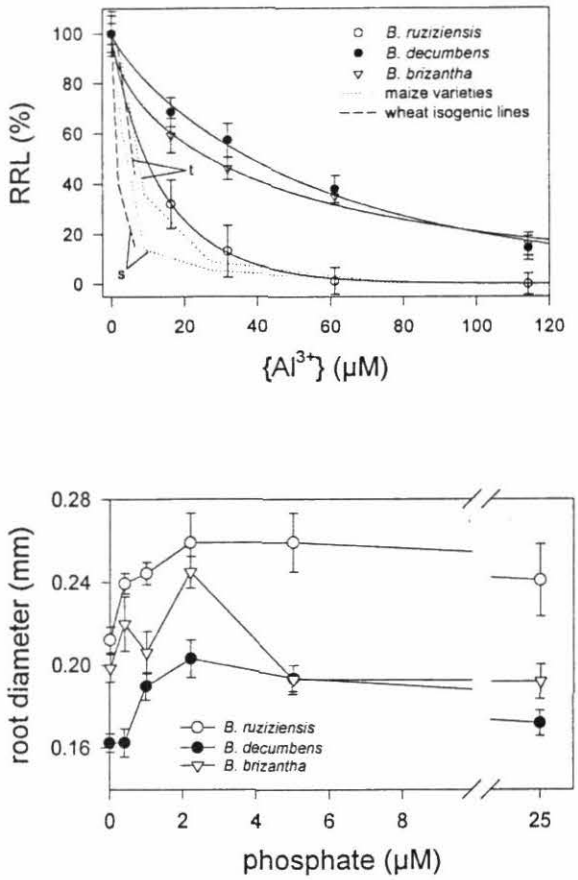


Figure 1. Differential adaptation to Al-toxicity and P-deficiency. *Left half:* relative root length (RRL) of three *Brachiaria* species as compared to maize and wheat varieties of contrasting Al-tolerance. Error bars denote SE (n = 28-36). Al-sensitive (s) and tolerant (t) maize varieties were Tuxpeño and South American 3. Al-sensitive (s) and tolerant (t) wheat isogenic lines were ES3 and ET3. Data of maize varieties and wheat isogenic lines were taken from the literature. *Right half:* mean root diameter of three *Brachiaria* species grown in nutrient solutions under decreasing P-supply. Error bars denote SE ($n = 8$).

The results demonstrated that *B.decumbens* has the finest root system, independent of the level of P in the nutrient solution, and that *B. ruziziensis* produced the thickest roots (Fig. 1; right half). Based on geometric considerations it can be estimated that *B. ruziziensis* needs 50 - 55 % more biomass than *B. decumbens* to construct a root system of a given length. Under P-deficient conditions this is a significant advantage, given the importance of active foraging for soil P-reserves.

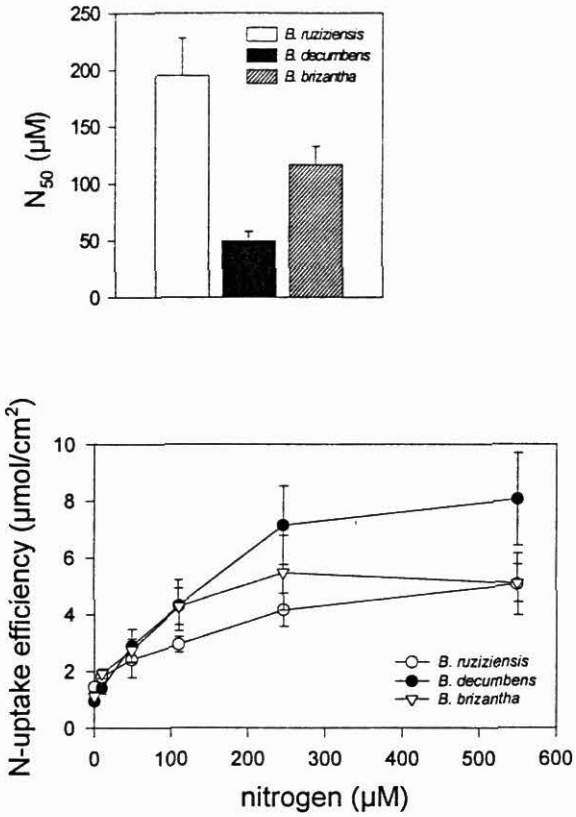


Figure 2. Differential adaptation to N-deficiency. *Left half:* comparison of the level of nitrogen at which 50 % of maximum growth was achieved (N_{50}). Values were calculated from plot of the concentration of N in the nutrient solution versus dry matter production, using non-linear curve-fitting (Marquardt-Levenberg algorithm) and a Michaelis-Menten-like function. Error bars indicate SD. Right half: N-uptake efficiency as calculated as the amount of nitrogen taken up per root surface area. Error bars denote SE (n = 8)

Adaptation to N-deficiency. Declining soil N-reserves might cause degradation of pastures over time. An experiment was conducted to evaluate whether interspecific differences in tolerance to N-deficiency exist. Plants were grown in nutrient solutions in the greenhouse under declining N-supply. Upon harvest, root systems were stained, scanned and analyzed in a similar manner as above. Significant variability among species was found for total dry matter production in response to N-supply (Fig. 2; left half). *B. decumbens* was the species which was best adapted to low N-supply. It was further shown that *B. decumbens* had a superior N-uptake efficiency, that is, it can take up the largest amount of N per root surface area (Fig. 2; right half).

Biochemical responses to acid soil stress

Aromatic metabolites. Secondary aromatic metabolites frequently accumulate under nutrient stress when root growth is stimulated at the expense of shoot growth. In some cases they have been demonstrated to be involved in fungal protection and signal exchange with soil microorganisms such as VAM and N_2-fixing bacteria. Using reverse-phase HPLC, two dominant aromatic metabolites were detected in roots of *Brachiaria* species. Their level increased under P- and N-deficient conditions. A purification protocol

was developed and their structure was elucidated by means of structure ^{1}H-NMR, ^{13}C-NMR, COSY, CD and positive-ion FAB mass spectroscopy.

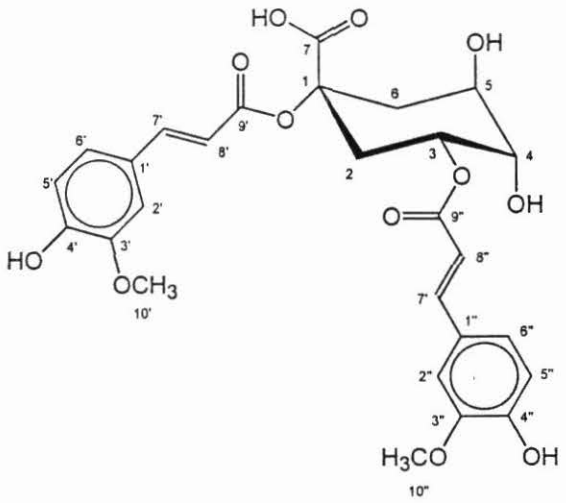


Figure 3. Structure of DFQA. FCQA lacks an -OCH_3 group of one of the two feruloyl moieties.

The compounds were shown to be L-1,3-di-*O-trans*-feruloylquinic acid (DFQA) and L-1-*O-trans-p*-coumaroyl-3-*O-trans*-feruloylquinic acid or L-1-*O-trans*-feruloyl-3-*O-trans-p*-coumaroylquinic acid (FCQA) (Fig. 3). They are new hydroxycinnamic acid conjugates of quinic acid. Both the hydroxycinnamoyl and the quinic acid portion are synthesized via the shikimate pathway. Shikimate kinase, an enzyme in the middle of the shikimate pathway, is subject to control by energy charge. Under energetically unfavorable conditions, a larger portion of shikimate molecules are diverted from the main trunk into secondary products such as quinic acid and its derivatives. This could explain their accumulation in roots of *Brachiaria* species under P- and N-deficiency.

Transgenic tobacco plants with suppressed levels of phenylpropanoid products showed increased disease susceptibility. This indicates that 5-*O*-caffeoylquinic acid (chlorogenic acid) might be a chemical barrier against microbial attack, since it is the major soluble phenylpropanoid in tobacco. In a similar way, DFQA and FCQA might accumulate in roots of *Brachiaria* species as preformed protectants against attacks of soil-born fungi and by this increase root lifespan. An longer root lifespan is expected to increase the amount of nutrients taken up per unit of biomass invested in roots. Under nutrient deficient conditions, when biomass is costly to construct, this might improve the plant's overall performance. Experiments are underway to evaluate whether these compounds inhibit growth of the pathogenic fungus *Rhizoctonia solani* or stimulate growth of VAM fungi *in vitro.*

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1. National Accelerator Centre (NAR), South Africa; 2. Michigan State University, USA; 3.CAMBIA, Canberra, Australia.

2.2.3 Validation of analitycal methods to quantify Vitamins A and E in *Jessenia bataua* Palm Oil

Introduction

Colombia has a wide variety of ecosystems. The tropical humid forest is one of them; it is characterized by a wide diversity of flora in wich palm trees occupies a very important place. In the Colombian Pacific region, considered as a tropical forest, the inhabitants obtain a diversity of products derived from the palm tress. In the present study we have selected the "Mil Pesos" palm tree *(Jessenia bataua)* because of its socioeconomical, cultural and enviromental importance. One of the products derived from *J. bataua* is an edible oil , extracted from the palm fruit and comparable to olive oil because of it's aroma, flavor and chemical composition. In spite of this, we analized the nutritional qualities based on the fact that the oil of *J.bataua* stored at room temperature conserves it's organoleptic quality for up to three years (Balick 1992).

It has been proved that the presence of natural antioxidants such as carotenes and tocopherols give stability to the oils (Nargiz 1991). These substances have elicited great interest in present days because of the possible role they play in the prevention of root damage and many degenerative process. They are also considered micronutrients as well as vitamins that act as buffers and reductors, and with this characteristic, they may be used for industrial production .

In this study we assess the methods reported in the literature for quantification of carotene and tocopherols of vegetable oils and we validate the two best analitical methods using UV-VIS spectrophotometry technique for carotenes determination and HPLC (High Performance Liquid Chromatography) for tocopherols. We applied these methods for quantification of carotenes and tocopherols for various samples of oil *J. bataua* and others vegetable oils.

Methods

Carotenes. Samples of oil, obtained from *J.bataua* palm fruits were extracted with a mixture of petroleum ether, acetone and water (1:1:1) at room temperature . This extract was saponified with methanol at a 1:4 ratio of oil to alcohol, catalysed by 10% potassium hydroxide. The reaction mixture was stirred (12 hours) until all the triglycerides were converted to alkyl esters. The concentration of carotenes was determined by UV-VIS spectrophotometry, using a shimadzu UV-VIS 160A recording spectrophotometer. UV detection was done at $\lambda = 455$ nm using oil previously decolored as blannk(2).

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Tocopherols. Aliquots of 1 g palm oil were dissolve in hexane. This mixture was homogenized by centrifugation and the tocopherols were analyzed by triplicates using normal-phase HPLC (AOCS 1992) using a Hewlett Packard 1050 chromatograph, column Lichrosorb SI 60, 10 μM (Merck), hexane:dioxane (94:6) as mobil phase, flow rate : 1.2 ml/min and fluorometric detection (λ ex = 295 nm and λ em = 330 nm).

Results.

Ten methods reported by literature to determine carotenes and five to characterize tocopherols were evaluated. The saponification method at room temperature permited a good extraction and characterization of carotenes. The quantity of the detected carotenes and tocopherols depends on factors as harvest, site, and extraction methods, and therefore may vary within samples.

Complete conversion of the triglycerides into alkyl esters took place without destroying the carotenoids during the saponification reaction. This was monitored by the internal standard method. There were not significant differences between concentrations of tocopherols and carotenes of *J. bataua* oil and the others comercial edible oils. The data were evaluated as mean ± standard deviation of triplicate determinations. Statistical significance within sets of data was determined by one-way analysis of variance. The significance level was in all cases $P < 0.05$. This study showed the carotenes vary in the following ranges: *J. bataua* oil (0.601 ± 0.02 mg carotenes/100 g oil.), sunflower oil (0.184 ± 0.08 mg carotenes/100 g oil) and soya oil (1.036 ± 0.08 mg carotenes/100 g oil). The average values of α-tocopherol content were: *J. bataua* oil (16.573 ± 0.8 mg/100 g oil), sunflower oil (16.777 ± 0.1 mg/100 g oil) and soya oil (38.969 ± 0.9 mg/100 g oil).

Palm oil obtained from the fruits of *J. bataua* showed to be as resistant to oxidation as the other edible oils because their unsaponificable componentes including tocopherols and carotenes. Since crude *J. bataua* oil is not refined, the natural oxidants are partly preserved and these compounds are reported responsible for their higher stability to oxidation.

The storage stability of β-carotene and α-tocopherol in the *J. bataua* oil were observed for a period of 5 months. The carotene were more stable at 10 °C than at 28-30 °C. There was a slight decrease (1.76%) in the carotene content of the *J. bataua* oil when stored in dark glass. However the bigger decrease (41.09%) in the carotene concentration of the oil when it was stored in clear glass could be due to greater exposure to light, leading to an increased oxidation and degradation of the carotenes.

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NARGIZ, C. and K. Unal. (1991). J. Sci. Food Agric. 56:79, pp. 208-212.

Collaborators: A.Rios[1], W.Roca, F.Villota, A.L.Chavez
1.Universidad del Chocó, 2.Universidad del Valle

Figura 1. UV Spectra of: (1) β-carotene standard (50ppm). (2) *J. bataua* saponified oil.

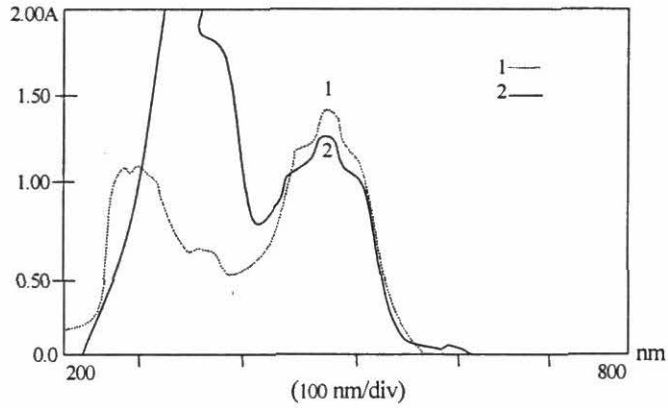
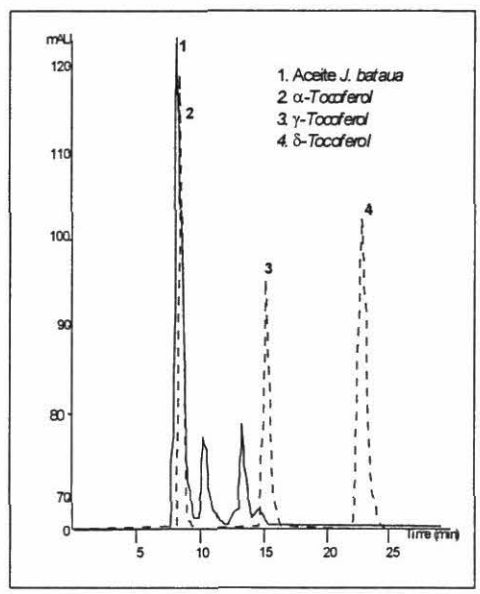


Figura 2. Comparison between the chromatograms of a mix of tocopherols and *J. bataua* oil.

Column: Lichrosorb SI 60 (250 X 4 mm),Flow: 1.2 ml/min, mobil phase: Hexane:Dioxane (94:6),detection: Fluorescente ex. 295 nm, em. 330 nm.



OUTPUT 3. COLLABORATION WITH PUBLIC AND PRIVATE SECTOR PARTNERS ENHANCED

ACTIVITY 3.1 Organization of Networks, Conferences/Workshops and Training Courses.

SUMMARY OF ACHIEVEMENTS

- DGIS accepted to fund a second phase of the Cassava Biotechnology Network (CBN) to facilitate the stakeholder-participatory process and develop a regional structure for LAC. CBN has also been involved in the development of projects for a low-cost, artesanal, cassava tissue culture scheme, and for restoring national cassava collections in Ecuador.
- In the period Oct. 1997- Sept 1998, Project SB-02 has organized/carried out 6 Workshops and Training Courses in Biotechnology and Agrobiodiversity, including tissue culture of fruit tress, genetic transformation, biodiversity, QTL analysis and IPR.

3.1.1 The Cassava Biotechnology Network

The Cassava Biotechnology Network (CBN) is a global voluntary research network, founded at CIAT in 1988. Members include CG centers, advanced laboratories, national programs, and farmers organizations in 35 countries.

CBN's goal is to mobilize the contribution of biotechnology tools to enhancing cassava's value for:

- food security;
- poverty alleviation;
- economic development via farm income and rural jobs; and
- natural resource management (particularly through providing resources and incentive for small-farm conservation practices)

CBN's principal objectives are to:

1. Integrate priorities of small-scale farmers and processors in cassava biotechnology research
2. Stimulate cassava biotechnology research on priority topics
3. Foster exchange of information on cassava biotechnology research, techniques, results, materials

From July 1992 to June 1997, the Government of the Netherlands, through DGIS, funded a CBN Coordination project. This year, from July 1997 to Aug 1998, DGIS accepted to fund an extension (retroactive; approved 11 Dec 1997) for a stakeholder-participatory

process to design a new regional structure for CBN. This added a fourth objective for the year, to (4) facilitate the stakeholder-participatory process to design a new regional structure. In 1999, DGIS anticipates funding a new three-year project, based at CIAT, for coordination and activities of the CBN Latin American regional network.

Regionalization of the network is expected to permit: closer contacts with end-users; better integration of farmer participation; enhanced regional ownership and accomplishment; enhanced responsiveness and cost-effectiveness; and expanded funding base. It is possible that regionalization will lead to enhanced sustainability of the network.

Some concerns have been raised concerning the lack of a global coordination facility after 1998: individual national or laboratory interests may dominate in a region; competition for scarce collaborative opportunities and funds may exclude late comers; public information in the North, about cassava and its importance to millions of people, may be neglected.

Outputs by objective:

1. Integrate priorities of small-scale farmers and processors in cassava biotechnology research

- Farmers' workshop in the Latin American regional stakeholders meeting (see appendix for the farmers' workshop recommendations to CBN)

- Farmer-participatory research, planned with farmers (Ecuador) or on the basis of farmers' stated priorities (Colombia)

  (i) Implementation began September: Low-cost tissue culture of traditional cassava variety and varieties from participatory selection; extension to other local staple crops with similar propagation problems (bananas, plantain); redevelopment of indigenous root crops; possible local micro-business; economic opportunity. Colombia; FIDAR (NGO), CIAT, community associations; supplemental funding anticipated SWG-PRGA

  (ii) Implementation beginning October: Restore national cassava collection from in-vitro back-up at CIAT; propagate and disseminate variety selected by farmers+national program; rescue, conserve (field + in-vitro), and use local cassava genetic diversity; reconstruct production-processing-market chain; restore food security and economic opportunity. Coastal Ecuador; CIAT, INIAP, Uni. Tec. Manabí, UATAPPY farmers' cooperative; El Nino disaster relief; funded by USAID

- Field day for CBN biotechnology members to visit small-scale cassava farmers in small groups, and plenary and working group sessions on farmer participatory research in CBN, organized in 1997-98; to be held at the CBN IV meeting 2-7 Nov 1998, in Brazil

2. Stimulate cassava biotechnology research on priority topics

New cassava biotechnology research projects developed and research begun (see above, Obj. 1):

- Planting material: need for responsive seed systems for high quality and sufficient quantity of desired varieties: Proposal for farmer-participatory research in collaboration with an NGO, FIDAR (see above)

- Conservation and use of cassava diversity; new and improved products: Participatory proposal design and implementation, with farmers in Ecuador for El Nino disaster relief (see above)

3. Foster exchange of information on cassava biotechnology research, techniques, results, materials

- Facilitated combined electronic mail listserver between CBN, SWP-PRGA Plant Breeding Group, and invited biotechnologists outside CBN, on selected issues related to feasibility, value, and organization to biotech-assisted farmer participatory research

- CBN IV fourth international scientific meeting organized with EMBRAPA (to be held 2-7 Nov 1998 in Salvador, Brazil).

4. Facilitate the stakeholder-participatory process to design a new regional structure

- Latin American stakeholders regional meeting and report
- Concepts developed for CBN-Asia and CBN-Africa, in collaboration with CIP and IITA
- Global stakeholders meeting and report
- CBN regionalization plan presented (donor briefing) CGIAR Mid Term Meeting, Brazil

Training courses/Workshops on agrobiodiversity and biotechnology organized in cooperation with LDC and DC CIAT partners

- CBN IV fourth international scientific meeting organized with EMBRAPA (to be held 2-7 Nov 1998 in Salvador, Brazil).

Collaborators:

- CIAT: Ann Marie Thro, Willy Roca, Martin Fregene, Luis Alfredo Hernandez

Principal partners 1997-1998:

- *Brazil*: EMBRAPA: Luis JCB Carvalho, Marcio Porto, Maria Jose Sampaio
- *Ecuador*: Uni. Tecnico Manabí, Hernan Caballero; FLACSO, Susan Poats
- *Colombia*: FIDAR (NGO), Jose Restrepo
- *CGIAR*: CIP, Gordon Prain; IITA, Mpoko Bokanga and Robert Booth; SWG PRGA: Charlie Spillane, Louise Sperling
- *Advanced research organizations*: Univ. of Bath: G. Henshaw; NRI: N. Poulter and A. Westby; AU Wageningen: R. Visser; ILTAB: C. Fauquet, N. Taylor
- *CBN regionalization process participants* – Latin American and African regions : Over 40 partners from national programs, private industry, CG centers; farmers' associations
- *CBN Scientific Advisory Committee; Regional Technical Committee; Local Organizing Committee* for CBN IV meeting organization: over 20 persons from national programs, advanced research institutions, and CG centers

3.1.2 Training courses/workshops on agrobiodiversity and biotechnology organized in cooperation with LDC and DC CIAT partners.

- Workshop on International Systems on Intellectual Property Rights Applied to Biotechnology and Biodiversity, 1998. Jorge A. Goldstein PhD., J.D. Attorney at Law, specialist IPR Director Sterne, Kessler, Goldstein & Fox P.L.C. Washington, D.C. CIAT. Cali-Colombia. April 27-28, 1998.
- Course/Workshop on Tissue Culture of Perennial Tropical Fruits. 1998. Proffessor Richard Litz and Pamela Moon. University of Florida, Homestead, USA. CIAT. Cali, Colombi. August 24-28.
- Training on rice genetic transformation and anther culture were given to two scientists from Venezuela (Ariadne Vegas, Fonaiap, and Ramiro de la Cruz, Universidad de Los Llanos); one scientist from Colombia (Fdiz Milena Quintero, Cenicafé); and one from Surinam (Jerry R Tjoe-Awie, ADRON).
- Course on Biodiversity: genetic diversity and phylogeny. Support/organization with Institute, Washington DC
- Workshop on QTL analysis for CIAT staff.
- Workshop on Genetic Diversity with participation of CIP and CIMMYT scientists. Collaboration of Cornell Univ. Institute of Genomic Research.

ACTIVITY 3.2 Assembling of data bases, genetic stocks, maps probes and related information.

SUMMARY OF ACHIEVEMENTS

- Project SB-02 has assembled a range of genetic stocks, which are product of its research, including anther culture-derived rice plants and lines, trangenic rice lines, transgenic cassava lines and micrografted soursop plants.

- The strain collection assembled in the BRU has increased to 101 *E. Coli* strains containing plasmids with genes of interest for genetic transformation; and *112 A. tumefaciens* and 17 *A. rhizogenes* strains for use in genetic transformation.

3.2.1 Genetic stocks.

(i) The rice anther culture laboratory generated total of 17,673 plants from October 1997 to September 1998. These lines includes material with cold tolerance, resistance to rice blast, sogata, rice hoja blanca virus, high yield, high milling grain quality. Resposible Z.Lentini:

- 1,711 anther culture derived plants for the wild QTL CIAT –USAID project.
- 7,764 plants from anther culture for FLAR breeding program (Brazil, Colombia, Costa Rica, Uruguay, Panama, Paraguay , and Venezuela);
- 3,758 somaclones for the Venezuelan National Plan of Rice leaded by Fundarroz with the participation of Fonaiap, Danac, and la Universidad de los Llanos.
- 4,440 somaclones plants for Fedearroz, Colombia.
- 815 doubled haploids lines from 1997 were in advanced trials of evaluations for disease send as part of the VIOFLAR for selection at Brazil, Paraguay and Uruguay.

(ii) Transgenic rice lines carrying *gus*, hygromycin resistance, and resistance to rice hoja blanca virus. Responsible Z. Lentini.

(iii) Transgenic cassava plants carrying bar gene (resistance to herbicide Basta).

(iv) . Micropropagated and micrografted (*annona muricata*) plants: 200 *in vitro*. Collaboration with Corporación BIOTEC, Cali.

3.2.2 *E. coli* and *A. tumefaciens* collections

The BRU Agrobacterium tumedaciens collection contains 112 strains. Among the most important strains of A. tumefaciens are LBA4404, AT650, C58C1 and EHA101, which are hypervirulent wild strain isolated from cassava.

The Escherichia coli collection contains 101 strains. Among them, the strains DH5a, JM 109, XL1-Blue and DH10B are often used in cloning. The collection contains many plasmids with different genes of interest for plant transformation like hygromicyn and

basta resistance genes, cry IA(b) (lepidoptera resistance), and metabolic enzyme such as rubisco. PEP carboxylase and starch branching enzyme.

The viability of bacteria collections was evaluated this year and three copies per strain were stored at –80oC.

Table 1. Collection of *E. coli* strains.

Escherichia coli	Number
Wild strains	30
Strains with plasmids	45
Strains with genes of interest	26
Total strains in the collection	101

Table 2. Collection of *A. tumefaciens* strains.

Agrobacterium tumefaciens	Number
Wild strains	32
Strains with plasmids	30
Strains with genes of interest	51
Total strains in the collection	112*

- The total number of *A. tumefaciens* strains may be smaller since few strains may have lost viability

Table 3. Collection of *A. rhizogenes* strains.

Agrobacterium rhizogenes	Number
Wild strains	12
Strains with plasmids	5
Total strains in the collection	17

Collaborators: L. Mancilla, P. Chavarriaga, V. Segovia, J. Ladino, Z. Lentini, W. Roca

ACTIVITY 3.3 Publications, project proposal development, and contribution to IRP and biosafety management.

SUMMARY OF ACHIEVEMENTS

- In the period Oct. 1997-Sept 1998, project SB-02 staff has produced: 19 refereed publications, 14 other publications as book chapters and conference proceedings, and has supervised 15 degree Theses.

- The project staff has also generated/prepared 13 Project Concept Notes and Proposals for seeking financial support.
- The Team has contributed to at least 5 activities (workshops, conferences, consultancies, etc) to develop IPR and biosafety management at CIAT in Colombia and the LAC region.
- In the period of this report, Project SB-02 has enjoyed the contributions of 10 donors through complementary funding.
- In Nov. 1997, Project SB-02 was the subject of a CIAT ICER. The review Panel's recommendations have been submitted to CIAT management.

3.3.1 Publications by SB-02 staff in the period Oct. 1997 – Sept. 1998.

3.3.1.1 Refereed Publications

Sanchez I., Angel, F., Grum M., Tohme, J., Lobo, M. and Roca, W. 1998. Variability of chloroplast DNA in the genus Passiflora L. In press Euphytica.

Sanchez, I. Angel, F. Grum, M. Tohme, J. Lobo, M. and Roca, W. 1998. Characterization molecular, base sólida para el mejoramiento genético de Passifloraceae Juss. Fitotecnia Colombiana, Organo de la sociedad Colombiana de Fitomejoramiento y producción de cultivos. In press.

Sanchez, I., Angel, F., Grum, M., Tohme, J., Lobo, M. and Roca, W. 1998. Estudios de la variabilidad genética de Passifloraceae – familia de plantas nativas promisorias. Revista Corpoica. In press.

Fajardo D., Angel, F., Grum, M., Tohme. , Lobo, M., Roca, W., Sánchez, I., 1998. Genetic variation analysis of the genus passiblora L. using RAPD markers. Euphytica 101:341-347

Restrepo S. and V.Verdier. 1997. Geographical differentiation of the population of Xanthomonas axonopodis pv manihotis in Colombia. Appl. Env. Microbial, 63:4427-4434.

Verdier, V., Mosquera, G. and Assigbetse K. 1998. Detecting the Cassava Bacterial Blight Pathogen, Xanthomonas axonopodis pv manihotis, using the polymerase chain reaction. Plant Disease, 82: 79-83.

Verdier, V., Restrepo, S., Mosquera, G., Duque, M.C., Gerstl A. and Laberry R. 1998. The Xanthomonas axonopodis pv manihotis populations in Venezuela: its genetic and pathogenic variation. Plant Pathology, 47: in Press

Thro, A.M., and M. Fregene 1998. The Cassava Biotechnology Network: Impact of the network and scientific advances in cassava biotechnology. Tropical Agriculture (Trinidad). In press.

Thro, A.M., N. Taylor, K., Raembarkers, R. Visser, J. Pounti-Kaerlas, C. Schopke, C. Iglesias, W. Roca, M.J. Sampaio, C. Fauquet, and I. Potrykus. 1998. Maintaining the Cassava Biotechnology Network: An agenda to make a difference. Nature Biotechnology 16(5):428-430.

Thro, A.M., W.Roca, G. Henry, C. Iglesias, and S.Y.C. Ng. 1998. Contributions of in vitro biology to cassava, a small farmer's crop. African J. Crop Sci. Kampala (accepted).

Lentini, Z., Martinez, C.P. and E. Nossa. 1997. Introgression of high response to anther culture into indica rice. International Rice Research Notes (IRRN). 22(1):18-20.

Jones, P., G.; Beebe, S.; Tohme, J. Galwey, N.W. 1997. The use of geographical information systems in biodiversity exploration and conservation.

Roa, A.C.; Maya, M.M.; Duque, M.C.; Tohme, J., Allem, A.C. Bonierbale, M. 1997. AFLP analysis of relationship among cassava and other *Manihot* species.

Fregene, M.; Angel, F. Gomez, R. Rodriguez, F. Chavarriaga, P. Roca, W. Tohme, J. Bonierbale, M. 1997. A molecular genetic map of cassava (*Manihot esculenta* Cranzt). Theor. Appl. Genet. 95:431-441.

Escobar, R. Mafla, G., Roca, W. 1997. A methodology for recovering cassava plants from shoot tips maintained in liquid nitrogen. Plant Cell Reports 16: 474-476.

Yeoh, H.H. Sanchez, T. and Iglesias, C. 1998. Large-scale screening of Cyanogenic Potential in Cassava Roots using the Enzyme-Base Dipsticks. J. Food Composition and Analysis 11: 2-10

Fregene, M.A., Ospina, J.A. and Roca, W. 1998. Recovery of cassava (*Manihot esculenta* Crantz) plants from inmature zygotic embryos. Plant cell and orgon culture (in press.

Thro, M.M., Roca, W. Henry, G. Iglesias, C. and Mg, SY.C. 1998. Contribution of in vitro biology to cassava, a small farmer's crop. African J. Crop Sci. (accepted)

Chavarriaga-Aguirre, P. Maya, M.M. Bonierbale M. Kresovich, S., Fregene, M., Tohme, Jkochert, G. 1998. Microsatellites in cassava (*Manihot esculenta* Crantz): discovery, inheritance and variability. Trends in Genetics (in press).

3.3.1.2 Other Publications

- **Book chapters and Conference Proceedings**

Thro, A.M., M. Fregene, N. Taylor, C.J.J.M. Raembarker, J. Pounti-Kaerlas, C. Schopke, R. Visser, I. Potrykus, C. Fauquet, W. Roca, C. Hershey. 1998. Genetic biotechnologies

and cassava-based development of marginal rural areas: In: T. Hohn and L. Leisinger 9eds). Gene and biotechnology of food crops in developing countries. Springer; Wien, New York (E.S. Dennis et al. (eds). Plant gene research: Basic Knowledge and applications. In press.

Roca, W.; Correa-Victoria, F. Martínez, C. Tohme, J. Lentini, Z. Levy, M. 1998. Development of durable resistance to rice blast: Productivity and environmental considerations. In Komen, J. Falconi, C. and Hernandez, H. (eds) Transforming Priorities into Viable Programs, Lima Peru. IBS//ISNAR, The Hague, The Netherlands.

Thro, A.M. and M.O. Akoroda (eds). 1997. Proceedings Third International Scientific Meeting, Cassava Biotechnology Network, Kampala, Uganda, 26-30 Aug. 1996. African J. Root & Tuber Crops, Vol II. Nos. 1&2.

Thro, A.M. 1998. The Cassava Biotechnology Network: Considerations for effective use of biotechnology tools for cassava. pp. 515-525 In: CIAT (R Howeler). Cassava breeding, agronomy, and participaroty research in Asia. Proc. Fifth Asian Cassava Research Workshop, Danzhou, Hainan, China, 3-8 Nov. 1996.

Restrepo S., Tohme,. J. and Verdier, V. 1998. Assessment of the AFLP technique for the genetic study of Xanthomonas axonopodis pv. Manihotis. *In Abstr. Of the proceedings* of the 7th International Congress of Plant Pathology. Edinburg, Scotland, 9-16 August 1998.

Restrepo, S. Velez C. Verdier, V. 1998. Measurement of the genetic diversity in Xanthomonas axonopodis pv manihotis within different field in Colombia. In Abstract of the proceedings of the 7th International Congress of Plant Pathology, Edinburgh, Scotland, 9-16 August 1998.

Lentini, Z. 1998. Biotechnology mediated genetic modification of rice for insect resistance: A component for integrated pest management. Latin American Rice Forum 4(1):14-16. In Conference Proceedings.

Lentini, Z., L.Calvert, I. Lozano, and E. Tabares. 1998. Resistance to hoja blanca virus encoded by the nucleocapsid transgene in tropical rice. IX International Congress on Plant Tissue and Cell Culture. Plant Biotechnology and In Vitro Biology in the 21st Century. Jerusalem, Israel.

Mora A. Z. Lentini, and C. Martinez. 1998. Rice breeding with anther culture. II Latin American Meeting on Plant Biotechnology. Havanna, Cuba.

Lentini, Z., L. Calvert, I. Lozano, and E. Tabares. 1998. Trangenic rice with RHBV resistance. II. Latin American Meeting on Plant Biotechnology. Havanna, Cuba.

Mora, A., Z. Lentini, and C. Martinez. 1998. Anther culture a tool for rice breeding. First International Meeting of Rice. Havanna, Cuba.

Lentini, Z. L. Calvert, E. Tabares, I. Lozano, M. Cuervo, W. Roca and BC Ramires, 1997. Resistance to rice hoja blanca in transgenic rice using the nucleoprotein gene. Fifth International Crongress of Plant Molecular Biology. Singapore.

Mejia-Jimenez, H.J. Jacobsen, 1998. Development of an in vitro regeneration system in common bean suitable for genetic transformation. Eucarpia Int. symposium, Partevedre, Spain.

Escobar, R.H., Palacio, J.D., Rangel, M.P. and Roca, W.M. 1998. Crioconservación de apices de yuca mediante encapsulacion-dehidratacion. In: III Latin American meeting on plant biotechnology, REDBIO, La Habana, Cuba.

- **Degree Theses**

Galindo, L. 1997. Transformación genética de la graminea forragera *Brachiaria spp* mediante la técnica de bombardeo de partícula. CIAT.

Rengifo J. 1997. Caracterización de germoplasma de *Phaseolus vulgaris* L. cultivado Andino por medio de AFLPs

Reina, G. 1997 Effecto de la congelación rápida de ápices de yuca (*Manihot esculenta* Cranzt) euphorbiaceae, a la crioconservación de nitrógeno líquido.

Giraldo M. 1997. Effecto del genotipo de yuca yuca (*Manihot esculenta* Cranzt) euphorbiaceae, a la crioconservación de nitrógeno líquido.

Mancilla L.I. 1997. Efecto del aluminio y bajos niveles de nutrientes sobre la expresión de genes y actividad de las enzimas H+ ATPasa y Fosfatasa acida y alcalina de tres especies de *Brachiaria.*

Suarez M.C. 1998. Desarrollo de la técnica cDNA/AFLPs para mapeo en *yuca Manihot esculenta* Crantz.

López A. F. 1997. Estudios de la bacteria *Xanthomonas campestris* pv *manihotis:* expresion de un gen de patogenicidad PthB.

Dávalos L. 1997. Mapeo de genes de resistencia al virus Hoja Blanca en arroz en la población Fanny x Oryzica Llanos 5.

Segovia, V. 1997. Optimización de la transformación genética de tejido de Yuca *Manihot esculenta* C. utilizando *Agrobacterium tumefaciens.*

Palacio, J.D. 1998. Crioconservación de ápices de yuca utilizando la técnica de encapsulación deshidratación. CIAT.

Hernández, A. 1998. Propiedades Químicas de paredes celulares de raíces de *Brachiaria spp* en relación con el bajo suministro de nutrientes y toxicidad de aluminio.

Almeida, A. 1998. Marcadores microsatelites asociados a QTLs de Rendimiento en Arroz, en un retrocruce avanzado entre una especie silvestre (*Oryza barthii*) y una variedad mejorada (Lemont).

Santaella, M. 1998. Hacia el aislamiento de genes de resistencia en Arroz a *Pyricularia grisea.*

Lasso, N.L. 1998. Efecto del nitrogeno, fósforo y alumnio en la estructura del sistema radical de tres especies del género *Brachiaria.*

Villota, F. 1998. Evaluación de métodos para la determinación de carotenos y tocoferoles.

3.3.1.3 Concept Notes and Project Proposals prepared

- Gene flow analysis at the ecological frontier: Setting risk assessment for genetically modified plants. Donor: BMZ. Participating institutions: CIAT; Federal Biology Institute, Germany; ICA and Institute Von Humboldt, (Colombia); UNALM (Peru); DANAC (Venezuela); UCR (Costa Rica). Responsible: Z. Lentini, W. Roca, D. Debouck.
- Expanding the range of uses of cassava starch: A source of employment and income Fedeyuca, Colombia; and Agropecuaria Mandioca, Venezuela. Responsible: Z. Lentini, C.Iglesias, W. Roca.
- Plant genetic engineering to incorporate tolerance to iron toxicity and increase iron nutrional institutions: CIAT; and IDEA (Institute of Advanced Studies), Venezuela. Z.Lentini (CIAT), R. Rangel (IDEA).
- Genetic transformation of venezuelan rice varieties for resistance to rice hoja blanca virus. Donor: Polar. Participating institutions: CIAT; Rutgers University; Danac and IDEA, Venezuela. Responsible: Z. Lentini (CIAT), Nilgun Tumer (Rutgers), Maria Angelica Santana (IDEA).
- Increasing cassava starch yields: Stability in synthesis and storage in the roots. Donor: Palmaven, Petroleum of Venezuela. Participating institutions: CIAT, IDEA (Institute of Advanced Studies), Universidad Central de Venezuela (UCV), and Agropecuaria Mandioca, Venezuela, Responsible: Z. Lentini and C. Iglesias (CIAT), Maria Angelica Santana (IDEA), Juscelino Tovar (UCV).

- Funding of CBN Second Phase: Latin American Network. Donor: DGIS. Responsible: A.M. Thro, W. Roca and partners from LAC countries.
- Producción de semilla de calidad para mejorar la economía de pequeños agricultores de la Costa Norte de Colombia. Donor: DGIS-Colombia. Collaboración con CORPOICA. Responsible: W. Roca, A.M. Thro.

- Genetic Improvement of Common Bean Exotic Germplasm and Biotecnology-Second Phase. Donor: AGCD. Collaborator: Univ. of Ghent, Belgium, Responsible: W. Roca, C. Cardona.
- Development of a pilot project for cassava under cryopreservation. Donor: to be identified. Collaborators: CORPOICA, EMBRAPA, CUBA, IITA, CIP. Responsible: W. Roca
- Fine mapping and cloning and engineering of apomixis gene from Brachiaria. Donor: private sector. Collaborators: Clemson Univ., USA; CAMBIA, Australia. Responsible: J. Tohme, W. Roca, J. Miles.
- Genetic improvement/clonal multiplication of tropical fruit crops. Donor: Fontagro. Collaborators: CORPOICA, FONAIAP, INIAP. Responsible: I. Sanchez (CORPOICA), W. Roca
- Development of an Artesanal Tissue Culture Propagation Lab. Donor: System Wide Program a Participatory Research and Gender Analysis. Collaborators: FIDAC (an DGO), small cassava growers, Cauca, Responsible: W. Roca, A.M. Thro.

DONORS CONTRIBUTING TO PROJECT SB-02 COMPLEMENTARY FUNDING IN 1997-98

The RF.

- Rice Biotechnology. (I) Molecular analysis of *Pyricularia* genetic structure and population dynamics; (ii) Mapping of rice blast resistance genes; (iii) transgenic resistance to RHBV.
- Cassava Biotechnology: (I) Saturation of cassava molecular genetic map; (ii) mapping of ACMF resistance genes.

USAID

- Mapping of QTLs from wild *Oryza* spp responsible for yield in rice(*)
  BMZ/GTZ Germany
- Common bean genetic transformation (*) AGCD, Belgium
- Transfer of economic traits from P. coccineus to common bean

DFID, U.K.

- Genetic analysis of cassava roots post-harvest deterioration
- Genetic transformation of cassava(*)

DGIS, The Netherlands

- Cassava Biotechnology Network (**)

SDC, Switzerland

- Microsatellite markers for cassava

COLCIENCIAS, Colombia

- Cloning CBB resistance genes

Institute A.V. Humboldt, Colombia

- Training Course

Fundación Polar

- Publication of Rice anther Culture Manual.

(*) To end in 1999.
(**) *Ended in mid 1998.*

INTERNALLY COMMISIONED EXTERNAL REVIEW (ICER) OF PROJECT SB-02

Date of Review:	Nov. 17-23, 1997
Review Panel:	B. Schaal, Washington Univ. St. Louis, MO, USA. (Chair)
	M.J. Sampaio, EMBRAPA, Brazil
	K.I. Hayashi, Japan
	W. Beversdorf, Novartis Co (CIAT BOT)

The review's outcome was very possitive. Project SB-02 staff, teaming with Project SB-01 staff, have prepared and submitted to the attention of the Review Panel a Strategy for future directions in agrobiodiversity and biotechnology research at CIAT. The document contain a proposal to focus CIAT biotech research at genomic level, with a Genome Research Lab comprising three outputs: genome characterization, genome modification and clonal propagation. An additional facility is proposed for regional partnerships in genomic research, particularly in dealing with crops outside CIAT's mandate and with the private sector.

PROJECT SB-02 STAFF (1998)

Name	Discipline	Area	Dedication %
J. Tohme	Genetics	(Molec. Markers)	1.0
S. Beebe	Breeding	(Gene mapping)	0.7
S. Singh	Breeding	(wide crossing)	0.3
A. Bellotti	Entomology	(IPM)	0.2
Z. Lentini	Cell Biology/genetics	Tissue culture/genetic transformation	0.8
M. Fregene	Molec. Genetics	(Molecular markers)	1.0
A. Mejia	Biology	(Tissue culture.transformation)	1.0
A.M. Thro	Breeding	(Nerworking)	1.0
C. Martínez	Breedings	(Germplasm-enhancement)	0.75
I. Sanchez	Genetics	(Diversity-CORPOICA)	1.0
V. Verdier	Molec. Pathology	Microbial diversity	1.0
D. Debouck	Botany	(Genetic Resource)	0.2
W. Roca	Physiology	(Tissue culture/transformation)	0.8

Genome Modification

L.F. Galindo	Young Research- COLCIENCIAS
F. Giraldo	Assistant
P. Chavarriaga	Associated Scientist
L.I. Mancilla	Assistant
V. Segovia	Young Research- COLCIENCIAS
J.J. Ladino	Student – Univ. Nacional
R. Escobar	Research Assistant
M.P. Rangel	Student – Univ. Javeriana
E. Tabares	Research Assistant
L. Duque	Student Univ. Nacional
C. Flores	Student Doctorado Univ. . Nacional
H. Ramírez	Student Doctorado Univ.. Nacional
Pablo Herrera	Téchnician
M. Valenciano	Téchnician

Genome Diversity

G. Gallego	Coordinador Investigación
A. almeida	Research Assistant
C. López	Research Assistant
A.C. Roa	Research Assistant
M.C. Suarez	Research Assistant
E.Gaitan	Research Assistant
E. Barrera	Research Assistant
J.P. Gutierrez	Research Assistant

J. Lopez	Research Assistant
A. Bohorquez	Research Assistant
J. Vargas	Research Assistant
M. Santaella	Student
D.F. Cortés	Student
S.M. Garzón	Student
N. Reyes	Technician

Plant-Stress interactions

P. Wenzl	Student, PhD.
A.L. Chaves	Associated Research
G. Patiño	Student
A. Hernández	Student
J.M. Bedoya	Student
F. Villota	Student

Administrative

O.L. Cruz	Bilingual Secretary
C.S. Zuñiga	Bilingual Secretary

A.V. Humboldt

J.D. Palacio	Visiting Researcher
P. Sanchez	Visiting Researcher
J.F.Fernández	Visiting Researcher

SINCHI

F. Rodriguez	Visiting Researcher
P. Toquica	Visiting Researcher

Corporación BIOTEC

N. Royero	Visiting Researcher