BIOTECHNOLOGY RESEARCH UNIT

ANNUAL REPORT 1988-1992

EXECUTIVE SUMMARY

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1. BIOTECHNOLOGY RESEARCH AT CIAT

The involvement of CIAT in biotechnology research should be viewed against the ever- increasing gap between the progress being made in developed countries and that which is evident in most of the tropical developing countries. As an international public research institution, CIAT shares responsibility for ensuring that the benefits of biotechnology will not fail to be achieved for long-term public goals including growth, equity and good environmental stewardship.

CIAT has constantly been on the alert to identify innovations in science and technology that can result in quick pay-offs in terms of technology development. In its long-term plan for the 1980s CIAT anticipated its involvement in monitoring and applying biotechnology. In fact work on tissue culture for conserving cassava germplasm and accelerated rice germplasm improvement were tackled in 1980-84. In 1985 CIAT established the Biotechnology Research Unit (BRU) in response to a recommendation by the 1984 External Program Review Panel. From 1988-92, the period included in this report, a significant evolution in biotechnology research at CIAT took place. The BRU's research and collaboration focused further on critical challenges in CIAT crop germplasm and expanded to include the study of selected microorganisms; modern biochemical and molecular techniques were incorporated, developed and used to address those constraints. Priorization of production and utilization constraints led to the strengthening of international collaborative research, which in the case of cassava and beans, took the form of biotechnology research networks. Practical biotechnology methods and techniques have been integrated into the activities of other CIAT Units and Programs for further implementation and use. A considerable portion of the activities in the BRU have been carried out with special project funding.

2. THE BIOTECHNOLOGY RESEARCH UNIT

2.1 Role and Objectives

The role of biotechnology research at CIAT is to develop applications of modern biological methodologies and tools for increasing the efficiency of CIAT strategic research. The BRU's work addresses three broad objectives: (i) characterization of genetic diversity, (ii) characterization of plant biotic and abiotic stress mechanisms, and (iii) broadening the genetic base of crops and germplasm conservation. CIAT

biotechnology research focuses on important constraints in cassava, *Phaseolus* beans, rice for Latin America, some tropical forages, and will also pay attention to other selected components of the biodiversity occurring in CIAT agroecological regions.

2.2 Strategies

To perform its role as a scientific bridge for developing modern cellular, molecular and biochemical methods and techniques, the BRU follows three strategies:

- Biotechnology methods and tools are integrated into ongoing CIAT Program and Unit research activities: constraints that are amenable to biotechnological solutions are identified; the BRU monitors basic research developments worldwide and brings to CIAT new information, and research is carried out to develop and utilize biotechnology. Collaboration with CIAT scientists and advanced labs is essential for implementing this strategy.

- The BRU cooperates with CIAT programs and units to bring prioritized research constraints to the attention of the world scientific community and donors, encouraging and establishing international cooperative efforts and effective research linkages between research organizations in developed and developing countries and the strategic research conducted at CIAT.

- To accomplish its bridging role with the national agricultural research systems (NARS) of developing countries, the BRU cooperates with CIAT programs to offer training for developing country scientists and to organize workshops and visits to the NARS. Awareness on issues such as biosafety and intellectual property rights are also addressed.

2.3 Organization of the BRU's Activities

In the last three years, the BRU has implemented three interrelated, interdisciplinary, research activities: (i) Molecular markers and mapping; (ii) Molecular biochemistry and (iii) Tissue culture and genetic transformation (gene transfer). The three research groups interphase with CIAT scientists and with responsibilities across CIAT germplasm for the characterization of genetic structure; characterization of mechanisms involved in resistance and tolerance to biotic and abiotic stresses in plants, and broadening of the genetic base of crops and germplasm conservation.

3. ACTIVITIES DURING 1988-92

3.1 Advanced Research Networks

The Cassava Biotechnology Network (CBN) was founded as a result of a workshop held at CIAT in 1988; and in a 1990 workshop, the Phaseolus Beans Advanced

Research Network (BARN) was formed. Since its inception the CBN has made steady progress in encouraging research projects addressing critical cassava topics; from 5 projects in 1988 to 25 in 1992. The network was consolidated in 1992 with the appointment of a Coordinator and the organization of the network's first scientific meeting. Plans are under way for the first BARN scientific meeting in 1993. In addition, CIAT participates actively in the Rice Biotechnology Program under the auspices of the Rockefeller Foundation.

3.2 Complementary research through special projects at CIAT

In cooperation with CIAT programs and units, the BRU developed and carried out the following complementary research through special project funding:

- A pilot project on cassava in vitro active genebank: 1988-90 (IBPGR)
- A pilot project on cassava cryopreservation: 1989-91 (IBPGR)
- Anther culture-derived haploids for rice germplasm improvement: 1989-91 (RF)[1]
- Gene tagging and mapping of rice hoja blanca virus (RHBV) and rice blast resistance genes: 1991-ongoing (RF)[1]
- Construction of a cassava molecular map: 1992-94 (RF)
- Gene tagging and mapping of common bean useful genes: 1991-94 (Belgian AGCD)[2]
- Construction of a molecular map of tepary beans: 1991-94 (Belgian AGCD)[2]
- Characterization of mechanisms involved in resistance to the bean weevil: 1991-94 (Belgian AGCD)[2]
- Operations and coordination of the Cassava Biotechnology Network (CBN): 1992-97 (Dutch DGIS)[3]

In addition, four PhD theses fellowships have been supported by the GTZ, CIDA and IDRC for the period 1990-93.

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[1] Two projects under a grant.
[2] Three projects under a grant.
[3] Cassava Program and BRU cooperation.

3.3 Collaborative Research Projects in Developed Countries

During the last five years, the BRU has collaborated in the following formalized research relationships with U.S. and European institutions:

- Cassava genomic studies. U. of Georgia, Athens, and Washington U., St. Louis: 1992-94 (RF)
- Rice Biotechnology: Cornell U., Ithaca; Purdue U., West Lafayette: 1990-onward (RF)
- Cassava cyanogenesis. Ohio State U., Columbus: 1990-92 (USAID)
- Cassava virus resistance. ILTAB, The Scripp Research Institute, La Jolla: 1990-93 (USAID, Rockefeller)
- Construction of a molecular map of common bean: U. of Florida, Gainesville: 1990-92 (USAID)
- Common bean molecular markers for evolutionary studies. U. of California, Davis: 1989-91 (USAID)
- Cloning of bean mexican weevil resistance gene and transformation of common beans. U. of Ghent, Belgium: 1991-94 (Belgium AGCD)
- Regeneration of common beans. U. of Bonn: 1990-92 (GTZ)
- Cassava cyanogenesis. U. of Newcastle Upon Tyne, UK: 1990-93 (RF, ODA, EC)
- Cassava somatic embryogenesis. U. of Bath, UK: 1988-90 (ODA)

In addition, nonformalized research relationships have been developed with a dozen US and European institutions.

3.4 Research Achievements and Future Plans

3.4.1 **Characterization of genetic structure.** In the past five years, the Unit has implemented various techniques to carry out detailed studies on the genetic structure of CIAT crops. Starting in 1988, seed protein and isozyme techniques were implemented and used to characterize the variability of the germplasm deposited in the Genetic Resources Unit (GRU). The questions to be addressed were different for each crop.

- In the case of **beans**, the molecular data available from research conducted at the U. of Wisconsin, Michigan State U. and Cornell U. provided a starting base for the research at CIAT. These studies were aimed at increasing our understanding of the variability of wild and cultivated *P. vulgaris*, the implications of domestication on the existing variability in cultivated beans, and the genetic structure of bean gene pools. Additional molecular markers such as RFLPs and RAPDs are currently being used either to complement the available data with a better resolution or address the same issues with more adequate tools. The use of mtDNA probes and minisatellites is being initiated. Additional emphasis will be placed on implementing or developing molecular markers to characterize the genetic diversity in order to manipulate said diversity through introgression from non-*P. vulgaris* species. The identification of species-specific probes and the use of markers from the common bean molecular map (constructed at the U. of Florida in cooperation with CIAT) to tag important bean genes will be areas of research in the coming years.

- The initial questions to be addressed in **cassava** were related to germplasm conservation. The collection contains an estimated 20% duplicates, resulting in increased costs of maintaining large field and *in vitro* collections. In order to detect possible duplicates, the collection was fingerprinted with isozymes. The survey initiated by the BRU was later implemented by the GRU in collaboration with the Cassava Program. More recently, DNA fingerprinting using both M13 and RAPD is being carried out to obtain better coverage of the genome. These markers will not only allow better identification of duplicates but will also provide information related to the stability of the *in vitro* collection and the characterization of the cassava gene pools and wild *Manihot*. The construction of a cassava molecular map using RFLPs and RAPDs has been initiated and will continue on the next few years, followed by the use of the map in gene tagging.

- Due to CIAT's regional mandate for **rice**, the focus of genetic characterization has been more toward understanding the genetic base. Isozyme analysis has already been conducted on elite and selected cultivars. To complement this study, an extensive survey is currently been carried out using RAPDs to understand the genetic base of elite cultivars of Latin American breeding programs and to link the molecular data with agronomic data. Using RFLPs and RAPDs on anther culture-derived doubled haploid populations, a resistance gene to RHBV has been tagged and located on chromosome 12; and a resistance gene complex to blast lineage SRL-1 was tagged and located on chromosome 4.

The direction of new research will focus on implementing and developing molecular markers that will provide ample information at a minimum cost. A Molecular Markers Applications Lab will be implemented for use by all CIAT

programs and units. An additional research area will be the fingerprinting of microorganisms and their characterization. The use of PCR-based markers will undoubtly be expanded. Oligonucleotide DNA fingerprinting using repetitive sequences or species specific sequences will be tested for evolutionary and taxonomic and biodiversity studies. One of the primary uses of these markers will be the already established core collections in beans and cassava. Sequence-characterized amplified regions (SCARs) will be developed to facilitate future screening. Implementation of non-radioactive labelling will be further emphasized in the immediate future.

3.4.2 **Characterization of mechanisms involved in resistance and tolerance to biotic and abiotic stress in plants**. Research being done in this area can be subdivided into three categories:

- characterization of resistance mechanisms to pests and pathogens
- characterization of physiological and biochemical processes in plants and bacteria
- development of methodologies

In the first category we are investigating resistance to bruchids, a major pest of beans, and antibiosis to the spittlebug in *Brachiaria*. The molecular bases of co-evolution of the common bean bacterial blight (CBB) pathogen and *Colletotrichum lindemuthianum*, the fungal agent that causes anthracnose in beans, are also being researched. In the second category, studies are being conducted on the stress tolerance of cassava to drought and the related $C0_2$ assimilation mechanisms. We are also quantifying organic acids and sugars of forage roots under aluminum stress as part of a project to develop glasshouse screening techniques for acid soil tolerance. The study of the bacterial amylolytic activities during cassava starch fermentation also falls into this category. In the methodology development category, we are searching for more convenient alternatives for DNA extraction and the development of a quick, reliable methodology for quantifying linamarin in cassava.

i. Characterization of resistance mechanisms to pests and pathogens:

- **Resistance to bruchids**. Breeding programs have not been successful in introgressing the complex resistance trait to the bean weevil *Acanthoscelides obtectus* from wild to cultivated beans. Identification of a resistance-conferring factor has been elusive. We have identified a proteinaceous fraction in the resistant accessions that induces death of first instar larvae feeding on artificial seed supplemented with the fraction at lowest doses. The experiment is being repeated with *Zabrotes subfasciatus*, the Mexican bean weevil, to assess the specificity of the particular fractions toward the bean weevil.

Inhibitors of α-amylases with specific-action spectra that may play a synergistic role together with other resistance factors (e.g., arcelin) have been identified in certain wild bean accessions. Specific proteinase inhibitors are being sought.

As an alternative to natural resistance, we have initiated work to clone the specific inhibitor of the insect's gut cysteine-type protease, cystatine, from egg white into beans. This transgenic approach could be the basis for multigenic resistance, which would be harder to break. We have shown in artificial seed that this could be a very effective way to control the insect.

- **Antibiosis to spittlebug in *Brachiaria*.** The spittlebug causes severe damage in *Brachiaria decumbens* fields. Resistance has been found in *B. jubata*, and there are indications that the factor involved could be a phytoecdysone. We are working on this hypothesis by developing an immunologic assay for ecdysone. The antibody would not only be used to identify the substance, but could also be used to assay for ecdysone in germplasm improvement programs. Ecdysone has been successfully bound to a carrier protein in order to produce antibodies, and antisera to the conjugate has been produced. We are in the process of isolating polyclonal monospecific antibodies for the assay.

- **Molecular bases of co-evolution in bean pests.** In collaboration with the Bean Pathology Section, genetic diversity in the CBB pathogen was demonstrated by DNA fingerprinting, using two probes isolated from the genome and the plasmid, respectively, of a *Xanthomonas campestris* pv *phaseoli* (XCP) isolate. After optimizing the conditions for generating the fingerprint patterns, most of the collection comprising over 300 isolates, has been characterized. The pigment-producing XCP var *fuscans* (XCPF) was clearly identified by the probes. Furthermore, the isolates were grouped into families by their fingerprints, which relate to their geographic origins. An attempt will be made to establish the relationship between fingerprints and pathogenicity. This may not be an easy task as resistance to CBB is of quantitative nature and thus no races exist for XCP or XCPF.

DNA fingerprint analysis of *C. lindemuthianum*, the fungal agent that causes anthracnose in beans, has been initiated. For that purpose we have conducted hybridizations of total genomic DNA from different races with a ribosomal DNA probe and with the M13 bacteriophage. Some degree of polymorphism was detected but not enough to provide a fingerprint pattern. Polymorphisms in the ribosomal intergenic regions will be analyzed using PCR. Our goal is to understand the molecular

basis of co-evolution of the fungus and beans. Our present model is based only on pathogenicity tests on differential bean lines.

ii. Characterization of physiological and biochemical processes in plants and bacteria:

- **Drought and heat tolerance in cassava**. The objective of this project is to understand the molecular mechanisms involved in photosynthesis that enable cassava to grow under drought and high temperature stress. The goal is to develop selection criteria for this trait in germplasm development programs. We are in the process of cloning genes involved in CO_2 assimilation and have also purified PEP carboxylase from cassava in order to carry out *in situ* hybridization studies and immunohistochemistry on thin sections of embedded leaf tissue. These techniques are being used to establish the cellular compartmentalization of the enzymes in the leaf and thus facilitate the elucidation of the underlying mechanisms.

- **Aluminum tolerance**. HPLC is being used to measure carboxylic acid and sugar levels in roots of plants treated differently with respect to aluminum and phosphorus concentrations. This is part of a project being carried out by the Tropical Forages Program (TFP) Nutrition Section, aimed at understanding the molecular mechanisms involved in tolerance to acid soils.

- **Cassava starch fermentation**. Solid-state fermented cassava is a natural product that is traditionally produced and consumed in Colombia and in Brazil. It can replace a certain percent of wheat flour in many products and is used as a unique starch source in several others. We are characterizing the amylolytic bacteria involved, at the enzymatic and molecular levels, in order to identify the strains best suited for the process and help define the fermentation parameters that will lead to reproducible high-quality fermented starch. Defined inocula would be a useful step toward upscaling of the process. This is part of a larger project, headed by SAR/CIRAD, to analyze the problem from the technological, microbiological, biochemical and market standpoints.

3.4.3 **Broadening the genetic base of crops and germplasm conservation**. In this area research has been conducted to develop and use methods and techniques for gene transfer by sexual and nonsexual means for expanding our access to a wider range of genetic variability from related and more distant sources. Development of methodologies for conserving genetic resources is another BRU activity, contributing toward broadening the genetic base of CIAT crop germplasm.

i. Building upon the Unit's early work on *in vitro* conservation of cassava, a collaborative project was established to develop and implement the technical and logistical aspects of this approach for running a large (i.e., over 4500 accessions) *in vitro* collection. The cassava *in vitro* active gene bank was transferred under the responsibility of the Genetic Resources Unit in 1989.

ii. Following the development of the *in vitro* active gene bank for cassava germplasm, a breakthrough was achieved in cassava cryopreservation. Complete plants have been recovered consistently from cassava shoot tips conserved in liquid nitrogen. Future work will emphasize expanding the protocol to a wider range of cassava genotypes and developing the logistics for a base gene bank of cassava in liquid nitrogen.

iii. With the objective of transferring important traits--i.e., resistance to bacterial blight and to *Empoasca*, and tolerance to drought--from tepary beans (in the tertiary gene pool) to common beans (primary gene pool of *Phaseolus*), an interspecific crossing system was implemented using recurrent and congruity backcrosses aided by embryo rescue and culture. Large numbers of recurrent and congruity mature, fertile hybrid plants have been obtained for field evaluation. Work continues to increase fertility of the hybrids further in order to develop a tepary x common bean gene pool and eventually expand this to other *Phaseolus* species so as to access traits not expressed, or inadequately expressed, in the common bean.

iv. We have developed a model system for transforming and expressing foreign genes using the forage legume *Stylosanthes guianensis*. Regeneration of *Agrobacterium*-mediated transformed stylo plants, expressing marker genes, was achieved. The presence of the introduced gene and its Mendelian inheritance have been demonstrated. In the case of cassava, regenerated somatic embryos are growing in a selection medium, following transformation with *A. tumefaciens*. In addition, expression of a reporter gene was obtained in cassava somatic embryos 60 days after particle bombardment.

Plant regeneration of five *Brachiaria* spp. through somatic embryogenesis and organogenesis and of *Arachis pintoi* through organogenesis was obtained. Likewise, regeneration of plants from isolated protoplasts of an *indica* rice variety was achieved. These results are critical for developing methodologies for future implementation of novel strategies of germplasm enhancement.

3.4.4 **Technologies transferred to programs and units**. As a means of enhancing the integration of biotechnology tools into CIAT program and unit activities, responsibility for the following activities was shifted from the BRU to the respective Program/Unit during the period 1988-90:

- Rice anther culture to the Rice Program: 1988-89
- Cassava *in vitro* genebank to the GRU: 1989
- Cassava germplasm exchange *in vitro* to the GRU: 1989
- Isozyme fingerprinting of cassava germplasm to the GRU/Cassava Program: 1988
- Bean germplasm characterization using phaseolin markers to the GRU: 1988
- The implementation of a Molecular Markers Applications Lab, will be carried out shortly as a means of increasing the application of molecular genetic markers at CIAT.

3.4.5 **Training**. Nearly 90 people from NARS of 20 countries in Latin America, Asia and Africa have participated in various training activities on tissue culture, biochemical and molecular characterization of genetic resources at CIAT. Future training will emphasize advanced degree theses and in-service research, linked as much as possible to research projects in the NARS.

3.4.6 **Biosafety**. Biosafety Guidelines and an Institutional Biosafety Committee (IBC) have been organized at CIAT to oversee and advise the Director General and Management on Center research using R-DNA organisms and their eventual release into the environment (small-scale testing). The Colombian Institute of Agriculture (ICA) has appointed a representative to the IBC. The guidelines have been prepared to adapt to the latest developments in the field and abide to the oncoming Colombian legislation.

3.4.7 **Complementary future initiatives**:

- The BRU's activities in the characterization of genomic structure and resistance/tolerance mechanisms for implementing genetic manipulation approaches and broadening the genetic base of crops will be complemented with activities for characterizing natural plant-microbial systems as a means to develop safe biocontrol methods.

- The Unit will develop molecular markers to analyze the genetic diversity in CIAT agroecosystems, and draw relationships between plant populations and gene pools, and describe gene flow from ancestral to cultivated species. The goal is to optimize strategies for the *in situ* and *ex-situ* conservation and utilization of critical components of the biodiversity. The biodiversity in CIAT agroecosystems.

- In the oncoming years the BRU will contribute to the Center's activities designed to enhance public awareness on issues related to biotechnology, such as environmental impact, equity, intellectual property protection and access to, and use of, genetic resources.

CONTENTS

INTRODUCTION

INTRODUCTION

1. BIOTECHNOLOGY RESEARCH AT CIAT

The recent evolution of biological sciences has added new dimensions to research by enhancing our ability to conduct detailed analyses of the genetic structure, diversity and processes of living organisms at the molecular and cellular levels. These new approaches, known collectively as biotechnology, are becoming powerful tools in contemporary research with plants and associated living organisms.

Most developments in biotechnology have begun in the developed world. There the speed of change in research is faster; the promise of the new research approaches and tools is becoming increasingly clear, and it is evident that the pay-off will be enormous, with the private sector playing an ever-increasing role in research and development. Most research in developed countries is built upon a wealth of information on the biology of commercially important plant species and is targeted to well-defined, organized markets. In the tropical countries, however, environments are less favorable, the people are poorer, and markets are smaller and more diffuse. For most crops important to developing countries, information is scarce; therefore, it is hardly surprising that contemporary biological research has, to a large extent, ignored developing countries' specific needs.

The international agricultural research system has a comparative advantage for providing germplasm-based technology that will be strengthened by applications of the new biology. Public research institutions in developed and developing countries can jointly approach researchable problems in tropical agriculture, with biotechnology improving their capacity to provide adequate solutions.

CIAT's involvement in biotechnology research should be viewed in light of the foregoing considerations and the ever-increasing gap between the progress being made in developed countries and that which is evident in most tropical developing countries. As an international public research institution, CIAT shares the responsibility of ensuring that the benefits of biotechnology be achieved for long-range public goals including growth, equity and good environmental stewardship.

2. EVOLUTION OF BIOTECHNOLOGY RESEARCH AT CIAT

CIAT has constantly paid attention to innovations in science and technology that can result in quick pay-offs in terms of technology development. In its long-term plan for the 1980s, CIAT anticipated its involvement in monitoring and applying biotechnology. In fact, work in tissue culture for the conservation of cassava germplasm had already begun in 1978-79. This work was followed in the early 1980s

by the introduction of anther culture techniques for accelerated rice germplasm improvement. The panel of CIAT's second External Program Review (1984) recommended establishing an interdisciplinary research structure comprised of those related disciplines that would interact increasingly with all commodity programs in the future. In its response to this recommendation, CIAT agreed on the concept of a cross-commodity effort in the form of a research unit devoted to the emerging field of biotechnology. The BRU was established in 1985 as a cross-center research support unit. During the period 1985-87, CIAT biotechnology research included cassava, common beans, rice and tropical forages, with applications of tissue culture and biochemistry-based techniques to research constraints in these crops. At the same time, CIAT began to develop research links in the form of collaborative projects with advanced research institutions.

The period 1988-1992, which is the time frame covered by this report, was significant in the evolution of biotechnology research at CIAT. Not only were the BRU research and collaboration efforts further concentrated on all CIAT crops, but they were expanded to include studies of selected microorganisms; and the most advanced biochemical and molecular techniques were incorporated, to address important research constraints (Table 1). Prioritization of crop production and utilization constraints led to the strengthening of international collaborative research which, in the case of cassava and beans, took the form of biotechnology and advanced research networks, respectively. As shown in Table 1, CIAT biotechnology research has been constraint-oriented, striving to develop practical methods and techniques for integration into other CIAT units and programs. A large portion of the BRU's activities have been carried out with special project funding.

2.1 Role, Objectives and Strategies of Biotechnology Research at CIAT

i. **Role**. The role of biotechnology research at CIAT is to develop applications of modern biological methodologies for increasing the efficiency of CIAT's strategic research.

ii. **Objectives**. Biotechnology research at CIAT addresses three broad objectives:

- characterization of genetic diversity and identification of gene pools and useful genes
- characterization of mechanisms involved in plant biotic and abiotic stresses for germplasm improvement
- broadening the genetic base of crops and germplasm conservation

CIAT biotechnology research focuses on the following germplasm: cassava (*Manihot esculenta* Crantz) and its wild relatives; common beans (*Phaseolus vulgaris* L.) and related cultivated and wild *Phaseolus* spp.; rice (*Oryza sativa*) for Latin America; selected tropical forage species such as *Brachiaria* spp, *Arachis* spp. and *Stylosanthes* spp.

iii. **Strategies**. To achieve these objectives in biotechnology research, CIAT has implemented the following strategies:

- **Creation of the BRU**. Organized in 1985, the BRU is designed to perform as a scientific bridge for developing and using cellular, biochemical and molecular methods and techniques, and for addressing priority constraints in CIAT germplasm development research.

- **Integration of biotechnology with strategic research**. First, constraints in CIAT germplasm research that may be amenable to biotechnological solutions are identified. The BRU monitors developments in basic research worldwide and brings new information to CIAT. Then constraint-oriented methodological research is carried out to develop and use biotechnology tools. Collaboration with CIAT scientists and advanced labs is essential for implementing this strategy.

- **Networking**. The BRU cooperates with CIAT programs and units to bring prioritized research constraints to the attention of the world scientific community and donors. International cooperative efforts and effective research linkages are established between research institutions in developed and developing countries and the strategic research done at CIAT.

- **Institution building**. CIAT's bridging role with the NARS of developing countries is accomplished through training of developing country scientists, advanced degree thesis research and specialized workshops, conferences and visits to the NARS. Development and stimulation of awareness on biotechnology-related issues such as biosafety and intellectual property protection are also included as part of CIAT's institution building in biotechnology.

3. ORGANIZATION OF THE BRU

In carrying out its bridging role to integrate biotechnology into CIAT germplasm development research, the BRU has, on the one hand, developed collaborative links with research organizations in developed and developing countries, on the other

hand, the BRU has strengthened its scientific and technological capabilities in order to take full advantage of new scientific advances and tools.

Access to adequate scientific, technical and physical resources has been essential to accomplish the Unit's objectives. Table 2 shows the evolution in core and complementary staffing of the BRU through the period 1980-92. The current three core senior positions in the areas of plant cell tissue culture and genetic transformation, molecular markers and mapping and molecular biochemistry have been considered the minimum core research team in the BRU for developing applications of biotechnology at CIAT. The participation of postdoctoral and visiting scientists has been essential to tackle specific research projects.

In the last 3 to 4 years, the BRU has developed three interrelated research activities, interfacing in research at the cellular, biochemical and molecular levels with responsibilities across CIAT crop germplasm:

- Characterization of genetic structure for assessing genetic variability and identifying gene pools and useful genes by using molecular linkage maps, gene tagging and DNA fingerprinting techniques. The objective is to characterize more accurately genetic variability and develop improved strategies for germplasm enhancement and conservation, as well as contribute to a more efficient management of natural resources. This activity is a responsibility of the molecular markers/mapping group.

- Characterization of mechanisms involved in resistance and tolerance to biotic and abiotic stresses in plants. To identify factors underlying resistance/tolerance in selected plant-pathogen/pests and physiological/quality relationships and develop efficient germplasm-screening methodologies, gene cloning and manipulation. This activity is a responsibility of the genes molecular biochemistry group.

- Broadening the genetic base of crops and germplasm conservation to develop novel sexual and nonsexual gene transfer strategies using hybridization, tissue culture and genetic transformation technologies, and *in vitro* germplasm conservation approaches. The objective is to achieve alien gene transfer, gene pool development and germplasm conservation to broaden the genetic base of CIAT germplasm adapted to specific agroecosystems. This activity is a responsibility of the tissue culture/genetic transformation group.

The three research groups of the Unit interact to approach selected constraints in cooperation with CIAT Programs/Units: Analysis and understanding of the genetic makeup of crops and associated microorganisms will provide tools for identifying useful genes and leads to more efficient genetic manipulation. The work to identify biochemical/molecular factors involved in plant-stress interactions will lead to the

identification of points for genetic intervention and will facilitate manipulation of these factors and genes. Work on the introduction of useful genes to selected crop germplasm background, and the analyses of their expression in tissues and full grown plants, and the assessment of their effects will contribute towards the broadening the genetic base and enhancing CIAT germplasm.

4. ACTIVITIES OF THE BRU DURING THE PERIOD 1988-92

Table 3 lists the research topics by commodity, presented under four main BRU research activities. Information is also given on the type of project (i.e., funded through CIAT core or complementary), its status (i.e., initiating, ongoing and whether information and/or technology have already been made available). Of the 34 activities in which the Unit has been involved in the period 1988-1992, 18 were complementary, i.e. financed through special projects. A list of special projects for the period 1988-1992 and the respective donor agencies is shown in Table 4. Table 3 also shows that, cropwise, *Phaseolus* beans and cassava have received highest, and about equal, attention by the Unit, followed by tropical forages and rice. An important observation in Table 3 refers to the six technologies that were shifted to the GRU, the Rice and Cassava programs for use within ongoing germplasm research activities of the programs.

A fundamental activity of the BRU is the implementation of complementary research on specific topics for each CIAT crop through special project funding. Table 4 summarizes such BRU activities for the period 1988-92. Of 14 activities, 7 corresponded to *Phaseolus* beans, 5 to cassava and 2 to rice.

Table 1. Evolution of biotechnology research at CIAT during the period 1980-1992.

Challenges	Methods and Tools	Periods		
		1980-84	1985-87	1988-92
1. Germplasm conservation & characterization	· *In vitro* culture	Cassava		Cassava
	· Cryopreservation			
	· Protein/isozyme fingerprinting		Cassava Beans Tropical Forages	
2. Slow genetic fixation, sterility	· Haploid induction	Rice		
3. Methodological	· Plant regeneration from somatic cells		Stylosanthes Cassava	Rice Brachiaria
4. Genetic structure · plants	· DNA fingerprinting			Beans Cassava Rice
· pathogens				Pyricularia Xanthomonas Colletotrichum
5. Identification (tagging) of resistance gene(s)	· Genetic maps · Gene tagging			Rice Beans Cassava Brachiaria
6. Mechanisms of plant stress interactions	· Biochemical/molecular tools · Characterization of genetic products · Gene cloning			Beans Cassava Trop. Forages
7. Broadening the genetic base of crops	·Interspecies/hybridization			Beans
	· Genetic transformation			Stylosanthes Cassava Beans
8. Advanced research networks				Cassava Beans

Table 2. Staffing of biotechnology research.

	1980-1984	1985-1987	1988-1992
1. Core			
1.1 Senior scientists			
· Tissue culture/Genetic transformation	1	1	1
· Molecular markers/mapping			1
· Molecular biochemistry			1
1.2 Postdoctoral scientists			
· Biology		2[a]	
· Genetics		1	
2. Complementary			
2.1 Postdoctoral scientists			
· Genetics/Tissue culture		1	0.5
· Molecular Biology			1[a]
· Biology			1[a]
TOTAL	1	5	5.5

a) Duration of appointments: 1-2 yr within the respective period.

Table 3. Summary of activities in *Phaseolus* beans, cassava, rice and tropical forages biotechnology (1988-92).

Activities	Type of Project	Project Status
1. Characterization of Genetic Structure		
Phaseolus Beans		
1.1 *P. vulgaris* wild-weedy complex	Core	Available(a)
1.2 *P. vulgaris* wild/cv core collection	Core	Ongoing
1.3 Construction *P. acutifolius* molecular map	Complementary	Ongoing
1.4 Tagging BGMV/*Acanthoscelides* resist. genes	Core/Complementary	Ongoing
Cassava		
1.1 Isozyme charact. of *M. esculenta* germplasm	Core/Complementary	Available[(a)]
1.2 DNA fingerprinting of wild *Manihot* spp	Complementary	Initiating
1.3 Construction cassava molecular map	Complementary	Ongoing
Rice		
1.1 DNA fingerprinting of L.A. blast isolates	Complementary	Ongoing
1.2 Tagging RHBV resist. genes	Complementary	Ongoing
1.3 Tagging blast resist. genes of L.A. rice elite lines	Complementary	Ongoing
1.4 DNA fingerprinting	Core	Ongoing
Tropical Forages		
1.1 Isozyme fingerprinting: *Brachiaria*, *Stylosanthes*, *Centrosema*, *Desmodium*	Complementary	Available[(a)]
1.2 Construction *Brachiaria* molecular map	Core	Initiating
2. Characterization of Mechanisms Involved in Resistance and Tolerance to Biotic and Abiotic Stresses in Plants		
Phaseolus Beans		
2.1 Resistance to *Acanthoscelides*	Complementary	Ongoing
2.2 DNA fingerprinting and pathogenicity (Xanthomonas)	Core	Ongoing
2.3 DNA fingerprinting and pathogenicity (Colletotrichum)	Core	Ongoing
Cassava		
2.1 Characterization and localization of photosynthetic enzymes	Complementary	Ongoing
2.2 Characterization of bacterial amylolytic activities	Core	Ongoing
Tropical Forages		
2.1 Resistance to spittlebug		

3. Broadening the Genetic Base of Crops and Germplasm Conservation

Phaseolus Beans		
3.1 Interspecies gene transfer (gene pool development)	Core	Ongoing
3.2 Genetic transformation	Core/Complementary	Ongoing
Cassava		
3.1 *In vitro* active gene bank	Core/Complementary	Available[a]
3.2 Cryopreservation	Core/Complementary	Ongoing
3.3 Genetic transformation	Core	Ongoing
Rice		
3.1 Haploids for germplasm improvement	Complementary	Available[a]
3.2 Plant regeneration/Transformation	Complementary	Ongoing
Tropical Forages		
3.1 *Brachiaria* germplasm transfer	Core	Available[a]
3.2 *Stylosanthes* intra accession variabil.	Core	Available
3.3 *Brachiaria* & *Arachis* plant regeneration	Core	Ongoing
3.4 *Stylosanthes* genetic transformation	Core	Available
4. Institution Building		
4.1 Biotechnology/Advanced Res. Networks		
Cassava	Complementary	Available[a]
Beans	Core	Ongoing
4.2 Training (all commodities)	Core/Complementary	Ongoing
4.3 Biosafety	Core	Ongoing
4.4 Biotech advances (Internal Seminars)	Core	Ongoing

a) Activities which were developed in the BRU and then to the respective CIAT Commodity Programs and Units.

Table 4. Complementary research activities financed through special projects in the period 1988-92: Collaboration of BRU with CIAT commodity programs/units.

Topic	Duration (yr)	Termination Date	Funding
1. Cassava *in vitro* conservation (pilot study)	3	Dec. 1990	IBPGR
2. Cassava cryopreservation	3	Feb. 1991	IBPGR
3. Cassava beans & tropical forages isozyme fingerprinting	3	Dec. 1988	IDRC
4. Rice haploid induction	3	Dec. 1991	RF[a]
5. Rice gene tagging/regeneration (RHBV, blast)	-	On going	RF[a]
6. Cassava molecular mapping	3	Sep. 1994	RF
7. Cassava biotechnology network	5	Aug. 1997	Dutch DGIS[b]
8. *Phaseolus* beans gene tagging[d]	5	Sep. 1996	Belgian AGCD[a]
9. Tepary bean molecular mapping[d]	5	Sep. 1996	Belgian AGCD[a]
10. Resistance mechanism to bean weevil[d]	5	Sep. 1996	Belgian AGCD[a]
11. *Phaseolus* bean regeneration	3	Aug. 1992	GTZ[c]
12. *Phaseolus* bean transformation	2	Dec. 1991	IDRC[c]
13. Common bean interpool recombination	2	Aug. 1992	CIDA[c]
14. Bean CBB molecular characterization	2	Dec. 1993	IDRC[c]

a) Two-three research projects covered under a grant.
b) Under Cassava Program and BRU.
c) PhD student fellowships.
d) After 3 years these projects will be followed by other priority research.

RESEARCH PROGRESS AND ACHIEVEMENTS

I. CHARACTERIZATION OF GENETIC STRUCTURE

1. MOLECULAR MARKERS

1.1 Understanding the Genetic Base of *P. vulgaris*: Studies on Wild, Wild-Weed-Crop Complex and Selected Cultivated Germplasm (Collaboration: D.G. Debouck (previously at the GRU, now at the IBPGR) and O. Toro (GRU)

The established techniques of one dimensional SDS/PAGE, two dimensional IEF-SDS/PAGE and isozymes were implemented at the BRU with certain modifications to improve the resolution of the gels (Vargas 1988). These techniques were transferred to the GRU (1991) and to the germplasm characterization section of the Bean Program (1992). The analysis of seed protein and isozymes were used to:

- study the genetic diversity of wild and cultivated common beans
- follow the genetic flow between wild and cultivated genotypes
- assess the variability of *P. vulgaris* within and between the two main gene pools
- look at dissemination pathways in selected African countries

Phaseolin was used as a molecular marker to characterize the genetic structure of *P. vulgaris*. Phaseolin, which accounts for ca. 40% of the total seed storage protein of common beans, is coded by a multigenic family of closely linked genes and is highly heritable (Brown et al. 1981). Gepts (1984, 1988) demonstrated that phaseolin is a good evolutionary marker in *P. vulgaris* because of its genetic characteristics. The advantages of using phaseolin as a marker are: (1) the probability of the same variant being produced several times during the history of the crop is extremely low given the complexity of the marker; (2) it is not influenced by the domestication process, the selection pressure being on other characters; and (3) it is not influenced by the growing conditions.

Phaseolin analyses conducted in various labs including CIAT (reviewed in Debouck and Tohme 1989) have provided additional evidence about a separate domestication in Mesoamerica vs the Andes. It has been possible to establish the origin of common bean cultivars and their dissemination pathways. This finding is also supported by isozyme analysis of Latin American landraces, which resulted in contrasting isozyme profiles for the two regions (reviewed in Gepts and Debouck 1991).

The survey conducted by the BRU from 1988 to 1991 concentrated on wild populations of *P. vulgaris* and selected landraces from the Andean regions. Wild common beans display a high variability in their most important storage protein

between the different regions and within them. This variation is of obvious interest when it can be used as a geographic marker in evolutionary studies. In this case the different variants found in the wild are checked for their presence in the cultivated germplasm (or vice versa), and deductions can be made about places of domestication. For instance, a particular phaseolin type 'Sd' was found in the most traditional landraces of Durango and Zacatecas, Mexico (Koenig et al. 1990), and the same phaseolin was identified in the wild bean growing in Durango (Tohme, personal observation, 1990). The 'C' phaseolin, once thought to be a hybrid type between the 'T' and the 'S' (Brown, 1981), was recently identified in the wild forms growing in Apurimac, Peru (Vargas et al. 1990).

Several new phaseolin variants were identified in both the Mesoamerican and Andean centers as more wild *P. vulgaris* germplasm were made available for screening. The phaseolin types from Mesoamerica have been extended from the previously characterized 6 phaseolins, M1 to M6 (Romero and Bliss 1986), to 19 (Toro et al. 1990 for a partial listing) (Fig. 1). Some phaseolin variants such as M1 are more widely distributed across Mexico; others are more localized geographically (Table 1). The diversity within populations was quite variable. Certain accessions had four phaseolin types within one accession. These data stress the need for sufficient sampling when running seed protein electrophoresis. In our lab we tried whenever possible to use 5-8 original seeds per population.

The survey also provided additional data as to the distribution of already identified phaseolin types. The range of distribution of accessions with S phaseolin was increased to several regions of Mexico and Guatemala. Sd phaseolin has also been identified in newly characterized accessions from Durango, Mexico. These data will be crucial to determine the possible site of domestication in Mesoamerica because all the cultivated Mesoamerican germplasm evaluated to date have only the S phaseolin and, to a lesser extent, Sd phaseolin.

Of particular interest in the Mesoamerican characterization is the presence in Guatemala of phaseolin CH previously identified in Colombian accessions (Fig. 2a). As mentioned in previous reports the available data do not clarify the relationship of the Colombian wild *P. vulgaris* with either Mesoamerican or Andean germplasm. We are currently investigating such questions with mtDNA probes and RAPD markers.

Two new phaseolins were identified in Colombian wild *P. vulgaris* from newly collected populations from Cundinamarca (Fig. 2b). The characterization of these new phaseolins, determined in one dimension, is being extended to two dimensional IEF-SDS/PAGE (Fig. 2d). The identification of these new variants is illustrative of the incomplete survey for both cultivated and wild materials. Additional collections and an ampler sampling strategy will be required in the future to fill gaps in the collection.

The characterization of wild populations from the Northern Andes was completed. Phaseolin I (Fig. 3a), identified previously in two populations from Cajamarca, Peru (Koenig et al. 1990), is present in the populations collected from Chimborazo and Azuay, Ecuador, and Piura, Peru. Phaseolin I, which lacks the 52 kD band frequent in T phaseolin, appears to be localized in wild accessions from that range of the western side of the Northern Andes. Most populations are quite uniform with respect to phaseolin. Only one weedy accession with phaseolin (from Ecuador) had the T phaseolin, suggesting a possible recent introgression with Andean cultivated germplasm. An initial survey on selected populations from that region--conducted at Michigan State U. in collaboration with the BRU, using mtDNA probes--indicates that such germplasm is unique and highly polymorphic. An extensive study is currently under way at CIAT using mapped mtDNA clones provided by S. Mackenzie from Purdue U.

In the southern Andes, phaseolin T, C and H were identified in several populations. Several subvariants of H phaseolin were characterized by two dimensional gels (Fig. 4a). Phaseolin A, a very rare type previously identified in one nuña genotype, was found in one wild population from Apurimac (Fig. 4b). Two new types, phaseolin To and Ta, were identified in the germplasm from Bolivia (Tohme et al. 1989) (Fig. 4c). To stands for Totora while Ta for the region Tarija where the collections were made. These two phaseolins have not yet been identified in wild populations or cultivated genotypes. Phaseolins from Argentina were also characterized. The polymorphic J group, classified by Koenig et al. (1990), was split into three new phaseolins J1, J2 and J3 as a result of the differences detected in one and two D gels (Fig. 5).

The results obtained demonstrate that wild beans are much more variable than cultivated common beans (Table 2). The characterization was also extended to the wild populations of *P. acutifolius*. The research was carried out in the BRU by researchers from the GRU. Nine more phaseolin types were identified in addition to the fifteen already known. A new phaseolin type was found in the cultivated genotypes (DGD-288 from Durango, Mexico). The reduction of genetic diversity in both gene pools--although to a different extent in *P. vulgaris* and in *P. acutifolius*--points to the amount of variability still untouched in the wild populations. The implications of the founder effect and its extent are still to be determined. Studies are being conducted using molecular markers such as RADPs and hypervariable probes to address such issues.

1.1.1 **Wild-weed-crop complex**. Recent collections, mainly in the Andes, suggest (1) a natural genetic flow between sympatric wild forms and primitive cultivars; and (2) the crossing between cultivated genotypes. In several sites in Apurimac and Cuzco, Peru a dozen weedy or intermediate types were also found in the same field with wild and truly cultivated types. That situation and the comments made by the farmers led to the hypothesis of a "wild-weedy-crop complex" (Debouck et al. 1989),

where the weedy types result from crosses between the wild and the cultivated forms. One possible outcome of such a complex is that the wild types could have enriched the primitive landraces.

Two such complexes from Apurimac and Cuzco, Peru were analyzed with phaseolin and seven isozyme systems (Gutierrez 1991). The data were analyzed by factor analysis of correspondence (SAS). The analysis separated the complex into wild, weedy, escapes, recombinant and cultivated forms. Of the five classes the wild group was the most diverse, with isozymes profiles not identified in the cultivated germplasm studied; e.g., alleles null and 100 for GOT_1, PRX 100 and DIAP1 100 (Tables 4 & 5). The cultivated forms were less variable but still had some unique profile; e.g., the allele PRX 98 and GOT_1 98. The weedy, escapes and recombinant forms were variable, suggesting a flow between the wild and the cultivated forms. We have started to use RFLPs, RAPDs and mtDNA to dissect the complex better and provide an understanding of the dynamics of *in situ* habitats of wild and cultivated forms in the Andes and the implication of such genetic flow from wild populations and/or early cultivars in recently domesticated stocks.

1.1.2 **Nuñas**. Nuñas are a class of *P. vulgaris* that are eaten toasted. It is an ancient group of cultivars, possibly domesticated in preceramic times for their ability to burst once heated. Their current range of distribution extends from Cajamarca, Peru, to Chuquisaca, Bolivia. Thus far, several phaseolin types have been identified in the nuñas (Table 3). Interestingly, the types found in the normal landraces of beans are also present in the nuñas in addition to certain types identified only in this group such as phaseolin A, Ko (Fig. 4a) and K. Ko was identified in a nuña from Bolivia. Nuñas in Bolivia are referred to as "kopuru". We are planning to expand the survey of the nuñas with other molecular markers in order to determine whether they represent a truly ancient group.

1.1.3 **Conclusions**

- There are more phaseolin types in the wild materials than in the cultivated ones. This implies that just a part of the genetic diversity existing in the wild was domesticated. As in several other crops, a founder effect has occurred in the Andes and Mesoamerican centers. The extent of reduction of variability for both centers is yet to be determined.

- The relationship of Colombian wild populations with the Andean and Mesoamerican centers needs additional studies. We plan on using mtDNA to address this issue.

- The Northern Andes wild populations have a unique molecular make-up.

- More variability exists in the southern Andes than previously thought. The genetic flow between wild and cultivated germplasm has been determined. The implication of such flow on the cultivated germplasm is being studied with molecular markers such as RAPDs and mtDNA.

- The nuñas as a group are quite diverse and might represent an ancient group.

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Table 1. Presence of phaseolin types in Mesoamerican wild and weedy populations of *P. vulgaris.*

	Country			
Phaseolin	Mexico	Guatemala	El Salvador	Costa Rica
M1	X			X
M2	X			
M3	X			
M4	X		X	
M5				
M6	X			X
M7		X		X
M8	X			
M9	X			
M10	X			
M11		X		
M12	X			
M13		X		
M14	X			
M15		X		
M16	X			
M17	X			
M18	X			
S	X	X		
CH		X		

Table 2. Phaseolin types found in wild-weedy and landraces of *P. vulgaris.*

Country	Wild-Weedy Populations	Landraces
Mexico	Ms, S, Sd	S, Sd, T
Guatemala	Ms, S, CH	S, B
Costa Rica	M1	S, B
Colombia	CH, B, S, Mu*	S, T, C, H, B
Ecuador	I, T	T, C
Peru		
North	I	T, C, H, S
Center	T, C	T, C, H, A, S
South	T, C, H, A, K	T, C, H, S
Bolivia	T, To, Ta	T, C, Ko
Argentina (NW)	T, H, C, J1, J2, J3	T, H, C

* New phaseolin.

Table 3. Distribution of phaseolin types in nuñas from Peru and Bolivia.

Country	Phaseolin Types							
	T	H	C	Ko	A	To	H , C polymorphic	Total
PERU								
Amazonas	2	1				1		3
Cajamarca	38	11	10	2				62
La Libertad	25	6	5					36
Ancash	7	2	3					12
Huancavelica		1				1		1
Huanuco	13	5	2					21
Junín	2	1						3
Apurimac	7		2		1	1	6	16
Ayacucho	7	3			1			11
Cuzco	1		3					4
Lima	1							1
BOLIVIA								
Chuquisaca							1	1
Cochabamba	33	1		2			2	8
La Paz			1				1	5
TOTAL	109	31	26	4	2	3	10	185

Table 4. Phaseolin and isozyme characterization of wild weedy complex from Apurimac, Peru.

Group	Phaseolin	Seed Size	Isozymes						
			GOT_1	RBSC	ACP_3	$DIAP_1$	$DIAP_2$	MDH	PRX
Wild	T	Small	nule	96	98	100	100	98	100
	C/H		100	98	100				
				100					
Weedy	T	Small	96	96	96	98	98	96	98
	C	Medium	98	98	98	100	100	98	100
		Large	100	100	100			100	
Wild-weedy	T	Small	98	96	98	98	100	98	98
	H	Medium	100		100	100		100	100
Wild-weedy	T	Small	98	98	96	98	100	98	100
escape	C	Medium	100	100	98	100		100	100
		Large							
Weedy	T	Small	98	98	98	100	100	98	98
escape	C	Medium	100	100				100	100
		Large							

Table 5. Phaseolin and isozyme characterization of wild weedy complex from Cuzco, Paruro, Peru.

Group	Phaseolin	Seed Size	Isozymes						
			GOT_1	RBSC	ACP_3	$DIAP_1$	$DIAP_2$	MDH	PRX
Wild	H	Small	100	96 98 100	100	100	100	98 100	100
Weedy	H	Small Medium Large	100	98	98	100	98 100	100	100
Wild-weedy	C	Small Medium	100	98	96 98	100	100	100	100
Wild-weedy cultivated	T C H	Small Medium Large	100	96 98 100	96 98	100	100	98 100	100
Weedy cultivated	T C H	Small Medium Large	98 100	100	98	100	100	98 100	100

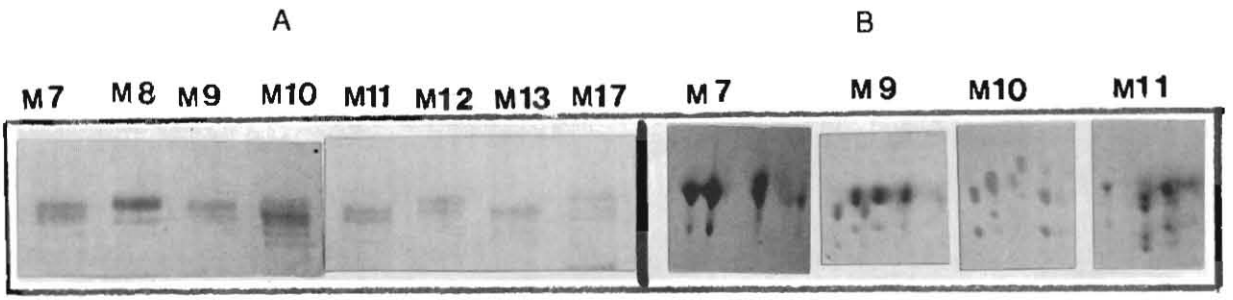

Figure 1. One-dimensional SDS/PAGE and two-dimensional IEF-SDS/PAGE of wild accessions from Mexico and Guatemala: (a) one D gel of M type phaseolin; (b) two D gels of M types.

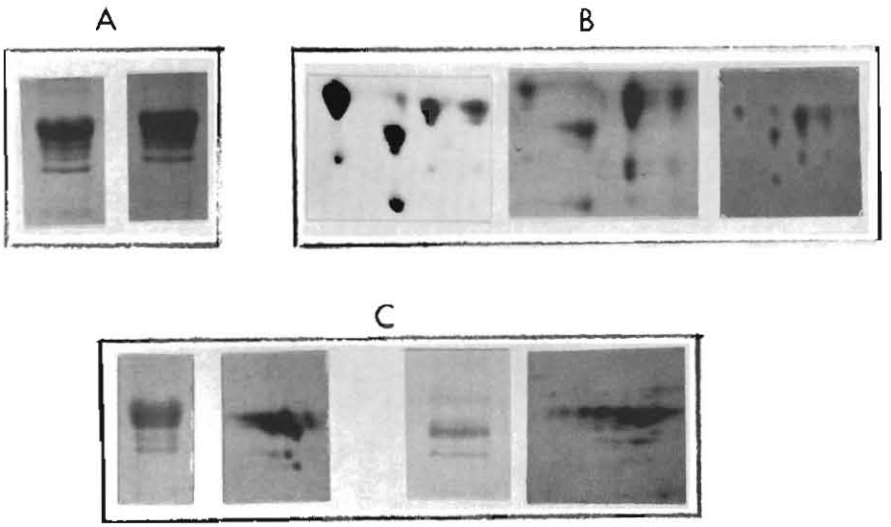

Figure 2. One-dimensional SDS/PAGE and two-dimensional IEF-SDS/PAGE of wild accessions from Guatemala and Colombia: (a) one D gel of CH phaseolin of G21113 (Colombia) and G 19907 (Guatemala); (b) two D gels of phaseolin CH of G21113 and G12855A and G12854 (Guatemala); (c) one and two D gels of standard Colombian B phaseolin, one and two D gels of novel phaseolin from Cundinamarca, Colombia.

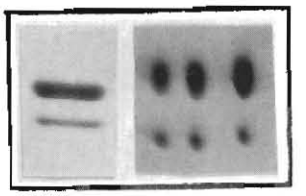

Figure 3. One-dimensional SDS/PAGE and two-dimensional IEF-SDS/PAGE of wild accession G23583 from Ecuador, Northern Andes.

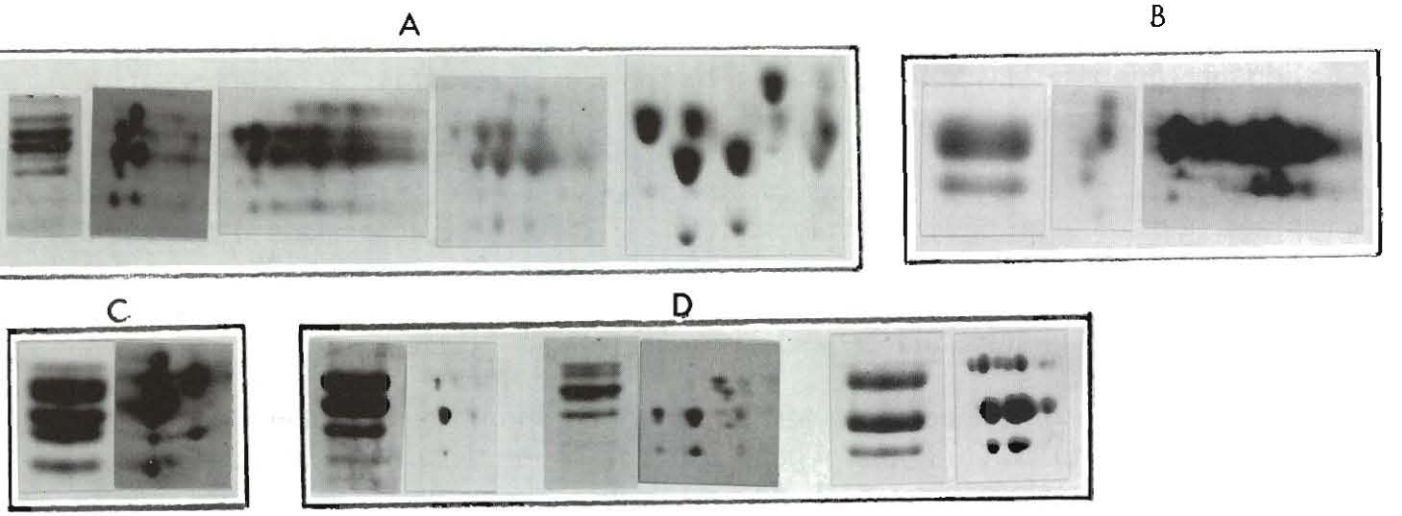

Figure 4. One.dimensional SDS/PAGE and two-dimensional IEF-SDS/PAGE of wild accessions from the southern Andes: (a) one D of standard H phaseolin and two gels of polymorphic H with G12199, DGD-2581, DGD-2580 and G23452; (b) one D and two gels of A phaseolin (G12058) and two D gels of DGD2161 from Peru; (c) Ko phaseolin in a cultivated genotype from Bolivia; (d) one D and two D gels of standard T phaseolin, To phaseolin and Ta phaseolin from Bolivia.

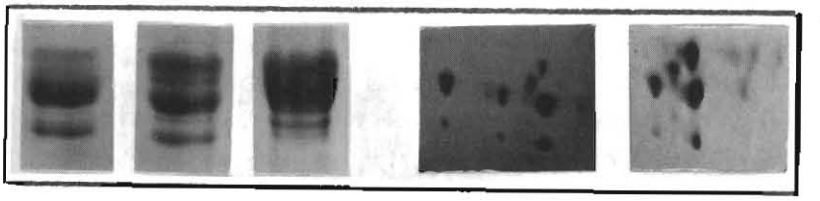

Figure 5. One-dimensional SDS/PAGE and two-dimensional IEF-SDS/PAGE of wild accessions from Argentina: one D gel of phaseolin J1, J2 and J3 from G19894, G19897 and G21197, respectively, two D gels of J1 and J2.

1.2 Formation and Molecular Characterization of a Common Bean Core Collection (Collaboration in the establishment of the core collection: M. Iwanaga and O. Toro, GRU; S. Beebe, Bean Program; and P. Jones, Land Use Program; in the characterization: S. Beebe, Bean Program)

1.2.1 **Background**. The *P. vulgaris* collection with ca. 24,000 accessions, stored at CIAT headquarters in Palmira, Colombia, is by far the largest collection of any food legume in the world. As part of CIAT's global mandate for conserving the *Phaseolus* cultivated species, the formation of a core collection (Brown 1989) is currently being pursued in order to promote the use of the germplasm in various breeding programs and facilitate the study of the crop's genetic diversity.

Using both an evolutionary and an agroecological approach, a *P. vulgaris* core collection was established, consisting first of a representative sample of the entire genetic variability of wild and cultivated germplasm of *P.vulgaris*. A collection of the complete genus will be considered at a later stage.

The wild core collection consists of 100 accessions selected to cover the whole range of distribution of wild *P. vulgaris* (Fig. 1). About 1200 accessions of cultivated common beans were identified from the primary centers. A sample of 300 accessions from the secondary centers was made based on seed characteristics and morphology. Elite lines and genetic stocks were also included.

The core collection will be characterized at the morpho-physiological and molecular levels in order to (1) study the genetic structure of *P. vulgaris* germplasm between gene pools and within gene pools and (2) assess the extent of the founder effect.

1.2.2 **Methodology**. The wild and cultivated core collections will be characterized differently. We plan on using initially only RAPD primers to analyze the cultivated core collection. The wild core collection will be studied initialy at the level of seed proteins (phaseolin and lectins) and RAPD primers. Minisatellite probes will also be used whenever available.

We looked at various components of the RAPD analysis that had already been implemented by the Unit for gene tagging to maximize the efficiency of screening a large number of genotypes. The first parameter was the selection of adequate primers. Some 300 primers (purchased from Operon Co., Calif.) are available at the BRU. The initial PCR profile is similar to Martin et al. (1991) and consists of 40 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min, followed by 7 min at 72°C. To be able to identify primers that will provide a maximum amount of information quickly, we screened the primers using two DNA bulks. Each bulk consisted of 15 genotypes from either the Andean or Mesoamerican gene pools (Fig. 2). Of the 60 primers screened thus far, 12 resulted monomorphic; 28 were polymorphic but with 1 or 2 amplification products; and 19 were polymorphic with more than 3 amplification products. Initial screening on the wild genotypes will be

carried using those primers having one amplification product. Screening more primers on the two bulks will also continue.

The second parameter looked at was the PCR profile itself. Several profiles have been tested at the BRU for rice fingerprinting. The profile selected for its time efficiency and resolution quality consists of 35 5-sec cycles for denaturation at 94°C, a 30-sec annealing at 36°C, and a 1-min extension step at 72°C, followed by 4 min at 72°C (Yu, K and K; Pauls 1992) (Fig. 3). The whole program takes about 3 hr on the Perkin Elmer--two h less than the regular PCR profile.

We are also looking at DNA concentrations, primer concentration, source of Taq (AmliTaq and Stoffel) and Mg^{2+} concentration. Screening of the wild accessions has already begun with the selected primers and the Yu PCR methods on DNA extracted from a single plant.

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Figure 1. Distribution of the wild core collection of *P. vulgaris* population selected for the RAPD fingerprinting.

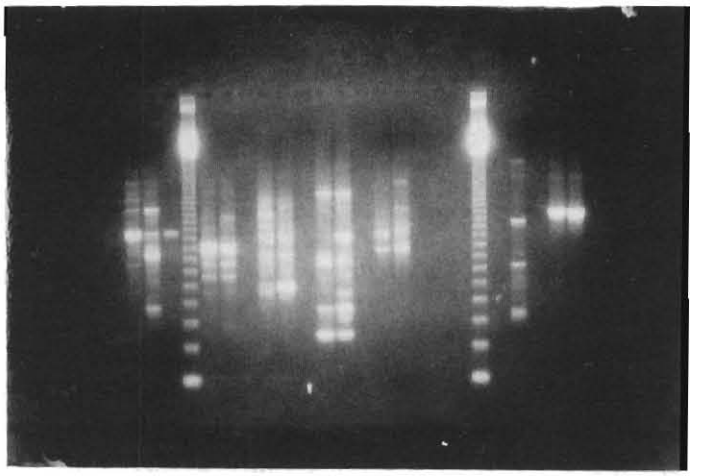

Figure 2. Primer performance on two DNA bulks from Andean and Mesoamerican genotypes; The primers used were A1, A2, A3, A4, A5, A7 and A8.

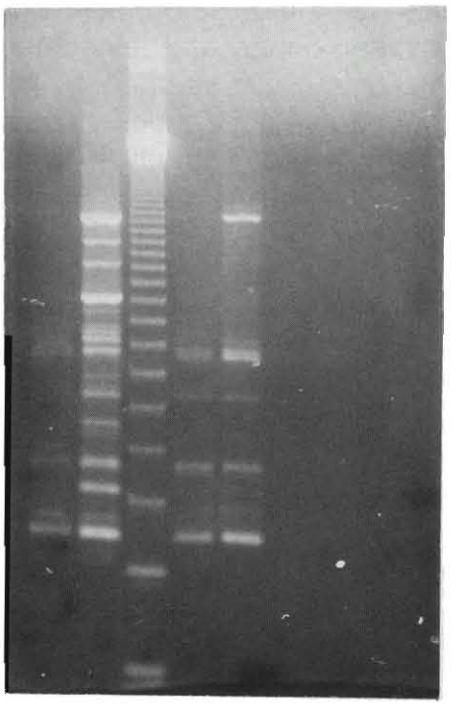

Figure 3. Amplification of Andean and Mesoamerican bulks using Yu and Pauls' profile; lanes 1, 4 and 5 are Mesoamerican DNA bulk at different DNA concentrations (12.5 and 25 ng); lane 2 is Andean DNA bulk at 25 ng DNA concentration; lane 3 is a molecular weight marker of 1 kb ladder.

1.3 Construction of a cDNA Library from Beans and Characterization of a Set of Clones for Polymorphism (Collaboration: J. Mayer, Molecular Biochemistry, BRU)

A small cDNA library from beans of about 1500 clones was constructed to saturate further the bean RFLP linkage map. A novel technique was used to generate the library: the primarily synthesized cDNAs were amplified by PCR using the adaptors as primers for amplification (Jepson et al. 1991); (Fig. 1). Inserts in the range of 500 bp were obtained. Then 64 clones were hybridized to digested DNA from three sets of parental lines. The clones were divided into 25 groups, 2 of which were repeated several times (11 and 8 times, respectively). Around 80% of the unique clones were single copy; 20%, low copy, as would be expected from a cDNA library.

The three pairs of parental bean lines were chosen for their agronomic traits and evaluated for polymorphisms (Fig. 2). G12952 is resistant to *Acanthoscelides obtectus*; APN 18, to anthracnose race Kappa and to *Apion*; DOR 60, to BGMV (bean golden mosaic virus). Polymorphism was highest as revealed by digestion with EcoRV (77%), followed by DraI (73%), EcoRI (63%) and HindIII (60%). The highest polymorphism was observed between the pair DOR 60 and APN 18, 71% for EcoRV and 57% for EcoRI, respectively (Table 1A and B).

Two clones from the two groups with the most repeated clones were analyzed by slot blot hybridization against the other clones and ribosomal DNA, to understand the origin of the repetitions. Only one clone seemed to be of ribosomal origin, as confirmed by the patterns obtained by hybridization to bean genomic DNA digested with HaeIII, implying that the whole group to which it belonged was of ribosomal origin. The repetition of clones is probably due to kinetic preferences of the Taq polymerase and the ratio of primers.

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Table 1. Summary of the level polymorphism of 35 representative clones (25 groups and 10 subgroups) using four restriction enzymes. A. Total polymorphism. B. Polymorphism for the individual pairs of selected parentals.

A

	EcoRI		EcoRV		HindIII		DraI	
++	9/35	25.7%	6/35	17.1%	1/35	2.8%	2/19	10.5%
+	7/35	20.0%	16/35	45.7%	4/35	11.4%	6/19	31.5%
±	6/35	17.1%	5/35	14.2%	16/35	45.7%	6/19	31.5%
-	13/35	37.1%	8/35	22.8%	14/35	40.0%	5/19	26.3%
Polym.	22/35	63.0%	27/35	77.0%	21/35	60.0%	14/19	73.0%

B

	EcoRI			EcoRV			HindIII			DraI		
	Calima x XR-235-1-1	DOR60 x APN18	Ica Pijao x G12952	Calima x XR-235-1-1	DOR60 x APN18	Ica Pijao x G12952	Calima x XR-235-1-1	DOR60 x APN18	Ica Pijao x G12952	Calima x XR-235-1-1	DOR60 x APN18	Ica Pijao x G12952
++	10	11	0	9	8	4	3	0	0	5	2	0
+	4	5	2	7	9	5	4	4	3	6	6	5
±	1	4	6	3	8	3	7	8	5	2	3	2
-	17	12	16	15	9	12	21	23	17	6	8	2
0	3	3	2	1	1	1	0	0	0	0	0	0
NE	0	0	9	0	0	10	0	0	10	16	16	26
Polym	15/35 42.8%	20/35 57.1%	8/26 30.8%	19/35 54.3%	25/35 71.4%	12/25 48%	14/35 40.0%	12/35 34.3%	8/25 32.0%	13/19 68.4%	11/19 57.9%	7/9 77.7%

++ Strong polymorphism; + Medium; ± Weak; - Polymorphism absent; NE Not evaluated; 0 Absence of bands.

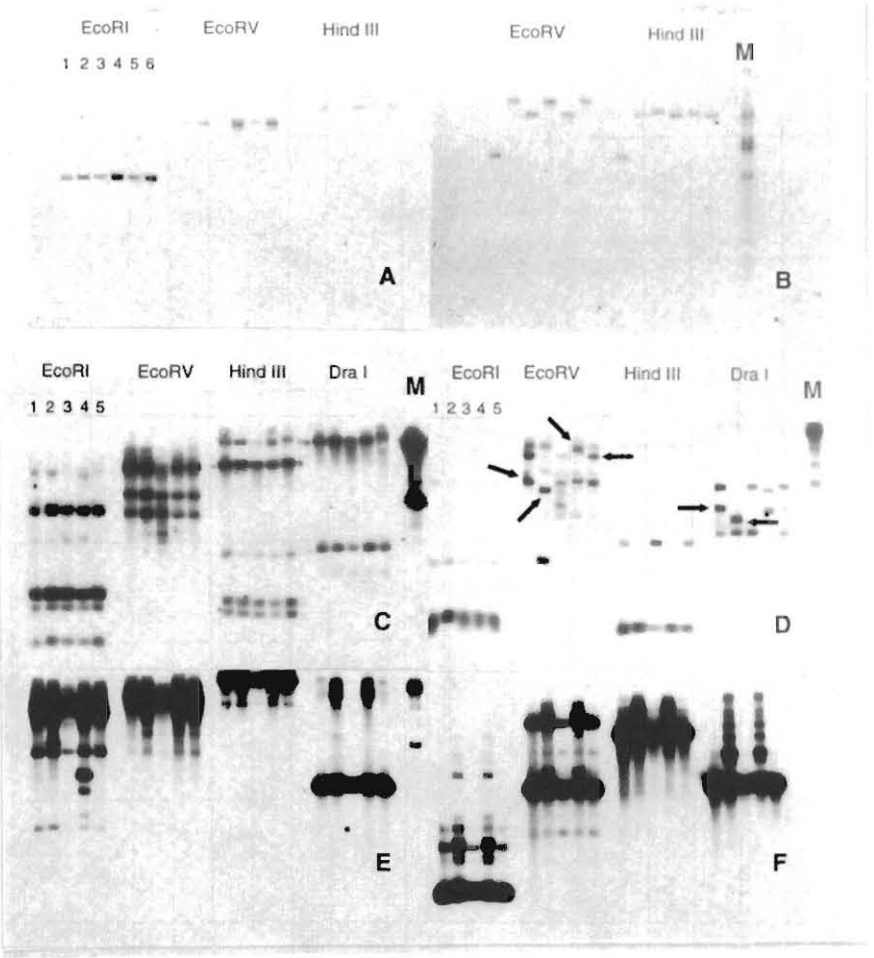

Figure 1. Southern blots of genomic DNA from the selected parentals hybridized with six different cDNA clones. (A-B) Single copy. (A) Non-polymorphic, (C-F) Low copy, (C) Non polymorphic, (D-F) Polymorphic.

1.4 Identification, Isolation and Characterization of Minisatellite Sequences in the Genus *Phaseolus*

1.4.1 **Background**. Multilocus DNA fingerprinting has several potential uses in germplasm characterization. Such DNA fingerprintings are based on the hybridization of plant DNA with specific repetitive DNA sequences known as minisatellites or VNTR (Variable Number of Tandem Repeats). The advantage of such probes over single-copy RFLP probes is obvious as they reveal information on several loci scattered through the genome, using only a single probe.

Minisatellite structures have been detected in a range of organisms and are now being studied much more frequently in plant genomes. Such minisatellites are a short core of consensus sequence, repeated in tandem and variable in number along the genome of a genotype. Several probes have already been isolated (Daly et al. 1991; Nybom 1991). The human minisatellites identified by Jeffreys have been used extensively to cross-hybridize to a wide range of plant species including beans (Stockton et al. 1992). These minisatellites contain a 10-15 bp GC rich core sequence. An internal sequence from bacteriophage M13 also detects polymorphic and hypervariable loci. More recently, oligonucleotides using simple repetitive sequences have been shown to reveal hypervariable regions in plants (Weising et al. 1991).

Possible applications of minisatellites in germplasm research include:

- identification and characterization of the genetic structure of a genus
- analysis of genetic relatedness of breeding materials and evaluation of genetic diversity of breeders' germplasm
- study and quantification of introgression between gene pools or between species
- selection tool in backcrossing programs to return quickly to the genetic makeup of the recurrent parent
- use as locus-specific probes for mapping and tagging

We have used M13 on wild and cultivated *P. vulgaris* genotypes (Fig.1); however, the resolution of the hybridizations was too low for adequate scoring. To address such limitations, we are trying to isolate minisatellite sequences in three genomic libraries from two *P. vulgaris* and one *P. acutifolius* genotypes. Based on the sequences of the minisatellite, we also plan to design oligonucleotides for PCR amplification (Jeffreys 1991). By being specific for the target genome, these sequences can give a better resolution and sharper bands than using repetitive

probes such as M13. They are also representative of many loci simultaneously and present a relatively low mutation rate (Jeffreys 1988; Wu 1992; Daly 1991), making them ideal for DNA fingerprinting.

Such sequences will be used to learn more about the *Phaseolus* genome organization and evolution. Initially these minisatellites will be used in DNA fingerprinting: to characterize further the Mesoamerican and Andean gene pools of *P. vulgaris*; to establish the degree of relatedness among bred lines; to facilitate the recovery of the recurrent parent genotype in backcrossing experiments; to study introgression in *P. vulgaris* x *P. acutifolius* recombinants from sexual crosses.

1.4.2 **Methodology**

i. *Phaseolus* DNA isolation and digestion. Two *P. vulgaris* and one *P. acutifolius* genotypes were chosen for the isolation of possible *Phaseolus* minisatellites: ICA Pijao, G23580B and G40110. ICA Pijao, which belongs to the Mesoamerican gene pool, is a common bean-bred material from ICA, the Colombian national agricultural research program. G23580B is a weedy *P. vulgaris* with phaseolin I, from Azuay, Ecuador. Phaseolin I is found only in wild or weedy populations from the Northern Andes. No cultivated common bean genotypes have been identified so far. G40110 is a cultivated *P. acutifolius* genotype form Campeche, Mexico.

Total genomic DNA was isolated from leaf material. After grinding 2-5 g of young leaf tissue with liquid nitrogen, three vol of a modified Dellaporta's buffer (Dellaporta 1983) was added (150 mM Tris-HCl (pH:7.5)/15 mM NaEDTA/1.05 M NaCl/1.5% CTAB/1.5% 2-mercaptoethanol/1% PVP) in a propylene tube and incubated for 20-30 min at 65°C. It was then extracted with phenol-chloroform 1:1 and with chloroform-octanol 24:1. DNA was precipitated with 1/10 vol of NaAc 3M pH:5.2 and 6/10 vol of isopropanol for one h at -20°C. After centrifugation, the DNA pellet was washed with 70% ethanol and re-suspended in TE buffer. It was incubated for 15 min at 37°C after adding 20 µg/ml of RNase. DNA was quantified using a Hoefer TKO 100 fluorometer. Digestion with the restriction enzyme EcoRI (Biolabs) was done using 6 units/µg of DNA.

ii. Vector DNA, ligation and packaging *in vitro*. Lambda NM1149 was used as an immune insertion vector. The vector has the capacity of receiving 0-8.5 Kb of foreign DNA. Minipreparations from lambda were done using a conventional method. Isolated DNA was digested with restriction enzyme EcoRI, obtaining two lambda arms. After dephosphorylation, DNA from each genotype was ligated to lambda by mixing lambda and bean DNA in a relation of 2:1 and 100 units of T4 DNA ligase (BRL). The ligation was carried out for 4 h at 16°C.

E. coli strains BHB 2690 and 2688 were grown, induced by temperature, and lysated by sonication (BHB 2690) and by freeze and thaw (BHB 2688) in order to obtain capsid protein extracts. For packaging, the extracts were thawed in an ice bath for 15 min. Then 5 μl of BHB 2690 extract, 2 μl of ligated DNA and 25 μl of BHB 2688 extract were mixed and incubated for one h at room temperature, after which 500 μl of TMG buffer (20 nM Tris-HCl (pH: 7.5)/100mM NaCl/ 10mM $MgSO_4$/0.1% gelatin) was added. Packaged bacteriophages were evaluated for plaque formation in *E. coli* k-803 (a strain for phage replication) and in *E.coli* C 600 Hfl A (a strain used for selecting recombinants). The plaques were transferred to nylon membranes for a replica plate. Bacteria were lysed and DNA was denatured. Then filters were neutralized, fixed by auto cross-linking, and covered with saran wrap for storage.

iii. **Hybridization**. Three different probes were used to detect possible minisatellites: Jeffrey's probes 33.15 and 33.6 (Jeffreys 1985) and a fragment of gene III from M13 (Vassart et al. 1987 and Westreat et al. 1988) obtained by digestion with BsmI and ClaI (Biolabs). Labeling and hybridization conditions for Jeffrey's probes were done according to the commercial house specifications (Cellmark Diagnostics). For the M13 probe, the hybridization method described by Westreat et al. (1988) was used.

iv. **Purification of recombinant bacteriophages**. Plaques that showed a positive signal on the autoradiography were localized on the plate, picked up with a sterile tip and placed in an Eppendorf tube. Chloroform was added in order to lysate bacteria, and TMG buffer was added for storing at 4°C. Each mixture was then titrated in order to obtain an amplified, pure bacteriophage. This procedure was repeated as many times as necessary to obtain a plate where all plaques showed a positive signal (Lachtman 1989).

1.4.3 **Results**. Three different genomic libraries were constructed in Lambda NM1149. Table 1 shows the titer obtained and how many plates (150 mm in diameter) were used for each library in order to obtain all possible isolated plaques.

The filters prepared from each plate were hybridized with Jeffrey's probe 33.6, obtaining 9 positive signals for G40110 (Fig. 2a), 3 for G23580B and none for Ica Pijao. Additional hybridizations were carried out with Jeffrey's 33.15 and M13 probes. Table 2 shows all positive signals obtained in the first screening with each probe. At this point clones are not completely pure; after isolation, it is possible to find in the same tube both positive and negative clones. Therefore it is necessary to purify them until a pure positive clone is obtained.

Of the 48 possible positive signals, all were isolated and spotted on an overlay of *E. coli* k-803. As clones evaluated with Jeffrey's probes 33.15 and 33.6 were not

evaluated with M13 probe, a first purification step was done using M13 as probe. Clones showing a very strong positive signal (Fig. 2b) were isolated for a second purification with M13 (Table 3). These clones were plated in 150-mm plates and two filters were made from each. A large number of strong positive signals were obtained for each clone after hybridization with the M13 probe (Fig. 2c); consequently, it was assumed that all clones were purified.

Minipreparations were made to isolate possible minisatellite sequences (Fig. 3). Size and internal restriction sites will be determined. The selected fragments will be subcloned in a convenient plasmid vector and evaluated on different accessions of *P. vulgaris* and *P. acutifolius* to detect possible multilocus DNA fingerprint and species-specific patterns. They will also be tested on an F_2 population to determine possible segregation patterns of the various bands. We plan to sequence the various fragments to determine their structure and to be able to design primers for oligonucleotide fingerprinting. We also plan to investigate the use of PCR-based methods to detect species specific probes (Skinner 1992).

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Table 1. Titer and number of plates of three genomic libraries.

Genomic Library		Titer u.f.p.	No. of Plates
P. acutifolius	G 40110	1×10^4	5
P. vulgaris	G 2350B	5×10^3	2
P. vulgaris	ICA PIJAO	4×10^3	2

Table 2. Positive signals in the first screening.

Genome Library	Probe	Jeffrey's 33.15	Jeffrey's 33.6	M13
	Plate 1	0	6-01, 6-02	RM1
	Plate 2	15-1	6-03, 6-04	RM2 - RM4
P. acutifolius	Plate 3	15-2	6-05	RM5 - RM12
G40110	Plate 4	0	0	RM13- RM18
	Plate 5	15-3, 15-4	6-06, 6-09	0
	Total	4	9	18
P. vulgaris	Plate 1	0	6-10 - 6-12	RM19, RM20
G23580B	Plate 2	15-5	0	RM21 -RM24
	Total	1	3	6
P. vulgaris	Plate 1	15-6, 15-7	0	RM25 -RM26
ICA PIJAO	Plate 2	15-8	0	RM27 -RM28
	Total	3	0	4
TOTAL		8	12	28

Table 3. First purification with M13.

Genomic Library		Positive Signals
P. acutifolius	G 40110	15-1, 15-2, 15-3, RM11, 6-05
P. vulgaris	G 2350B	RM20, RM24
P. vulgaris	ICA PIJAO	0

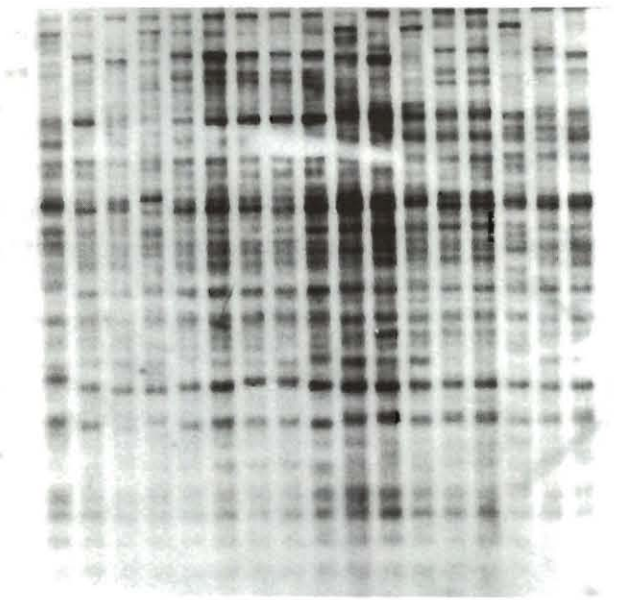
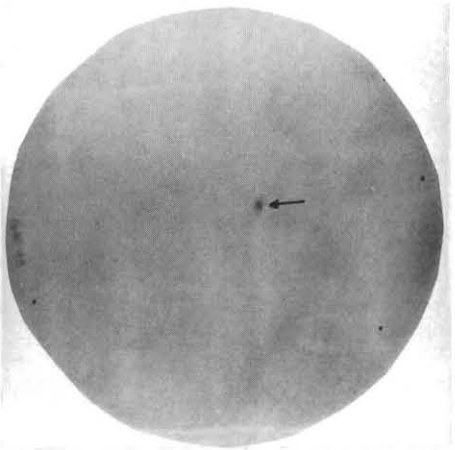
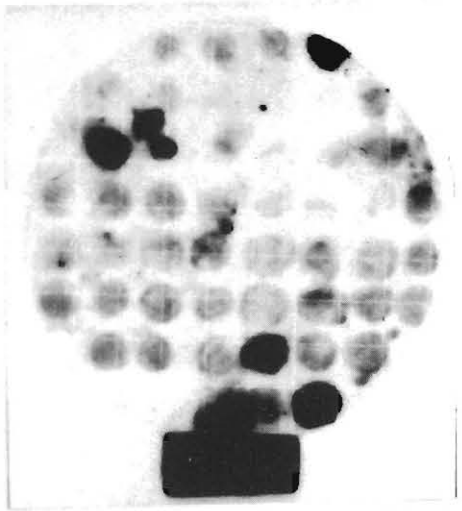


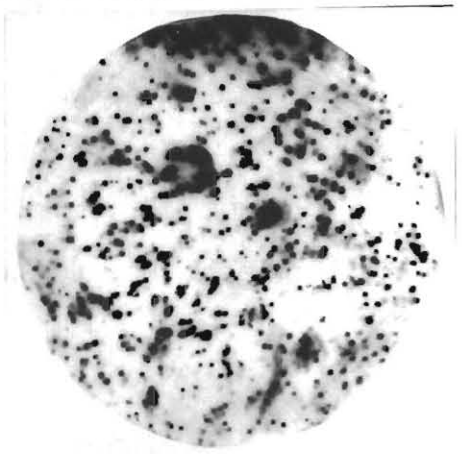
Figure 1. Genomic DNA from wild and cultivated common bean genotypes digested with HaeIII and hybridized with M13 probe.



a) Arrow points to a positive signal from the first screening obtained with Jeffrey's probe 33.6 in *P. acutifolius*; similar signals were obtained with the other probes and the two *P. vulgaris* genotypes.



b) Spots from each amplified plaque evaluated with M13 probe; intensive black spots were selected for an additional amplification step and a second purification with M13.



c) Second hybridization with M13 probe; it is assumed that these purified clones are homologous to M13 and will not require additional purification.

Figure 2. Purification of Positive Plaques from Recombinants: In the first screening 48 positive signals were obtained using Jeffrey's probes (33.6, 33.15) and M13.

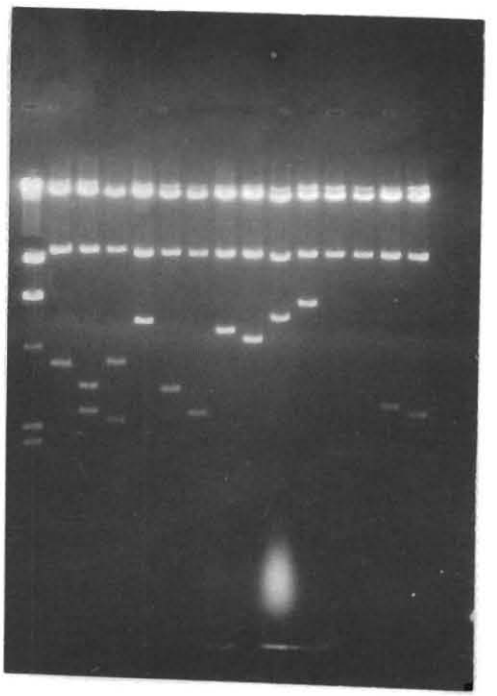


Figure 3. Minipreparation of 14 isolated and purified clones. Lane 1 is lambda digested with HindIII, lanes 2-7 clones from G40110 with Jeffreys 33.15; lanes 8-11 clones from G23580B with M13; lanes 12-13 clones from G40110 with M13; lanes 14-15 clones from G40110 with Jeffreys 33.6.

2. GENETIC FINGERPRINTING OF PLANTS AND MICROORGANISMS

2.1 Fingerprinting of the Genetic Base of Rice in Latin America and the Caribbean (Collaboration: F. Cuevas, INGER-Rice Program)

2.1.1 **Background**. Rice programs in Latin America have depended on a small genetic core in their breeding efforts. While the situation is different for each country, there is a concern that the genetic base of Latin American rice programs is narrow. A recent study based on pedigree analysis indicated that a group of 14 landraces account for nearly 70% of the genes in released cultivars (Cuevas et al. 1992). The understanding of the relationship among the core genotypes at the molecular level could contribute to a better selection of parental combinations and to the evaluation of the efficiency of newly released cultivars.

The BRU started a RAPD fingerprinting project with INGER-Rice Program to:

- analyze the genetic structure of the released cultivars and elite germplasm in rice
- assess the degree of relationship among the different genotypes
- compare the molecular data with the coefficient of parentage calculated based on pedigree

2.1.2 **Methodology**. The 150 rice genotypes released in various Latin American countries from 1979-89 will be used for the RAPD fingerprinting. These genotypes have already been analyzed by coefficient of parentage (Cuevas et al. 1992). DNA was obtained from all the genotypes. Efforts were dedicated to optimize the PCR conditions. The parameters looked at included primer selections, DNA concentrations, source of Taq, electrophoresis systems and PCR profiles. Primers were selected based on previous screening at the BRU in the gene tagging project. These primers produced at least four robust and scorable amplification products.

Several PCR profiles published in the literature were tested (Table 1). The protocol reported by Yu and Pauls (1992) was considered the most appropriate for its short amplification time (3 h) and the good resolution of the amplified products. The other parameters looked at were the DNA concentration, source of Taq and type of gel systems. To determine the optimum DNA template, amplifications with several primers were carried with DNA ranging from 12.5 ng to 200 ng (Fig. 1). Results show that DNA template of 25 ng gave good reproducible patterns.

Amplitaq and Stoffel Taq obtained from Perkin-Elmer were tested. The Stoffel Taq is an engineered DNA polymerase from Thermus Aquaticus lacking 289 amino acids from the N-terminal portion and 5' to 3' -exonuclease activity. Stoffel has been

reported to give sharper bands and is less sensible to Mg concentrations. Initial results seems to confirm such reports (Fig. 1).

Agarose and polyacrylamide gel electrophoresis were evaluated. The agarose gel electrophoresis consisted of 1.2% ultra-pure agarose stained with ethidium bromide. Polyacrylamide gel electrophoresis consisted of 5% acrylamide, 50% urea gel prepared in 1 x TBE buffer. Staining was prepared based on a improved silver-staining protocol (Blum et al. 1987). More bands were detected using the polyacrylamide gel systems (Fig. 2); however, not all the bands could be scored and the cost of the system is higher than the agarose gel electrophoresis. We are currently looking at the possibility of combining both fingerprinting systems to maximize the scoring of the various fragments. Besides the selected 10-mer primers we plan to use pairwise primers as described by Welsh and McClelland, 1991 and oligonucleotide primers such as (GATA)4 and (GACA)4.

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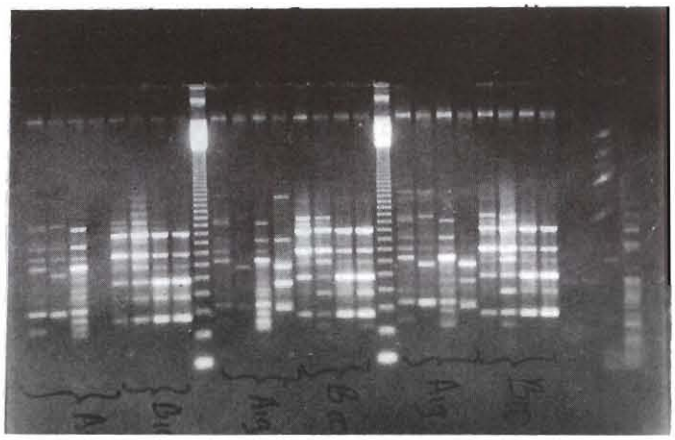

Figure 1. Effects of DNA concentrations and source of Taq obtained from the amplification of two rice cultivars and two primers. DNA concentration: Lanes 1-8 were from DNA with 12.5 ng; lanes 9-16 with 26 ng; and lanes 18-25 with 50 ng. Source of Taq: Lanes 9, 10, 13, 14 are with AmpliTaq; lanes 11, 12, 15, 16 with Stoffel fragment.

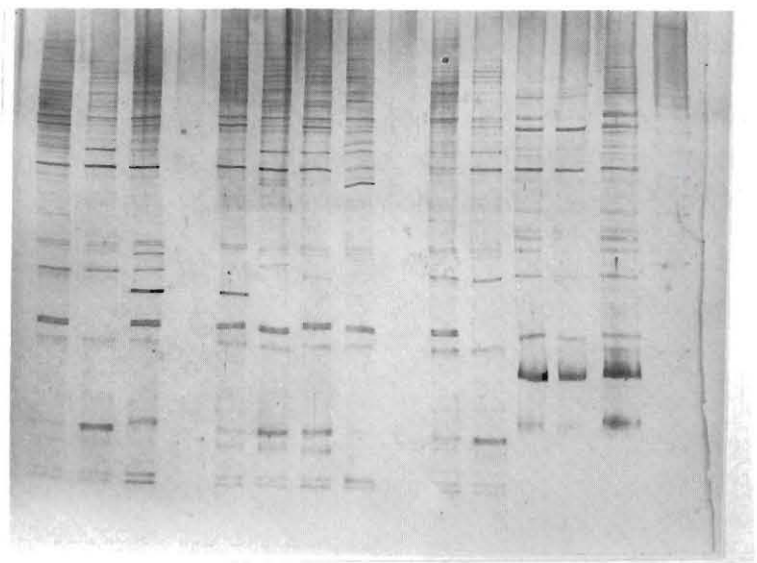

Figure 2. Polyacrylamide gel silver stained with 15 rice genotypes; gel thickness is 0.5mm.

2.2 Genetic diversity of the rice blast fungus in Colombia. (Research conducted by the rice pathology section in collaboration with the BRU).

Rice blast caused by the fungus *Pyricularia grisea*, expresses a large number of virulent forms or pathotypes. Genetic resistance to blast is short lived, rarely being effective for more than 2 to 3 years, due to the presumed diversity of the fungus. A novel and integrated strategy is being developed by an interdisciplinary and institutional team from CIAT's- rice program and Biotechnology Research Unit in cooperation with scientists at Purdue University. The strategy components combine classical pathology, breeding and molecular biology.

DNA probes developed by Dr. J. Hamer (previously at Dupont, currently at Purdue University), were shown to reliably identify the genetic backgrounds of the full spectrum of rice blast fungus pathotypes. One such probe consists of cloned fragments of repeated DNA obtained from the rice blast fungus genome and is called MGR586. The MGR586 restriction fragment profiles observed in the natural clones of the pathogen define multilocus haplotypes and are referred to as MGR-DNA fingerprints. Such DNA fingerprinting can identify both genetic diversity and relatedness among field isolates.

A preliminary study of Colombian rice blast diversity was conducted by Dr. Morris Levy from Purdue University in collaboration with CIAT to analyze the population structure in a Colombian blast disease "hot spot" where local pathotype diversity is five times greater than in the entire USA. The study site at Santa Rosa Station is a 30-hectare resistance breeding farm located in the savannas of eastern Colombia. The isolates diversity was subsumed in only six distinct MGR-defined lineages. Isolates within each lineage had a 92% or greater average fingerprint similarity, with no significant subclustering among lineage members. Each lineage was associated with a specific subset of cultivars and a specific subset of pathotypes; 90% of all pathotypes were lineage-specific and most were cultivar-specific within a lineage.

Based on this study we have initiated a project to characterize and identify all possible genetic lineages of the fungus from isolates collected from different rice genetic backgrounds and from various locations in Colombia. The analysis of the structure of the rice blast will also be incorporated in the gene tagging project to target specific lineage resistance genes. So far, the probe MGR586 has been obtained from the Dupont company and the technique has already been implemented (Fig. 1). Virulence and DNA fingerprinting will be studied from commercial varieties as well as other sources like introduced landraces, sources of resistance and breed lines.

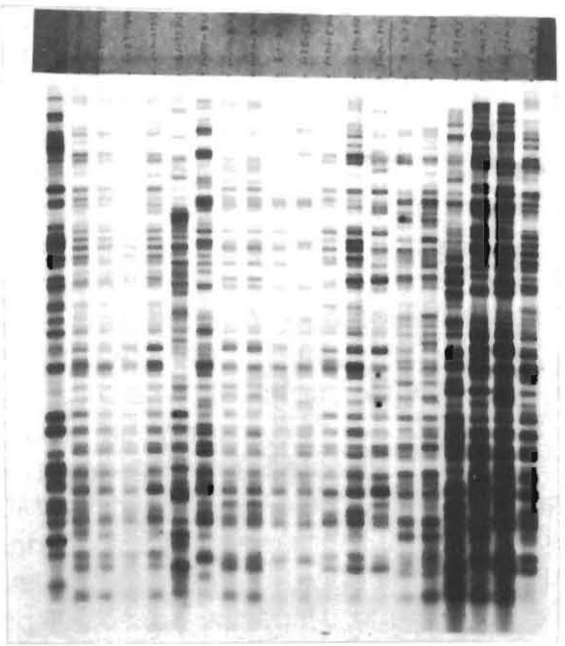

Fig 1. DNA fingerpinting with MGR 586 of Colombian isolates of *P. grisea.*

2.2 Genetics of Esterase and Glutamate Oxaloacetate Transaminase Isozymes in Cassava (Collaboration: C. Hershey, Cassava Program and C. Ocampo, GRU)

2.2.1 **Background**. Few studies on the genetics and cytogenetics of cassava have been reported. Large populations of homozygous individuals are required for useful genetic studies. In cassava, however, problems such as high heterozygosity, low seed set through controlled pollination, and strong inbreeding depression after selfing make it quite difficult to obtain sufficient numbers of individuals that are homozygous at all loci or even a given locus. Consequently, the ploidy level is undetermined, and the diploid or tetraploid status of this crop remains to be defined. Isoenzyme electrophoresis can provide additional data for genetic studies (tomato: Tanksley and Rick 1980; potato: Quiros et al. 1985; apple: Mangaris and Alston, 1987 and Weeden et al. 1987). The differences in electrophoretic mobility of isoenzymes are usually the result of changes in the structural genes coding for the polypeptides. Electrophoretic polymorphisms are thus the direct result of genetic differences.

Earlier work in the BRU established the methodology for fingerprinting cassava cvs. by electrophoretic isozyme patterns of a, β-esterase (EST), glutamate oxaloacetate transaminase (GOT), diaphorase (DIA), phoglucoisomerase (Pi) (Hussain, et al., 1987). The methodology was transferred to the GRU in 1989 for implementation in cassava germplasm studies, including: description of germplasm accessions based on EST banding patterns, identification of duplicates in the collection, and the distribution of isozyme polymorphism in the collection. The GRU has characterized over 4000 cassava accessions for their EST isozyme patterns, and identified a number of putative duplicates in the collection.

The main objectives of this work were to determine the genetics of the esterase anodic region and the glutamate oxaloacetate transaminase (GOT) cathodic region in cassava root tips.

2.2.2 **Methodology**. A total of 9 crosses using 11 different cultivars were used for progeny studies for both esterases and GOT isozymes (Tables 1 & 2).
The methodology for determining isozyme patterns in cassava by polyacrylamide gel electrophoresis was developed by Hussain et al. (1986). Stakes from mature plants (6-9 mo in age), were potted in the greenhouse in a 1:1 sand:soil mixture. After 3 weeks, 0.5 g of root tips were harvested and proteins extracted in 1 ml of ice-cold 0.05 M Tris-HCl buffer (pH 8.3). The crude extract was centrifuged at 27,000 x g for 15 min. The supernatant (ca. 25 μl) was directly used for isozyme electrophoresis in a 10% polyacrylamide gel using 0.05 M Tris-borate (pH 9.0) as running buffer. Samples were run for 6 h at 4°C and 250 V.

For esterase activity detection, aβ-naphthyl acetate diluted in acetone are used as substrates and fast blue RR salt for staining. For GOT substrates are aspartic acid,

a-ketoglutaric acid and pyridoxal-5-phosphate diluted in 1 M Tris-HCl buffer (pH 8.0) and for staining fast blue BB salt is used.

2.2.3 **Results**. EST-1 (Fig. 1) is a single locus with 5 alleles, including one null allele, and is expressed as a monomeric enzyme. For this locus a diploid inheritance model is proposed (Table 1). None of the individuals exhibits a three-band phenotype. A total of 11 different phenotypes represented by 15 different genotypes were classified from the studied progenies. These findings were supported by the esterase characterization of the cassava germplasm collection.

GOT-1 (Fig. 2) is a single locus with three alleles, including one null allele. Polypeptides behave as monomers. The analysis of the two populations leads us to propose a simple diploid Mendelian inheritance for this locus (Table 2).

Although the genetics of the two isozyme loci could be interpreted, other complex regions of the zymograms could not be explained by a simple diploid inheritance and remain to be characterized, using highly contrasting parents.

For EST-1 the most complex region for analysis was the cathodic region of the zymogram although the parents have a simple phenotype. It would be useful to check for the occurrence of interactions between loci, as well as a possible tetraploid inheritance model.

In the case of GOT-1, the analyzed anodic region has not been considered for the GOT zymogram of cassava. The central region of this zymogram is very complex with characteristics similar to the esterase loci.

2.2.4 **Conclusions**. Genetic studies of two isozyme loci were carried out in root tips of 8 cassava F_1 progenies using polyacrylamide gel electrophoresis. Two loci--esterase-1 (EST-1), the most distant cationic region, and glutamate oxaloacetate transaminase-1 (GOT-1)--the most proximal anionic region, were evaluated among the segregating populations.

EST-1 locus has five multiple alleles, including one null allele, and behaves as monomer, with a diploid inheritance pattern with only two alleles present in each individual. These data were confirmed by esterase characterization of CIAT cassava germplasm bank accessions; a total of 11 different phenotypes representing 15 different genotypes for this locus were demonstrated. GOT-1 locus comprises two alleles with diploid inheritance.

This work provides evidence for a diploid inheritance pattern of the two isozyme loci supporting the allotetraploid nature of cassava. Many other regions of the zymograms are still to be elucidated. This information will be valuable for interpreting studies on isozyme fingerprinting of cassava genetic diversity.

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Table 1. Crosses used to study inheritance of locus EST-1 in cassava.

Cross	Parental Genotype		No. of Genotypes Expected	No. of Genotypes Observed	Ratio	X_2
	Female	Male				
1X1	A_oA_4	A_oA_4	A_4A_-: 22.5 A_oA_o: 7.5	A_4A_-: 23 A_oA_o: 7	3:1	0.04
1X2	A_oA_4	A_oA_3	A_3A_4: 7.25 A_oA_3: 7.25 A_oA_4: 7.25 A_oA_o: 7.25	A_3A_4: 8 A_oA_3: 6 A_oA_4: 7 A_oA_o: 8	1:1:1:1	0.4
2X1	A_oA_3	A_oA_4	A_3A_4: 7.5 A_oA_3: 7.5 A_oA_4: 7.5 A_oA_o: 7.5	A_3A_4: 10 A_oA_3: 6 A_oA_4: 8 A_oA_o: 6	1:1:1:1	1.46
3X4	A_1A_1	A_2A_3	A_1A_2: 13 A_1A_3: 13	A_1A_2: 11 A_1A_3: 15	1:1	0.62
5X6	A_2A_4	A_1A_3	A_1A_2: 6.5 A_1A_4: 6.5 A_2A_3: 6.5 A_3A_4: 6.5	A_1A_2: 7 A_1A_4: 6 A_2A_3: 5 A_3A_4: 8	1:1:1:1	0.77
7X6[a]	A_4A_4	A_1A_3	A_1A_4: 13 A_3A_4: 13	A_1A_4: 16 A_3A_4: 10	1:1	1.38
8X9	A_2A_4	A_4A_4	A_2A_4: 13 A_4A_4: 13	A_2A_4: 12 A_4A_4: 14	1:1	0.15
10X11	A_oA_4	A_oA_2	A_oA_o: 20.25 A_oA_2: 20.25 A_oA_4: 20.25 A_2A_4: 20.25	A_oA_o: 17 A_oA_2: 18 A_oA_4: 19 A_2A_4: 27	1:1:1:1	3.1
10X10[b]	A_oA_4	A_oA_4	A_4A_-: 7.5	A_4A_-: 7	3:1	0.14

a) Cross showed in Fig. 2.

b) Cross showed in Fig. 1.

1: M Col 1505
2: M Col 1468
3: M Col 948C
4: CM 847-11
5: M Col 72
6: CM 996-6
7: M Col 1495
8: M Tai 1
9: M CR 2
10: CM 681-2
11: CM 2177-2

Table 2. Crosses used to study inheritance of locus GOT-1 in cassava.

Cross	Parental Genotype		No. of Genotypes Expected	No. of Genotypes Observed	Ratio	X_2
	Female	Male				
1X2	A_1A_2	A_1A_o	A_1A_2: 20.25 A_1A_-: 40.5 A_oA_2: 20.25	A_1A_2: 21 A_1A_-: 42 A_oA_2: 18	1:2:1	0.304
3X4[(a)]	A_1A_2	A_1A_1	A_1A_1: 13 A_1A_2: 13	A_1A_1: 12 A_1A_2: 14	1:1	0.154

a) Cross showed in Fig. 3.

1: CM 681-2
2: CM 2177-2
3: M Col 1495
4: CM 996-6

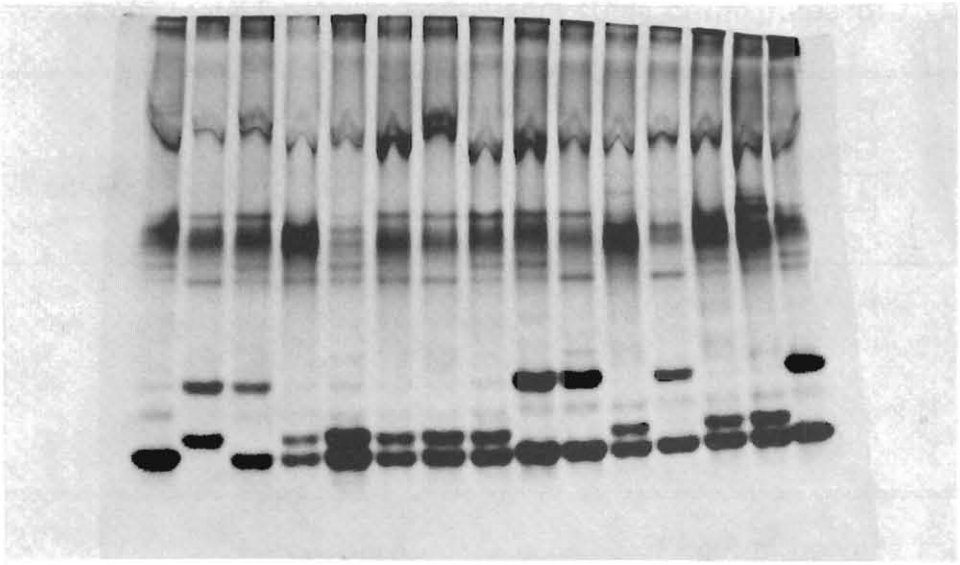

Figure 1. EST-1 analysis in the cross M Col 1495 x CM 996-6 and its progeny. Alleles involved are A_1, A_3 and A_4. Inheritance data of this cross are shown in Table 1.

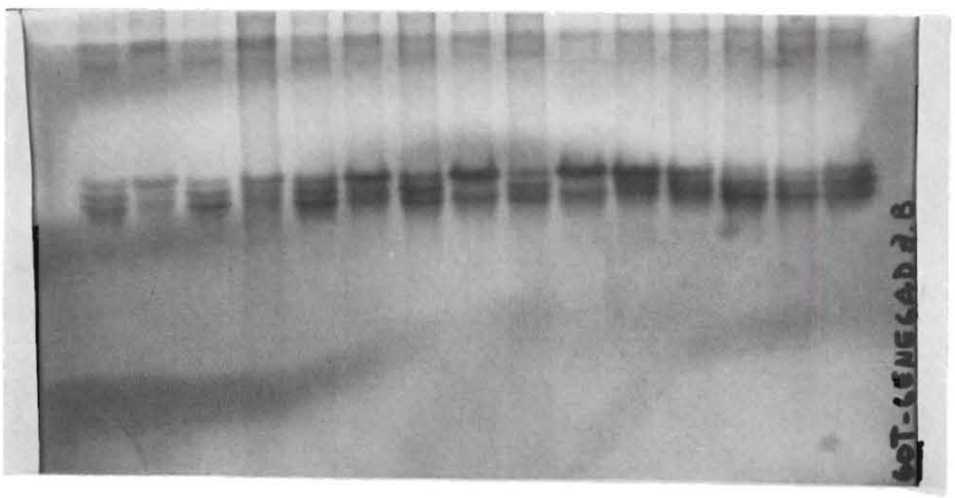

Figure 2. GOT-1 analysis in the cross M Col 1495 x CM 996-6 and its progeny. Alleles involved are A_1 and A_2. Inheritance data of this cross are shown in Table 2.

2.3 DNA Fingerprinting to Confirm Possible Duplicates in Cassava Germplasm (Collaboration: C. Ocampo, GRU)

A research project is under way to study the use of DNA fingerprinting to confirm suspected groups of duplicates; that is, detect genotypic differences among these groups that otherwise appear identical in their morphology and isozyme-banding patterns.

Unlike morphological and/or isozyme characterization, DNA fingerprinting techniques directly characterize the genome, unaffected by external environmental or developmental factors. Although it is probably more sensitive in detecting genotypic differences, it is more expensive and more complex than morphological and isozyme characterization. Therefore the project goal is to (1) reduce operational costs, perhaps by selecting adequate probes, and (2) overcome the technical difficulties of producing enough high-quality DNA, and enough good quality autoradiographies, with high banding definition.

The Cassava Program provided 90 clones grouped in 45 pairs according to morphological and isozymatic similarities. This study should complement studies made with morphological and isozymatic descriptors. Because the methodology for RFLP detection has already been standardized for cassava by the BRU, this technique was selected for the project.

Different probes are being selectively tested to identify at least one that can show polymorphism among accessions that are similar morphologically and isozymatically. The probes being tested include the gene for the protein III of the bacteriophage M13 (DNA minisatellite) and the human-derived DNA minisatellites, Jeffrey's probes 33.6 and 33.15. Preliminary studies indicated that these three probes were useful for discriminating between closely related individuals. Probe M13 will probably be more practical, however, because it is readily available while Jeffrey's probes are very expensive.

DNA isolated from 15 different varieties was digested with HaeIII restriction enzyme and probed with the smallest ClaI/BsmI fragment of the M13 bacteriophage. This probe discriminated between different cultivars used here, indicating that M13 is a good probe to carry out DNA fingerprinting in cassava (Fig. 1). M13 will be used to screen a larger number of cassava genotypes.

We are also looking at RAPD markers. Several primers tested thus far differentiate among different varieties, indicating the feasibility of this technique to carry out DNA fingerprinting studies (Fig. 2). Selected cassava genomic probes (from the DNA cassava library) will also be tested.

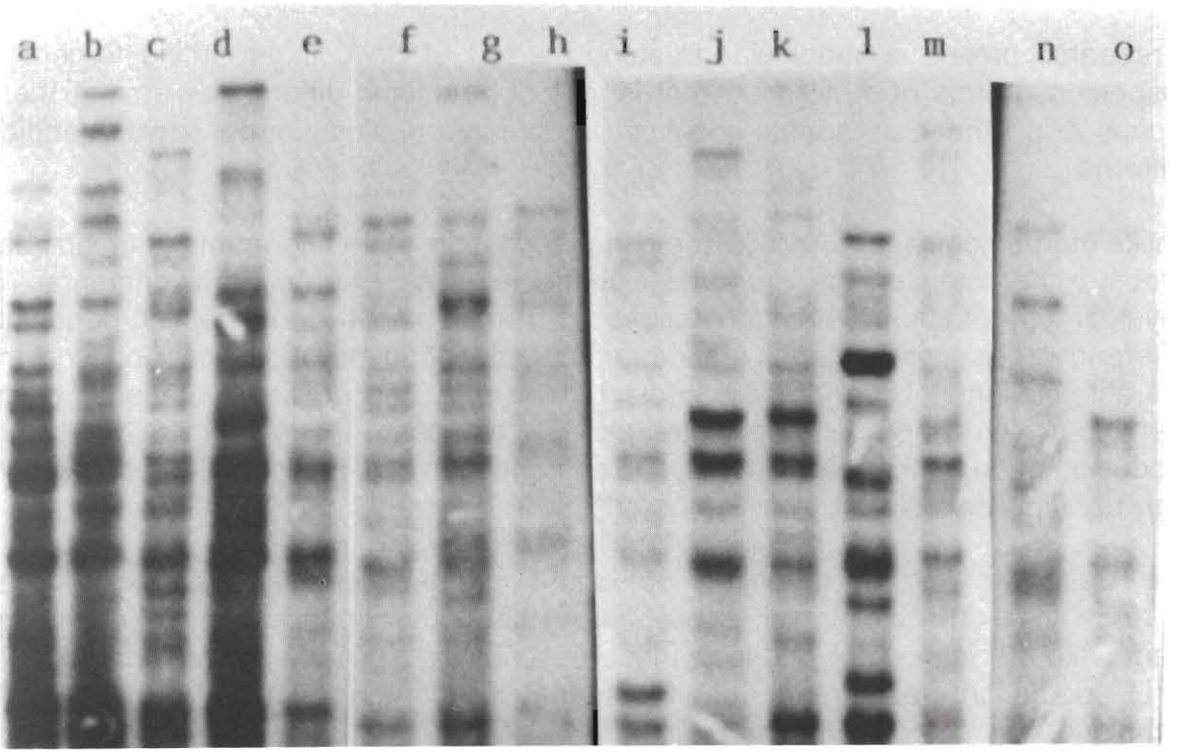

Figure 1. Fingerprinting of 15 cassava varieties using M13 as probe.

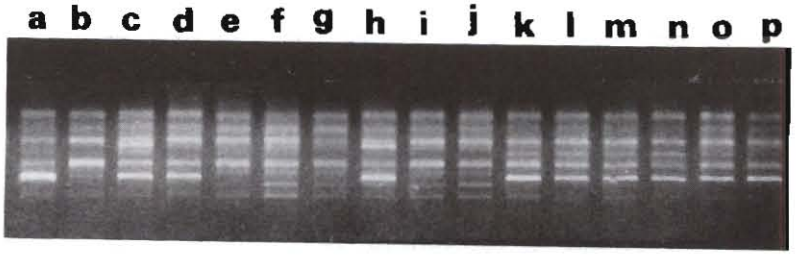

Figure 2. DNA fingerprinting of 16 cassava varieties using RAPD system.

2.4 Assessment of Genotypic Stability of Cassava Stored in Vitro

In vitro conservation has to be considered part of the overall conservation strategy for a particular plant species, as a valuable adjunct to genetic resource conservation. Genetic stability in cultures has long been a matter of concern in the potential application of *in vitro* techniques for germplasm conservation.

Monitoring the genetic stability of *in vitro* plant cultures of crop species is becoming of great interest. Molecular criteria for detecting genetic changes have been proposed, monitoring genetic stability of cultures by DNA fingerprinting.

Ten varieties from the world cassava germplasm collection at CIAT, stored *in vitro* under minimal conditions of growth for ten years, have been tested by RFLPs and RAPDs. Preliminary results have indicated no variation at the evaluated regions of the genomes tested (Fig. 1). An extensive survey using several RAPD primers will be conducted to test the genetic stability of *in vitro* germplasm collections, maintained under both slow-growth and cryogenic conditions.

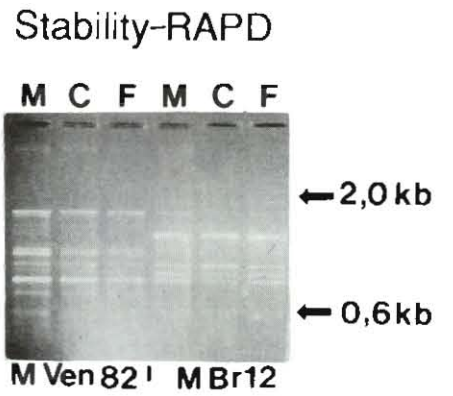

Figure 1. DNA stability monitored in two different cassava varieties after long *in vitro* storage.

3. MOLECULAR MAPS AND GENE TAGGING

3.1 Construction of a molecular map of Cassava using RFLPs and RAPDs markers (Collaboration: Carlos Iglesias, Cassava Program)

We have initiated a research project to construct a molecular map of cassava using Restriction Fragment Length Polymorphism (RFLPs) and Random Amplified DNA (RAPDs) markers. The construction of a detailed genetic map of cassava will contribute significantly to the understanding of cassava genetics. It will be useful to facilitate introgression from wild species for targeted traits and to tag agronomic important traits, simply and quantitatively inherited. Eventually the map will be useful to isolate and clone cassava genes.

3.1.1 **Methodology**. As a first step in the construction of a detailed genetic map of cassava we evaluated cloned nuclear sequences from five different genomic libraries generated, at the BRU, with different restriction enzymes. Different random genomic probes were compared in their ability to detect polymorphism in several cultivated lines and in one wild *Manihot* species, *M. aesculifolia*. Cloned nuclear sequences and different primers selected previously on the basis of their ability to detect polymorphism (Angel et al. 1991), are being screened in the offspring from different controlled crosses. DNA polymorphism in a wild *Manihot* species and some cassava cultivars as well as segregation of markers using RFLPs and RAPDs were evaluated.

Nuclei from green leaves were isolated as described (Vayda et al. 1986). Nuclear and genomic DNA isolation procedures were similar to that reported previously (Dellaporta, S.L. et al. 1983). Nuclear DNA was divided in five fractions and each one was digested with one of the following restriction endonucleases (Pst I, Eco RI, Bam HI, Hind III, Xba I). Fragments between 0.5 and 3.0 Kb were cloned into the polylinker site in the pUC 19 plasmid and recombinant colonies were selected based on X-gal and IPTG screening procedures. Low copy number inserts were preselected by hybridization in dot-blot of each insert with ^{32}P-labeled total genomic DNA.

DNAs of eleven cassava genotypes from different geographical origins (three from Colombia, two from Brazil, one from Argentina, two from Thailand, one from Nigeria and two hybrids) were digested with ten different enzymes and probed with the whole plasmid including cassava inserts. A wild *Manihot* species from Mexico, *M. aesculifolia*, was included in this study.

Amplification reactions for the RAPDs analysis were performed in a Perkin Elmer Cetus DNA thermocycler programmed for 40 cycles of 1 min at 94°, 1 min at 36°, 2 min at 72° (Williams et al. 1991).

3.1.2 **Results**. The polymorphism among cultivated varieties evaluated was extremely low, except for MCol 22. Nevertheless, the higher polymorphism was found

when MCol 22 was compared with the other ten genotypes evaluated. The percentage of polymorphic probes in intraspecific comparison is presented between MCol 1505, which represents the group of ten cultivated varieties and M Col 22 (Table 1, Fig. 1).

In interspecific polymorphism, when patterns of hybridization between M Col 1505 and *M. aesculifolia* were compared, the percentage of polymorphic probes were, in all cases, higher than those detected among cultivated genotypes (Table 1, Fig. 2). If we compare the ability of the probes to detect polymorphism in both cases, intraspecific and interspecific, we can classify Pst I, Xba I and Hind III genomic probes in a first group, detecting higher polymorphism levels, and Eco RI and Bam HI genomic probes in a second group, detecting low polymorphism levels.

Furthermore, different restriction enzymes were compared in their ability to detect polymorphism among the same genotypes (Table 2). Eco RI was the best for displaying polymorphism in both cases. Eco RV and Hae III also detected high polymorphism levels. Bam HI in intraspecific but not in interspecific and Xba I in interspecific but not in intraspecific groups also detected high polymorphism levels (Table 2).

F1 progenies from an intraspecific cross is being evaluated in order to observe the segregation of genomic probes and polymorphic primers. Autoradiograph (Fig. 3) shows RFLP segregation displayed by a Pst I genomic probe after hybridization with Eco RV digested DNA from an F1 population. 43 plants showed the female parent pattern and 40 plants the male parent pattern. This probe detect two loci, one with a double banded allele and the second single banded with two alleles, the second locus is heterozygous in the female parent and homozygous in the male parent (Fig. 3).

Several primers have been screened in the same cross. Segregation of two primers is shown (Fig. 4), J12 detecting one locus, and B11 detecting two loci in the parental lines (Fig. 4, see arrows). Those bands segregate close to the expected ratio 1:1.

3.1.3 **Summary**

- Polymorphism detected between cultivated genotypes and *M. aesculifolia* was higher than that among cultivated genotypes.

- Hind III, Pst I and Xba I probes detected higher polymorphism than Bam HI and Eco RI probes.

- Cassava DNA digested with Eco RI, Eco RV and Hind III displayed more polymorphism.

- Four cutter restriction enzymes displayed less frequency of polymorphism when compared with six cutter restriction enzymes among cultivated genotypes.

- Polymorphism displayed by Dra I was extremely low indicating that regions rich in adenine and thymine may not be hot spots for mutations in cassava.

- RFLPs and RAPDs segregation indicated that those markers will be very useful in the construction of a molecular map of cassava.

RFLPs and RAPDs studies will be continued for the cross shown here and other crosses generated by the cassava program at CIAT, in order to construct a molecular map of cassava.

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Table 1. Genomic probes compared for their ability to detect polymorphism with at least one restriction enzyme.

INTRASPECIFIC		INTERSPECIFIC	
Probe	% Polymorphism	Probe	% Polymorphism
Pst I	60	Hind III	95
Xba I	60	Pst I	85
Hind III	55	Xba I	85
Eco RI	40	Eco RI	60
Bam HI	30	Bam HI	45

Table 2. Comparison of different restriction enzymes for their ability to detect polymorphism for all probes tested.

INTRASPECIFIC		INTERSPECIFIC	
Restriction Enzyme	% Polymorphism	Restriction Enzyme	% Polymorphism
Eco RI*	34	Eco RI*	69
Eco RV*	29	Xba I	61
Bam HI	22	Eco RV*	53
Hind III*	20	Hae III	51
Pst I	20	Hind III*	49
Xba I	16	Bam HI	44
Hae III	15	Pst I	44
Taq I	6	Taq I	N.D.
Dra I	3	Dra I	N.D.
Hpa II	1	Hpa II	N.D.

N.D.= Not determined.
* Enzymes detecting high polymorphism levels in both cases.

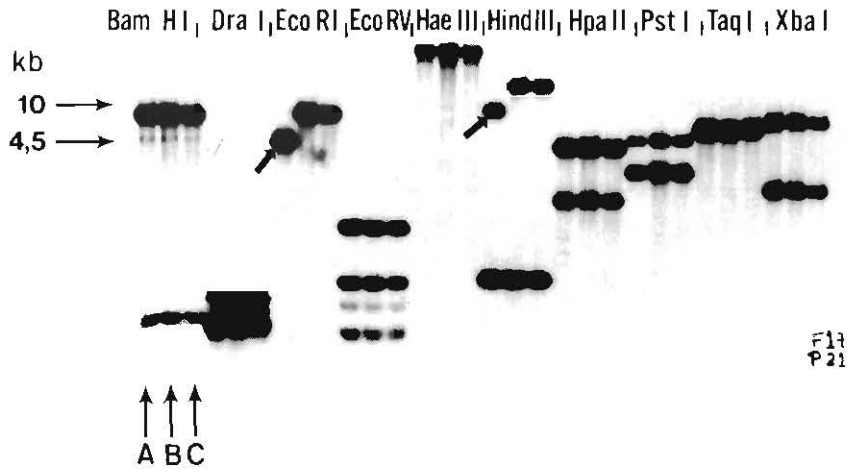

Figure 1. Polymorphism detected with a Pst I probe in three cultivated genotypes. Restriction endonucleases Eco RI and Hind III detect DNA variation in M Col 22.
A= M Col 22, B= M Col 1505, C= CM 507-37.

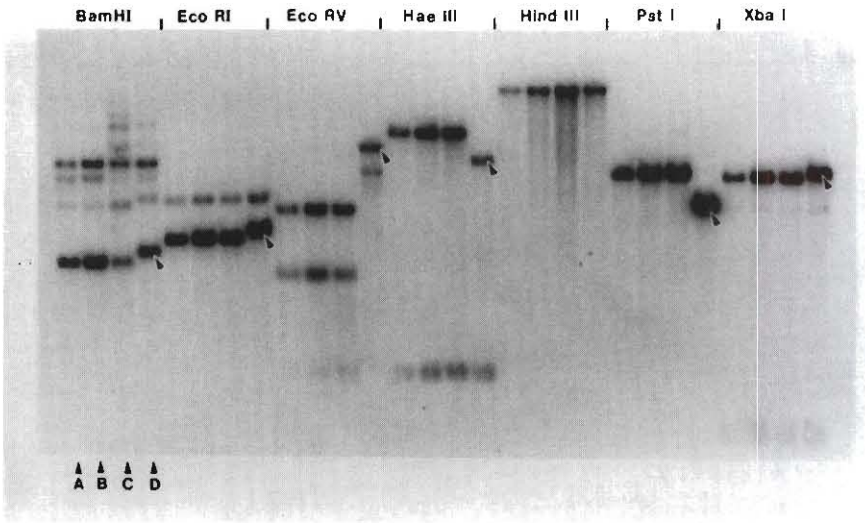

Figure 2. Polymorphism detected between three cultivated genotypes and *M. aesculifolia* with six out of seven restriction enzymes used.
A= M Thailand 8, B= M Col 1505, C= M Nigeria 5, D= *M. aesculifolia.*

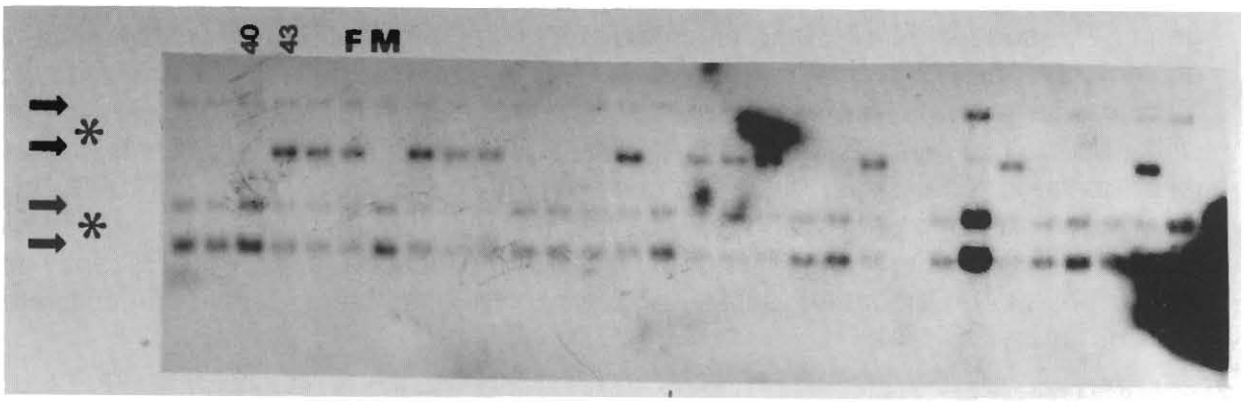

Figure 3. RFLP pattern for a segregating probe that hybridize to two loci, one of them homozygous in the male parent (M) and heterozygous in the female parent (F). Segregation of this locus in F1 population is evident.

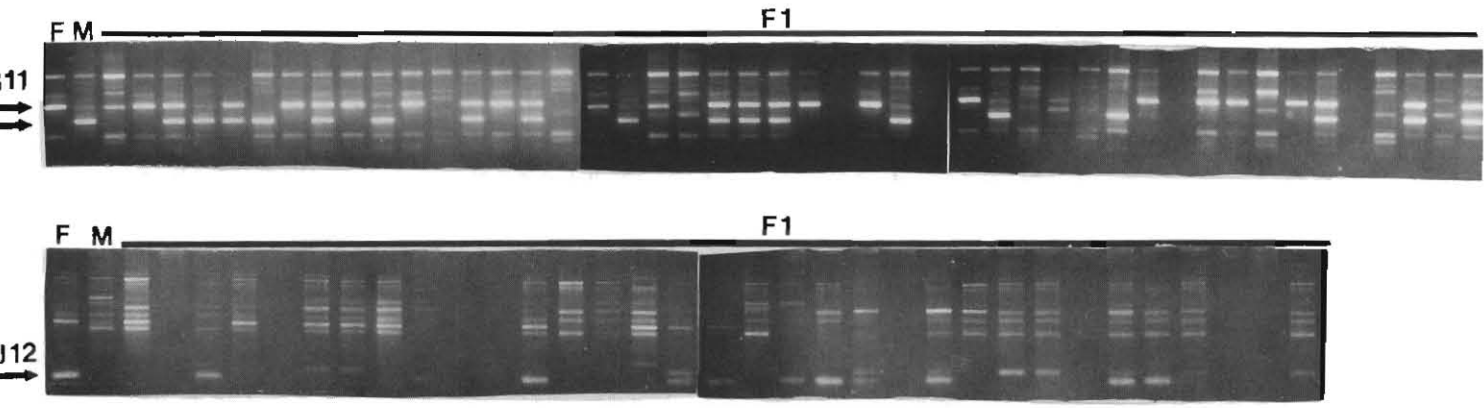

Figure 4. RAPD segregation of two polymorphic primers in the parental lines. B11 primer detecting two segregating loci (see arrows) and J12 detecting one segregating locus.

3.2 Construction of a molecular map of *P. acutifolius* and tagging of common bacterial blight resistance genes (Collaboration: Masa Iwanaga, Orlando Toro, Genetic Resources Unit and Steve Beebe, Bean Program)

The tepary bean (*Phaseolus acultifolius*), one of the five cultivated species of the genus *Phaseolus*, is of Mesoamerican origin. Its distribution covers two distinct ecological zones: Semi arid regions mainly in Southwestern USA and Northern Mexico with a rainfall of less than 400 mm per year and lowland tropical America with a rainfall of 1000-2000 mm per year. A strong founder effect seems to have taken place in tepary bean as in the case of *P. vulgaris*. Schinkel et al. (1998) have shown that only one phaseolin type was found in cultivated teparies versus 15 in wild *P. acutifolius* species, suggesting a single domestication site. More recently the survey have been extended at CIAT and two more types were identified in cultivated form of tepary.

The tepary bean is well known for its high tolerance to a biotic streseses like drought, heat and salinity. The species is also a source of resistant to several diseases and pests like common bacterial blight and empoasca. High levels of resistance to CBB (*Xanthomonas campestris*) have been reported and the resistance controlled by a dominat single gene gene (Drijfhout and Blok 1987)

We are working on the construction of a molecular linkage map of the tepary bean with the same RFLPs markers used in the *P. vulgaris* map to better understand the relationship of the two species and to develop a series of markers to assist in directed introgression of desirable traits from *P. acutifolius* into *P. vulgaris*.

3.2.1 **Methodology**. A selection of parental materials to generate mapping populations was made based on origin of collections and seed characteristics. Two populations were selected from a cross between zones but within the same morphotype (G40065 x G40110) and a cross between zones and between morphotypes (G40013 x G40065) (Table 1). The success of obtaining enough F1 and F2 seeds from the two crosses and their reciprocal suggested the lack of any genetic barriers between the parental used. Leaf tissue was obtained from individual F2 plants. Single seed descent being carried to established fixed recombinant inbred lines.

3.2.2 **DNA extraction**. High quality genomic DNA is essential for RFLPs and RAPDs studies. Several DNA extraction protocols have been tested to isolate total DNA from 4 to 5 g of bean leaf tissue. A modified Dellaporta procedure gave the best results. A liquid nitrogen powder from young leaf tissue is mixed with three volumes of extraction buffer [150mM Tris.HCl (pH: 7.5)]/15mM EDTA (pH:8.0)/1M NaCl/1.5% CTAB/1.2% mercaptoethanol/1.0% PVP (Polivynilpirrolydone) in a polypropylene tube and incubated at 65°C for 30 min.

The mix is then extracted with 1 volume of Phenol: Chloroform (1:1) ($CHCl_3$: octanol 24:1) and the aqueous phase separated by centrifugation and reextracted with 1 vol. Chloroform: octanol (24:1) The aqueous phase is separated again by centrifugation. The DNA is precipitated from the aqueous phase by the addition of 2/3 vols. cold isopropanol/1/10 vols. 3M sodium acetate (pH: 5.2) and kept in the freezer at -20°C for 1 hour to overnight. The DNA is pelleted by centrifugation and washed once with ice-cold 70% Ethanol. The DNA is well dried and dissolved in T.E. buffer or water. The quantification of the DNA is estimated in a fluorometer Hoefer TKO-100 as described in the manufacturer's manual. The yield of this extraction procedure is about 50 μg DNA/g of tissue. The quality of the DNA was evaluated successfully by digestion with the restriction enzymes BamHI, EcoRI, EcoRV, HindIII, XbaI.

3.2.2 **Source of probes and survey of polymorphism**. The source of probes is a PstI genomic library enriched for single copy sequences. The probes used were already mapped by Vallejos in the linkage map of *Phaseolus vulgaris* (Vallejos et al. 1992). The 244 probes received from the University of Florida were cloned in the PstI site of the plasmid pTZ and dissolved in T.E. buffer. Transformed bacteria were obtained by standard protocols and the probes were recovered by mini preparations of plasmid using the Lysis by boiling method. The remaining aliquot of plasmid DNA received from Vallejos is stored in the freezer at -20°C. The transformed bacteria, containing the recombinant pTZ plasmid, are stored in a glycerol buffer at -70°C freezer. A working solution of plasmid DNA is maintained in T.E. buffer at -20°C and a stock of transformed bacteria, at -70°C. Having stocks, of transformed bacteria and plasmid DNA at -70°C and -20°C respectively, which are never used as working stocks, guarantee the availability of all the probes not only for our works, but for additional requests.

We have also received clones from the mapping project of Dr. Gepts, University of California, Davis. The clones are also being used in the mapping and tagging projects at CIAT. They will be also used to merge the two existing maps.

Initial survey of polymorphism between the parental lines indicate the feasibility of the mapping project (Table 2). The level of polymorphism is almost complete. Based on these results we have started screening the two F2 populations with proper probe/enzyme combinations. The restriction enzyme BamH1 has been dropped from the analysis since it does not contribute much to the level of polymorphism. The F2 plants from the G40065 x G40110 cross are being screened for CBB. The data will be used for gene tagging. We are also using RAPDs marker to complement the RFLP data.

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Table 1. Characterisitics of the tepary genotypes used in the mapping and tagging study.

Genotype	Geographical Origin	Seed Morphology	Reaction to CBB
G40013	Nicaragua	Prismatic, flat	Intermediated
G40065	USA-Arizona	Rounded	Highly ressitant
G40025	USA-Arizona	Prismatic, flat	Not available
G40110	Mexico-Campeche	Rounded	Susceptible

Table 2. Percentage of polymorphism detected per restriction enzyme for the two tepary mapping populations.

Restriction enzyme	% Polymorfism	
	G40025 x G40013	G40065 x G40110
Bam HI	17.3	35
Eco RI	47.8	50
Eco RV	43.4	50
Hind III	56.5	40
Xba I	43.4	50
Accumulated freq.	41.6	45
Without Bam HI	48.6	47.5

3.3 Tagging the gene (s) of resistance to the bean weevil, *Acanthoscelides obtectus* (Say). (Collaboration: C. Cardona and C.E. Poso, Entomology Section, Bean program)

3.3.1 **Background**. The bean weevil, (*Acanthoscelides obtectus*), is with the Mexican bean weevil (*Zabrotes sufasciatus*) one of the main seed storage pest. The weevil is widely distributed and can cause extensive damage affecting cooking quality, seed germination and seedling vigor (Schoonhovn and Cardona 1980). No source of resistance in cultivated *P. vulgaris* has been identified despite an extensive screening. However high level of resistance have been found in some wild *P. vulgaris* populations from Jalisco, Mexico (Schoonhoven *et al.* 1983) . The resistance is also found in several wild *P. acutifolius* accessions. In lima bean (*Phaseolus lunatus*) the resistance is found in both wild and cultivated accessions. The resistance mechanisms has been reported to be due to two recessive genes (Kornegay and Cardona 1991).

3.3.2 **Methodology**. A susceptible commercial cultivar, ICA Pijao, was crossed with the accession G12952. A known source of resistance to the bean weevil, G12952 is a wild *P. vulgaris* from Arandas county in Jalisco, Mexico. The resistance of this accession is expressed as antibiosis causing delayed and reduced adult emergence (Cardona et al. 1989). Individuals seeds from the parental lines and 90 F2 seeds were placed into separate vials and infested with three *A. obtectus* eggs. Days to adult emergence was taken 30 days after infestation. Four classes were identified; susceptible, intermediate, resistance and highly resistant (no emergence at 69 days).

The F2 plants were also scored for the I gene of bean common mosaic virus (BCMV) using the detached leaf methods. Single seed descent (SSD) is currently being carried to generate recombinant inbred lines (RIL). These recombinant lines will be tested with the bean weevil to confirm the inheritance of the trait.

We are currently using RFLPs and RAPDs markers to obtain a possible linkage. The level of RFLP polymorphism obtained by screening the two parents is around 57%, a good level for any linkage mapping project. The screening of the F2 plants has already started.

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3.4 Tagging of tolerance-resistance genes to Bean Golden Mosaic Virus (Collaboration: S. Beebe and S.H. Orosco, Bean Program)

3.4.1 **Background**. Bean golden mosaic virus (BGMV) is a gemini virus transmitted by the whitefly insect *Bemisia tabaci*, is the most important bean disease in Latin America. The incidence and severity of the BGMV are increasing in various countries due to the change in agricultural practices favoring the whitefly vectors. We have initiated last year a RFLPs linkage analysis on backcross inbred lines carrying the tolerant genes from the tropical black source. The overall goal of the project is to study the genetics of BGMV resistance and tag BGMV resistance-tolerance genes (simply or quantitatively inherited) using RFLPs and RAPDs primers

3.4.2 **Methodology**. Backcross inbred lines from the cross Dor 60 x APN 18 have been obtained and screened under fields conditions for BGMV in Guatemala and Apion in Honduras. The cross also segragates for anthracnose resitance to the Kappa race. Recombinant imbred lines from the cross Dor 364 x A 686 also being tested. The same methodology described previously for the tepary bean project was followed with respect to DNA extraction, Southern blotting and hybridization.

The two parents used belong to the same mesoamerican gene pool. So it was not surprising to find a low level of polymorphism of about 25%. The restriction enzymes Hind III, EcoRI and EcoRV gave the highest level of polymorphism. Same results were obtained with the mapping population in tepary and for the bruchid project. In addition to RFLPs we have tried so far some 60 RAPDs primers. Both markers will be used on the backcross inbred lines. In conjuction with the RFLPs analysis we are planning to use the RAPD bulk segregant analysis to determine possible loci linked to the level of tolerance.

3.5 Toward the tagging of the apomixis gene (s) and mapping *Brachiaria* using RAPDs markers (Collaboration: J. Miles, Tropical Forages Program)

Until recently, artificial hybridization of *Brachiaria* has not been possible as important commercial species within this genus are polyploid apomict, i.e. reproduction is by seed whose embryo arises by mitosis from maternal tissue. An apomictic progeny is genetically uniform and identical to the mother plant. The potential of apomixis is that, if hybridization can be achieved, the resulting heterozygous genotypes can be multiplied as true breeding lines. Tetraploidy has been induced in the naturally sexual, diploid species *B. ruziziensis*. The resulting sexual tetraploid has allowed hybridization with the commercial species *B. decumbens and B. brizantha*, natural

tetraploid apomict. Owing to a high level of self-sterility, simple techniques of open pollination in the field yield a high proportion (in excess of 90%) of hybrid progeny. Putative hybrids show paternal electrophoresis bands that are absent in the female parent: strong evidence that these progenies are indeed true interspecific hybrids. It would be now possible to combine spittlebug resistance with other desirable agronomic traits in heterotic, true breeding, apomictic *Brachiaria* cultivars. An understanding of the genetic mechanism of apomixis and its inheritance would be extremely valuable and it is classified by the Tropical Forages Program as a high strategic research priority.

The Genetics section of the TFP and the BRU have initiated a joint project to tag the apomixis gene(s) in *Brachiaria*. Potential genotypes have already been identified and a large number of first generation hybrids of the type tetraploidy induced *B. ruziziensis* by *B. decumbens* or *B. ruziziensis* by *B. brizantha* have been made and are being characterized by reproductive mode.

The specific objectives of the project are to :

1- develop the tools to tag the apomixis gene (s) using the Random Amplified Polymorphic DNA (RAPDs) techniques and the bulk segregant analysis. Initial PCR amplification suggests that it will be possible to identify primers that will segregate 1:1 (Fig. 1). The tagging will permit the identification of apomictic plants in hybrid (segregating) progenies at seedling stage, rather than after flowering (by embryo sac analysis) or in a subsequent growing season by progeny test.

2- identify random primers that could be used in the construction of a molecular map for *Brachiaria*. The map will be used to study the genetics of tetraploid apomictic and sexual species. The map would also allow genes tagging of important agronomic traits. Finally the map would facilitate the detection of quantitative trait loci (QTL's) for complex, difficult to evaluate attributes (such as forage quality dry matter digestibility) and will assist in the breeding program for the improvement of these traits.

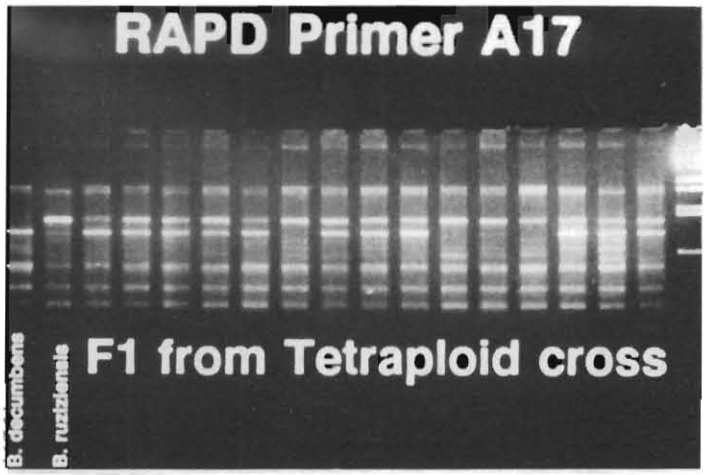

Figure 1. Amplification with RADPs primer A17 of *B. decumbens*, *B. ruziziensis* and F1 plants.

3.6 Tagging resistance genes to Rice Hoja Blanca virus and Colombian lineages of rice blast with RFLP and RAPD markers (Collaboration: F. Correa, C. Martinez and Z. Lentini, Rice Program)

Rice blast caused by the fungus *Pyricularia grisea* Sacc. is the most widespread and damaging disease of rice in the tropical and temperate zones. The fungus is known for expressing a large number of virulent pathotypes. The preferred strategy to control this disease rely on deployment of resistant blast cultivars and a heavy chemical control. The breeding effort to incorporate durable resistance has been slow due to the inability to identify the various genes in a segregating population. Released resistant varieties have been so far short lived, rarely effective for more than 2-3 years.

Recently the diversity and genetic structure of Colombian rice blast isolates from the Santa Rosa station, hot spot for rice blast, have been analyzed with MGR fingerprinting. The study, conducted by M. Levy from Purdue University in collaboration with CIAT, was able to resolve the genetic structureof Santa Rosa 50 races into six distinct MGR lineages. The isolates from each lineage were associated with a specific subset of cultivars and a specific subset of pathotypes. Of particular interest was the case of the cultivar CICA 9. Virulent isolates from Lineage 3 to 6 did not exhibit any virulence reaction on CICA 9. Wheresas isloates virulent on CICA 9 belong to the genetic lineage SRL-1 and 2.

To develop more durable resistance we are currently trying to integrate pathotyping, MGR-fingerprinting and molecular marker assisted gene tagging to efficiently incorporate appropriate resistance genes.

The objectives of this study are:

1- to identify and characterize of currently available and novel resistance genes against Colombian lineage of blast.

2- to tag gene (s) of resistance to rice blast Colombian lineages and RHBV.

3- to develop a strategy to integrate molecular makers in a breeding selection program for durable rice blast resistance.

3.6.1 **Plant materials**. Hundred doubled haploid lines were derived from anther-culture of field grown F1 hybrid from the cross IRAT 13 x Fanny. Irat 13 is cultivar with African origin, highly resistant to most of the rice blast isolates in Colombia. Fanny is highly susceptible to many blast isolates in Colombia and 90 % of the international blast nursery. Recent evaluation have however shown that IRAT 13 is resistant to isolates from lineage 1 and sucseptible to isolates from lineage from Altillanura (ALL). Fanny has the exact opposite reaction; being susceptilbe to isolates

from lineage 1 and 6 and resistant to isolates from ALL7. Each doubled haploid line used in this study was generated from a single callus (CIAT, 1989). Only normal looking and highly fertile plants (suggesting dihaploidization) were selected.

3.6.2 **Blast isolates selection and inoclation**. Isolates were collected from leaves infected in the field with naturally occurring inoculum. Cultures are derived from single conidial isolates and maintained in V-8 juice agar and multiplied for inoculations on rice -polish-agar. Each isolates was screended with the eight International blast differentials to detemine the race, and then characterized by MGR fingerprinitng.

Seedlings from the doubled haploid (DH) lines were grown to the 3-4 leaf stage (18-21 days after planting) in the greenhouse in plastic pots (15 cm diameter) and fertilized with ammonium sulphate in 3 equal fractions (time of planting, one week later, and one day before inoculation) to the equivalent of 180 kg of Nitrogen per hectare.

All DH lines were inoculated simultaneously with each isolate. Ten plants per pot per DH line were inoculated 18-21 days after sowing by spraying (10-15 PSI) a constant volume (1 ml/pot) of conidial suspension (1-5x10^5/ml) in 0.5% gelatin. Two replicates were used. Inoculated plants were then incubated in plastic chambers under >90% relative humidity at night and opened during the day (20-32°C). Disease reaction was evaluated after seven days. Lesion types and percentage leaf area affected were evaluated for each seedling. A cultivar was considered susceptible when more than 20% of the inoculated seedlings exhibited either typical compatible lesions (3mm or longer with heavy sporulation) or lesion type 3 (1-3 mm in diameter) covering 8% or more of the leaf area. All the isolates used showed a clear susceptible ot resistant reaction on the parental lines, a set of differential and on the DH lines.

3.6.3 **Plant DNA extraction, Southern blotting and hybridization**. Fresh-frozen of freeze-dried leaf tissue was powdered in liquid nitrogen using a mortar and pestle. The frozen powdered tissue was mixed with warm extraction buffer composed by 0.15M Tris-HCl pH 7.8, 15mM EDTA, 1.05M NaCl, 1.5% CTAB and 1,5% B-mercaptoethanol and incubated at 65°C for 15 min. One volume of chloroform was added and mixed gently until and emulsion was formed, then the phases was separated by centrifugation (4000g*30 min) and the aqueos phase transfered to a new tube in where 2/3 vol of cold isopropanol was added to precipitate DNA. The DNA precipitate was hooked and incubated in 5 ml of EtaOH/NaCl for 30 min or overnight at -20°C. The DNA was rinsed in EtaOH/NH4Acet, dried and dissolved in 500 μl of TE, treated with RNAse and quantified on a TKO Hoefer fluorometer.

DNA from parental lines, doubled haploid lines, and F2 progeny were digested with five enzymes that showed the highest degree of polymorphism in the rice mapping work (McCouch et al. 1988): Xbal, Dral, EcoRI, EcoRV and HindIII (BRL or New

England Biolabs). BstNI, HaeIII, HindI, MspI, TaqI (BRL) was used whenever these main enzymes failed to reveal polymorphism with an interesting clone. Spermidine (4mM) was added to promote complete digestion (McCouch et al. 1988). Electrophoresis (2,5-3μg DNA per line) was done in 0.8% agarose gels. Hybond-N$^+$ membrane (Amersham) was used in Southern blotting according to manufacturer's recommendations.

Random genomic rice clones (RG clones in pUC8 or pGEM4Z), rice cDNA clones (RZ clones in pGEM4Z) and oat cDNA (CDO clones in pBluescript SK) provided by by the Tanksley' group at Cornell University were used. These clones were propagated in *E. coli* DH5 and extracted by alkaline lysis procedure. Whole plasmids including inserts were labeled with ^{32}P-dATP using random primer labeling kit from Amersham, and were used as probes on filters of rice DNA. The highest stringency condition reached was 0.5X SSPE at 65°C.

3.6.4 **RAPDs screening and Bulk segregant Analysis**. A total of 300 random 10-mer oligonucleotides from the Operon company was used. The RAPDs markers were amplified on a Coy and a Perkin-Elmer thermocyclers. The PCR reaction conditions were as reported by Martin et al. (1991). The amplification productes were resolved on a 1.2% ultrapure agarose stained with ethidium bromide. The bulk segregrant method (Michelmore et al. 1991) was used on DNA from the two parents and on two seprate DNA samples pooled from resistant and susceptible DH lines to a specific blast isolate. This method offer the advantage of not requiring the use isgenic lines and allow the quick screening of a large number of primers to detect possible linked markers with the taget trait.

3.6.5 **Sequence Characterized Amplified Regions (SCARs)**. Sequence Characterized Amplified Regions (SCARs) are PCR based markers that represent a single and genetically defined locus. This technique complement RAPD markers as they detects only a single locus, their amplification is less sensitive to reaction conditions and they can potentially converted into codominant markers (Paran and Michelmore, 1992).

SCARs are obtained from the specific amplification of the DNA with primers of 24 bp designed from the sequence of the only RAPD fragment that co-segregates with the trait of interest. To generate SCARs, RAPDs reaction is performed using the regular PCR protocol. On average, the number of bands obtained from each primers is of 5 bands. The amplification product of interst like the cosegragating band is eluted from low melting point agarose gel using Glass Max DNA Isolation spin Cartridge System (Gibco BRL) and reamplified with the same 10 bp primer.

The amplification product is then blunt end ligated into the pBluescriptII KS vector, that had been linearized with SmaI. The identity of the cloned RAPD product can be verified by restriction analysis or by its hybridization with progeny individuals that

segregate for the progenitor RAPD marker. Double stranded sequencing could be done by dideoxy chain termination method using the M13 universal and T3 primers.

For the cloned RAPD amplification product two oligonucleotides are designed to be used as SCARs primers. Each primer contains the original ten bases of the RAPD primer plus the next 14 internal bases from the end. The primers to be synthesied will be then used with the following profile; 30 cycles of 1 min at 94°C, 1 min at 60°C and 2 min at 72°C. The data generated by SCARs correspond to only one locus.

3.6.6 **Linkage Analysis**. The combined data of the isolates, of the RFLPs and RAPDs data were analyzed with MAPMAKER to determine any possible linkage. A LOD score of $\geq$ 4, recombination value of 30 % and the Kosambi function were used.

3.6.7 **Results**. Data from screening 120 F2 plants from the cross IRAT 13 x Fanny with CICA 9-31-4 indicated a single dominant gene. The reaction was similar for the 6 CICA 9 isolates used, suggesting that the resistance is controlled by the same gene. The reaction to two additional isolates from SRL-6 was also similar with the exception of 4 DH lines, suggesting either two tightly linked genes or the same gene with some escapes during the screening evaluation.

Out of 200 primers tested on the bulk DNA, 6 primers co-segregated between the bulk DNA and the matching parents. These primers were then screened on the whole DH lines (Fig. 1). Three primers: B10, B8 and C15, were found to be linked to the gene of resistance, whereas at least one primer (J1) resulted in a false positive and was unlinked (Fig. 2).

The amplified co-segregating products of B10, B8, C17, the gene(s) of resistance to lineage SRL-1 (CICA 9 isolates) and two isolates from SRL-6 are located on chromosome 4 (Fig. 3). Although IRAT 13 is resistant to almost all isolates from SRL-6, more testing is needed to determine the extent of resistance of the tagged gene to the entire SRL-6 lineage.

We have identified Fanny as a new source of resistance to RHBV. The tagging of one gene conferring resistance to RHBV was achieved in 1991 using RFLPs markers (Fig. 6). The resistance gene to RHBV and two RFLP clones located on chromosome 12, RG-190 and RG-396 were identified to be tightly linked in mapping population of 100 doubled haploid lines from a cross between Fanny and Ceysvoni. The linkage was confirmed in an another doubled haploid population. However all the clones around RG-190 from the interspecific map have resulted monomorphic and we were unable to get an orientation of the gene. Similar results have been obtained with these two clones (Mc Couch 1988). The two clones were placed on chromosome 12 based on trisomic evaluation, but the linkage to the rest of chromosome 12 markers was not resolved probably due to the low level of polymorphism.

3.6.8 **Summary**. We are currently screening a third population from a cross between Fanny and Carreon for RHBV. The objective of the additional screening is to be able to place the gene on chromosome 12. The level of polymorphism of the cross has already been evaluated and is around 70%. This should allow a better resolution of the linkage. Since the sequence of RG-190 has already been published, we plan on designing primers to test if PCR based markers can be used as a screening tools for RHBV. We are also looking at a cross between Fanny and Colombia 1 to try to tag a second gene to RHBV from Colombia 1.

The gene tagging of blast is oriented toward the tagging of specific genes taking into account the knowledge generated from the MGR-fingerprinting and the pathotyping. We are currently generating several mapping populations to dissect the resistance of either whole lineage or part of a lineage using both RFLPs and RAPDs and to incorporate markers assisted selection into a blast breeding program.

We are also incorporating SCARs (Sequence Characterized Amplified Region) by sequencing RAPDs product (Fig 4) to generate longer and more specific which will be better suited for screening of populations in different background.

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Table 1. Source and characteristics of part of the Colombian *P. oryzae* isolates used in the screening.

	ISOLATES				
	Cica 9-35-1	Cica 9-6	Cica 9-11	Fanny 29-1	Ory 1-84
Site	Santa Rosa	Santa Rosa	Santa Rosa	Altillanura	Santa Rosa
Race	IA-128	IA-128	IA-128	IA-102	IA-103
Lineage	SRL-1	SRL-1	SRL-1	SRL-6	SRL-6

Table 2. Pathogenicity under greenhouse conditions of the Colombian isolates of *P.oryzae* tested on the blast international differential set, Colombian released cultivars and the two parental lines.

Genotypes	Isolates				
	Cica 9-25	Cica 9-6	Cica 9-11	Fanny 29-1	Ory 1-84
Int. Differential					
CALORO	R	R	R	R	S
SHATIAOTSAO	R	R	R	S	R
KANTO 51	R	R	R	R	R
DULAR	R	R	R	S	S
USEN	R	R	R	S	S
NP-25	R	R	R	R	R
ZENITH	R	R	R	R	R
RAMINAD	R	S	S	S	S
Local Differ.					
CICA 9	S	S	S	R	R
IR 8	S	S	S	S	R
METICA 1	R	R	R	S	S
ORYZ 2	R	R	R	R	R
CICA 6	R	R	R	R	R
IR 22	R	R	R	S	S
ORYZICA 3	R	R	S	R	S
ORYZICA 1	R	R	R	S	S
CICA 8	R	R	R	R	R
Parental Lines					
IRAT 13	R	R	R	R	R
FANNY	S	S	S	S	S

RAPD SCREENING FOR BLAST RESISTANCE TO COLOMBIAN LINEAGE SRL-1

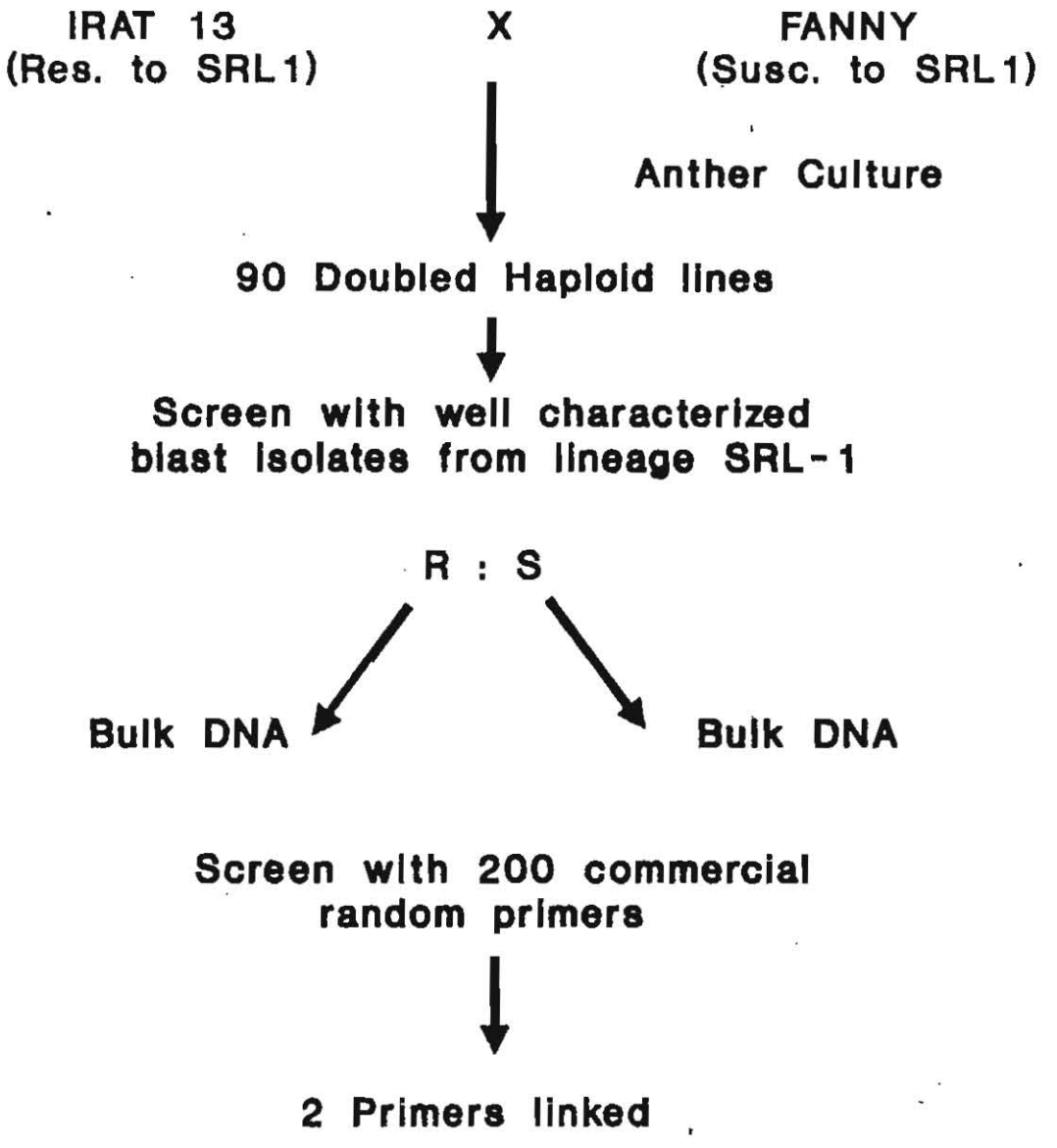

Figure 1. Scheme used to screen for blast resistance to lineage SRL-1.

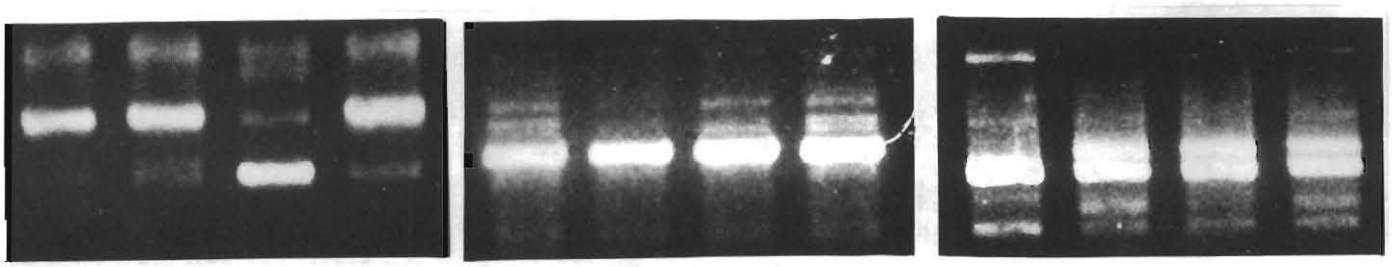

Figure 2. Primers showing no co-segregation. Lane 1 is the susceptible parent (Fanny), lane 2 is the resistant parent (Irat 13) to blast isolate CICA9 from SRL-1, lane 3 and 4 are the bulk DNA from resistant and susceptible lines respectively.

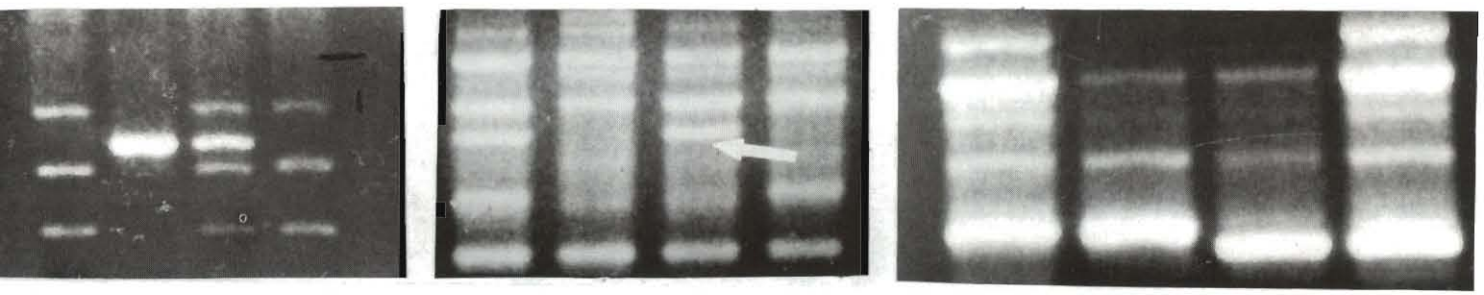

Figure 3. Primer J1,B10 and C15 showing co-segregation between the resistant parent and the bulk DNA from resistant doubled haploid lines and between the susceptible parent and the bulk DNA from susceptible DH lines. Primer J1 resulted as a false positive when tested on the DH mapping population.

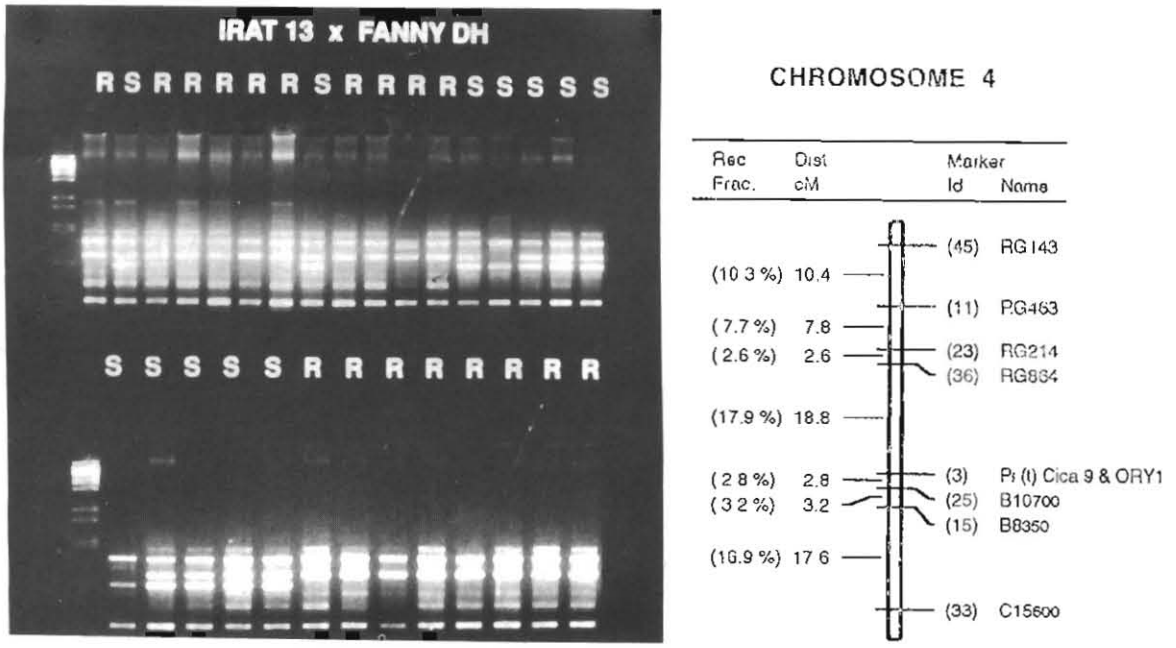

Figure 4. Screening of DH lines with primer B10 that co-segregate in the bulk analysis with the resistant parent and localization of the resistance gene to SRL-1 on chromosome 4 of the rice map. Linkage analysis was made with Mapmaker.

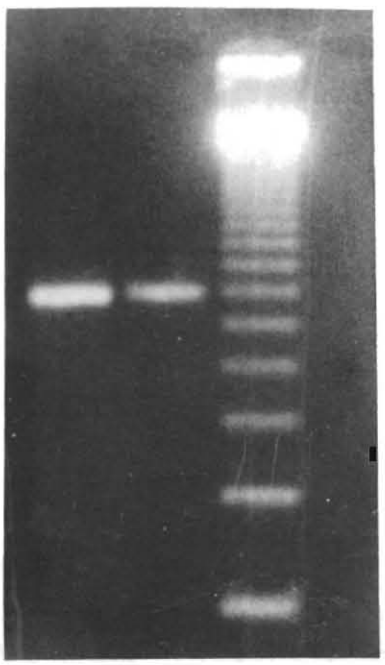

Figure 5. Cloning for subsequent sequencing of the product of B10 that co-segregate with SRL-1 blast reaction. Lane 1, 2 amplification with B10 of the cosegregating fragement B10 primer. Lane 3 1 kb ladder marker.

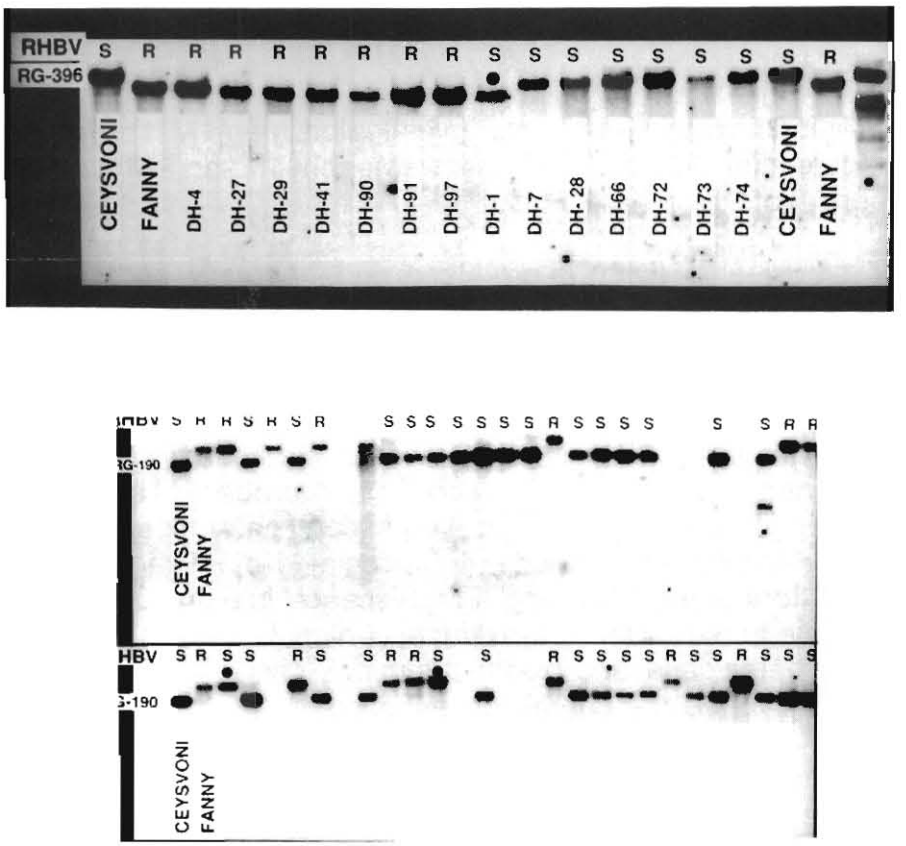

Figure 6. Southern blot of DH lines from the Fanny x Ceysvoni cross hybridized with clones RG-396 and RG-190 from chromosome 12 to show the linkage with RHBV and these two clones. Dot points to recombinant DH lines.

3.7 Nonradioactive methods in RFLPs analysis. (Technique implemented by M. Fregene from the International Institute of Tropical Agriculture, Oyo road, PMB 5320 Ibadan, Nigeria, as part of two months intership at CIAT)

Restriction fragment length polymorphism analysis has proven to be a powerful tool in the analysis of plant genomes. It depends on the hybridization of labelled probes to target DNA immobilized on nylon membranes. Traditional methods of labelling involves the enzymatic incorporation of radioactively labelled probes into a probe. This requires lengthy precautions and disposal procedures that makes an already crowded protocol, cumbersome and harzadous.

In recent years nonradioactive labelling and detection systems have increased in importance because their sensitivity have become comparable to the radioactive ones. In general, they consist of enzyme linked immunosorbent assay (ELISA) detection of a chemically labelled deoxynucleotide enzymatically incorporated into a probe.

3.7.1 **Methods**. In this study, to asses sensitivity, specificity, and ease of filter reuse, two nonradioactive systems were investigated; namely, the Digoxigenin labelling and detection kit of Boehringer Mannheim and the Enhanced chemiluminescence of Amersham PLC.

The cardenolid digoxigenin (DIG) is produced endogenously and soley by the plant *Digitalis purpurea*. Detection of the hapten is by Fab-fragment of a highly specific polyclonal sheep antibody, in an ELISA based method involving the enzymatic oxidation of 5-bromo,4-chloro,3-indolyl phosphate(BCIP) and coupled reduction of nitroblue tetrazolium salt(NBT), to a blue precipitate, which forms on the filter. Probe labelling is by random primed incorporation of the novel nucleotide analogue, DIG dUTP into the probe. The enhanced chemiluminescence protocol employs the fluorescent properties of the hapten fluorescein, coupled to a deoxynucleotide, fluorescein-11-dUTP and incorporated into a probe by random primed labelling. Fluorescence is enhanced by the anti-fluorescein antibody horse radish peroxidase conjugate; which enzymatically reduces hydrogen peroxidase and oxidizes luminol, giving off light. A sheet of blue film is used to capture the chemiluminescence.

3.7.2 **Results**. Results reveal that while the digoxigenin method of Boehringer Mannheim gave highly specific and sensitive hybridization with low or no background (Fig. 2). Non-complete removal of hybridization signals complicated the ease of filter reuse. Nevertheless the method is rapid and 20-30 filters can be conveniently handled in 2 days. The Amersham ECL technique gave promising results with respect to filter reuse, good sensitivity and specificity (Fig. 1) but had lesser degree of success with probe reuse compared to the Boehringer kit. Manipulations were also more than those encountered with the Boehringer system; particularly due to the autoradiography addition to the ELISA step.

We plan on increasing our effort to acheive non radioactive labelling. Testing new systems like BRL Photogene will be carried next year in addition to the two kits already mentionned. Our goals is to implement a system that will allow an efficient striping of labelled probe and repeated resue of a filter.

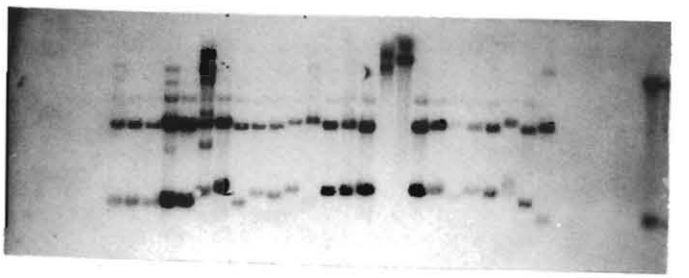

Figure 1. ECL protocol Amersham. *M.esculenta* genotypes digested with EcoRI and hybridized with *V. radiata* chloroplast DNA probe.

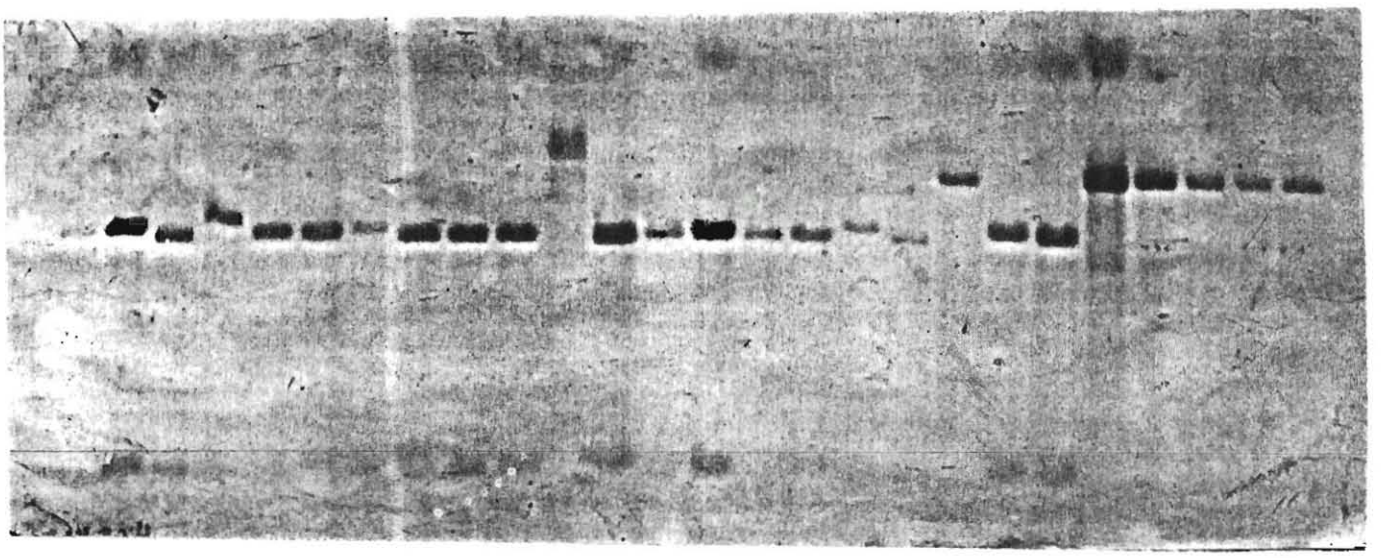

Figure 2. Boehringer Mannheim system. *M.esculenta* genotypes digested with HaeIII and hybridized with nuclear DNA probe.

II. CHARACTERIZATION OF MECHANISMS INVOLVED IN RESISTANCE/ TOLERANCE TO BIOTIC AND ABIOTIC STRESS IN PLANTS

Research in the BRU's Molecular Biochemistry Laboratory can be subdivided into three areas:

- Resistance mechanisms to pests and pathogens
- Physiological and biochemical processes in plants and bacteria
- Methodology development

In the first area, resistance to bruchids, a major pest of beans (*Phaseolus vulgaris*), and antibiosis to the spittlebug in *Brachiaria* are being investigated. Furthermore, the molecular bases of virulence evolution of the bean common bacterial blight (CBB) pathogen and *Colletotrichum lindemuthianum*, the fungal agent that causes anthracnose in beans, are being studied using molecular markers.

In the second area, studies are being conducted on CO_2 assimilation mechanisms in cassava under drought stress. Organic acids and sugars of forage roots under aluminum stress are also being quantified as part of a project to develop screening techniques for acid soil tolerance in pastures. The study of bacterial amylolytic activities during cassava starch fermentation also falls into this category.

With respect to methodology development, cost effective alternatives for DNA extraction and the development of a quick, reliable, HPLC based methodology for the quantification of linamarin, the cyanoglucoside of cassava, are presented.

The objective of this research is to provide an insight into biochemical/molecular mechanisms in order to develop efficient germplasm screening methodologies and identify points for genetic intervention and identify novel sources of genetic variability.

1. RESISTANCE MECHANISMS TO PESTS AND PATHOGENS

1.1 Biochemical Basis of Resistance to the Bean Weevil in Phaseolus (Collaboration with C. Cardona, Bean Entomology)

The bean weevil *Acanthoscelides obtectus* (AO) and the Mexican bean weevil *Zabrotes subfasciatus* (ZS)--both of the family of the Bruchidae (Coleoptera)--are major pests of stored beans in the Americas and in Africa. Resistance has been found only in a few Mexican wild bean accessions. Resistance to ZS has been attributed to arcelin, a family of lectin-like proteins, which has already been introduced into commercial cultivars. Resistance to AO, which is expressed as

reduced and delayed adult emergence with high levels of mortality, has been elusive to classical breeding thus far. Resistance, which seems to be of complex nature, is lost even when crossing wild, resistant accessions among themselves.

This work was started with the aim of understanding the biochemical basis of resistance to AO in wild beans. Several classes of substances (proteins, alkaloids, polysaccharides, flavonoids) have been investigated in this regard at the Natural Resources Institute (NRI)-London, without success; nevertheless, we considered that the problem deserved a second chance given the importance of the pest. The combination available at CIAT--namely, accessibility to a huge germplasm collection and the possibility of combining the expertise of the entomology, biochemistry and genetics groups--is appropiate for tackling this challenge. The aim is to provide geneticists with tools to identify resistance in beans to AO or otherwise provide them with novel sources of resistance.

As shown earlier at CIAT, purified phaseolin, phytohemagglutinin (PHA) or arcelin are nontoxic to AO when added to artificial seed. We proceeded to incorporate acetone-fractionated protein extracts from a resistant bean accession (G 12954), an intermediate accession (G 12880) and two susceptible checks, a cultivated (Pijao) and a wild susceptible accession (G 10019), into artificial seed on a susceptible background flour (Pijao). Highly inhibitory levels of unknown proteinaceous factors were detected, especially in the 0-20% and in the 20-40% acetone fractions as expressed by the extremely low emergence numbers (Fig. 1A and B).

Protease inhibitors and α-amylase inhibitors have been described as sources of resistance to bruchids. We have detected differential inhibition of porcine pancreatic vs bruchid α-amylases among 50 bean accessions including resistant, intermediate and susceptible wild beans, as well as susceptible cultivars (Table 1). Inhibitory behavior was correlated with specific protein-banding patterns in the inhibitors zone (ca. 15 kD on SDS PAGE) (Figs. 2 and 3).

Ionic strength in the insect's gut is dictated by diet and the dilution factor reached in the lumen of the gut. Insects feeding on seeds have adapted to low water content and high concentrations of certain ions such as potassium, calcium and magnesium (Table 2). The Malpighian tubules are responsible for recycling the water; together with the intestinal membrane, they also regulate the uptake of ions from the diet.

α-Amylases and proteases secreted into the lumen have to work under the given dietary conditions. Thus the activity optima of these enzymes have adapted to the high ionic strengths of seed. Protein conformation is highly dependent upon ionic strength. This also applies, of course, to any specific inhibitor that may have developed in the plant, which might be the reason why it has gone undetected thus far.

Under assay conditions used by several researchers in earlier publications, no inhibition of α-amylases was found in bean seeds resistant to AO. We have found some specific *in vitro* inhibition of α-amylase in the resistant accessions of wild beans when mimicking the cationic composition of dry beans by raising potassium and calcium concentrations.

One explanation why breeding for resistance has been elusive is that two or more factors acting synergistically become separated during the breeding process. Resistance to AO probably includes several mechanisms that potentiate the defensive effect. The arcelin example shows how a general defense mechanism such as a lectin has evolved into a specific resistant factor. On the other hand, arcelin 4 does not perform well when bred into commercial lines; in the original accession it may have acted in conjunction with an α-amylase or protease inhibitor (AI or PI). We have found specific inhibition of ZS α-amylase in wild accessions containing arcelin 4 (no inhibition of porcine pancreatic α-amylase, nor of AO α-amylase). Advanced arcelin 1-containing lines have not lost their resistance-conferring trait. It remains to be elucidated whether in this case the arcelin and the AI co-segregated (RAZ 2 and RAZ 25).

Protease and α-amylase inhibitors have been reported in several bean species. It is conceivable that some inhibitors have developed further to become specific to certain pests, which is what our work with AI seems to indicate. Dissecting the mechanisms of resistance will be crucial for breeding. Knowing the targets, every component can be followed separately in the breeding process.

Although serine proteases are the commonest kind of proteases found in insect guts, Profeases of the cysteine or thiol type, are found in the midguts of several families of the Hemiptera and Coleoptera. This is also the case with AO and ZS. Why some insects do not use serine proteases as major digestive enzymes similar to most other insects and higher animals is of a speculative nature. The diversity among different species (or families) may have been the consequence of the evolution of digestive physiology of the insects in response to their environments in which potentially toxic serine protease inhibitors could be used as food proteins. One hypothesis is that the ancestors of insect species that use thiol or aspartic proteinases used to live in an environment that did not require digestive proteolysis. When proteolysis became necessary for survival later on, they may have been isolated in niches where the serine proteases were not useful; e.g., in the presence of high levels of serine protease inhibitors. Under these conditions, the lysosomal enzyme systems--which use thiol and aspartic enzymes in acidic, compartmentalized environments--may have been shifted from a strictly intracellular role to include a secretory digestive role as well.

We have shown that white egg proteins are extremely toxic to AO. The egg lowest white concentrations incorporated into artificial seed caused 100% mortality of AO

at first instar. Egg white contains cystatin, a specific thiol protease inhibitor, which probably explains its toxicity to the bruchid. The DNA sequence of egg white cystatin is available from the DNA data bank. Using specific primers, the cDNA clone will be cloned from a chicken library generated from chicken oviduct using polymerase chain reaction (PCR). The cystatin leader sequence, which directs export of the protein in the oviduct, will be omitted.

The cystatin structural gene will be combined with the phaseolin promoter plus leader peptide, thus directing expression of the construct specifically to the bean grain endosperm. Only low levels of expression should be necessary to confer resistance to AO, as indicated by the feeding trials; moreover, the expression of cystatin alone will probably suffice to create resistant beans. In order to prevent its breakdown, however, it is always recommendable to have more than one resistance gene at hand. A combination of cystatin expression plus some natural resistance gene might yield the ideal combination.

The cystatin construct could be made available to other scientists working with crops susceptible to pests that also use thiol proteases in their digestive tracts; e.g., the flour beetle *Tribolium castaneum*, the Mexican beetle *Epilachna varivestis*, the Colorado potato beetle *Leptinotarsa decemlineata*, and the cowpea weevil, *Callosobruchus maculatus*.

The next hurdle to jump will be the transformation of the common bean, which although reported by Agracetus and by Herrera-Estrella (De la Fuente-Martinez et al., 1992), is very difficult to achieve and highly dependant upon the cultivar. Herrera-Estrella's transformation protocol involves the combined use of microwounding of regenerative tissue with the particle gun and *Agrobacterium tumefaciens*. He has been able to introduce a phaseolotoxin-detoxifying gene, which leads to halo blight-resistant plants. Z. Lentini spent a few months in the lab at the ETH (Federal Technical University) Zurich this year, where she used I. Potrykus' new particle-accelerating device, designed for microtargeting DNA-coated particles. Within a collaborative project with Prof. M. van Montagu's group at the U. of Ghent, bean transformation using several cultivars is being pursued.

At Ghent, G. Angenon and R. Geremia are working on cloning arcelin 5, a very potent variant of the arcelin family, conferring high levels of resistance to ZS. Partial sequencing of the N-terminal amino acids of proteolytic fragments of arcelin 5 was carried out after transferring the electrophoretically separated proteins to a PVDF membrane. Sequence comparison analysis was performed with members of the lectin family; and some characteristic sequence motifs found seem to confirm that arcelin 5, like the other arcelins, also belongs to this group. One of the next steps involves synthesis of oligonucleotides for cloning the arcelin 5 gene and characterizing it further.

1.1.1 **Methodology**

- **Protein fractionation**. Bean flour was extracted with 0.3 M NaCl Ph 3. The extract was precipitated with acetone to different levels of saturation, and the pellet was then lyophilized. Basic proteins were extracted with acetic acid and precipitated with 80% acetone. The latter fraction was practically noninhibitory to bruchid development.

- **Artificial seed**. Increasing levels of isolated protein fractions from a susceptible bean cultivar were added to flour. Artificial seeds were reconstituted in teflon molds through lyophilization and the pellets were coated with gelatin. Percent emergence of controls surpassed 98%.

- **Rearing of bruchids**. Each concentration of the fractions was tested (five repetitions) by applying 3 larvae to each of 5 artificial seeds in a vial, and evaluating percent emergence and days to adult emergence until the last insect emerged.

- ***α*-amylase activity and inhibition**. Amylase activity was assayed by iodometric quantification of starch hydrolysis. Inhibition was demonstrated by preincubating the inhibitor extract with porcine pancreatic amylase or extracts from midguts of fourth instar larvae and the subsequent measurement of amylolytic activity.

1.1.2 **Conclusions**. Strong inhibition of bruchid growth is caused by specific flour protein fractions from resistant bean accessions. The experiment is being repeated with ZS to establish specificity toward AO.

The 0-20% and the 20-40% acetone fractions derived from the resistant accessions G 12880 and G 12954 appear to contain a potent antibiotic factor to AO as expressed by the strong negative effect on emergence. The 60-80% fraction probably contains some general resistance factors common to all bean accessions tested. Amylase and protease inhibitors are normally contained in this fraction.

Resistant sources show two types of mechanisms: one affecting percent emergence only (G 12880) and the other (G 12954) acting additionally on days to adult emergence (DAE). The 40-60% acetone fraction of G 12954 causes a considerable delay of DAE, indicating that the two mechanisms are independent.

Differential, ionic strength and buffer-dependant *α*-amylase inhibitory activity toward bruchid and porcine pancreatic *α*-amylase were detected in wild bean accessions.

The cystatin-type protease inhibitors of thiol proteases--but not the trypsin inhibitors from soybeans and lima beans--probably inhibit development of AO at the lowest

concentrations tested. A protease inhibitor of this type might also be involved in resistance to the bruchid, which is a subject of ongoing research.

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De la Fuente-Martinez, J.M.; Mosqueda-Cano G.; Alvarez-Morales, A. and Herrera Estrella, L. 1992. Expression of a bacterial phaseolotoxin-resitant ornithyl transcarbamylase in transgenic tobacco confers resistance to *Pseudomonas syringae* pv *phaseolicola*. *Bio/Technology* 10:905-909.

Mayer, J.E.; Fory, L.F.; Tenjo, F.A.; Posso, C.E. and Cardona, C. 1992 α-Amylase and protease inhibitors in wild beans as related to bruchid resistance. Molecular Crop Agriculture for the Pacific Rim, U. California Davis/Sacramento, June 20-24.

Mayer, J.E.; Fory, L.F.; Tenjo, F.A.; Posso, C.E. and Cardona, C. 1992 α-Amylase and protease inhibitors in wild beans as related to bruchid resistance. Biotechnology for Crop Improvement in Latin America, Nov 1-7, Caracas.

Table 1. Differential inhibition of α-amylases. Every accession is characterized by it's original site of recollection (MX, Mexico; GT, Guatemala; PE, Peru; CV, cultivar; plus a three letter code for the department), it's resistance to the bruchids (*Acanthoscelides obtectus* (AO), *Zabrotes subfasciatus* (ZS); R, resistant; S, susceptible; I, intermediate), the presence/absence of arcelin variant (ARC), the inhibition of porcine pancreatic (PA), AO and ZS amylase in calcium succinate (cs) or calcium acetate buffer (ca).

					AMYLASE INHIBITION			
ACC NR	ORIGIN	AO	ZS	ARC	AO	ZS	PA	PA
					cs/ca	ca	cs	ca
G 12866	MX JAL	S	R	2				-
G 12880	MX GUE	R	S		+	-	+	+
G 12882	MX GUE	S	R	1		-		-
G 12922	MX JAL	S	S	3		+		-
G 12947	MX JAL	R	R	4	+ -	+	-	+
G 12949	MX JAL	R	R	4	+ -	+	-	+
G 12950	MX JAL	R	R	4	-	+	-	+
G 12951	MX JAL	R	R	4	+ -	+	-	+
G 12952	MX JAL	R	R	4	-	+	-	-
G 12953	MX JAL	R	R	4	-	+	-	-
G 12954	MX JAL	R	R	4	+ -	+	-	-
G 12955	MX JAL	I	R	4	-	+ -	-	+
G 02771	MX NAY	S	R	5	-	-	-	+
G 09989B	MX JAL	S	S		+	-	+	+
G 10022	MX DUR	S	S		-	+	+	+
G 11051	MX JAL	S	I	6		-	+	+
G 12851	GT SRO	I	S		-	-	+	+
G 12867	MX NAY	I	S		-	-	+	+
G 12886	MX	S	I		+ -	-	+	+
G 12891	MX JAL	I	I		+	+	-	+
G 13016	MX GUE	I	S		-	-	+	+
G 10002	MX GUE	S	S		-	-	+	+
G 10005	MX GUE	S	S		-	+ -	+	+
G 10006	MX GUE	S	S		-	-	+	+
G 10019	MX MIC	S	S		-	-	+	+
G 11027	MX DUR	S	S			-		+
G 11034	MX DUR	S	S		-	-	+	+
G 11050	MX MIC	S	S		-	-	+	+
G 12858	PE JUN	S	S		-	+ -	+	+
G 12862A	MX JAL	S	S		-	-		+
G 12862B	MX JAL	S	S		-	+	+	+
G 12876	MX OAX	S	S		-	-	+	+
G 12910	MX GUA	S	S		-	-	+	+
G 12923	MX JAL	S	S		-	+ -	-	+
G 12928	MX JAL	S	S		-	+	-	-
G 12935	MX JAL	S	S		-	-	-	+
G 12942	MX JAL	S	S		-	+	+	+
G 12957	MX JAL	S	S		-	+	+	+
G 12976	MX JAL	S	S		-	-	+	+
G 12981	MX JAL	S	I		-	-		+
G 12985	MX JAL	S	S		-	-	+	+
G 13017	MX MOR	I	S		+ -	-		+
G 13018	MX MOR	S	S		+	-	+	+
Calima	CV	S	S		-	-	+	+
Pijao	CV	S	S		-	-	+	+
NEP 2	CV	S	S		-	-	+	+
EMP 218	CV	S	S		-	-	+	+
MCM 5005	CV	S	S		-	-	+	+
RAZ 2	CV	S	R	1		-		+
RAZ 25	CV	S	R	1		-		-

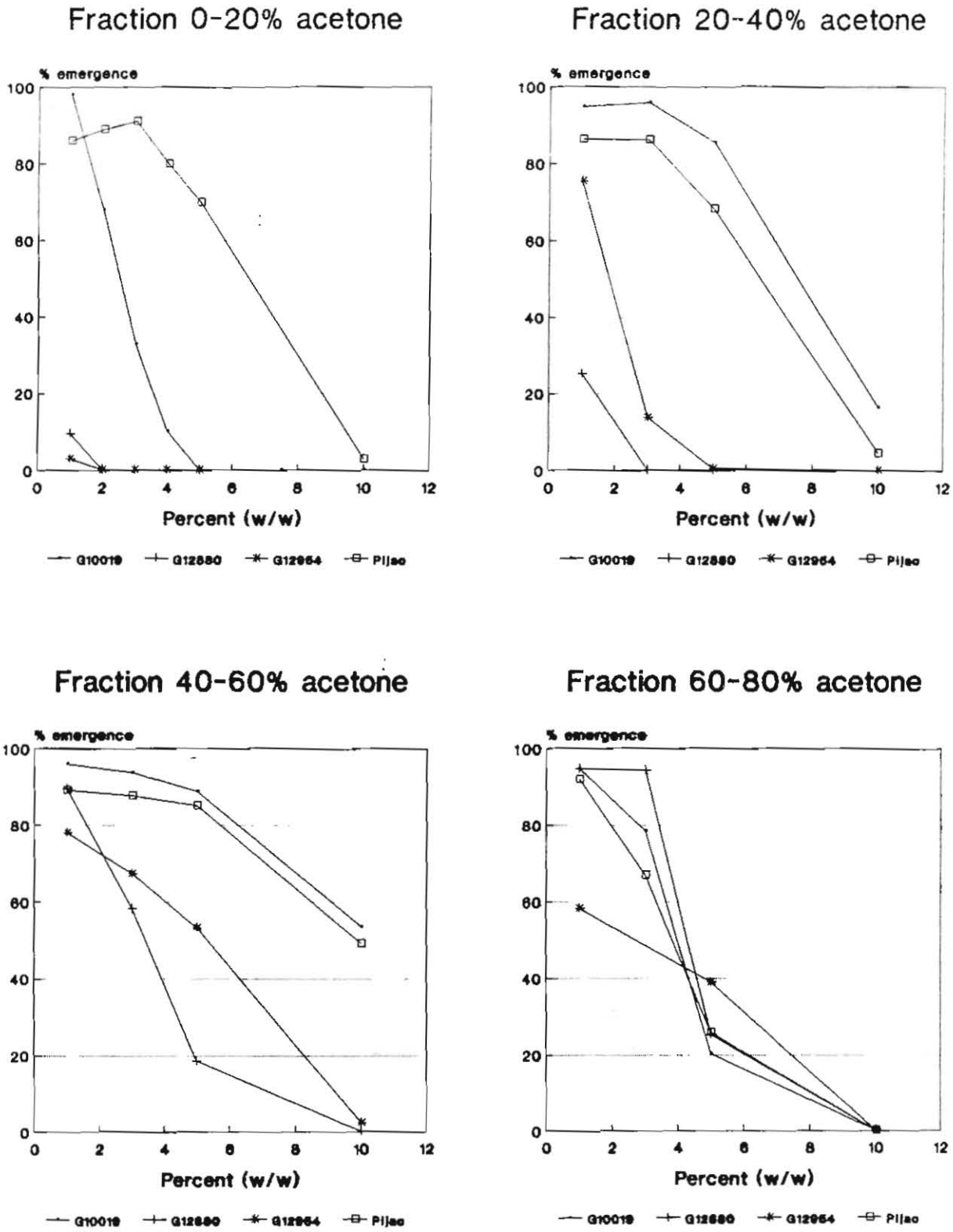


Figure 1. A. Effect of proteic fractions added to artificial seed on percent emergence of *A. obtectus.*

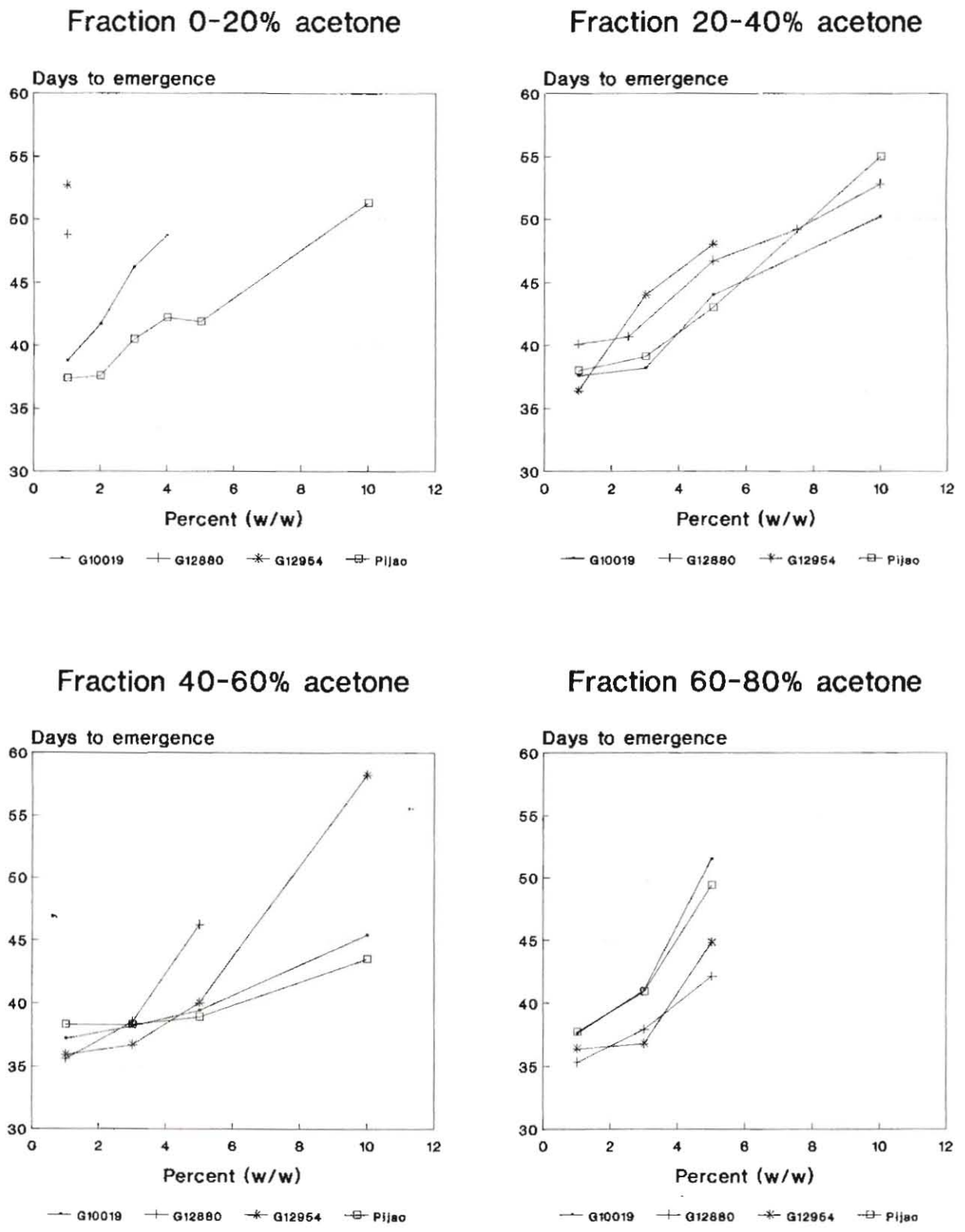


Figure 1. B. Effect of proteic fractions added to artificial seed on lifecycle elongation of *A. obtectus*.

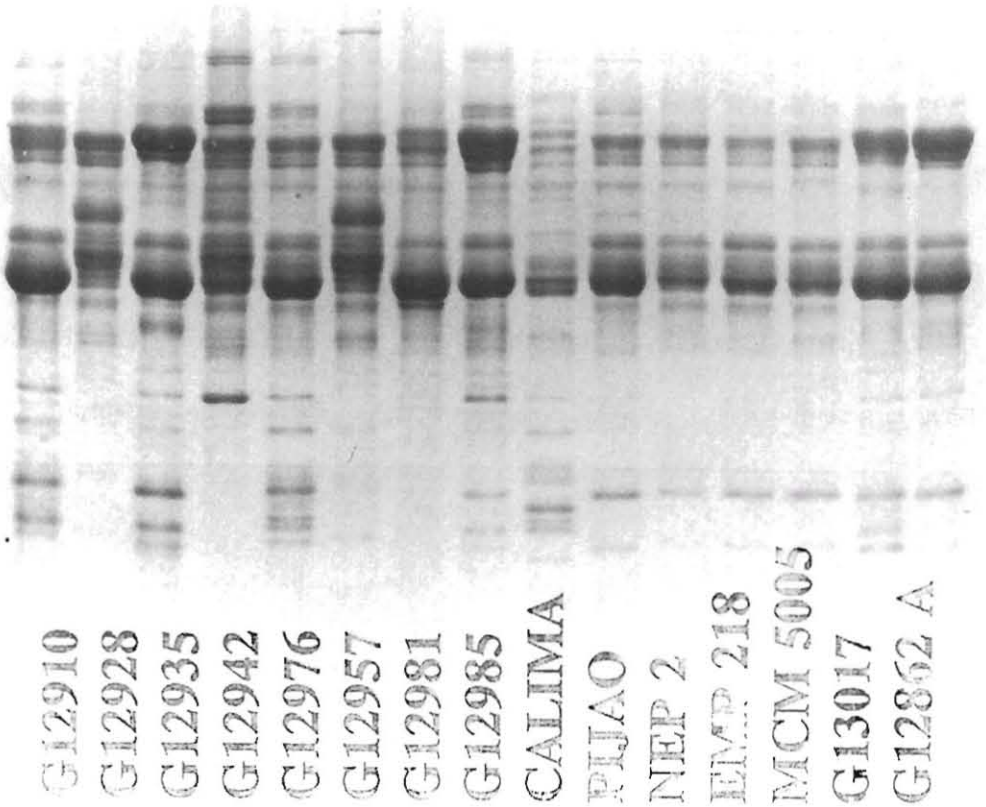


Figure 2. Total soluble proteins of bean accessions used in this study. Accessions can be classified according to Phaseolin type, lectin, arcelin, and polymorphisms in the amylase and protease inhibitor zone.

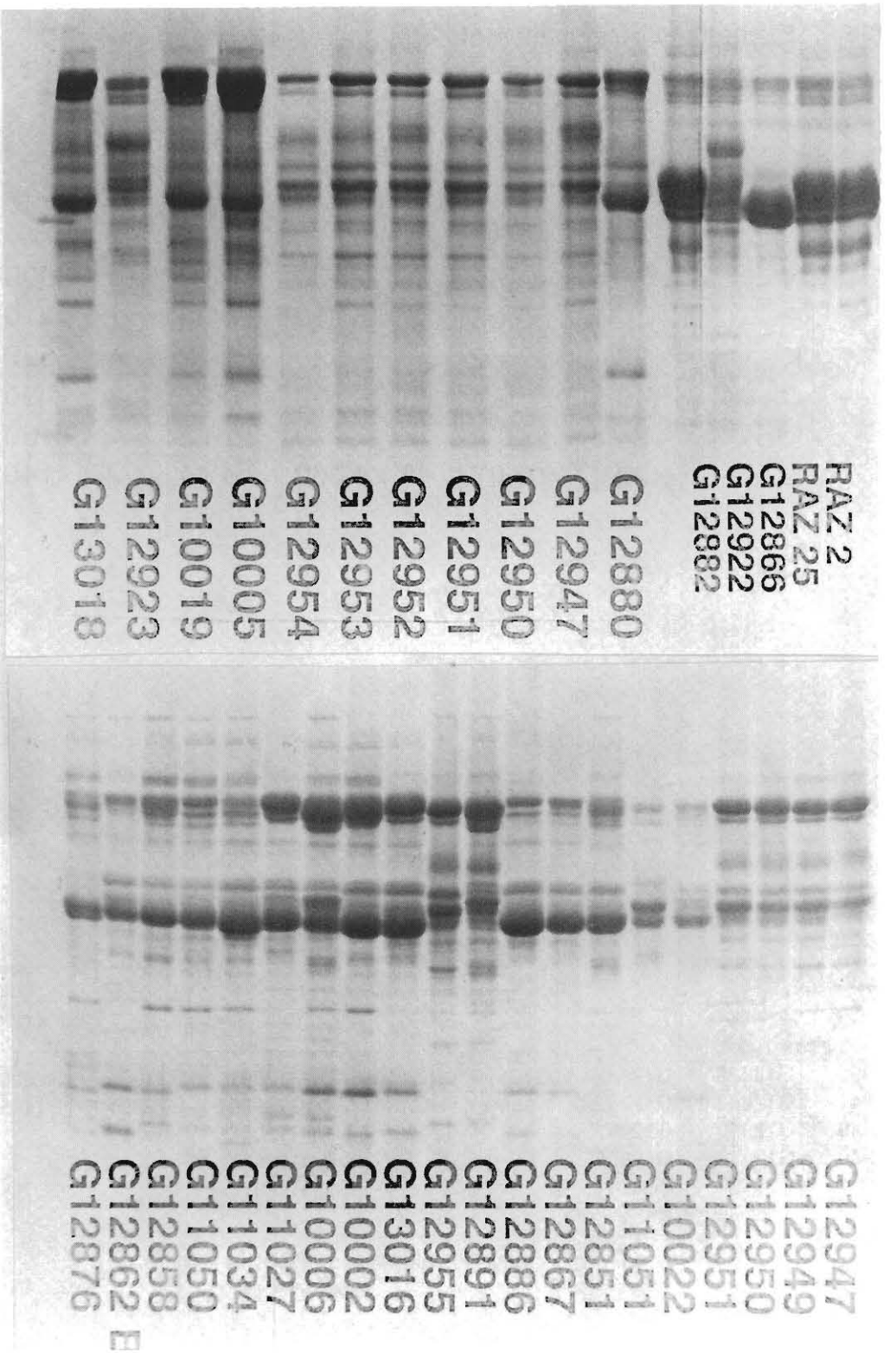
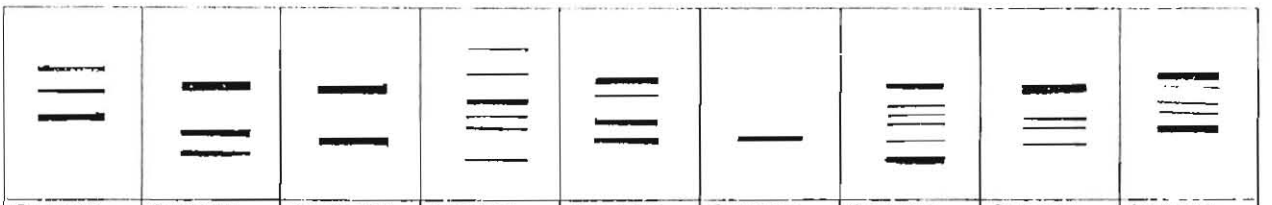


Figure 2. Cont'd.

Table 2. Elementary composition of bean grain (14% water w/w).

		Field Grown		Pot Grown	
	AMU	%	mMol/kg	%	mMol/kg
N	14	3.4	2400	2.9	2000
P	31	0.33	110	0.33	110
K	39.1	2	520	2	520
Ca	40.1	0.4	100	0.36	90
Mg	24.3	0.4	170	0.2	80
S	32.1	0.87	270	0.44	140



G12923	G10022	G12862A	G12876	G12985	G12891	G11034	G10002	G10006
G12947	G12858	G12862B	Calima	G13017	G12928	G12851	G11050	G11027
G12949	G12910	G12867			G12942			G11050
G12950	G13018	G12886			G12957			G12935
G12951		Pijao			G12976			
G12952		NEP2						
G12953		EMP218						
G12954		MCM5005						
G12955								

Figure 3. Electrophoretic inhibitor patterns.

1.2 Antibiosis to Spittlebug in *Brachiaria*

Spittlebug infestation in susceptible *Brachiaria* fields is devastating. These xylem-sucking insects produce yellowing and drying out of huge areas planted with this pasture. Probably due to the low contents of nutrients in the xylem sap, large amounts of the fluid are sucked by the insects, which also protect themselves from desiccation by a spittle cover.

Antibiosis has been detected in *B. jubata* accessions. The antibiotic factor interferes with ecdysis of the nymphs. These effects have been reproduced in a bioassay by adding ecdysone to the nutrient solution of plantlets growing in tubes (S. Lapointe, TFP Entomology). Phytoecdysones have been observed in other plant families, which makes this hypothesis plausible.

Work done by J.C. Steffens at Cornell U. with HPLC indicates that insects feeding on the resistant *B. jubata* excrete a substance that co-migrates with 20-hydroxyecdysone (ß-ecdysone = ECD), but data are inconclusive. Amino acid composition might be in antibiosis; this is not the case when feeding on the susceptible *B. decumbens*.

The production of **anti-ECD antibodies** (α-ECD-Ab) as a complementary project could accelerate the understanding of the resistance mechanism as well as provide a tool for further research. The availability of α-ECD-Ab will permit the identification and quantification of ECD in *Brachiaria* spp., using either radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA) techniques. Positive correlation of ECD existence and resistance to spittlebug infestation would provide one more proof for the proposed mechanism of resistance; namely, interference of a phytoecdysone with the insect's larval development. Furthermore, once the relationship is established, the immunological assays would be used to select ECD-producing plants in a breeding program.

As a hapten ECD is not sufficiently immunogenic to induce the immune response in a rabbit; the molecule has to be chemically coupled to a carrier protein. This has been achieved by attaching a succinyl bridge to the hydroxy groups of ECD using succinic anhydride, preferentially at the A-ring of the steroid molecule (Fig. 1).

The succinylated intermediate was purified and desalted by chromatography through QAE Sephadex (quaternary anion exchanger). Succ-ECD was further activated with ethylchloroformate for deriving reactive lysine ϵ-amino groups of BSA. The separation of the BSA-ECD from the unreacted ECD was achieved chromatographically. The product was identified by spectrophotometric analysis (Fig. 2). Fraction 2 shows an ECD/BSA molar ratio of about five, while fraction 4 contains the free, unreacted ECD.

The conjugate with the exposed ECD molecules was injected into a rabbit. One intramuscular injection containing Complete Freund's Adjuvant, followed by two intradermal boosters with Incomplete Freund's Adjuvant, were applied over two months. The isolated serum obtained after bleeding showed a strong reaction against BSA in double immunodiffusion tests.

To obtain a polyclonal monospecific antiserum, the serum was precipitated stepwise with increasing amounts of BSA over the course of several days. The specificity of the antiserum has been confirmed, using Western Blot analysis.

Although an RIA for ECD has been described in the literature (Delaage et al. 1982; Borst and O'Connor 1972), an ELISA is preferred as it seems more appropriate for a breeding program to do the testing on microtiter plates, obviating the use of radioactively labeled ECD. The RIA relies on competitive binding of IgG to labeled vs unlabeled ECD; whereas in the ELISA test, a BSA-ECD complex would be bound to microtiter plates. Alkaline phosphatase (AP)-labeled goat-α-rabbit-IgG antibodies (GAR-AP) would then be used to calculate the amount of free IgG; i.e., still able to bind to the plate after binding to ECD from the plant extract. Alternatively, BSA-ECD labeled with AP will compete for binding to α-ECD-IgG, adhered to the plates. Thus both methods rely on the quantification of AP by colorimetric determination of hydrolyzed substrate.

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Delaage, M.A.; Hirn, M.H. and deReggi, M.L. 1982. Radioimmuno-assay of ecdysteroids. *Meth Enzymol* 84:350-358.

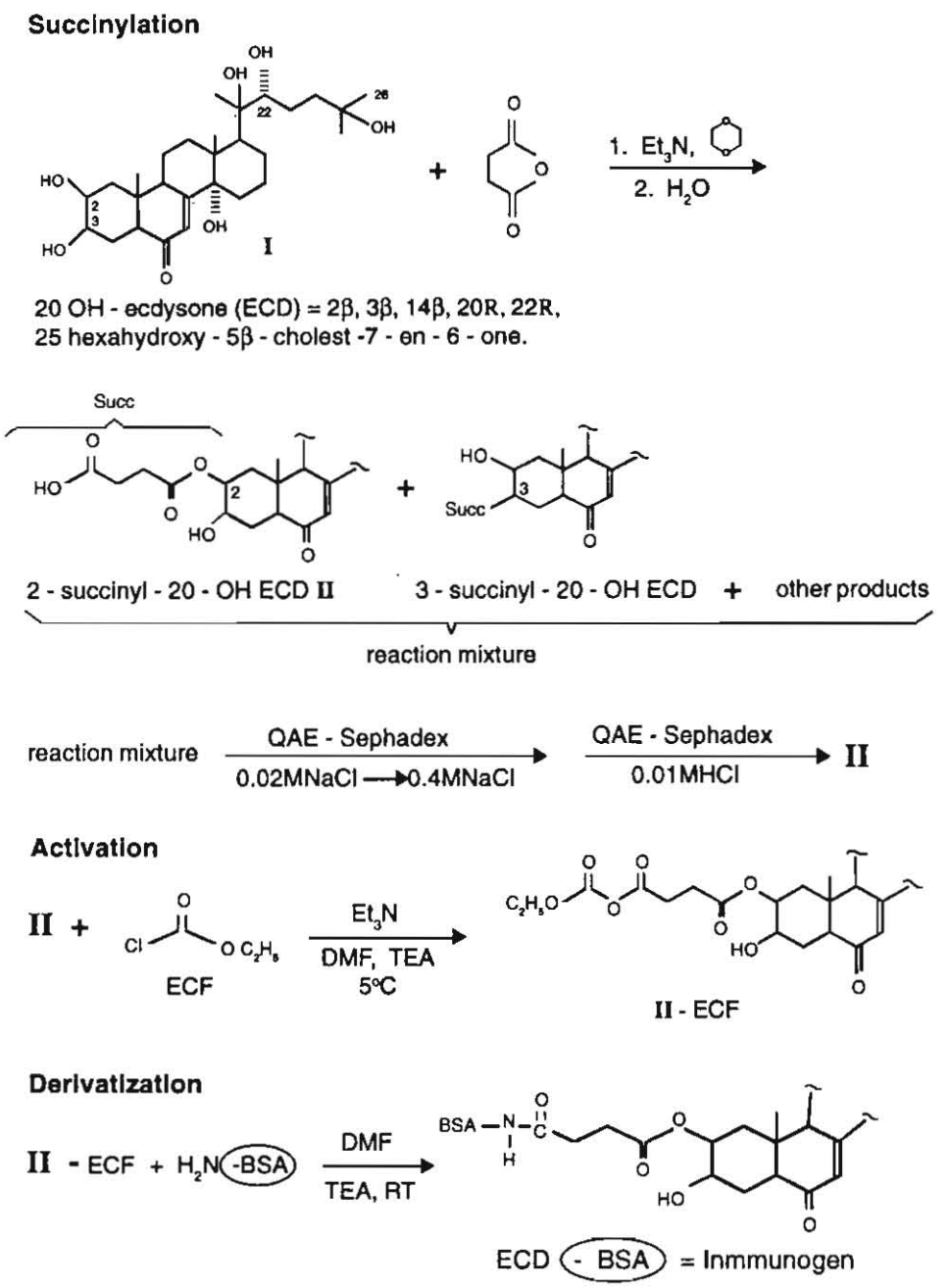
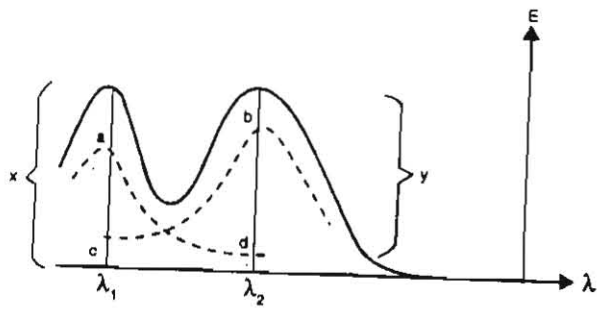


Figure 1. Synthesis of BSA-Ecdysone.



a, b : E of ECD at λ1 and λ2
c, d : E of BSA at λ1 and λ2

λ1= 248 nm λ2= 280 nm

$$\frac{\epsilon_{ECD\lambda 1}}{\epsilon_{ECD\lambda 2}} = \frac{a}{b} = \frac{11600}{654}$$

$$\frac{\epsilon_{BSA\lambda 1}}{\epsilon_{BSA\lambda 2}} = \frac{c}{d} = \frac{22105}{35425}$$

$$conc = \frac{E}{\epsilon}$$

$$conc_{ECD} = \frac{a}{\epsilon_{11}} = \frac{b}{\epsilon_{12}}$$

$$conc_{BSA} = \frac{c}{\epsilon_{21}} = \frac{d}{\epsilon_{22}}$$

a + c = x; b + d = y

							ECD	BSA	
FR	x	y	a	b	c	d	c248	c248	ECD/BSA
2	6.56E-01	3.02E-01	4.84E-01	2.73E-02	1.72E-01	7.26E-01	4.17E-05	7.78E-06	5.36E+00
3	5.55E-01	1.60E-01	4.72E-01	2.66E-02	8.32E-02	1.33E-01	4.07E-05	3.76E-06	1.08E+00
4	1.23E+00	2.02E-01	1.15E+00	6.47E-02	8.56E-02	1.37E-01	9.91E-05	3.87E-06	2.56E+01
5	1.57E-01	1.33E-01	7.67E-02	4.32E-03	8.03E-02	1.29E-01	6.61E-06	3.63E-06	1.82E+00
6	1.70E-01	1.38E-01	8.69E-02	4.90E-03	8.31E-02	1.33E-01	7.49E-06	3.76E-06	1.99E+00
BSA	4.21E-01	6.71E-01	2.38E-03	1.34E-04	4.19E-01	6.71E-01	2.05E-07	1.90E-05	1.05E-02

Figure 2. Spectrophotometric determination of ECD/BSA molar ratio. Top: Spectral multicomponent analysis. Bottom: Determination of ECD/BSA molar ratio in fractions of QAE-Sephadex column.

1.3 The Molecular Basis of Virulence Evolution of *Colletotrichum lindemuthianum* in Latin America (Collaboration with M.A. Pastor Corrales, Bean Pathology; work conducted in the Bean Pathology Section. Technical advise and interpretation of molecular genetic data by J. Mayer)

C. lindemuthianum (CL) is the causal agent of anthracnose, causing severe losses in bean production throughout the mountainous humid tropics and subtropics. Susceptible cultivars are usually destroyed--in contrast with bean common bacterial blight (CBB), where susceptible cultivars suffer losses but are not generally killed.

Also in contrast to CBB, races for CL are well defined, and a set of differentials has been successfully used by the Bean Pathology group at CIAT to characterize 351 isolates from Latin America into races (Table 1). This is an excellent basis for in-depth analysis of virulence evolution. In preparation for the genetic analysis, an evolution model is being developed based on the geographic distribution of the CL races. In this model it is assumed that every race can give rise to another/several others through acquisition of an additional virulence gene in a forward fashion. Alternatively an avirulence gene could be lost in every step. The diagrams for Mesoamerica and the Andes show some very clear regional subgrouping patterns (Figs. 1 and 2). In some cases the introduction of CL races into new production areas can be inferred. Some races can theoretically originate from two or more races; this may not be the case in nature, but genetic analysis should provide more insight.

Linkage of virulence patterns with seed size, which seems to indicate the co-evolution of Mesoamerican and Andean races with their respective regional bean pools, has been proposed by the Bean Pathology Section.

The search for a repetitive probe for the fingerprint analysis of CL has been initiated. A DNA probe derived from the M13 bacteriophage, which has shown polymorphic repetitive patterns with several organisms, led to a multiband pattern but did not show polymorphism for the isolates tested. A soybean ribosomal probe showed polymorphism, but only a few bands; hence it can probably be used to define only the major lineages (Fig. 3).

Restriction fragment length polymorphism (RFLP) analysis of variable ribosomal regions will be performed using ribosomal DNA primers for PCR. RFLP analysis will be enhanced by digesting the amplified fragments with four-cutter enzymes (Vilgalys and Hester 1990). Utilization of the Stoffel fragment of the Taq polymerase (an N-terminal deletion of 289 amino acids from the enzyme lacking 5'-3' exonuclease activity) will also be pursued, as this variant leads to the production of more bands, which can be used to generate fingerprints (Erlich et al. 1991).

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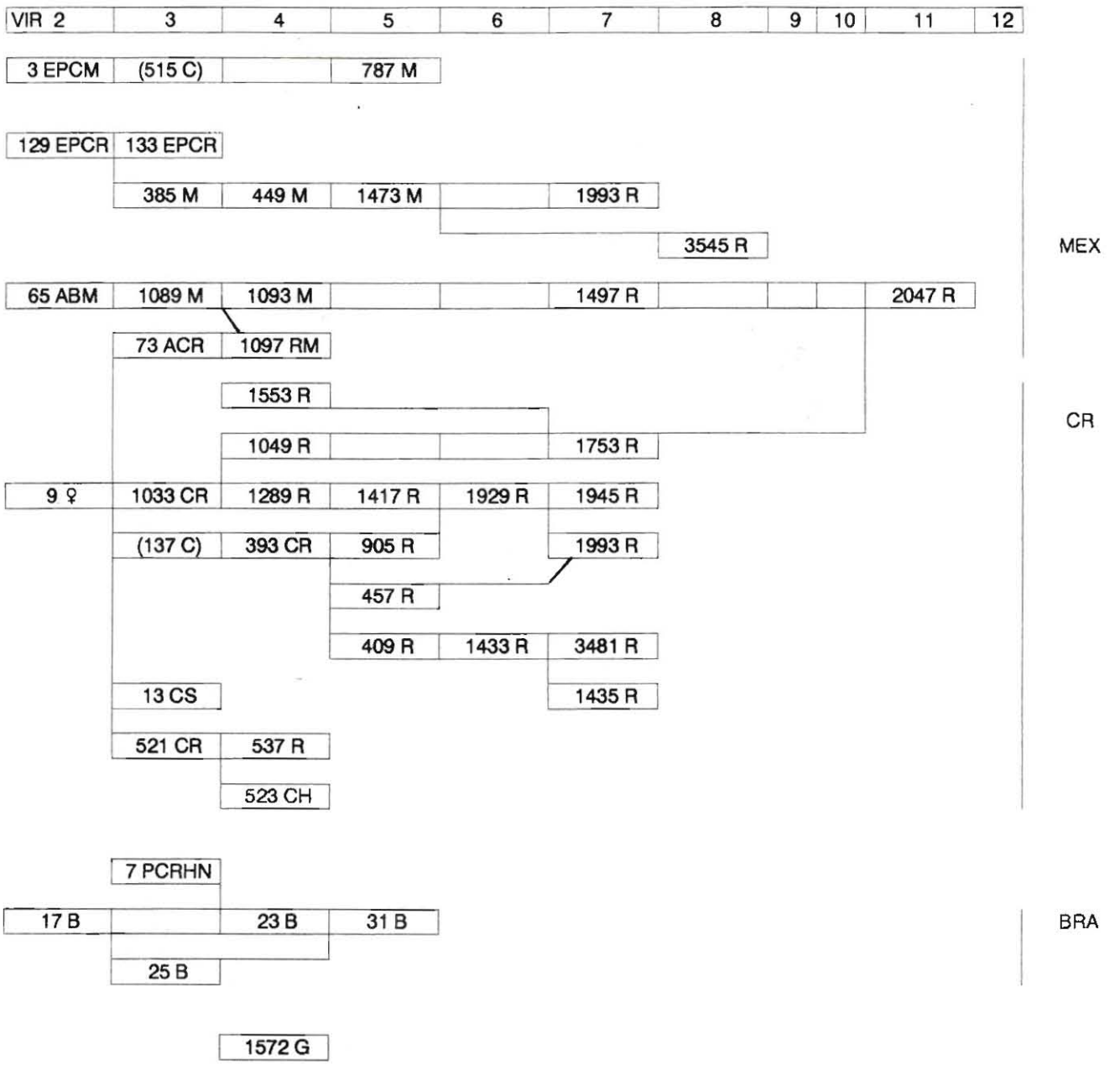

Figure 1. Evolution of virulence of *Colletotrichum lindemuthianum* in Mesoamerica and Brazil.

A=ARG; B=BRA; C=COL; E=ECU; G=GTA; H=HON; M=MEX; N=NIC; P=PER; R=CR; S=SAL
9♀= 9EACBGHMS

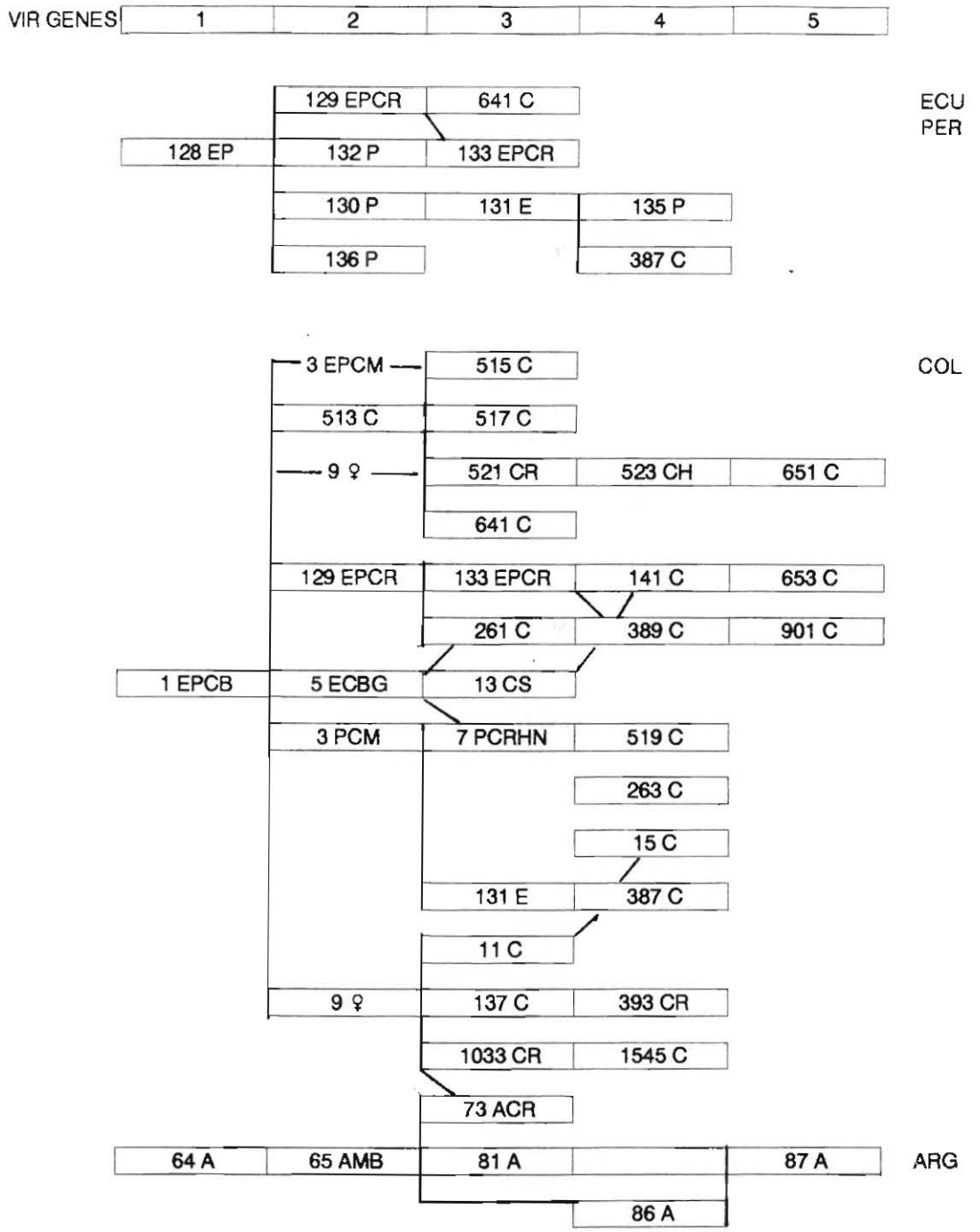

Figure 2. Evolution of virulence of *Colletotrichum lindemuthianum* in the Andes.

A=ARG; B=BRA; C=COL; E=ECU; G=GTA; H=HON; M=MEX; N=NIC; P=PER; R=CR; S=SAL
9♀= 9EACBGHMS

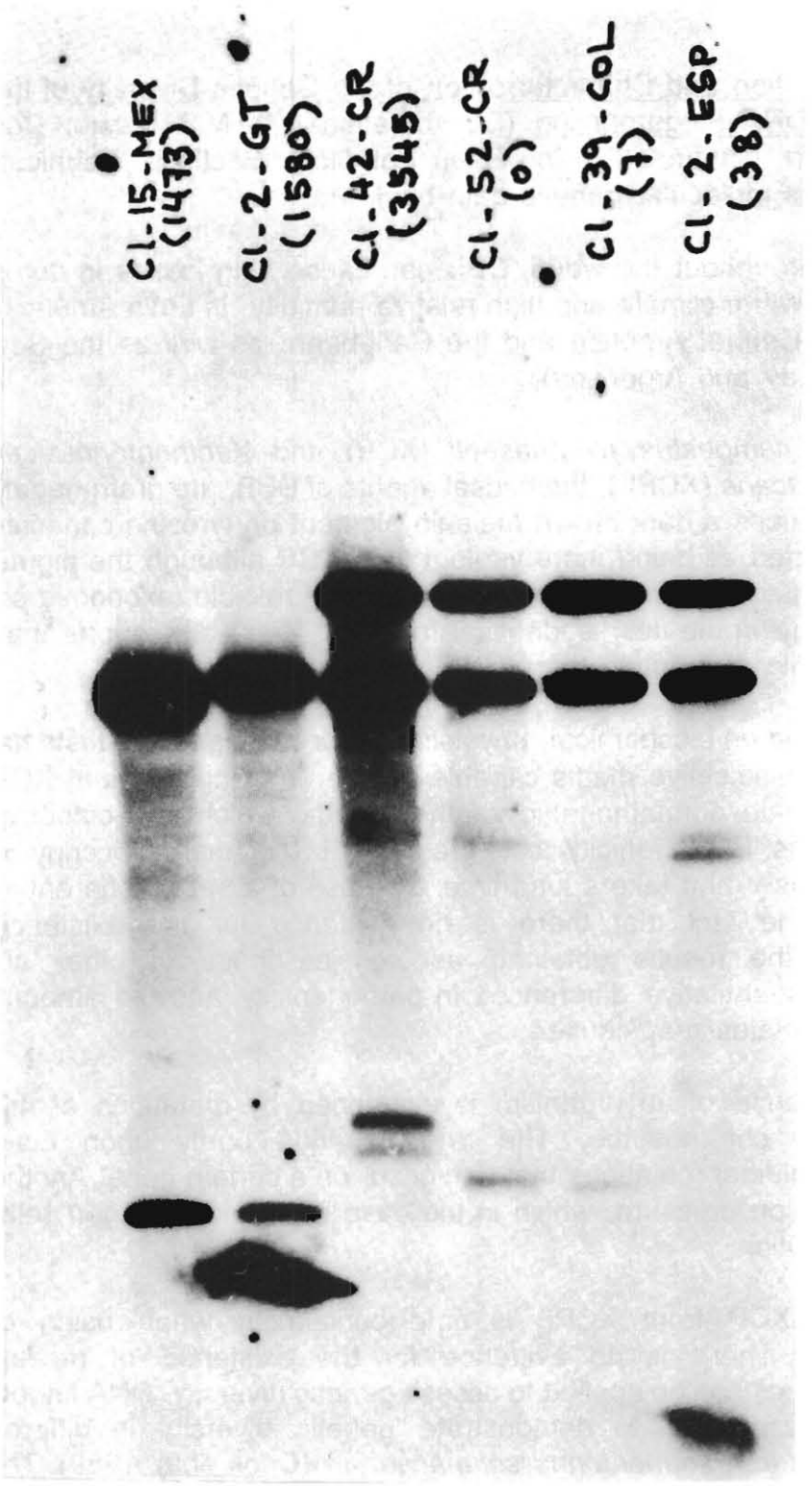

Figure 3. RFLPs of *Colletotrichum lindemuthianum* isolates revealed by a soybean ribosomal probes (pGmr1).

1.4 Demonstration and Characterization of the Genetic Diversity of the Bean CBB Pathogen by DNA Fingerprinting (Collaboration with M.A. Pastor Corrales, Bean Pathology; work conducted in the Bean Pathology Section. Technical advise and interpretation of molecular genetic data by J. Mayer)

Widespread throughout the world, CBB can cause high losses in dry bean yield in regions with a warm climate and high relative humidity. In Latin America the disease is endemic in Central America and the Caribbean, as well as the Southern Cone (Brazil, Paraguay and Argentina).

Xanthomonas campestris pv phaseoli (XCP) and *Xanthomonas campestris pv phaseoli var fuscans* (XCPF), the causal agents of CBB, are gram-negative bacteria. The latter produces a dark brown melanin pigment on tyrosine-containing media. It has been reported as being more virulent than XCP although the pigment does not seem to be related to its pathogenicity and is not a reliable taxonomic characteristic. Symptomatology in the field is identical for both. There are reports that XCP has a wider geographic distribution than XCPF.

Taxonomy based on biochemical, physiological or pathogenicity tests has been very limited. The semiselective media capable of differentiating between XCP and XCPF do not discriminate nonpathogenic xanthomonads, which also colonize fresh bean tissue and debris. Pathogenicity tests are very labor-intensive, occupy a lot of space in the greenhouse, and take a long time. The use of a set of differential varieties is restricted by the fact that there is no evidence for the existence of races. Nevertheless, the results obtained as well as those of other authors have demonstrated quantitative differences in pathogenicity and the amount of disease that different isolates may cause.

Genetic divergence of an organism is evidenced by mutations at different rates throughout the chromosome. The rate depends partly upon the number of permissible nonlethal mutations that can occur on a certain gene. Another important aspect is selection pressure, which in the case of a host/pathogen relationship will lead to co-evolution.

Discriminating XCP from XCPF is time consuming when using conventional methodologies. There is no evidence for the existence of races; hence no pathogenicity tests can be applied to assess genetic diversity. DNA fingerprinting has been used successfully to demonstrate genetic diversity in different bacterial pathogens such as *Pseudomonas solanacearum* (Cook et al. 1989). This approach is also appropriate for XCP and XCPF (Gilbertson et al., 1989). The objective of this work was to develop a probe that would serve a dual purpose: quickly identify XCP and XCPF for diagnostic purposes and study their genetic diversity in Latin America.

The ideal case for DNA fingerprinting analysis is to find a single probe that will detect several changes at a time. This occurs in some cases with medium-repetitive DNA probes. Looking at disperse DNA sequences makes it possible to detect more than one mutation at a time.

The XCP-specific probes were isolated from a genomic EcoRI library and a plasmid-derived EcoRI library (Gilbertson et al., 1989). The genomic DNA was sized on an agarose gel after digestion, and the 1-5 kb fragments cloned into pUC12. Clones P2 and P7, which contain a 3.6 and a 3.4 kb fragment, respectively, show the kind of complex pattern on digested genomic DNA required for fingerprinting.

The ends of both clones were sequenced using the dideoxy method and the universal primers. To analyze the internal sequence information, Hae III and Sau 3A subclones were generated. The resulting sequence information was compared with the GenBank database. A partial sequence has revealed homology to a Pseudomonas avirulence gene ant to a transposon. As the probes hybridize only to pathogenic isolates, the sequence information could have some relation to pathogenicity (e.g., transposable elements), which could turn virulence genes on/off by jumping out/into virulence loci. The sequence information can be used further to generate primers for PCR analysis of seeds. This application for seed quarantine will be developed in 1993 in Bean Pathology.

Most of the collection of over 300 XCP and XCPF isolates has now been fingerprinted. The isolates analyzed thus far were clearly separated into two groups that coincided with the XCP and XCPF groups (Fig. 1). The banding of these two groups is very characteristic and can be observed by simple inspection. This is in contrast to the time-consuming procedure of growing the bacteria on selective media to observe the formation of melanin pigments by XCPF.

The clear-cut separation of XCP and XCPF into two groups indicates that they are two divergent xanthomonads, as postulated by some researchers. It is possible to make some inferences about co-evolution of host and pathogen. It has been reported, for example, that only XCPF is pathogenic on cowpeas.

Within the two large fingerprint subgroups, several pattern families can be observed, whereby all the criteria used seem to correlate well with geographic origin (Table 1). This demonstration of genetic diversity, linked to geographic origin, substantiates the need for further analysis of differential pathogenicity, which in turn would lead to defining different resistance sources. As reported previously, the nonexistence of XCPF in the Caribbean has been confirmed so far. Furthermore, a clustering of certain more related groups (by pattern) can be observed throughout Latin America; e.g., families 5 and 6 in Central America (Figs. 2 and 3).

Thus far the probes have not been characterized as to their role or linkage to pathogenicity, but the fact that only pathogenic isolates are identified by the probes makes them extremely useful. Pathogenicity tests are being conducted with selected isolates (16 tested to date) belonging to the different fingerprint families on 24 bean genotypes in order to assess the relationship between pattern and pathogenicity. The quantitative behavior of pathogenicity will demand an exhaustive analysis of the host/pathogen interaction as well as any edaphoclimatic factors involved.

Table 2 shows the result of an evaluation of experiments of the type shown in Fig. 4. Percent affected leaf tissue within a zone circumscribed by the initial wounding and inoculation is representative of the level of plant resistance. Defining 10% damage as the limit of full resistance and 50% as the beginning of susceptibility, the isolates can be classified according to their virulence at different times after inoculation. An attempt is being made to develop an evaluation procedure that takes into account the quantitative character the host/pathogen interaction, by distinguishing among highly resistant (R), moderately resistant (r), intermediate (I), moderately susceptible (i), susceptible (s) and highly susceptible (S) levels of reaction in time. The shaded interactions in the table highlight those isolates that induce resistance and intermediate levels of resistance, suggesting the existence of avirulence genes. We might speculate that some isolates have specific avirulence genes, as they induce resistance in otherwise highly susceptible cultivars. Of course, there are very resistant bean genotypes like XAN 112, which has *Phaseolus acutifolius* resistance genes incorporated.

The results may help identify pathogenicity and resistance genes. Isolates devoid of such genes can be transformed with plasmid libraries from virulent or avirulent isolates. Transformants that acquire the corresponding characteristics can be further analyzed. This knowledge will facilitate the correct choice of resistance genes in the design of breeding programs adapted to specific regional needs.

The probes are also being used to quantify XCP and XCPF on leaves of infected plants in the search for some correlation between the supporting of cell growth and host resistance. The cell number of pure bacterial suspensions can be estimated accurately, using the probe on bacteria bound to a nylon membrane (Fig. 5). It has not been easy to develop a simple extraction procedure for bacteria from infected tissue as some plant component seems to interfere with the binding to the membrane. Only extraction of total DNA with phenol/chloroform and ethanol precipitation has worked thus far. Sucrose gradients designed to separate the plant cell debris from the bacteria were unsuccessful. Efforts are still under way to develop a quick extraction procedure amenable to large screening experiments.

1.4.1 **Methodology**

- **Bacterial isolates**. All isolates used in this study are from the CIAT Bean Pathology collection, which is kept lyophilized. They have been characterized by entry number and geographic origin. Most were isolated from common beans, some from cowpeas (*Vigna unguiculata*) and lima beans (*Phaseolus lunatus*). Bacteria were grown on MXP and YCDA media. Liquid cultures were inoculated from single colonies on nutrient broth.

- **Genomic DNA extraction**. Cells from 30-ml cultures grown in nutrient broth for 16-18 h under continuous agitation were sedimented by centrifugation at 10,000 rpm for 10 min and the pellet washed with 1M NaCl. The cells were re-suspended in 3 ml 50 mM Tris-HCl pH 8.0, 150 mM NaCl. To this suspension 200 µl of 10% SDS and 15 µl proteinase K (50 mg/ml) were added, followed by incubation at 50°C for 1 h. The aqueous phase was extracted twice with phenol/chloroform. The DNA was precipitated with 1/10 vol 3M NaAc and 2 vol ethanol. The centrifuged pellet was washed with 70% ethanol and re-suspended in 200 µl TE plus RNase A (20 µg/ml).

- **Electrophoresis**. For fingerprinting purposes approximately 3 µg of DNA were digested overnight with restriction enzymes at 37°C; 0.8% agarose gels were run in 0.5 x TBE buffer at 2-3 V/cm for 48-72 h. DNA was transferred to nylon membranes by capillary absorption, overlaying the gel with the membrane and absorptive paper without buffer tank.

- **DNA hybridization.** Probes were labeled with α^{32}P-ATP by random priming. Membranes were prehybridized for 4 h in bottles with 20 x SSPE, Denhardt solution, salmon sperm carrier DNA and 20% SDS. Hybridization with the labeled probe took place for 16-18 h at 65°C. Membranes were kept moist in Saran wrap. For re-probing the membranes were stripped by boiling 3 times for 30 min in 0.1% SDS.

- **Pathogenicity assays.** Bacteria were re-suspended in water, the titer adjusted to $5x10^7$ cfu/ml (A_{600} = 0.5) and inoculated onto 24 bean lines using two razor blades that press into a sponge imbibed with the inoculum.

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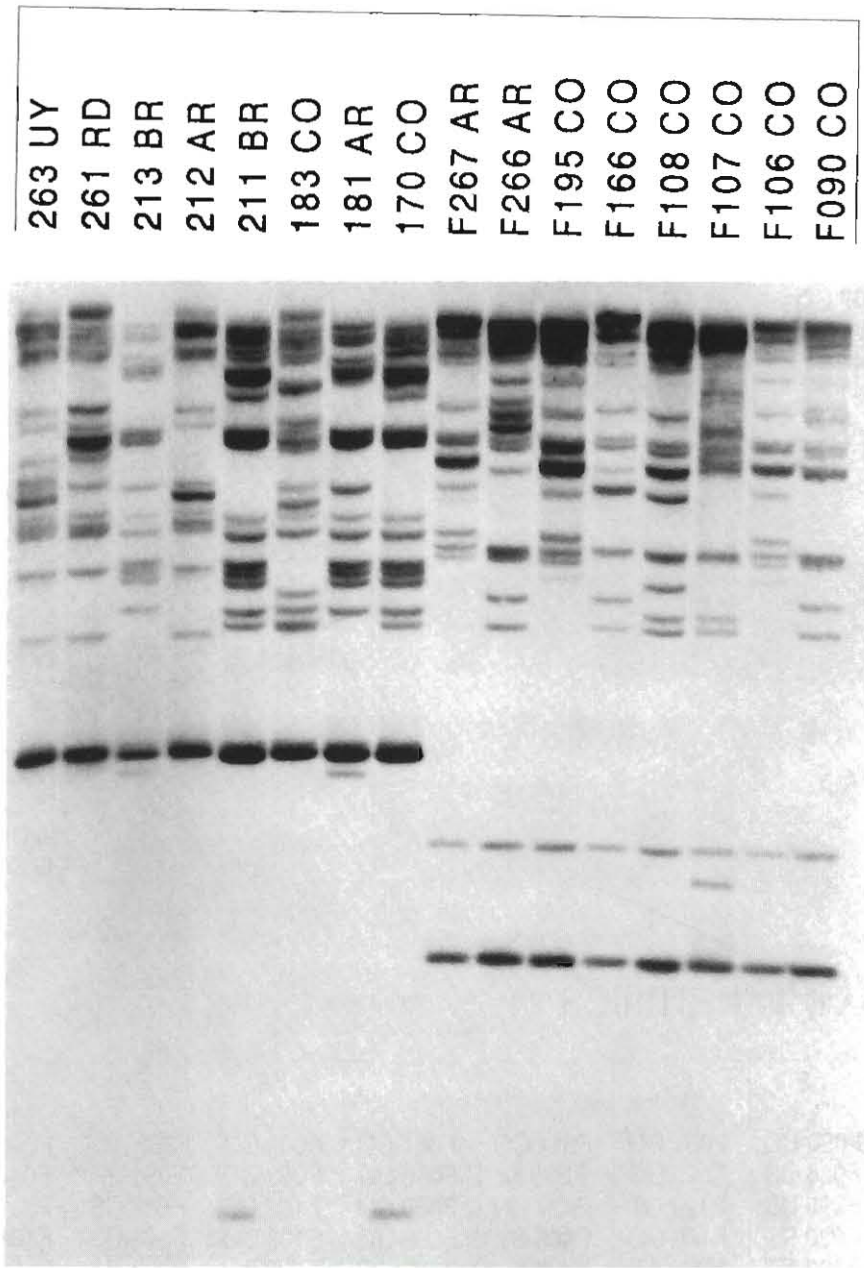


Figure 1. DNA fingerprints of XCP and XCPF obtained with probe P2. XCP isolates in the first half, XCPF in the second. The genetic diversity within the two groups is clearly demonstrated, as well as the formation of groups (see for example 213 BR, 211 NR, 181 AR and 170 CO and F106 CO.

Table 1. XCP and XCPF families as defined by their DNA fingerprints.

XCP Families

1	2	3	4	5	6	7	8	9	10
364 NI	001 CO	003 UG	183 CO	038 PR	040 PR	148 BR	004 US	002 HN	033 PR
024 PR	131 CO	028 PR	418 DO	403 CR	109 CO	151 BR	029 HT	042 HT	037 PR
027 PR	141 CO	035 PR	419 DO	422 HN	165 CO	152 BR	057 US	043 JM	275 DO
	167 CO	269 AR			170 CO	159 BR	066 CO	045 MX	
	261 DO	076 US			181 AR	160 BR	071 CO	047 HT	
	340 PY	167 CO			211 BR	272 CO	074 US	054 MX	
	341 PY	212 AR			213 BR	339 PY	079 CO	055 MX	
					280 PY	411 GT	081 CO		
					281 PY	412 GT	082 CO		
					282 PY	440 BR	093 CO		
					306 AR	442 BR	097 CO		
					334 BR	447 BR	123 CO		
					402 CR	450 BR	139 CO		
					412 GT	451 BR	145 CO		
					416 HN	455 BR	183 CO		
						456 BR	274 DO		
						458 BR	295 ES		
							296 ES		
							312 CA		
							315 CA		
							414 DO		
							436 CO		

XCPF Families

F1	F2	F3	F4	F5	F6	F7	F8	F9
F408 GT	F060 CO	F061 CO	F311 CO	F061 CO	F062 CO	F36ATCC	F006 CO	F305 AR
F409 GT	F065 US	F090 CO	F366 NI	F078 CO	F063 CO	F051 PR	F044 GT	F441 BR
F423 HN	F095 US	F126 GT	F424 HN	F080 CO	F102 US	F068 US	F081 CO	F443 BR
F433 HN	F280 PY	F166 CO	F426 SV	F090 CO	F108 CO	F072 US	F106 CO	F445 BR
	F402 GT	F266 AR	F430 SV	F180 CO	F299 BU	F074 US	F166 CO	F446 BR
	F404 GT	F367 NI	F457 BR		F302 BU	F077 CO	F195 CO	F452 BR
	F405 GT	F406 GT			F413 GT	F082 CO	F267 AR	F453 BR
	F454 BR					F100 US	F305 CO	
						F102 US	F308 CO	
						F107 US	F372 CO	
						F185 CO		
						F313 CA		
						F375 CO		
						F413 GT		

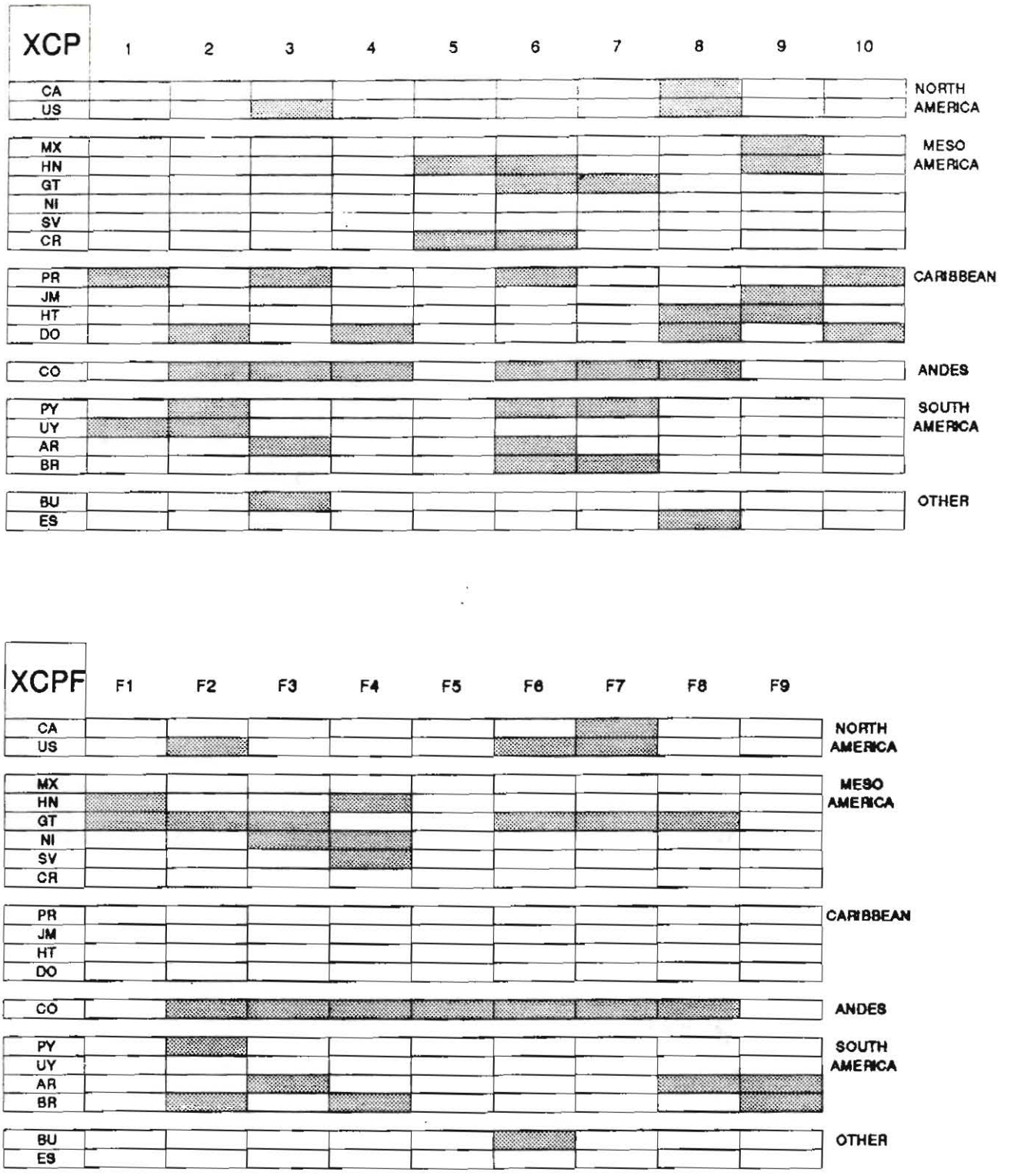


Figure 2. Occurrence of XCP and XCPF families in the Americas.

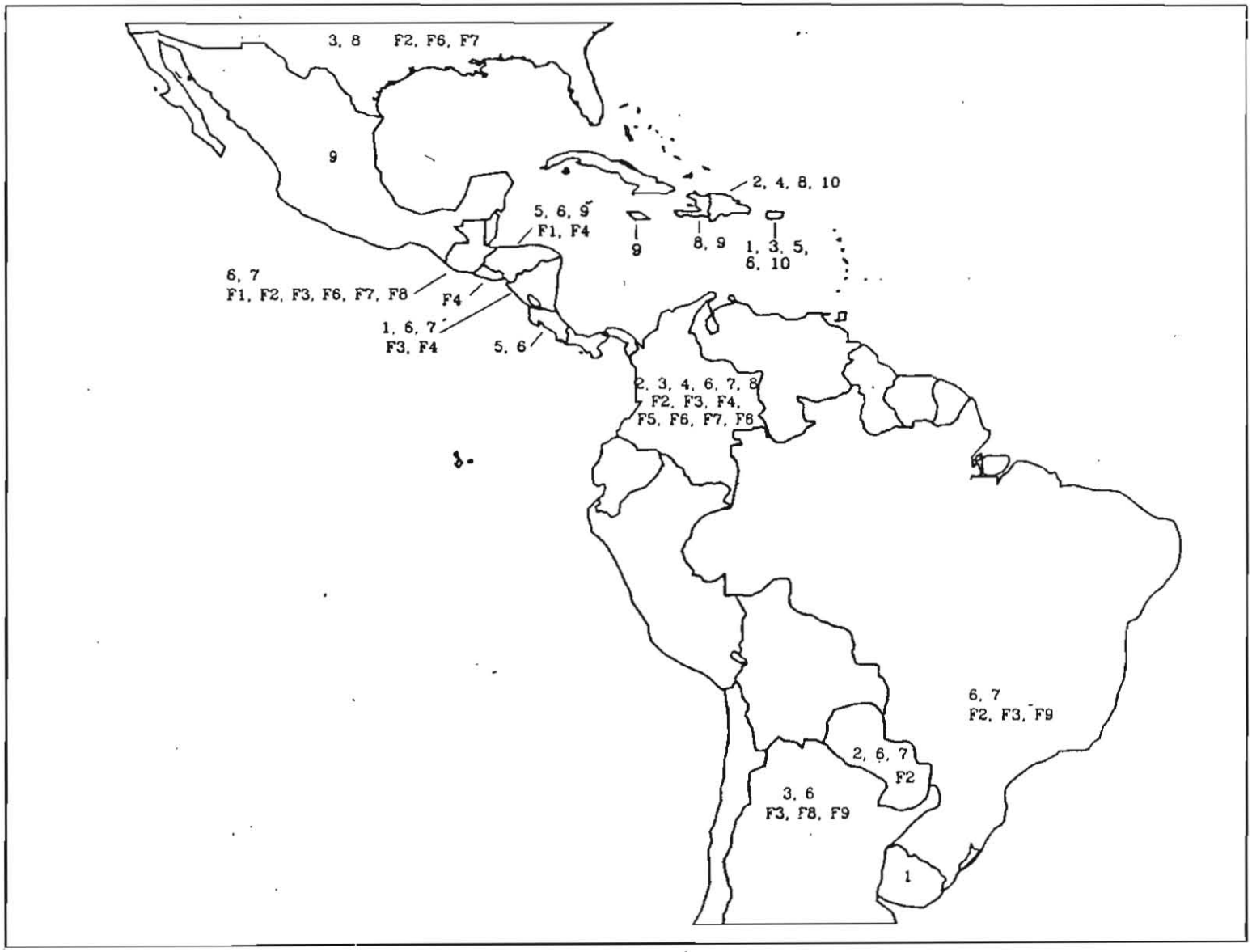


Figure 3. Occurrence of XCP and XCPF in the Americas.

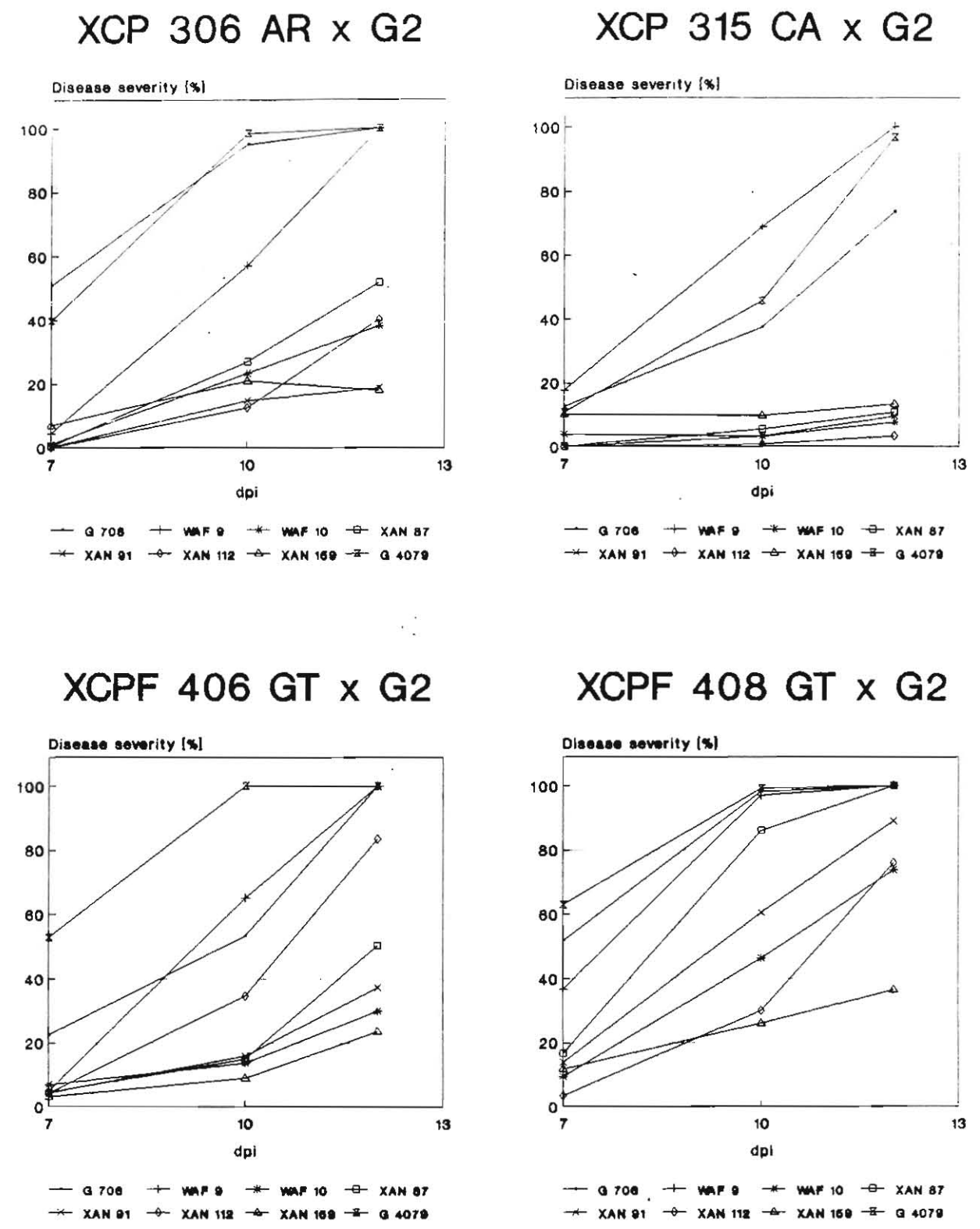


Figure 4. Pathogenicity tests of selected isolates on different bean genotypes.

Table 2. Pathogenicity tests with selected XCP and XCPF isolates.

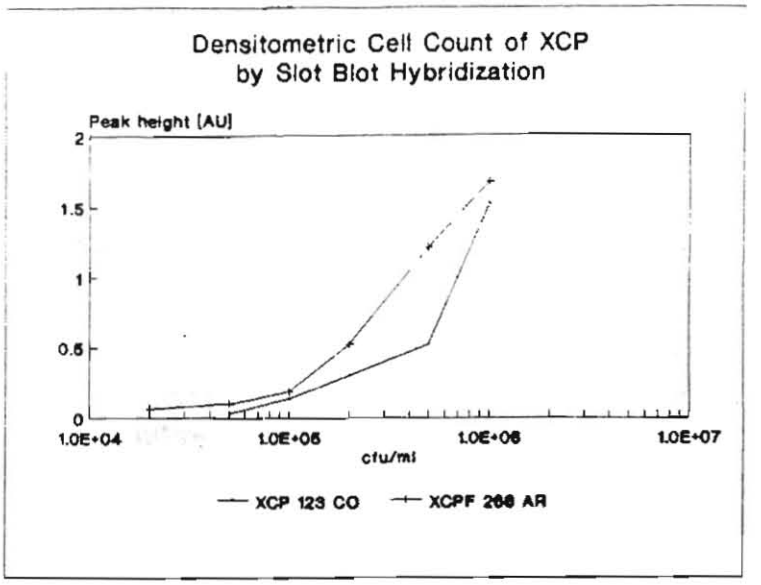
ISOLATE	364NI	306AR	181AR	315CA	123CO	F408GT	F407GT	F266AR	F406GT	262UY	F195CO	F333BR
FAM / VAR	1	6	6	8	8	F1	F2	F3	F3		F8	
AFR569												
AFR603												
AFR618												
BAT41												
BAT76												
BAT93												
BAT1297												
DRK56												
G706			---									
WAF9												
WAF10												
XAN87												
XAN91												
XAN112												
XAN159												
G4079												
G4081												
G5377												
G6392												
G6414												
G6415												
G10060		---		---		---			---			
ICA L24												
PORRILLO												

susceptible

disease severity 60–80%

intermediate

resistant



	Isolate	Cell count [cfu/ml]	Location [mm]	Height [AU]	Area [AU*mm]	Rel Area [%]
1-	XCPF 266AR	5.0E+05	19.12	1.217	2.017	15.4
2-	XCPF 266AR	1.0E+06	28.04	1.674	4.052	31.0
3-	XCP 123CO	5.0E+05	45.96	0.524	0.743	5.7
4-	XCP 123CO	1.0E+06	54.88	1.503	2.873	22.0
5-	XCP 123CO	1.0E+06	81.52	1.535	3.374	25.8

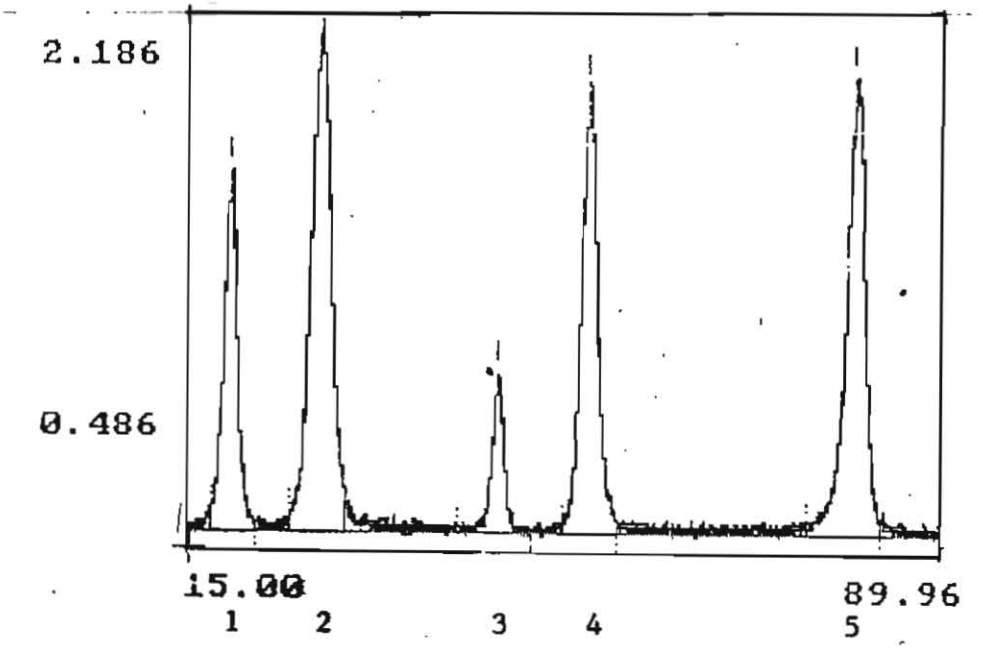


Figure 5. Quantification of XCP and XCPF by densitometric analysis after slot blot hybridization.

2. PHYSIOLOGICAL AND BIOCHEMICAL PROCESSES IN PLANTS AND BACTERIA

2.1 Molecular Mechanisms of CO_2 Assimilation in Cassava (Collaboration: M. El Sharkawy, Cassava Program)

2.1.1 **Understanding the subcellular compartmentalization of the enzymes involved in CO_2 assimilation**. The cassava plant accumulates starch in the storage roots even under harsh climatic conditions of high temperatures and drought stress, making it only one of a few crops for critical areas such as the sub-Sahara. Biomass and root yield of cassava are correlated with photosynthetic rate (El-Sharkawy et al. 1990). Cultivated species cover a wide range of photosynthetic activities, making it necessary, on the one hand, to develop diagnostic tools to evaluate this trait for breeding purposes and, on the other hand, important to characterize it at the molecular level as part of the tool development strategy.

Several enzymatic parameters allow us to classify cassava as a C3-C4 intermediate species, which include the activities of PEPC, malic enzyme (ME) and the ratio PEPC/RuBPC (Table 1). Cassava shows a high photosynthetic rate and a low compensation point for CO_2, low photorespiration and high water use efficiency (WUE). The appearance of C4 acids is not yet conclusive and is a matter of controversy.

The C4 syndrome is found in around 20 different families, where it seems to have evolved separately. Examples exist where C3, C4 and C3-C4 intermediates can be found within one species; e.g., *Flaveria*. This is also indicated by the different C4 mechanisms that have evolved. Three main mechanisms can be classified according to the C4 compound shuttled (malate or aspartate) and the decarboxylating enzyme in bundle sheath cells [NAD-ME, NADP-ME or PEP carboxykinase (CK)] (Langdale and Nelson 1991). The compartmentalization of these enzymes has not developed parallel to that of the C4 anatomy, as can be deduced from the study of several intermediate species (Edwards and Ku 1987; Nelson and Langdale 1991). Thus C3-C4 intermediates provide very useful for evolutionary studies.

El-Sharkawy (Cassava Physiology) has developed a working model for cassava based on leaf cell ultrastructure and physiological data, which include some degree of enzyme compartmentalization in the leaf tissue. Some of the observations on which the model is based include the positioning and number of mitochondria, chloroplast dimorphism and large gas vacuoles going deep into the palisade cell-layer, which is composed of very long cells funneling into bundle sheath cells. This arrangement could serve as a novel, efficient mechanism of CO_2 recycling and concentration.

Recycling of CO_2 has been postulated by Schuster and Monson (1990) as a means of improving photosynthesis rates (PR) at high temperatures. Without evolving new biochemical pathways or altering the compartmentalization patterns of photosynthetic reactions, the net PR of C3 plants could be increased by raising intercellular CO_2 through increased stomatal conductance. This would lead to a higher PR per unit of RuBPC [higher photosynthetic nitrogen-use efficiency (NUE)], but at the expense of higher transpiration (lower WUE). The alternative would be to increase RuBPC concentration, which would result in lower NUE and WUE, as stomatal conductance would have to be raised to deliver higher CO_2 concentrations to the higher enzyme concentration. The evolution of a C3-C4 mechanism involving the recycling of CO_2 has the potential for increasing PR at warm leaf temperatures, without incurring the aforementioned costs.

In situ hybridization techniques using antisense RNA on histological sections allow the analysis of the subcellular expression pattern of specific genes. This technique should shed light on the mechanisms involved in cassava, as gene expression of the relevant enzymes could be located in their respective organelles. In situ hybridization is done with homologous probes because heterologous probes are prone to result in high background noise due to the low stringency hybridization conditions needed when homology is below 90%. To date, not many genes involved in CO_2 fixation from different plant species have been cloned. ME has been isolated from *Flaveria trinervia* and maize; MDH from watermelons, maize and sorghum; PEPC from *F. trinervia*, maize and tobacco; RuBPC from peas, petunias, *Pinus thunbergii*, potatoes, tobacco, sorghum, spinach and maize. A homology of about 70% can be found in some regions of the PEPC genes of maize and tobacco--a monocot and a dicot species, respectively. This homology would not be sufficient for in situ hybridization studies but would allow fishing the corresponding genes from a cassava DNA library.

Gene probes for PEPC, NADP-ME, NADP-MDH and RuBPC from maize were received from T. Nelson (Yale U.). The probes are being sequenced to locate them within the corresponding genes. After locating the PEPC probe to exon 9 of maize, we used it to fish out two clones from a lambda GEM11 genomic library, which contained ca. $2x10^5$ cassava clones. The two clones underwent three rounds of purification and have been partially sequenced. No homology to PEPC from maize has yet been found. We might be sequencing part of an intron (they can be very divergent through evolution); another possibility is that we got the wrong clone. We have to go through the process of isolating more clones in order to increase the probability of having the right one.

Glycine decarboxylase (GDC) is a high molecular weight multienzyme complex located within the matrix of plant mitochondria. GDC may play a central role in the recycling of CO_2 as it is involved in the respiratory conversion of two glycine molecules into serine, ammonia and CO_2. The multienzyme complex is composed

of four component enzymes: the P-protein (a pyridoxal 5-phosphate-dependent amino acid decarboxylase), the H-protein (a lipoamide-containing carrier protein), the T-protein (a tetrahydrofolate transferase) and the L-protein (lipoamide dehydrogenase). Only the H-protein of peas and *Arabidopsis thaliana* has been sequenced. A PCR approach utilizing this sequence information is envisioned for cloning cassava GDC. Sequence homology between peas and *Arabidopsis* is 73%.

Within this project the techniques for generating high output lambda phage libraries at CIAT were established (Figs. 1 and 2). The adaptation of the in situ hybridization techniques is a very delicate task. We have contacted M. Hughes at Newcastle, who has a good working protocol using nonradioactively labeled probes. We already have some expertise for embedding the tissue and preparing microsections using the microtome.

We are in the process of cloning and sequencing additional clones for PEPC and will soon start doing the same for the other enzymes. Southern blots of several cassava cultivars will be performed with the Nelson probes to establish the degree of expected homology and possibly get an estimate of the number of gene copies per genome.

The characterization of an isolated clone will involve:

- Physical mapping with restriction enzymes
- Subcloning of restriction fragments or generation of staggered deletions with exonuclease III (lambda clones are too large for direct sequencing)
- Assembly of the sequenced fragments
- Comparison with the DNA database
- Location of coding sequences and signals
- Northern blot hybridization to RNA from different tissues after different treatments of the plant to study regulation of gene expression
- Generation of probes for in situ hybridization

2.1.2 **Purification and characterization of PEPC from cassava**. A parallel approach to isolating genes involved in CO_2 assimilation involves the purification of phosphoenolpyruvate carboxylase (PEPC) from cassava. PEPC plays the central role in the primary fixation of CO_2 in C4 plants. Purification of the enzyme will make it possible to produce antibodies for quantification and subcellular localization of the enzyme using immunofluorescence techniques on histological sections, analog to the in situ hybridization with antisense RNA.

PEPC from cassava was purified to >95% purity by liquid chromatography (fractionated ammonium sulfate precipitation, desalting by Sephadex G-25, DEAE Sepharose anion exchange chromatography, and gel filtration through Sephacryl S-300 HR). One peak of activity was eluted from DEAE Sepharose by salt gradient at 0.125 M ammonium sulfate. Gel filtration yielded two peaks of 350 and 400 kDa, respectively. Specific activity of the main peak was 5.5 units/mg protein. The K_m of PEPC from cassava (0.18 mM) is lower than that of maize (1.5 mM), indicating higher substrate affinity. If this can be substantiated, it could explain in part the efficiency of the CO_2-assimilating mechanisms.

Phosphoenolpyruvate carboxylase (PEPC)--a key enzyme of primary photosynthetic CO_2 fixation by C4 and CAM plants--is also present in C3 plants, but at much lower levels. Levels of PEPC in leaves of C4 plants are nearly 20 times higher than in C3 plants on a chlorophyll basis. In maize PEPC constitutes 10-15% of leaf total soluble proteins and is mainly localized in the cytosol of the mesophyll cells. PEPC activities from maize, beans and cassava leaves were compared using a spectrophotometric assay and Fast Violet detection on polyacrylamide gels. PEPC relative content and activity in cassava have intermediate values between maize and beans under stress conditions which drop to normal C3 values under nonstress conditions (Table 1).

A maize PEPC-specific antiserum cross-reacts with cassava PEPC, indicating homologous antigenic determinants. It is necessary to produce a specific antiserum for conducting immunofluorescence experiments on leaf sections. To produce PEPC antiserum, the protein stemming from the last DEAE Sepharose purification step will be separated by PAGE, the band containing PEPC will be cut out, and the finely powdered gel will be re-suspended in buffer plus Freund's Adjuvant for producing rabbit antiserum.

2.1.3 **Materials and methods**

- **Purification of PEPC.** PEPC was isolated using a combination of fractionated ammonium sulfate precipitation and anion exchange chromatography on DEAE Sepharose CL-6B (Fig. 3), followed by gel filtration chromatography on Sephacryl S-300 HR (Fig. 4). PEPC activity precipitated between 40 and 60% ammonium sulfate saturation. The desalted fraction was loaded onto the DEAE column and eluted with an ammonium sulfate gradient (0.05-0.4 M). The peak fraction was concentrated by ammonium sulfate precipitation and passed through the Sephacryl column. Tris-SO_4 buffers were used throughout as chloride is deleterious to the enzymatic activity of PEPC.

- **Enzyme assays:**

  - PEPC. The oxidation of NADH in a coupled enzymatic reaction with MDH was quantitated photometrically at 340 nm. In this reaction PEP is converted to oxaloacetate and further to malate.

- RuBPC. The conversion of phosphocreatine to phosphoglycerate and diphosphoglycerate in a coupled enzyme assay utilizing PGDH and PGK is quantitated as with PEPC.

- NAD-ME. The reduction of NAD in the conversion of malate to pyruvate and CO_2 is quantitated photometrically.

- **Immunologic and electrophoretic analysis.** Protein preparations were assayed using the double immunodiffusion technique on agarose gels buffered with 0.05 M barbital pH 7.6. The precipitin reaction was stained with Coomassie Blue R-250 in methanol/acetic acid. Proteins were also analyzed by Western blot of non-denaturing gels. Primary antibodies were detected with antirabbit-IgG coupled to peroxidase. PEPC activity was also detected in situ on gels by Fast Violet stain (detects oxaloacetate).

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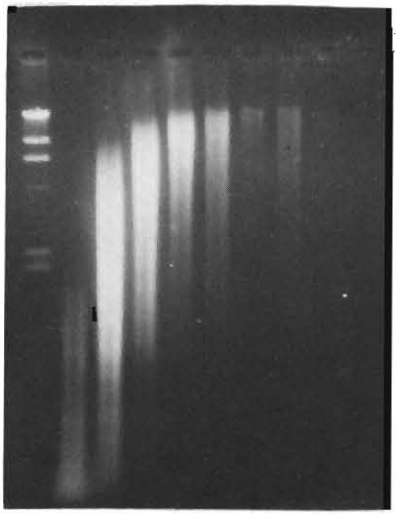
Table 1. Activities of PEPC, RuBPC and NAD-ME in cassava leaf extracts.

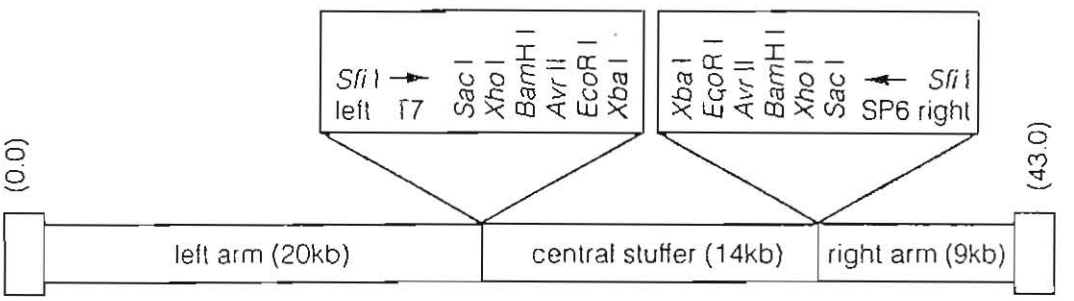
VARIETY	PEPC			
	$\frac{\mu mol}{gFW\ min}$	$\frac{\mu mol}{mg\ CHL\ min}$	$\frac{\mu mol}{mg\ PROT\ min}$	$\frac{PEPC}{RuBPC}$
CM 523-7	5.58±0.53	1.57±0.10	0.068±0.007	0.43
CM 507-37	2.96±0.16	1.91±0.10	0.049±0.003	0.28
MCOL 1684	4.28±0.27	2.90±0.19	0.058±0.003	0.42
MCOL 1468	4.33±0.39	3.07±0.27	0.052±0.005	0.36
C4				1.7-5.0
C3				0.05-0.10
C3-C4				0.25-0.50

	RuBPC		
CM 523-7	12.84±2.20	3.62±0.62	0.156±0.026
CM 507-37	10.60±1.02	6.84±0.66	0.174±0.017
MCOL 1684	10.22±1.74	6.96±1.18	0.138±0.023
MCOL 1468	12.18±1.88	8.16±0.71	0.146±0.022

	NAD-ME		
CM 523-7	1.94±0.29	0.55±0.08	0.024±0.004
CM 507-37	2.01±0.16	1.30±0.10	0.033±0.003
MCOL 1684	2.27±0.15	1.54±0.10	0.031±0.002
MCOL 1468	2.10±0.17	1.48±0.12	0.025±0.002
C3		0.1-0.3	
C4		5.0-9.0	
Cassava/C3		5.0-15.0	
Cassava/C4		0.2-0.3	



Sizing of DNA 9-20 kb fragments by NaCl gradient



Structural map of the uncut lambda GEM-11 vector.

Figure 1. Generation of lambda phage libraries.

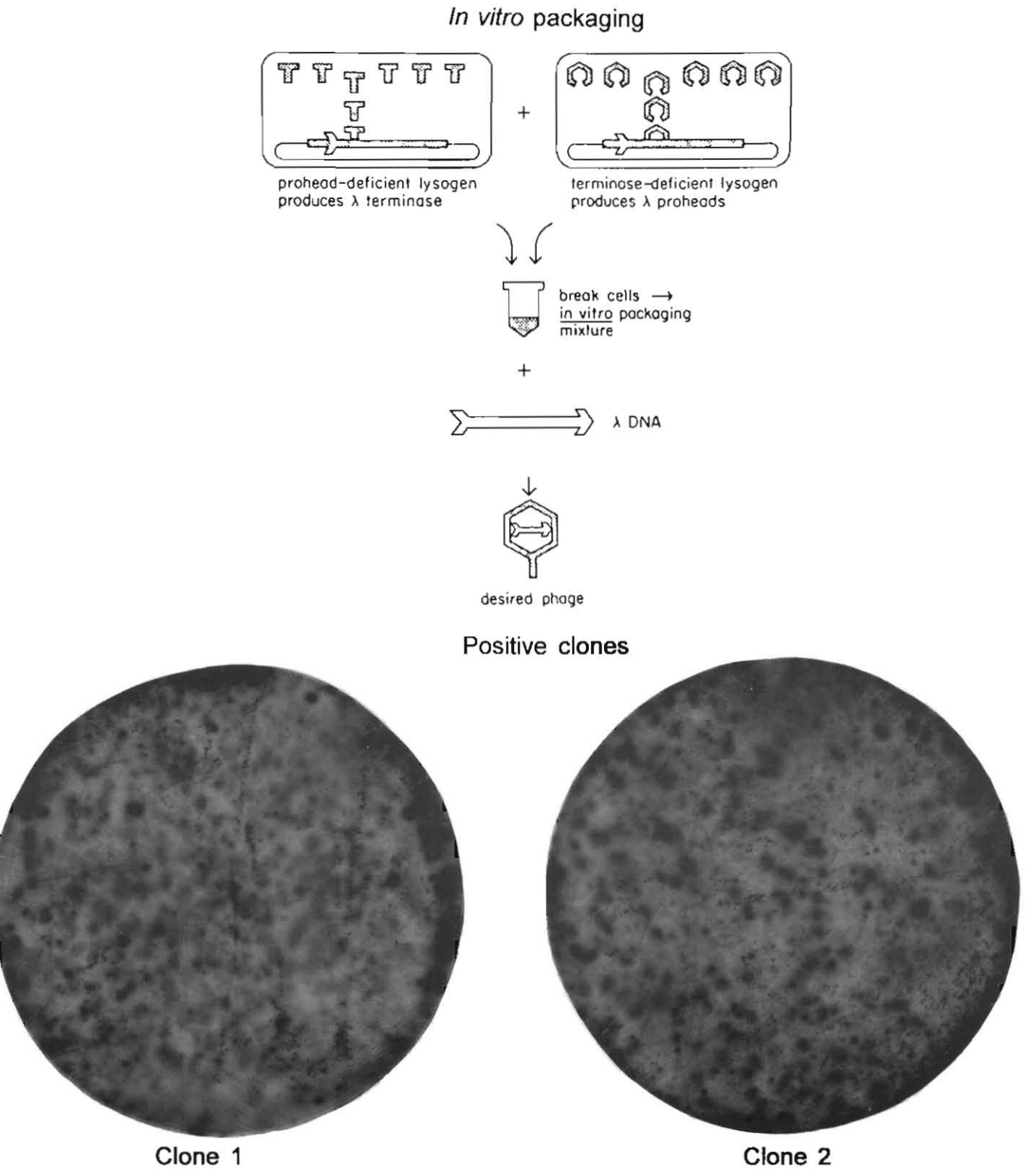


Figure 2. Cloning of the PEPC gene. *In vitro* packaging principle and purification of positive clones.

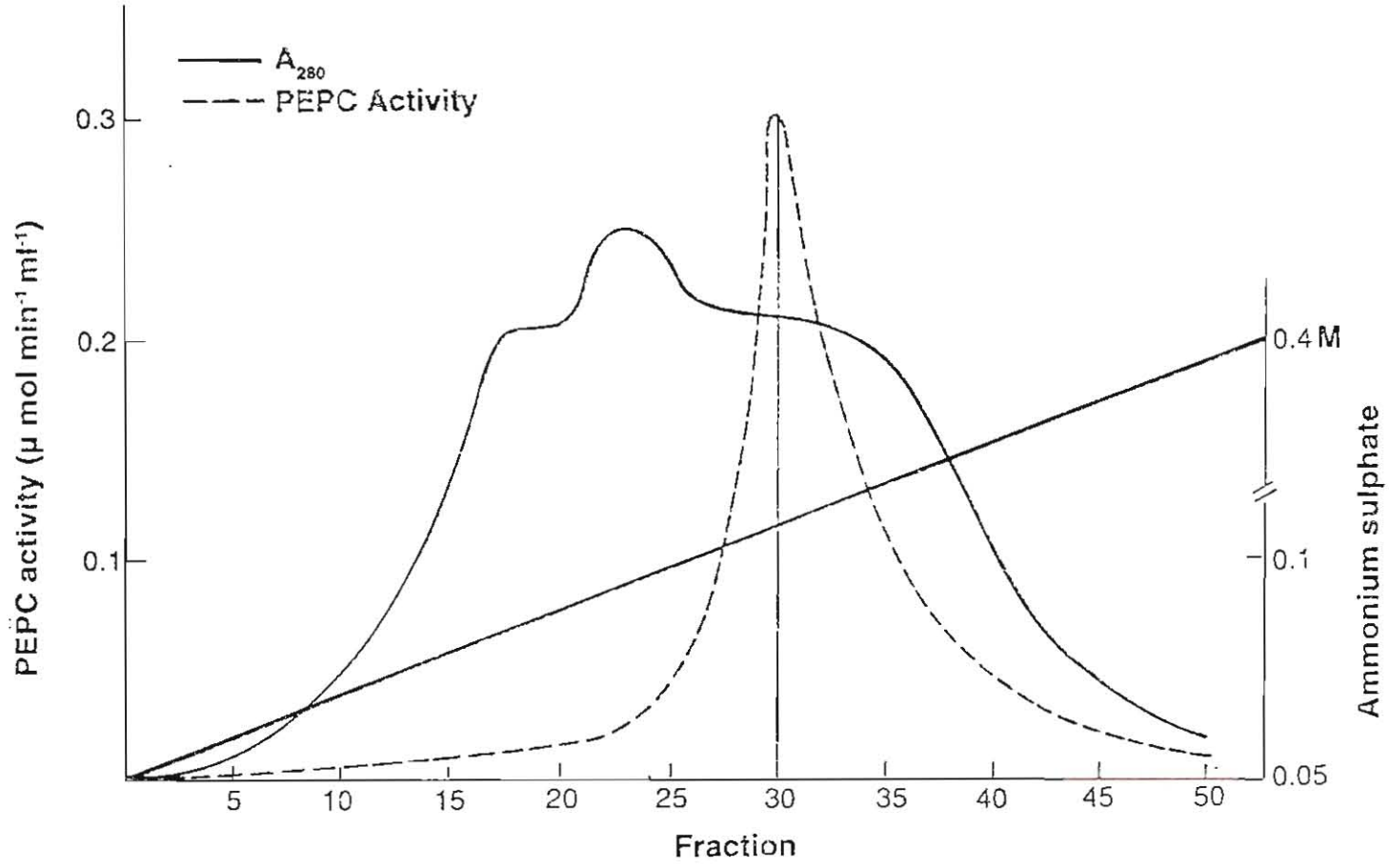


Figure 3. Ion exchange chromatography on DEAE-Sepharose CL-6B.

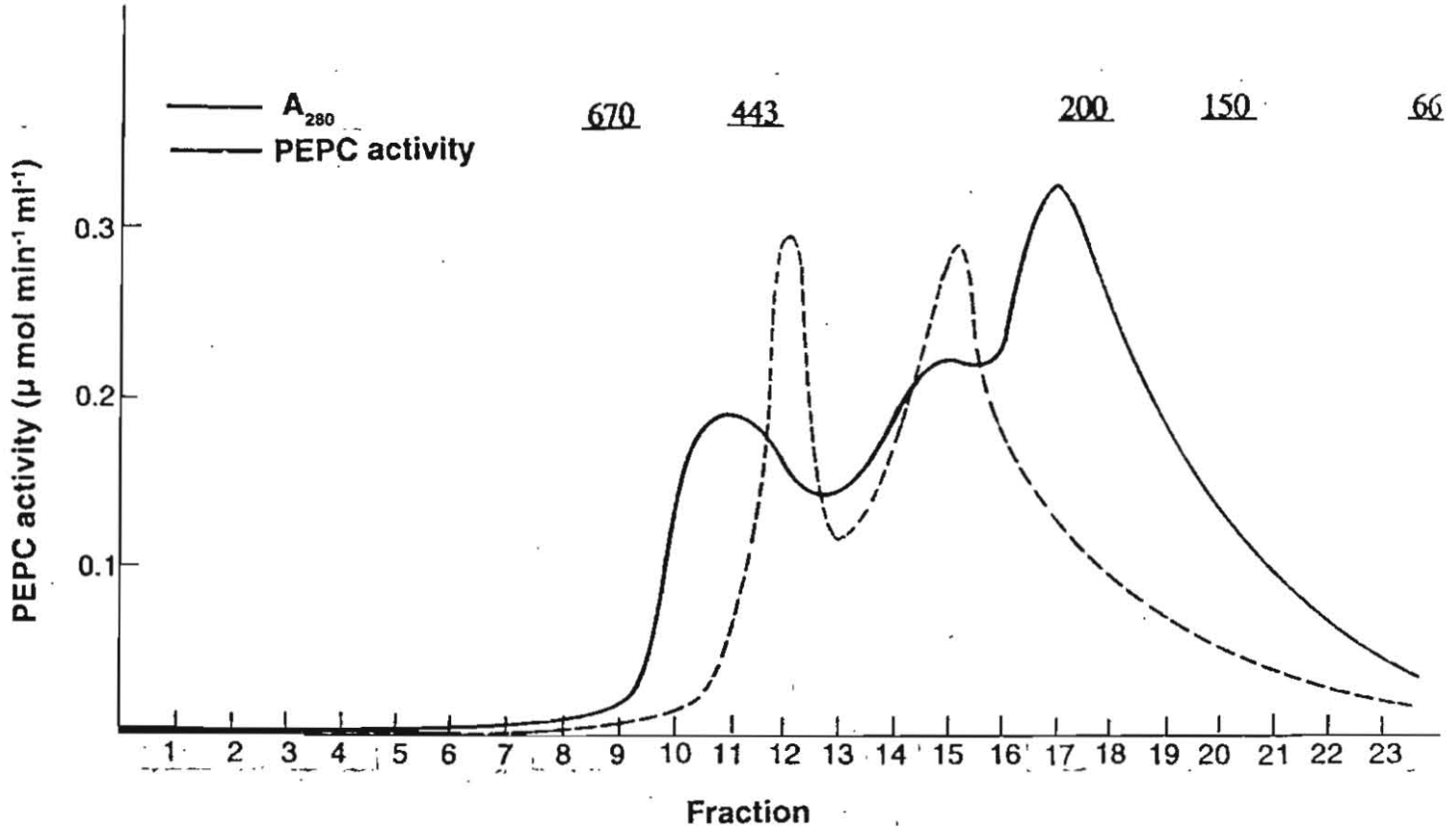


Figure 4. Gel filtration chromatography on Sephacry S-300-HR.

2.2 Mechanisms of aluminum tolerance

We are involved in a project of I. Rao (TFP) to determine organic acids and sugars in roots of plants that have been exposed to different treatments with aluminum and phosphate. The toxic effects of aluminum are exerted at several levels; thus tolerance mechanisms probably act at the exclusion or deposition levels. Inheritance of the tolerance trait is complex. Glasshouse studies under controlled environmental and soil conditions are indispensable to define parameters for identifying tolerant germplasm in breeding programs.

Sugars and organic acids are the main chelators of metal ions and regulators of pH homeostasis--important mechanisms of aluminum tolerance. We have adjusted the isolation and separation techniques for both groups of substances using HPLC analysis (Scott et al. 1991). We start from 1 g of fresh root tissue. After grinding, the tissue is extracted with 80% ethanol at 75°C, followed by rotaevaporation. The extract is then freed from amino acids by chromatography through an A6 50W-X8 H^+ cationic exchanger. The flow-through is passed through an A6 1-X-8 anionic exchanger as formate; and the sugars are collected in the flow-through. The organic acids are eluted with 6M formic acid, and the eluate rotaevaporated. Both fractions are analyzed separately by HPLC using an Aminex HPX-87H ionic exclusion column under isocratic conditions with 6 mM sulfuric acid.

It was necessary to adjust the detection methodology in order to distinguish between acids and sugars in mixtures. As we have no refraction index (RI) detector for sugars, the UV diode array detector is used to detect sugars and acids at 192 nm (at 210 nm only the acids are detected) (Fig. 1). Although UV detection of sugars is much less sensitive than RI detection, it appears to be adequate for these experiments.

References

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Column	:	Aminex HPX-87H ionic exclusion
Flow	:	0.6 ml/min
Temp	:	65°C
Eluent	:	6 mM sulfuric acid
Inj vol	:	20 µl
Detector:		Diode array UV 195 and 210 nm

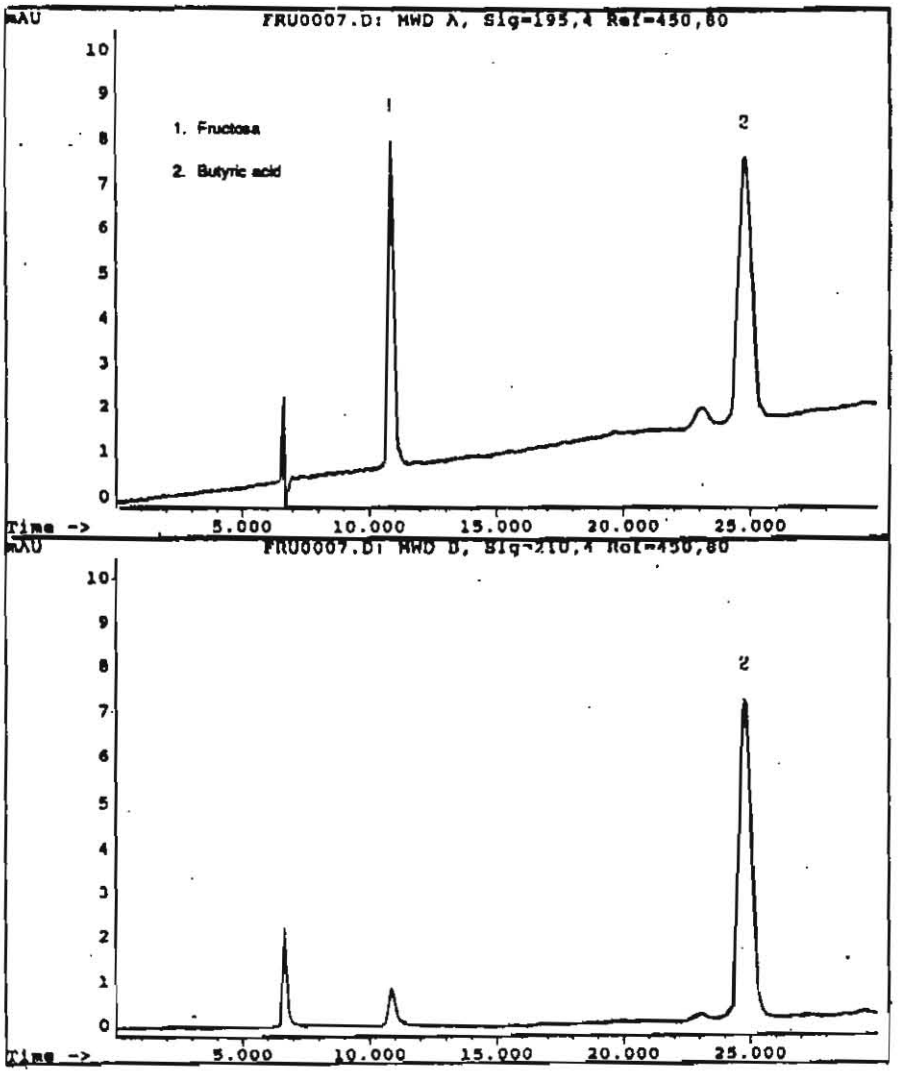

Figure 1. UV detection of monosaccharides and organic acids after HPLC separation.

2.3 Characterization of Amylolytic Bacteria and Associated Enzymatic Activities during Cassava Solid State Fermentation (Collaboration: G. Chuzel, Cassava Utilization, SAR/CIRAD)

Sour starch from cassava is a naturally fermented product of some economic importance in the food industry, especially in Colombia and Brazil, where it is used to make several traditional, flour-based products (e.g., "pandeyuca" and "rosquillas"). It can replace wheat flour to a great extent, which could greatly reduce imports of this grain.

Fermentation and drying under the sun give the product its specific baking properties (bread-making power, flavor and aroma). Market acceptability is related to partial amylolytic digestion of the starch grains by bacterial exoamylolytic activities. Strains of *Lactobacillus, Leuconostoc* and *Streptococcus* constitute the bulk of bacteria found in the fermentation process.

The product has a high potential for broader industrial applicability, but present processing methodology is rudimentary and slow (20-30 days). The main problem is fluctuation in product quality. Low efficiency and irregular quality are the consequence of the lack of control parameters. In Colombia most production is carried out in rural units ("rallanderías") in Northern Cauca.

SAR/CIRAD (France) has initiated a project to define the needed standard fermentation parameters, improve production practices, and eventually develop starter inocula. The Universidad del Valle (UniValle) in Cali, Cassava Utilization (CIAT), the Office de la Recherche Scientifique et Technique d'Outre-Mer (ORSTOM-France) and the Molecular Biochemistry Laboratory (BRU) are collaborating. The isolation and microbiological characterization of strains from the natural fermentation process were carried out at UniValle. We are characterizing selected strains with respect to amylolytic enzymes; then molecular markers will be developed for strain identification.

Most of the fermentation process takes place under anaerobic and low pH conditions. Acidification of the medium is a selective process, in which most other competing bacteria are killed. The production of lactic acid correlates well with the quality of the product. Several lactic acid bacterial strains (LAB) were isolated on selective MRS starch medium with aniline blue. After Gram, catalase, sugar metabolism (API) and mobility analysis, the 12 best growing bacterial strains were selected for further analysis. Most belong to the genus *Lactobacillus* and are homofermentative.

Amylolytic activities of these selected strains were chromatographically enriched from the medium, and their enzymatic parameters (K_m, V_{max}) determined, as well as their pH and temperature optima (Table 1, Fig. 1). The isolated amylolytic activities were thermostable and acid tolerant--two qualities that make them especially interesting

for industrial applications. The 12 strains showed varying degrees of amylolytic activities, which was readily demonstrated on activity polyacrylamide gels, as were the isozyme patterns (Fig. 2). Protein fingerprinting using SDS/PAGE was performed on these strains, again demonstrating the nonidentity of the strains (Fig. 2B).

The techniques for characterizing LAB are now well established. The next task is to characterize the whole collection of 75 strains isolated from the natural fermentation process. Initially all strains were raised under strictly anaerobic conditions. The 12 strains characterized in detail were rather indifferent to aerobic conditions. We are in the process of establishing whether this also applies to the other strains in the collection.

Having conducted HPLC analysis of the organic acids produced by the LAB, we will now compare the production of acids under aerobic and anaerobic conditions, as well as with changing carbon sources in the medium. Glucose is known to inhibit the production of amylolytic activity; whereas glutamate has been reported to increase activity several-fold (Rothstein et al. 1986).

Physiological fermentation parameters of the isolated strains have to be established under varying conditions to determine their potential industrial applications. Growth under optimal conditions is required to establish parameters such as biomass conversion and maximal growth rates, but the growth conditions for inducing specific enzymatic activities in each case have yet to be established. Small-scale natural fermentation with selected strains will be also characterized to establish the performance of potential starter inocula.

DNA probes for identifying interesting strains will be developed. The variable regions of ribosomal DNA will be amplified by PCR using primers complementary to the conserved border regions. The amplified fragments will be further characterized by sequencing, and phylogenetic studies will be performed by sequence comparison to the data base. Ribosomal sequences have been used extensively for this kind of study (Klijn et al. 1991). Once sequenced, the information can be used to produce specific probes.

2.3.1 **Methodology**

- **Isolation and characterization of amylolytic enzymes.** Lactic bacteria were grown in MRS-starch broth under aerobic conditions for 72 h at 30°C. Proteins were precipitated from the supernatant with 4 vol of acetone and separated on Laemmli polyacrylamide gels. Amylolytic activities were specifically identified by incubation in citrate-phosphate buffer containing 1% starch and posteriorly stained with iodine. Isolated proteins were also analyzed on denaturing and non-denaturing gels using silver stain. Amylolytic activities were quantified using

0.3% starch as substrate and dinitrosalicylic acid (DNS) as a specific reagent for reducing sugars.

- **Isolation of α-amylases from the fermentation mass.** Fermenting starch was extracted with acetate buffer (50 mM pH 6.5), which was then precipitated with 80% ammonium sulfate. The precipitated proteins were separated by anion exchange chromatography (DEAE-Biogel Agarose). The amylolytic activity-containing peak was concentrated with polyethylene glycol.

- **Fingerprinting using total protein patterns.** Bacterial cells were cracked by ultrasonic treatment and separated by electrophoresis on denaturing polyacrylamide gels. Protein bands were revealed by silver stain.

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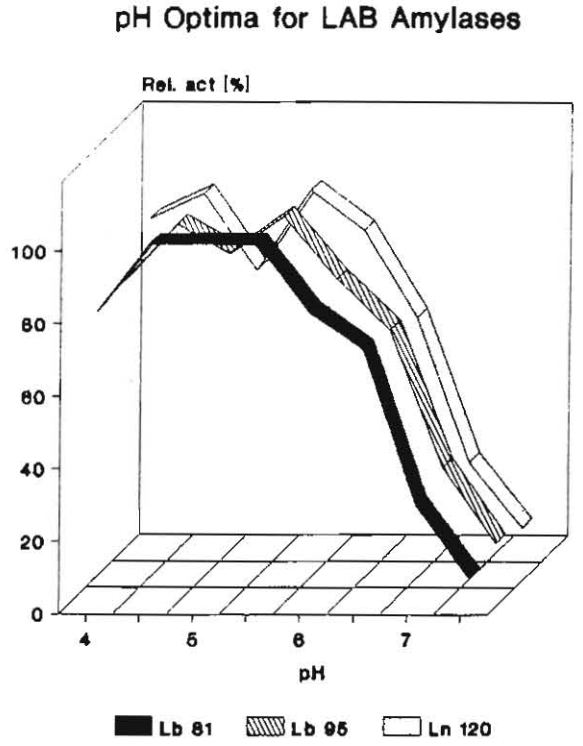
Table 1. Amylolytic activity of selected lactic acid bacterial strains.

Strain	Amylose	Starch	Gel	pH	T °C
Lb 01	+	+	+	6.0	
05	–	–	–		
07	+	+	+	6.0–6.4	
12	–	–	–		
81	+	+	+	4.4–5.4	60
95	+	+	+	4.4–5.4	60
105	+	+	–		
160		+	+	4.0	
214		+	+	6.4–7.4	
Ln 120		+	+	4.4–5.4	80
B. cereus		+	+	7.0	

Lb Lactobacillus
Lu Leuconostoc
B Bacillus



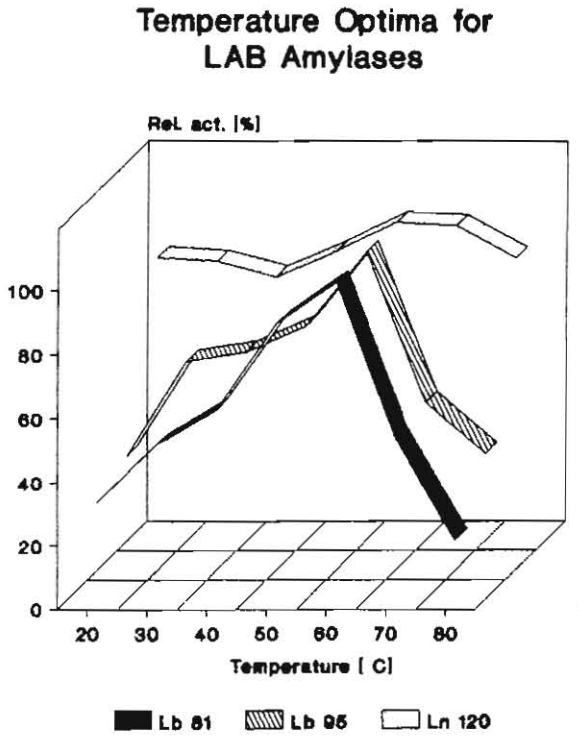


Figure 1. pH and temperature optima for lactic acid bacterial strains amylases.

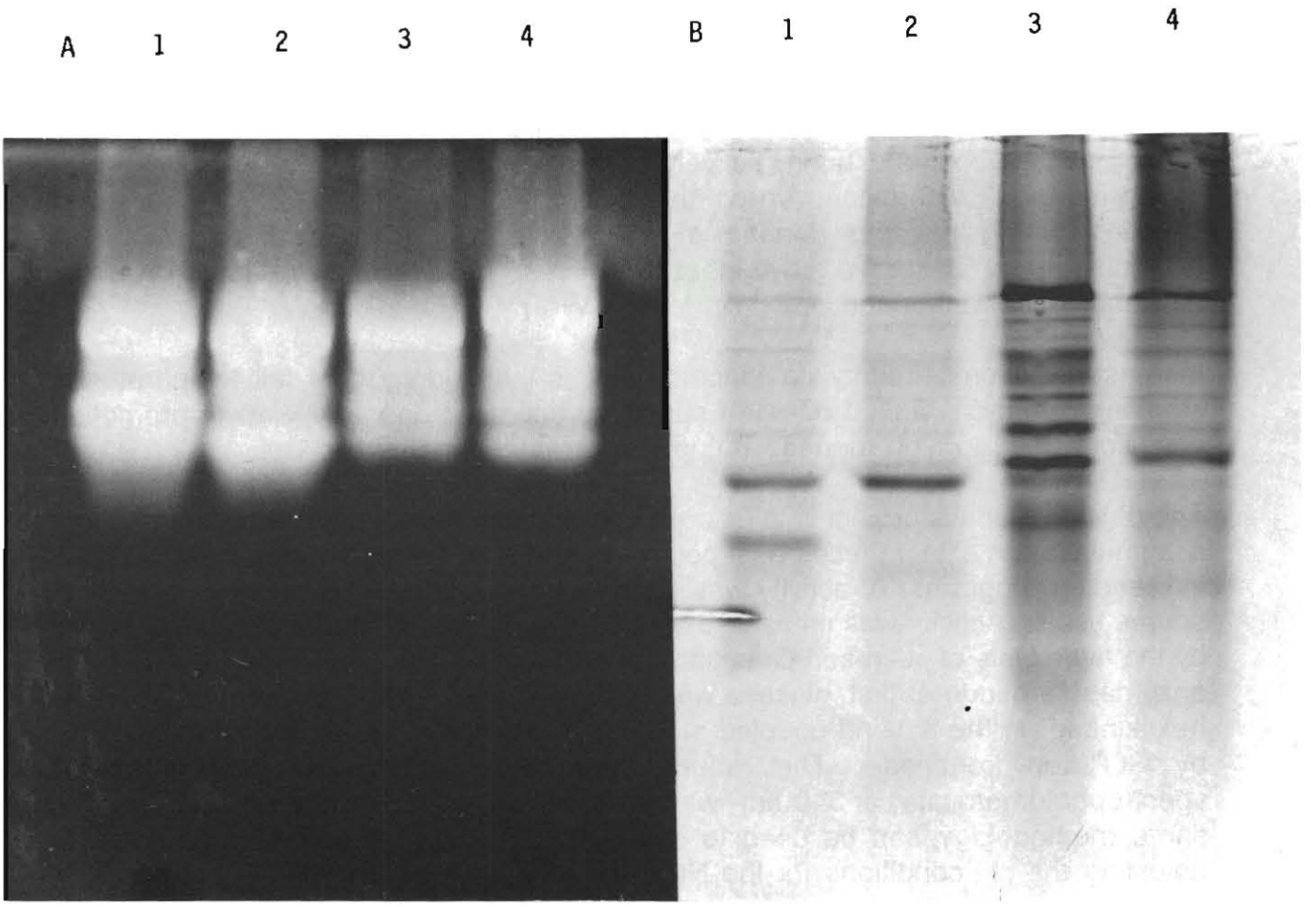


Figure 2. Electrophoretic separation of extracellular bacterial amylases. (A) activity staining with iodine; (B) silver stain: (1) Lb 01, (2) Lb 8, (3) Lb 95, (4) Ln 120.

2.4 Acid Invertase as an Indicator for Sugar Accumulation

The purpose of this project, which is being done in collaboration with Cenicaña, is to establish a methodology for measuring critical enzymatic activities which is also relevant to sucrose transport and carbohydrate accumulation in cassava.

Acid invertase (AI) is a vacuolar enzyme involved in hydrolysis of sucrose. This process is thought to play a central role in the so-called futile cycling between sucrose and hexose (Huber 1989; Geigenberger and Stitt 1991). This cycle plays a central role in the regulation of photosynthetic processes. An excess of sucrose will lead to the increased liberation of hexoses, which in turn will act as feedback inhibitors of photosynthesis (Fig. 1). If sucrose is transported efficiently to sink and storage tissues, AI activity will be low, and photosynthesis will not be inhibited.

AI in the apoplasmic free space and in the vacuole is responsible for providing hexose for respiration, sucrose transplant gradients and vacuolar osmotic turgor-related cell expansion. Young, actively growing tissues are high in AI activity; e.g., during internode elongation. A drop in AI activity has been reported for high-yielding varieties during ripening (Madan et al. 1991). Also involved in transport and accumulation processes are two other invertases: the cytoplasmic neutral invertase (NI) and a cell wall-bound acid invertase (CWAI).

Increase of sucrose purity or concentration is induced by cool fall temperatures, stopping irrigation, limited nitrogen supply or chemical ripeners. Growth-promoting conditions lead to high tonnage but low sugar concentration.

The objective of this project is to establish correlations between AI activity and sugar accumulation in elite varieties as a possible parameter for screening advanced lines in breeding programs. AI activity was determined in crude leaf extracts, which is the source tissue. Activity was measured by determining the amount of glucose liberated by the hydrolysis of sucrose. Quantification of glucose was performed by a two-step enzymatic procedure: first, glucose was converted to glucose-6-phosphate (G6P) by hexokinase; in the second coupled step, G6P was converted to phosphogluconate by G6P-dehydrogenase. The reduction of NADP during this step--quantified spectrophotometrically at 340 nm--was proportional to the hydrolyzed sucrose. The same methodology can be used to measure the other two invertases, simply by adjusting the pH conditions for the NI or by isolating the cell walls for the CWAI.

This methodology worked perfectly with young cassava leaves; but in sugarcane activities were either too low or there was some kind of inhibitor, as we were unable to increase activity by adding more sample or by concentrating it. We will try to confirm one or the other hypothesis by measuring AI in cane juice and by adding sugarcane leaf extracts to cassava leaf extracts and looking for inhibition of activity.

Low activities could also be due to the specific growth conditions as compared to other regions. where well-defined seasons exist.

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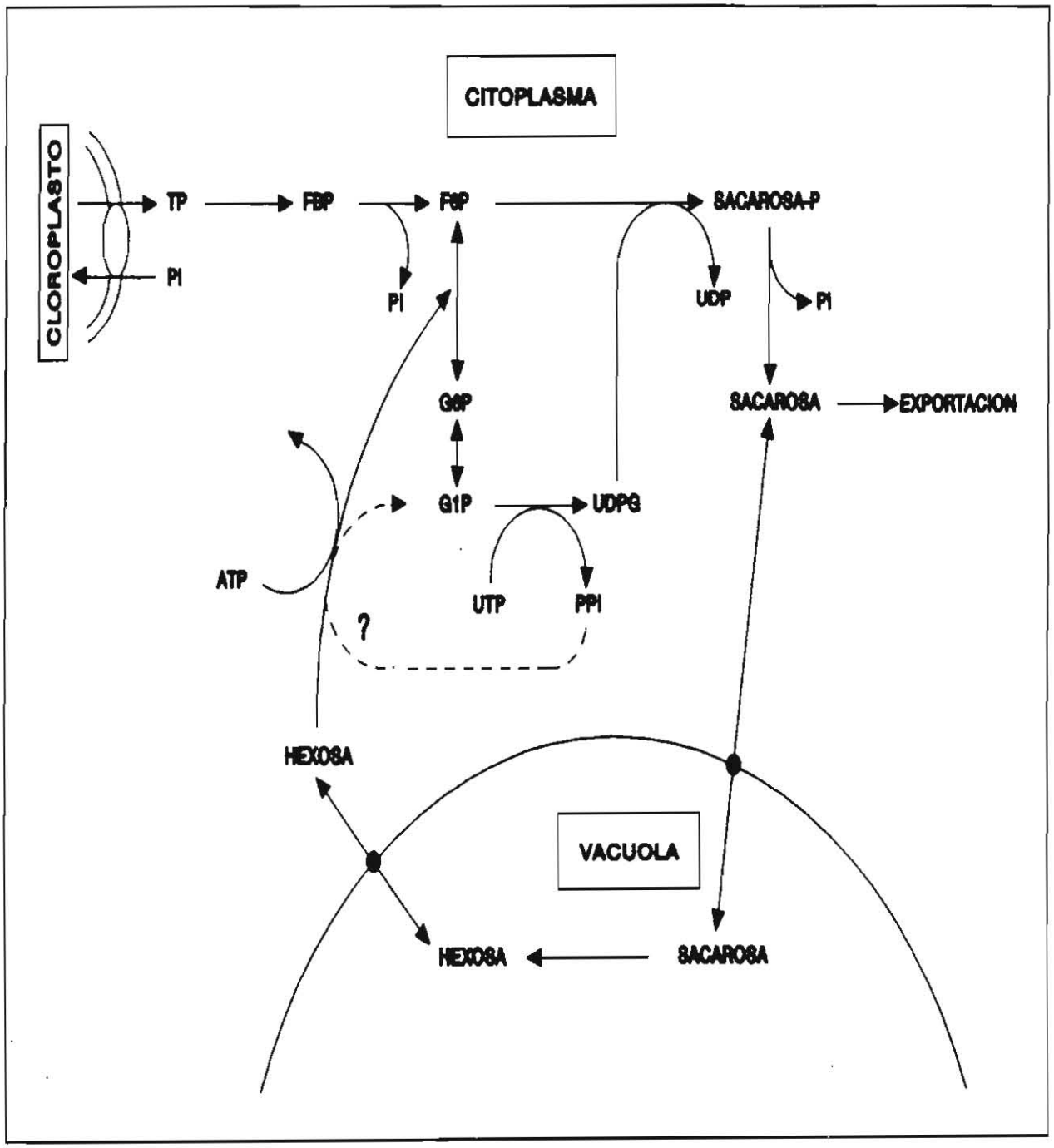

Figure 1. Regulation of photosynthesis through cyclic hydrolysis of sucrose.

3. METHODOLOGY DEVELOPMENT

3.1 Dichloromethane as an Economic Alternative to Chloroform for Extracting DNA from Plant Tissue

One major drawback to the application of RFLP and RAPD in extensive crop improvement programs is logistical in nature. The goal is to process thousands of samples in a short time, using reproducible, simple techniques at a low cost. RAPD analysis is a step in this direction as it obviates blotting DNA from agarose gels to nylon membranes and the subsequent hybridization with radioactive probes. After the direct evaluation of band patterns on the gel, the data are processed by potent mapping software (e.g., Mapmaker).

The next step where savings can be obtained is in extracting DNA from plant tissues. It is important to save on the amount of tissue that needs to be extracted, as well as on the chemicals used. Based on our experience with rice, beans and cassava, extraction procedures must be adjusted for each crop, depending upon the disturbing substances in the tissue. The DNA must be digestible by restriction enzymes or amplifiable by PCR. The classical methods make use of phenol and chloroform for extracting proteins; other methods use some specific precipitation steps (Dellaporta et al. 1983). In the former, phenol and chloroform constitute an important cost factor, which can be partially cut by reducing the extraction volumes.

We have tried an additional way to save on chloroform by replacing it with dichloromethane on the following grounds: similar polarity index, less toxic and about half the price. Table 1 presents the main characteristics of both solvents. The low boiling point and the high vapor pressure of dichloromethane restricts its use to room temperature applications, which is mostly the case in this process.

To demonstrate the applicability of dichloromethane for DNA purification, comparisons were carried out with respect to its protein- and phenol-extracting power and its compatibility with restriction enzymes and PCR. No interference with enzymatic reactions was detected in the different crops analyzed. The amount of protein extracted in consecutive steps was about the same with both solvents (Fig. 1).

Purity of the preparations and extracting power were checked applying three criteria:

- The absorbance ratio 260/280 nm of pure DNA should be between 1.8 and 1.9.
- The absorbance ratio 230/280 nm should be ca. 1; higher ratios denote contamination with protein.

- Second-derivative UV spectrum of DNA detects changes in slope of the zero-order spectra, otherwise undetectable to the eye (Mach et al. 1992), as demonstrated by the detection of phenol traces after extraction with chloroform and dichloromethane (Fig. 2). Problems arising during restriction digestion may derive from contamination by phenol that has passed undetected. This analysis has once again shown that the partition coefficient of phenol in water makes it necessary to re-extract or precipitate and wash the DNA carefully in order to assure a phenol-free sample.

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Table 1. Chloroform vs dichloromethane for DNA extraction.

Properties	Chloroform	Dichloromethane
Formula	$CHCl_3$	CH_2Cl_2
FW (g/mol)	120.4	84.9
▸ bp [°C]	61	40
mp [°C]	-63	-95
d [g/ml]	1.483	1.327
n_D	1.4457	1.4242
▸ Polarity $E^\circ(Al_2O_3)$	0.40	0.42
Viscosity [$mPas_{20}$]	0.57	0.44
UV cut-off [nm]	245	230
▸ P_{vap} [mm Hg]	160	340
App.	colourless liq	colourless liq
Sol. in water [v/v]	1:200	1:50
Flammability	no	no
▸ Toxicity	high, mutagenic, carcinogenic	high, mutagenic
▸ Exposure limit [ppm]	10	100
▸ Price [US$] [$Col]	31 23'200	21 12'800

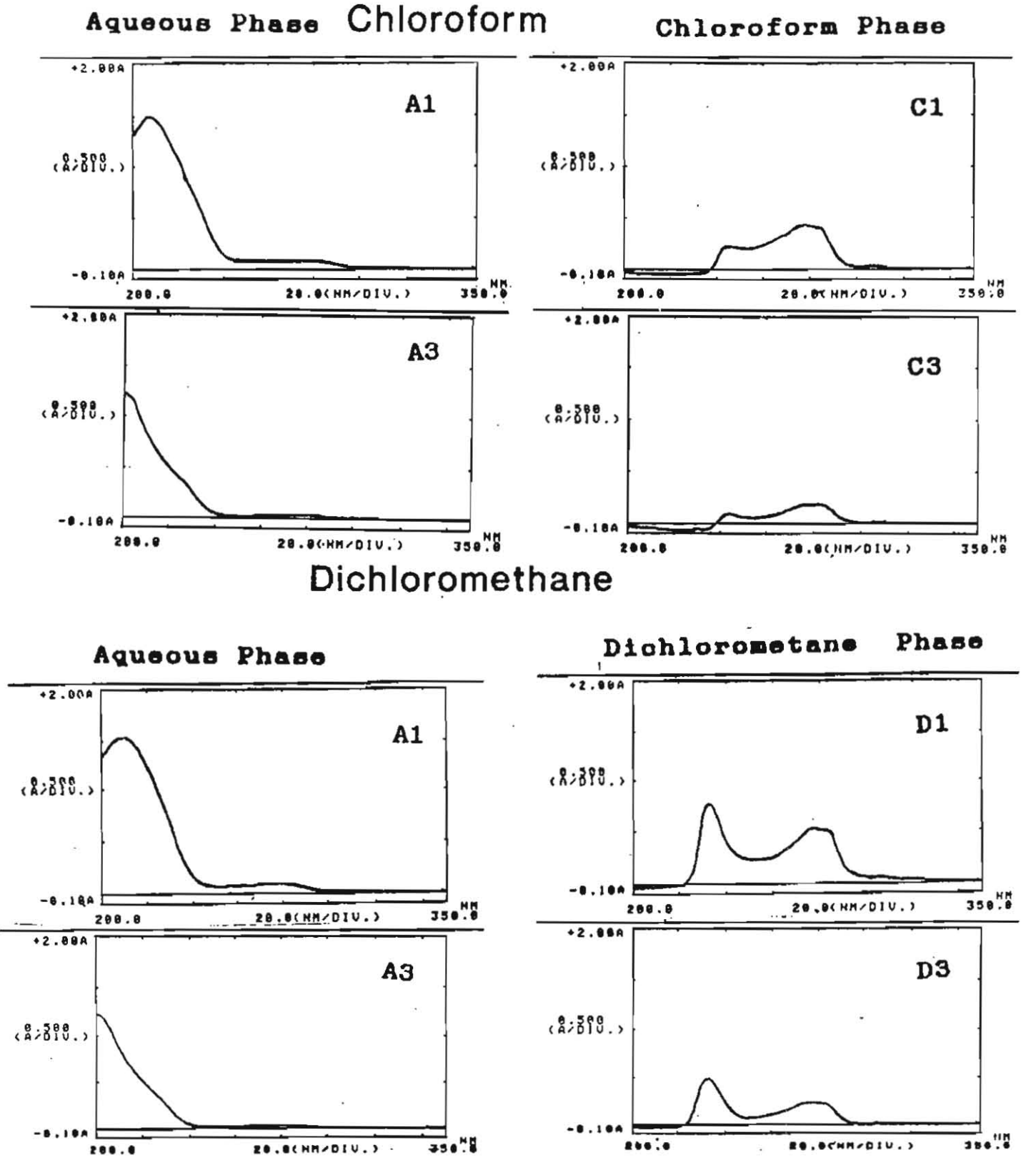


Figure 1. Extraction of proteins with organic solvents.

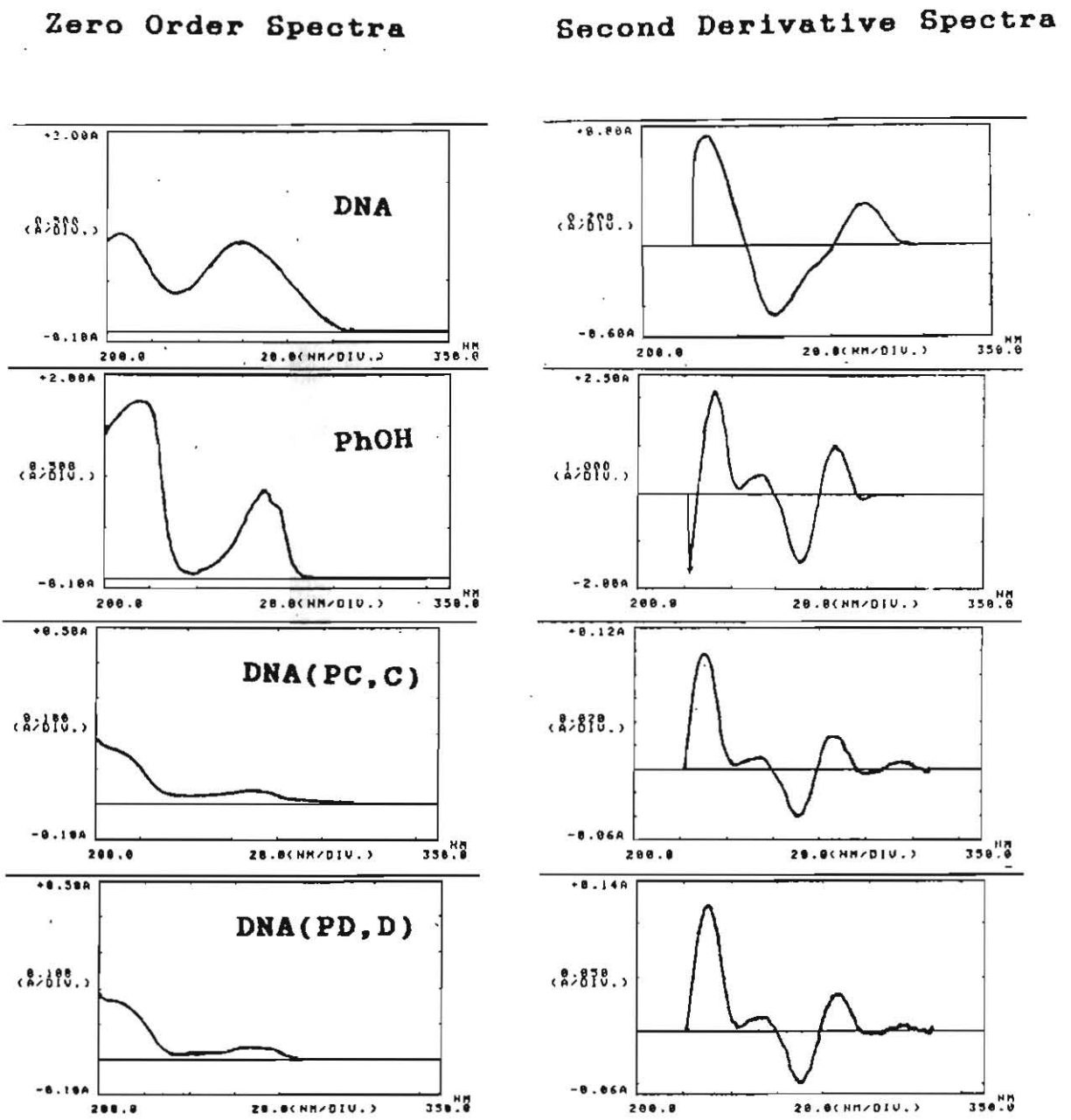


Figure 2. Phenol detection in DNA second derivative spectra.

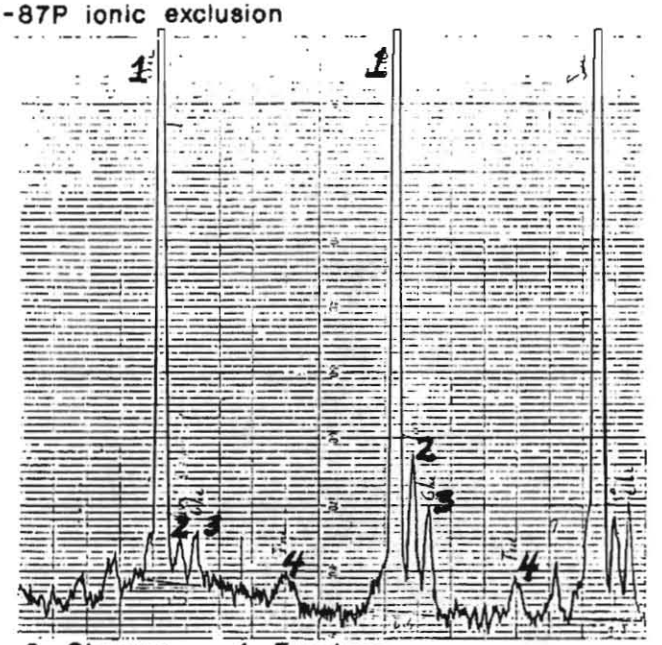
3.2 HPLC Determination of Linamarin in Crude Cassava Extracts (Tests carried out by Z. Bainbridge, NRI, London in a research stage at CIAT)

Determination of linamarin in crude extracts is crucial to screening for low-cyanide varieties in cassava germplasm. Chemical analysis methods are available; however, in some cases they require expensive reagents, in others they are inaccurate or time consuming. G. O'Brian from NRI is developing a simple chemical methodology at CIAT; while Z. Bainbridge, a visiting scientist from NRI, recently spent a couple of weeks in the Molecular Biochemistry Laboratory developing an HPLC technique for determining linamarin. Such a methodology has to be fast, robust and accurate.

Two different columns were used to separate the extracts: one Aminex HPX-87P cationic exchanger and a Chrompack C-18 reverse phase column (RP-18). To detect the cyanoglucoside, she used a refraction index detector, brought from the UK for that purpose. Refraction index detection is the most sensitive methodology for sugars. As can be observed in Fig. 1, nonsugars practically do not interfere with the determination. Both separation criteria work well. The RP-18 resolves the linamarin peak better above the background.

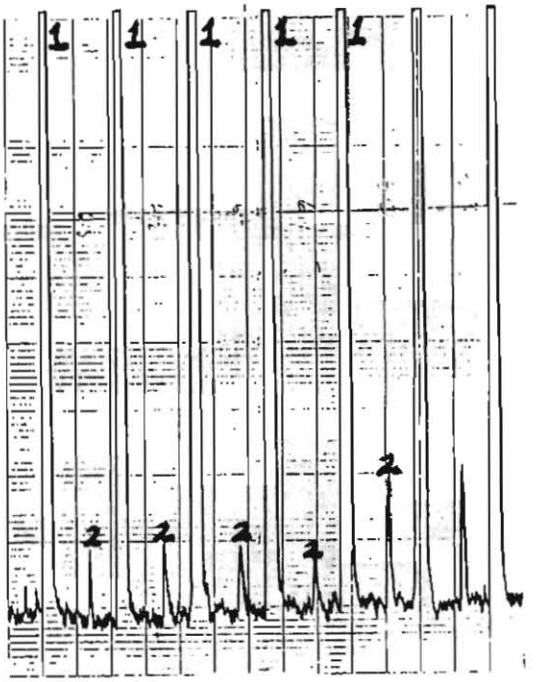
The procedure can be automatized using an autosampler. Every determination cycle takes less than 10 minutes as can be observed in the other two photos. Fig. 2 shows a calibration curve with pure linamarin. It should be noted that this is a very sensitive detection method (we are in the ppm range) and that about 10 ng of linamarin were applied to the column for the lower values of the calibration curve. Repetitions with changing concentrations of linamarin are practically identical. The plot of concentration against peak height yields a perfect linear relationship. Cassava Utilization plans to acquire an HPLC apparatus for such routine determinations.

Column : Aminex HPX-87P ionic exclusion
Flow : 0.3 ml/min
Temp : 80 C
Eluent : water
Inj vol : 40 µl
Detector : RI



1: Sucrose 2: Linamarin 3: Glucose 4: Fructose

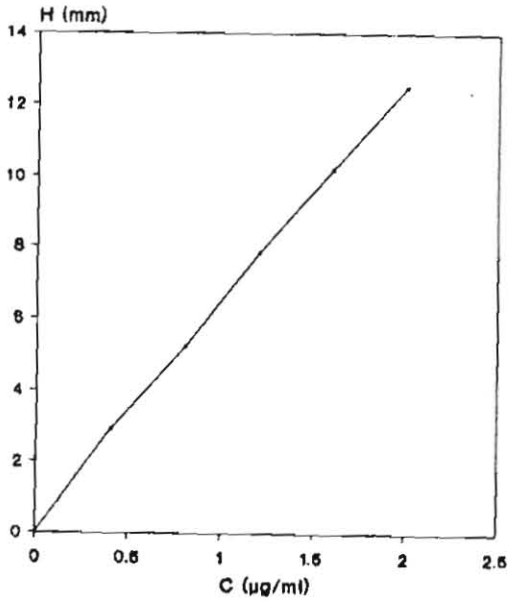
Column : Chrompack C-18 reverse phase
Flow : 0.7 ml/min
Temp : ambient
Eluent : water
Inj vol : 40 µl
Detector : RI



1: Carbohydrates 2: Linamarin

Figure 1. HPLC analysis of linamarin content in cassava root extracts.

Column	:	Aminex HPX-87P ionic exclusion
Flow	:	0.3 ml/min
Temp	:	80 C
Eluent	:	water
Inj vol	:	40 µl
Detector	:	RI



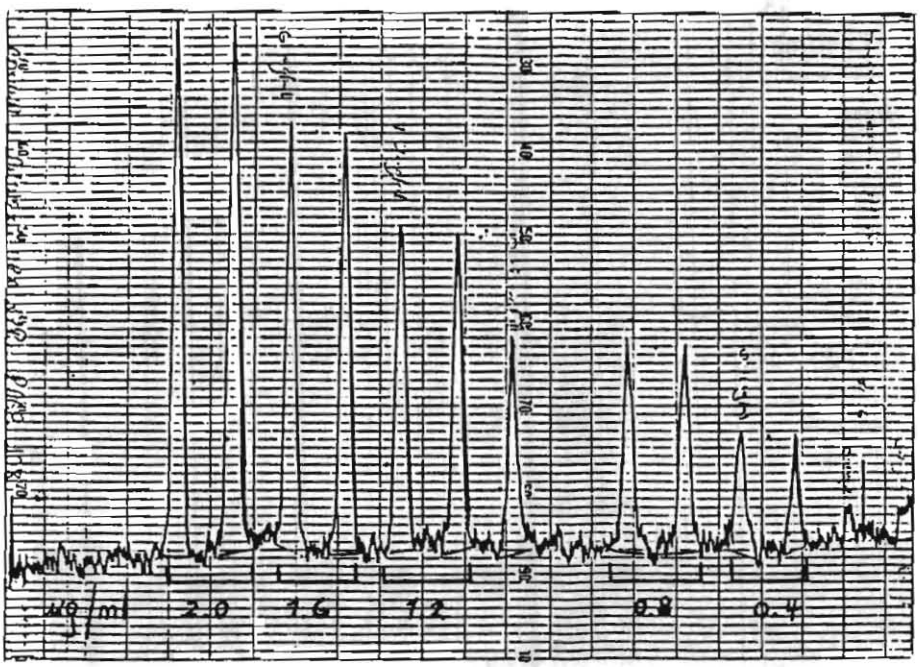


Figure 2. Linamarin HPLC calibration curve.

III. BROADENING THE GENETIC BASE OF CROPS AND GERMPLASM CONSERVATION

Many of the BRU's activities contribute directly or indirectly to the development of broad-based germplasm. The basic steps involved in this activity include: trait identification, gene isolation and gene transfer. Genetic conservation is another important activity of a broad-based germplasm development approach. The BRU's work is characterizing genetic variability through genetic mapping, gene tagging and fingerprinting contributes to the identification of useful traits from a range of gene pools. Research on the characterization of mechanisms involved in plant-stress interactions will ultimately contribute to gene isolation and cloning.

The BRU's activities in broadening germplasm base and germplasm conservation in the period 1988-1992, included: (i) interspecific hybridization in *Phaseolus* aided by embryo culture; (ii) rapid fixation of genetic variability and overcoming of sterility by haploid/doubled haploid induction in rice; (iii) development of methodologies for *Agrobacterium* and particle bombardment-mediated genetic transformation of CIAT crops; (iv) conservation of cassava genetic resources through *in vitro* culture and cryopreservation.

1. ENHANCING SEXUAL GENETIC RECOMBINATION

1.1 Interspecific Hybridization Between Common and Tepary Beans (Collaboration: S.P. Singh, A. Gutierrez, A. Criollo, Bean Program Genetics Section)

The tepary bean (*P. acutifolius*) is known to possess high levels of resistance to several abiotic and biotic stresses. Successful gene transfer for most of the desirable traits--except for resistance to common bacterial blight (CBB) from the teparies to true breeding lines of common bean--has seldom been realized. The causes for limited success include: (a) use of relatively few genotypes from either species for hybridization; (b) F_1 hybrid lethality associated with certain genotypes of either species; (c) use of few flower buds for pollination; (d) postzygotic embryo abortion; (e) F_1 hybrid sterility; (f) rapid reversion of hybrid progenies to parental types; (g) limited number of hybrids produced for evaluation; and (h) inadequate evaluation of interspecific hybrid progenies.

Haghighi and Ascher (1988) proposed the use of congruity backcrossing (i.e., backcrossing alternately with tepary and common beans) to facilitate genetic recombination between the two species and increase hybrid fertility; however, they presented no evidence for increased gene recombination between the two species.

The objective of our work, which began in 1989, is to transfer multiple desirable traits from selected genotypes of tepary into elite lines of common bean. A modified embryo culture technique was essential to obtain congruity backcross hybrids. The final goal of CIAT's *Phaseolus* wide-crossing program is to develop an interspecific *P. vulgaris x P. acutifolius* gene pool for facilitating gene transfers between these two and other *Phaseolus* species.

The tepary and common bean genotypes utilized for interspecific hybridization were evaluated and selected for agronomic traits. All crosses were made in the greenhouse by hand emasculation and pollination to prevent selfing and contamination from foreign pollen. The parental sequence used in the crossing program is shown in Table 1.

Young pods resulting from interspecific crosses were harvested as soon as the first symptoms of embryo abortion were apparent.
Using aseptic techniques, immature embryos including the embryonic axis and cotyledons were extracted from pods and transferred to the surface of the culture medium. For initial F_1 hybrids and the first recurrent backcross (RBC_1) embryos, a single medium named SE (for small embryos) was used.

Hybrid origin of F_1 plants of the initial cross was verified with polyacrylamide gel electrophoresis (PAGE) of diaphorase isoenzymes, using young trifoliolate leaves (Hussain et al. 1986).

To assess introgression of tepary alleles, the F_3 of the RBC_1, CBC_3 and CBC_5 hybrids was analyzed for seed protein to common bean, by a modified SDS-PAGE. Moreover, additional studies on introgression of tepary DNA fragments were carried out using a soybean ribosomal DNA probe. DNA was extracted from leaves of parental genotypes and hybrid populations using phenol-chloroform; extracted DNA was double digested with the restriction enzymes EcoRI and BamHI.

Hybrid plants with superior performance were propagated vegetatively by means of stem cuttings in order to carry out larger numbers of backcrosses or produce larger numbers of F_2 seed.

1.1.1 **Results**

- **Embryo culture**. All embryos from the initial interspecific cross, the first recurrent backcross, the first and second congruity backcrosses, as well as most of the embryos from the third, fourth and fifth congruity backcrosses, aborted from 8 to 27 days after pollination. Immature embryos had to be rescued aseptically from ovules and cultured in vitro as soon as abortion symptoms were detected.

- Establishment in soil of initial interspecific F_1s and first recurrent backcross mature hybrid plants was carried out without problems, following immature embryo culture in the SE medium.

- CBC_2 embryos were on average the smallest of the entire backcross program; culturing in SE medium gave rise to weak plantlets that could not survive transplanting into soil.

  A two-phase embryo culture protocol was adopted for the CBC_2 and subsequent backcross generations (Table 2). The first-phase medium (SE) had a high sucrose concentration (6%) and was supplemented with organic ammonium; incubation of cultures was carried out in the dark. The second-phase medium (LE) had a similar composition to the SE medium, but with reduced sucrose and mineral salt levels. When the medium for small embryos contained low sucrose (2%), the embryos either germinated prematurely, giving rise to very weak plantlets; or formed only callus without shoats. The two-phase embryo culture methodology developed in this study permitted the recovery of the CBC_2 plants from embryos as young as 12 days with an efficiency rate of 38% from some parental combinations and 35% efficiency when embryo growth averaged 8.5 days in other parental combinations.

- **Growth of hybrid embryos in situ and recovery efficiency of mature hybrid plants**. All F_1 hybrids of the initial cross resulted self-sterile. Rescuable embryos were made available only after backcrossing the F_1 hybrids with *P. vulgaris*. The RBC_1 embryos grew more than the F_1 embryos, but produced fewer mature plants (Table 3). Backcrossing of the initial F_1 hybrids with tepary did not yield any rescuable embryos. In the RBC_2, embryo growth increased and reached the most advanced developmental stage in the pods, to the extent that most of the backcrosses produced mature RBC_2 seeds with more than 30 days of growth in situ (Table 3). In contrast, the second congruity backcross (RBC_1 x *P. acutifolius*) gave rise to the smallest, underdeveloped and most-difficult-to-culture embryos of the entire backcross program, with a mean of 11 days' growth. Growth of embryos from congruity backcrosses increased or decreased depending upon whether the last male parent was *P. vulgaris* or *P. acutifolius*, respectively. At the fourth congruity backcross (CBC_4), it is noteworthy that, although the gene dosage of tepary increased with respect to the CBC_2, there was an increase in the growth reached by these embryos before rescue. A similar relationship occurred for the CBC_5 embryos with respect to the CBC_3 generation (Table 3). Whereas not a single seed was obtained from the second congruity backcross, 4.6% of all the embryos cultured in the fourth congruity backcross reached the seed stage. Most seeds did not mature fully and either had to be germinated in vitro or their embryos extracted and cultured on the LE medium. Similarly, in the CBC_5 generation there was an increase in the proportion of embryos that formed seed in situ with respect to the CBC_3 generation, and a much higher increase

occurred in the CBC_3 and CBC_5 generations with respect to the first recurrent backcross (Table 3).

- **Effect of common and tepary bean genotypes**. Throughout the interspecies crossing program, the genotype of common and tepary parents determined, to a great extent, the ability to obtain viable F_1 hybrids, as well as the efficiency of using F_1 hybrids as female parents in subsequent backcrossing.

  Use of the common bean cultivar ICA Pijao in the initial cross with tepary bean was more successful than Sacramento light red kidney. Tepary bean accession G 40001 gave higher hybrid plant recovery than G 40066 and G 40063 when crossed to ICA Pijao (Table 4). No first recurrent backcross hybrids were obtained from the single F_1 plant between Sacramento common bean and tepary accession G 40063. All common bean genotypes used in the first recurrent backcross were capable of yielding mature fertile hybrid plants, but with differences in efficiency (Table 6).

  In the second congruity backcross, the most efficient tepary genotype in generating mature hybrid plants was again G 40001 as in the initial interspecific cross. The most striking differences among tepary genotypes, however, were found in the frequency with which the CBC_2 hybrids could be backcrossed again with common bean to generate CBC_3 hybrids. Only 1% of the CBC_3 embryos obtained from the backcross [(RBC_1 x G 40023) x *P. vulgaris*)] developed into mature plants vs 27% of the embryos from the backcross [(RBC_1 x G 40001) x *P. vulgaris*].

  ICA Pijao was not only the best common bean genotype for generating interspecific hybrids, but hybrids involving ICA Pijao were the most efficient for continuing the subsequent congruity cycle following backcrossing with *P. acutifolius*.

- **Fertility of hybrids**. Fertility, measured as the percent of mature F_1 hybrid plants that produced at least one F_2 fertile plant, was recovered in the first recurrent backcross, increasing greatly in the second recurrent backcross. In congruity backcrossing, fertility was lost cyclically when a *P. acutifolius* genotype was the last male parent (i.e., CBC_2 and CBC_4), but was recovered when *P. vulgaris* was the last male parent (i.e., CBC_3 and CBC_5) (Table 3). Fertility recovery efficiency increased as the congruity backcross generations advanced (i.e. CBC_5 > CBC_3 > CBC_1). Likewise, seed production per plant increased as recurrent and congruity backcrossing advanced. Thus a single hybrid plant of the fifth congruity backcross yielded over 100 F_2 seeds after 5 mo in the greenhouse--well above the total seeds obtained from 11 first recurrent backcross hybrids after 8 mo.

- **Morphology of interspecific hybrid plants**. All F_1 plants of the initial cross had plant height, overall morphology and growth habit of tepary parents (Fig. 1 a-c). These plants also had pink flowers and leaf and bracteole size intermediate between common and tepary parents.

  Congruity hybrids that had tepary as the last male parent displayed more tepary than common bean characteristics, even more than the initial F_1 hybrids. These characteristics included overall plant morphology and growth habit (Fig. 1d), small bracteoles and leaves, and flowers with variable pink and occasionally white color of standards and wings. Similarly, congruity backcross hybrids with *P. vulgaris* as the last male parent showed more characteristics of common bean (Fig. 1e). In advanced generations of congruity backcrossing the vigor of hybrid plants was higher than in earlier generations.

  Traits not seen in the parental genotypes but expressed in hybrid progeny included (1) inflorescences bearing multiple pods, a trait mostly of common beans of Middle American origin; light brown seed like some wild genotypes; new seed protein electrophoretic patterns; and leathery leaves.

- **Verification of initial hybrids and evidence of gene introgression from tepary bean**. Presence of a low molecular weight band of isozyme diaphorase from the male parent *P. acutifolius* confirmed the hybrid origin of F_1 plantlets obtained in the initial cross (Fig. 2a).

  Monitoring of introgression of genes into the F_3 progenies of RBC_1, CBC_3 and CBC_5 hybrids was carried out by analyzing seed protein electrophoretic patterns and by hybridizing hybrid plant DNA with a ribosomal DNA probe. Introgression into the hybrid progenies was shown for all the protein and rDNA fragments (Fig. 2b & Fig. 3). To assess the stability of introgression, seeds from different F_3 plants of RBC_1 showing the four tepary seed protein bands were analyzed. Introgression was ascertained in all individuals. Table 5 shows the estimated frequency of occurrence of seed protein alleles in the progenies of RBC_1, CBC_3 and CBC_5 hybrids. Assuming a free combination of alleles for the expected frequencies, in the RBC_1 there is a high observed frequency of the alleles in homozygotes but very low in heterozygotes. In contrast, in the F_3 progenies of CBC_3 hybrids, the observed allelic frequency in homozygotes decreases, but there is an increase in the frequency of heterozygotes.

- **Hybrid progeny generated for agronomic evaluation**. Mature F_2 hybrid seed was produced from the first and second recurrent and from the third and fifth congruity backcrosses. Intercrossing of highly fertile F_1 and F_2 congruity hybrids (as male parents) with F_1 plants of CBC_2 and CBC_5 produced large amounts of mature seed (Table 6). The material generated for field evaluation, the genetic contributions of *P. vulgaris* and *P. acutifolius*, and the list of common bean

genotypes used as male parent to generate the respective hybrid, in order of efficiency, are shown in Table 6.

Progenies of the RBC_1, RBC_2 and CBC_3 are currently being grown in the field for advance generations and for seed increase to evaluate agronomic traits.

1.1.2 **Discussion**. As in previous interspecific hybridization between *P. vulgaris* and *P. acutifolius*, these results would not have been possible without in vitro embryo culture. This study has shown that successful transplanting of rescued embryo-derived plantlets to soil was possible after subculturing small germinated embryos in a second medium, under decreased sucrose and salt concentration, especially to allow more vigorous growth of the root system.

The use of common bean cultivar ICA Pijao in the initial cross greatly facilitated the production of vigorous interspecific F_1 hybrids, which could be backcrossed efficiently with other common bean cultivars to yield fertile first recurrent backcross hybrids.

Parker and Michaels (1986) identified ICA Pijao as a noncarrier of an incompatibility allele that interacts with a factor in the *P. acutifolius* genome, producing lethal F_1 hybrids; thus they proposed the use of ICA Pijao as a bridging parent in interspecific crosses with *P. acutifolius*. When difficulties in congruity backcrossing with other common bean genotypes were encountered in our work, use of ICA Pijao helped overcome such difficulties. Thus ICA Pijao not only facilitated the initial cross but also the latter congruity backcrosses.

The tepary bean genotype was also critical to the success of recurrent and congruity backcrosses. G 40001 was the most efficient tepary genotype for the initial interspecific cross and for the CBC_2; it was also the only tepary giving rise to hybrids that could be used effectively in subsequent congruity backcrossing. Other, initially less efficient tepary genotypes gave rise to fertile progenies when used as parents of advanced congruity backcrosses, apparently after genotype G40001 has helped overcome the interspecific incompatibility barrier. The perenniality habit of G40001--the result of increased indeterminacy of the Type IV growth habit--could partly explain the success of the tepary genotype G40001 in our congruity backcrossing program.

All common bean genotypes used in the fifth congruity backcross gave rise to mature fertile hybrids. As the majority of common bean genotypes studied are carriers of interspecific incompatibility genes in crosses with *P. acutifolius*, it is likely that most of the common bean genotypes used in this study would be incompatible when used directly in the first hybridization. The generation of viable hybrids could have possibly occurred through segregation of viable and lethal gene-carrying gametes in later generations. These results indicate that congruity hybrids can serve as a bridge for

the transfer of tepary traits to common beans when either is a carrier of interspecific incompatibility genes.

Analysis of electrophoretic patterns of seed proteins and fingerprinting with a ribosomal-DNA probe suggested high introgression frequencies of tepary alleles in the lectin region; the observed values are close to the ideal values to be expected if the alleles had combined freely after a cross. Likewise, increase of introgression of tepary seed protein alleles in advanced congruity generations shows the superiority of congruity over recurrent backcrossing. A gradual increase in fertility, growth of hybrid embryos in situ, and production of mature hybrid plants was observed as the congruity backcrosses increased. For example, the requirement of embryo rescue diminished toward advanced congruity backcrosses. Increase of embryo growth in situ, vigor of F_1 plants and improved fertility of hybrids can be attributed to various possible causes: (a) increase in structural congruity of the homologous chromosomes of the two species, resulting from the exchange of chromosomal segments as backcrossing advances; and (b) accumulation of complementary favorable gene combinations of the two species, which confers greater fertility and vigor to the hybrids. Gene combinations present in fertile and vigorous plants are then transferred to congruity hybrids in the subsequent backcross.

Hybrid sterility can be attributed, to a large extent, to the structural incongruities between the chromosomes of common bean and tepary bean. Using highly fertile and vigorous congruity hybrids as parents in subsequent crosses in lieu of common bean genotypes, produced fertile, vigorous CBC_5 hybrids with greater frequency than with the best common bean genotypes. This modification of the scheme proposed by Haghighi and Ascher (1988) may prove useful for transferring tepary traits using a lower number of congruent backcrossing cycles and thus deserves more attention in the future. Intercrossing among congruity hybrids, using the highly fertile ones as male parents and progenies of self-sterile hybrids as females, also made it possible to obtain a large amount of seed. This "intermating" of hybrids has been proposed as a means of combining different donor alleles and thus allowing greater genetic recombination. Congruity backcrossing thus seems to overcome gradually *P. vulgaris* x *P. acutifolius* hybridization barriers such as genotype incompatibility, early embryo abortion, hybrid sterility and lower frequencies of hybridization.

In this work we have generated the largest number of *P. vulgaris* x *P. acutifolius* fertile hybrids to date. We have also demonstrated high frequencies of tepary allele introgression. The major factors contributing to the success of this work have been knowledge about facilitator genotypes such as ICA Pijao (Parker and Michaels 1986) and G 40001 (this study), the adoption of the congruity backcross strategy (Haghighi and Ascher 1988),and modification of the embryo culture technique to allow adult hybrid plant recovery from small congruity backcross embryos (this study). We have also developed a pool of congruity hybrids that can be used as a bridge to introgress

germplasm from other incompatible tepary to common beans and vice versa. This pool might also facilitate hybridization with other *Phaseolus* species that are phylogenetically next to *P. acutifolius*, but distant from *P. vulgaris*.

Currently, advanced generation lines from the first and second recurrent backcrosses and the first cycle of congruity backcrosses are being selfed to produce inbred lines. This will be followed by seed multiplication and evaluation to identify lines with desirable traits from both species.

As shown in this study, fertility, embryo growth in situ and recovery efficiency in mature plants from congruity hybrids have not yet apparently reached their maximum possible level. Consequently, our congruity backcrossing program will continue.

References

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Hussain, A.; Bushuk, W.; Ramirez, H. and Roca, W.M. 1986. Field bean (*Phaseolus vulgaris*) cultivar identification by electrophoregram of cotyledons storage proteins. *Euphytica* 35:729-732.

Parker, J.P. and Michaels, T.E. 1986. Simple genetic control of hybrid plant development in interspecific crosses between *Phaseolus vulgaris* L. and *P. acutifolius* A. Gray. *Plant Breed* 97:315-323.

Table 1. *Phaseolus vulgaris* x *P. acutifolius* recurrent and congruity backcrosses carried out at CIAT.

Type of Cross		Resultant Hybrid
Recurrent Backcrossing		
Initial hybridization	P. vulg x P. acut	F_1
First recurrent backcross	F1 x P. vulg	RBC_1
Second recurrent backcross	RBC_1 x P. vulg	RBC_2
	RBC_2-F_1	
	-F_2	
	etc.	
Congruity Backcrossing[a]		
First congruity backcross	F_1 x P. vulg	CBC_1
Second congruity backcross	CBC_1 x P. acut	CBC_2
Third congruity backcross	CBC_2 x P. vulg	CBC_3
Fourth congruity backcross	CBC_3 x P. acut	CBC_4
Fifth congruity backcross	CBC_4 x P. vulg	CBC_5
	CBC_5-F_1	
	CBC_5-F_2	
	-etc.	

a) After Haghighi and Ascher (1988).

Table 2. Effect of embryo culture involving one- and two-phase protocols on the production of mature hybrid plants in two *P. vulgaris* x *P. acutifolius* second-congruity backcrosses (CBC_2).

	Second Congruity Backcrosses[a]			
	CBC_1 X G40023		CBC_1 X G40138	
	One-Phase Culture[b]	Two-Phase Culture[b]	One-Phase Culture	Two-Phase Culture
No. embryos cultured	29	202	2	11
Embryo growth[c]	14.5±5.2	12.4±3.3	15.5±3.4	10.3±1.7
No. mature plants	0	37	0	4
% CBC_2 plants	0	18	0	36

a) G40023 and G40138 are the *P. acutifolius* genotypes used in backcrossing as male parents.
b) One-phase culture in medium SE; two-phase culture in media SE followed by LE.
c) No. of days from pollination to embryo rescue and culture.

Table 3. Overall results of the *Phaseolus vulgaris* x *P. acutifolius* recurrent and congruity backcross programs.

Type of Cross	Resultant Hybrid	Gene Dosage vulg:acut	% Embryos Forming Seeds	% Embryos Rescued	Embryo Growth[a]	Mature Hybrid Plants		
						No.	%	Fertility[b] %
P. vulg x P. acut	F_1	1:1	0.0	100.0	17.8 ± 1.8	64	21	0
F_1 x P. vulg	$RBC_1 = CBC_1$	2:1	0.9	99.1	27.6 ± 4.1	24	11	46
RBC_1 x P. vulg	RBC_2	3:1	98.3	2.7	32.9 ± 3.4	332	92	88
CBC_1 x P. acut	CBC_2	2:2	0.0	100.0	11.9 ± 3.2	56	22	0
CBC_2 x P. vulg	CBC_3	3:2	22.2	77.8	18.7 ± 6.6	61	17	48
CBC_3 x P. acut	CBC_4	3:3	4.6	95.4	16.7 ± 4.7	130	25	0
CBC_4 x P. vulg	CBC_5	4:3	25.1	74.9	21.2 ± 6.9	202	23	55
					TOTAL	869		

a) No. of days from pollination to embryo rescue and culture.
b) % mature F_1 hybrids that produced at least one F_2 fertile plant.

Table 4. Efficiency of *P. vulgaris* and *P. acutifolius* parental genotypes from the initial interspecific hybridization aided by embryo rescue and culture *in vitro*.

Parental Genotypes		No. Embryos Cultured	No. Mature Plants	% F_1 Hybrid Plants
P. vulgaris	*P. acutifolius*			
ICA Pijao x	G40001	63	27	43
	G40066	154	35	23
	G40063	19	1	5
Sacramento x	G40001	21	0	0
	G40066	34	0	0
	G40063	14	1	7

Table 5. Introgression of lectin alleles of *Phaseolus acutifolius* into *P. vulgaris* x *P. acutifolius* recurrent and congruity backcross hybrids.

Hybrid Generations & Frequencies	No. Individuals Evaluated	Alleles[a]		
		VV	VA	AA
RBC_1-F_3				
Expected freq.	66	0.68	0.12	0.19
Observed freq.	66	48(0.73)	4(0.06)	14(0.21)
CBC_3-F_3				
Expected freq.	66	0.61	0.15	0.23
Observed freq.	66	53(0.80)	7(0.11)	6(0.09)
CBC_5-F_3				
Expected freq.		0.59	0.16	0.25
Observed freq.				

a) VV = homozygous *P. vulgaris* alleles; VA = heterozygous; AA = homozygous *P. acutifolius* alleles.

Table 6. *Phaseolus vulgaris* x *P. acutifolius* recurrent and congruity backcross hybrid material generated in this study for agronomic evaluation.

Hybrid Generation	Genetic Contribution vulg:acut	No. Mature Fertile Hybrids	*P. vulgaris* Genotypes Used as Last Male Parent to Generate Respective Hybrids[a]
RBC_1	75:25	11	A798(4), MAM38(3), A775(2), A779(1), A797(1)
RBC_2	87.5:12.5	281	ARA9(56), SC83(41), MAR(39), A769(38), PEF14(32), A800(32), PVA1111(14), Carioca(12), CTC1(9), IPAG(6), A429(6), A798(4), A797(1)
CBC_3	68.75:31.25	27	ICA Pijao(14), MAR1(6), A800(3), ARA9(1), A429(1), PEF14(1), A797(1)
CBC_3-F_1 x CBC_3-F_2		183	Various combinations of CBC_3-F_1 and F_2 hybrids
CBC_5	67.19:32.81	109	ICA Pijao(78), A800(9), CBC_3-F_2(8), A775(6), MAR1(4), ARA9(3), A798(1)
CBC_5-F_1 x CBC_5-F_1/F_2		> 600	Various combinations of CBC_5-F_1 and F_2 hybrids

a) Figures in parentheses show the no. of fertile hybrid plants obtained by using the respective parents.

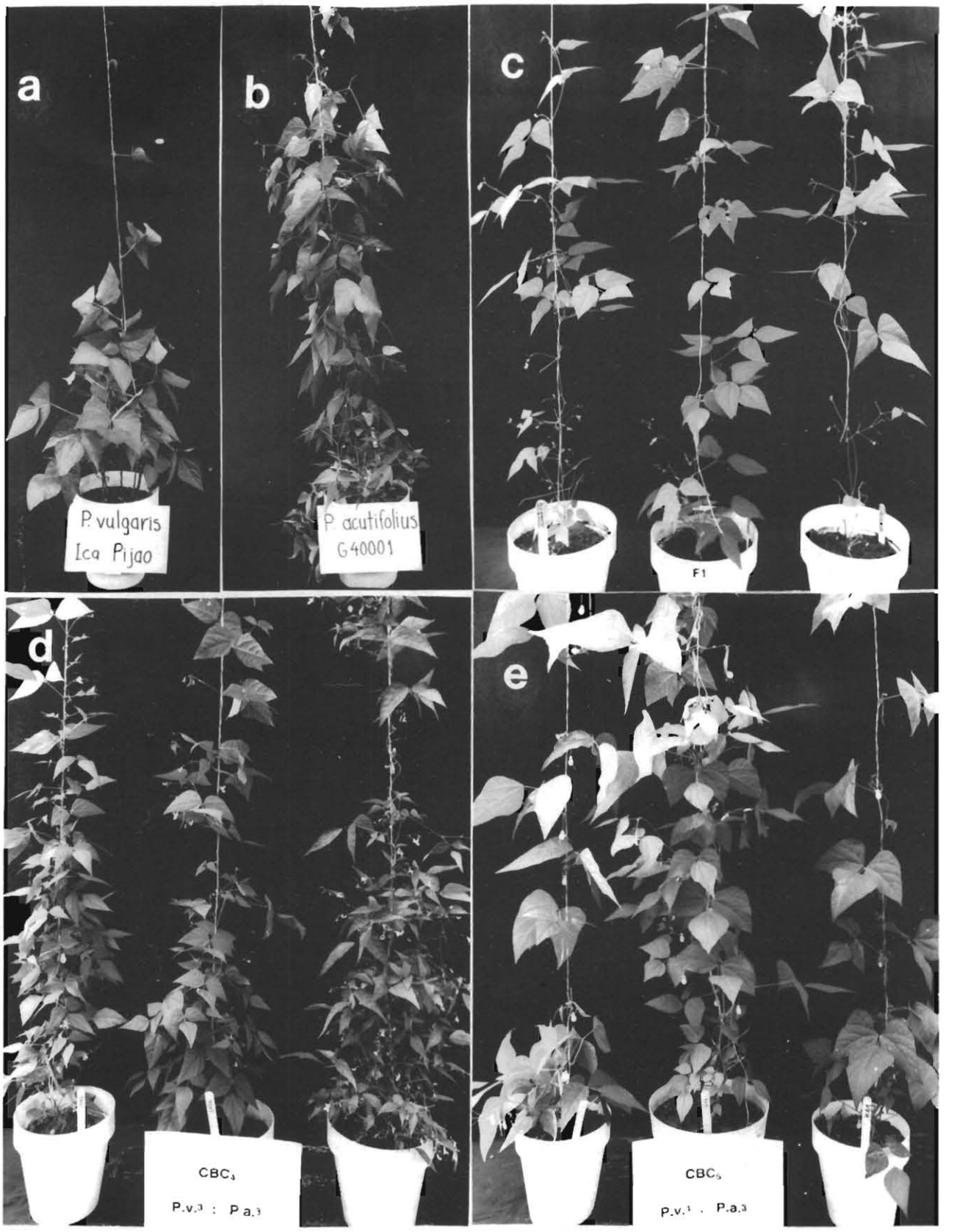

Figure 1. Morphology of parental genotypes and interspecific *P. vulgaris* x *P. acutifolius* mature hybrids: (a) common bean parent ICA Pijao; (b) tepary bean parent G 40001; (c) F_1 hybrids of initial *P. vulgaris* x *P. acutifolius* cross; (d) fourth congruity backcross hybrids (gene dosage 3 Pv:3 Pa); (e) fifth congruity backcross hybrids (gene dosage 4 Pv:3 Pa).

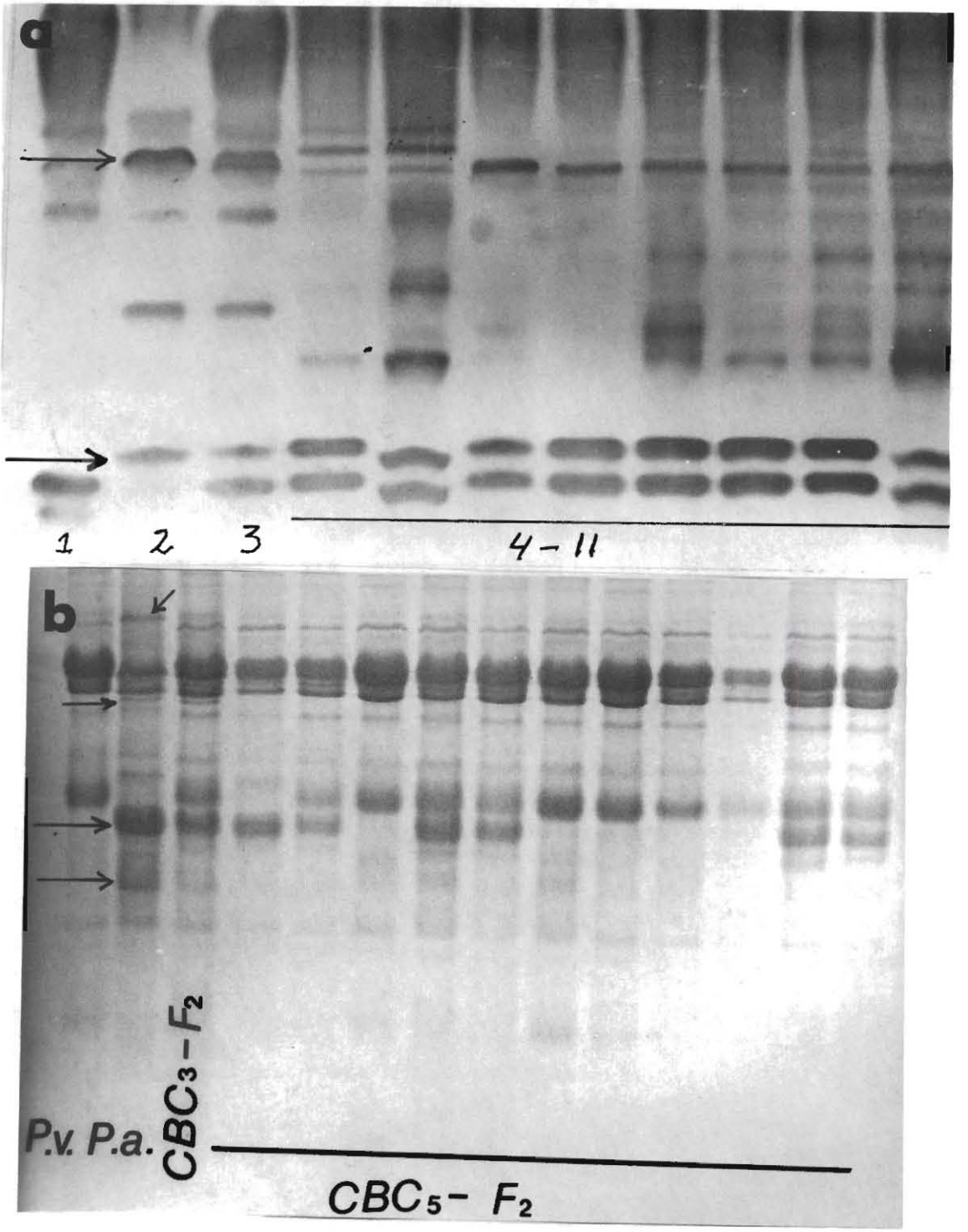

Figure 2. (a) Verification of initial interspecific F_1 hybrids by diaphorase isozyme electrophoresis. Lanes: (1) ICA Pijao (*P. vulgaris* female parent; (2) G 40001 (*P. acutifolius* male parent), (3) tissue extract mixture of ICA Pijao + G 40001; (4-11) interspecific ICA Pijao x G 40001 hybrids. Arrow shows a tepary diaphorase isozyme band inherited by the hybrids. (b) Evidence of allele introgression from tepary bean by seed protein electrophoretic separation. Lanes: (1) ICA Pijao (*P. vulgaris* parent), (2) G 40001 (*P. acutifolius* parent), (3-6) third congruity backcross hybrids; (7-10) fifth congruity third congruity backcross hybrids; (7-10) fifth congruity backcross hybrids. Arrows show the tepary alleles whose introgression is monitored in the congruity hybrids.

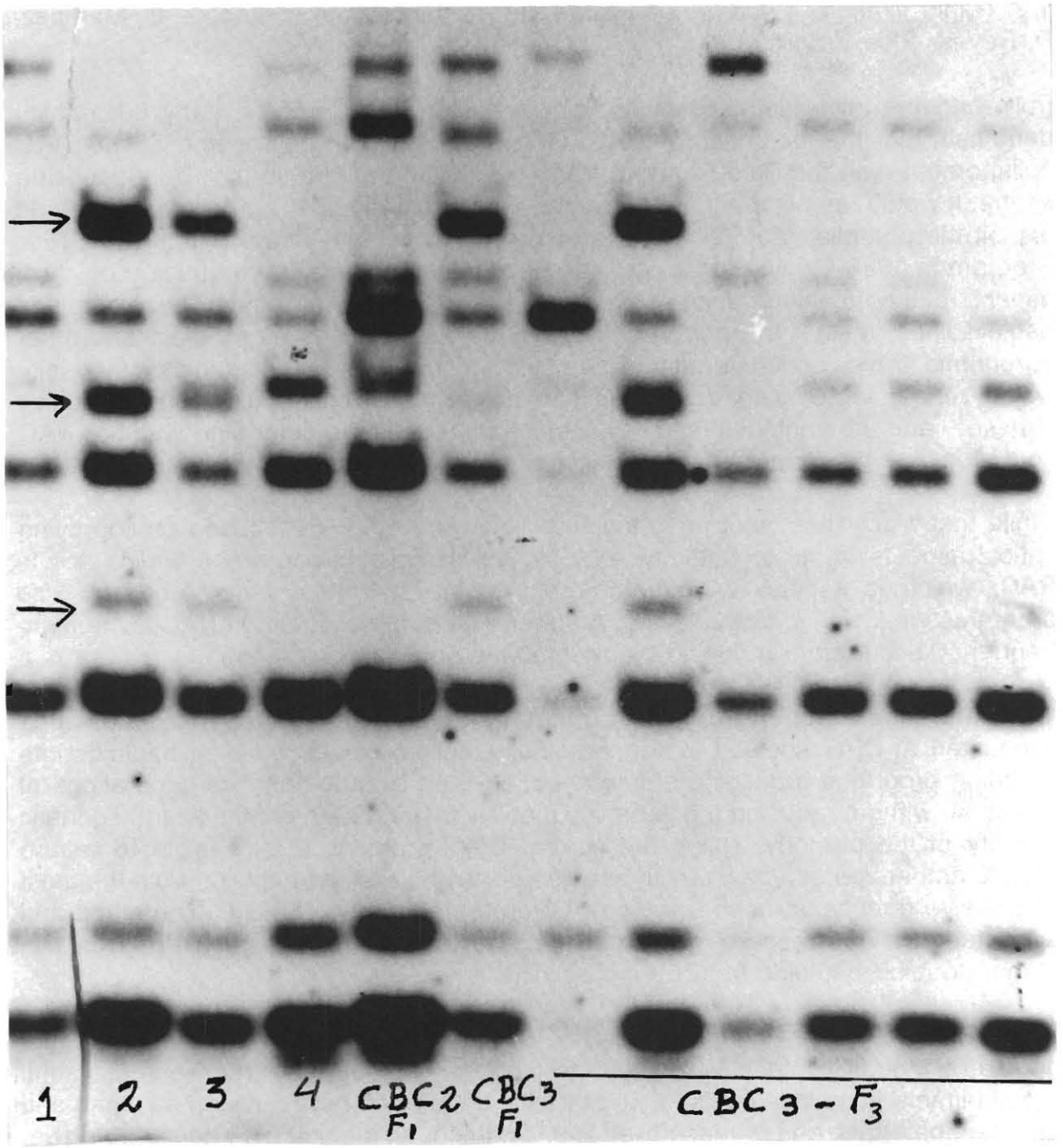

Figure 3. Introgression of ribosomal DNA fragments from tepary into third and fifth congruity backcross hybrid progenies: lane 1) *P. vulgaris* parent ICA Pijao; lane 2) *P. acutifolius* parent G 40001; lane 3) *P. vulgaris x P. acutifolius* F_1 hybrid; lane 4) another *P. vulgaris* genotype (MAM 38); lane 5) F_1 of second congruity backcross; lane 6) F_1 of third congruity backcross; lanes 7-12) F_3 of third congruity backcross.

1.2 Rapid fixation of rice genetic variability (Collaboration: Z. Lentini, C. Martinez, P. Reyes, Rice Program)

Following the initial activities in rice anther culture (RAC) of the early 80s, which demonstrated the potential of RAC in rice germplasm improvement, the BRU collaborated with the Rice Program from 1986-1988 to improve the RAC technique so that it would be suitable for a large germplasm improvement program. In order to exploit the potential of RAC fully, the responsibility for RAC was shifted to the Rice Program in 1988-89, where lab facilities were developed for that purpose. Logistical aspects of the technique such as production of sufficiently large numbers of doubled haploid lines, testing the performance of RAC-derived lines as for yield and other agronomic traits, and determining whether the high degree of genetic uniformity would render large-scale rice production more prone to periodic biotic or abiotic stresses, and the implementation of cost/benefit studies to determine whether RAC could feasibly be adopted by national rice programs in Latin America.

While these activities went on in the Rice Program, the BRU focused on improving critical aspects of the technology such as the known recalcitrancy of *indica* rice to RAC, which is especially important considering that most Latin American rice programs work with *indica* cultivars. Another important constraint in RAC is the high-frequency occurrence of albino plants in many genotypes.

1.2.1 **Increased generation of doubled haploids from *indica* rice**. Research conducted at CIAT showed that incorporating pollen-derived doubled haploids into breeding programs reduces the time to obtain fixed lines to only two generations of selection, without reducing the genetic variability in grain yield or affecting the genetic stability of the progeny. Using the current CIAT protocol, it is possible to culture 10,000 anthers/person/day, which can generate 440 doubled haploids from the most responsive genotypes (*japonica* type). Given the efficiency of this RAC method and a lab team of one research assistant and five technicians, the Rice Program can obtain doubled haploids from 60 crosses/mo.

Anther culture is been used by the Rice Program to introgress early maturity and good grain quality into cold-tolerant germplasm; increase the recovery of useful recombinants from wide crosses; facilitate the transfer of traits between savanna and irrigated materials; and produce fixed lines for the gene tagging of *Pyricularia oryzae*, rice hoja blanca virus (RHBV) and *Togasodes oryzicola* resistance genes.

Large numbers of doubled haploids are obtained from crosses which involve at least a *japonica* or upland parent. As *indica* types have proved unresponsive to anther culture, the use of this technique, particularly in breeding irrigated rice, is greatly restrained. This collaborative work between the Rice Program and the BRU has resulted in a mean increase of 35-fold in green plant production from anther culture of the *indica* types.

A total of 7 nonresponsive *indica* genotypes were selected to include the genetic diversity that may be present in Latin American varieties. Callus induction was evaluated on Potato-2 medium (commonly used at CIAT, Nuñez et al. 1989) and on modifications of N6 medium; i.e., macronutrients, micronutrients, vitamins and growth regulators (Chu et al. 1975). The effects of maltose, $AgNO_3$ (an anti-ethylene compound), heat pretreatment of panicles, activated charcoal, L-cysteine, citric acid, anther density and plantlet age for transplanting from the greenhouse into the field were also examined (Lentini et al. 1991). The regeneration of green plants from the various treatments was determined using ½ medium concentration of standard CIAT protocol (Nuñez et al. 1989).

Efficiency of response of various of the recalcitrant (i.e., *indica*) genotypes was increased 3- to 9-fold by replacing the potato-2 callus induction medium with a modified N6 basal medium (Table 1). Further enhancement in callus induction was obtained by replacing sucrose with 5% maltose and by adding 10 mg/l $AgNO_3$ (Fig.1). A clear-cut increase in callus embryogenesis was noted when 2,4-D (2 mg/l) and picloram (0.07 mg/l) were added to the induction medium. Similar callus induction was obtained with kinetin (0.5 mg/l) or zeatin (0.1 mg/l). These modifications increased callus induction from 0-2% up to 40%, depending on the genotype. This protocol also enhanced the mean regeneration of green plants/anther from 0.06% to 0.98% for *indica*, and from 3.2% to 8.6% for the most responsive genotype (Table 2). These modifications increased the generation of green plants from 0.39% to 4.4% green plants/anther of an otherwise recalcitrant genotype. Improvement in anther culture response was not noted with the other parameters evaluated.

An economic analysis conducted by the Rice Program Genetics and Economics Sections indicates that this level of response results in a savings of about US$70,000 per variety developed in half the time required in standard breeding.

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Lentini, Z.; Martinez, D.P. and Roca, W.M. 1991. Generation of doubled haploids feasible for a rice breeding program. 5th Annual Meeting of the Rockefeller Foundation's International Program on Rice Biotechnology. p. 34.

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Table 1. Modifications of N6 medium.

2. ENHANCING NONSEXUAL GENETIC RECOMBINATION

This section deals mainly with the BRU's efforts to develop genetic transformation techniques for CIAT crops. Genetic transformation offers a direct means to introgress genes into plants from other species, genera, taxa or artificially made gene constructs. This section also includes the BRU's activities in plant regeneration, an essential step for most genetic transformational approaches and other biotechnological applications. Owing to its nature, this research is basically methodological. Given the high efficiency of *Stylosanthes* plant regeneration from cell/protoplast cultures demonstrated in the Unit (Szabados and Roca 1986), this important forage legume was used as a model for developing a gene transfer method through transformation using available marker genes and for studying the expression and inheritance of the introduced genes.

2.1 Agrobacterium-Mediated Transformation and Production of *Stylosanthes guianensis* Transgenic Plants

Prerequisites for *Agrobacterium*-mediated transformation include the availability of a plant regeneration protocol and the identification of bacterial strains that can infect and introduce the desired genes into plant cells and tissues. Methodologies for assessing stable expression and inheritance of the introduced genes in the regenerated plants and in their progeny are also necessary.

The objective of this research was to develop a transformation system for the stable introduction of foreign genes into *S. guianensis*. This is the first report demonstrating efficient transfer of three genes to *S. guianensis* (Aubl.) Sw. CIAT 184, using a disarmed strain of *Agrobacterium tumefaciens*, harboring a selectable marker, the bact bar gene. Inheritance data from the progeny of selfed transformed plants were also obtained. The ultimate goal is to use transformed plants for studying the effect of expression of alien genes on growth and agronomic performance of a pasture legume and for improving economically important *S. guianensis* characters.

For transformation experiments, leaf discs from in vitro germinated seedlings were cultured on regeneration MS medium (basal MS supplemented with 5.3 μM NAA and 17.6 μM BAP) (Szabados and Roca 1986) and infected with *A. tumefaciens*.

A. tumefaciens disarmed strain EHA101 containing the plasmid pGV1040 (Calderon-Urrea 1988), provided by Plant Genetic Systems, Belgium, was grown overnight in 5 ml of Luria broth (LB) containing 50 mg/l Kanamycin (Km), 10 mg/l Rifampicin (Rif), 100 mg/l Spectinomycin (Sp) and 300 mg/l Streptomycin (Sm). Overnight cultures were pelleted and re-suspended in fresh LB medium by vortexing.

The plasmid pGV1040 contains two selectable marker genes between the T-DNA borders (Fig. 1): the bar gene for phosphinotricin resistance and the kanamycin

resistance gene, npt II; and as a screenable marker, the uid A gene which encodes the enzyme ß-glucuronidase.

The bar and npt II coding sequences are under the control of the bidirectional PTR1'-2' promoter of the octopine TR-DNA, the termination and polyadenylation signals of the T-DNA gene 7, and the octopine synthase gene, respectively. The uid A gene is controlled by the cauliflower mosaic virus 35S promoter and the 3'-end of the nopaline synthase gene.

Leaf disc explants were inoculated by swirling for ca 1 min in an overnight culture of bacteria. After 48 h co-cultivation on MS regeneration medium in the dark, explants were rinsed with liquid basal medium containing 250 mg/l carbenicillin (Cb), blotted dry on sterile filter paper and cultured on the regeneration medium, supplemented with 250 mg/l Cb and 50 mg/l Km. After 10 days all the growing calli were transferred back to the regeneration medium containing 1 mg/l phosphinotricin (PPT).

Calli grown on the selective regeneration medium were transferred, for organogenesis induction, to MS basal medium solidified with 0.5% agar without hormones until shoots appeared. Regenerants were then transferred to basal MS containing 0.53 μM NAA and 1.76 μM BAP for elongation; rooting was carried out on basal MS. Cb was maintained during all regeneration steps; PPT was not present during the rooting step.

To determine whether any bacteria remained in the regenerated plantlets, Cb was removed from the medium 1 month before transplanting to the greenhouse, and tissues of each plant were used as inoculum in liquid LB at 28°C for 1 wk to detect bacterial growth.

GUS tests were carried out at different regeneration stages according to the standard histochemical assays of Jefferson (1987) and Kosugi (1990). Tissues from primary transformed plantlets and their selfed progeny were also tested with GUS to identify transformants.

The plasmid pGV1040, maintained in DH5 library efficiency cells, was isolated by the alkaline extraction procedure of Birnboim and Doly. The probes used for detecting the introduced genes were a Bgl II/ 6 Kb fragment, which comprises the regions of the three introduced genes and allowed us to determine the integrity of introduced genes, and the Bgl II/Pst I 1.2 kb fragment (Fig. 1), located on the right border of the T-DNA, which represents fragments of the bar and npt II genes, allowing us to determine the copy number of introduced genes. The Bgl II/Pst I 1.2 Kb probe was cloned in pUC19 and maintained in DH5 α cells.

To obtain the probes for radioactive labeling, plasmid pUC 19 containing the 1.2 kb fragment was PCR-amplified in a Perkin Elmer Cetus DNA thermal cycler using the

universal M13 primers; while the 6 Kb fragment was obtained by eluting the fragment from the Bgl II-digested pGV1040, separated by electrophoresis in 0.6% low-melting point agarose gels, freeze-fractured at -70°C using phenol extraction, cleaned 3 times with sec-butanol and precipitated with 3M sodium acetate and 2 vol of 100% ethanol at -70°C; the pellet was then washed with 70% ethanol.

Using the method of Weeks et al. (1986), DNA was extracted from 3 g of mature leaves from glasshouse-grown plants. These plants were regenerated from 4 different transformation events and one non-transformed control plant. Extracted DNA was then treated with RNAse. A DNA concentration was estimated fluorometrically in a Hoefer TKO 100 fluorometer, using Hoechst dye 33258. DNA was digested with Bgl II for 3 h at 37°C. Electrophoretic separation was carried out on 0.6% agarose horizontal gels in TBE buffer at 40 V for 16 h and transferred to Hybond-N^+ nylon membranes (Amersham) by capillary blotting. DNA was immobilized by UV crosslinking. Prehybridization of filters was carried out with 5X SSPE (pH 7.0), 5X Denhart's solution, 1% SDS and 114 ng/ml herring sperm. For hybridization, 50 ng of probe were labeled using the Amersham multiprime DNA-labeling system. After overnight hybridization, filters were washed once in each solution (2X, 1X, 0.5X SSPE/0.1 SDS) for 30 min at 65°C. Kodak-X-Omat AR X-ray film was used to expose the hybridized filters for 2 days at -80°C.
Seeds from selfed, transformed and nontransformed plants were germinated in soil in the glasshouse and in vitro to study segregation ratios of the foreign genes in the progeny. Twenty days after germination, seedlings grown in the glasshouse were sprayed with different concentrations (equivalent to 1 to 10 l/ha) of the commercial herbicide Basta (phosphinotricin; Hoechst), which contains 200 mg/ml PPT as the active ingredient. Seedlings germinated in vitro for 3 days were also challenged with 3 mg/l of the herbicide in basal MS medium.

2.1.1 **Results**

- **Selection and regeneration of transformants**. The methodology used included 50 mg/l Km in the selection medium, resulting in 10% of explants forming calli after 10 days (Table 1). These calli were then transferred to a second selective medium with 1 mg/l PPT. In this medium half the putative transformed calli showed stable expression of the uid A and bar genes, with a transformation efficiency of 5%, based on the total number of explants. Transfer of transformed calli to hormone-free medium with low agar concentration facilitated the development of regenerative structures. After rooting, the number of plants obtained per callus varied from 10-20. Leaves of the transformed plants were capable of callus induction and plant regeneration in the MS medium containing up to 160 mg/l PPT (Fig. 2).

- **Primary transformed plants**. No changes in morphological characters were detected in the primary transformed plants. Under in vitro conditions,

transformed regenerated plants showed resistance to Basta at levels 160 times higher than the MLD dose of nontransformed control plants. Under biosafety conditions in the glasshouse, 0.25 l/ha of Basta was found as the MLD for control plants. Transformed plants resisted up to 10 l/ha of the selection agent, equivalent to 40 times the MLD for nontransformed control plants (Fig. 3a).

- **DNA analysis**. A limited number of primary transformed plants were transferred to pots in the glasshouse under biosafety conditions. Evidence for physical DNA transfer was provided by Southern blot analysis. DNA of transformed plants, digested with Bgl II, and hybridized with 6 Kb-Bgl II fragment of PGV1040, gave the expected monomorphic pattern (Fig. 4) equivalent to the size of the intact T-DNA of PGV1040. Control plants did not hybridize, while plant D_1 exhibited a ca-14 Kb fragment.

- **Progeny of transformed plants**. Primary transformants flowered 8 mo after planting in the glasshouse. Flowers were bagged to ensure autopollination. Four progeny families--each derived from a plant from a different transformation event--were sown in the glasshouse for inheritance studies of the foreign genes. Data obtained from progeny plants (Table 2) after spraying with 1 mg/l Basta (Fig. 3B) in the glasshouse and germinated on PPT-containing *in vitro* culture (Fig. 3B), showed Chi-square values with acceptable probabilities for a 3:1 phenotypic segregation.

  The GUS test was done on leaves from each of the progeny plantlets of the transformed plant of event A_3, prior to the Basta selection. Data obtained showed co-segregation of the bar and npt II genes (Table 2).

  Work is under way to determine the gene copy number in each progeny plant and identify individuals that are homozygous and heterozygous for the introduced genes. Progeny of the transformed plant of event D_1 included dwarf individuals; this character is directly correlated with the presence of the foreign genes, at the same frequency as the homozygous individuals for the introduced genes. Studies are in progress to determine the genetic nature of the dwarfs.

2.1.2 **Discussion**. In this work we have shown the successful transformation and regeneration of transgenic plants of *S. guianensis* using a disarmed *A. tumefaciens* strain. The transgenic nature of the *S. guianensis* plants was also demonstrated through inheritance analysis of the trait into the progeny.

According to our results, two factors have favored the production of transformed *S. guianensis* plants: the use of PPT as selection agent instead of Km and the modification of the regeneration medium originally reported by Szabados and Roca

(1986). The medium composition was changed to induce organogenesis

Table 1. Results of two distinct transformation experiments of *S. guianensis* carried out under the same conditions; no. of selected calli with kanamycin was reduced to half after selection on PPT-containing medium.

Expt.	Treatment	No. of Explants	Km+	GUS+/PPT +	No. of Regenerated Calli
1	Control	10	0	0	0
	EHA101	10	0	0	0
	EHA101(pGV1040)	100	10	5	4
2	Control	10	0	0	0
	EHA101	10	0	0	0
	EHA101(pGV1040)	100	9	6	5

Table 2. Chi-square values (1 DF) for F_1 progenies of four different transformed plants, each belonging to a different transformation event.

Event	No. Progenies	O.R.	E.R.	O.P.P.	Chi Sq.	P(%)
A_3 [(a)]	28	14:4	13,5:4,5	3,5:1	0,2222	50-70
B_2	53	42:11	39,8:13,3	3,81:1	0,6478	30-50
C_2	33	25:8	24,8:8,3	3.13:1	0,0707	70-80
D_1	13	9:4	9,8:3,3	2,3:1	0,3333	50-70

O.R.= Observed Ratio: resistant:susceptible to the herbicide Basta; E.R.= Expected Ratio; O.P.P.= Observed Phenotypic Proportion; Chi Sq.= Chi square values; P(%)= Probability of occurrence of the event.

a) This progeny showed the same inheritance data for uid A gene.

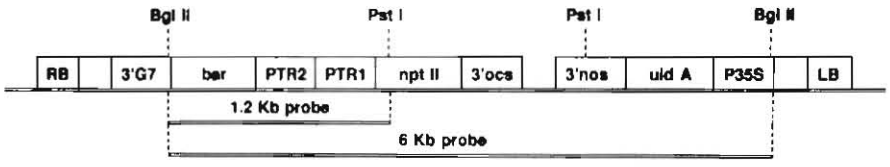

Figure 1. T-DNA region of the plasmid pGV1040[13], Bgl II/Bgl II 6 Kb and the Bgl II/Pst I 1.2 kb fragment used as probes for DNA analysis; RB = right border, LB = left border of T-DNA.

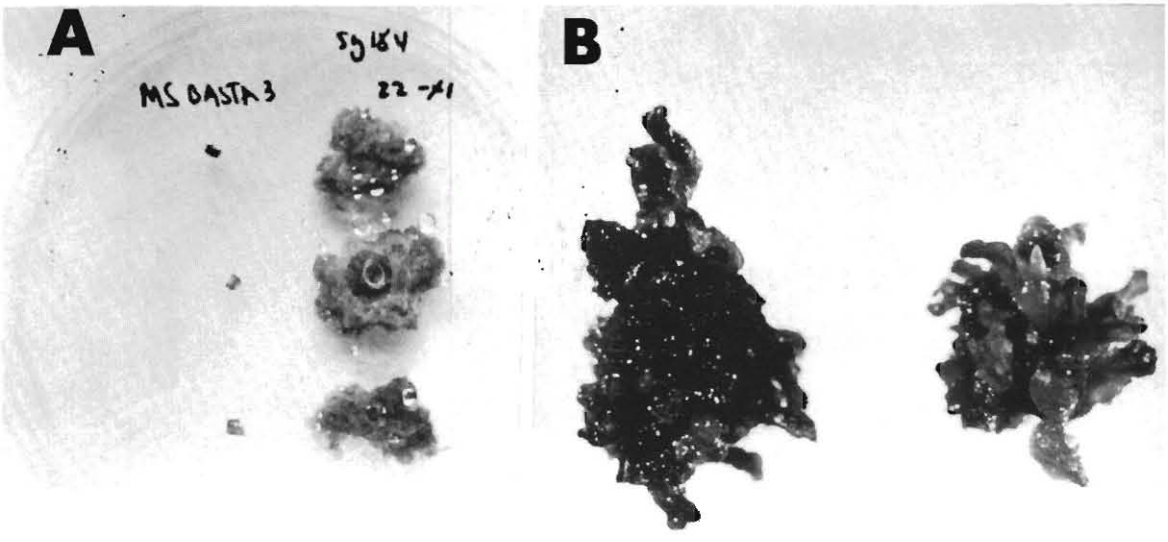

Figure 2. Leaves from primary transformed plants were induced to produce calli and to regenerate under different concentrations of Basta: A. Control (left) and transformed (right) leaves on MS regeneration medium containing 160 mg/l of PPT; B. Regeneration of callus grown on medium containing Basta.

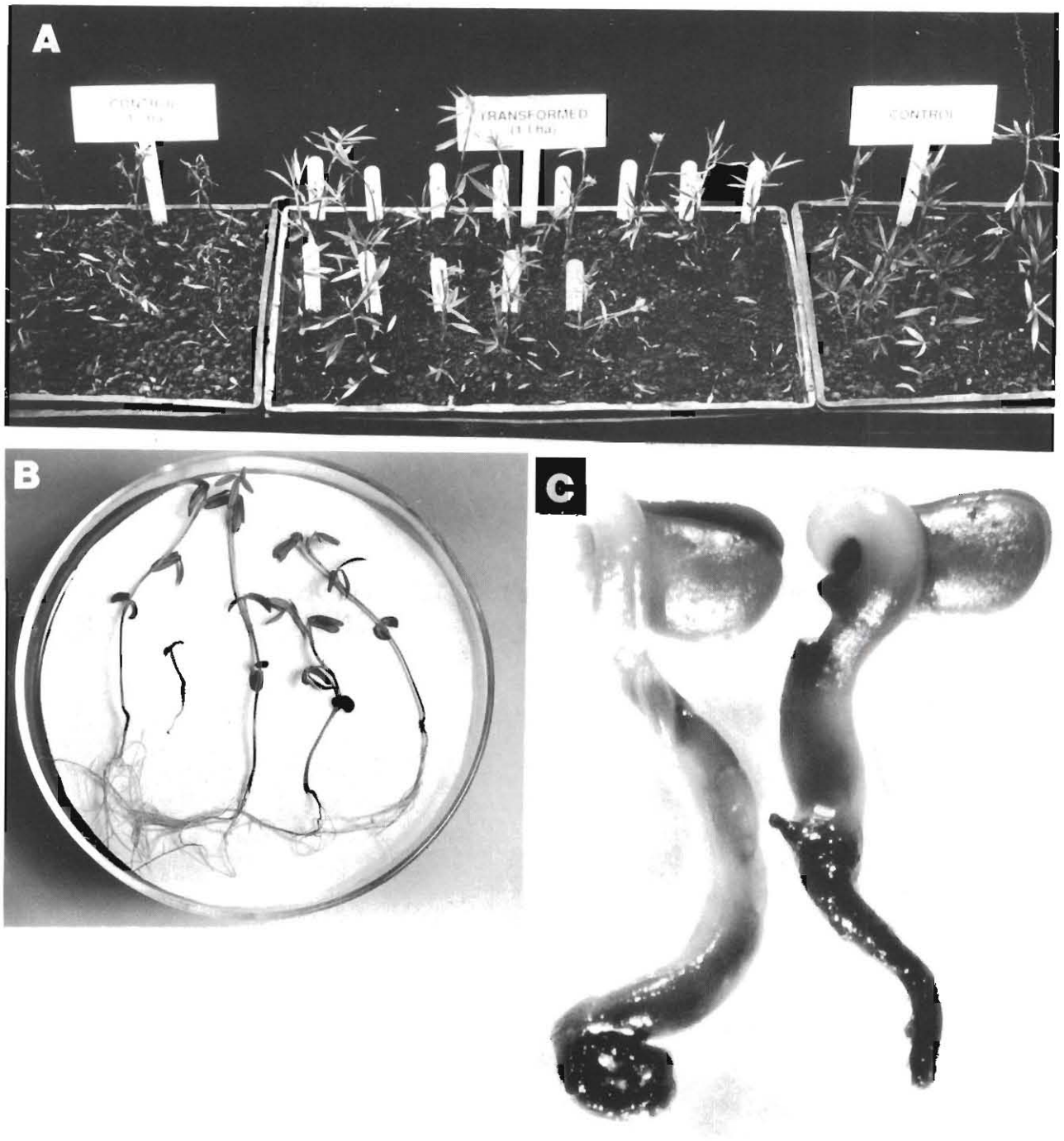

Figure 3. (A) Applications of Basta, the selection agent utilized, under glasshouse conditions: for primary transgenic plants concentrations of 1 l/ha of Basta were used. Left = treated control plants (5 days); center = transformed plants (5 days); right = nontreated control plants. (B) Plants of the progeny of the transgenic plant A_3 after selection in medium containing PPT to determine the inheritance of the trait; second plantlet from left showed susceptibility to basta. This progeny was previously GUS tested, and segregation data of these two characters could be correlated. (C) GUS test of germinated seeds of the primary transformed plants; hypocotyl region exhibit the lowest GUS activity.

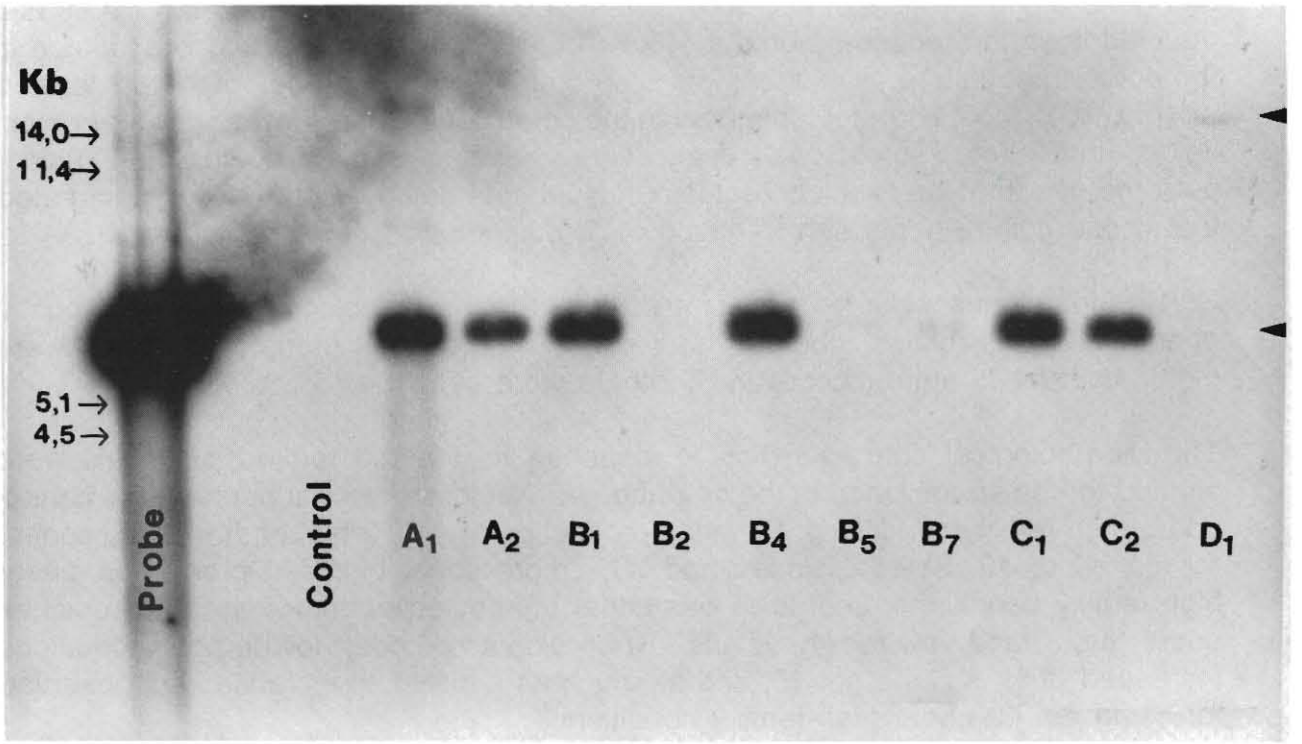

Figure 4. Southern blot of some of the glasshouse transformed plants. DNA was Bgl II-digested for obtaining the intact T-DNA introduced to the *Stylosanthes* plants and hybridized with the Bgl II/Bgl II 6 Kb probe. The monomorphic pattern indicates that T-DNA was integrated into the genome without losses, plant D_1 exhibited a fragment of 9 kb, which is larger than the expected 6 kb. Progeny of this individual also included dwarf individuals. Faint bands could be due to nonhomologous hybridization or to contamination of the plasmid DNA containing the border regions.

Control: Non-transformed plant
A_1-A_2; B_1-B_7; C_1-C_2; D_1: Transformed plants obtained from different transformation events.

2.2 Intra-Accession (Somaclonal) Variation in *Stylosanthes guianensis* as a Research Tool (Collaboration: I. Rao, V. Borrero, TFP)

Early work in the BRU demonstrated that *S. guianensis* cell cultures displayed great variability in chromosome number: from 20 (normal 2x chromosome no. of the species) to 100 (Fig. 1A). A line of cells became habituated to grow without hormones (Fig. 1B); however these cells were unable to differentiate organs in contrast to the non-habituated lines. Over 100 plants were regenerated from leaf-derived callus of a single seedling (CIAT 2243). Field progenies of these plants displayed great variability in morphology, seed yield, fresh and dry weight, leaf area and general vigor (Miles et al. 1989). Twenty per cent of the regenerated plants had doubled their chromosome number (4 x = 40). Chromosome doubling was related to the duration of culture, with linear increase up to 60 days and then gradually leveled off (Fig. 1C). Most changes observed in the somaclones were toward low agronomic value, but some somaclonal lines were selected for their individual higher performance than the check or for their unusual morphology. This work demonstrated that tissue culture-generated variation in *S. guianensis* can be heritable.

Of 14 somaclones selected with characteristic features including dwarf and bushy phenotypes, tetraploids, chlorotic and variable number of leaflets, 3 clones were highly tolerant to anthracnose and 2 others were sensitive.

The morphological characteristics in response to different fertilization levels were studied for the somaclones in the greenhouse. All clones were superior to the control under high-fertility conditions. Biomass production was 60% higher than in the control for clone no. 40. Several clones had 30% more shoot biomass production under high-fertility conditions. Leaf area was either unaffected or improved in most clones under high- and low-fertility levels. Root biomass under low-fertility conditions increased 85% in clone no. 15; and as much as a threefold increase was observed for clone no. 40 under high-fertility conditions.

A dramatic increase in nutrient uptake efficiency NUE was observed in the tetraploid somaclone no. 5, in comparison to the check line under high fertility: the increase was 1.8-fold for N uptake, 3.1-fold for P, and 2.8-fold for K. The tetraploid root biomass was not too large. NUE in chlorotic somaclone no. 36 was markedly decreased in comparison to the check. The high root biomass of clone no. 40 resulted in a drop in NUE. The data obtained (1991 TFP Ann. Report) suggest that: (1) genetic diversity exits in the partitioning of fixed carbon between shoot and roots in *S. guianensis* somaclones; (2) root production in relation to nutrient supply may be genetically controlled; and (3) genetic analysis of clones no. 40 and no. 5 (4x) in comparison to check line may provide clues to understanding the genetic basis of root production and NUE in *S. guianensis*.

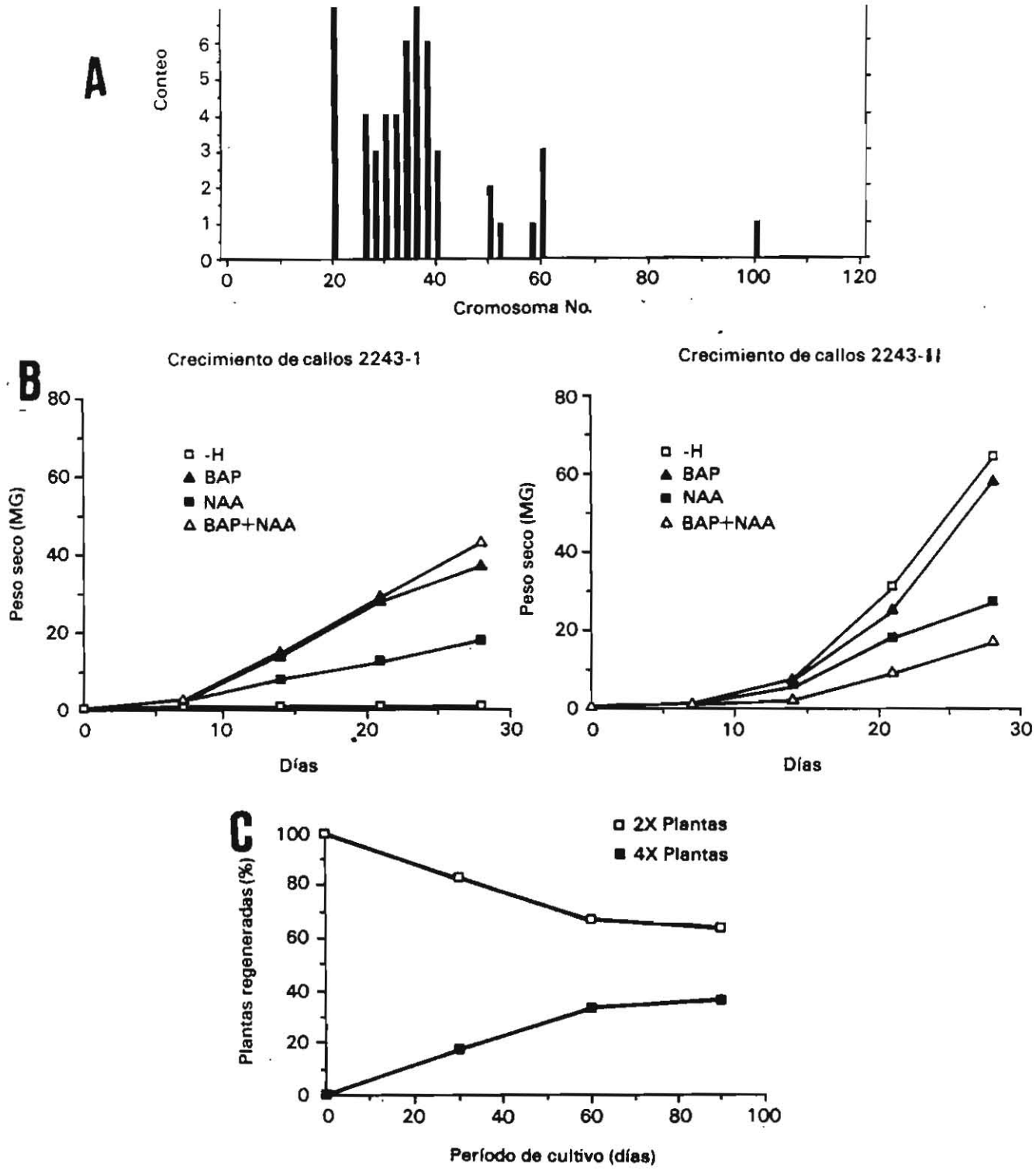

Figure 1. Variability of *S. guianensis*, CIAT 2243, cell cultures and regenerated plants: A. Variation in chromosome number of cell cultures; B. Hormonal habituation of cell line 2243-1 vs. normal cell line 2243-1; C. Chromosome doubling (tetrapolidization) of regenerated plants from normal cell line 2243-1.

2.3 Development of *Agrobacterium tumefaciens* and Particle Bombardment-Mediated Cassava Transformation

The objective of this work is to develop a transformation system for cassava, using two strategies: the *Agrobacterium*-mediated transformation system and the particle delivery system. The plasmid vector used in both cases was pGV1040 (Calderon-Urrea 1988), containing the bar, the npt II and the uid A genes. Promising results have been obtained with both the *A. tumefaciens* and the particle bombardment-mediated systems.

In our *Agrobacterium*-mediated transformation experiments, cv. M Per 183 (a cv. sensitive to *Agrobacterium* infection) was used as a source of cotyledonary leaves from somatic embryos for inoculations with the bacterium. These were used as initial explants due to their high response to embryogenesis and their similarity to immature leaves. In previous experiments we showed the sensitivity of this explant to *Agrobacterium* infection. The primary source of somatic embryos was apical meristems of 1-mo greenhouse-grown plants, cultured in basal MS containing 8 mg/l of 2,4-D, under low light conditions (100 $\mu E.s^{-1}.m^{-2}$) (Szabados et al. 1987).

For particle bombardment, 1-mo-old somatic embryos at the globular stage of the cv. M Col 1505 are being used. These somatic embryos were induced from apical meristems as for M Per 183. The embryogenic clumps used were about 0.5 cm^2 in size.

Experiments with 25 different *Agrobacterium* strains for identifying highly infective strains in cassava were conducted under both greenhouse and in vitro conditions, using five different cassava cvs.

As a result of these experiments, the strain selected for infecting explants was *A. tumefaciens* CIAT 1182, an agropine/mannopine strain, carrying the plasmid pGV1040 in a binary vector system.

In experiments with immature leaves of M Per 183, bacterium strain 1182 was able to induce tumor formation on hormone-free medium; and synthesis of agropine and mannopine was demonstrated by paper electrophoresis (Fig. 1A).

Inoculations were carried out by dropping an overnight culture (OD_{550}: 0.8) of 1182pGV1040 containing 100 μM acetosyringone on each explant, the borders of which were cut to leave a surface exposed to *Agrobacterium* infection.

Co-cultivation was carried out during 24 h at 28°C in darkness. Then explants were rinsed in basal liquid MS containing 500 mg/l Carbenicillin and 250 mg/l Cefotaxime, blot-dried on filter paper and cultured for embryo induction.

Our device for particle bombardment is based on the original system developed by Sanford (Klein et al. 1987). The bombardment cocktail contained tungsten particles, $CaCl_2$, spermidine and the desired DNA (pGV1040): 5μl pGV1040 (2μg/μl), 10 μl spermidine (0.1M), 20 μl tungsten (100 mg/ml) and 25 μl Ca_2Cl_2. Tissues were bombarded twice at a distance of 22 cm from the specimen and with 680 mm Hg of vacuum. A mesh was used to disperse the particles, thereby avoiding tissue damage.

Selective conditions for PPT were determined in previous experiments. It was found that LD_{50} for embryos is 16 mg/l (10-15 days) while for green tissues it is 1 mg/l. For our *Agrobacterium* experiments, we used 1 mg/l PPT before embryo formation and then 16 mg/l for the proliferation stage.

In this step, explants were cultured on basal MS containing 8 mg/l 2,4-D, 1 mg/l phosphinotricin (PPT), 500 mg/l Carbenicillin and 250 mg/l Cefotaxime, under low-light conditions (100 $\mu E.S^{-1}.m^{-2}$). After 4-5 days, tissues were transferred to the same medium containing 16 mg/l PPT, under the same light conditions. Once embryo induction occurred, tissues were maintained in the same medium, in the dark, for proliferation.

Torpedo-stage embryos from the *Agrobacterium* experiments were transferred for germination to basal MS medium, without hormones, containing 500 mg/l carbenicillin, 250 mg/l cefotaxime and 16 mg/l PPT at high-light conditions (300 $\mu E. S^{-1}.m^{-2}$). Germinated embryos were transferred to basal MS medium containing 0.5 mg/l BAP, without PPT or antibiotics.

Three months after bombardment, the embryos were induced to germinate, under the same light conditions, on basal MS with 16 mg/l PPT, basal MS containing 0.5 mg/l BAP, without antibiotics or PPT. For elongation the embryos were transferred to the same medium containing 1 mg/l PPT and 0.5 mg/l BAP.

2.3.1 **Results and discussion**

- ***Agrobacterium* experiments**. A low frequency of embryo induction was obtained in the tissues infected with the bacterium strain CIAT 1182 carrying pGV1040. Seven explants produced embryos from a total of 120 infected explants, 20 days after selection. Embryo formation started 7-10 days in inoculated explants under selective conditions and 3-5 days in control, nontreated explants under nonselective conditions. In general, proliferation of infected explants was slower than in the control tissues, possibly because of the selection pressure produced by PPT. Kanamycin was not used for selecting putative transformed embryos due to the inconsistency of results.

  Three embryos have now been germinated and transferred to the BAP-containing media for elongation (Fig. 1B). PPT has not been included at these stages.

Regenerated plantlets will be tested for GUS expression using the Kosugi protocol and will also be DNA analyzed.

This is a progress report of a novel strategy to generate transformed cassava plants. Because infection of somatic embryos with *Agrobacterium* has always been difficult, we are using the cotyledonary leaves of young somatic embryos as explants for inoculation with *Agrobacterium*. These explants are highly embryogenic; and, as demonstrated, can be infected using the CIAT 1182 *Agrobacterium* strain.

- **Bombardment experiments**. GUS expression was monitored in bombarded tissues at 1, 30 and 60 days after bombardment, using the Kosugi protocol for GUS detection. This test diminished the GUS endogenous expression about 10 times as compared to the Jefferson protocol (Table 2).

  GUS-expressing foci 30 and 60 days after bombardment were larger than those observed after one day. The percent of GUS-expressing regions in the tissues at different times are presented in Table 2.

  The results are quite promising. Expression of the introduced gene (GUS) was detected at 60 and even 90 days after bombardment (Fig. 1C). The GUS expression observed after this time is likely to be stable. Further assessment of transformation will use PPT selection.

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Table 1. Percent endogenous GUS activity in different cassava tissues, using the Kosugi (1990) and Jefferson (1987) protocols. Only embryogenic tissues exhibited endogenous activity.

Tissues	Jefferson	Kosugi
Mature/immature leaves		
Stems	0.0	0.0
Roots		
Somatic embryos	30	3

Table 2. GUS expression detected in cassava somatic embryos after 1, 30, 60 and 90 days after bombardment.

Days	Embryogenic Groups Tested	GUS Spots/ Embryogenic Group	% Remaining GUS Spots in Relation to 1-Day Evaluation	% Embryogenic Groups with GUS Expression
1	140	15.6	-	97.9
30	203	1.6	10.8	45.8
60	263	1.0	6.5	36.8
90	94	0.2	1.0	10.6

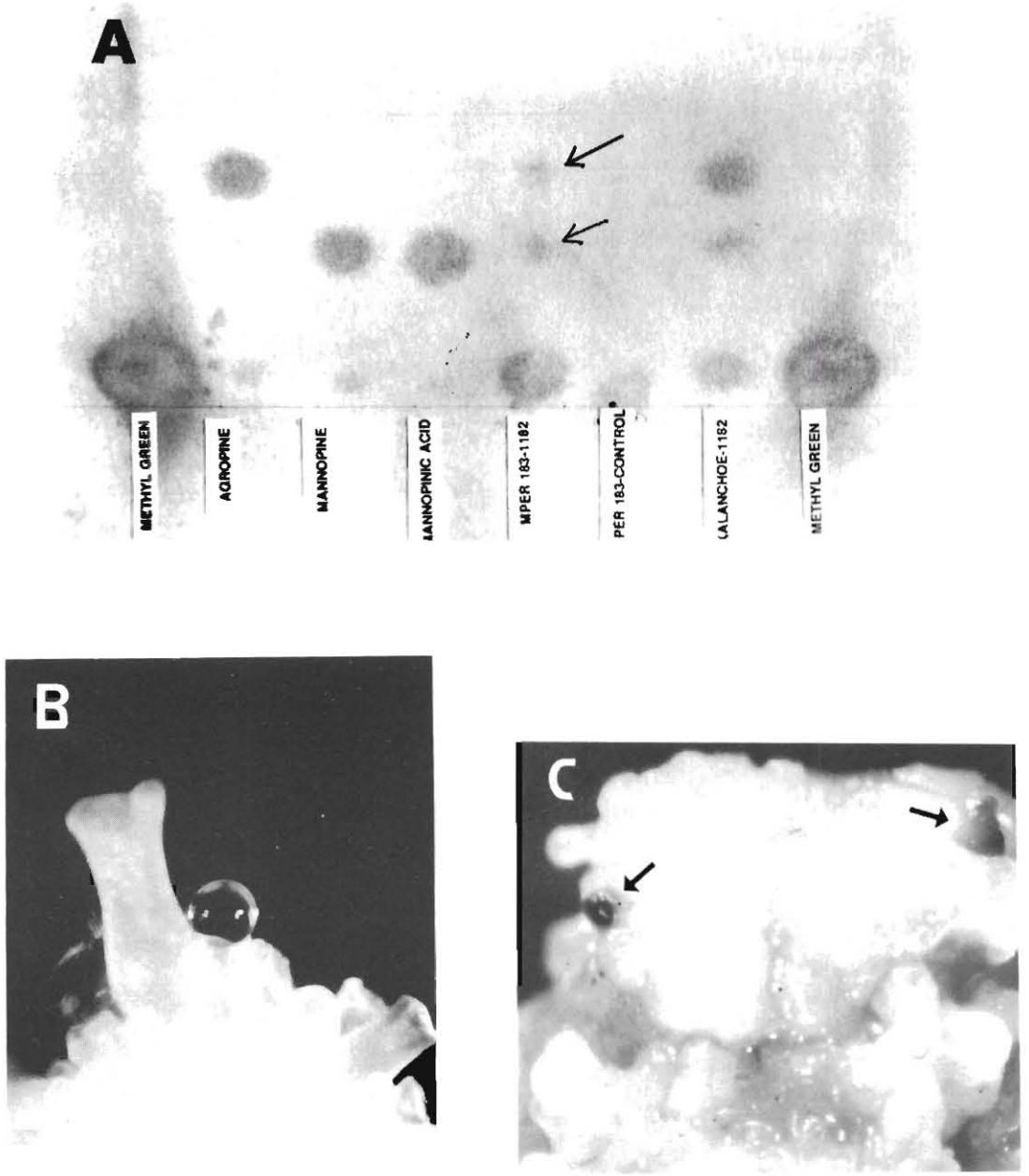

Figure 1. Results of transformation of cassava mediated by *A. tumefaciens* and particle bombardment: (A) Silver-staining paper electrophoresis showing the presence of opines after infection with CIAT 1182 *A. tumefaciens* strain; *Kalanchoe* sp. was used as a positive control. (B) Elongating cassava somatic embryo from selection medium containing 16 mg/l Basta. (C) Expression of GUS activity in cassava somatic embryos 60 days after bombardment.

2.4 Isolation and *In Vitro* Culture of Cassava Immature Pollen and Zygotic Embryos
(Collaboration: C. Iglesias, Cassava Program Genetics Section)

Efficient systems for the isolation and in vitro culture of cassava pollen would be a way to avoid the possible detrimental effect of anther wall tissue on microspore development observed in previous cassava anther culture research. In vitro germination of cassava zygotic embryos will be useful in designing protocols for germinating and growing interspecific hybrid embryos as well as for the former to contribute to the construction of a cassava molecular map. The methodology is as follows:

- **Isolation and in vitro culture of pollen**. Mature and immature male inflorescences from four cassava var. (HMCI, CM91-3, CM523-7 and CM507-37) were used. Flower buds 0.8 to 2.5 mm. in length, corresponding to tetrad to late uninucleate microspore stages (Fig. 1) were gently macerated in 5% sucrose solution sterilized by filtration. According to pollen size (40-100 μm), the slurry was passed through 2 filters of 750 and 150 μm to eliminate somatic tissue. The slurry suspension was collected in a centrifuge tube. The filtrate was allowed to sediment and the supernatant discarded, followed by 3 washes (re-suspensions) with sucrose solution and finally with culture medium. The contents of 50 anthers in a vol of 5 ml sucrose solution resulted in a density of 10,000 microspores ml^{-1} (as determined by hemocytometer count), cultured in 15-mm petri dishes, in a hanging drop system, at 26°C, dark and high humidity conditions (Fig. 2).

- **Zygotic embryo isolation, culture and growth.** Immature seeds obtained from 3 var. (M Col 122, M Cub 18 and M Cub 62) were collected in the field at different stages of fruit development. Under aseptic conditions the seeds were split along the raphe, with the aid of forceps and scalpels to remove embryogenic axes. Immature embryo axes (with their cotyledons separated) were cultured between 25 to 45 days after pollination (torpedo to cotyledonary stage).

  We have developed a very efficient system for the rapid isolation and in vitro culture of large quantities of mature and immature cassava pollen in a very short time, starting with male flower buds. About 50 flower buds processed with 5 ml of sucrose solution will permit the manipulation of thousands of pollen grains at a time. Isolation and in vitro culture of immature cassava pollen has allowed us to obtain cell proliferation from microspores cultured at the tetrad stage. Induced microcallus was obtained by direct pretreatment of the isolated microspores with high osmoticum (Fig. 3).

  We have also developed a technique for growing cassava immature zygotic embryos in sterile, *in vitro*, conditions. The technique has been used to recover plants from inter-specific crosses used in the molecular mapping project, as well as for difficult-to-germinate *M. esculenta* and wild *Manihot* spp. (Fig. 4).

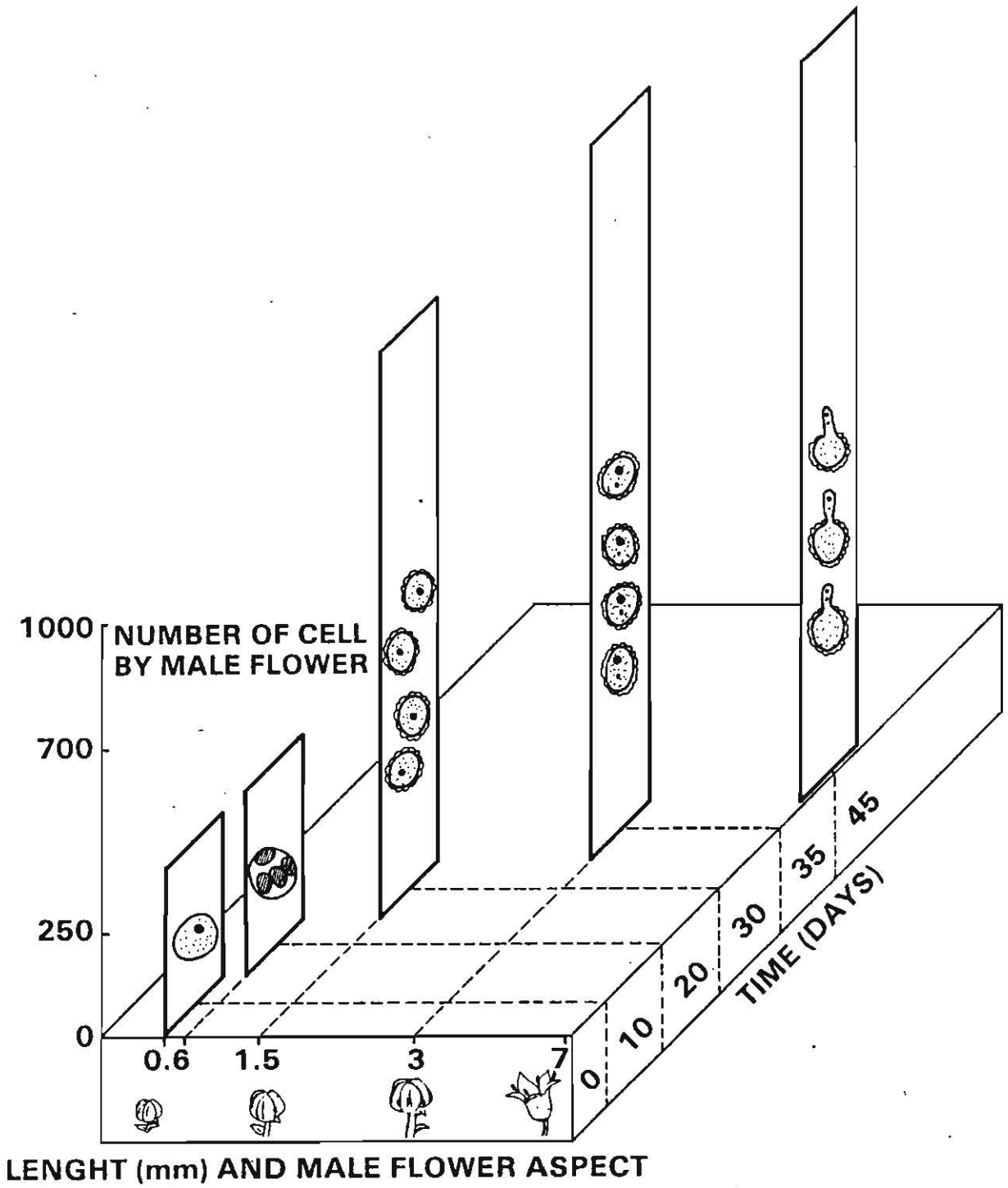

Figure 1. Developmental stages of cassava pollen grains (microsporogenesis): relationships of microspore-pollen stage to the morphology of flowers.

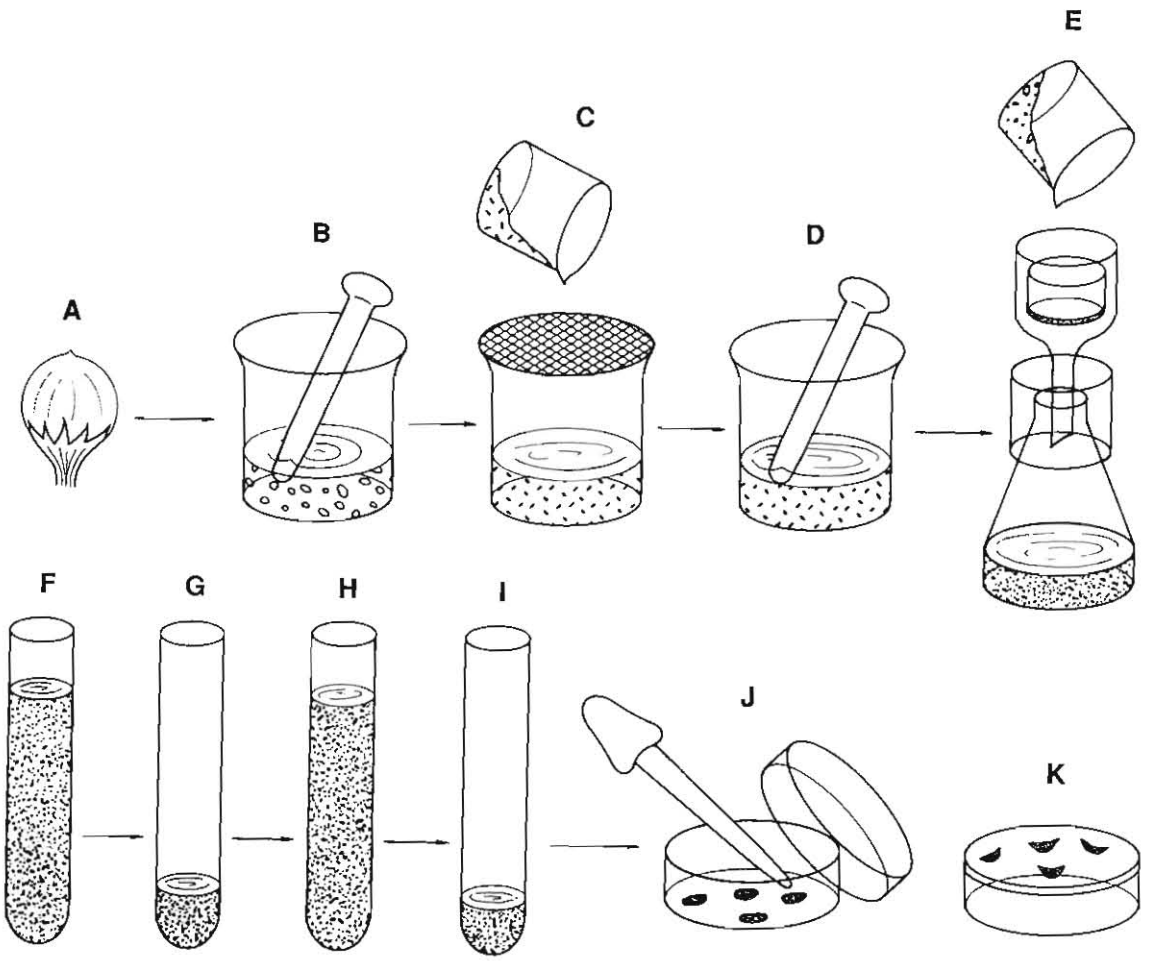

Figure 2. Steps A-K followed to isolate large numbers of cassava microspores at the tetrad stage.

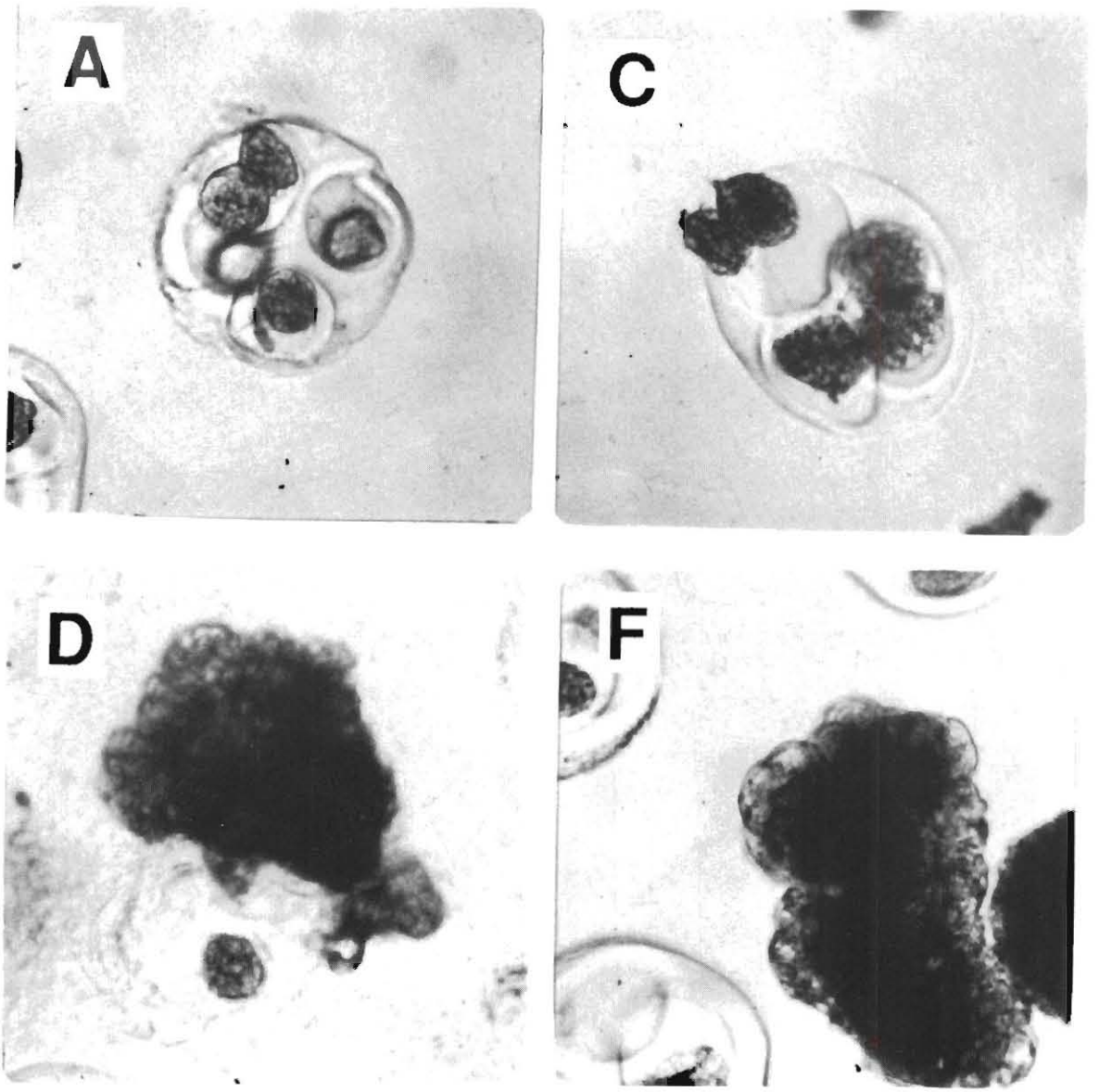

Figure 3. Induction of mitosis and cell proliferation from isolated cassava microspores at the tetrad stage: A. isolated tetrad-stage microspore; C. first mitosis of tetrad cells; D. and F. micro-calli grown from microspores.

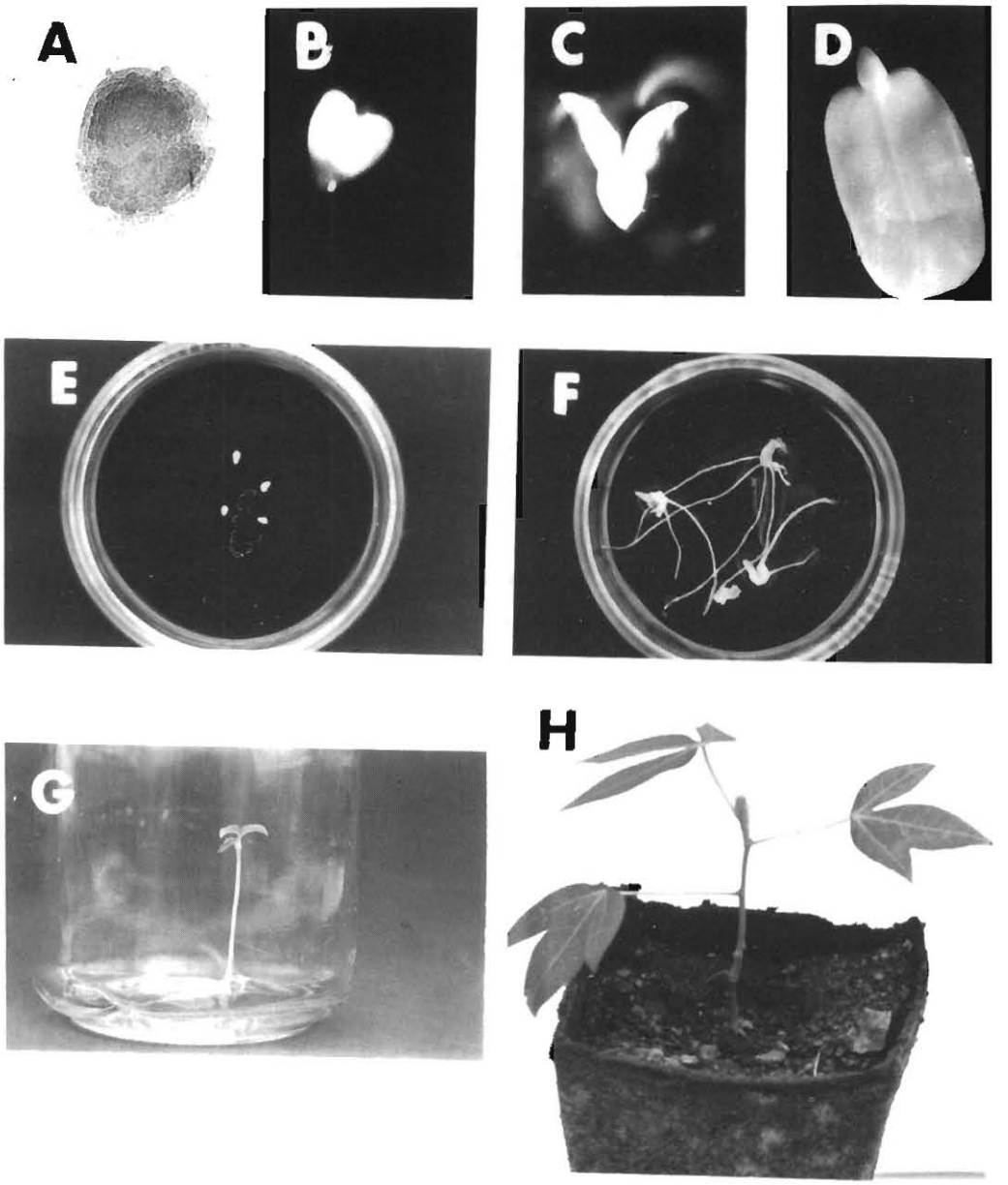

Figure 4. Isolation, culture and growth of cassava immature zygotic embryos: (A & B) heart-shaped embryos isolated from fertilized ovaries; (C & D) torpedo and cotyledonary-shaped embryos; (F-G) growth of embryos in vitro; (H) seedling transplanted into soil from a zygotic embryo.

2.5 Progress Toward the Genetic Transformation of *Phaseolus* Beans

2.5.1 ***Agrobacterium*-mediated transformation systems**. Plant regeneration from tissue culture is still the main bottleneck for *Agrobacterium*-mediated transformation of the common bean. Earlier work in the BRU succeeded in inducing somatic embryo differentiation and plantlet growth from embryogenic axes of five wild *Phaseolus* species: *P. neglectus, P. scrabellus, P. xanthotrichus, P. pedicelatus* and *P. glaucocarpus* (Fig. 1). More recently, organogenesis and bud differentiation were obtained as part of a PhD thesis (A. Mejía) in *P. acutifolius*, and induction of organogenic calli was observed in *P. coccineus, P. polyanthus, P. lunatus, P. filiformis* and *P. xanthotrichus*, but not in a range of *P. vulgaris* genotypes tested nor in 10 wild relatives from Mexico and Argentina. Recently, the segregation of two types of callus from leaf tissue was identified in our work on *P. vulgaris x P. acutifolius* third congruity backcross-F_1 and fourth congruity backcross-F_1 hybrid plants. One type of callus resembled the typical *P. acutifolius* organogenic callus; the other resembled the non-organogenic *P. vulgaris* callus.

Work toward regeneration will continue using the interspecific germplasm now available at CIAT. Transformation of the best responding wild species will be investigated as well.

Research on genetic transformation of the common bean using the *Agrobacterium* system was initiated in the BRU as part of a PhD thesis (M. Korban). A first step in this study--the relative susceptibility of bean tissues to infection by strains of *Agrobacterium*--was determined. Screening of wild *Agrobacterium* strains (including *A. tumefaciens* and *A. rhizogenes*) was carried out on four greenhouse-grown bean varieties (ICA Pijao, Nuña Pava, ICA Viboral and Calima) as well as on in vitro-derived seedlings. Virulence was scored as the ability to form opine-producing tumors on the inoculation site for plants, and growth on hormone-free medium for explants.

After selecting *A. tumefaciens* C58 as the most virulent strain on beans and ICA Pijao as a very susceptible variety, we concentrated on transformation. A regeneration protocol from bean cotyledonary nodes was adopted to carry out transformation experiments (McClean and Grafton 1989). Multiple (adventitious) shoot formation at a meristematic ring structure around the cotyledonary node explant was achieved and is reproducible.

The virulent *Agrobacterium* strain C58 was transformed with a binary vector containing marker genes of interest. The pGV1040 plasmid, which has two selectable marker genes--the npt II gene and the bar gene, which confers resistance to Basta--was used. This plasmid also carries a scorable marker gene--the uid A or GUS A gene, which codes for ß-glucuronidase, detectable in transformed tissues using histological assays.

Agrobacterium was transformed with pGV1040 directly by rendering *Agrobacterium* cells competent through a $CaCl_2$ treatment combined with freezing and thawing the bacteria. Transformed bacteria were selected on Sm/Sp-containing plates.

Basta-sensitivity tests were carried out on the shoots regenerated from bean cotyledonary nodes of the var. ICA Pijao to determine the minimal lethal concentration. The lethal dose was established at 1 mg/l Basta in the medium. At 0.8 mg/l growing shoots were still observed 7-10 days after treatment.

Transformation experiments on half cotyledonary nodes of the common bean variety ICA Pijao were carried out by testing two inoculation methods: dipping or pricking. The effect of acetosyringone (AS) was tested by applying it to the bacteria either before or at the time of infection at concentrations ranging from 25 to 200 μM.

The best antibiotic treatment for eliminating bacteria was achieved by combining cefotaxim (a cephalosporin) at 800 μg/ml and carbenicillin (a penicillin) at 500 μg/ml. In addition, the regenerative capability of the explants was not hindered. Control shoots showed no GUS expression.

Bean shoot regeneration is decreased when inoculated with C58 (pGV1040); many explants necrotized during the procedure. Results of five separate experiments are presented: (a) 14 buds out of 161, stemming from 28 explants; (b) 6 buds out of 37; (c) 4 out of 15; (d) 2 out of 27, containing 200 μM AS; and (e) 5 out of 24, containing 25 μM AS.

These preliminary transformation experiments have permitted the selection of pricking as an inoculation method in transformation experiments. Expression was detected in buds, thereby indicating that bean cotyledonary nodes are amenable to transformation and subsequent regeneration.

Future work will include a study on the early events of *Agrobacterium*-mediated transformation by determining the spatial arrangement of regenerable cells of bean cotyledonary nodes amenable to transformation using an improved GUS gene construct, the GUS-intron construct. The use of an intron-containing gene completely eliminates background expression by the bacteria, which could hamper evaluation of GUS expression.

References

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2.5.2 **Particle bombardment-mediated transformation systems: Microtargeting of bean apical meristems**. *Phaseolus vulgaris* is a species recalcitrant to plant regeneration; transformation of beans through biolistics with the particle gun was first reported by Russell (Agracetus) in 1991 using the US var. "Seafearer." However, it apparently has not been reproducible with other bean varieties. A novel method for microtargeting microprojectiles to target areas in the micrometer range was described by Sautter et al. (1991) and Potrykus (1992) (Potrykus' Group ETH, Zurich).

We have explored the microtargeting device (collaboration with Z. Lentini) in cooperation with the ETH group in order to evaluate the comparative advantage(s) of this system over the particle gun; to determine if this system is feasible for transforming beans by bombarding the apical meristem; and to initiate a collaborative project toward bean transformation by microtargeting where CIAT plays an active role.

Material and Methods

- **Seed sterilization**: 1 min in 70% ethanol, rinsed (critical), 8 min in 1.5% calcium hypochlorite + tween, rinsed thoroughly. Note: Do not use higher concentration of bleach or considerable seed damage will occur.

- **Seed imbibition**: Immerse seeds in sterile dH2O for 1-2 h, 25-28°C, dark. Add just slightly more water than needed to cover the seeds and leave sufficient air above.

- **Embryo dissection and incubation**: Only use unwrinkled, healthy-looking seeds. Do not let seeds dry out; otherwise, it is hard to open and oftentimes, the apical region will be damaged. After dissecting the seed, discard those showing a brownish-yellowish film on the embryo. Remove one cotyledon, leaving the embryo attached to the other (easier to work). Then remove the primary leaves with care not to damage the apical area. The easiest way is to place the tip of the blade at the inner edge of the petiole base and make a quick movement toward the outside. Cut the radicle below the cotyledonary area, leaving a 1- to 2-mm-long segment, and remove the embryo from the remaining cotyledon by softly moving the embryo sideways with the aid of the blade. Make sure the apical area was not damaged by the dissection. Rinse embryo briefly in sterile dH2O, eliminate excess water by tapping on paper towel, and culture on medium (Fig. 2A). Incubate in the dark overnight at 25°C. The meristem will be covered by leaf primordia if incubation is more than 1 day. Use the same incubation conditions after shooting.

- **Media**: For embryos before and after shooting: MS salts + B5 vitamins + 200 mg/l casein hybrodyzate enzymatic + 200 mg/l glutamine + 6% sucrose + 0.6% agarose; for mounting embryos for shooting: the same medium but with 3% sea plaque agarose.

Results and discussion

Experiments were conducted to optimize the targeting of bean meristems (Fig. 2B). Meristems were fixed and cleared after bombarded, and the distribution of the particles through L1, L2 and L3 cell layers was quantified under interference contrast using an inverted microscope (Figs. 2C and 2D). Evaluations on the effect of gold particle size, particle concentration and shooting pressure to target bean meristem (Fig. 2B) indicated that the maximum number of cells showing particles is obtained when using 130 shooting pressure, and 1.3 μm gold particles at a concentration of 0.5 X 10 6 particles/μl (Tables 1 and 2). The shooting pressure (Table 1) seemed to have a more important effect on particle distribution through the various cell layers than the particle size (Table 2). Although 1.5 μm particles increased the number of targeted cells, this larger size of particles seemed to cause more cell damage as well. These results are comparable to those obtain on the first attempts with wheat. At present, it is possible to target up to 10-12 L2 cells/wheat meristem (C. Sautter, ETH, personal communication).

The GUS transient expression was evaluated using pGV1040 under the control of the 35S promoter, and a similar contruct but with the prom A promoter isolated from a bean lypoxigenase gene. Total of 12-14% of targeted meristems showed GUS expression 1-2 days after bombardment (Table 3). Similar results were obtained when embryos were mounted on medium containing either 6% or 10% sucrose. A reduced number of meristems showed GUS expression when prom A promoter was used (Table 3). These preliminary experiments indicate that is possible to target bean meristems following protocols already established for wheat, and the targeted meristems elongate and develop into normal looking seedlings (Fig. 2E). Further experiments are needed to optimize the system. Microtargeting could be a promising alternative for transformation, which circumvents the problem of non-regeneration of beans.

References

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Sautter C. et al. 1991. Biotechnology 9: 1080-1085.

Potrykus I. 1992. Nature 355: 568-569.

Table 1. Effect of shooting pressure on particle distribution in ICA Pijao meristematic cell layers using 1.3 μm particles and 0.5 X 10^6 particles/μl.[a]

Pressure (bars)	No. of Cells per meristem with at Least One Particle[b]								
	L1			L2			L3		
	Mean	S.E.	Range	Mean	S.E.	Range	Mean	S.E.	Range
90	3.8	1.4	5-10	0.3	0.2	0-2	0	--	--
110	6.6	1.1	1-10	1.5	0.6	0.5	0	--	--
130	6.1	1.3	1-12	2.1	0.9	1-8	0	--	--

a) All meristems were targeted showing particles on surface.

b) Average of at least 50 meristems/variable. Evaluations were done under inverted microscope with interference contrast at 40X, tissue previously cleared.

Table 2. Effect of particle size on particle distribution in meristematic cell layers using 110 bars and 0.5 X 10^6 particles/μl[a].

Size (um)	No. of Cells with at Least One Particle[b]								
	L1			L2			L3		
	Mean	S.E.	Range	Mean	S.E.	Range	Mean	S.E.	Range
1.1	6.5	2.8	0-14	0	--	--	0	--	--
1.3	8.0	2.0	0-16	1.8	0.9	0-7	0	--	--
1.5	9.5	2.1	4-17	1.2	0.5	0-4	0	--	--
1.7[c]	--	--	--	--	--	--	--	--	--

a) All meristems were targeted showing particles on surface.

b) Average of at least 50 meristems/variable. Evaluations were done under inverted microscope with interference contrast at 40X, tissue previously cleared.

c) Problems with appearance of yellowish film on meristem, which restricted particle penetration; the same happened in experiment on particle concentrations.

Table 3. GUS transient expression on bean apical meristems. [a]

Promoter	Sucrose (%)	Days after Shooting	Meristems with GUS Expression/total meristem bombarded	GUS Expression[b]
35S	6	1	7/50	14% weak, on leaf primordia
		2	12/100	12% " "
		4	0/50	0%
	10	2	15/150	10% weak, on leaf primordia
prom A[c]	6	2	3/50	6% weak, on leaf primordia

[a] 1.3 um particle, 0.5 X 10^6 particle/ul, 130 bars shooting pressure.
[b] According to Kosugi et al. 1990.
[c] Promoter isolated from lypoxigensase gene of bean.

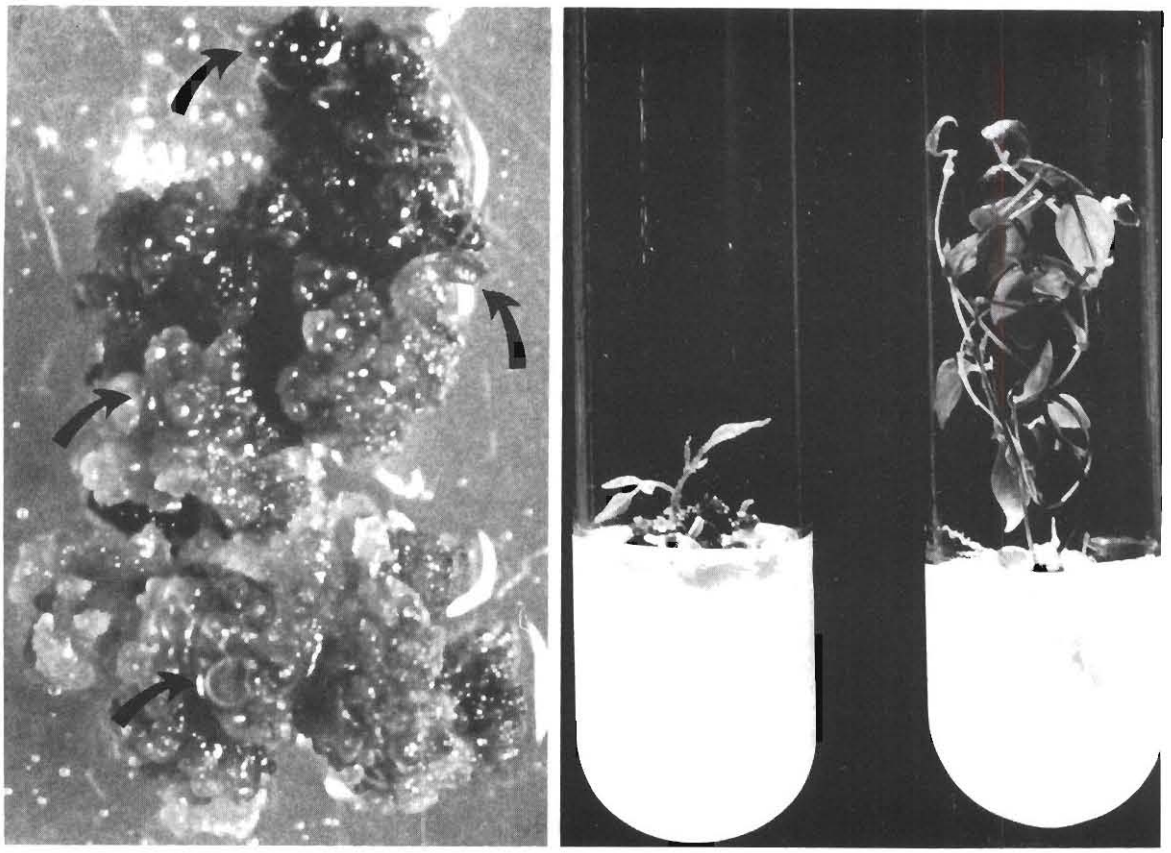

Figure 1. Plant regeneration of wild *Phaseolus* species: *P. scrabellus, P. neglectus, P. xantotriclus, P. pedicelatus* and *P. glaucocarpus*. Left: nodular callus from embryogenic axis showing shoot primordia differentiation (arrows); Rigth: shoot development and rooting of individual shoots prior to potting.

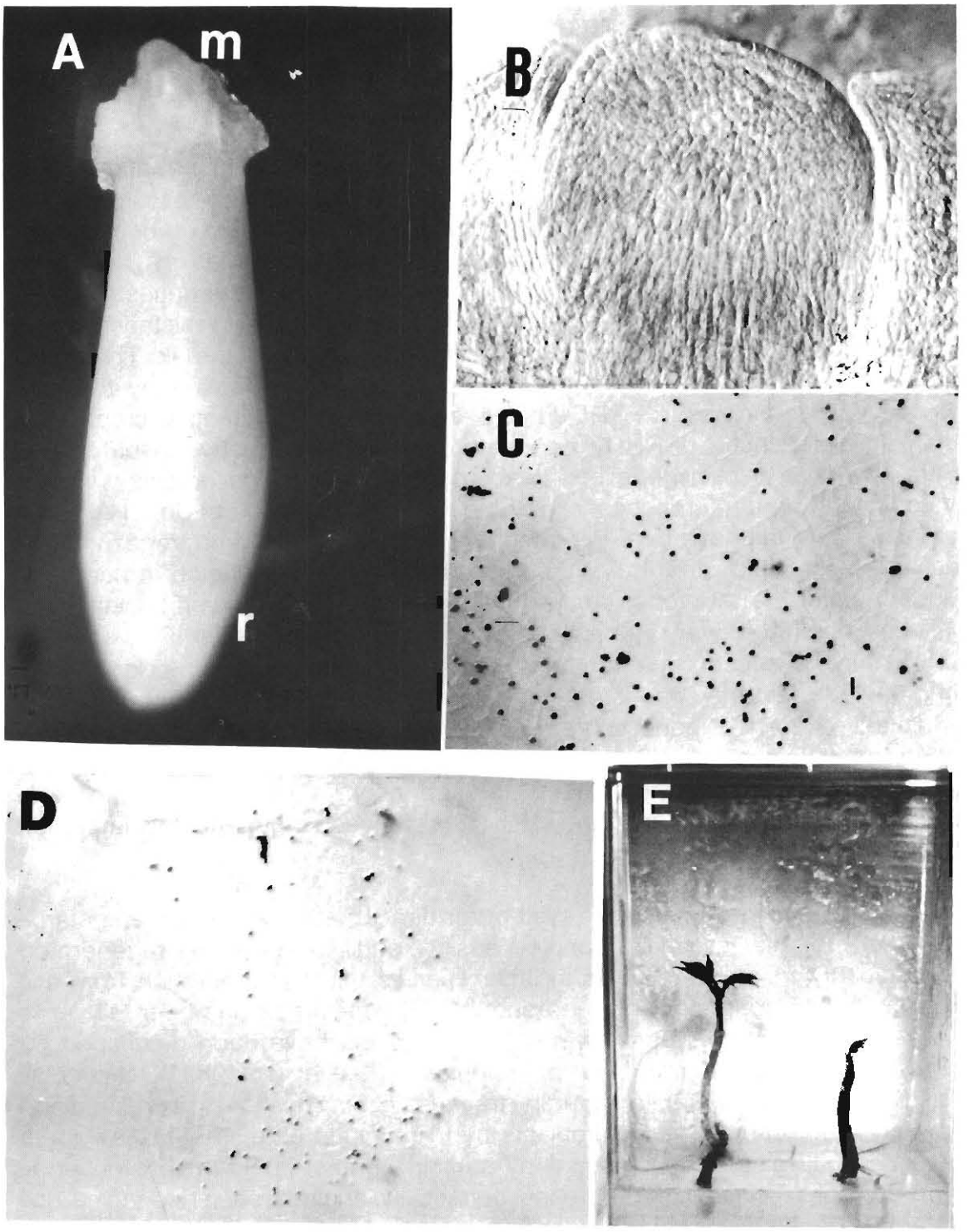

Figure 2. Microtargeting of *Phaseolus vulgaris* (Ica Pijao) mersitems using the device developed by Sautter et al. (1991) at ETH, Zurich. (A) Bean zygotic embryo without cotyledons, m = meristem, r = radicle. (B) Interference constrant view of apical meristem under inverted microscope, 40X. (C) Distribution of gold particles 1.3 um on meristem surface and (D) on L1 meristem-cell layer. (E) Elongated shoots 2 weeks after bombardment.

2.6 Establishment of *In Vitro* Culture Systems in Rice Suitable for Genetic Transformation

In Latin America there are two diseases in which genetic transformation could facilitate the development of germplasm adapted to the diverse rice ecosystems. The rice hoja blanca virus (RHBV) causes 100% yield losses. This disease and its vector are restricted to Latin America and the Caribbean. Recent studies at CIAT have indicated that the same gene is controlling the resistance in most varieties released in the region. Therefore there is an urgent need to incorporate new resistance genes in breeding populations to minimize the possibility of a disease outbreak. The RHBV coat protein gene could be used for this purpose. Cross protection by the coat protein gene to RNA viruses like RHBV has been demonstrated in other crops. The molecular characterization of RHBV has been done by the Virology Research Unit (VRU) and the coat protein gene has been recently cloned. *Rhizoctonia solani* is already causing important crop losses in Southern Cone countries of South America and increasing spreads have been reported in Colombia, Mexico and Venezuela. All varieties are susceptible, and there is no known source of resistance in rice. IRRI has tried to transfer the resistance from wild species, but it is lost when backcrossed into cultivated rice. Moreover, attempts at using biological control by IRRI were not successful either. At present the control depends on heavy use of fungicides. CIAT recently obtained the barley ribosome inactivating protein (RIP) gene from the Max-Planck Institute. The RIP gene confers higher levels of resistance to *Rhizoctonia solani* than the chitinase gene (Logemann et al. 1992). The direct incorporation of the RIP gene into CIAT germplasm by transformation would facilitate the rapid transfer, through conventional breeding, of this resistance to other varieties important in Latin America.

Protoplast culture is a highly efficient system for direct gene transfer because large populations of protoplasts can be handled easily, and plants can be regenerated from the selected individual cells. As in other species, plant regeneration from rice protoplasts is highly dependent on the genotype. *Japonica* rice has been easier to regenerate than the *indica* type; however, two protocols have been developed for efficient plant regeneration from *indica* protoplasts (Lee et al. 1989, Datta et al. 1990a and Datta et al. 1992), and fertile transgenic *indica*s have been obtained through direct gene transfer into protoplasts by PEG (Datta et al. 1990b). Although transgenic rice has also been obtained by particle bombardment (Christou et al. 1991), the current efficiency of generating genetically engineered fertile *indica* rice from protoplasts is higher (Datta et al. 1990b).

Most varieties grown in Latin America are of the *indica* type. The objective of this collaborative project between the BRU and the Rice Program is to optimize a methodology for plant regeneration from *indica*s to be used for the genetic transformation of important varieties in the region. Because the in vitro response depends on the genotype, we are initiating a work--in addition to protoplast culture--

directed at optimizizing systems amenable to particle bombardment as well, such as regeneration from immature embryos and immature panicles. This report refers to progress made on regeneration from protoplasts of BR-IRGA 409, one of the most widely grown *indica* varieties in Brazil.

2.6.1 **Materials and Methods**. Cell suspension cultures of 5 Latin American var. [BR-IRGA 409, CICA 8, Oryzica 1, Oryzica Llanos 4 and Oryzica Llanos 5 (*indica*)] and the breeding line CT 6241-15-1-7-1 (*japonica*, control) were initiated from mature seeds and maintained according to Lee et al. (1989). Cell suspensions lines of IR 54-2 (*indica*, a known responsive genotype to protoplast culture) and IR 52 (*japonica*, a line reported to be used as feeder layer) were supplied by T.K. Hodges, Purdue U. (USA). Protoplast isolation and culture were conducted using the protocols by Lee et al. (1989) and Datta et al. (1990a). Cell and protoplast viability was determined by fluorescent staining with FDA (450-490 nm). Undigested cells were detected in the protoplast preparations with the fluorochrome Calcofluor White (330-380 nm), a specific stain for cell walls. Several factors were evaluated for inducing differentiation from the protoplast-derived calli. Modifications of N6, MS and LS basal medium compositions (macronutrients, micronutrients, vitamins) and the effect of NO_3^-:NH_4^+ on the development of embryogenic calli were tested. Plant regeneration was evaluated with different hormone combinations (AIA, NAA, 2,4-D, kinetin, zeatin) and concentrations (from 0.011 to 1 mg/l for auxins and from 0.5 to 5 mg/l for cytokinins). The osmotic level and type (sucrose, glucose, manitol and sorbitol at 1, 2, 3 and 8%), light intensities (dark, dim, direct) and callus age (1, 2, 3 mo) were also examined to optimize plant formation.

2.6.2 **Results and discussion**. Calli were formed from mature seeds of all the materials tested. Fine and rapidly growing cell lines were developed from BR-IRGA 409 and CT 6241-15-1-7-1. The other varieties responded poorly to the cell suspension induction treatments and the cell type was not embryogenic, which is unsuitable for protoplast culture (Table 1).

Initial attempts at protoplast isolation and culture were made using cell line IR 54-2. The protocols reported by Datta et al. (1990) and Lee et al. (1989) were tested. The latter resulted in greater efficiency of protoplast isolation and protoplast viability (Table 2). Further attempts were made using BR-IRGA 409.

Modifications of Lee (1989) protocol were implemented, resulting in greater protoplast culture efficiency. Cell suspensions composed of small cell aggregates (ca. 50 cells each) (Fig. 1A) were critical for enhancing the release of viable protoplasts (from 10^4 to 10^8 protoplasts/g fresh weight/ml with > 80% viability) (Table 2 and Fig. 1B). Fine cell suspensions were maintained by sifting the cultures through a 500 μm mesh before subculturing. An increase from 4 to 5 h incubation with the enzyme solution reduced the number of cells with undigested cell walls (cell contamination) from 0.7-1.0 % to 0.01-0.5 %, without affecting protoplast viability (Table 2). The number of

BR-IRGA 409 protoplasts dividing (Fig. 1C) and giving rise to colonies (plating efficiency) (Fig. 1 D) was enhanced from 1.2 %± 0.3 colonies/protoplast to 10.7%± 4.8 colonies/protoplast by increasing the concentration of the feeder layer about 3-fold as recommended (Table 3). Plating efficiency of the *japonica* line CT 6241-15-1-7-1 was 5.3% ± 1.2 colonies/protoplast (Table 3). Lee et al. (1989) reported a plating efficiency of 3% for IR 54-2.

NO_3^-:NH_4^+ of 80 mM:20 mM, as in N6 and Kao media, was optimal for ensuring the development of protoplasts into embryogenic calli (ca. 60% survival). Higher levels of ammonium [NO_3^-:NH_4^+ of 75:25, 70:30 and 60:30 (as in MS medium)] totally inhibited calli development. Calli survival was highly reduced by BAP (Table 4). An increase of sucrose up to 8% was needed to induce coleoptile emergence (Fig. 1E and Table 5), and concentrations of 0.5 mg/l and 0.05 mg/l NAA in combination with 1 mg/l kinetin were optimal for differentiation induction and further plant development, respectively (Table 5 and Fig. 1F). With these modifications 12 of 52 (23%) calli tested gave rise to shoots, plants rooted (Table 5 and Fig. 1G) and moved to the greenhouse (Fig. 1H). Experiments directed to test regeneration from a larger population of protoplast-derived calli and to establish a higher regeneration efficiency are under way.

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Table 1. Establishment of cell suspension lines from mature rice seeds.

Rice type	Genotype	Callus induction (%)	Cell suspension	Cell type	Protoplast culture
Indica	Cica 8	35	+	NE	-
	BR-Irga 409	80	+++	NE	+
	Oryzica 1	32	+	NE	-
	Oryzica Llanos 4	60	++	NE	-
	Oryzica Llanos 5	80	+	NE	-
Japonica	CT 6241-15-1-7-1	97	+++	E	+

E = embryogenic; NE = nonembryogenic.

Table 2. Protoplast isolation from two *indica* varieties.

Genotype	Enzymatic incubation (hr)	Protoplasts/gr fresh weight/ml	Protoplast[a] viability (%)	Cell[b] contamination(%)
IR 54-2	4[c]	1.5×10^4	---	---
	4	1.7×10^5	---	---
	4	2.1×10^8	85	0.7
BR-IRGA 409	4	6.0×10^6	80	1.0
	4	2.0×10^7	90	0.7
	5	2.7×10^7	90	0.3
	5	2.8×10^8	90	0.5
	5	3.0×10^8	92	0.3

[a] Determined with FDA (450-490 nm).
[b] Cell wall stained with Calcoflour White (330-380 nm). Cell contamination (%) = viable cells (with undigested cell wall) X 100/viable protoplasts + viable cells.
[c] Cell aggregates > 50 cells each.

Table 3. Plating efficiency from protoplast-derived calli of BR-IRGA 409-1 and CT 6241 1-15-1-7-1.

Genotype	Pp/g Fresh weight/ml	Cell (%)[a] contamination	Colonies[b]	Plating[c] efficiency (%)
BR-IRGA 409	3.0 X 10^7	0.3	372 - 5985	26.5 ± 6.7 n = 8
	5.6 X 10^7	0.1	114 - 2804	6.8 ± 2.9 n = 8
	6.8 X 10^7	0.01	300 - 3210	10.5 ± 1.7 n = 6
	4.4 X 10^7	0.1	58 - 1710	3.9 ± 1.7 n = 6
	3.8 X 10^7	0.01	282 - 2039	6.1 ± 1.8 n = 6
				10.7 ± 4.8 ~ 1600 col/plate
CT 6241-15-1-7-1	5.7 X 10^7	0.05	205 - 1069	3.6 ± 0.9 n = 6
	2.6 X 10^7	0.00	373 - 1440	4.2 ± 1.7 n = 10
	2.8 X 10^7	0.01	490 - 2540	7.3 ± 1.1 n = 12
				5.3 ± 1.2 ~ 755 col./plate

[a] Undigested cells.
[b] Microcalli obtained from 10^4 pp/plate.
[3] Colonies X 100/pp cultured.

Table 4. Effect of benzyl-amino purine (BAP) on development of embryogenic protoplast-derived calli of BR-IRGA 409.

BAP (mg/l)	Embryogenic calli	Healthy calli	Survival (%)
0	50	25	50
0.5	50	10	20
1.0	40	5	13
1.5	40	4	10
2.0	40	0	0

Table 5. Culture conditions for plant regeneration from protoplasts of BR-IRGA 409.

Culture Phase	Medium	Carbon Source	Light
Protoplasts	Kao + 0.5 mg/l 2,4-D + 1 mg/l NAA + 0.5 mg/l zeatin	7% glucose	dark
Microcalli	LS + 2 mg/l 2,4-D + 50 mg/l tryptophan	3% sucrose	dark
Differentiation induction	1/2 R2 + 0.5 mg/l NAA + 1 mg/l Kin	1% sucrose 2% glucose	dark
Coleoptile emergence	R2 +0.05 mg/l NAA + 1mg/l Kin	8% sucrose	dim
Shoot development	R2 + 0.05 mg/l NAA + 1 mg/l Kin	3% sucrose	direct
Rooting	R2	3% sucrose	direct

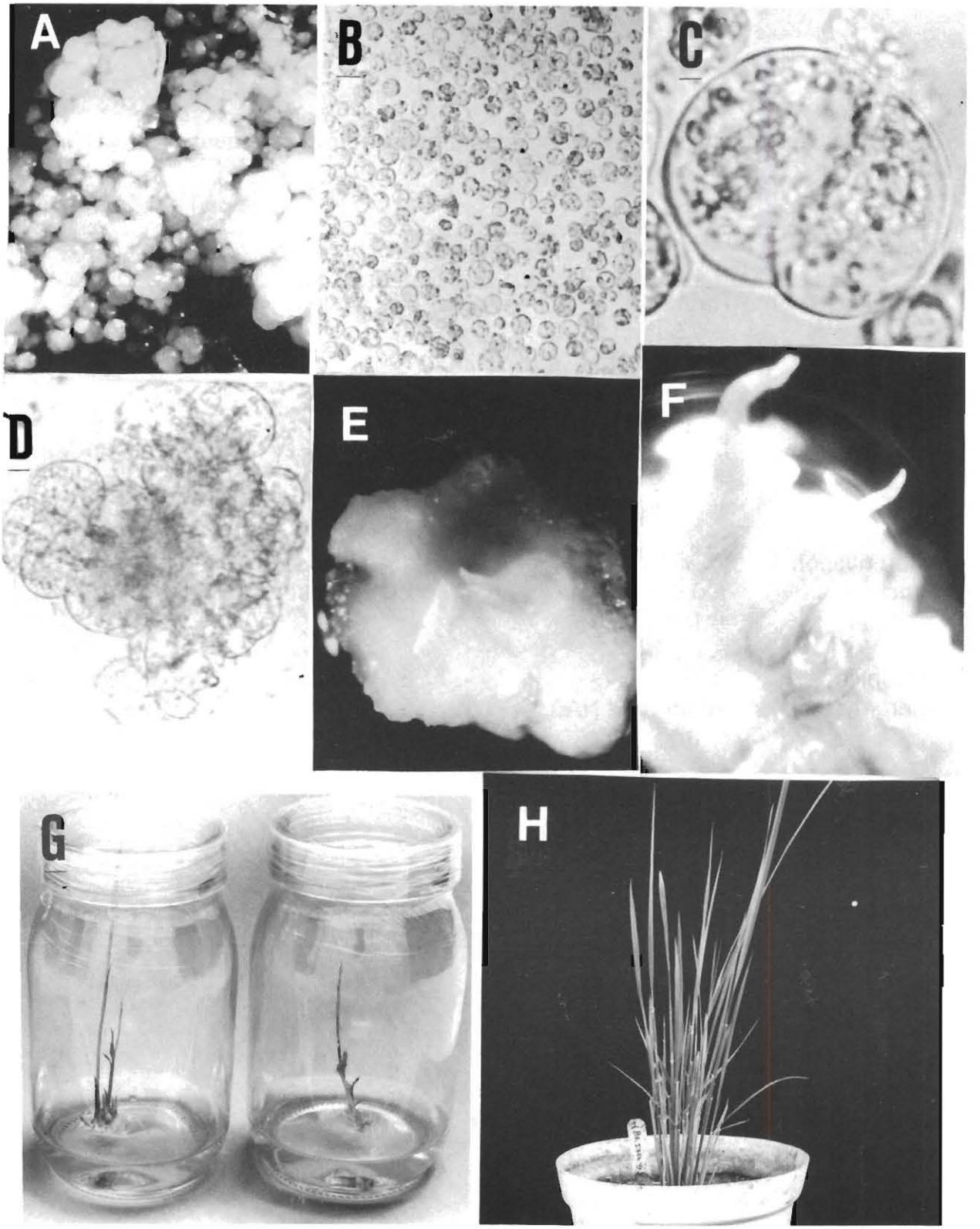

Figure 1. Protoplast culture and plant regeneration of BR-IRGA 409: (A) Cell suspension aggregates; (B) freshly isolated protoplasts; (C) initial cell divisions 7-13 days after isolation; (D) microcalli (colonies) onto millipore filter at 4 wk; (E) coleoptile emergence from embryogenic callus at 8 wk; (F) shoot development; (G) rooted regenerated plants; (H) regenerated plant in soil.

2.7 Regeneration of *Brachiaria* spp. and *Arachis pintoi*

A range of media components were tested for callus induction and regeneration of 5 *Brachiaria* spp.: *B. decumbens* (CIAT 606; apomictic, tetraploid), *B. brizantha* (CIAT 6780; apomictic, tetraploid), *B. dictyoneura* (CIAT 6133; apomictic, hexaploid), *B. humidicola* (CIAT 679; apomictic, hexaploid), and *B. ruziziensis* (CIAT 16551; sexual, tetraploid).

Callus induction from immature panicles was best with the MICP (Parker 1988) and the PCCM (Linsimaier and Skoog 1965) media (Table 1); and PCCM and M1 media for mature seed (Table 2). A common characteristic was the presence of 2,4-D; 2 mg/l was the best level of this auxin, and casein hyfrolizate at 100-300 mg/l. All species responded well to callus induction from immature panicles and mature seed (Tables 1 and 2).

After 30-40 days, embryogenic callus were transferred to the regeneration medium. For generation the rice anther culture regeneration medium gave best results. Following 20-25 days in the regeneration medium, somatic embryos were clearly differentiated from the callus (Fig. 1A). Amount of callus depended on the medium and the genotype (Tables 1 and 2). Germinated embryos were transferred one week later to test tubes for further growth and finally into soil in the greenhouse (Fig. 1B).

Given that regeneration of *Brachiaria* has been achieved, the system can be used for studies on generation of interaccession (somaclonal) variability as well as for genetic transformation.

Plant regeneration of *A. pintoi* accessions (nos. 18745, 18748, 18747, 18746, 18744, 18751, 17434) was obtained through organ differentiation from leaf-derived callus (Fig. 1C). These results also open the way to studying nonsexual means of inducing genetic variability in this important forage legume. Genetically stable lines can then be used as tools for studying basic mechanisms of adaptation to acid soils, especially in the ar ea of P uptake efficiency.

Table 1. Callus induction, proliferation and plant regeneration from immature panicles of five *Brachiaria* spp.

Species	Medium	Total expl. with callus rate (%)	Final callus Rate (%)	Regeneration Rate (%)
B. brizantha	MICP	20/40 (50)	32/20 (160)	32/16 (50)
	PCCM	6/40 (15)	7/6 (116)	2/7 (28.5)
B. decumbens	MICP	13/40 (32.5)	17/13 (130)	5/17 (29.4)
	PCCM	9/40 (22.5)	11/9 (122)	2/11 (18.8)
B. humidicola	MICP	12/40 (30)	23/12 (191)	12/23 (52.1)
	PCCM	6/40 (15)	7/6 (116)	4/7 (57.1)
B. dictyoneura	MICP	6/40 (15)	8/6 (133)	0
	PCCM	2/40 (5)	3/2 (150)	1/3 (33.33)
B. ruziziensis	MICP	11/40 (27.5)	15/11 (136)	6/15 (40)
	PCCM	4/40 (10)	4/4 (100)	3/4 (75)

Table 2. Callus induction, proliferation and plant regeneration from mature seeds of five *Brachiaria* species.

Species	Medium	Total explt. with callus rate (%)	Final callus Rate (%)	Regeneration Rate (%)
B. brizantha	M1	49/100 (49)	114/49 (232)	43/114 (37)
	PCCM	34/100 (34)	76/34 (223)	0/76 (0)
B. decumbens	M1	19/100 (19)	53/19 (278)	6/53 (11)
	PCCM	22/100 (22)	56/22 (254)	1/56 (1.7)
B. humidicola	M1	0/100 (0)	0	0
	PCCM	0/100 (0)	0	0
B. dictyoneura	M1	7/100 (7)	15/7 (214)	10/15 (66)
	PCCM	0/100 (0)	0	0
B. ruziziensis	M1	26/100 (26)	78/26 (300)	19/78 (24)
	PCCM	18/100 (18)	44/18 (244)	1/44 (2.2)

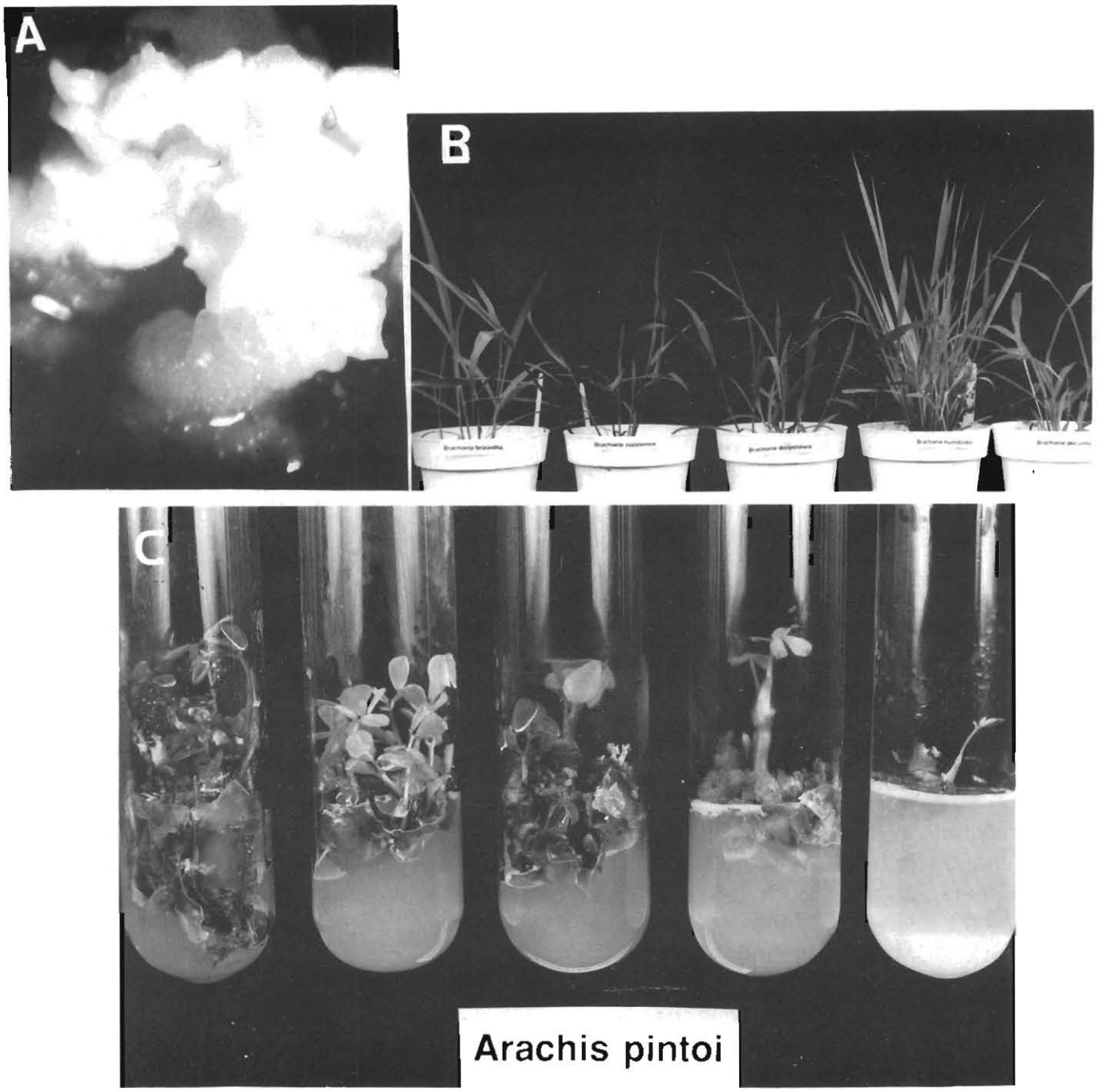

Figure 1. Regenerating plants from immature panicle and mature seed-derived callus of 5 *Brachiaria* spp. and from leaf segments of *Arachis pintoi*: (A) Induction of somatic embryos on callus derived from *Brachiaria* mature seed: (B) regenerated plants of 5 *Brachiaria* spp. transplanted into soil; (C) *Arachis pintoi* regenerated plants from leaf-derived callus.

3. IN VITRO CONSERVATION AND CRYOPRESERVATION

3.1 The Cassava *In Vitro* Active Gene Bank - IVAG (Collaboration: G. Mafla, GRU; C. Iglesias, Cassava Program)

Up to 1989, when this activity was transferred from the BRU to the GRU, the cassava IVAG comprised over 4300 clones in a 6x7 m room.
The current cassava IVAG under the responsibility of the GRU, is probably the largest, most complete *in vitro* collection for any crop in the world; it comprises nearly 5000 accessions. Plantlet cultures derived from meristem tips are available for micropropagation and distribution from the IVAG at any time. The conditions of the IVAG are: 22-24°C, 1000 lx illumination and 12-h photoperiod. The subculture period can be extended to 12-18 months.

Given CIAT's experience in in vitro conservation, a collaborative pilot project with the IBPGR was undertaken from 1987-1989. The objective of this project was to evaluate critical technical and logistical aspects of establishing and running an IVAG, using cassava as a model. The Pilot-IVAG project has provided valuable information on procedures for:

- entry of material to storage from the field, *in vitro* exchanges including pathogen-tests, and determination of the number of samples per accession
- monitoring cultures (viability and stability) and facilities for *in vitro* conservation
- management of data including passport, fingerprinting, pathogen tests, subculturing and distribution of cultures

Significant increases in yield have been reported as a consequence of virus elimination from cassava cultivars. The technique consists in the culture of 0.1-0.2 mm meristem tips from terminal buds grown on stakes at 40-42°C and 35°C day/night for 3-4 wk. Indexing by cassava virologists has confirmed the elimination of FSD, CCMV, CsXV and latent viruses.

International germplasm exchange has been facilitated by the use of pathogen-tested material. Thus, up to 1989, nearly 2000 clones had been introduced to CIAT from 13 countries in the form of *in vitro* cultures; and many pathogen-tested clones were distributed *in vitro* from CIAT to 35 countries in the same period.

CIAT hybrid CM 321-188 was selected and named "Nan-Zhi 188" in Guangzhong, China. This hybrid has been propagated massively using in vitro techniques, and the planting material distributed to 15 sites in Southern China. Similarly, national agricultural programs in Panama, Venezuela, Mexico, Peru and the Philippines have advanced cassava micropropagation to the field-testing stage.

3.2 Cryopreservation of Cassava Shoot Tips (Collaboration: G. Mafla, GRU; C. Iglesias, Cassava Program)

Currently, ex-situ conservation of cassava at CIAT is carried out both in the field and in the laboratory as shoot tip cultures. The in vitro gene bank of cassava at CIAT presently comprises nearly 5000 clones. This is an active collection in the sense that clones have to be transferred every 12-18 mo to fresh culture medium. Long-term conservation of cassava clones in a small space, free of genetic changes and at low cost, can be achieved by cryopreservation. Suspended animation, prevention or delay of processes of cell deterioration and indefinite preservation of cassava genotypes at a low cost are the main objectives of cryopreservation.

The project, which began at CIAT in 1988, consists of three phases:

- The first phase, carried out in cooperation with the IBPGR (1988-90), resulted in the recovery of plants from frozen shoot tips in liquid nitrogen (-196°C);

- The second phase of the research (1990-present) was designed to improve the previous protocol in order to increase the recovery rate of plants;

- The third phase of the research will focus on developing further the technique, especially with regard to genotype response and evaluation of genotypic stability, as well as critical logistical aspects of cassava cryopreservation.

3.2.1 **First phase of the project**

Main outcome: A methodology for cassava cryopreservation was developed:

- extraction of shoot tips between 2-4 mm.
- preculture in C4 medium (1M sorbitol and 0.1M DMSO) for 3 days
- cryoprotector (1M sorbitol, 10% DMSO and 0.1M sucrose) addition for 2 h on ice
- drying for 1 h
- control freezing rate:Wait to 5°C
  0.5°C to -15°C
  -15°C to -20°C
  -20°C to -17°C
  1°C to -40°C
  End
- transfer to liquid nitrogen (-196°C)
- thawing, swirl in 37°C bath
- reculture medium: (a) Equilibrium media (R1 & R2) for 2 days each (b) Normal proliferation medium (4E)
- evaluation of viability and shoot formation (1 mo)

With this methodology it was possible to obtain consistently, for the first time, plants from frozen shoot tips of cv. M Col 22. The parameters evaluated included viability (i.e., tissue that survives and shows capacity to grow) and shoot formation (i.e., explants that can form shoots capable of growing into plants). Recovery rates ranged from 20%-40%. Several other cassava cv. tested, however, showed only a low response or did not respond at all (Table 1).

3.2.2 **Second phase of the project**

The objective was to improve the methodology developed in the first phase. It was found out that lower temperature and higher illumination of donor cultures increased the recovery from liquid nitrogen. Moreover, use of high (0.75M) sucrose concentration in lieu of sorbitol and DMSO in the preculture stage gave high rates of plant recovery from frozen shoot tips.

- **Pre-culture phase**. In this phase 3 media were tested; and when compared with the C4 medium (preculture medium of the first protocol), the rate of plant recovery increased significantly (Table 2).

- **Effect of light and temperature**. When variation of growth temperature and light intensity of donor cassava shoot tip cultures were compared, we found increased recovery from liquid nitrogen at 75$\mu Em^{2}s^{-1}$ intensity and 21-23°C (Table 3).

- **Effect of rapid freezing**. Ultra-rapid freezing (i.e. direct immersion of shoot tips into liquid nitrogen) resulted in similar or higher recovery rates than slow freezing (Table 4).

- **Recalcitrant genotypes**. Recovery rate of otherwise unresponsive genotypes has significantly increased with the improved technique. The main modification consisted in varying the osmotic level in the preculture medium (Table 5).

- **Freezing curves**. Freezing injury to plants and the mechanisms that they have devised to avoid or tolerate freezing are, of course, subject to fundamental physical and biological principles. When we tested the behavior of pure water, preculture medium and cryoprotecting medium during the freezing program named "Program 2", we observed that the response curve of culture media didn't follow the expected values.

  The objective of this experiment was to observe the stage and time necessary to freeze various solutions, and determine the relations with the freezing

program of the first protocol (first phase of CIAT project), named "Program 4". The program includes six steps, out of which we believe the most important phase in the process are steps 3 and 4. These are related to the initiation of ice crystal formation, when ice nuclei are present. During these steps the energy release by the formation of ice must be dissipated. According to the freezing curve, the point of supercooling of the cryoprotector (the temperature of energy released) occurs at -18°C, and not at -15°C as it was used in the first protocol. Hence we are planning to shift the temperature of steps 3 and 4 to -18°C to -20°C in future experiments. The thermo-dynamics of these temperature are safer for the tissue.

The range of temperature drop in the solutions is normally around 10 degrees, which decreases by about 3 to 5 degrees after autoclaving, probably due to hydrolytic processes. This reduction must also be taken into account when designing new freezing protocols.

The costs of maintaining a germplasm collection are due largely to the labor required in the field, as well as in the IVAG. Cryopreservation should decrease these costs, as well as reduce the space needed to preserve a collection as large as the one maintained at CIAT. With the current protocol many cassava genotypes have successfully been cryopreserved and shoot tips recovered and grown to maturity (Fig. 1 A-D). Further development of this technology will make it possible to implement cryopreservation for cassava shoot tips. Cryopreservation of pollen, seeds (or zygotic embryos), somatic embryos and other cells and tissues will be fundamental and complementary for achieving the same goal and for genetic manipulation of cassava using novel techniques.

Changes continue to be made in the re-culture (i.e., culture of shoot tips recovered from LN). We have found that using 0.5mg/l Zeatin, 0.2mg/l GA_3 and 0.5mg/l, AIA the tissue remains green for a longer time with respect to 4E (standard recovery medium).

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Table 1. Effect of cassava genotype on viability and shoot formation after cryopreservation in LN.

Cultivar	Viability(%)[a]	Shoot(%)[b]
M Col 22	92.72(a) [c]	52.22(a)
CM 922-2	81.39(ab)	48.65(a)
M Col 1468	73.73(b)	10.00(bc)
MARG 2	67.72(b)	37.45(a)
M Par 193	51.66(c)	13.97(bc)
M Cub 27	45.31(dc)	11.42(bc)
M Ecu 48	32.94(de)	21.03(b)
M Pan 125	24.83(ef)	12.92(bc)
M Gua 14	1.22(efg)	2.00(c)
M Per 303	15.26(fgh)	0.00(c)
M CR 113	14.44(fgh)	2.77(c)
M Bra 12	11.00(fgh)	0.00(c)
M Dom 2	5.25(gh)	3.12(c)
M Ven 232	5.02(gh)	0.00(c)
M Mex 71	3.90(h)	0.00(c)

a) Viability: tissue that survives and show capacity to grow.
b) Shoot formation: explants that can form shoot capable of growing in plants.
c) Averages with the same letter do not show significant differences at 0.05 level.

Table 2. Effect of prefreezing culture media on viability and shoot development from shoot tips (cv. M Col 22) after freezing in liquid nitrogen.

Preculture Medium	Viability(%)	Shoot(%)
0.75M sucrose, 0.01% AC[a]	88(a)[d]	56 (a)
1M sorbitol, 0.1M DMSO[b]	90(a)	32(b)
0.35M sucrose,1g m-inositol[c]	56(b)	26(b)
Micropropagation medium	0	0

a) Without basal salt nor hormones; AC = active charcoal.
b) Basal medium 4E.
c) ½ basal medium, without hormones.
d) Averages with the same letter do not show significant differences at 0.05 level.

Table 3. Effect of light intensity and temperature of source cultures (cv. M Col 22) on tissue viability and shoot development from shoot tips after freezing in LN.[a]

Temp	Light Intensity	Viability	Shoot
(°C)	($\mu E.m^2.s^{-1}$)	(%)	(%)
21-23	75	80a[b]	60a
	45	44b	24b
26-28	75	52ab	20b
	45	48b	24b

a) Preculture medium: Basal medium 4E, 1M sorbitol, 0.1M DMSO, 0.1M sucrose.
b) Averages with the same letter do not show significant differences at 0.05 level.

Table 4. Effect of freezing rate on tissue viability and shoot development from shoot tips after freezing in LN.

Freezing Rate	Viability	Shoot
	%	%
Moderate[a]	68	28
Ultra rapid[b]	86	58

a) 0.5°C/min: +5 to -15°C
direct: -15°C to -20°C; -20°C to -17°C
1°C/min: -17°C to -40°C
-40°C to LN
b) Direct transfer from room temp to LN.

Table 5. Effect of prefreezing culture medium on tissue viability and shoot development of recalcitrant cvs. after freezing in LN.

Medium[a]	M Col 22[b]		M Mex 71		M Ven 232	
	Viabil	Shoot	Viabil	Shoot	Viabil	Shoot
1	88	72	40	8	32	18
2	84	56	32	4	40	20
3	92	52	4	0	5	0

a) 1:Basal 4E, 0.5M sorbitol, 0.01M DMSO, 0.1M sucrose
2:Basal 4E, 0.5M sorbitol, 0.001M DMSO, 0,25M sucrose.
3:Control medium: basal (4E), 1M sorbitol, 0.1 M DMSO.
b) Control cv.

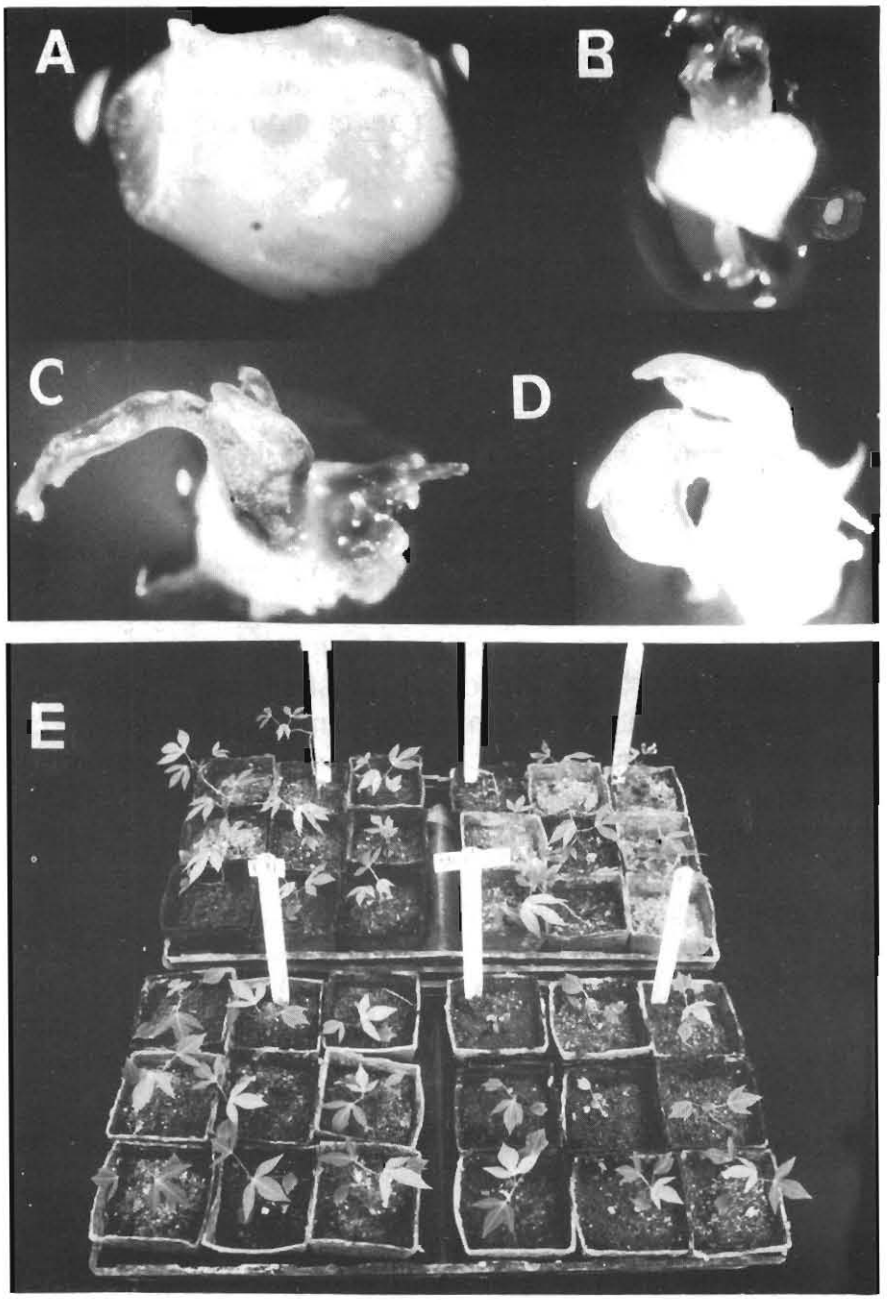

Figure 1. Recovery of cassava plants from cryopreserved shoot tips in liquid nitrogen: A. Meristem tip (arrow) which survived freezing and started to grow; B. Young bud; C. Bud branching; D. Further growth; E. Potted plants.

IV. INSTITUTION BUILDING

This section of the BRU report includes the activities of the Unit in developing crop-specific advanced research networks, training of national agricultural research institutions' (NARS) scientists, the Unit's participation in developing CIAT's biosafety guidelines and other activities geared to developing biotechnological applications at CIAT.

1. INTERNATIONAL ADVANCED RESEARCH NETWORKS TO ADDRESS BIOTECHNOLOGY FOCUSED ON CIAT CROPS

One of the cornerstones of CIAT's strategy for biotechnology is the organization of international networks to address the development of biotechnology focused on CIAT germplasm. In this context the BRU has played an important role in cooperation with the Cassava and the Bean Programs.

Several advantages are inherent in organizing crop-specific advanced research networks: (1) merging of the interests and experiences of researchers from developed and developing countries, policymakers and technology users; (2) concentration of efforts on the crop's most relevant production and utilization constraints that are recalcitrant to conventional methodologies; (3) more effective and efficient integration of developing countries into the networking process through research projects and training programs; and (4) stimulation of financial support for priority research topics identified by the network, as well as for other critical activities.

1.1 The Cassava Biotechnology Network (CBN)

With the collaboration of CIAT, the International Institute for Tropical Agriculture (IITA), and several national and international agricultural research institutions interested in cassava, the network was organized in a founding meeting held at CIAT in September 1988. Originally the network was called Cassava Advanced Research Network; in 1989 the Steering Committee decided to change its name to Cassava Biotechnology Network (CBN).

The objective of the CBN is to facilitate the implementation of modern biological approaches and tools that can contribute to solving priority problems in cassava production and utilization that are recalcitrant to traditional solutions. In this process priority is given to the participation of developing country NARS and their scientists, and to the various types of cassava end users.

In the founding workshop several critical topics for biotechnology and more basic research were identified in cassava; e.g., cyanogenesis, virus resistance, starch

quality, photosynthesis under stress, postharvest root deterioration, true seed propagation and insect resistance. Technological constraints identified included plant regeneration and transformation, molecular mapping and gene tagging.

In the last few years, new research priorities have been added to the CBN's agenda, while others have been de-emphasized. The network approach to cassava biotechnology has received wide acceptance by the scientific community and national and international funding agencies. Since its foundation, the number of projects has steadily increased; currently some 25 projects are under way in developed and developing country institutuions (Table 1). In early 1992 the Directorate General for International Cooperation of the Netherlands (DGIS) approved CIAT's proposal for funding the CBN's operations and activities. Activities include: Steering Committee and scientific meetings of the network, a full-time network Coordinator, communications media for the network, and training of developing country scientists. The CBN Coordinator was appointed by CIAT in August 1992.

An important activity of the BRU was the organization of the **first scientific meeting of the CBN**, held in Cartagena, Colombia, in August 1992. The meeting was attended by 128 people from 29 countries (19 developing and 10 developed countries); over 70 papers were presented on a range of cassava biotechnology and related aspects. Proceedings of the meeting will be published in early 1993. Advances were reported in methods for increasing the effectiveness of germplasm conservation and characterization via biotechnology--for example, cryopreservation research and fingerprinting techniques at CIAT's BRU and GRU. Construction of a molecular map of the cassava genome is in progress. Several laboratories including CENARGEN (Brazil), the U. of Georgia and Washington U. (USA), the U. of Newcastle upon Tyne (UK), IITA (Nigeria) and CIAT/BRU and the Cassava Program, have identified germplasm with adequate polymorphism for phylogeny studies and molecular mapping of cassava, and have developed cassava genomic and cDNA libraries. Important steps toward a repeatable protocol for transforming and regenerating cassava were reported by CIAT/BRU, the ILTAB/Scripps Laboratory (USA) and the U. of Wageningen (Netherlands). Improvements in cooking and processing quality could increase cassava's potential to generate rural income by opening new markets to the crop. New information was presented in the areas of protein metabolism (U. of Bristol, UK) and carotene content (Australian National U.). Genetic variation and biochemistry of starch quality of cassava is under study at the CTCRI (India), the U. of Nottingham (UK), the NRI (UK) and the CIAT Cassava Utilization Section. Results of this work will be used to direct crop improvement research and to design transgenic approaches to quality manipulation when transformation/regeneration protocols are implemented. A new project--proposed jointly by the FAO/Field and Food Crops Group, CIAT and NRI--will address the problem of rapid postharvest deterioration of fresh cassava through a multidisciplinary effort involving biochemistry, molecular genetics, crop science and socioeconomics.

Table 1. Research projects under way in Cassava Biotechnology (Oct. 1992).

Subject	Institutions	Funding
Cyanogenesis	U. of Newcastle upon Tyne (UK)	RF/EC/ODA
	Royal Vet. & Agric. U. (Denmark)	F/EC
	Mahidol U. (Thailand)	
	Ohio State U. (Columbus, USA)/CIAT	RF/EC
	Free U. (Amsterdam, Neth.)	USAID
	Royal Vet. Agric. U. (Denmark)/IITA	
		-
		DANIDA
Virus resistance	ILTAB/Scripps Res. Institute	ORSTOM/RF/U
	(La Jolla, USA)	SAID/
		GTZ
	Free U., Amsterdam/U. of Zimbabwe	DGIS
Insect resistance	Washington State U. (Pullman, USA)/CIAT	RF
Photosynthesis	Australian Nat. U. (Canberra)	AIDAB
	U. of Georgia (Athens, USA)	USAID
Plant regeneration	U. de Paris (Orsay, France)	EC
	U. of Bath (UK)	ODA
	U. of Zimbabwe	DGIS
	CIAT	Core
	South China Inst. Botany	RF
	U. Wageningen	DGIS
	IITA	Core
Genetic transformation	U. Nottingham (UK)	RF
	U. of Guelph (Canada)	RF
	CIAT	Core
	CENARGEN (Brazil)	-
	IITA	Core
DNA fingerprinting and molecular mapping	Washington U. (St. Louis, USA)	RF
	U. of Georgia (Athens, USA)	RF
	CIAT, IITA	RF
Cryopreservation	CIAT	Core
CBN activities	CIAT	DGIS
Other activities	CIAT, IITA	Core

RF = Rockefeller Foundation; EC = European Community; ODA = Overseas Development Administration; USAID = US Agency for International Development; ORSTOM = French Institute of Scientific and Technical Research Collaboration Abroad; AIDAB = Australian International Development Assistance Bureau.

As a result of thoughtful, wide-ranging dialogue on CBN issues, a consensus has emerged that research on farmer, processor and consumer priorities must play a part in the continuing definition of the CBN's overall research agenda. One example of these discussions concerned cyanogenesis in cassava. Low root-cyanide cultivars of cassava are known to be needed for fresh consumption; however, reports to the CBN meeting suggested that low root cyanide may not be required in all cassava-growing regions. The International Child Health Unit, Uppsala U. (Sweden) and CEPLANUT (Zaire) presented findings that cyanide toxicity in Africa may be related to crisis circumstances that prevent proper processing of cassava rather than to normal consumption of traditional cassava foods. The CIAT Cassava Entomology Section presented preliminary data that may implicate root cyanide in natural defense against burrowing insects. Participants concluded that more anthropological and biological information is needed before cyanide in cassava is understood and its genetic manipulation can be optimized for the most efficient production and utilization of this crop in each of the environmentally and culturally diverse regions where it is grown.

The CBN has now been consolidated through the support received to carry out its major activities, the appointment of a Coordinator and the organization of the first scientific meeting.

1.2 The Bean Advanced Research Network (BARN)

A *Phaseolus* bean advanced research network (BARN) was organized in a workshop held at CIAT in September 1990. Nearly 50 scientists from Europe, the USA, Latin America and Africa attended the workshop. Research constraints, prepared by CIAT bean scientists, were discussed and agreement reached regarding priorities for attention by the network. Possible research strategies were proposed to approach the constraints in a cooperative, multi-institutional fashion. Participants elected a Steering Committee to guide the operation of the network. Among the priority areas identified for biotechnology attention or more basic research are plant/soil relationships including root structure and function, especially in relation to drought and P uptake; yield potential; a range of pathogen and pests; and utilization quality constraints. Technological topics for priority attention include construction of the bean molecular map and techniques for plant regeneration and genetic transformation.

An international BARN workshop has been planned for September 1993. The main objective of this workshop will be to stimulate collaborative efforts, facilitate exchange of information on modern biotechnologies, and strengthen NARS involvement in the network.

1.3 Training

In the last few years, nearly 90 people from 20 countries have received training at CIAT in a range of biotechnology topics. From 1988-1990 most training involved tissue culture techniques such as somatic embryogenesis (cassava) and haploid induction (rice), and fingerprinting of cassava, beans and tropical forages by isozyme and protein electrophoretic analyses. In the last two years, training topics gradually moved to molecular biology technologies such as DNA fingerprinting and genetic mapping and tagging, as well as genetic transformation.

Various training modalities have been implemented by the BRU: (1) in-service training, short-medium term, mostly for NARS; (2) two international intensive courses: one for Latin American scientists, in cooperation with ICRO (UNESCO) and another joint activity for Latin America with the International Potato Center (CIP) and IITA; (3) thesis research (biology and Ag.Eng.) for one year each for local (Colombian) university students; and (4) advanced thesis research (MSc and PhD) for local (Colombian) and foreign students. Table 2 summarizes PhD training at the BRU.

Table 2. PhD thesis students and topics of research in the BRU (1989-92).

Name	Topic	Institution
M. Korban	Bean genetic transformation	McGill U.; Canada
A. Mejía	Bean interspecific hybridization	U. Bonn, Germany
W. Welsh	Bean interpool recombination	U. Manitoba, Canada
C. Constabel	Bean bacterial blight fingerprinting	U. of Guelph, Canada

1.4 Inter-Center Cooperation

The BRU cooperated with IITA and CIP in organizing a training course on cassava, potato and sweet potato tissue culture for Latin American NARS; with ILCA, in developing grass pasture tissue culture techniques for international exchange of germplasm; with the IBPGR on two pilot projects: the in vitro active gene bank (1987-90) and the cryopreservation pilot project (1988-91) using cassava as a model; with IITA on DNA fingerprinting of wild *Manihot* spp. (M. Fregene), and on organizing the founding workshop and the first scientific meeting of the Cassava Biotechnology Network in 1988 and 1992, respectively.

1.5 Awareness of Biotechnology Issues

1.5.1 **Biosafety**. In 1990-91, the BRU played an active role in CIAT's efforts to develop its Biosafety Guidelines. These Guidelines comprise peer-approved norms for research with R-DNA and the planned testing (small scale) of transgenic organisms in the greenhouse and field. A CIAT Institutional Biosafety Committee (IBC) has also been appointed by the Director General to oversee all CIAT research with R-DNA organisms. The IBC is comprised of CIAT senior staff members representing different disciplines plus one representative from ICA-Colombia. Provisions have been made to incorporate new developments in the field at the international level and to abide by future Colombian legislation on the subject. The IBC meets on an ad-hoc basis and recommends to the Director General any actions agreed upon.

1.5.2 **Intellectual property rights**. Because recent developments in biotechnology research have exacerbated the privatization of agricultural research, mainly in industrialized countries, the BRU has been contributing to the Center's actions to develop awareness and to develop a policy on the issue through CIAT's IPR Working Group.

1.5.3 **Seminars on molecular genetics**. The BRU has organized introductory seminars/discussions on molecular genetics for CIAT Program scientists. The objective is to strengthen the integration of biotechnology into ongoing disciplinary research at CIAT, discuss the potentials and limitations of the new biological technologies, and strengthen the collaborative links of the BRU with CIAT Programs and Units. The series of seminars began with the Bean Program, followed by the Cassava, Tropical Forages and Rice programs. Given their success, these seminars will be repeated in the immediate future.

1.5.4 **Relations with the private sector**. The BRU has developed links with the biotechnology private sector to gain access to genetic constructs (PGS) and to develop protocols for non-radioactive labelling of genomic and cDNA probes (BRL).

APPENDICES

APPENDIX 1:

BRU PUBLICATIONS 1988-1992

Refereed and Other Publications

1. Angel, F.; Arias, D.I.; Tohme, J.; Iglesias, C. and Roca, W.M. 1992. Towards the construction of a molecular map of cassava: Comparison of restriction enzymes and probe sources in detecting RFLPs. *J. Biotechnology*. (Accepted for publication) (In press)

2. Angel, F.; Giraldo, F.; Gómez, R.; Iglesias, C.; Tohme, J. and Roca, W.M. 1992. Use of RFLPs and RAPDs in cassava genomic studies. Proceedings, First Scientific Meeting of the Cassava Biotechnology Network, CIAT, Cali-Colombia. (In press)

3. Cataño, M.L.; Mornan, K.; Plazas, J. and Roca, W.M. 1992. Development of methodologies for the isolation and culture of cassava immature pollen and zygotic embryos. Proceedings, First Scientific Meeting of the Cassava Biotechnology Network, CIAT, Cali-Colombia. (In press)

4. Escobar, R.; Mafla, G. and Roca, W.M. 1992. Cryopreservation of cassava shoot tips. Proceedings, First Scientific Meeting of the Cassava Biotechnology Network, CIAT, Cali-Colombia. (In press)

5. Flórez, C.; Chuzel, G. and Mayer, J.E. 1992. Characterization of bacterial amylolytic activities during cassava solid state fermentation. Proceeding, First Scientific Meeting of the Cassava Biotechnology Network, CIAT, Cali-Colombia. (In press)

6. Kartha, K.K. and Roca, W.M. 1992. The role of plant biotechnology in crop improvement. Proceedings, First Scientific Meeting of the Cassava Biotechnology Network, CIAT, Cali-Colombia. (In press)

7. Kipe-Nolt, J.A.; Montealegre, C.M. and Tohme, J. 1992. Restriction of nodulation by the broad host range *Rhizobium tropici* strain CIAT 899 in wild accessions of *Phaseolus vulgaris* L. *New Phytol.* 120:489-494.

8. Lynch, J.; González, A.; Tohme, J. and García, J. 1992. Variation in characters related to leaf photosynthesis in wild bean populations. *Crop Sci.* 32:633-640.

9. López, Y.; Vélez, W.; El-Sharkawy, M. and Mayer, J.E. 1992. Biochemical characterization of PEPC from cassava. Proceedings, First Scientific Meeting of the Cassava Biotechnology Network, CIAT, Cali-Colombia. (In press)

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11. Roca, W.M.; Henry, G.; Angel, F. and Sarria, R. 1992. Biotechnology research applied to cassava improvement at the international center of tropical agriculture (CIAT). Ag Biotech News and Information. C.A.B. International, Wallingford. (In press)

12. Roca, W.M. 1992. The role of biotechnology in Latin American agriculture. Hi-Tech Agriculture and Food, Milano, Italy. (In press)

13. Roca, W.M.; Angel, F.; Sarria, R. and Mafla, G. 1992. Future initiatives in biotechnology research for tropical agriculture: the case of cassava. In D.K. McCorwick, (ed.), Advances in Gene technology: Feeding the World in the 21st Century, 1992 Miami Bio/technology Winter Symposium, Miami, FL.

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15. Sarria, R.; Ocampo, C.; Ramírez, H.; Hershey, C. and Roca, W.M. 1992. Genetics of esterase and glutamate oxaloacetate transaminase isozymes in cassava. Proceedings, First Scientific Meeting of the Cassava Biotechnology Network, CIAT, Cali-Colombia. (In press)

16. Tenjo, F.A., Mayer, J.E. 1992. Cloning and sequence analysis of PEP-carboxylase from cassava. Proceedings, First Scientific Meeting of the Cassava Biotechnology Network, CIAT, Cali-Colombia. (In press)

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18. Dietrich, A.; Mayer, J.E. and Hahlbrock, K. 1990. Phytophtora megasperma elicitor induces rapid, transient, and specific protein phosphorylation in parsley cell suspension cultures. *J Biol Chem* 265:6360-6368.

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27. Miles, J.W.; Roca, W.M. and Tabares, E. 1989. Assessment of somaclonal variation in *Stylosanthes guianensis*, a tropical forage legume. In: A. Mujeeb-Kazi and L.A. Sitch (eds.), Review of Advances in Biotechnology. Mexico, D.F., CIMMYT and IRRI.

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5. Lentini, Z.; Martínez, C. and Roca, W.M. 1991. Generation of doubled haploids feasible for a rice breeding program. Fourth Annual Meeting of the Rockefeller Foundation International Program on Rice Biotechnology, Tucson, Arizona.

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7. Tohme, J.; Montenegro, M.V.; Tenjo, F.; Correa, F.; Martínez, C.; Zeigler, R. and Roca, W.M. 1991. Tagging resistance genes to rice hoja blanca virus and Colombian isolates of rice blast with RFLP and RAPD markers. Rockefeller Foundation. International Rice Biotechnology Meetings, Tucson, Arizona.

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blanca virus by RFLP linkage analysis. Abstracts: Symposium on Plant Breeding in the 1990s. North Carolina State University, Raleigh.

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10. Lynch, J.; Gonzalez, A. and Tohme, J. 1989. Characters related to leaf photosynthesis in wild bean. *Agron. Abstr.* 81:116.

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Manuscripts in Preparation

1. Mejía-Jiménez, A.; Muñoz, C.; Jacobsen, H.J.; Roca, W.M. and Singh, S.P. Interspecific hybridization between common and tepary beans: Increased hybrid embryo growth, fertility, and efficiency of hybridization through congruity backcrossing.

2. Pastor-Corrales, M.A.; Otoya, M.M.; Mayer, J.E.; Leong, S.A.; Maxwell, D.P. and Gilbertson, R.L. Demonstration of genetic diversity of the bean common bacterial blight pathogen by DNA fingerprinting.

3. Sarria, R.; Calderón, A.; Thro, A.M. and Roca, W.M. *Agrobacterium*-mediated transformation and regeneration of *Stylosanthes guianensis* transgenic plants carrying the bar, nptII and uid A genes.

4. Tohme, J.; Montenegro, M.V.; Tenjo, F.; Correa, F.; Martinez, C.; Zeigler, R. and W. Roca. 1991. RFLP and RAPD tagging of genes for resistance to Colombian lineage of rice blast.

5. Tohme, J.; Montenegro, M.V.; Correa, F.; Martínez, C.; Zeigler, R. and W. Roca. 1991. RFLP mapping of Rice Hoja Blanca virus resistance genes.

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Supervision of Theses

1. Constable, C. (ongoing). Molecular screening of bean bacterial blight. **PhD** thesis, Guelph Univ., Canada.

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3. Fory, L.F. (ongoing). Study of resistance factors against the bruchid *Acanthoscelides obtectus* in *Phaseolus vulgaris*.

4. García, A. 1992. Acid invertase as an indicator for sugar accumulation in sugarcane.

5. Gómez, A.M. 1992. Transformation of cassava by the particle bombardment device. Univ. del Valle, Cali.

6. Gómez, R. (ongoing). Cassava molecular map. **MSc** thesis, Univ. Javeriana, Bogota.

7. Lenis, S. 1992. Plant regeneration of *Brachiaria* and *Arachis* for tissue culture. Univ. Santiago de Cali.

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9. Villaquirán, R. 1992. Adjustment of the particle bombardment device for the transformation of common bean. Univ. Nac., Palmira.

10. Vélez, W. (ongoing). Isolation, purification and immunological characterization and quantification of phosphoenolpyruvate carboxylase of cassava (*Manihot esculenta* Crantz).

11. Welsh, W. 1992. *Phaseolus vulgaris* interpool recombination, **PhD** thesis, Univ. of Manitoba, Canada.

12. Zambrano, A.P. (ongoing). Enzymatic and biochemical characterization of amylolytic activities in the fermentation of cassava starch.

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14. Gutiérrez, J.P. 1991. Análisis de isoenzimas y faseolinas en el complejo "silvestre maleza-cultivo" del frijol común (*Phaseolus vulgaris* L.). Univ. del valle, Cali.

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17. Muñoz, L. 1991. Efecto del ácido acetil salicílico en conservación *in vitro* de quince variedades de yuca (*Manihot esculenta* Crantz). Univ. Santiago de Cali. 107p.

18. Plazas, J. 1991. Respuesta al cultivo *in vitro* de microsporas aisladas de variedades de yuca (*Manihot esculenta* Crantz) con fertilidad diferencial. Univ. del Valle, Cali. 60 p.

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21. Sarria, R. 1989. Estudio de herencia y variabilidad genética de isoenzimas en poblaciones F1 de Yuca (*Manihot esculenta* Crantz). Univ. del Valle, Cali. 72p.

22. López, G.P. 1988. Crecimiento de cultivos de tejidos de *Stylosanthes* spp. en medio conteniendo extractos de *Collecotrichum gloesporioides*. 68p.

23. Tello, L.M. 1988. Análisis histológico e isoenzimático durante la inducción de la embriogénesis somática en yuca (*Manihot esculenta* Crantz). Univ. del Valle, Cali. 91p.

24. Triana, M. 1988. Estudio electroforético de germoplasma de frijol (*Phaseolus vulgaris* L.) en centros secundarios de diversidad genética: El caso de Africa Suroriental.

25. Vargas, J. 1988. Caracterización de los acervos genéticos de *Phaseolus vulgaris* L. por medio de electroforesis. Universidad del Valle, Cali. 73 p.

APPENDIX 2:
BRU PERSONNEL 1992

1. Broadening the Genetic Base of Crops and Germplasm Conservation

William M. Roca	Physiologist, Unit Head
Zaida Lentini	Postdoctoral, Rice Program (½*)
Alvaro Mejía	PhD student, Bonn, FRG, Beans(*)
Marta Lucía Cataño	Research Assistant II, Cassava
Rodrigo Sarria	Research Assistant II, Cassava, TFP
Roosevelt Escobar	Laboratory Assistant, Cassava
Pablo Herrera	Technician III, Cassava, Beans
Germán Martínez	Technician III, Beans
Eddie Tabares	Laboratory Assistant, Rice
Ana María Gómez	Student, Cassava
Sandra Lenis	Laboratory Assistant, TFP
Ramiro Villaquirán	Student, Beans
Luz Amparo Cartagena	Bilingual Secretary
Pilar Torres	Bilingual Secretary
Marlene Valenciano	Technician III
Régulo Arias	Technician III

2. Characterization of Genetic Structure

Joe Tohme	Geneticist
Fernando Angel	Postdoctoral SRF, Cassava(*)
Rocio Gómez	MSc student, Cassava(*)
Faustina Giraldo	Rural year, Cassava(*)
Bill Welsh	PhD Student, Beans
Alfredo Badillo	Research Assistant III, Rice(*)
María V. Montenegro	Research Assistant II, Rice(*)
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3. Characterization of Mechanisms Involved in Resistance and Tolerance to Biotic and Abiotic Stresses in Plants

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APPENDIX 3:
ACRONYMS

AGCD	Administration Générale de la Cooperation au Dévelopment (Belgium)
AIDAB	Australian International Development Assistance Bureau
BARN	Bean Advanced Research Network
BRU	Biotechnology Research Unit (CIAT)
CBN	Cassava Biotechnology Network (CIAT)
CEEMAT	Centre d'Etudes et d'Experimentation du Machine Agricole (CIRAD-France)
CENARGEN	Centro Nacional de Recursos Genéticos (EMPASC-Brazil)
CEPLANUT	National Nutrition Planning Center (Zaire)
CIAT	Centro Internacional de Agricultura Tropical (Colombia)
CIDA	Canadian International Development Agency
CIP	Centro Internacional de la Papa (Peru)
CIRAD	Centre de Coopération Internationale en Recherche Agronomique pour le Développement (France)
CTCRI	Central Tuber Crops Research Institute (India)
DANIDA	Danish Ministry of Foreign Affairs
DGIS	Netherlands Agency for International Cooperation
EC	European Community
EHT	Eidgenössische Technische Hochschule (Switzerland)
EPR	External Program Review
GRU	Genetic Resources Unit (CIAT)
GTZ	Deutsche Gesellschaft für Technische Zusammenarbeit (Fed. Rep. of Germany)
IBC	Institutional Biosafety Committee (CIAT)
IBPGR	International Board for Plant Genetic Resources (Italy)
ICA	Instituto Colombiano Agropecuario (Colombia)
ICRO	International Cell Research Organization (UNESCO)
IDRC	International Development Research Centre (Canada)
IITA	International Institute of Tropical Agriculture (Nigeria)
ILCA	International Livestock Centre for Africa (Ethiopia)
ILTAB	International Laboratory for Tropical Agricultural Biotechnology
INGER	International Network for the Genetic Evaluation of Rice
IRRI	International Rice Research Institute (Philippines)
IVAG	In Vitro Active Gene Bank Project (CIAT)
NARS	National Agricultural Research Systems
NRI	Natural Resources Institute (UK)
ODA	Overseas Development Administration (UK)
ORSTOM	Office de la Recherche Scientifique et Technique d'Outre-Mer (France)
RF	Rockefeller Foundation
SAR	Sistèmes Agro-Alimentaires et Ruraux (CIRAD)

TFP	Tropical Forages Program (CIAT)
UNIVALLE	Universidad del Valle (Colombia)
USAID	United States Agency for International Development
VRU	Virology Research Unit (CIAT)

APPENDIX 4:
ABBREVIATIONS

AC	active charcoal
AcP	acid phosphatase
$AgNO_3$	silver nitrate
AI	amylase inhibitor
IAA	indole aceticacid
AO	*Acanthoscelides obtectus*
AS	acetosyringore
ATP	adenosine triphosphate
BAP	6-benzylaminopurine
bp	boiling point
BRL	biological research labs
BGMV	bean golden mosaic virus
ca.	circa
$CaCl_2$	calcium chloride
Cb	carbenicillin
CBB	bean common bacterial blight
CCMV	cassava common mosaic virus
cDNA	complementary DNA
CK	carboxykinase
CL	*Colletotrichum lindemuthianum*
cm	centimeter(s)
CO_2	carbon dioxide
col.	colonies
CsXV	cassava X virus
cv.	cultivar
CWAI	cell-wall-bound acid invertase
2,4-D	2,4 cichlorophenoxtacetic acid
DAE	days to adult emergence
D/F	degrees of freedom
dH_2O	distilled water
DIAP	diaphorase
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNS	dinitrosalicylic acid

ECD	beta-ecdysone
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EST	esterase
FOA	fluorecein di-acetate
FSD	frog skin disease
g	gram(s)
GA_3	gibberellic acid
GC	gas chromatography
GDC	glycine decarboxylase
GOT	glutamate oxaloacetate transaminase
GUS	beta-glucuronidase
h	hour(s)
ha	hectare(s)
HCl	hydrochloric acid
Hg	hectogram(s)
HPLC	high-pressure liquid chromotography
IEF-PAGE	isoelectric focusing-PAGE
IPR	Intelectual Property Rights
J	joule(s)
Kb	kilobase(s)
kD	kilodalton(s)
Km	Michaelis constant
l	liter(s)
LB	Luria broth
LD_{50}	median lethal dose
LN	liquid nitrogen
lx	lux
m	meter(s)
M	molar concentration
MDH	malate dehydrogenase
ME	malic enzyme
$MgSO_4$	magnesium sulfate
min	minute(s)
ml	mililiter(s)
MLD	median lethal dose

mM	millimole(s)
mo	month(s)
MPFTS	multiple-purpose forage trees and shrubs
MS	mass spectrometry
mtDNA	mitochondrial DNA
N6	chinese medium N6
NAA	naphthalene acetic acid
NaAc	sodium acetate
NaCl	sodium chloride
NADH	reduced nicotinamide adenine dinucleotide
NaEDTA	ethyl-diamino tetra acetic acid, sodium salt
ng	nanogram(s)
NI	neutral invertase
no.	number
NUE	nitrogen-use efficiency
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEP	phosphoenolpyruvic acid
PEPC	PEP carboxylase
PHA	phytohemagglutinin
PI	protease inhibitor
ppm	parts per million
PPT	phosphinotricin
PrX	peroxidase
PVP	polyvinyl pyrrolidone
QAE	quarternary anion exchanger
RAC	rice anther culture
RAPD	random amplified polymorphic DNA
RBSC	rubisco
RDNA	recombinant DNA
RFLP	restriction fragment length polymorphism
RHBV	rice hoja blanca virus
RI	refracion index
RIA	radioimmunoassay
rif	rifampicin
RIP	ribosome inactivating protein
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RUBISCO	ribulose bi-phosphate carboxylase

SCARs	sequenced characterized amplified regions
s	second(s)
SDS/PAGE	sodium dodecyl sulfate/PAGE
SE	small embryo
S.E.	standard error
Sm	streptomycin
Sp	spectinomycin
sp./spp.	species
t	metric ton(s)
T-DNA	*Agrobacterium* T-DNA region
temp	temperature(s)
U.	university
ul	microliter(s)
UV	ultraviolet
V	volt(s)
var.	variety
VNTR	variable number of tandem repeats
vs	versus
wk	week(s)
WUE	water-use efficiency
XCP	*Xanthomonas campestris* pv *phaseoli*
XCPF	*Xanthomonas campestris* pv *phaseoli* var *fuscans*
YAC	yeast artificial chromosomes
yr	year(s)
ZS	*Zabrotes subfasciatus*