Biotechnology Research Unit

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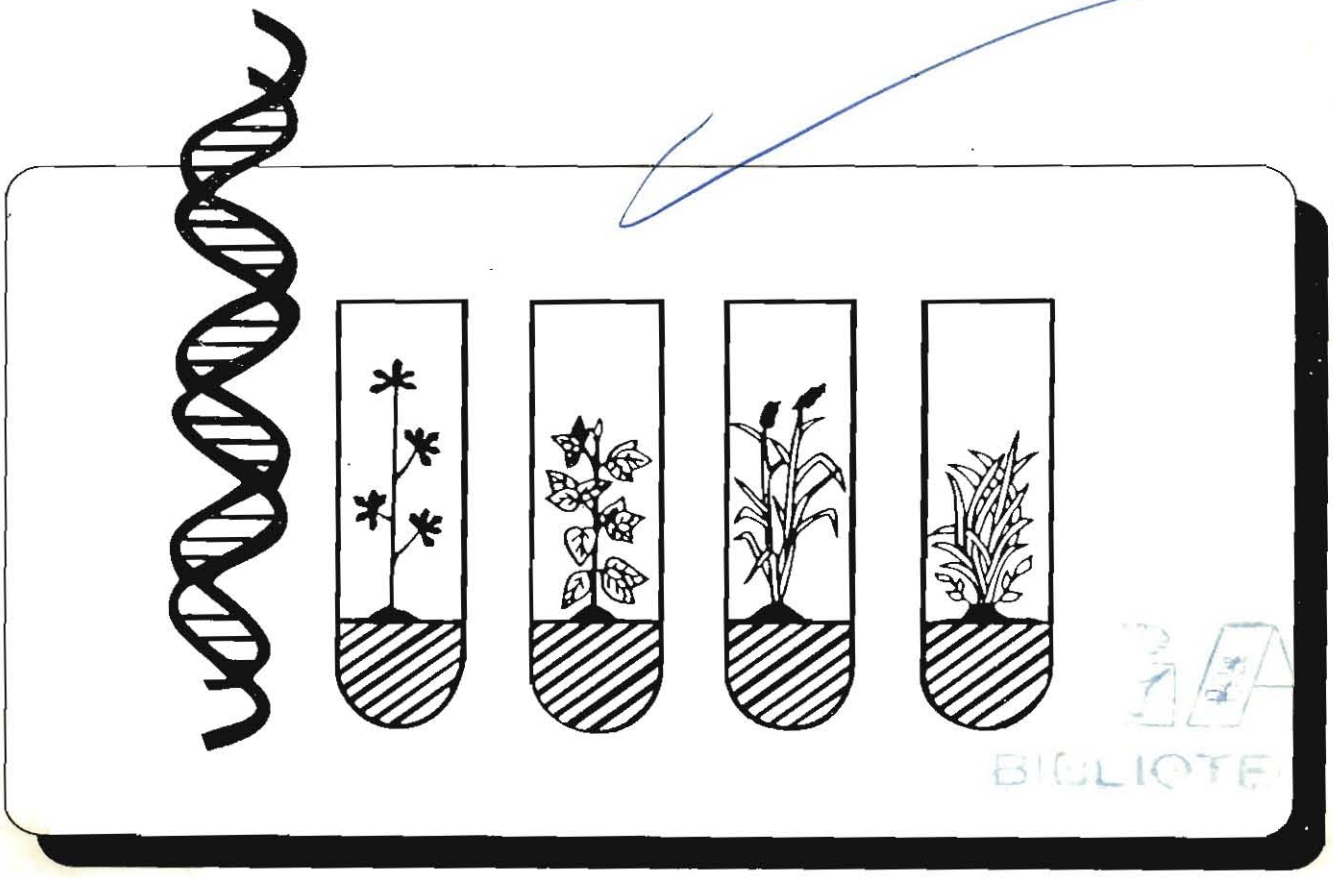

CIAT

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EXECUTIVE SUMMARY

In 1991, the Biotechnology Research Unit (BRU) has successfully continued to integrate efforts with CIAT's programs in the constant pursuit of developing germplasm well adapted to selected agroecosystems, the specific role of the BRU being the application of modern biological and biochemical technology to address critical research constraints in productivity and stress tolerance. The activities of the BRU can be subdivided into the following interrelated areas: (1) characterization and utilization of genetic variability, (2) research on crop productivity, (3) mechanisms of plant adaptation to the environment, (4) institution building.

1. Characterization and Utilization of Genetic Variability

1.1 Molecular Mapping of Crops

A solid basis has been worked out for the upcoming project of the molecular mapping of the **cassava** genome. A basic set of polymorphic markers from cassava genomic libraries has been selected within the frame of an IBPGR sponsored project. Now, with support from the Rockefeller Foundation, we are going into the generation of the linkage map using these markers. The map will be useful in the characterization of genetic variability in cassava, and will assist in future breeding projects.

A hundred clones from a common **bean** cDNA library have been selected to complement the existing bean maps generated by C.E. Vallejos (Gainesville, FL) using genomic clones (this map contains about 200 markers by now), and P.Gepts (Davis, CA). The saturation of the map is an important goal, as it is a prerequisite to get tight linkages in future gene tagging projects. The level of polymorphism of the selected clones is currently being analyzed using selected parental lines. The genome of tepary bean will be mapped as part of a project, in collaboration with the University of Ghent, Belgium, which includes the characterization of resistance factors against bruchids in beans. RFLPs (Restriction Fragment Length Polymorphism) as well as RAPDs (Randomly Amplified Polymorphic DNA) will be used for this purpose.

The genome size is an important parameter in mapping studies. In a collaboration with the Rice Program (C. Martínez) and the University of Cornell, flow cytometry was used to assess nuclear DNA contents of *P. vulgaris*, *P. acutifolius* and *P. lunatus*. The genome size seems to be smaller than expected. Differences

were observed between wild and cultivated accessions, while no relationship was found between genome and seed size, as had been speculated.

The mapping of Brachiaria has been recognized as a high priority project by the **Pastures Program**, apomixis being one of the targets for gene tagging. Contacts have been established with the Salamini group at the Max-Planck-Institute in Cologne for this mapping project. We will start by selecting parentals and providing a set of polymorphic markers. The RAPD technique will be used to generate a primary map.

1.2 Molecular Fingerprinting of Pathogenic Microorganisms

The fingerprinting of **rice blast** (*Pyricularia oryzae*), which is a cooperation between **Rice Pathology** and Purdue University, has been a very successful project, opening the doors to detailed studies on genetic variability and stability of the rice blast fungus.

Another very successful ongoing project is the fingerprinting of *Xanthomonas campestris pv phaseoli* utilizing molecular probes, developed through a cooperation between **Bean Pathology** and Wisconsin.

Both projects are being continued at CIAT in close collaboration with the BRU. The results achieved so far and the experience gained by the programs have led to new initiatives concerning three fungal pathogens, which are the causative agents of Angular Leaf Spot Disease and Anthracnose in **beans**, and Anthracnose in **Stylosanthes**, respectively. The high degree of observed variability of these pathogens justifies the efforts being undertaken. A better understanding of the epidemiology and phylogeny will assist in the design of new resistant varieties and integrated pest management.

1.3 Cryopreservation

Cryopreservation has reached a very advanced stage now. We are able to regenerate up to 70% of material stemming from frozen shoot tips. The experimental development of this IBPGR sponsored project was successfully finished this year. We have gone now into testing the applicability of the methodology to multiple genotypes of the collection. The importance of this project resides in the stable long-term storage of such a unique germplasm collection as CIAT's, thus solving a logistic problem and conserving potentially useful germplasm for future breeding programs.

2. Crop Productivity Research

2.1 Gene Tagging

The final application of any genetic map is its use in breeding. We have been very successful in using molecular markers from the **rice** map (acquired from S. Tanksley, Cornell University) to map resistance loci for the Rice Hoja Blanca Virus (RHBV) and Rice Blast. For tagging resistance genes to blast, bulk analysis of susceptible and resistant doubled haploids was performed. The RAPD markers linked to blast resistance are currently being checked on larger F2 populations and additional doubled haploid lines from the same cross. We have gone very early into the utilization of newly developed techniques, e.g. RAPD, which are very promising as to their direct applicability in breeding programs because of their simplicity and their potential to handle large numbers of crosses, a major constraint to other technologies.

In **beans** we are aiming at tagging the resistance loci against BGMV and the Common Bean Weevil. Another important issue will be the merging of the **bean** maps generated by Vallejos (Gainesville, FL) and Gepts (Davis, CA), which will lead to a more saturated map. The RAPD technique, combined with the bulk screening method, is currently being used to tag the resistance locus to the Kappa race of anthracnose.

2.2 Interspecific Hybridization

The introgression of tepary bean (*Phaseolus acutifolius*) characteristics into common bean using a congruity backcross approach, has advanced further this year. The fourth congruity backcross generation (CBC4) has now been crossed with *P. vulgaris* and the first resulting embryos from that cross are presently being grown in vitro. The goal of this recurrent crossing scheme, is to force recombination between the two genomes as to attain fertile and commercially useful plants with important tepary bean traits, like drought and heat tolerance, as well as *Empoasca* and bacterial blight resistance. We have already morphological and biochemical evidence for stable introgression of tepary bean into common bean.

2.3 Genetic Transformation

Genetic transformation is a prerequisite for the non-classical introduction of useful traits into plants. Such traits include at the moment genes for insect resistance, protection against viral infection, and herbicide resistances. Sources of resistance against fungal pathogens, genetic manipulation of protein contents, genes

leading to cold-tolerance, these are some of the upcoming new possibilities for the future.

The forage legume ***Stylosanthes guianensis*** is CIAT's first transgenic plant. It was transformed using a construct which includes two selectable marker genes that confer resistance to an antibiotic and a herbicide, and a scorable marker gene whose expression can be detected by a histochemical assay. Regenerated plants have conserved their herbicide resistance trait. The Mendelian segregation of the trait is presently being studied in the F1 progeny in the greenhouse.

Another pasture, ***Brachiaria***, is passing through the first step that is needed for successful transformation: regeneration. *Brachiaria* has responded very well to preliminary experiments dealing with somatic embryogenesis, thus we can now start phase number two, which is the introduction of foreign DNA and selection of the transgenics. Another crop that is responding well to preliminary regeneration experiments is **rice**. In a collaboration between the **Rice Program** and the BRU, viable protoplasts have been successfully isolated from indica type rice embryogenic suspension cultures stemming from mature seeds. Regeneration experiments are underway. Protoplasts are amenable to direct DNA transfer, and have been used to produce transgenic rice of the japonica type.

In **beans** we have adapted a powerful regeneration protocol starting from cotyledonary nodes. The organogenic meristematic ring in the cotyledonary area will be a target for Agrobacterium-mediated or direct DNA transfer. Virulent Agrobacterium strains have been selected after a meticulous screening procedure, as well as susceptible bean cultivars. Adventitious shoot formation has been histologically demonstrated. Transient gene expression of a scorable marker gene has been detected after infection with *Agrobacterium* and after particle bombardment with DNA coated tungsten microprojectiles.

Cassava can be regenerated from secondary embryos forming on embryogenic calli stemming from apical meristems. This is presently our target tissue for transformation experiments using *Agrobacterium* and the particle gun.

2.4 Doubled Haploids

The incorporation of pollen derived doubled haploids into breeding programs reduces the time to obtain fixed lines to two generations of selection without affecting genetic variability and stability. The **Rice Program** is using anther culture to introgress early grain maturity and good grain quality into cold tolerant germplasm, to increase the recovery of useful recombinants from wide crosses, to facilitate the transfer between savanna and irrigated materials, and to produce fixed lines for the mapping of resistance gene loci. We have been able to achieve a 35 fold enhancement in the production of green plants from indica types, which had proved

unresponsive to anther culture up to now. This has been a constraint, particularly in the breeding for irrigated rice.

2.5 Somaclonal Variation

14 ***Stylosanthes guianensis*** somaclonal lines generated through in vitro regeneration have been tested for their agronomic performance. A main goal is the search for plants tolerant to acid soils. Some of the lines have shown a superior agronomic performance than the check, others have shown some special morphological characteristics, like dwarfism, chlorotic foliage, 1-2 leaflet leaves, and one tetraploid line. These traits have shown to be inheritable through four generations.

2.6 Utilization

Another topic that is related to productivity, is the fermentation of **cassava** starch to produce bitter starch. This artisanal product is very important for Colombia, and needs input with respect to well defined parameters and possibly inocula, to enhance starch quality and reproducibility of the process. Together with the Cassava Utilization Unit in a collaborative project with CIRAD/CEEMAT (France), the BRU has been working on the characterization of microbial amylolytic activities involved in the process. This results, added to the studies being done at the microbiological and physicochemical levels, will provide a more accurate picture of the fermentation process, which is not a simple one, as we are dealing with a solid state fermentation and mixed microbial populations.

3. Mechanisms of Plant Adaptation to the Environment

3.1 Resistance to Pests

A major constraint in **bean** storage is the bruchid *Acanthoscelides obtectus* or Common Bean Weevil. Very few resistant accessions have been found in wild beans collected in only a small number of locations in Mexico. Resistance has also been found in wild lima and tepary beans. A few years ago resistance to the Mexican Bean Weevil (*Zabrotes subfasciatus*) was found in a similar way. After identification of the factor involved, a protein from the lectin family named arcelin, an immunoassay was developed that accelerated breeding enormously. We have set for the identification of the factor involved in the resistance to the Common Bean Weevil. Some promising results have been obtained with a protein fraction stemming from the resistant accessions; artificial seeds enriched with this fraction lead to high mortality rates in insects feeding on them. Other approaches include the analysis of

inhibitors of digestive enzymes, as well as other types of biomolecules, using separation techniques like HPLC and other analytical tools, combined with enzymology and feeding experiments.

In a similar way we are looking for an antib otic factor involved in the resistance against the spittlebug in ***Brachiaria***, together with the **Pastures Entomology** group. A phytoecdysteroid has been postulated as the putative resistance factor, due to the observed effects on the insect, which resemble ecdysone action on the development of the insect. We are trying to develop an immunoassay for positive identification of the substance as well as for the development of a screening procedure to assist in breeding programs.

3.2 Photosynthesis

Cassava is well known for its drought and heat tolerance. Together with the **Cassava Physiology** group, we are interested in the adaptation mechanisms of cassava for dealing with this climatic constraints without loosing productivity. Cassava has shown some photosynthetic characteristics that range between typical C3 and C4 plants. Additionally to the physiological experiments being performed, we want to analyze possible compartmentalization of photosynthetic enzymes in the leaf by in situ hybridization, using labelled gene probes. We have shown that a high degree of homology exists between maize and cassava photosynthetic genes by hybridization techniques (maize clones from T. Nelson, Yale). We are in the process of generating a genomic cassava library from where we want to fish out the correspondent cassava clones, as heterologous probes are not useful for in situ hybridization. A mechanistic explanation of the phenomenon will aid us in searching for better-performing genotypes in the cassava collection, as there is variation of the physiological parameters among them.

4. Institution Building

4.1 Networking

Financiation of the Advanced Research Network for Cassava by the Dutch Government has been confirmed. The budget includes the position of a coordinator, who will be placed at CIAT, the publishing of a newsletter, and bridging funds for the initiation of projects. A meeting of the members of the network is scheduled for August 1992 in Cartagena.

Some members of the Steering Committee of the Advanced Phaseolus Beans Research Network (founded in September 1990) met at the BIC Meeting in

November in Nebraska, to discuss the needs of the network. A recommendation was made for CIAT to actively seek for potential donors for the network.

4.2 Training

Four PhD students have been doing practical research at the BRU. **Alvaro Mejía** (Bonn University, FRG, funded by the BMZ), who is working on the tepary bean introgression into common bean using congruity crosses and embryo rescue techniques, and on regeneration from embryogenic suspension cultures. **Martine Korban** (McGill University, Montreal, funded by IDRC), who has been working on bean regeneration and transformation. **Bill Welsh** (University of Manitoba, Winnipeg, funded by CIDA) is working on the characterization of recombinant inbred lines from crosses between Mesoamerican and Andean gene pools. **Rodrigo Hoyos** (Michigan State University) spent some time working on regeneration of bean plants from embryogenic suspension cultures.

It is already a tradition for biology and agronomy students from the Universidad del Valle and the Universidad Nacional de Colombia, Seccional Palmira, Universidad de San Buenaventura and Universidad Santiago de Cali to do their thesis research at the BRU. Their one year research work usually reaches levels of MSc thesis elsewhere.

The BRU has organized introductory courses to Molecular Genetics for CIAT's programs as to acquire a common language that will facilitate exchange of ideas, and to make clear what kind of contributions the BRU could make to their specific problems. On the other hand, we have been offered introductory courses to different areas of the programs, like breeding and pathology, for the same purpose. Courses have also been held at the Universidad Nacional
in Bogotá and Medellín.

4.3 Biosafety

The BRU assumed a major responsibility in the editing of the Institutional Biosafety Guidelines. These have been approved by external highly qualified reviewers, thus representing the state-of-the-art situation of biosafety regulations in the world. We are already in contact with representants of the Colombian government, in order to develop national regulations in the spirit of reaching uniform regulations all across Latin America, observing the highest standards of developed countries.

PHASEOLUS BEANS

1. Interspecific Hybridization *P. vulgaris* x *P. acutifolius*

This is a collaborative project between the Bean Breeding Program (Dr. S.P. Singh) and the BRU that was initiated in 1989. The main objective of this project is to transfer multiple desirable traits such as drought, heat, Empoasca and bacterial blight resistance, from cultivated tepary bean lines (*P. acutifolius*) to common bean genotypes possessing desirable agronomic traits.

Two different backcross strategies are being followed to achieve this goal: The classical or introgressive backcross and the congruity backcross which consists of backcrossing the F_1 hybrids alternatively with lines of each species (Haghighi and Ascher, 1988). The partial results of this project can be seen in the accompanying Table 1. (See also BRU Annual Report 1989 and 1990). Because hybrid embryos were aborted following interspecific crosses, it was necessary to apply embryo rescue techniques in most of the crosses to allow embryo germination and growth *in vitro*.

The choice of the right parental genotypes, mainly that of the *P. vulgaris* seed parent, was decisive for obtaining mature F1 plants. Using ICA Pijao as proposed by Parker and Michaels (Parker and Michaels, 1986), in combination with the *P. acutifolius* varieties G40001 and G40066, 62 vigorous growing hybrids were obtained. All the F1 hybrids were self-sterile, but embryos that could be rescued and grown to plants were obtained when the hybrids were backcrossed with several advanced breeding varieties of *P. vulgaris*. Thirty four BC1 plants were obtained, 16 of these were self-fertile and produced mature BC1-F2 seeds. With the second backcross with *P. vulgaris* lines, large numbers of mature BC2 seeds were produced.

These materials have been planted in Santander de Quilichao and a large number of BC1-F_4 and BC2-F_3 progenies are presently being evaluated by the Bean Breeding Program. Stable introgression of *P. acutifolius* traits has been detected, both morphologically and biochemically, by the analysis of total seed protein with SDS-PAG-Electrophoresis of BC1-F_5 seeds.

After the second backcross to *P. vulgaris*, BC1 plants were backcrossed with *P. acutifolius* lines to generate congruity backcross 2 (CBC2) hybrids. The embryos of these crosses were aborted very early, 8 to 12 days after pollination. The embryo rescue technique used earlier to obtain F1 and BC1 plants was not adequate for obtaining plantlets of the CBC2 embryos, that could otherwise survive the transfer to the greenhouse. Only after a modification of the embryo rescue technique (BRU Annual Report 1990), 60 CBC2 hybrids could be established.

All the CBC2 plants were self-sterile but immature embryos and mature CBC3 seeds were obtained when backcrossed to *P. vulgaris* lines. Initially in this cross, problems with abnormal embryo growth and plantlet lethality appeared, but these could be solved by changing the parental lines used. Sixty five CBC3 plants were established in the greenhouse. Most of them have shown to be self-fertile, producing mature CBC3-F_2 seeds.

Currently, we are backcrossing the CBC3 plants with the best *P. acutifolius* lines and we are obtaining immature embryos and mature CBC4 seeds (the first after a backcross with *P. acutifolius*) which reached a greater development and have presented fewer difficulties for *in vitro* culture and their transfer to the greenhouse as the homologous embryos of the past congruity backcross cycle (CBC2). Since they have the same *P. acutifolius* parentals, this has been interpreted as being caused by an improved recombination ability between *P. vulgaris* and *P. acutifolius* genomes. Twenty-three CBC4 hybrids are now flowering in the greenhouse and we hope that with the present embryo rescue efficiency, we will reach our goal of obtaining 200 flowering CBC4 hybrids in a few weeks.

The CBC4 x *P. vulgaris* backcross has been initiated and the first resulting embryos are presently being grown *in vitro.*

References

Haghighi KR and Ascher PD (1988) "Fertile, intermediate hybrids between *Phaseolus vulgaris* and *P. acutifolius* from congruity backcrossing". Sexual Plant Reproduction 1, 51-58.

Parker JP and Michaels TE (1986) "Simple genetic control of hybrid plant development in interspecific crosses between *Phaseolus vulgaris* L. and *P. acutifolius* A. Gray". Plant Breeding 97, 315-323.

2. Recombination Between *P. vulgaris* Gene Pools

The common bean (*Phaseolus vulgaris* L.) is an example of a non-centric crop in that it can be divided between two centres of domestication: Middle America and Andean South America. Morphologically, the Middle American bean is characterized by its small size relative to that of Andean material, typically characterized by larger seed sizes.

A system of landrace classifications for the common bean has been established by Singh, et al. (1988) with respect to site of origin, environmental adaptability, as

Table 1. Summary of partial results of the *P. vulgaris* x *P. acutifolius* interspecific hybridization program 1989-1991.

Type of Cross	Resultant Hybrids	Genic Dosis Vulg: Acut.	Embryo[1] Develop.	Viability[2]	Mature[3] Plants	Fertility[4]	Actual Status	Parentals[5]
P. vulg. x P. acut.	F1	50:50	24.54	20.51	64	0%	Concluded	ICA,PIJAO,G40001, G40066
F1 x P. vulg.	BC1	75:25	27.47	10.85	34	50%	Concluded	A775,A798,A752,MAM38, A797
BC1 x P. vulg.	BC2	87.5:12.5	<35	91.76	332	89.50%	Concluded	ARA9,SC83,MAR1,A769, A800,PEF14
BC1 x P. acut.	CBC2	37.5:62.5	11.69	22.4	58	0%	Concluded	G40001,G40023
CBC2 x P. vulg.	CBC3	68.75:31.25	19.48	14.28	65	<50%	Concluded	ICA PIJAO,A800,MAR1, ARA9,PEF 14
CBC3 x P. acut.	CBC4	34.38:65.63	16.15		23	?	Initiated	G40001,G40023
CBC4 x P. vulg.	CBC5	67.19:32.81					Initiated	
CBC5 x P. acut.	CBC6	33.59:66.41						
CBC6 x P. vulg.	CBC7	66.79:33.20						

[1] Average days of development of the embryo
[2] % of mature plants from cultivated embryos
[3] Plants which reached flowering
[4] % of mature plants which produced F2 seeds
[5] Varieties utilized efficiently as parentals: in order of efficiency

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well as the other following agronomic traits: growth habit, days to maturity, yield/per hectare (kg/ha). This classification system conforms to six landraces: Middle America (M), Durango (D), Jalisco (J), Nueva Granada (N), Chile (C), Peru (P). The first three landraces correspond to the Middle American centre of domestication, while the latter three belong to the Andean group.

There exists an average yield differences of 1500 kg/ha between the two main centres, or groups, thus emphasizing the need to develop seeds possessing a higher yield potential. Nevertheless, genetic incompatibility problems (F1 hybrid weakness, dwarfism, heterosis) between the larger-seeded Andean material and its small-seeded, higher-yielding Middle American counterpart have prevented successful crossing of materials between the two groups.

The purpose of this study is to evaluate recombinant inbred lines (RILs) derived from four contrasting populations. Each population differs from the other in terms of its morphological characters and agronomic traits. This study is being conducted to determine the existence of correlative relationships of these morpho-agronomic traits with existing polymorphisms of the seed proteins (phaseolins, lectins, albumins) and selected isoenzymes.

This work is a Ph.D. thesis (W. Welsh, University of Manitoba, Canada) carried out at CIAT in collaboration with the Bean Breeding Program (S.P. Singh).

In this study, 79 recombinant lines (RILs) have been developed for each of the four following populations:

Population 1.	Canadian Wonder x A486 (79 RILs and 2 parents)
Population 2.	G-76 x MAM4 (79 RILs and 2 parents)
Population 3.	ABA58 x G4830 (79 RILs and 2 parents)
Population 4.	ICA L23 x G3807 (79 RILs and 2 parents)

Population "One" involves two cultivars of determinate growth habit I, both possessing large seed size. This is a cross between two Nueva Granada landraces. Therefore no genetic incompatibility is expected as this population represents the control.

Population "Two" represents a cross between G-76 (Nueva Granada landrace) and MAM4 (Durango landrace). The former possesses a determinate type I growth habit and large-sized seeds. The latter parent possesses a type III indeterminate growth habit and medium-sized seeds. The Durango landrace is known to possess generally higher yielding capacities than other lines of the common bean.

Population "Three" is a cross between a parent of a determinate type I growth habit possessing large-sized seeds (ABA-58, Nueva Granada) and a parent of

indeterminate type II growth habit possessing small-seeded material (G4830, Middle America). Of all the populations, this population represents a cross between two parents with the greatest genetic distance between them. This population contrasts with population "One" in terms of genetic distance.

Population "Four" represents a cross where growth habit has been maintained between the two parents, but where seed size is different: ICA L23 (large seed-Nueva Granada) x G3807 (small seed-Middle America).

The recombinant inbred lines (RILs) of each population represent F2 generation-derived plants of the F6 generation. All four populations were planted in duplicate randomly ranked rows (approximately 40 plants/row) at two localities over two growing seasons. The first site was Popayán (1750 m.a.s.l.) on May 02/90 and Oct 03/90. The second site was Palmira (1000 m.a.s.l.) on May 29/90 and Oct 09/90. Average growing periods for Popayán and Palmira are four and three months, respectively.

Evaluation of Morpho-Agronomic Characteristics

The following morpho-agronomic traits were evaluated: days to flowering, flower colour, bracteole type, bracteole size, leaf type, chlorophyll content, 5^{th}-6^{th} internodal length, days to maturity, grainfilling trait, number of seeds/pod, 100 seed weight, harvest index, growth habit, seed yield.

Biochemical Markers

- Isoenzymes

All 79 RILs and their respective parents were analyzed electrophoretically on 10% starch gel system in a lithium borate buffer system.

The tissue material evaluated was taken from growing two 115 days old plants. Extraction buffer to tissue ratios were: 1:1 (root tissue), 1:2 (leaf tissue). The buffer used was a Tris-Malate based extraction buffer (see CIAT Working Doc No. 40).

The following isoenzymes were analyzed in the root material: diaphorase (DIAP), malic enzyme (ME), malate dehydrogenase (MDH), shikimic dehydrogenase (SKDH).

For the leaf tissue: acid phosphatase (ACP), rubisco (RBSC), glutamate oxalo-acetate transaminase (GOT).

- Seed proteins

The seed proteins were separated on one-dimensional SDS-PAGE systems and stained with Coomassie-Blue G. The parental materials for each population were used as standards. Each RIL was run in triplicate, therefore a subtotal of 243 lines per population were run. Consequently, an overall total of 992 lines were analyzed on the SDS-PAGE system.

Results

- Isoenzymes

The isoenzymes that demonstrated the greatest polymorphism in the evaluated populations were the following:

In the root tissue: DIAP, SKDH, and MDH
In the leaf tissue: ACP and RBSC

- Seed proteins

Pattern differences were occasionally encountered within the lines, each run in triplicate. In population 'Two', 12 lines presented this problem. In population 'Three', three lines, and in population 'Four', 23 lines. These differences are most likely attributed to the slight occurrence of mechanical mixture and/or a small degree of segregation.

Lectins and albumins

- Some differences within certain lines were found for the lectin patterns (e.g.: population 'Two', RIL 157).

- There seems to be a relationship between the pattern of the lectins and the albumins. If the lectin profile of one RIL was similar to that of the second parent, the albumin profile of the same RIL would have the albumin profile of that second parent.

- It was also revealed that there were combinations or contributions of both parents in the formation of bands in a region in the gel, above the albumins.

Current Work

At present, statistical analysis of both the morpho-agronomic data and biochemical data is being conducted in order to visualize existing correlations between the biochemical markers and the agronomic characters studied in order to properly select parental materials that will permit effective crosses between landraces, avoiding the genetic incompatibility problem.

Concurrent with the statistical analysis, DNA is presently being extracted from each of the RILs and their respective parents to be used in restriction fragment length polymorphism (RFLP) studies. This approach will address the problem of indirect genomic analysis (proteins, isoenzymes) and bypass those problems associated with analyzing a (possibly post-translationally modified) product of gene expression by focussing directly on the genome itself.

3. Plant Regeneration of *Phaseolus* spp from Cell Cultures

Within a collaborative project with the University of Bonn (Prof. H.J. Jacobsen, now at the University of Hannover, Germany), and as a part of a Ph.D. Thesis (A. Mejía), we have been conducting work on the regeneration of *Phaseolus* bean plants from cell cultures.

Development of *in vitro* regeneration techniques is a pre-requisite for the application of genetic transformation, *in vitro* selection, haploid induction, and somatic hybridization for the improvement of beans.

For the first time, in 1991 we have been able to initiate morphogenic cell suspension cultures and differentiate buds on callus cultures of the cultivated *P. acutifolius* genotype G40043. The methodology is similar to the one developed by Kumar et al. (1988) for the regeneration of *Vigna unguiculata*, and involves the following steps:

- Induction of callus from root or hypocotyl of 7 day old seedlings in the medium M-MS with 2 mg/l 2,4-D and BAP.

- Selection of green, compact, callus and transfer to the medium M-MS with 1 mg/l thidiazuron. In this medium, a more friable granular callus is formed and used to initiate cell suspensions.

- Cell suspensions are initiated in liquid M-MS medium, with 2 mg/l 2,4-D and BAP, and supplemented with 5 mg/l L-asparagine.

- Weekly subculturing of the dense suspension fraction (cell clusters which can be readily decanted). Green, compact, calli are formed in this suspension.

- Plating of green, compact calli (clusters larger than 5 mm) on the medium M-MS with 2 mg/l BAP.

We have obtained bud induction on callus in two independent experiments and using different separate cell suspension lines. We are now developing medium conditions for shoot elongation. Elongated shoots will be rooted for transfer to soil.

We have screened a number of genotypes from cultivated *Phaseolus* species and their wild relatives for the formation of organogenic callus similar to *P. acutifolius*. Organogenic callus is compact, light yellow-greenish or green in color, comprising small spherical, non-vacuolated cells. Genotypes of most of the *Phaseolus* species tested (*P. coccineus, P. polyanthus, P. lunatus, P. filiformis* and *P. xanthotrichus*) formed organogenic type calli, with the exception of the *P. vulgaris* genotypes. The latter formed white or brown soft, spongy callus comprising large vacuolated cells. Among the *P. vulgaris* genotypes tested we included 10 wild genotypes from Mexico down to Argentine.

This work has shown that the induction of organogenic callus is highly dependent on the genotype, with *P. vulgaris* being the only bean species not forming organogenic callus.

Furthermore, we have found that induction of callus from tissues of interspecific *P. vulgaris* x *P. acutifolius* hybrids (congruity backcross 3-F1 and congruity backcross 4-F1 plants from our work on interspecific hybridization) showed the segregation of two callus types: an organic callus similar to *P. acutifolius* and a non-organogenic callus like *P. vulgaris*. This suggests the feasibility of transferring regenerability genes from *P. acutifolius* to *P. vulgaris* by sexual crossing.

Our future work will focus on:

- optimization of the protocol for bud differentiation, shoot elongation and rooting.

- adapt the technique to the *Phaseolus* spp.

- select recombinant lines from the project on interspecific hybridization which respond to the treatment.

- because our results with the species indicate that the *P. vulgaris* callus may lack a critical regeneration factor, we will design a series of systematic experiments to modify the culture medium and provide the required factor(s).

References

Kumar AS, Gamborg OL, Nabors WM (1988) Plant regeneration from cell suspension cultures of *Vigna aconitifolia.* Plant Cell Rep 7, 138-141.

4. Genetic Transformation of Common Beans

Research on the genetic transformation of common beans using the *Agrobacterium* and the particle gun systems was initiated last year in the BRU as part of the PhD thesis of Martine Korban, from McGill University, Montreal, thus linking the efforts of Alvaro Mejía and Rodrigo Hoyos, on regeneration of beans from suspension cultures.

Several grain legumes, including beans, have been reported to be susceptible to *Agrobacterium* infection. However, little is known about bean genotype vs *Agrobacterium* strain specificity in this crop species. Plant compounds can interact with the virulence functions of an *Agrobacterium* strain, and their presence may change according to the wounding response and the stage of development of the plant. Phenolic compounds, like acetosyringone, have been reported to enhance virulence functions of *Agrobacterium tumefaciens* (Stachel et al., 1985).

Therefore, as a first step in this study, it was important to determine the **relative susceptibility of bean tissues** to infection by strains of *Agrobacterium.* Screening of a collection 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 **plant regeneration and 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 reproducibly achieved, as shown by histological analysis. Preformed shoots were eliminated early during the treatment.

The virulent **Agrobacterium strain C58 was transformed with a binary vector** containing marker genes of interest. The resident Ti megaplasmid of *A. tumefaciens* (around 200 kb, and therefore difficult to manipulate) can provide the transfer functions in trans, i.e. any DNA within the "25 bp Border Sequences" of the T-DNA will be integrated into the plant genome by the vir-gene functions. These

border sequences, called Left and Right Borders, are incorporated into a small plasmid (around 13 kb), which can be easily manipulated. We used the pGV1040 plasmid, which has two selectable marker genes, the **aph II (npt II)** gene, which confers resistance to kanamycin, and the **bar** gene which confers resistance to Basta, a non-specific herbicide, whose active component phosphonitricin, a tripeptide analog, inhibits glutamine synthase in plants. This plasmid also carries a scorable marker gene, the **uidA** or **gus** A gene, that codes for ß-glucuronidase, which can be detected in transformed tissues using histological assays. The plasmid also includes resistance genes against the antibiotics streptomycin (Sm) and spectinomycin (Sp) to select the plasmid-containing bacteria on selective media.

Agrobacterium was transformed with pGV1040 using a novel methodology that obviates conjugation of the binary vector from *E. coli* to *Agrobacterium* (Höfgen and Willmitzer, 1988). Direct transformation was achieved by rendering *Agrobacterium* cells competent through a $CaCl_2$ treatment combined with freezing and thawing of 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 variety Ica Pijao to determine the minimal lethal concentration that should be used during the selection phase after transformation with pGV1040. The lethal dosis was established at 1 mg/l Basta in the medium. At 0.8 mg/l growing shoots were still observed after 7-10 d of 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. Transformation efficiency was scored by histological visualization of gus gene expression in the regenerated shoots.

The complete dipping of the half cotyledonary node tissue was not a good inoculation treatment, since it hindered shoot regeneration and permitted excessive multiplication of the bacteria. Bacteria could be better controlled following pricking of the node area. The best antibiotic treatment for the elimination of 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.

No significant difference was observed when AS was added to the bacteria, with respect to the regeneration or the transformation efficiency of the inoculated explants as compared to controls. Regeneration of bean shoots is decreased by inoculation with C58 (pGV1040), many explants necrotized during the procedure. In the average, 5-10 adventitious buds arose from these explants. Of those which

survived the treatment, GUS positives were scored as the number of regenerating buds showing positive GUS activity. Results of five separate experiments are presented: (i) 14 buds out of 161, stemming from 28 explants. (ii) 6 buds out of 37. (iii) 4 out of 15 (iv) 2 out of 27, containing 200 µM AS. (v) 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. Moreover, the addition of AS did not particularly increase transformation efficiency. Expression was detected in buds, and hence indicate that bean cotyledonary nodes are amenable to transformation and subsequent regeneration.

Transient gene expression using the particle gun has been achieved on cotyledons as well as around isolated apical meristems of germinating beans. For this purpose the plasmid pGV1040 was directly coated by $CaCl_2$ precipitation onto 1 µm tungsten microprojectiles. We have been working on the improvement of the impact density on target tissues, which is extremely important as to increase the probability of hitting a cell that will give rise to a regenerated plant.

Future studies include the following:

1. Study the early events of *Agrobacterium*-mediated transformation by determining the spatial arrangement of regenerable cells of bean cotyledonary nodes that are amenable to transformation using an improved gus gene construct, the gus-intron construct (Vancanneyt et al., 1990). The use of an intron-containing gene completely eliminates background expression by the bacteria, which could hamper evaluation of GUS expression. Only eukaryotic cells are capable of splicing, which is the act of assembling exons to give rise to the mature mRNA. The GUS assay also allows for the analysis of DNA transfer events independently of tumor formation. Tumor formation is the result of phytohormones produced by the T-DNA gene products, and the resulting hormonal imbalance in the transformed tissue. An efficient DNA transfer could pass inadverted if the hormonal imbalance does not lead to tumor formation.

2. Finally achieve genetic transformation of common beans and study the stable incorporation of the genetic information into the genome by molecular and genetic means. These include measuring gene expression at the transcriptional and translational levels, as well as studies on Mendelian inheritance of the traits.

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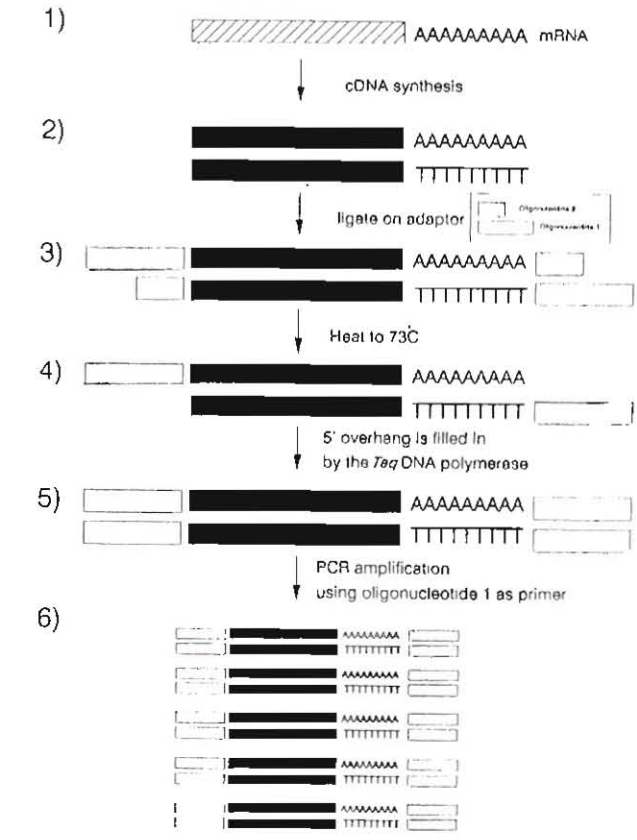
Vancanneyt G, Schmidt R, O'Connor-Sánchez A, Willmitzer L and Rocha-Sosa M (1990) Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in Agrobacterium-mediate d plant transformation. Molec Gen Genet 220, 245-250.

5. RFLP Linkage Mapping of *Phaseolus vulgaris*

The synthesis of a cDNA library from *Phaseolus vulgaris* was accomplished this year, after solving some problems encountered in the last step of the procedure, described in last year's BRU Annual Report. This library will serve as the source of genetic markers to further saturate the linkage map generated by C.E. Vallejos at the University of Florida, which is made up mostly of genomic DNA markers. The genetic map of *Phaseolus* currently comprises around 250 markers covering about 800 cM. Our work is part of the MSc thesis of H. Ramirez, UNC Palmira, aimed finally at the mapping of valuable agronomical traits, like resistance genes to the bruchid *Acanthoscelides obtectus*, to Anthracnosis (*Colletotrichum lindemuthianum*), to Apion, or to Bean Golden Mosaic Virus (BGMV).

The problems encountered in the production of the cDNA library seem to have been in the low amount of cDNA obtained before ligation into the plasmid vector. We overcame this inconvenience by utilizing a recently published protocol which uses the Polymerase Chain Reaction (PCR) to amplify the synthesized cDNA by several orders of magnitude (Jepson et al., 1991). Usually some sequence information is needed in order to synthesize the oligonucleotides which will prime the reaction catalyzed by the thermostable Taq Polymerase. In this case the longer of the adaptors, which are used to generate sticky ends at both ends of the cDNA, is used as primer, after filling the cohesive ends (see Fig. 1). The cDNA was amplified over 35 cycles, that is equivalent to an amplification factor of 2^{35} or more than 10^{10}. After the amplification, cohesive ends were regenerated by digestion with Eco RI, the 3'-ends were dephosphorylated with alkaline phosphatase to prevent self-ligation, and finally the cDNA was ligated into the pUC19 plasmid vector and transformed into *E. coli*.

100 insert-containing cDNA clones, with an average size of 600 base pairs, have been selected. Their level of polymorphism is currently being assessed by hybridization to DNA of the bean parental lines selected for the tagging studies.



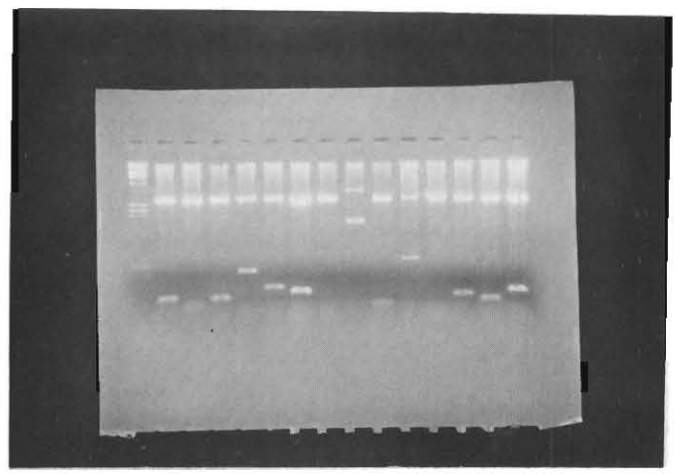


Figure 1. Top: Schematics of the PCR driven cDNA amplification procedure used for the generation of the bean cDNA library.
Bottom: Electropherogram showing cDNA inserts that have been cut out of the vector by Eco RI digestion. Left lane shows a molecular weight marker; the lowest band has 564 bp.

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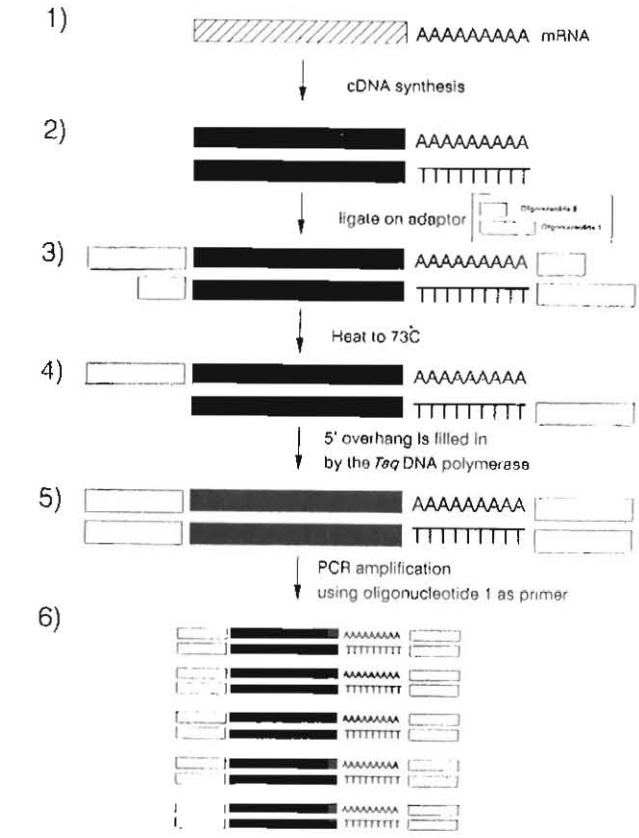

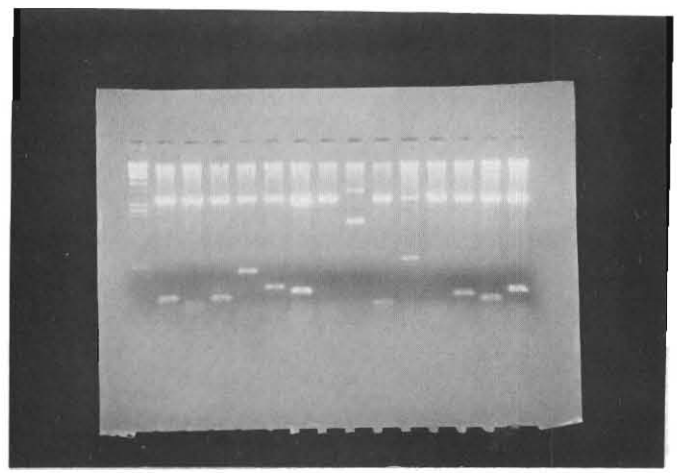

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The parental lines selected are:

Resistance to	Parentals
Acanthoscelides obtectus	G12952
Anthracnose (race Kappa)	APN 18
Apion	APN 18
BGMV	DOR 60 (tolerant)

References

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6. **Tagging Bean Golden Mosaic Virus Resistance Genes**

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 molecular markers. BGMV is a geminivirus transmitted by the whitefly *Bemisia tabaci.* It 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. The preferred strategy for dealing with BGMV has been the screening of germplasm and the deployment of tolerant genotypes.

Breeding for resistance has been slow and laborious due to the lack of true BGMV resistance sources. The sources of tolerance initially used belong to the tropical black beans gene pool such as the landrace "Porrillo". Additional sources of tolerance have been recently identified. They belong to: 1) Mexican highland germplasm such as the Pinto 114 and Garrapato, 2) Andean germplasm, 3) *P. coccineus* germplasm. Combining some of the different sources has resulted in the release of several lines in Guatemala and other countries of Central America. The lines tested in the field (e.g., DOR 364, DOR 500 etc.) have consistently outyielded previous sources of tolerance and local landraces.

Screening of populations is frequently complicated by difficulties in managing the *Bemisia* vector and in controlling extraneous problems in the field. Recent developments in the area of biotechnology have produced molecular tools that could assist breeders in managing the BGMV constraint. Molecular markers such as restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNA (RAPD; Williams et al., 1990) could provide additional tools for plant

breeders to better characterize the progenies generated and to select lines with the desired gene combinations.

RFLP markers from the already constructed bean map are being used on two populations: 1) a backcross recombinant population and 2) a recombinant inbred population. The backcross recombinant population was derived from a cross between DOR 60 (Porrillo source) and APN 18-1 while the recombinant inbred was derived from a cross between DOR 364 (Pinto and Porrillo source) and A 686. The screening for BGMV has already been conducted under field conditions in Guatemala.

In addition to RFLP, preliminary work is being initiated with random primers to look at the feasibility of using the random amplified polymorphic DNAs (RAPD) technique on resistant and susceptible bulk for the mapping (Michelmore et al., 1991).

The DOR 60 by APN 18-1 population is also being used for two additional traits, Apion and anthracnose, race Kappa. The Apion characterization will be conducted in Honduras, and like BGMV is a trait difficult to screen for and does not occur in Colombia. Molecular markers will facilitate the breeding effort, specially by combining them with BGMV markers. Tagging the anthracnose resistance gene to race Kappa will allow a pyramiding scheme for disease resistance.

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7. Tagging *Acanthoscelides obtectus* resistance genes

The seed storage pests *Zabrotes subfasciatus* and *Acanthoscelides obtectus* are major constraints for small farmers in all bean production areas of the world. To provide an alternative to the use of costly and dangerous chemicals, genetic resistance research has received major priority at CIAT. Resistance to these bruchids was not found in cultivated *P. vulgaris*, but high levels of antibiosis to both bruchid species have been identified in a number of wild *P. vulgaris* populations of

Mexican origin. The resistance is also found in wild *P. acutifolius* (tepary bean) but not in the cultivated forms. In Lima Beans (*P. lunatus*) the resistance is found in both wild and cultivated accessions but only to *A. obtectus*.

While Arcelin, a novel bean seed protein present only in resistant wild accessions, has been associated with resistance to *Z. subfasciatus* in *P. vulgaris*, no information has been obtained for *Acanthoscelides obtectus*. Initial studies suggested that resistance could be due, at least in part, to two recessive genes. The tagging project is aimed at identifying the genes responsible for resistance using RFLP and RAPD markers. A F2 population derived from the cross G 12952 (wild resistant) by Ica Pijao (cultivated susceptible) has already been screened with *Acanthoscelides obtectus* and RFLP work has been initiated. F3 families will also be screened with molecular markers as well as with the insects to confirm the inheritance.

8. Measurement of nuclear DNA content

A flow cytometric procedure was used by Dr. Cesar Martinez (Rice program) in collaboration with the BRU during his sabbatical at Cornell University to characterize and measure nuclear DNA contents of *P. vulgaris*, *P. acutifolius* and *P. lunatus*. The mean genome size of cultivated *P. vulgaris* from Mexico and Peru is 1.449 pg, and 1.361 pg for wild accessions from Mexico and Peru. The data suggest that the genome size is smaller than previously thought. Statistical analysis for the *P. vulgaris* accessions suggests differences between the cultivated beans from Mexico and the wild from Peru, Mexico and cultivated from Peru. No relationship with seed size was found.

9. Mapping *Phaseolus acutifolius*

The molecular map of *P. acutifolius* has been initiated as part of a collaborative project with Ghent University. RFLP and RAPD will be used for that purpose. The map will serve for comparative evolutionary studies between *P. vulgaris* and *P. acutifolius* in collaboration with the GRU. Two F2 populations derived from crosses of wild by cultivated are being used for that purpose.

10. Biochemical Approaches to Bean Weevil Resistance in *Phaseolus*

The Bean Weevil (*Acanthoscelides obtectus*, Bruchideae) is a major pest of stored beans in America and Africa, accounting for the greatest postharvest losses of this crop. Resistance has been found in only very few Mexican wild bean accessions. It is expressed as reduced and delayed adult emergency with high

levels of mortality in the first instar and diminished female fertility. The genetic is turning out to be more complex than it is the case with arcelin, a protein from the lectin family leading to high levels of resistance against the other important bruchid, the Mexican Bean Weevil (*Zabrotes subfasciatus*). Arcelin stands for four, probably five, related allelic genes of simple inheritance, which are mutually exclusive in the homozygotes. The resistance to the Bean Weevil seems to be inherited as two recessive genes, and no protein product has been identified so far that goes with the resistance trait (Kornegay and Cardona, 1991), but the data are not conclusive yet. Cytoplasmic inheritance is one of the possibilities that remains to be studied, still.

Together with the Bean Entomology Group, C. Cardona and coworkers, we have started the search for factors involved in the resistance to the Bean Weevil in order to understand the underlying mechanisms and thus develop breeding strategies and tools for that purpose, like biochemical or serological tools for screening programs.

So far, purified proteins like phaseolin, phytohemagglutinin or arcelin have not shown to confer resistance when added to artificial seed. In a first attempt to pinpoint down a possible resistance factor, we have fractionated protein extracts. Protein was extracted with 0.3 M NaCl pH 3, and the extract either fractionated by acetone precipitation in three steps (0-20, 20-40, 40-80 vol% acetone) or precipitated as a single 80 vol% acetone step. Another 0.5 M acetic acid extract was precipitated directly with 80 vol% acetone. The precipitates were then lyophilized for inclusion into artificial seed on a susceptible background flour (Ica Pijao). This type of fractionation gives a rough separation of proteins by molecular weight; the solubility in acetone depends largely on hydrophilicity parameters (Fig. 1).

Extracts and protein fractionation were done from a resistant bean accession (G12954), an intermediate (G12880) and a susceptible one (Ica Pijao). The 80 vol% fraction from the resistant accession led to high levels of mortality as compared to the corresponding fraction from the susceptible one. Other fractions showed a dosis response, this is also true for the susceptible cultivar, which can be interpreted as a hyperproteic diet. The results are entomologically relevant and highly significant on a statistical basis (see Fig. 2 for percent emergence data).

The feeding trials and its evaluation took several months. After thorough evaluation and discussion we went into a second round of experimentation. A new wild susceptible accession was included (G10019). The interesting 80 vol% acetone fraction has been further fractionated (40-60 and 60-80 vol% acetone) to narrow down the antibiotic factors. This time more flour was extracted as to obtain enough protein from each fraction to be able to test several concentrations in the artificial seed.

Inhibitors of the insect's digestive processes have been described as resistance factors for other crops. Protease inhibitors are a main source of resistance (Ryan, 1990), α-amylase inhibitors have been cited in connection with resistance to bruchids (Huesing et al., 1991; Ishimoto and Kitamura, 1991). The subunits of the α-amylase inhibitor are small, 15-18 kD glycoproteins, which is the range of proteins in the 80% fraction. Individually isolated middle guts of the Bean Weevil are the source of α-amylase activity to check for inhibitory activities in extracts from resistant vs susceptible accessions. This survey has just been started.

Several non-proteinaceous factors conferring resistance against bruchids have been extensively studied in legume seeds (Gatehouse et al., 1990). Complementary research on aromatic compounds and sugars using HPLC technology to look for factors specifically involved in the resistance to the Bean Weevil is being carried out (see HPLC, the Multipurpose Approach).

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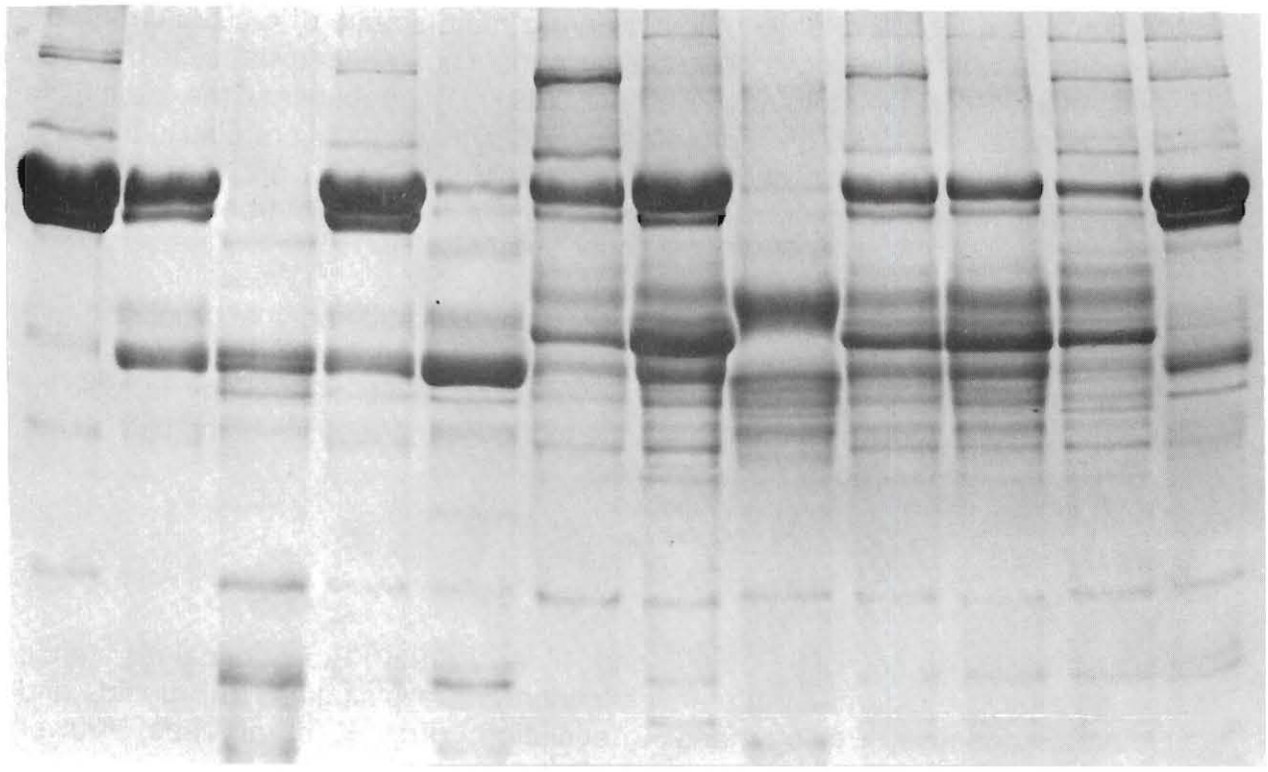

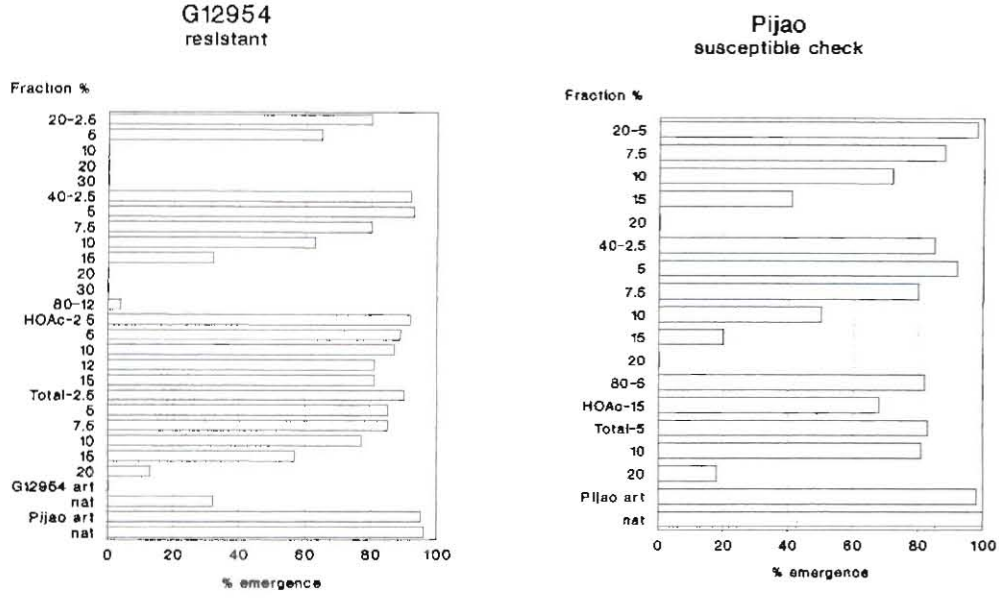

Fig. 1 Bean protein fractions incorporated into artificial seed. Numbers represent % acetone for precipitation; Ac, acetic acid extract; T, total protein (Ac and T were precipitated with 80% acetone; n, non-precipitated). P, Pijao, susceptible check; R, G12954, resistant accession.

Fig. 2 Percent emergence of Acanthoscelides obtectus feeding on artificial seed enriched with protein fractions from a resistant and a susceptible bean accession respectively.

CASSAVA

1. Cryopreservation of shoot-tips

Cryopreservation provides a means for the long-term storage of cassava clones, seed and pollen. With the collaboration of the IBPGR, that provided support for a post-doctoral fellow (Ma. Luisa Marin) and research assistance (R. Escobar) research was conducted on cassava cryopreservation between 1988-90.

Due to the lack of reproducibility of previous cryopreservation reports, we decided to use seeds as specimens for freezing experiments. In 1989, 90-100% seed germination was obtained after rapid immersion of whole seeds into liquid nitrogen. Thawing had to be slow to prevent seed shattering. In 1988 very few shoot tips survived cryopreservation, and in 1989 shoot tips consistently survived freezing to -25 to -35°C. Only in 1990 we were able to obtain on average 70% shoot tip survival and 20% plant formation from cryopreserved shoot tips in liquid nitrogen (-196°C).

The strategy applied consisted first in developing a protocol with var. MCol22 and then test the system with other cassava genotypes. We studied every single step of the process, paying special attention to conditions which could prevent or minimize growth of ice crystals within the tissue, such as composition of the culture medium and the kind and amount of cryoprotectant used prior to freezing. Use of sorbitol, sucrose and DMSO in the pre-culture medium, and the elimination of water from the surface of the explants, contributed to increase tissue survival to 90% and shoot formation to 50%.

The use of small shoot tips and purified cryoprotector increased shoot formation. Interestingly, direct immersion into liquid nitrogen gave higher recovery rate as well as higher shoot formation than slow freezing (Table 1).

We tested the cryopreservation response of 14 cassava genotypes, with MCol 22 as a check. Viability and shoot formation obtained varied from 4-80% and 0-50%, respectively. Our work in 1991 focused on improving the pre-culture, cryoprotection and after- freezing culture conditions. We have significantly increased the response of low responding genotypes and obtained shoot formation from non-responding ones. It seems that genotypic differences in response are not due to the actual freezing step, but to the culture conditions prior and after freezing. Recently, we have also succeeded in the cryopreservation of cassava pollen. This concerted effort should lead to the development of a cassava base gene bank.

Table 1. Effect of shoot tip size, cryoprotection and freezing rate on viability and shoot formation after freezing in liquid nitrogen (cv M Col 22).

Treatment	Average values (%)	
	Viability	Shoots
Shoot tip size		
small (0.5-1 mm)	71A	57A
large (2-3 mm)	14B	0B
Cryoprotection		
sterile DMSO (ampules)*	90A	44A
standard DMSO*	83AB	33A
without cryoprotection*	60B	11B
cryoseeds	67AB	0B
Freezing rate**		
fast	86A	57A
slow	68B	29B

* Shoot tips were dried prior to freezing.
** Fast = direct immersion in liquid nitrogen
Slow = 0.5°C/min.
Averages with the same letter are not significantly different at 0.05 level.

2. Towards a haploid method: culture of immature pollen

Haploids and double haploids will be of significant value to cassava as a research tool and for various applications in genetics, evolutionary studies for the expression of important recessive genes, and for developing a breeding system for true cassava seed.

Initially we carried out background research on the reproductive behavior of cassava, including *in situ* and *in vitro* pollen germination, pollen tube growth and isolation and culture of young zygotic embryos. The microsporogenesis of cassava was also characterized and macroscopic parameters have been defined which correlate with critical microspore developmental stages.

The *in vitro* culture of whole anthers has only lead to the formation of callus from anther cells. Therefore in the last year we have focused our attention to devicing a technique for culturing isolated immature pollen. The technique utilizes some 50 flowers, i.e. 50,000 microspores, per petri dish in hanging droplets.

Immature pollen at the mitosis stage did not divide in culture as it would be expected. The pollen rapidly formed exine components which rendered the pollen impermeable to the medium.

Therefore we have shifted our work to the culture of microspores at the tetrad stage. Optimal culture density was 10^5 microspores per ml medium. Tetrad-stage microspores cultured under high osmotic conditions, and then transferred to a medium with lower osmolarity, were able to divide mitotically and gradually pushed their way out of the tetrad's callose. Outside, the tetrad division continued, but at a slower rate. Microcalli would then become visible. Microspores isolated from highly fertile varieties responded better than those from highly sterile ones.

In summary we have been able to change the gametophytic fate of cassava microspores to initiate a sporophytic development. This is the first necessary step towards androgenetic development. We will focus our future activities on enhancing callus induction from tetrad microspores and monitor this development cytologically.

3. Genetic transformation

Development of genetic transformation requires the availability of:

- A genetic construct containing appropriate selectable and/or reporter markers, a constitutive or tissue specific promoter;
- A DNA transfer methodology;
- An efficient plant regeneration protocol;
- Technology to monitor the transcriptional and post-translational products of gene expression and the inheritance of the introduced gene.

We have demonstrated earlier the regeneration of cassava plants by somatic embryogenesis on immature leaves or apical meristems (Fig. 1).

Agrobacterium-mediated genetic transformation

- Because the efficiency of *Agrobacterium* infection is genotype dependant, we have first screened four cassava varieties (M Col 22, M Col 1505, M Mex 55 and M Cub 74) with 25 *A. tumefaciens* strains. M Col 1505, which also regenerates well through somatic embryogenesis, was highly susceptible to the strains 1182, 1183, C58C1, B6S3 and EHA 101.

- We used the plasmid construct pGV1040 provided by PGS, Belgium. This construct contains two selectable markers (the bar gene and the nptII gene) and one reporter gene (uid A) which are driven by strong promoters (NOS and CaMV 35S).

- Cassava somatic embryos produced endogenous GUS activity, which did not occur in leaves and stems. Using Kosugi's technique, which includes methanol (Kosugi et al., 1990), somatic embryos were still GUS positive at higher substrate (X-Gluc) concentrations with respect to the original GUS assay (Jefferson et al,. 1986, 1987), but not detectable at lower X-Gluc concentrations.

- Leaves and stems were much more susceptible (8 mg/l) than somatic embryos (32 mg/l) to the herbicide BASTA.

- The antibiotic Kanamycin was highly detrimental to somatic embryogenesis.

We will now define the conditions for efficient *A. tumefaciens* infection of MCol 1505 in order to initiate the work on transformation and regeneration. We intend to use both a cointegrate vector approach as well as the binary vector system.

Genetic transformation by particle bombardment

The particle gun available at the BRU accelerates DNA-coated metallic microprojectiles to high speed, in such a way that they can penetrate cell walls, thus being a vehicle for the introduction of foreign DNA into plant cells. This can result in stable integration of the DNA into the plant genome with low frequency.

- Transient GUS activity was demonstrated in cassava tissues 3 days after bombardment.

- Vacuum and distance for the particle bombardment have been optimized.

- Recently we have obtained GUS expression on the tips of somatic embryos 3 days after bombardment of embryogenic callus at the globular stage (Fig. 1).

Progress has been made with respect to the frequency and intensity of GUS expression on somatic embryos. Stable genetic transformation of somatic embryos will be sought for by selecting on herbicide containing media.

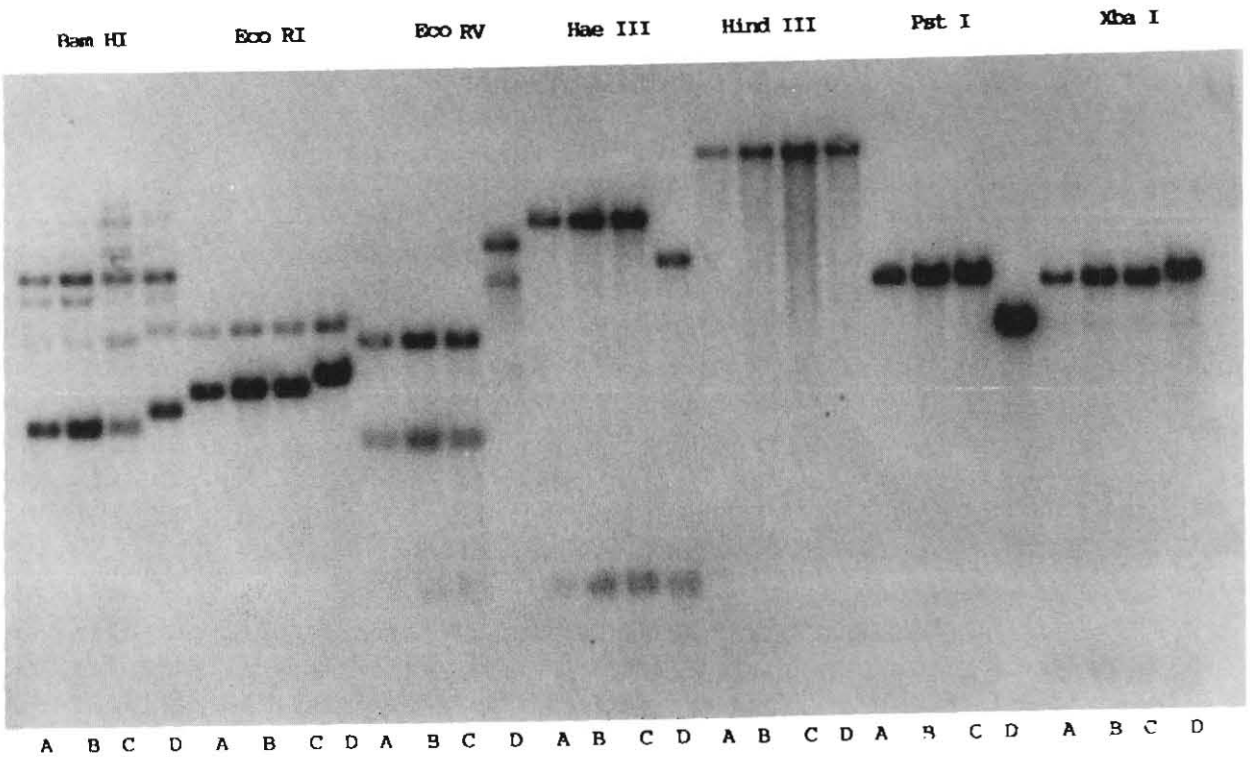

Figure 1. Restriction fragment length polymorphism (RFLP) with 7 restriction enzymes between A= M Thai 8, B= M Col 1505, C= M Nig 5 and D= *A. aesculifolia*. Note polymorphisms only with *M. aesculifolia*.

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4. **DNA fingerprinting**

In contrast to other methods, DNA fingerprinting covers the genome extensively, detects variation in coding and non-coding regions of the genome and is insensitive to developmental and environmental variations. In a collaborative project with the IBPGR we have been developing DNA-based techniques for the analysis of genetic diversity of *Manihot*. The construction of a genomic library has been achieved using the var M Col 22. DNA was digested with each one of the restriction enzymes: Pst I, Eco RI, Bam HI, Xba I and Hind III. Digested DNA was ligated into the plasmid pUC 19 and the constructs transformed into *E. coli* DH5α; insert sizes ranged from 0.2 to 7 Kb.

DNA isolated from varieties M Col 22, M Col 1505 and CM 507-35 was digested with the enzymes Apa I, Bam HI, Dra I, Eco RI, Eco RV, Hae III, Hind III, Hinf I, Hpa I, MspI, Pst I, Taq I and Xba I. We found that methylation sensitive-enzymes like Apa I and Pst I cut cassava DNA very ineffectively. Polymorphisms were detected with several clones; one Hind III clone was able to discriminate between the three varieties.

We have also used other probes for detecting polymorphism in cassava. The human minisatellite probe (Jeffrey's probe) was ineffective in detecting polymorphism; however, the phage M13 probe (Vassart et al., 1987) and several randomly amplified polymorphic DNA markers (RAPD) (Williams et al., 1990) did yield sufficiently variable band patterns as to differentiate between the varieties tested (Fig. 2B and C). We are testing those probes to analyze genetic stability of morphologically and isoenzymatically similar varieties and cassava clones which have been under *in vitro* storage for over 10 years.

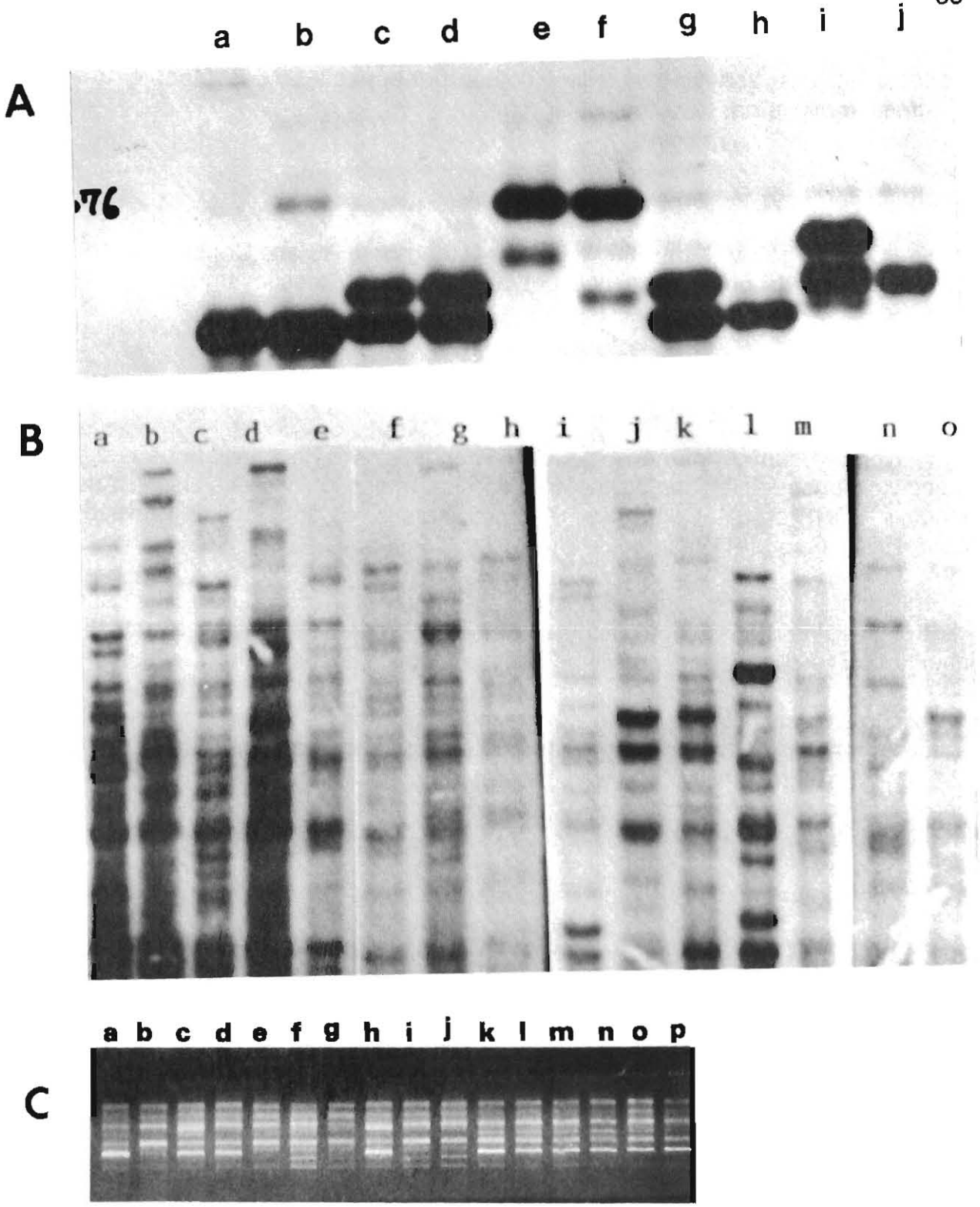

Figure 2. Molecular fingerprinting of cassava varieties; (A) EST isozyme fingerprints of 10 varieties (a-j); (B) M13 probe DNA fingerprints of 15 varieties (a-o); and (C) RAPD fingerprints of 16 varieties (a-p).

Genotyping of cassava by isozyme and DNA fingerprinting techniques will be useful to assess genetic variability, define gene pools and contribute to the construction of the cassava molecular map.

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5. **Molecular mapping**

With the support of the Rockefeller Foundation we have initiated a research project to construct the molecular and physical maps of cassava using random genomic, cDNA and YAC libraries. The project involves cooperation between the University of Georgia, Washington University, IITA and CIAT.

The maps will be useful to analyze the genomic structure of cassava and its wild relatives, and to tag agronomically important traits, simply and quantitatively inherited. Eventually the map will be useful to isolate and clone cassava genes.

The project builds up on the experience gained at CIAT in the last year. Thus, protocols for DNA extraction, digestion and hybridization have been implemented. A random Pst I genomic library was generated using the var M Col 22. Some 200 single copy clones of a total of 500 have been characterized, insert sizes ranging from 0.2 to 3 Kb.

Recent developments in this project include:

- Cassava DNA digested with Eco RI, Eco RV, Xba I and Hind III libraries provide probes with highest polymorphism rates.

- Polymorphism between cv M Col 22 and its wild relative *M. aesculifolia* was dramatically higher than the polymorphism found between two cultivated genotypes, as expected (Table 2, Fig. 1). Four-cutter restriction enzymes displayed less frequency of polymorphism than six-cutter enzymes for cultivated genotypes for fragments larger than 2 kb.

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

Table 2. Degree of polymorphism detected with at least one restriction enzyme* using five genomic libraries as source of probes [%].

Library	M Col 22 vs. M Col 1505	*M. aesculifolia* vs. M Col 1505
Pst I	60	85
Xba I	60	85
Hind III	55	95
Eco RI	40	60
Bam HI	30	45

* Best restriction enzymes in order of polymorphism are: Eco RV, Xba I, Eco RI, Hind III.

6. **Biochemistry of Cassava Starch Fermentation**

Sour cassava starch is a regionally very important small industry product, the departments of Valle and Cauca producing almost 80% of the national total. The product is consumed mainly locally as pandeyuca, besitos, rosquillas, and other local classics. The fermentation process still takes place under quite rudimentary conditions in the rallanderias. Inspite of some improvements in the machinery and sedimentation tanks over the last few years, the main problem encountered remains the reproducibility of the process, the lack of inocula, and well defined marketing criteria.

Gerard Chuzel, from CIRAD/CEEMAT-Cassava Utilization Section CIAT, has been pursuing a holistic approach to this problem, and putting together the Cassava Utilization Section, the BRU, and UniValle, microbiological, physicochemical, and biochemical studies have been performed (see also BRU Annual Report 1990; and Abstracts of the Workshop "Avances sobre almidón de yuca" Jun 17-20, 1991, CIAT). A very thorough microbiological analysis is well documented in the thesis work of Carlos Figueroa (1991). The fermentation process, which is mainly anaerobic, is carried out to a great extent by lactic amylolytic bacteria of the genera Lactobacillus, Streptococcus, Leuconostoc, Pediococcus, and Lactococcus.

Last year we reported the electrophoretical separation of amylolytic activities and their transfer to starch containing gels (SCGs) for their characterization. This year we set for the detailed characterization of amylases from the principal bacteria

involved in the process. It is the partial amylolytic degradation of the starch granules, as shown by electron microphotography, that confers to the starch its rheological properties for panification.

Excreted amylases were extracted from the fermentation mass in acetate buffer, as to preserve the optimal acidic conditions. Proteins were then precipitated from the supernatant by ammonium sulfate, and after dialysis of the resuspended sample, chromatographed on DEAE anion exchanger resin. The enriched amylases were then quantified and characterized on SCGs by iodine staining and by enzymatic assays (colorimetric assays either with iodine after hydrolysis of starch or after the liberation of p-nitrophenol from PNP-heptaglucoside).

Strains with strong amylolytic activities have been selected using the methods described, after analysis of supernatants from pure liquid cultures (see Fig. 3). Characteristic patterns of supernatant proteins have also been obtained by silver staining of polyacrylamide gels. In the next step the amylolytic enzymes of these strains will be characterized as to their pH and temperature optima, substrate preferences, cofactors, inhibitors, and kinetic properties, and also the elaboration of an amylolytic profile throughout the fermentation process will be pursued.

References

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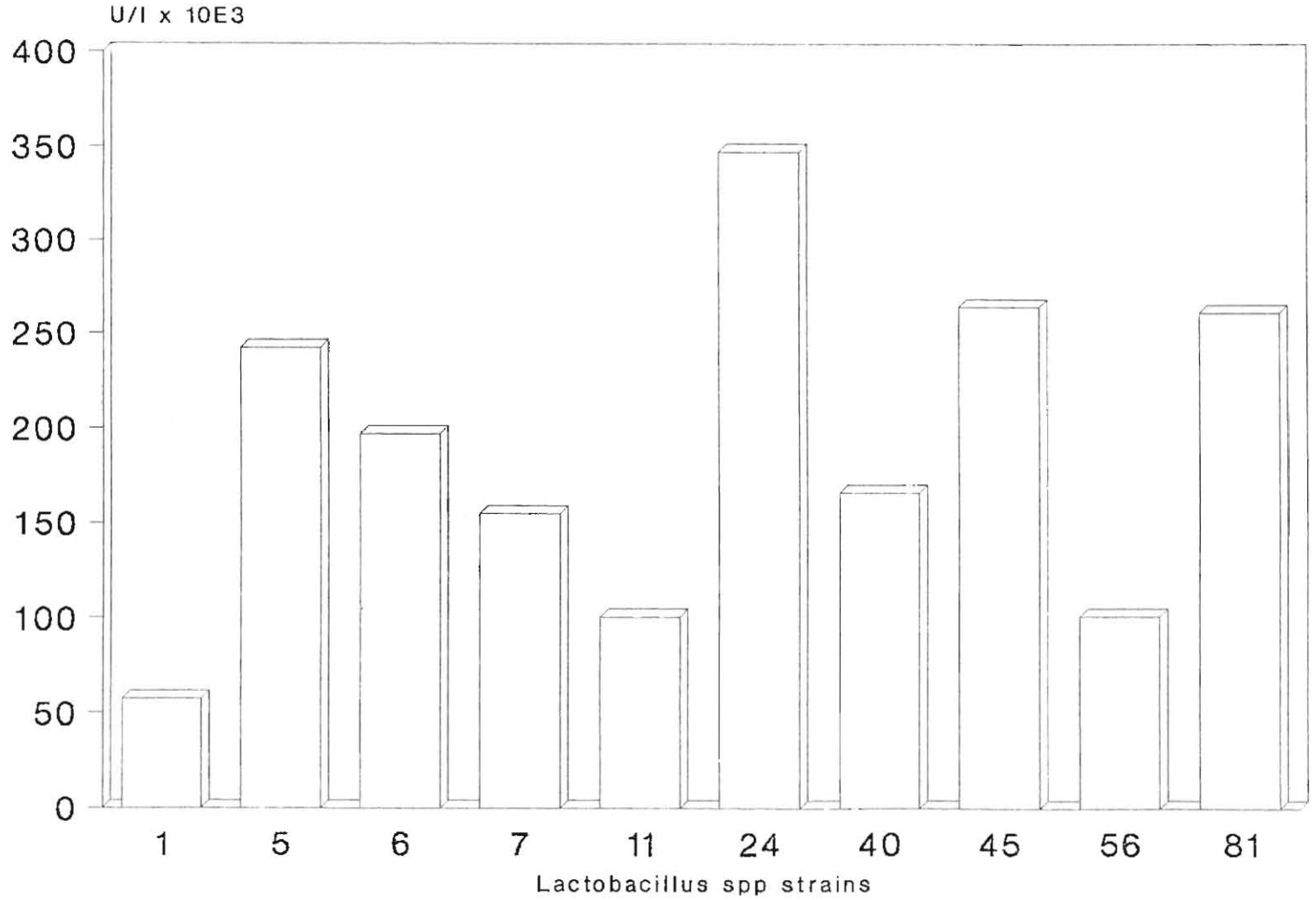
7. **Molecular Mechanisms of CO_2 assimilation**

Certain cassava photosynthetic parameters seem to lie between C3 (bean) and C4 (maize) plants (El-Sharkawy and Cock, 1987). This suggests that cassava might have developed mechanisms for efficient photosynthesis, especially under drought and high temperature conditions (Schuster and Monson, 1990). Earlier work at CIAT has demosntrated that there is a wide range of genotypic variability concerning this physiological behavior. Now, it remains to be established which are the actual mechanisms underlying these characteristics. The final objective is to use this knowledge for developing screening methodologies which can be incorporated into breeding programs searching for improved biomass production, water and nitrogen use efficiency.

One approach we are initiating is to develop *in situ* hybridization techniques on leaf tissue using photosynthetic gene probes to assess their compartmentalization patterns. For this purpose, homologous genes from cassava have to be used; therefore we must first isolate these genes from cassava gene libraries using heterologous probes from maize (provided by T. Nelson, Yale University). We are

Amylolytic Activity of Lactic Bacteria



1U = 1 µeq reducing ends/min at 37 C

in the process of constructing a genomic library from cassava. We have observed high homology between the maize genes and cassava by hybridization of total DNA with the maize probes, which indicates that these genes are highly conserved, hence, facilitating the task of isolating the cassava genes. The genes we are working on are: RUBISCO (rbcs), malate dehydrogenase (mdh), malic enzyme (me) and PEP-carboxylase (ppc). Another target would be the glycine decarboxylase, an enzyme that is involved in CO_2 recycling, a mechanism that maybe related to cassava's C3-C4 intermediate characteristics (Hylton et al., 1988).

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RICE

1. Increased Generation of Doubled Haploids from Indica Rice

An anther culture protocol was developed at CIAT to generate large numbers of doubled haploids for the Rice Breeding Program. The current protocol allows the culture of 10,000 anthers/person/day, which can generate 440 doubled haploids from the most responsive genotypes (japonica type). This anther culture efficiency, and a laboratory team of one research assistant and five technicians, allows the Rice Program to obtain doubled haploids from 60 crosses/month.

Research conducted at CIAT indicates that the incorporation of pollen derived doubled haploids into breeding programs reduces the time to obtain fixed lines to two generations of selection, without reducing the genetic variability in grain yield or affecting the genetic stability of the progeny. These results led to explore the potential uses that doubled haploids populations could have in rice breeding.

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

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

A total of 7 non-responsive indica genotypes were selected to include the genetic diversity that may be present in Latin American varieties (Table 1). The japonica (upland) breeding line CT 6241-17-1-5-1 was used as a check, because it is the current most responsive CIAT material to anther culture. Plants were grown in the field following standard procedures. Panicles were harvested and pretreated, and anthers were cultured according to Nuñez et al. (1989). Callus induction was evaluated on Potato-2 medium (medium commonly used at CIAT, Nuñez et al., 1989) and on modifications of N6 medium (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. The regeneration of green plants

from the various treatments was determined using the standard CIAT protocol (Nuñez et al., 1989).

The efficiency of response of various of the recalcitrant genotypes was increased 3 to 9 fold by replacing the potato-2 callus induction medium with a modified N6 basal medium (Table 2). Further enhancement in callus induction was obtained by replacing sucrose with 5% maltose, and by adding 10 mg/l $AgNO_3$ (Fig.1). A clearcut 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 0.02% to 0.70% for indica, and from 3.2% to 8.6% for the most responsive genotype (Table 3). Improvement in anther culture response was not noted with the other parameters evaluated. At present, work is directed to promote further plant regeneration from the indica types. A study to determine the economic feasibility of using anther culture for the development of irrigated variety is under progress.

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2. Plant Regeneration from Protoplasts of an Important Latin American Indica Rice Variety

Protoplast culture is a highly efficient system for genetic manipulations such as direct gene transfer. Large populations of protoplasts can be easily handled and plants can be regenerated from 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. Two protocols have been recently developed for regeneration of indicas from protoplasts (Lee et al., 1989 and Datta et al., 1990a), and genetically engineered fertile indica rice has also been obtained (Datta et al., 1990b). Most varieties grown in Latin America are of the indica type. This collaborative project between the BRU and the Rice Program pursues to optimize a methodology for plant regeneration from indica protoplasts to be used for the genetic transformation of important varieties in the region.

Table 1. Characteristics of materials used in this study.

Rice type	Genotype	Source	Anther culture response
Indica	CICA 8	Colombia	no callus
	Oryzica 1	Colombia	no callus
	BR-IRGA 409	Brazil	no callus
	Inti	Peru	no callus
	IR 8	IRRI	no callus
	P5446-6-6-1-13	CIAT	poor regeneration
	GZ864-2-3-1	Egypt	poor regeneration
Japonica	CT 6241-17-1-5-1	CIAT	highly responsive, check

Table 2. Modifications of N6 medium.

Compound	Concentration
$(NH_4)_2SO_4$	231.5 mg/l
KH_2P0_4	540-800
H_3BO_3	6
$MnSO_4.4H_2O$	22
$ZnSO_4.7H_2O$	10
$Na_2MoO_4.2H_2O$	0.25
$AgNO_3$	10
Thiamine-HCL	2.5
Nicotinic acid	2.5
Pyridoxine-HCl	2.5
Glycine	2.5
2,4-D	2.0
Picloram	0.1
Kinetin	0.5
Maltose	50.0 g/l

Table 3. Green plant regeneration efficiency of calli induced on modified N6 medium.

Rice type	Genotype	Callus induction (%)	Green plants/ anthers (%)
Indica	CICA 8	6.4	1.60[a]
	Oryzica 1	21.5	0.30
	BR-IRGA 409	8.9	0.18
	INTI	3.3	0.58
	GZ-864-2-3-1	2.4	0.06
	P5446-6-6-1-13	35.2	2.45
	IR 8	0.9	0.09
Japonica	CT 6241-17-1-5-1	64.8	8.62

[a] The mean regeneration of green plants/anther of calli induced on potato-2 medium is 0.02% and 3.28% for indica and japonica genotypes respectively.

Cell suspension cultures of the Latin American varieties BR-IRGA 409, CICA 8, Oryzica 1, Oryzica Llanos 4 and Oryzica Llanos 5 were initiated and maintained according to Lee et al., 1989 (Fig. 2A). Cell suspension 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 kindly supplied by Dr. T.K. Hodges, Purdue University. Protoplast isolation and culture, and plant regeneration (Fig. 2B) were conducted using a modification of the protocol by Lee et al., 1989. Cell and protoplast viability was determined by fluorescent staining with FDA (450-490 nm). Cell contamination was measured using the fluorochrome Calcofluor White (330-380 nm), a specific stain for cell walls.

Highly embryogenic calli were formed from mature seeds of the five varieties tested. Fine and rapidly growing cell lines were developed from BR-IRGA 409, Oryzica Llanos 4 and Oryzica Llanos 5. The Oryzica 1 line is not yet well established, and CICA 8 did not respond to the cell suspension induction treatments. Initial attempts of protoplast isolation and culture were conducted using the cell line IR 54-2. The protocols reported by Datta et al. (1990) and Lee et al. (1989) were tested. The latter protocol resulted in a high efficiency of protoplast isolation and viability (Table 4). Further attempts were performed using BR-IRGA 409, the most important Brazilian rice variety.

Several modifications of Lee's protocol (Lee et al., 1989) were implemented resulting in a higher protoplast culture efficiency. Cell suspensions composed by small cell aggregates (about 50 cells each) were critical to ensure a high yield of

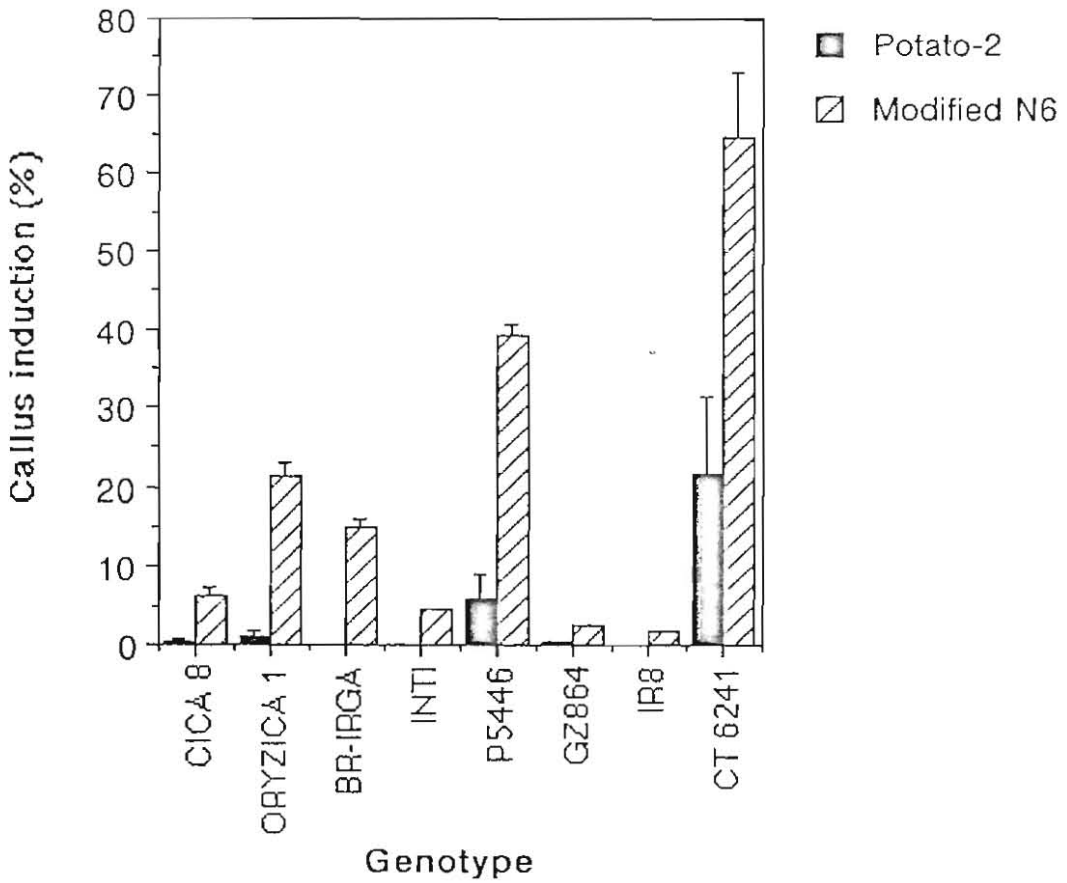

Figure 1. Callus induction six weeks after culture on potato-2 and modified N6 media.

viable protoplasts (Table 4). Fine cell suspensions were maintained by sifting the cultures through a 500 µm mesh before subculturing (Fig. 2A). An increase from 4 to 5 hours of incubation with the protoplasting enzyme solution reduced the number of cells with undigested cell walls (cell contamination) from 0.7-1.0 % to 0.3-0.5 % without affecting the protoplast viability (Table 4). The number of protoplasts dividing and giving rise to colonies (plating efficiency) was highly enhanced by increasing the concentration of the feeder layer about 3 fold over the recommended cone (Table 5). Lee et al. (1989) reported a plating efficiency of 3%. Embryogenic calli were selected to induce plant differentiation. Plant regeneration from these calli is still in progress and at present, two green plants have already been regenerated. Experiments directed to establish a high regeneration efficiency are underway. Future plans are to use the most efficient protocol for direct gene transfer, leading to genetic transformation of rice.

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3. **Characterization of Irrigated Rice Varieties and Upland Breeding Lines by Isozyme Analysis**

Rice varieties have been classified traditionally by morphological characters as well as by geographical location. Recent isozyme studies indicated that these biochemical markers detect intraspecific variation within *Oryza sativa*, aiding to clarify discrepancies in the classification of varieties. Glaszmann (1987) analyzed 1688 Asian rice varieties and found six enzymatic groups which correspond to various local ecotypes. Two major groups, I and VI corresponding to indica and japonica types. Group I is more polymorphic than group VI. Two minor ones, groups II and V, and two satellite groups III and IV. Groups II and V are genetically closer related to groups I and VI respectively. This isozyme classification corresponds with DNA analysis by RFLP (Wang, 1990). Thus, isozyme analysis could complement the morphological characterization of the rice germplasm. This collaborative work between the Rice Program and the BRU pursued to determine whether some

Table 4.Protoplast isolation from two indica varieties.

Genotype	Digestion of cell walls (hr)	Protoplasts/gr fresh weight/ml	Protoplast viability (%)	Cell[(1)] contamination (%)
IR 54-2	4	1.7×10^4	-	-
	4	1.7×10^5	-	-
	4	1.7×10^8	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	2.9×10^8	92	0.3

[(1)] Cell contamination (%) = $\frac{\text{viable cells (with undigested cell wall)} \times 100}{\text{viable protoplasts + viable cells}}$

Table 5.Protoplast plating efficiency.

Cell line	Protoplast/gr fresh weigh/ml	Cell contamination (%)	Culture density (protoplasts/ml)	Freeder layer ml/20 ml medium	Colonies	Plating[(1)] efficiency (%)
BR-IRGA 409-5	2.8×10^8	0.5	10^5	1.5	167-1715	1.2±0.3 n=7
BR-IRGA 409-1	2.7×10^7	0.3	10^5	5.0	372-5985	26.5±6.7 n=8

(1) Plating efficiency (%) = $\frac{\text{colonies (microcalli)} \times 100}{\text{protoplasts cultured}}$

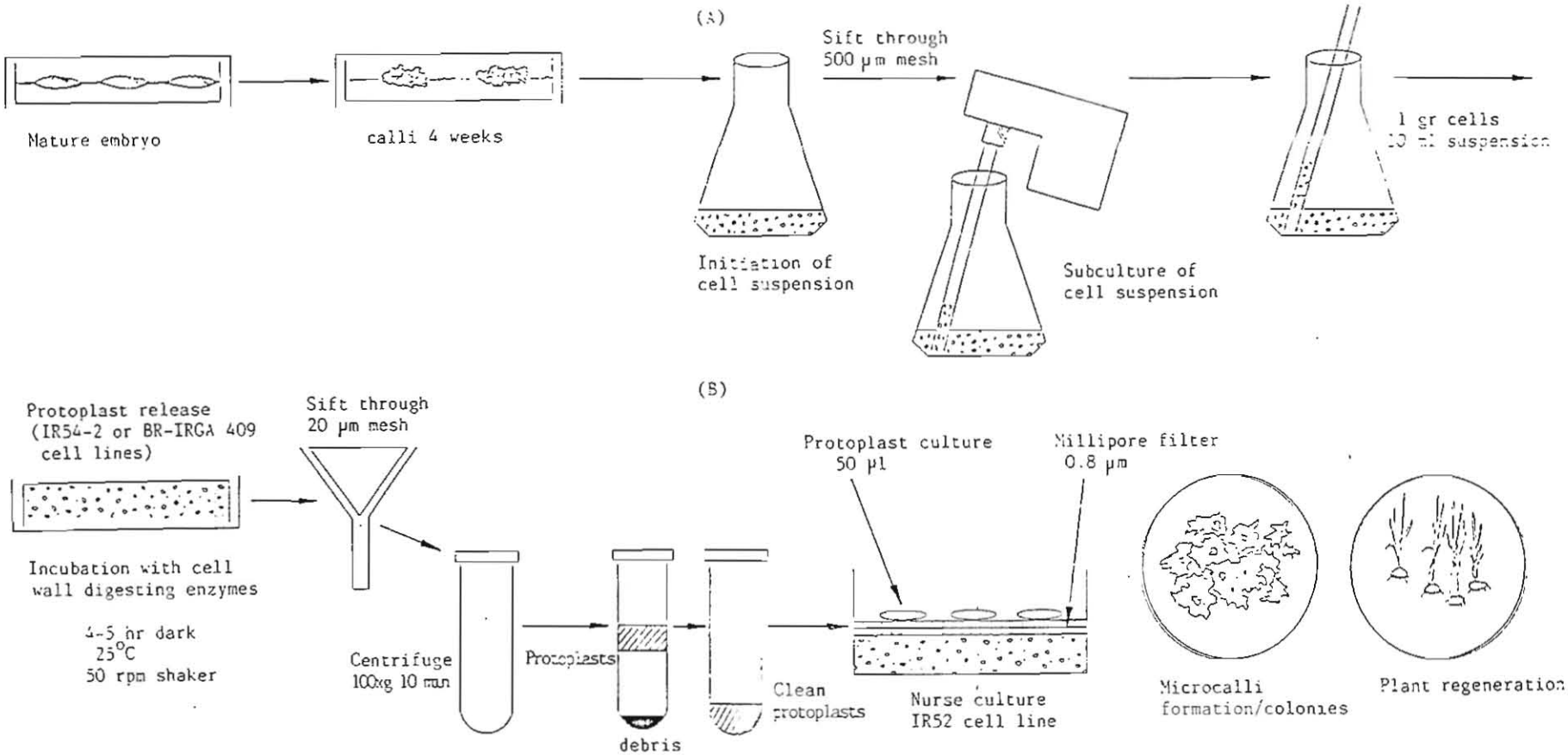

Figure 2. A. Initiation and maintenance of cell suspension cultures.
B. Isolation, culture and plant regeneration of rice protoplast.

irrigated varieties important for the rice breeding program could be discerned by isozymes, and to identify the rice type of upland materials developed at CIAT.

A total of 24 irrigated varieties from eight Latin American countries, 49 advanced breeding lines adapted to Colombian upland conditions, and 29 varieties used as parents in the upland population were analyzed. Twenty plants from three irrigated varieties and seeds produced in four locations were analyzed individually. Five indicas and five japonicas, according to Glaszmann (1987) and Wang (1990), were included as checks in the analysis of upland materials. Seeds were germinated and soluble protein extracts from the plumule and coleoptile of seedlings were prepared according to Glaszmann (1987). Three enzymes of the eight used by Glaszmann (1987), esterase (EST), phosphoglucose isomerase (PGI) and acid phosphatase (ACP), were analyzed. Protein extracts were subjected to polyacrylamide gel electrophoresis (EST) or starch gel electrophoresis (PGI and ACP) (Hussain et al., 1986). These enzymes permitted surveying seven polymorphic loci, from a total of 14 alleles. Data were analyzed using a Factor Analysis of Correspondence (SAS version 5, 1985).

The multivariate analysis of the data indicated that 19 of the 24 irrigated varieties (79.2%) are distinguishable by the isozyme system used (Fig. 3). The other five varieties could be discerned by the electrophoretic patterns in polyacrylamide gels of total proteins extracted from coleoptiles. Thus, the 24 irrigated varieties could be fully distinguished by a combination of both types of analysis. No differences in patterns were seen between plants within a variety from the various seed sources.

It appears that this type of analysis could also facilitate the verification of varietal homogeneity.

Analysis of the upland breeding lines and their parents identified two major groups, which explains 20% of the total variability. Group A clustered 18 materials (23.1%) and group B included the other 60 lines (76.9%) (Fig. 4). The five indica and five japonica checks were clustered in group A and group B respectively (Fig.4). These two groups were also formed when the data were plotted on the plane defined by the 2 axes which account for the largest part of the whole variation, but 11 lines were not included in either group (undefined) (Fig. 5). Following Glaszmann's analysis (1987), it appears that 81.6% (40 lines) of the upland breeding lines studied are of the japonica type (group B), 4.1% (2 lines) of the indica type (group A), and 14.3% (7 lines) are undefined (Fig. 6). These results agree with the analysis of 1680 Asian rice varieties which indicated that most Asian upland rices were of the japonica type (Glaszmann, 1987). Data suggest that adaptability to upland conditions may come from japonica germplasm. The two lines clustered in the indica group may be useful to maintain genetic variability in the population.

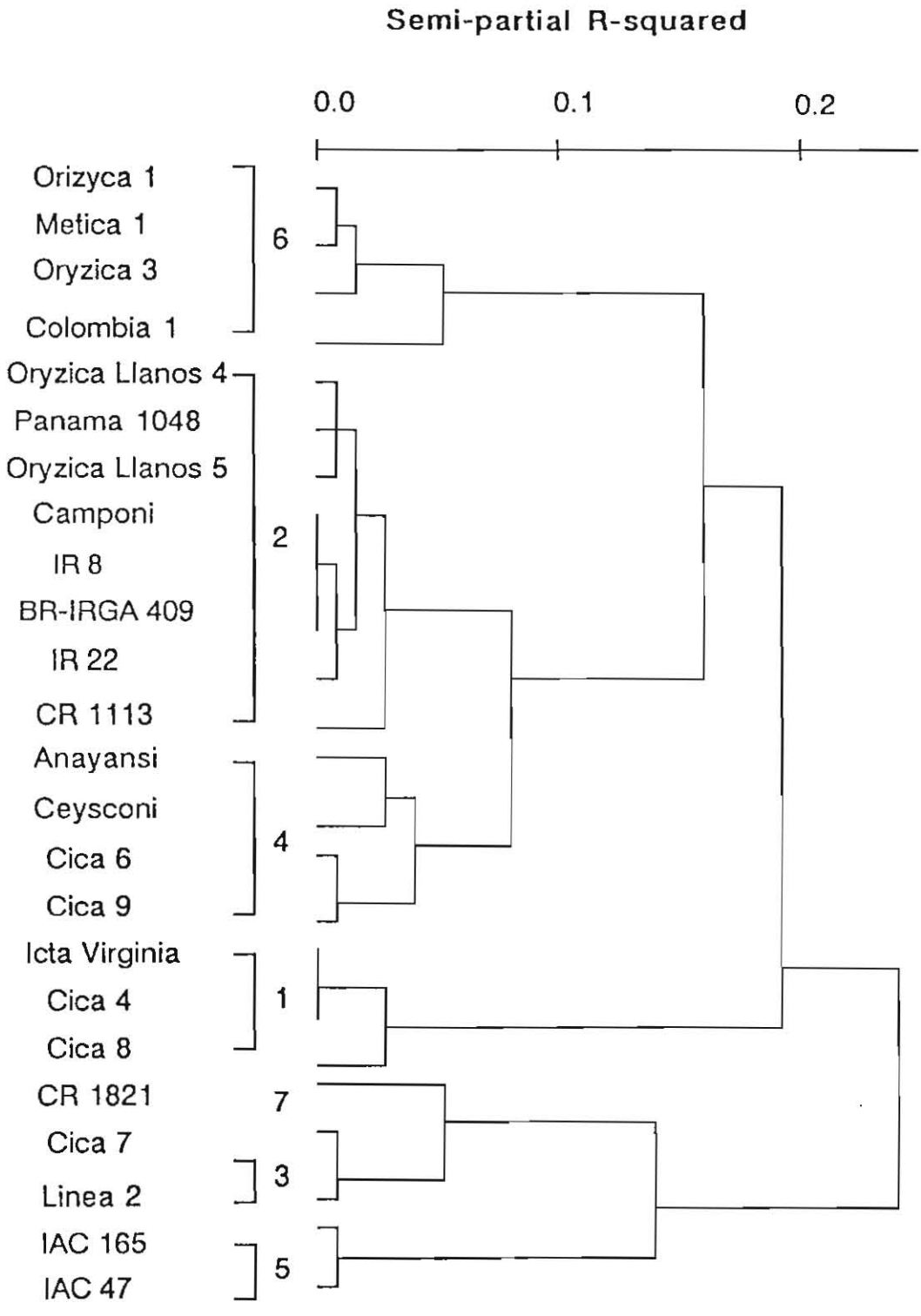

Figure 3. Factor Analysis of Correspondence (SAS) of EST, PGI and ACP enzyme patterns presented in 24 irrigated varieties.

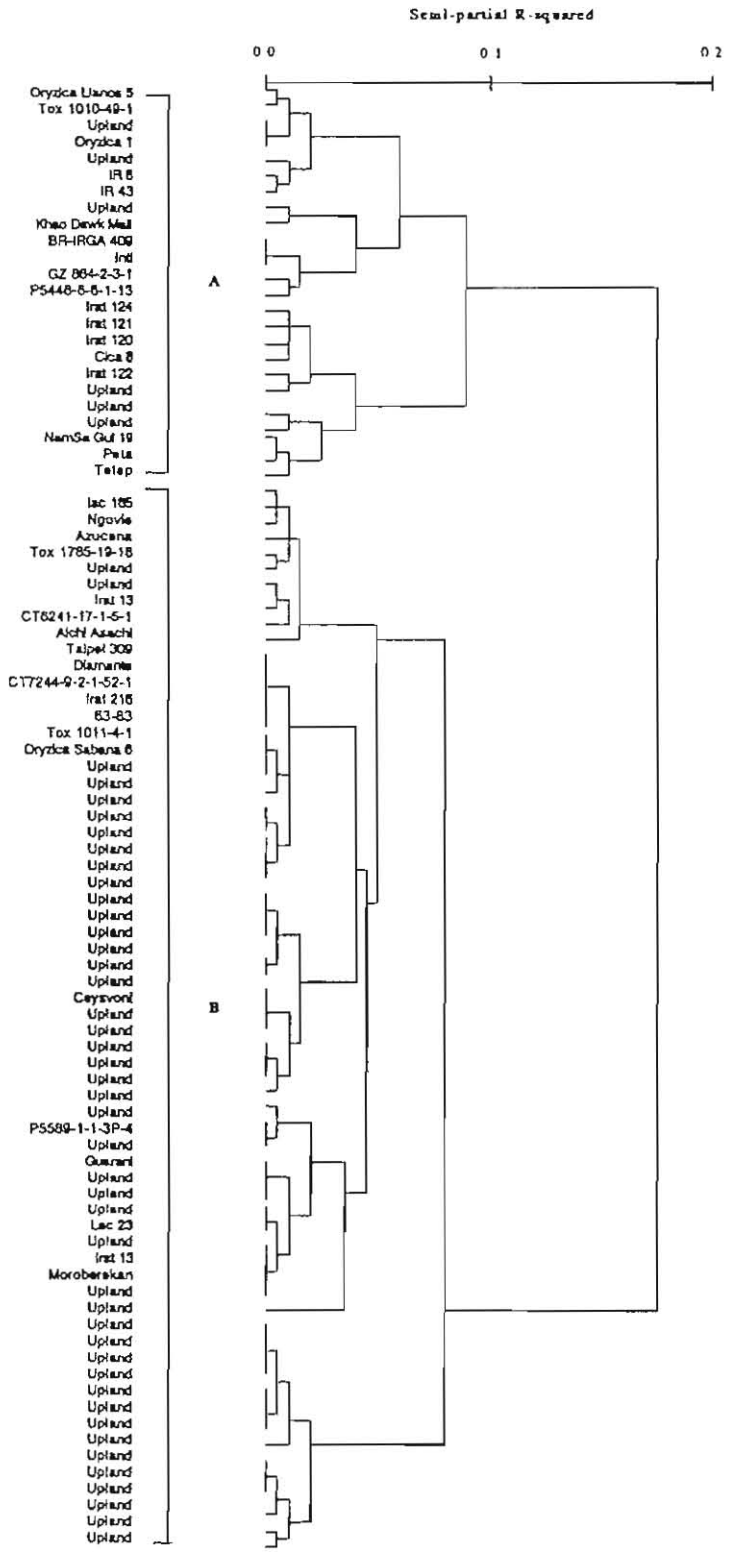

Figure 4. Factor Analysis of Correspondence (SAS) of EST, PGI and ACP enzyme electrophoretic patterns of upland breeding lines adapted to Colombia conditions (upland) and their parents. IR 43, Khao Dawk Mali, Nam Sa Gui 19, Peta and Tetep were used as indica checks; Aichi Asachi, Azucena, IRAT 13, Moroberekan and Taipei 309 were used as japonica checks.

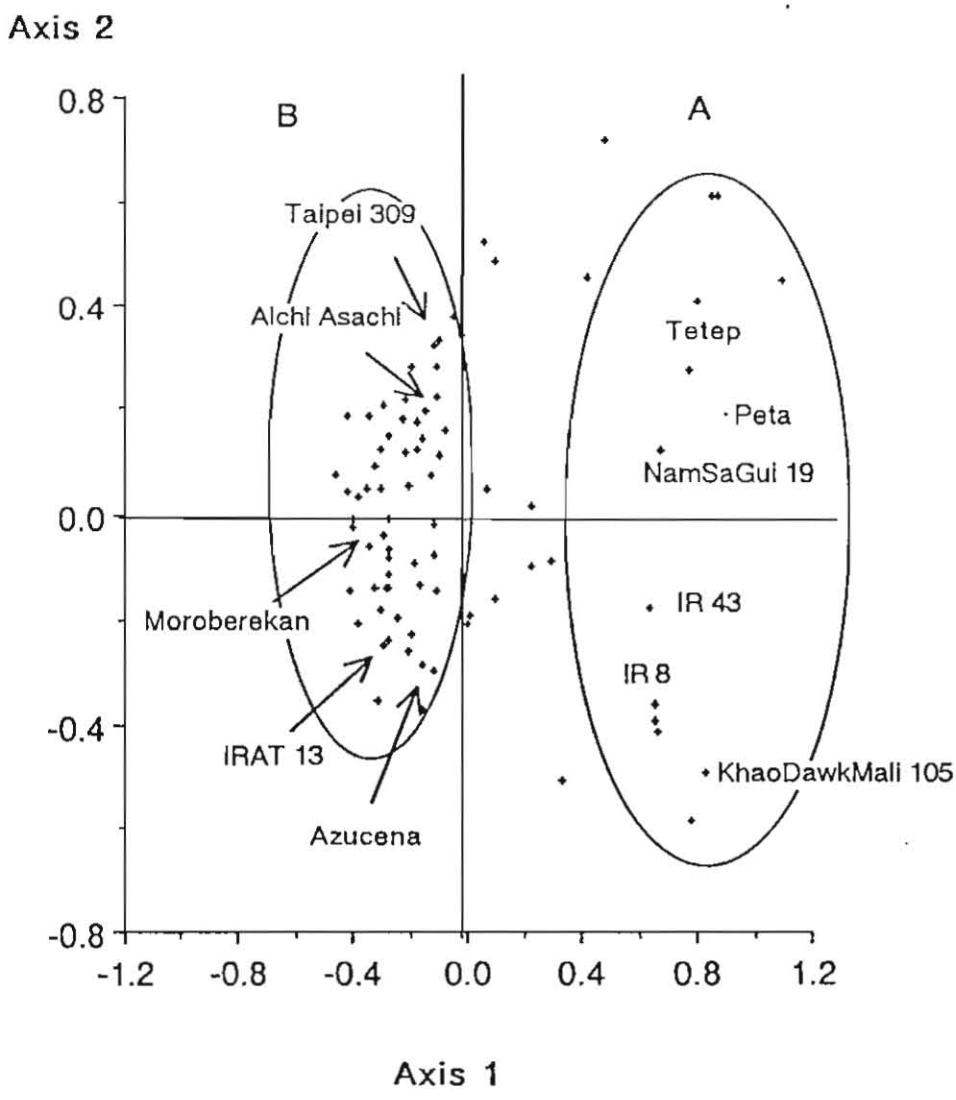

Figure 5. Data distribution by a Factor Analysis of Correspondence (SAS) on axis 1 accounting for 16.6% of variability and axis 2 for 9.12% .

Further analysis including more enzymes and/or DNA fingerprinting will increase the genetic differentiation within each group.

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4. Tagging Resistance Genes to Colombian Isolates of Rice Blast with RFLP and RAPD Markers

Molecular markers offer an efficient means to locate and monitor disease resistance gene transfer in rice breeding programs. Two types of molecular markers are being used at CIAT (a collaborative work between the BRU and rice pathology) to identify, tag and characterize currently used, as well as novel resistance genes against Colombian isolates of blast. The first type, RFLP, has been used previously in tagging Rice Hoja Blanca Virus gene from the cv. Fanny (CIAT Annual Report, 1990). A new type of molecular marker, RAPD (random amplified polymorphic DNA) (Williams et al., 1990) has recently been demonstrated to be effective in gene tagging in tomato and lettuce. RAPDs offer an inexpensive and efficient means for identification and characterization of genetic components. The technique provides new opportunities for the incorporation of molecular markers into breeding programs. Effort was dedicated this year to implement the RAPD technique for the tagging of rice blast resistance loci.

Plant Materials and Blast Isolates

Forty doubled haploid lines were derived from anther-culture of F1 hybrid from the cross IRAT 13 x Fanny. Fanny is highly resistant to the RHBV but susceptible to most of the rice blast isolates in Colombia. IRAT 13 is highly susceptible to the RHBV but resistant to many blast isolates. Each doubled haploid line used in this

study was generated from a single callus. Only normal looking and highly fertile plants (suggesting dihaploidization) were selected.

Seven well characterized blast isolates obtained from Santa Rosa and the Altillanura were used (Table 6). Fanny was susceptible to all the isolates from Santa Rosa but not to the isolates from the Altillanura, while IRAT 13 reaction was quite the opposite. The doubled haploid lines were planted under greenhouse conditions in four inch pots, 10 plants per pot, 20 plants per line. High nitrogen fertilization (150 kg N/ha) was given to the plants to enhance blast development. The plants were inoculated at the three leaf stage with a spore suspension of 5 x 10^5/ml by spraying. Inoculated plants were incubated under high relative humidity and at a temperature of 20-30°C for eight days before evaluation. Plants were evaluated using the standard evaluation system for rice. Lesion types as well as percent leaf area affected were recorded.

Table 6. Reaction of the parental genotypes to blast isolates from Santa Rosa and the Altillanura, Colombia.

GENO-TYPES	FY 29-1[1]	CE 53-1[1]	OR 1-84[1]	OLL 5-14-1[1]	C 9-31-4[1]	L 2-35-1[2]	I 165-2-2[2]
FANNY	S	S	S	S	S	R	R
IRAT 13	R	R	R	R	R	S	S

[1] Isolates from Santa Rosa
[2] Isolates from the Altillanura.

DNA Extraction, Southern Blotting and Hybridization

Plant DNA was prepared from frozen tissue as described (Rogers and Bendich, 1988). Total genomic DNA was digested with five restriction enzymes (XbaI, EcoRI, EcoRV, HindIII and DraI). These five restriction enzymes were previously reported to yield the highest levels of polymorphism among rice cultivars.

Southern blotting was performed on nylon membranes. Whole plasmids containing rice genomic inserts were multiprime labeled with ^{32}P. Hybridization and autoradiography were carried out according to McCouch et al. (1988). One hundred and ten single copy mapped probes obtained from Dr. Tanksley's Lab (Rice RFLP Framework Map, October 1989) were screened on the parental lines to determine

the proper restriction enzyme-probe combinations for screening the doubled haploid (DH) lines. Polymorphic clones were then used to screen the DH lines.

RAPD Analysis

One hundred random decanucleotides obtained from the Operon Co. were used for the RAPD analysis. Amplification was performed using a Coy thermocycler. The PCR reactions and the cycling parameters were similar to the one described by Martin et al. (1991) and consisted of one cycle at 94°C for 30 seconds followed by 40 cycles of 1 min at 94°C, 1 min at 35°C, 2 min at 72°C followed by 5 min at 72°C. The amplification products were resolved by electrophoresis using 1.2% ultrapure agarose stained with ethidium bromide. Rather than screening the whole segregating population with each primer, bulk segregants from resistant and susceptible doubled haploid lines were established (Michelmore et al., 1991). The bulked segregants were used with the parental genotypes to detect any co-segregation between the primer product of the resistant parent and the resistant bulk. Putative co-segregating primer products were then screened on the whole population.

Linkage Analysis

RFLP, RAPD and disease reaction data from the DH lines were analyzed using the program MAPMAKER. A LOD score of 4, the Kosambi function and a recombination fraction of less than 20% were used for the linkage analysis.

Results and Discussion

The blast reaction of the DH lines exhibited either type I or type IV lesions. No intermediate reaction was observed. The genetics of one isolate from Santa Rosa, CICA 9-31-4, was studied using 120 F2 plants from the cross IRAT 13 x Fanny. The results suggest a single dominant gene. The blast data from that isolate were used for the linkage analysis with the RFLP and RAPDs data. The reaction of two other isolates from Santa Rosa, Fanny 29-1 and Oryzica 1-84, were closely similar to the reaction of the doubled lines reaction with CICA 9-31-4 isolate, suggesting a close linkage. Another possibility would be that one gene is involved, with the difference in reaction due to escapes or "background noise" in the evaluation. The reaction of the two isolates from the Altillanura were identical.

The level (of polymorphism) of the RFLP was not high (about 39%) but still adequate for gene tagging. With respect to the RAPD data from the bulk segregants, four types of primer products were obtained; 1) no polymorphism between the parental genotypes as well as between the two bulks, 2) polymorphisms between Fanny and IRAT 13 but both bulks cosegregate with the susceptible parent,

3) polymorphisms between Fanny and IRAT 13 but both bulks cosegregate with the resistant parent, 4) cosegregation of Fanny with the bulk susceptible, whereas IRAT 13 cosegregated with the resistant bulk (Fig. 6). Out of the hundred primers tested 30 primers resulted polymorphic among Fanny and IRAT 13. On the average 4 bands were obtained per primer. Six primers showed cosegregation between the bulk and their matching parents. These primers were then tested on the whole population. Three primers (B10, B8, C15) showed cosegregation (Fig. 7) whereas the others were false positives. The linkage analysis with MAPMAKER placed the gene and the three primers on chromosome 4 (Fig. 8). Both B8 and B10 primers were tightly linked to the blast resistance gene pi-(t).

Additional work is currently being conducted on a larger population of DH lines to confirm the location and distance of the pi-(t) as well as to locate other genes. The combination of RFLPs and RAPDs proved quite useful in tagging a blast resistance gene. The RAPDs offer great potential to truly apply molecular markers in a breeding program, because potential of dealing easily with large numbers of samples.

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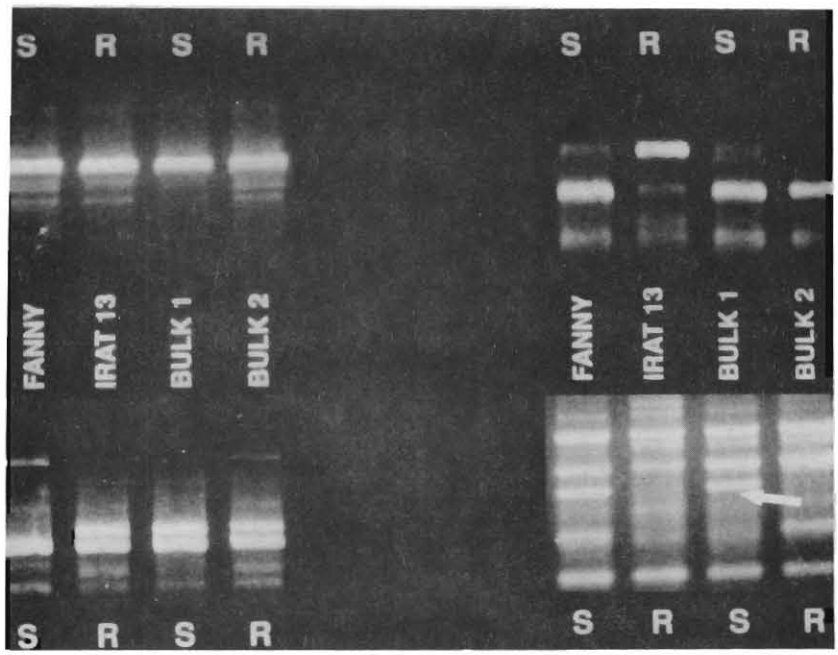


Figure 6. Possible amplification results using the two parents (IRAT 13 and Fanny) and the susceptible and resistant bulk from the doubled haploid lines.

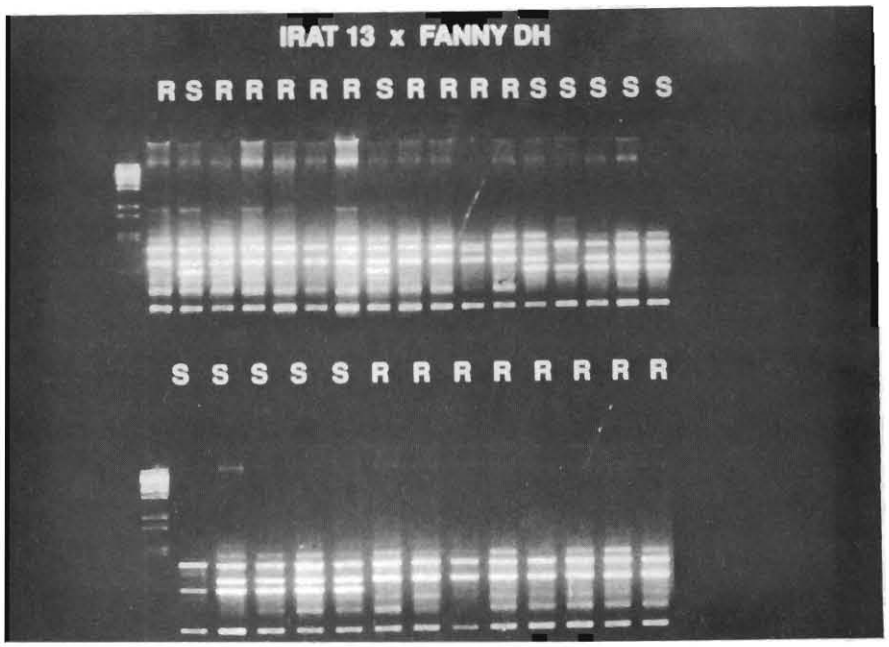
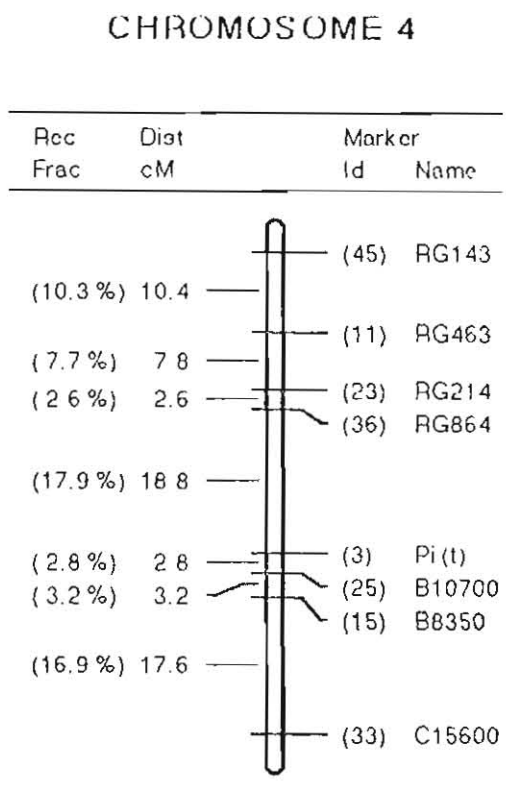


Figure 7. Amplification of the doubled haploid lines from the cross IRAT and Fanny with the primer B10. The reaction to blast isolate Cica 9-31-4 shown on top co-segregates with the primer product of 700 bp.



Mapping parents: IRAT 13 x FANNY
Population: 42 DH LINES
Kosambi function, LOD score = 4

Figure 8. RFLP and RAPD map of chromosome 4 using 42 doubled haploid lines form IRAT 13 by FANNY, showing the putative location of the blast gene indicated as pi-(t), (t for temporary identification). The mapping was conducted with a LOD score of 4 and a recombination fraction of less than 20%.

TROPICAL PASTURES

1. Genetic Transformation of *S. guianensis*

Genetically transformed plants of the forage legume *Stylosanthes guianensis*, CIAT 184, were obtained by regeneration of leaf-disc explants inoculated with the disarmed strain of *Agrobacterium tumefaciens* EHA101 and with the wild strain R1000 of *Agrobacterium rhizogenes*, both containing the plasmid pGV1040 in a binary disarmed vector system (7). The T-DNA region of this vector contains the selectable gene neomycin phosphotransferase II (npt II), which confers resistance to the antibiotic Kanamycin; the reporter gene ß-glucuronidase (uid A) and the herbicide resistance gene (bar) which confers resistance to the herbicide commercially known as "Basta" (Hoechst). Minimum lethal doses were determined for both Kanamycin and the herbicide (Basta) and used for selection during regeneration of transformed plants.

The GUS assay was carried out on leaves of transformed plants as described by Jefferson (1987). Percentage of transformation with the disarmed strain was about 10% but only 5% could be regenerated, while with the wild strain transformation was rate 5% and regeneration 2% respectively. In a second experiment carried out with the *A. tumefaciens* strain EHA101, transformation percentage was 11% and regeneration 5%. Regenerated plants were planted in the glasshouse.

DNA extracted from leaves was hybridized to a portion of the plasmid pGV1040, stemming from the region between the T-DNA border sequences. Results of this test provided evidence of physical DNA transfer for each of the transformation events of the first experiment.

As in the *in vitro* step, minimum lethal dosis of the herbicide was determined for use under glasshouse conditions; the results showed that a concentration of 0.5 l/ha produced death of rooted cuttings of non-transformed check-plants after 5 days. Rooted cuttings of transgenic plants were challenged with herbicide concentrations of 1 and 2 l/ha without severe damage. Work is underway to determine the inheritance of the bar gene to the next generation.

2. Somaclonal Variation for Plant Adaptation to Acid Soils

Fourteen *S. guianensis* CIAT 2243 derived somaclonal lines were selected from our early work on somaclonal variation generated through *in vitro* culture. Some of these lines displayed superior agronomic performance than the check line and others showed special morphological characteristics such as dwarf (bushy) plant habit, chlorotic foliage, 1-2 leaflet leaves, and tetraploidy. This material was grown

in the field for two generations and in the greenhouse for one generation. The observed variations remained throughout the four generations.

The objective of this work is to select somaclonal lines with plant characteristics (shoot-root biomass) suitable as tools in studies of plant adaptation to acid soils.

The results of this research have been presented and discussed in the TPP Annual Report, Section on Plant-Soil Relations and Nutrient Cycling (I. Rao).

3. Construction of a *Brachiaria* molecular linkage map

The overall goal of the proposal is to construct a highly saturated molecular map for *Brachiaria*, using random genomic and cDNA clones and random primers. The map will be used to tag important agronomic traits (simple or quantitatively inherited traits), to study the genetics of diploid-tetraploid species, to estimate the level of variability and heterozygosity within and between gene pools, to understand the evolution of the species, as for Salamini's group is working on the mapping of potato.

A random genomic library will be constructed from leaf tissue. Preliminary work will be initiated with random primers to look at the feasibility of using the random amplified polymorphic DNA (RAPD) technique (Williams et al., 1991) for mapping, in addition to the random genomic clones. If promising, a set of arbitrary primers (200 primers presently available at CIAT) would be used to initiate the mapping.

The random genomic library and arbitrary primers will be screened on genotypes involved in controlled interspecific crosses at the diploid level to detect polymorphisms among the parental genotypes. As polymorphic markers are identified, appropriate crosses would be carried out. Once probing with a number of RFLP clones or arbitrary primers has been completed, results will be used in the segregation analysis.

While most of the work could be carried out at CIAT it is suggested to write a joint project proposal with the Max-Planck Institute in Cologne (Dr. Salamini's group). This collaboration will provide CIAT with complementary expertise in genome mapping of diploid-tetraploid species.

4. DNA Fingerprinting of *Colletotrichum gloeosporoides*

Considerable pathogenic variability exists in populations of *Colletotrichum gloeosporoides*, particularly in Brazil, the center of diversity of *Stylosanthes capitata*.

Both the importance of *S. capitata* and the variability of *C. gloeosporoides* make it important to seek better biological characterization of this pathogen.

Genetic fingerprinting is nowadays the method of choice for taxonomic and phylogenetic studies in bacteria or fungi, the advantages being accurateness, independence from growth status of the pathogen, and the biological implications concerning variability and stability of the pathogen. This has been well demonstrated through work done in the Rice Program together with M. Levy and J.M. Hamer at Purdue University on *Pyricularia*, and the work on *Xanthomonas campestris* pv phaseoli in the Bean Program and the University of Wisconsin.

The necessary molecular techniques have been fully taken over by the programs. Bean Pathology is working now also on *Phaeoisariopsis griseola* and *Colletrotrichum lindemuthianum* the causative agents of angular leaf spot disease and anthracnose, respectively. This gave us the opportunity to establish a collaborative link between Bean Pathology, Pastures Pathology and the BRU. We are basically involved in the planning and conduction of initial experiments. We consider it a good investment to introduce the Pastures Pathology personnel into such widely applicable techniques.

Preliminary experiments with a few contrasting isolates have shown a good level of polymorphism using heterologous DNA probes, such as ribosomal DNA and a DNA fragment stemming from the M13 phage which has already shown high levels of polymorphism in different organisms. Our aim is to isolate a homologous semirepetitive DNA fragment from a *C. gloeosporoides* genomic library, which is presently being constructed.

5. Identification of the Spittlebug Resistance Factor in *Brachiaria jubata* and Development of a Screening Procedure

A phytoecdysteroid has been postulated as the resistance principle against spittlebug infestation in *Brachiaria jubata*. This stems from S. Lapointe's observations of the effects on the insect of feeding on resistant accessions. The interference with morphogenesis of the insect strongly supports some kind of hormonal disturbance, the picture favoring the production of a phytoecdysteroid. This has also been partially corroborated by HPLC analysis of spittlebug excreta by J.C. Steffens at Cornell University. Steffens is also working on aminoacid contents of different *Brachiaria* accessions with varying degrees of resistance to splittlebug.

As an alternative project we have initiated the production of antibodies against 20-hydroxyecdysone (ß-ecdysone). The immunological approach would lead us first to the identification of the resistance factor. If the hypothesis is correct, the antibodies would then be used for the immunological detection of phytoecdysteroid producing plants in a breeding program using ELISA techniques. Antisera have

successfully been used in the transfer of arcelin into bean commercial cultivars for bruchid resistance.

To generate antibodies, ecdysone has to be linked to bovine serum albumin (BSA) to make it immunologically active, as the molecule alone is too small to show good immune stimulation in rabbits. This will be achieved by binding a succinate bridge to the steroid using the hydroxyl groups in the A-ring. After activation with ethyl chloroformate, the link with BSA can be formed. This complex will then be used in an immunization scheme. After purification of the IgG fraction by affinity chromatography through Protein G Agarose Beads, the IgGs will be used to develop an ELISA test for ecdysone as an alternative to the RIA described in the literature, thus obviating the use of radioactivity.

Some comparative studies between resistant and susceptible *Brachiaria* accessions will be performed using HPLC as to complement Steffens' work. Different groups of substances can be monitored using this technique.

OTHER RESEARCH ACTIVITIES

1. High Performance Liquid Chromatography, the Multipurpose Approach

Liquid chromatography is probably the most wide-spread chromatographic technique globally. It is based on the partition of the components to be separated between a mobile liquid phase and a stationary phase. The sample is ideally dissolved in the mobile phase and carried along the stationary phase by a continuous flow generated by a pump or hydrostatic pressure. The component with the higher affinity for the stationary phase is delayed at the end of the column, thus we achieve separation from the other components.

The principles for separation are manifold: ion exchange, by size using gel filtration, affinity matrices, chromatofocusing which separates in dependence of the isoelectric point of molecules, or by hydrophobic interactions. The separated molecules can then be further analyzed by specialized detectors (UV-VIS, fluorescence, refraction index, electrochemical potential) or other analytical machinery (MS, IR, NMR) for their positive identification, or quantified using standards. Most biological substances, from simple molecules to complex polymers, can be brought into solution, and thus be separated by liquid chromatography, even without loss of their biological activities.

Reduction of particle size of the stationary phase enormously increases the separation efficiency of chromatographic columns, but with particle sizes of 5-10 µm, pressures of up to 400 atm (40 MPa) are required in order to push the mobile phase through the column, hence High Performance Liquid Chromatography (HPLC) was created, and has eversince revolutionized the analytical world. The theoretical plate number, which is a measure of the resolution capacity of a chromatographic column, reaches values of up to 50'000/m, while a classic column only reaches values of 200/m, thus a typical HPLC column of 10 cm will have up to 5000 plates, as compared to 100 for a 50 cm classic column.

Our HPLC apparatus was installed in August 1991. It consists of an Autosampler for multiple sample processing, the Quaternary Pump to generate the high pressures necessary and accomplish the different solvent mixing protocols, a Multidiode Array UV-VIS Detector which allows peak detection at various wavelengths at a time, and a PC to control the software that runs the machine and evaluates the data acquired. The heart of the machinery is composed of a basic set of analytical columns that allow separations following different principles (ion exchange, reverse phase, hydrophobic interaction, an Aminex HPX 87H cation exchanger specialized in volatile fatty acids separations). We hope to be able to expand our system by a fluorescence detector for the detection of aromatic

compounds, and a refraction index detector for the more sensitive detection of sugars, in the next future.
We have been optimizing the separation of several groups of biomolecules already within the frame of relevant projects. One of these projects involves the quantification of phytohormones in dormant *Brachiaria* seeds, a small project that was started by the Pastures Program together with the Chemistry Dept. of UniValle. For this purpose we have been using a methodology that allows the separation of ethanol soluble substances, which include several flavonoids (e.g. pigments), phenylpropanoids (e.g. phytoalexins), phytohormones (indoleacetic acid, giberellins, abcisic acid, cytokinines) (Graham 1991). We want to use the same methodology to generate profiles of ethanolic extracts of beans resistant to the bruchid *Acanthoscelides obtectus* as compared to susceptible lines, as a complement to the experiments being carried out at the protein level (see there).

In a collaboration with the Bean Nutrition Lab we have started the analysis of aminoacids of phaseolin and oligopeptides derived therefrom by enzymatic digestion. This analysis is within the frame of a project for the evaluation of digestibility and nutritional quality of beans. As the aminoacids cannot be directly detected, the analysis involves the derivatization of the aminoacids after acid hydrolysis with phenylisothiocyanate (PITC) to generate the phenylthiohydantoin amino acids (PTH-amino acids) (Bronzert 1986), which are then detected in the pMol range by the UV-detector (see PTH-amino acids chromatogram)

Another group of substances is being analyzed within the project of cassava starch fermentation together with the Cassava Utilization Group from CIRAD/CEEMAT (see also Biochemistry of Cassava Fermentation). The fermentation process is accomplished by a large number of microorganisms which generate fatty acids, glucose, ethanol and other substances. The fatty acids are one of the main flavour components of sour cassava starch, their qualitative and quantitative composition defining basically the quality. The microflora is composed in part by homolactic microorganisms, i.e. those that produce only lactic acid, and heterolactic bacteria, which also produce acetic, propionic, and butyric acids. In an attempt to follow the development of the microorganisms throughout the fermentation process, we have adjusted the conditions for the separation of fatty acids (see chromatogram). We hope to count on a Refractive Index Detector next year for the detection of sugars and starch.

References

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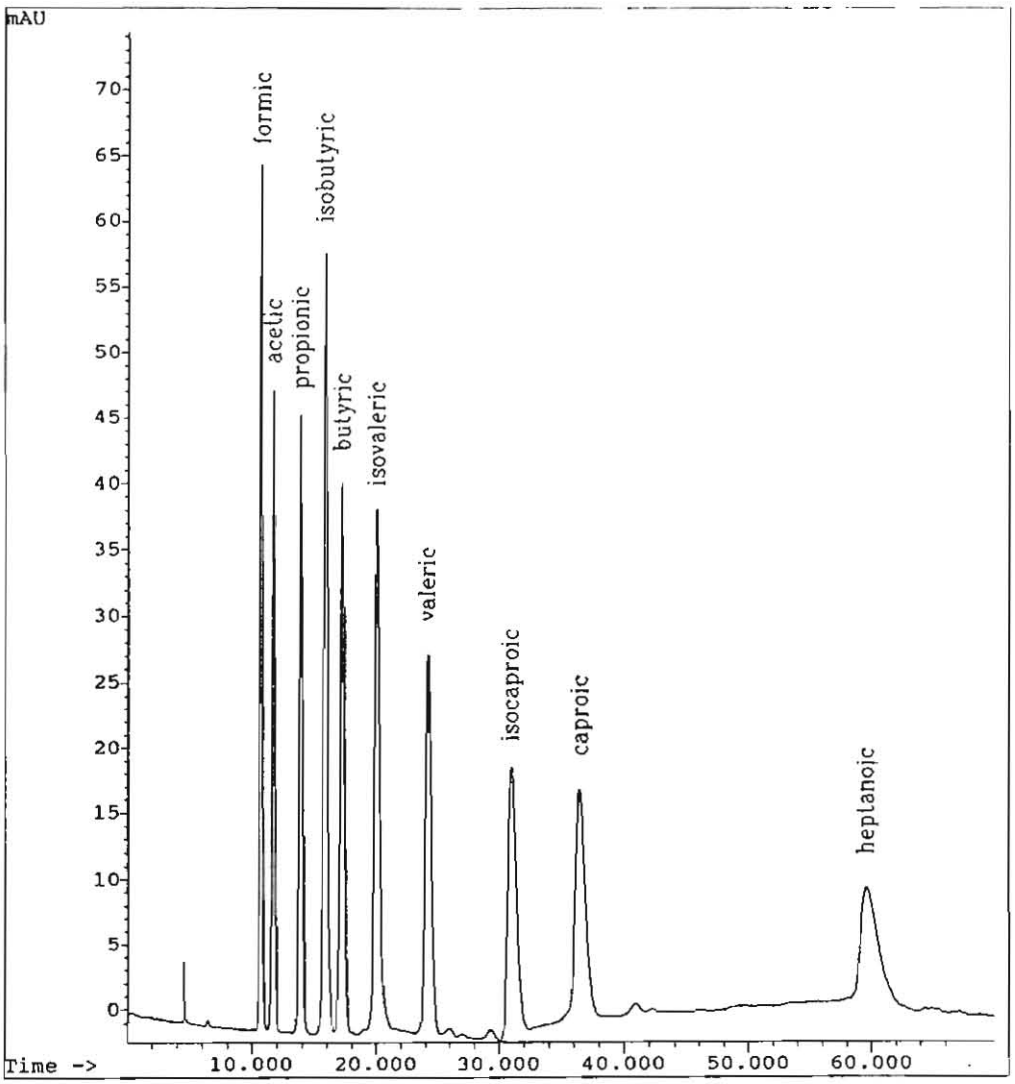
Data File Name : Fatty acids
Instrument : UV-VIS
Acquired on : Fri Nov 01 1991
Sample amount : 500 nMoles each
Column : Aminex HPX-87H cation exchanger
Mobile phase : 6 mM sulfuric acid
Pressure : 85 atm

Operator : CB-MPO
Wavelength : 210 nm



Pk#	Ret Time	Area	Height	Width	Area %
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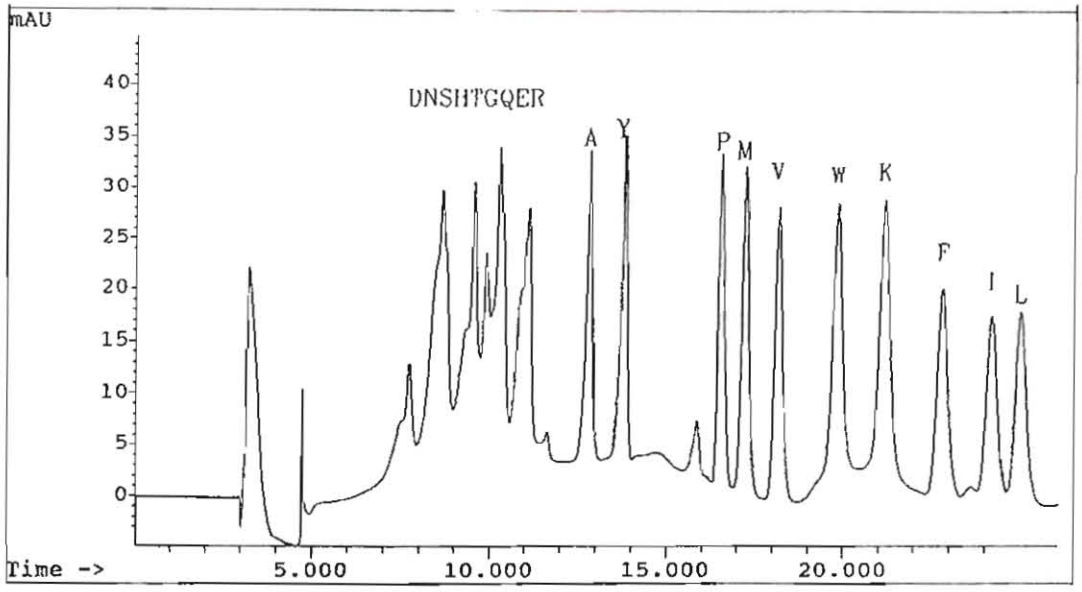
2	10.660	883.307	65.844	0.197	9.1135
3	11.666	717.178	48.570	0.222	7.3995
4	13.868	834.990	46.791	0.273	8.6150
5	15.851	1286.069	59.295	0.333	13.2690
6	17.197	964.953	41.721	0.357	9.9559
7	20.028	1178.487	39.571	0.457	12.1590
8	24.206	980.537	28.799	0.529	10.1167
9	30.973	947.747	20.380	0.720	9.7784
10	36.431	1001.987	17.544	0.875	10.3380
11	59.587	879.524	8.774	1.508	9.0745

Total area = 9692

AMINO ACIDS

Sample	: PTH-Amino acids	Operator	: MPO
Instrument	: UV-VIS	Wavelength	: 269 nm
Acquired on	: Fri Oct 25 1991		
Sample Amount	: 400 pMoles each		
Column	: Ultrasphere C18 reverse phase (5um,4.6x250mm)		
Mobile phase	: A) 93% water/ 7% B (+1.4ml TEA/100ml; pH 4.25 with HOAc) B) IPA/ACN 2.5:1		
Gradient	: 2min 2% B; from 2-10min to 20% B; 10-22min 20% B; from 22-27min to 100% B; 27-32min 100% B		



Pk#	Ret Time	Area	Height	Width	Area %
1	3.213	413.344	25.630	0.224	0.9758
2	4.725	35.140	12.706	0.040	0.0830
3	7.731	235.976	9.125	0.336	0.5571
4	8.662	794.447	25.477	0.387	1.8756
5	9.561	526.666	23.872	0.287	1.2434
6	9.900	213.061	16.670	0.177	0.5030
7	10.281	382.495	28.849	0.178	0.9030
8	10.382	202.336	26.802	0.112	0.4777
9	11.112	431.174	19.256	0.285	1.0179
10	12.859	328.600	27.677	0.166	0.7758
11	13.834	283.166	24.074	0.166	0.6685
12	15.854	45.741	3.446	0.188	0.1080
13	16.546	274.042	23.762	0.181	0.6470
14	17.236	312.509	22.862	0.205	0.7378
15	18.181	335.296	21.998	0.228	0.7916
16	19.853	702.145	25.380	0.381	1.6577
17	21.182	975.986	32.617	0.414	2.3042
18	22.815	355.472	15.352	0.342	0.8392
19	24.207	323.225	13.913	0.360	0.7631
20	25.032	333.761	14.941	0.343	0.7880
21	28.803	26081.546	1096.438	0.310	61.5748
22	30.302	6935.454	147.280	0.620	16.3736
23	31.105	1835.904	55.207	0.415	4.3343

Total area = 42357

Graham TL (1991) A rapid, high resolution High Performance Liquid Chromatography profiling procedure for plant and microbial aromatic secondary metabolites. Plant Physiol 95, 584-593.

2. Isoenzymatic Identification of Vesicular Arbuscular Micorrhizae

In an attempt to rescue the more than 1000 VAM isolates of the CIAT collection, maintenance as well as classification are badly needed. Only 50 isolates have been classified so far. Classification is tedious and a specialist in the area is needed to do the work. Within the frame of the thesis work of D. Mejia (UniValle), the analysis of VAM electrophoretic isozyme patterns was investigated as an alternative for taxonomy.

From 188 isolates initially taken into the study, only 46 were in good shape and yielded enough spores for the isozyme analysis. The following enzymes were evaluated as to the quality of bands and polymorphism levels: ACP, ADH, AKP, α,ß-EST, G1PT, G6PDH, GDH, GOT, HK, MDH, ME, PEP, PEPC, PRX, PGI, PGM (see also Working Document No. 40: A practical guide for electrophoretic analysis of isoenzymes and proteins in cassava, field beans and forage legumes, CIAT 1988). Activity was detected for ACP, AKP, α,ß-EST, G6PDH, GOT, MDH, PEPC and PRX, however only α,ß-EST showed clearly analyzable and highly polymorphic bands, thus allowing statistical treatment of the results. Casually esterase also works well in cassava and mites. Spores of many accessions are presently being multiplied to establish whether the non-detection in some cases was due to the bad condition of the spores.

The α,ß-EST survey done with 46 accessions include four of the six known VAM genera:

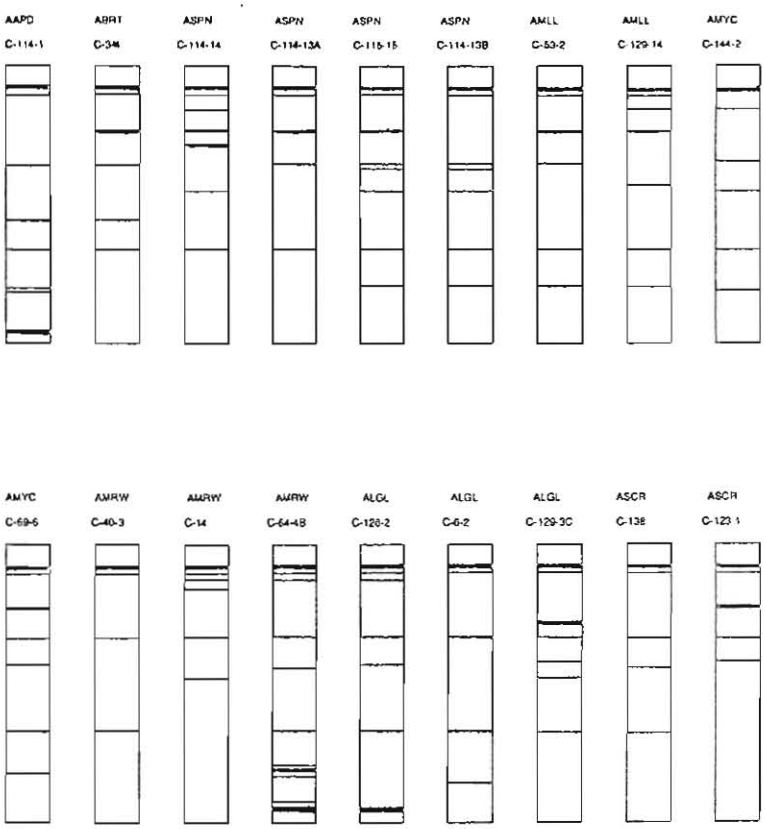
Genus	Species
Acaulospora:	A. appendicula, A. bireticulata, A. foveata, A. laevis, A. longula, A. mellea, A. morrowiae, A. myriocarpa, A. scrobiculata, A. spinosa, A. spp.
Entrophospora:	E. colombiana
Glomus:	G. albidum, G. caledonium, G. clarum, G. manihot, G. mosseae, G. occultum
Scutellispora:	S. biornata, S. calospora, S. gilmoreii, S. heterogama, S. pellucida, S. spp.

The cluster analysis of the esterase data does not always coincide with the taxonomy stemming from morphologic analysis (see dendrogram). In some cases isoenzymatic divergence does not seem to correlate with morphologic relatedness. On the other hand, some bands were very characteristic of some isolates, what makes them very useful for taxonomic studies.

This should be considered a preliminary study. Further studies should include more enzymes, if the refreshening of the bank allows, to give a more clear-cut result. MDH is a possible candidate, as has been published elsewhere (Rosendahl 1989). A very extensive characterization of CIAT's collection is sought, which could be the most complete characterization of this type worldwide. Direct analysis of genetic polymorphism as revealed by the RAPD technique could be a viable alternative (see polymorphism analysis of rice).

References

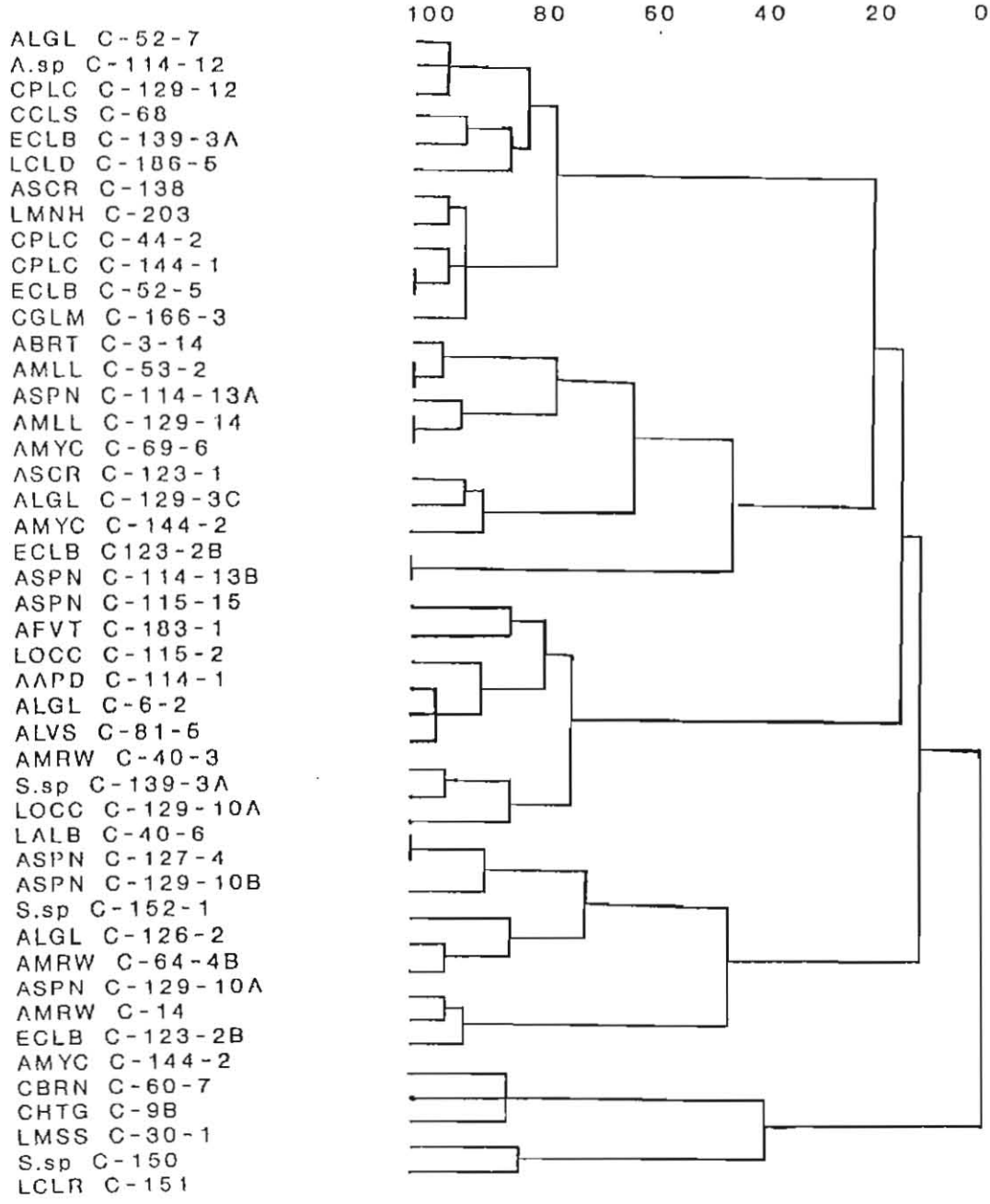
Rosendahl S (1989) Comparisons of spore-cluster forming Glomusspecies (Endogonaceae) based on morphological characteristics and isoenzyme banding patterns. Operaotanica 100, 215-223.



Esterase zymogram of eighteen species within the genus ***Acaulospora***

Schematic of a zymogram containing all esterase bands found in VAM species so far tested, used to identify bands for Cluster Analysis

Cluster Analysis of VAM Species using Esterase



INSTITUTION BUILDING

1. Networking

Funding of the **Cassava Biotechnology Network** has been confirmed in principle by the DGIS, Ministry of Foreign Affairs, the Netherlands. The proposal includes the position of a coordinator, who will be placed at CIAT, the publishing of a newsletter, and bridging funds for the initiation of projects. The first scientific meeting of the network is scheduled for August 1992 in Cartagena, and the third Steering Committee meeting for February 1992 at the Scripps Institute, La Jolla, California.

Members of the Steering Committee of the **Advanced Phaseolus Beans Research Network** (founded in September 1990) met at the BIC Meeting in November in Nebraska, to discuss the needs of the network. A recommendation was made for CIAT to actively seek for potential donors for the network.

2. Training

Four PhD students have been conducting their thesis research at the BRU. **Alvaro Mejía** (Bonn University, FRG, funded by the BMZ), is working on the **tepary bean** introgression into common bean using congruity back crosses and embryo rescue techniques, and on regeneration from embryogenic suspension cultures. **Martine Korban** (McGill University, Montreal, funded by IDRC), has been working on **bean** regeneration and transformation. **Bill Welsh** (University of Manitoba, Winnipeg, funded by CIDA) is working on the characterization of recombinant inbred lines from crosses between Mesoamerican and Andean gene pools. **Rodrigo Hoyos** (Michigan State University) spent some time working on regeneration of **bean** plants from embryogenic suspension cultures.

Biology and Agronomy students from the Universidad del Valle and the Universidad Nacional de Colombia, Seccional Palmira, San Buenaventura and Santiago de Cali have carried out thesis research at the BRU. Their one year research work usually reaches levels of MSc thesis elsewhere.

The BRU has organized introductory courses to **Molecular Genetics** for CIAT's Programs, as to acquire a common language that will facilitate exchange of ideas, and to discuss the potential contributions from the BRU to specific problems. On the other hand, we have been offered introductory courses to different areas of the programs, like breeding and pathology, for the same purpose.

3. Technology Transfer

As the bridging role for technology transfer and capacitation also play an important role in our work, it is noteworthy recalling the kind of activities done during 1991 in this respect.

W. Roca:

L.A. Meeting on Biosafety; Brasilia; organized by IICA and EMBRAPA.

Cassava Biotechnolgy Network Steering Committee meeting, ISNAR, The Hague. April 19, 1991.

CGIAR meeting on Biosafety and IPR, ISNAR, The Hague. August 31, 1991.

Seminar on biotechnology for agricultural production and agro-industry. FUNDEAGRO/INIAA, Lima. November 5, 1991.

J. Mayer:

La nueva biotecnología: Aplicaciones en la agricultura de los países en desarrollo. UNIBAN and UNC Medellín. Medellín Oct 5, 1990.

La genética molecular en el estudio de la fisiología vegetal y el control de malezas. In "XXI Congreso Anual y Primer Simposio Nacional sobre Fisiología de la Nutrición Mineral". Manizales Feb 20-22, 1991.

Aplicación de la ingeniería genética en fisiología y patología vegetal. In "Segundo Simposio de Ciencias Biológicas de la Asociación Colombiana de Ciencias Biológicas". Ibagué May 30-Jun 1, 1991.

Genética molecular: Entender para manipular. In "Segundo Congreso Nacional de Biotecnología de Plantas". Bogotá Jun 18-21, 1991.

I. Introducción y envío de materiales in vitro y organismos transgénicos en el CIAT. II. Intercambio y liberación de plantas transgénicas. In "Primer Encuentro Nacional sobre Control de la Calidad en Procesos Biotecnológicos". Medellín Sep 5-6, 1991.

Introductory courses in Molecular Genetics:

UNC Medellín Oct 4-6, 1990.
UNC Bogotá Mar 18-19, 1991.

BRU PERSONNEL 1991

Tissue Culture, Genetic Transformation and Germplasm Conservation

William M. Roca	Physiologist, Unit Head
Zaida Lentini	Postdoctoral Scientist, Rice Program
Martine Korban	PhD Student, Beans
Alvaro Mejía	PhD student, Beans
Marta Lucía Cataño	Research Assistant II, Cassava
Rodrigo Sarria	Research Assistant II, Cassava, TPP
Roosevelt Escobar	Technician I, Cassava*
Pablo Herrera	Technician III, Cassava
Germán Martínez	Technician III, Beans
Aquileo Rodríguez	Technician I, Cassava
Eddie Tabares	Laboratory Assistant, Rice
Ana María Gómez	Student, Cassava
Sandra Lenis	Student, TPP
Ramiro Villaquirán	Student, Beans

Molecular Markers and Germplasm Characterization

Joe Tohme	Geneticist
Fernando Angel	Postdoctoral Scientist, Cassava*
Bill Welsh	PhD Student, Beans
Alfredo Badillo	Research Assistant III, Rice*
María Victoria Montenegro	Research Assistant II, Rice*
Jaime Vargas	Research Assistant II, Beans
Claudia Vergara	Research Assistant III, Beans*
Delkin O. González	Rural Year, Beans*
Nidia Reyes de Brand	Technician, Rice
Vicky Barney	Student, Cassava
Janeth Gutiérrez	Technician I, Beans*

Molecular Biochemistry

Jorge E. Mayer	Biochemist
Yamel López	Associate Professor, UNC Palmira
María del Pilar Ospina	Research Assistant II, HPLC
Fernando Tenjo	Research Assistant I, Beans, Cassava
Hernando Ramírez	Research Assistant I, Beans
Camilo J. Flórez	Student, Cassava
Diego F. Mejía	Student, Micorrhizae
Walquiria Vélez	Student, Cassava

Supporting Staff

Luz Amparo Cartagena	Bilingual Secretary
Pilar Torres	Bilingual Secretary
Marlene Valenciano	Technician III
Régulo Arias	Technician III
Piedad Flórez	Technician III

* Special Project