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

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

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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.

References

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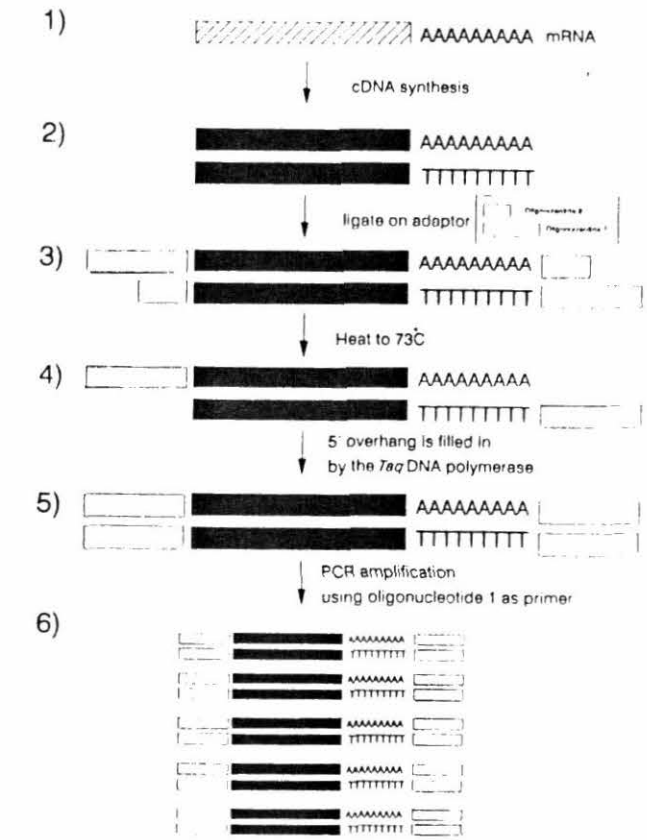
5. **RFLP Linkage Mapping of *Phaseolus vulgaris***

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.



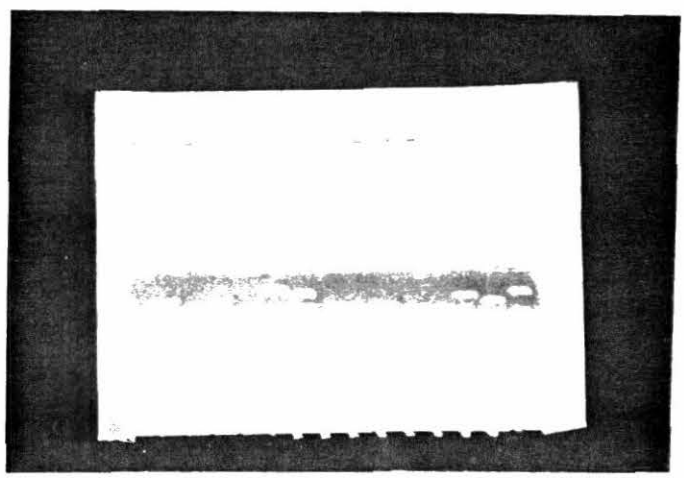


Figure 1. Top: Schematics of the PCR driven cDNA amplification procedure used for the generation of the bean cDNA library.
Bottom: Electropherogram showing cDNA inserts that have been cut out of the vector by Eco RI digestion. Left lane shows a molecular weight marker; the lowest band has 564 bp.

The parental lines selected are:

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

References

Jepson I, Bray J, Jenkins G, Schuch W and Edwards K (1991) A rapid procedure for the construction of PCR cDNA libraries from small amounts of plant tissue. Plant Molec Biol Rep 9, 131-138.

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.

References

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Williams JGK, Kubelik AR, Livak KJ, Antoni Rafalski AJ and Tingey SC (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl Acids Res 12, 6531-6535.

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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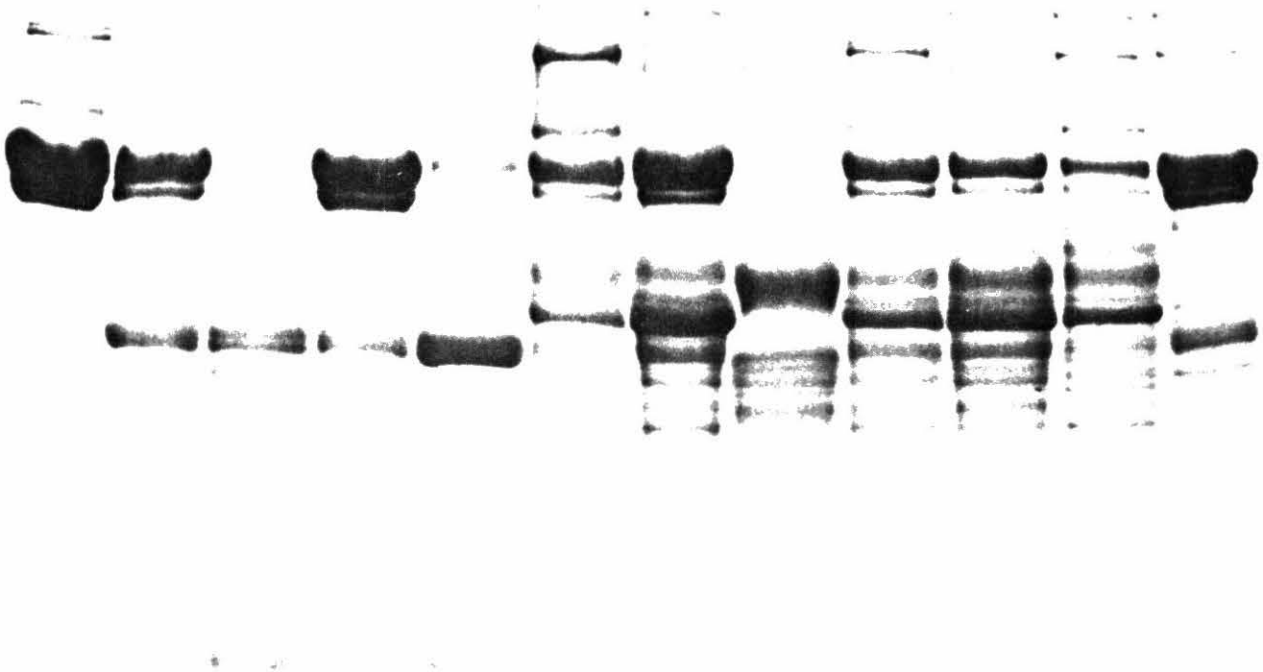

P20 P40 P80 PT PAc R20 R40 R80 RT RAc RTn PTn

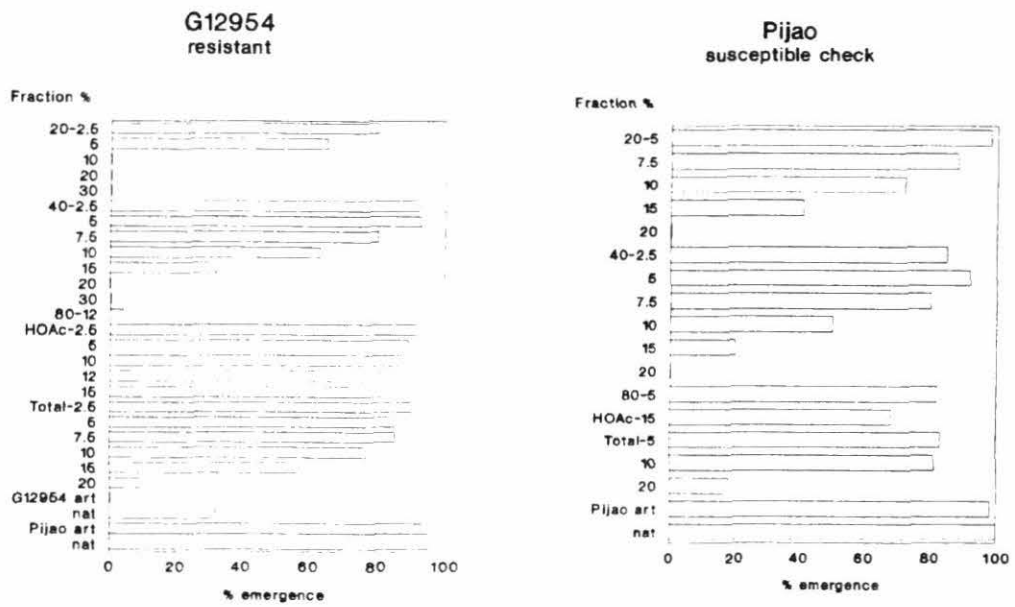

Fig. 1 Bean protein fractions incorporated into artificial seed. Numbers represent % acetone for precipitation; Ac, acetic acid extract; T, total protein (Ac and T were precipitated with 80% acetone; n, non-precipitated). P, Pijao, susceptible check; R, G12954, resistant accession.

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