

SUMMARY

ANNUAL REPORT

2001

PROJECT SB-2

**ASSESSING AND UTILIZING
AGROBIODIVERSITY
THROUGH BIOTECHNOLOGY**

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SUMMARY ANNUAL REPORT

SB-2: ASSESSING AND UTILIZING AGROBIODIVERSITY THROUGH BIOTECHNOLOGY

a. SB2 Investigators: Name, discipline, position and time fraction

Name	Discipline	Position	Time dedication %
Beebe Steve	Bean Breeding	Senior Staff	30
Beeching John	Plant Molecular Biology	Bath University. Visiting Scientist CIAT from Oct. 17/2000 - Oct 16/2001	100
Bellotti Anthony	Cassava Entomology	Senior Staff	20
Blair Mathew	Bean Genetics and breeding	Senior Staff	70
Ceballos Hernan	Cassava Breeding	Senior Staff	40
Debouck Daniel	Botany	Senior Staff	20
Fregene Martin	Cassava Genetics and breeding	Senior Staff	60
Lentini Zaida	Biology/Genetics	Senior Staff	80
Martínez César	Breeding	Senior Staff	49
Mba Chikelu	Cassava genomics	Visiting Research Fellow	100
Mejía Alvaro	Cell Biology	Consultant	20
Restrepo Silvia	Molecular Plant Pathology	Research fellow from July 27/2001	100
Roca William	Cellular Physiology	CIP. Part time CIAT - Dec, 22/2001	10
Tohme Joe	Genomics	Project Manager	100
Verdier Valerie	Molecular plant Pathology	IRD. CIAT Staff till - July 5/2001	100

b. Cooperators and location

Within CIAT

Genetic resources, bean, forage, cassava, rice, IPM, GIS, and Participatory research projects.
CLAYUCA and FLAR

Outside CIAT

CEGA, Colombia, Cenicafé, Colombia, Center for Applied Molecular Biology in International Agriculture (CAMBIA), Australia, Centro Tecnológico Polar, Venezuela, CIB, Colombia, CIP, Perú, Clemson University, US, COLCIANCIAS, Colombian Ministry Agriculture and Rural Development, Colombia, Cornell University, US, Corpoica, Colombia, Corporacion Biotech, Colombia, Danforth Center, US, FAO, Italy, FIDAR, Colombia, ICARDA, Syria, ICRISAT, India, Instituto Humboldt, Colombia, INVIT, Cuba, IPGRI, IRD Montpellier, France, IRRI, Philippines, Iwate Biotech Research Center, Japan, Kansas Sate University, Michigan State University, US, Ministerio de Agricultura, Nicaragua, National Center for Genome Research, National Root Crops Research Institute, Nigeria, Purdue University, US, REDBIO, Latin America, Rutgers University, US, Smithsonian molecular systematic lab, US, The International Institute for Tropical Agriculture (IITA), Nigeria, UniAndes, Colombia, UniValle, Colombia, Universidad Nacional Palmira, Bogotá, Colombia, Universite de Perpignan France, University of Bath, UK, University of Freiburg, Germany, University of Hanover, Germany, Univesity of Hoeiheimm, Germany, University of Nebraska, US, USDA, Plant genomics, US, USLU, Uppsala Sweden, Yale University, US.

c. Budget: resource allocation by research

LINE ITEM	TOTAL US\$
Unrestricted	1,216,640
Unrestricted carryover	222,478
Sub-Total	1,439,118
Unrestricted Substitution	27,956
Carryover	9,843
Sub-total	37,799
Restricted	2,250,172
Total Project	3,727,089
All sources:	
Personnel	1,743,577
Operations	1,983,512
Total	3,727,089
Number of IRS	7.89
Number of NRS	45.90
Total personnel	53.79

d. Highlights of outputs

The project has pursued successfully all three outputs:

- Output 1 Genomes of wild and cultivated species of mandated and non mandated crops, associated organisms characterized.
- Output 2: Genes and genes combination made available for broadening the base of mandated and non mandated crops.
- Output 3: Collaboration with public and private partners enhanced

Only the following achievements are summarized.

Microsatellites developed and implemented for common bean:

Microsatellites are polymerase chain reaction (PCR) based markers that detect length polymorphisms at loci with simple sequence repeats. They are also single-locus markers that are specific to a given place in the genome. Microsatellites are advantageous because they are readily amenable to relatively high throughput marker assisted selection (MAS) strategies. Microsatellites have been developed for a wide range of plant species, however few were available for common bean before we started this work.

Various techniques exist for discovering new microsatellite markers from anonymous genomic sequence. All of these techniques rely on the availability of DNA libraries. In our first experiments we developed microsatellites from an enrichment method that produced two libraries for di-nucleotide motifs. This year we have screened four additional gene and DNA libraries and over 120,000 individual clones, to investigate the normal frequency with which different microsatellite motifs occur in common bean and to develop a new set of genomic microsatellites for mapping and tagging projects in common bean. We have collected over 3000 sequences in this effort, representing at minimum a 600% increase in the number of sequences available for common bean in the Genbank public database. We have also screened a large number of microsatellite markers that were developed for soybean and cowpeas to try to adapt the microsatellites available for other *Phaseoleae* legume crops to common bean.

The microsatellite markers have been useful in the evaluation of genetic diversity in the CIAT germplasm collection and for various species of *Phaseolus* beans, especially for tepary beans (*Phaseolus acutifolius*) for which little variation is detected by other methods. The microsatellites are able to distinguish taxonomic relationship between species. The genetic diversity of microsatellite alleles has also been evaluated for two parental surveys of common bean that provide the basis for mapping and genetic tagging experiments for a range of important traits; including biotic and abiotic stress resistance / tolerance, micronutrient accumulation. This information is being incorporated into a new molecular genetics database.

Our ultimate goal has been to create a genetic map for common bean consisting entirely of microsatellites and to use these in MAS breeding. Genetic maps are needed to determine where microsatellites are located in the genome and what genes they may be linked to, which is a prerequisite first step for applying microsatellite markers to MAS selection. For now, we have implemented a set of over one hundred and fifty microsatellite markers in genetic mapping studies for common bean at CIAT. The microsatellites have been found to be well-

distributed throughout the genome, making them very appropriate for genetic mapping and selection. We hope that these second-generation markers will be easy to assay and will enable a large number of segregating individuals to be analyzed in gene and QTL tagging studies. We will be studying the potential of specific microsatellites to be used in MAS selection for specific genes with which they are linked.

The genetics of micronutrient accumulation in common beans:

Legumes provide essential micronutrients that are found only in low amounts in the cereals or root crops. An ongoing project, has shown that bean seeds are variable in the amount of minerals (iron, zinc and other elements), vitamins and sulfur amino acids that they contain and that these traits are likely to be inherited quantitatively. With the hope of selecting for higher mineral content on a regular basis, we were interested in tagging quantitative trait loci (QTLs) controlling the accumulation of these minerals. In our initial studies we have used two populations representing an Andean x Andean and a Mesoamerican x Mesoamerican cross. The phenotypic data was obtained by analyzing both the parents and the recombinant inbred lines for iron and zinc content by ICP (Inductive coupling plasma). The QTLs were mapped with microsatellite and RAPD markers that were used to construct separate genetic maps for each of the populations.

QTLs were found for iron and zinc content in both populations. The positive markers varied in their level of significance and the proportion of variance in mineral content that they explained. The most significant QTLs explained up to 12% of the variance in mineral content. In some cases the QTLs for both minerals occurred jointly at the same marker, in other cases there were QTLs specific for each mineral. The QTLs were generally found in similar locations of the same chromosomes in both populations. The majority of the positive QTLs were associated with alleles from the high mineral parent, therefore some of the QTLs for the accumulation of both minerals may be genetically linked or pleiotropic, controlling both traits at once. If the same QTLs contribute simultaneously to both iron and zinc content, it may be easy to select for these traits jointly. It also appears that high mineral content parents provide most of the genes for high mineral content to their progeny, while low mineral parents provide only a few additional genes for mineral content.

We have also begun to study the common bean genes involved in getting iron and zinc from the root zone to the grain. Several genes from other legumes or from model species, could qualify as candidate genes for the control of micronutrient accumulation and storage in the bean seed. Among the most likely is phytoferritin which is the major storage form of iron in all tissues including seeds. We studied both the expression and genetics of phytoferritin production in beans. The protein was identified in ground bean seed extracts and a sequence characterized region marker was developed to map the gene or genes encoding phytoferritin in beans. Other candidate genes that we will be considering are involved in the production and processing of Nicotianamine, a polyamine that is known to function in scavenging iron from the root zone in grasses and may be involved in other iron related functions in plants more generally, including possibly the transport of iron.

The present work will hopefully permit us to focus on certain parts of the genome to determine if desirable alleles for higher mineral content are located at the same loci in additional populations developed specifically for this purpose. We also plan to integrate the information about the map locations of QTLs for micronutrients with those for other agronomic traits that we have been studying, so that we can select for the best advanced lines from crosses with high micronutrient lines using marker assisted selection.

Overview of cassava SSR markers at CIAT

CIAT has continued to provide leadership in the development and deployment of cassava molecular genetic tools. We have by the development and deployment of microsatellites or simple sequence repeats (SSR) markers built up on the gains made with the publication of the RFLP-anchored framework map in 1997. Following however from the recognition of the shortcomings of the RFLP technology, our Unit shifted focus to the development of PCR-based molecular genetic tools, essentially SSR markers. The RFLP techniques are expensive, require the use of hazardous radioactive probes that are not available to many resource-poor developing country research programs, and these probes must be physically transferred from one site to another under strict safety protocols. In contrast, PCR-based markers are robust, inexpensive to assay, easily shared among researchers and readily accessible in public and private domains, making this a much more appropriate approach in these countries. With access to a simple text file containing the sequences of the oligonucleotide primers for the PCR-based markers of interest, a breeder can rapidly and efficiently evaluate the germplasm under study. This technology can significantly improve the efficiency of cassava varietal development programs and reduce the time and cost by up to 50 percent, effectively doubling the capacity of existing research programs. Some highlights of some of the achievements made in the development and deployment of SSR markers include:

- **Development:** Over 500 microsatellite (SSR) markers have been developed. Many of these have been placed on the framework map. It is expected that by the end of 2001, no less than 200 PCR-based markers would have been mapped.
- **Deployment:** These SSR markers have been made publicly available at <http://www.resgen.com/resources/apps/mappairs/mp.php3> and through publications
- **Capacity building:** Young cassava scientists from Uganda, Nigeria, Ghana, Brazil, and Ecuador have been trained in the use of cassava SSR markers as they are applying these markers in their work in their respective countries.
- **Application:** SSR markers have been successfully applied in the following:
 - Tagging of the cassava genome loci involved in resistance to cassava mosaic disease (CMD), a most virulent disease causing extreme yield losses;
 - The QTL mapping of the cassava genome loci controlling cassava bacterial blight (CBB), another cassava disease and the cassava whitefly, an important disease vector;
 - The QTL mapping of earliness and several other agronomic traits; and
 - The mapping of resistance to cassava green mite, an obnoxious cassava pest.

Marker-Assisted Breeding of Resistance to Cassava Mosaic Disease (CMD)

The discovery of a qualitative and high level of resistance to the devastating cassava mosaic disease (CMD) and molecular markers linked to it have made conceivable marker-assisted breeding for CMD resistance at CIAT. Progress made this year includes the establishment of a sexual hybridization scheme between resistant donor lines received last year from IITA and CIAT elite parents. Included for genetic crosses are high carotene lines, to combine high carotene and CMD resistance, targeted to sub-Saharan Africa. Furthermore, field experiments in Uganda have revealed that the novel source confers resistance against the

Ugandan variant (UgV), an aggressive recombinant strain of the virus that caused a disease epidemic that swept through Uganda and is now spreading into the Democratic Republic of Congo, Kenya, Tanzania and Rwanda. A marker-assisted selection (MAS) scheme has also been initiated for the rapid verification of CMD resistant selections in resistance breeding to contain and prevent the spread of the epidemic. Progress is also being made on the identification of candidate genes that may mediate the molecular basis of CMD resistance.

Genetic diversity characterized

Progress continue to be made in the understanding of the genetic diversity of *Phaseolus* species, Cassava landraces and *X. axonopodis* pv. *Manihotis* strains using a wide range of molecular markers (AFLP, RAPD, microsatellites and sequencing). This year the core collections of *Phaseolus coccineus* and *Phaseolus polyanthus* was evaluated with AFLP markers, demonstrating that very little structure exists in these two species, although Mexican and Guatemalan accessions of *P. coccineus* separate slightly, and an ecotype of *P. polyanthus* exists in South America.

Diversity in the CIAT collection of tepary beans (*Phaseolus acutifolius*) was analyzed with AFLP and microsatellites to distinguish taxonomic relationships with *P. parvifolius* as well as within the species between *P. a. var. acutifolius* and *P. a. var. tenuifolius*.

Genetic diversity of microsatellite alleles was determined for wild and cultivated bean genotypes, and accessions of wild and cultivated accessions of *P. coccineus*, *P. Polyanthus*, *P. acutifolius* and *P. lunatus* providing the basis for using the microsatellites over a wide range of genetic diversity studies.

The study of cassava land races using microsatellites was extended to assessing genetic diversity and differentiation of cassava land races from 5 countries in South America, 2 in Central America, and 2 in Africa, and to African cassava genotypes resistant to the Cassava Mosaic Disease (CMD). The analysis showed a substantial amount of genetic diversity in CMD resistance germplasm appropriate for the genetic improvement of CMD resistance as well as other traits, particularly yield.

RHBV Transgenic Resistance

Rice hoja blanca virus (RHBV) is a major virus disease of economic importance affecting rice in northern South America, Central America and the Caribbean. Few genes control the resistance to the RHBV, but most commercial varieties with this source are resistant when plants are older than 20 days. To ensure stable and durable resistance, additional sources need to be identified and incorporated into rice. Transgenic plants with the RHBV nucleoprotein (N) viral gene were generated and are resistant even at 10-day-old. The rustic variety Cica 8 used by small farmers in tropical America was used as a target. Cica 8 is also a good parent as gene donor for the breeding program. Field evaluations over two seasons indicated that six fixed transgenic lines were more resistant than Fedearroz 2000, the most RHBV resistant commercial variety. The resistance genetic gain obtained with transgenesis is a reaction score 7-9 for Cica 8 to a reaction score of 1-3 for the transgenic lines. The transgenic lines express low levels of RNA only detectable by RT-PCR, RHBV nucleoprotein is not express in these plants which suggest a very low risk, if any, for environmental as well food biosafety concerns. A genome conversion project is in progress, and F3 lines derived from crosses with three other popular commercial varieties suggest that the transgenic resistance could be used to complement the natural resistance source to the virus. Plants with transgenic derived

RHBV resistance are currently being advanced jointly with agronomic selection traits in the field. This project has been a learning experience on how to scale up the use of genetic transformation at CIAT from the lab to the field and next step is to the farmers. The project also delivered the first field test of transgenic plants developed at a CGIAR Center where the gene inserted was sequenced and cloned at the Center itself, and the protocol used for transformation contains innovations applicable to Intellectual Property Rights according to the CIAT IP Audit disclosure. The field trials has also become an experimental plot to conduct research on environmental biosafety and the National Colombian Biosafety Council has suggested to use CIAT experience as a model for Colombia.

Cassava Transformation and Friable Embryogenic Callus (FEC) Development

Genetic transformation of cassava is being used as a tool that will support conventional breeding programs at CIAT. The aim has so far focused on genes (like insect resistance) not available in cassava germplasm, although the modification of metabolic pathways (like starch modification and β -carotene content) to improve cassava are also sought as targets. Transgenic plants were produced at CIAT –via *Agrobacterium*- more than seven years ago. They are currently being tested for long term stability and expression of inserted genes; a critical issue in an asexually propagated crop were chimeras may results after transformation. With the latest results we confirm that transformation is achievable at CIAT. Transgenic plants, containing a gene for insect resistance, are now growing in the greenhouse. Molecular tests for efficient gene expression, and bio-assays to test efficacy of protection against Lepidoptera will be carried out this coming year. The gene for insect resistance has been cloned in expression vectors that will allow its protein purification and massive production. Colonies to rear insects not available at CIAT have also been set up.

This time transformation was achieved with a novel tissue culture system called Friable Embryogenic Callus or FEC, with a model cultivar that has proven easier to transform, useful to test constructs of interest. However, the target for transformation must be cultivars required by farmers. We have therefore established cell lines of FEC for four cultivars, two of which are for the Northern Coast and one for the Inter Andean Valleys of Colombia. FEC has also been identified (but not proliferated yet) in two cultivars for the Eastern Planes of Colombia. We have therefore set up a system for scaling up transformation of cassava at CIAT. Experiments are done 2-3 times a month using *Agrobacterium* and/or biolistics, with at least four cultivars (TMS60444, SM1219-9, CM2306-4, MCol2215). More cultivars and more genes of interest are expected to enter the system in the near future.

Cryo-preservation of Germplasm: Cassava and its Wild Relatives, Tissue Culture Cell Lines, and Tropical Fruits

More than 10 years ago the BRU, together with the Genetic Resources Unit, set goals to develop methods on cryo-preservation for safer, cheaper and long term conservation of genetic resources. Methods to cryo-preserve cassava germplasm were developed 4 years ago using classic protocols (chemical dehydration and programmed freezing). They were published in 1997. New protocols, encapsulation dehydration and rapid freezing, have now being developed and validated with more than 43% of the entire cassava core collection. More than 82% of the accessions tested have recovery rates above 30%, the minimal required to be considered for cryo-preservation. The protocols are now being adjusted to the wild relatives of cassava, species that some times behave very poorly in vitro or even in the field, making their conservation troublesome. Plants have been recovered for *M. esculenta* subsp. *flabellifolia*, *M. esculenta* subsp. *peruviana* and *M. carthaginensis*.

Cryo-preservation is also being used as a tool to support transformation of cassava. The development of Friable Embryogenic Callus cell lines is time consuming, with the inherent risks of genetic instability and low plant recovery with time. Cryo-preserving FEC cell lines is therefore a viable alternative. FEC cell lines have been frozen and recovered in two cassava cultivars (TMS60444 and MCol2215). Since transformation of cassava requires the development of FEC for each specific cultivar, we expect to establish a cryo-preserved bank of FEC cell lines developed for transformation.

Since CIAT's mandate crops include tropical fruits, we initiated studies on conservation strategies for tropical fruit germplasm. We selected Tree Tomato (*Solanum betaceae*) as model crop to run preliminary experiments on cryo-preservation of sexual seeds. The results were very encouraging. Plants were recovered, and moved to the greenhouse, from frozen seeds of three commercially available cultivars. We expect to make adjustments to the methodology, compare it to medium-term conservation protocols (at 5, -20°C), and transfer it to National Programs.

Micropropagation to satisfy small- and medium-to-large scale cassava growers

Cassava importance as a cash crop has raised in the last five years in Colombia. A limiting factor to make cassava a cash crop is the availability of enough, best quality "seed". This is true for all cassava growers and all crops. As a response to the need of certified, planting material, we established two propagation methods: one for small-scale, poor farmers, and another for medium-to-large cassava growers (semi-industrial farmers). Using a participatory research methodology, we developed low cost propagation techniques with a women farmer group, from Santa Ana (Cauca, Colombia), who played a key role in the establishment, maintenance and running of the system. The project was initiated using one cultivar of farmer's preference (MCol1522, Algodona). Today, three more cultivars have been incorporated into the propagation scheme. In vitro plants, produced and handled by farmers, are currently planted in farmers' fields for evaluation. Besides training farmers in tissue culture to produce their own seed, we lowered the costs of implementing rural tissue culture facilities. We built a transfer hood, with locally available materials, at a cost 12 times lower than the price in the market.

The second propagation method was set up to scale up the process, keeping in mind the needs of larger farmers. We implemented the RITA[®] system to increase propagation rates from 1:3 (conventional, solid system) to 1:6-10 (Temporal Immersion System). We validated the system with 16 commercial clones for all cassava growing regions in Colombia. Following a request from farmers of the Northern Coast in Colombia, we have incorporated three local clones (*Yema de huevo*, *Ramirana*, *Por encima*) for propagation in RITA[®].

Finally, we are trying to lower the costs of implementing RITA for rural tissue culture labs, public schools, and National Programs. Several parts of the equipment can be replaced by locally available pieces.

e. Problems encountered and their solutions

One of the major problems facing by the project is the lack of laboratory space. With the purchase of new major equipment and new assistants and students, the bench space is becoming a limiting factor. We are exploring the possibility of moving the autoclave and storage facilities to the basement as a temporary measure.

Another problem is still the lack of a full time or part time person on bioinformatics, the lack of adequate access to computing facilities to handle advanced searches for gene sequences and the lack of a lab data management system. We are exploring with the information unit possible way to increase their involvement and to establish a Linux system that will allow us to set up a low cost system for local GeneBank searches. We are also trying to set up a Laboratory Information Management system (LIMS) by outsourcing the development of the package to a software company in Cali.

The third limitation is related to the inaccessibility of genes and genes constructs for relevant genes for the transformation system. While some genes are becoming available for research purpose, still the freedom to operate and to release the transgenic lines to NARS is a major challenge. The same is relevant to access the rice sequence database from the major private companies. More time negotiating as well as legal advice will be needed We have initiated some discussions with Syngenta and Monsanto and have seeked advice from the CG CAS office at ISNAR.

f. Plans for next year

Based on the success of the last few years in the area of marker assisted selection and genetic transformation additional emphasis will put to scale up both areas and to continue the integration of marker assisted selection with breeding activities.

In the area of new tools, microarray technologies already implemented, will be used as high through put molecular screening of germplasm, genome analysis and gene expression.

The vacant position will be filled. An assessment of the needs of the project and CIAT strategic plans was considered to prepare the job description.

Team members will continue the coordination for the formulation and implementation of the breeding and biotech components of the biofortification CG project. A new project on biofortification funded by USAID will be initiated in the area of MAS in bean (iron and Zinc) and genetic transformation in Cassava (beta carotene).

Members of the project will continue their active participation in the coordination of: 1) a legumes genomics initiative between the CG and US universities and institutions and 2) a Global Cassava Biotech consortium between CIAT, IITA, Danforth Center and Embrapa. Also members of the project are participating in a Cereal genomics initiative between the CG and US universities and institutions. Such initiatives will result in proposals to be submitted to donors.

Training of NARS will be pursued in the area of genetics, genomics, tissue culture and genetic transformation. Public awareness for policy makers and journalists will continue.