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AGRICULTURE IN ALLIANCE WITH NATURE:

CATIE's recent advances in Breeding and Conservation of Plant Genetic Resources

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DEDICATION

To our loving wives and children, without whose support this work wouldn't have come to reality.

To CATIE`s scientists and graduate students whose long and painstaking efforts we attempted to summarize in this work.

To farmers, professors, and environmental practitioners, who are the ones testing the relevance of new science and technology generated in the context of sustainable agriculture.

To Jorge León, who contributed to the enhancement of CATIE`s plant genetic collections.

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PRESENTATION

Agricultural research in Turrialba began in the late 1930's and early 1940's on the production of strategic raw materials, such as rubber, timber, and other raw materials. Most of that research was commissioned by the United States Department of Agriculture and the U.S. Navy. With the creation of the Inter-American Institute for Agricultural Sciences, IICA, in 1943, emphasis was switched towards plantation cash crops, such as cacao, coffee, bananas & plantains, and later on fruits and vegetables. Germplasm collections were established ever since for these crops, and for breeding endeavors. Therefore, taking advantage of those materials to discover new varieties that are much more productive but simultaneously much more tolerant to pests, so as to minimize the use of agrochemicals, have been continuously implemented since then.

An institutional split of IICA brought the creation of CATIE in 1973, which retained the traditional site and functions in Turrialba. Soon after, the research agenda was expanded to include topics such as agricultural production using a systems approach, integrated pest management as well as the management of natural forests, protected areas and watersheds, and the use of biotechnology and molecular biology.

The tendency to highlight a balance between agricultural production and environmental protection has been a foremost objective in the institution during the last decade, resulting from strategic decisions: the accords from UNCED 1992, and to continue providing sustainable alternatives to top priority agricultural issues for Member Countries, and thus maintaining its leadership among Regional research institutions.

But in keeping its tradition, CATIE has continued generating information and developing new methodologies and technologies for preserving and improving the genetic resources of agricultural and forestry crops considered strategic for the development of the Region.

In the present book, the authors guide us through key findings and developments achieved in themes such as conservation and evaluation of plant genetic resources, as well as in breeding and propagation of improved genetic material, during the last decade of the Twentieth Century. Chapters in this book are organized per crop, so as to provide the readers with a birds' eye view of the set and sequence of scientific studies implemented in coffee, cocoa, bananas & plantains, and other promissory species.

In my view, the authors were successful in summarizing a tremendous amount of technical information, putting it in a more plain language, and in making it accessible to a larger audience. They were also accurate in highlighting remarkable contributions to science and production, such as those described for cryoconservation, breeding and propagation of cof-

fee genetic materials, the majority of them led by scientists assigned to CATIE by the French Cooperation (CIRAD and ARD – formerly ORSTOM), and within the framework of the PROMECAFE network; or the application of state-of-the-art technologies for cacao and *Musa* breeding. Their book also contributes to call the scientific community's attention on the importance of CATIE's germplasm collections, many of which are among the world's largest for diverse commercial or promising crops.

It is thus a pleasure to present the following book to all of those holding a stake in the sustainable development of agriculture in tropical America. It is my hope that a synthesis such as this one, will highlight the valuable contributions CATIE makes to the sustainable agricultural development of the tropical world.

Dr. Pedro Ferreira Rossi
Director General

FOREWORD

Research at CATIE addresses the need for greater agricultural and forest productivity, along with a commitment to sustainability, by generating knowledge, methods and technologies with the ultimate aim of fighting poverty and arresting the deterioration of natural resources and the environment.

Research efforts are structured into five strategic Research Lines:

- Germplasm improvement and conservation for selected agricultural crops and forest species
- Integrated pest management in agroforestry and forestry activities
- Agroforestry systems for hillsides, frontier and degraded lands.
- Sustainable management of forests and their biodiversity
- Socioeconomic evaluation and analysis of management systems for goods and services provided by tropical ecosystems

Results of those efforts brought recognition to the institution and helped maintaining CATIE's prestige as a knowledge- and technology-generating Center during the last decade. These results have been published in a variety of media such as scientific and technical papers, books and monographs.

But a need for compiling those results into a more coherent report, that summarizes the core findings of each paper and allows readers to understand how each individual study fits into a broader research endeavor, was strongly felt at the end of the 1990's. Such a report should help in filling the information gap existing for key stakeholders, such as donors and policymakers, which do not have the time to keep track of all technical advances published in journals, neither get sufficient details of those advances from abstracts or executive reports.

The present work aims at presenting an structured summary of the main achievements and advances obtained by diverse research projects and activities grouped within the first Research Line cited above. For convenience reasons, this compilation was limited to the main agricultural crops studied at the Center during the 1992-1999 period. It is the hope of the authors that this effort will represent the starting point of a whole new series of summary reports which will cover the progress made in all remaining research lines in future editions.

ACRONYMS

ANACAFE	National Coffee Association, Guatemala
ANAM	National Environmental Authority, Panama
AVRDC	Asian Vegetable Research and Development Center, Taiwan
BIOTROP	Plant Biotechnologies and Genetic Resources Programme, CIRAD, France
CATER	Andean Center of Rural Technology, Ecuador
CATIE	Tropical Agricultural Research and Higher Education Center
CEMARE-BSF/ANAM	Forest Seed Bank from the Training Project on Renewable Natural Resources Management, National Environmental Authority, Panama
CENARGEN	Genetic Resources and Biotechnology Research Center, EMBRAPA, Brazil
CENIAP	National Center for Agricultural Research, Venezuela
CENTA	National Center for Agricultural and Forest Technology, El Salvador
CIAT	International Center for Tropical Agriculture, Colombia
CIRAD	International Agricultural Research and Development Center, France
CNPAF	National Rice and Beans Research Center, Brazil
COHDEFOR	Honduran Forestry Development Corporation
CONSEFORH	Project for the Conservation and Genetic Improvement of Honduran Forest Resources, COHDEFOR, Honduras
CORBANA	Costa Rican National Banana Corporation
CORPOICA	Colombian Corporation for Agricultural and Livestock Research
CRU/UWI	Cocoa Research Unit, University of West Indies, Trinidad & Tobago
CUNSUROC/USAC	South Western Center, University of San Carlos, Guatemala
DAL	Department of Agriculture & Livestock, Papua New Guinea
EMBRAPA	Brazilian Corporation for Agricultural Research, Brazil
FAO	Food and Agriculture Organization of the United Nations
FHIA	Honduran Foundation for Agricultural Research
FRANCERECO	Nestlé Tours Research Center, France
IAC	Campinas Agronomic Institute, Brazil
ICAFE	Costa Rican Coffee Institute
ICGR-CAAS	Institute of Crop Germplasm Resources, Chinese Academy of Agricultural Sciences
IHCAFE	Honduran Coffee Institute
IICA	Interamerican Institute for Cooperation on Agriculture
INIA	National Institute for Agricultural Research, Peru
INIAP	Autonomous National Institute for Agricultural and Livestock Research, Ecuador
INIBAP	International Network for the Improvement of Banana and Plantain
INIFAP	National Institute for Forestry, Agricultural and Livestock Research, Mexico
INPA	National Institute for Amazonian Research, Brazil
IPA	Pernambuco Enterprise of Agriculture and Livestock Research, Brazil
IPGRI	International Plant Genetic Resources Institute
IPK	Institute for Plant Genetics and Crop Plant Research, Germany
IRD	Development and Research Institute of France (formerly ORSTOM)
ISRA	Senegalese Institute for Agricultural Research
MAE	French Ministry of Foreign Affairs
MAG/CR	Ministry of Agriculture and Livestock, Costa Rica
NBPGR	National Bureau of Plant Genetic Resources, India
ORSTOM	French Institute for Scientific Research and Development Cooperation

OTS	Organization for Tropical Studies
PROCAFE	Coffee Research Foundation, El Salvador
PROMECAFE	Coffee Improvement Program in Central America, Mexico and the Dominican Republic
RVAU	Royal Veterinary & Agricultural University, Denmark
UCR	University of Costa Rica
UFCO	United Fruit Company
UPLB	University of Philippines at Los Baños
USAC	University of San Carlos, Guatemala
USDA	United States Department of Agriculture

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INTRODUCTION

Agricultural productivity in tropical America has traditionally been low, especially in the case of native crops. This stems largely from not selecting better genotypes and from the need to improve technological packages to manage them. At its headquarters in Turrialba, Costa Rica, CATIE possesses one of the largest germplasm collections of tropical and exotic species in Latin America, whose origins can be traced back to the early 1940's, to IICA's beginnings. These collections could serve as a key source for selecting and developing superior genotypes that could, in turn, be used to increase productivity.

The importance of genebanks in crop breeding has been widely recognized since Vavilov published his seminal works in 1935[1]. Genebanks are essential if genetic diversity of commercial crops and their relatives is to be perpetuated. Presently, there are more than 300 species of current or potential importance in CATIE's germplasm collections. They are preserved in field collections as well as in cold storage facilities. Field collections, required for species with recalcitrant seeds, have been planted on a 50 ha farm with approximately 260 species and 4400 introductions. On the other hand, utilizing holding chambers for species with orthodox seeds, makes it possible to adjust conditions to allow for short, medium and long-term preservation. Around 6900 introductions plus a duplicate set of CIAT's bean germplasm collection (over 23,000 introductions) are maintained in holding chambers, including species from the genera: *Capsicum, Cucurbita,* and *Lycopersicon* as well as many other crops.

On the other hand, the characterization of preserved materials is the starting point for further research by providing descriptions and classifying a wide diversity of materials with potential in breeding programs. Characterization efforts were implemented during the 1990's in relevant crops such as Cocoa, Coffee, *Musa* spp. and several horticultural crops, as well as in other promising crops such as tropical fruits, peach palm and tree species.

Conservation and breeding efforts at CATIE during the period studied were supported by parallel advances in biotechnology research. Tissue culture and cryopreservation became a complementary tool to germplasm preservation. Traditional genetic improvement was accelerated by applying genetic engineering and molecular biology techniques, such as androgenesis, biolistics and molecular biology. In addition, *in vitro* propagation and regeneration of valuable species were successfully developed and refined on valuable species.

CATIE's mandate stresses the goal of alleviating poverty in tropical America. In carrying out this task, the Center has traditionally concentrated its germplasm and breeding research on commodities with potential for improving small farmers' income. Along the 1992-99 period, these species have ranged from plantation crops to horticultural ones. The present volume summarizes the progress made and the best practices found on three crops considered as essential to the region's economy: coffee, cocoa and *Musa* species. Relevant achievements in other crops are consolidated into the final chapter.

[1] Vavilov, N. I. 1935. The origin, variation, immunity and breeding of cultivated plants. Chron. Bot. 13,6 volumes.

CHAPTER ONE
COFFEE

CONSERVATION.

Increasing Preserved Stocks and Improving Preservation Methods.

CATIE preserves one of the most important coffee genebanks in the world, and definitely the largest in Latin America. It comprises a large field collection of almost 9,000 individuals. Recent introductions to this collection as well as advances in the techniques applied for their preservation, are reported in this section.

Field collections

Germplasm preservation in Turrialba started in 1948 when field collections of rubber, coffee and cocoa were established. Coffee materials initially came from Guatemala, El Salvador and Puerto Rico. Later, accessions from Brazil, Mexico, Portugal, India and several African countries were introduced (Morera *et al.*, 1993).

By 1993, CATIE's coffee collection contained 1,768 accessions whose distribution is shown in Table 1. At that time, the most recent accessions had been introduced between 1985 and 1987, including materials from France, Cameroon and Brazil (*Op.cit.*).

In 1997, a new inventory was undertaken, showing a total of 1,832 accessions (Anthony & Astorga, 1997). It included 133 new genotypes introduced from IRD's (formerly ORSTOM) *in vitro* collections. These were regenerated and added to CATIE's field collection between 1995-1997 as part of a regional Coffee Genetic Improvement Program which has been in operation since 1991 (PROMECAFE, 1999). They represented the diversity of 8 taxa: *C. arabica* (58 individuals); the West African genetic group of *C. canephora* (9); *C. canephora* var. *'Caféier de la Nana'* (3); *C. racemosa* (5); *C. eugenioides* (12); *C. pseudozanguebarie* (11); *C. sessiliflora* (16) and *C. brevipes* (19)[2].

TABLE 1. Number of accessions preserved in CATIE's coffee field collections.

Species	Number of Accessions		
	1993	1997	1999*
C. arabica	1,661	1,663	1,623
C. canephora	49	66	56
C. liberica	23	15	15
C. racemosa	4	8	8
C. salvatrix	2	1	1
C. congensis	5	0	0
C. eugenioides	1	13	13
C. stenophylla	2	1	1
C. bengalenses	1	0	0
C. pseudozanguebarie	0	11	11
C. sessiliflora	0	16	16
C. brevipes	0	19	19
C. spp.	3	0	0
Interspecific hybrids	17	19	19
TOTAL ACCESIONS	**1,768**	**1,832**	**1,782**

* Estimates by C. Astorga (1999, *Pers. Comm.*).

[2] Last three taxons introduced into Latin America for the first time.

Comparison of data sets in Table 1 provides evidence of genetic erosion occurring in CATIE's collections. In some cases, the individuals lost were the only representatives found in Latin America (*e.g. C. congensis* and *C. bengalenses*), making the cost of such losses even greater. Germplasm erosion occurs as a result of poor adaptation of materials to local conditions and/or pest and disease problems, but also as a result of the age of those resources (more than 30 years in a large segment of the collection). According to Anthony & Astorga (1997), the CATIE collection suffers the loss of some 250 individuals every year, which amounts to a general genetic erosion rate of 3%, (4.8 % for the wild genotypes). The expense of maintaining these collections, as well as the reticence of sponsors to finance such activities, are perhaps the most important factors affecting this erosion.

Table 2 presents an additional description of Coffee materials preserved at CATIE, now grouped by type of material and according to the latest formal inventory (Anthony & Astorga, 1997).

TABLE 2. Classification of *Coffea* materials preserved in CATIE's field collections (Data from 1997).

Type of material	# of Origins	# of Introductions	# of Individuals
Wild Materials			
-FAO collecting mission in Ethiopia	432	433	1,650
-IRD/ORSTOM collection in Ethiopia	69	148	420
-IPGRI collection in Yemen	11	11	20
-Diploid species	10	150	470
SUBTOTAL:	**522**	**742**	**2,560**
Varieties, Mutations and Selections			
-Varieties from Ethiopia	115	191	950
-Typica and Bourbon Varieties	107	288	1,810
-*C. canephora*-introgressed varieties	6	300	1,780
-Mutations and other selections	30	84	650
-Non-classified varieties	25	43	250
SUBTOTAL:	**283**	**906**	**5,440**
Hybrids			
-Interspecific Hybrids	5	19	90
-Hybrids among Arabica varieties	107	165	820
SUBTOTAL:	112	184	910
TOTAL	**917**	**1,832**	**8,910**

Anthony & Astorga (1997) indicated that those materials belonging to the first group (wild materials) are of utmost importance for breeding purposes. Special efforts should be taken to preserve and care for these resources. The other two groups, Commercial Materials and Hybrids respectively, are less relevant though still represent an important asset that should be preserved.

Genetic resources information

CaféBase, a database compiling data on the genetic resources preserved at CATIE, was launched in 1998. It contains fundamental information like passport data of all accessions, their origin and location within the collection, and key data on material evaluations undertaken at CATIE as well as in other Research Centers. These evaluations included data regarding resistance to diverse pests and diseases, fertility, productivity, architecture, vigor, and size of leaves and beans. With the aid of the database, information on coffee genetic resources preserved at CATIE can be grouped according to diverse criteria, such as genetic and geographic origin, phenotypic characteristics, etc. CaféBase was created in cooperation with IRD and represents a very powerful tool for selecting genitors for breeding programs (Anthony, 1998a).

In vitro conservation

Field genebanks have long been the basic germplasm conservation device for species with recalcitrant seeds such as Coffee[3]. Nevertheless, significant problems such as genetic erosion (due to poor adaptation to local conditions, or attacks by pests and diseases), large space requirements and maintenance costs, have reduced their expansion and effectiveness (Dussert *et al.*, 1997b).

The *in vitro* preservation of germplasm has been utilized as a good alternative to field and *in situ* conservation for recalcitrant-seed species. Plants can be maintained in reduced spaces, while being protected from contamination, pests and diseases. International exchange of materials is enormously facilitated using this method as well (Noirot *et al.*, 1994; Abdelnour-Esquivel & Escalant, 1994).

Diverse elite materials generated by the Coffee Breeding Program (*e.g.* selected F1 hybrids and the Nemaya rootstock variety) are being conserved *in vitro* at CATIE so that they may be multiplied regularly and disseminated to partner institutions (Etienne *et al.*, 1999 a.).

Cryopreservation

As a complement to *in vitro* conservation, the possibility of preserving coffee germplasm in the long-term *via* cryomethods was studied at CATIE during the 1990's. These methods allow genebanks to overcome genotypic selection and genetic drift problems that arise in *in vitro* collections. Moreover, given that somaclonal variation risk increases with culture duration, cryopreservation is, indeed, the only long-term conservation option available for recalcitrant and intermediate species (Engelmann *et al.*, 1995).

Zygotic embryos

Initially, cryopreservation methodologies were successfully developed and adapted for long term conservation of *Coffea* embryos at CATIE. Excised zygotic embryos of *C. arabica, C. canephora* and the *'Arabusta'* hybrid were successfully cryopreserved when desiccated to moisture contents between 8 and 30%, prior to direct immersion in liquid nitrogen (LN) (Abdelnour-Esquivel *et al.*, 1992; Abdelnour-Esquivel, 1993; Engelmann *et al.*, 1995).

Best results (96% survival) were obtained for embryos excised from mature coffee beans (*i.e.* 1 week before harvest) and that showed water contents of 0.20 g H_2O g dw^{-1} after desiccation in a controlled environment (Engelmann *et al.*, 1995; Abdelnour-Esquivel *et al.*, 1992). Embryos extracted from green coffee beans (2 months before harvest) seemed to be more susceptible to desiccation and freezing. Nevertheless, lower survival rates of those immature embryos was overcome by adding 100 mg/l gibberellic acid (GA_3) to the recovery medium, producing an 83% survival rate (Engelmann *et al.*, 1995; Abdelnour-Esquivel *et al.*, 1992).

Somatic embryos

On the other hand, Abdelnour-Esquivel (1993) and Tortós *et al.*, (1993), were able to cryopreserve somatic embryos of *C. canephora* and the Catimor Hybrid, after either encapsulation in alginate, or cryoprotective treatments with sucrose and dimethyl-sulphoxide (DMSO), or both encapsulation and cryoprotection. Slow pre-cooling down to -40 °C was tested. In all cases, desiccation with sterile air was utilized. Best results were obtained with encapsulation (70%) before embryos were incubated for 24 hrs in a 0.5M sucrose solution and desiccated for 2 hrs. Precooling did not provide additional benefits. Encapsulation also made direct germination of embryos possible, reducing manipulation and contamination problems.

[3] Several authors argue that Coffee seeds should be considered as having 'intermediate' seed storage behavior, since they can withstand desiccation but remain cold-sensitive (Dussert et al., 1998).

Seeds

In addition, cryopreservation of seeds has been suggested as the easiest and cheapest method for long term conservation of coffee genetic resources. Recent studies conducted by IRD and IPGRI (Dussert *et al.*, 1997a & 1998) using some material input from CATIE, assessed the possibilities of cryopreserving seeds of diverse *Coffea* species. All species but *C. arabica* survived after direct LN immersion and developed into normal seedlings. *C. arabica* seedlings could only be recovered (at a rate of 30%) when seeds were desiccated to 0.20 g H_2O g dw^{-1} and slowly pre-cooled (2°C min^{-1}) to –50 °C, prior to LN immersion (Dussert *et al.*, 1997b; Dussert *et al.*, 1998).

Nevertheless, the same authors also found that excising zygotic embryos from cryopreserved *C. arabica* seeds, allowed very high recovery rates (97%), even if direct immersion in LN was used (Dussert *et al.* 1997a).

Based on these experiences, a protocol to simplify the cryopreservation of coffee seeds was defined, including the following steps:

- Dry seeds under controlled RH (78%)
- Direct seeds LN-immersion
- Excise and germinate zygotic embryos *in vitro* after thawing

This protocol has been recently adopted at CATIE in several trials, cryopreserving coffee seeds and recovering excised zygotic embryos in 14 *C. arabica* genotypes. Excellent recovery rates (60-100%) were obtained for Typica, Maragogipe, E531 and Bourbon materials. Those trials are currently being replicated with 30 cultivars in order to refine the protocol and to assess its possible application to large-scale germplasm conservation (Vásquez *et al.*, 1999).

In addition, and following recent developments by Dussert *et al.* (1999), another simplified recovery procedure for cryopreserved seeds has been successfully tested at CATIE. It includes slow precooling of seeds, osmopriming of seeds for 2 weeks after thawing on a PEG (polyethyleneglycol) solution adjusted to a –1 MPa osmotic potential, and *in vitro* seed germination. To date, a survival rate of 16% has been obtained for Typica seeds. Several refinements to the protocol have been suggested (*e.g.* disinfection during osmopriming and germination phases proved much more effective when utilizing calcium hypochlorite (12 % w/v) before the priming treatment) (Vásquez *et al.*, 1999).

Given these promising results, efforts have been launched to establish a Core collection[4] of coffee germplasm to be cryopreserved at CATIE, thus ensuring the conservation of a backup collection of genetic material, by which the risks of genetic erosion and maintenance costs can be drastically reduced.

4 Core collections are a complementary strategy to field germplasm conservation, aimed at achieving small size plus maximum diversity *via* eliminating redundancies and accounting for alleles that are common locally though rare in the overall base collection (Hamon *et al.*, 1995; Noirot *et al.*, 1996; Dussert *et al.*, 1997c)

GERMPLASM EVALUATION.

Broadening our Knowledge of Preserved Stock

Cultivated coffee has a narrow genetic base, which has been evidenced by a generalized susceptibility to pests and diseases among varieties since the 1960's. For that reason, expanding the coffee genetic pool has been a major priority of crop improvement projects since that time. Collecting missions directed by FAO (1964-65) and IRD (1966), gathered wild and subspontaneous[5] materials from Ethiopia which were distributed to germplasm collections all over the world, including CATIE's (Anthony *et al.*, 1999b).

Despite having preserved the most important Coffee collection in Latin America for several decades, the knowledge generated about the structure of the diversity of CATIE's germplasm was meager at the onset of the 1990's and for that reason, those materials had scarcely been utilized in regional breeding programs (Anthony *et al.*, 1995; Anthony *et al.*, 1999 a & c). Efforts undertaken during the 1993-99 period to structure and characterize the genetic diversity of those materials are described in this section.

Analysis of C. arabica genetic diversity

An assessment of the genetic diversity of *Coffea arabica* germplasm preserved at CATIE was undertaken in the 1990's by means of phenotypic and genotypic evaluations, whose results are summarized below.

Phenotypic Evaluation

The first step in structuring the genetic diversity of a germplasm collection involves the methodological observation of key characteristics for each individual in the field. Multiple phenotypic markers are related to selection criteria, and include:

- *Morphological descriptors*: Architectural (*e.g.* ramification degree, number of internodes, and length of plagiotropic branches); and Physical (dimensions and colors of leaves, flowers and fruits, flush color, stem diameter, etc.).
- *Phenological descriptors:* flowering dates, fructification cycle duration.
- *Ecological adaptation descriptors:* altitude, dry or humid regions, resistance to pests and diseases.
- *Productivity descriptors:* productivity levels and early or late flower and fruit set.
- *Technological descriptors:* coffee quality, weight of 100 beans, 'caracoli'[6] rate, etc. (PROMECAFE, 1999; Anthony & Dussert, 1996; Anthony *et al.*, 1995; Bertrand *et al.*, 1995; Noirot *et al.*, 1994).

A phenotypic evaluation was implemented on 300 wild *C. arabica* individuals collected in 8 Ethiopian locations and accessed in 1985 into CATIE collections. High variability was observed in terms of fruit maturation length (130 - 258 days), caracoli rate (1 – 71 %), leaves' size, internode length and bean size. A correlation was also noticed among morphologic variables (*e.g.*, the lower the ramification of the tree, the bigger the leaves and the beans it produces) (Bertrand *et al.*, 1993 & 1995b).

[5] Subject to human interference given that they were collected on farms.

[6] Coffee beans containing only one grain, resulting from a certain form of sterility. The spherical shaped beans are called 'caracoli' or peaberries. (Noirot *et al.*, 1994).

BOX 1: The *Coffea* genus

Coffee trees (family Rubiaceae) are classified in two genera, *Coffea* and *Psilanthus*. *Coffea* species are woody, ranging from small shrubs to robust trees, and originate in the intertropical forests of Africa, Madagascar and islands of the Indian Ocean. The subgenus *Coffea* encompasses more than 80 taxa, including the two species of economic importance: *C. arabica* and *C. canephora*. *Coffea* species are diploid (2n = 22) and generally self-incompatible, except for *C. arabica*, which is considered a segmental self-fertile allotetraploid (2n = 4x = 44)[1]. Although showing considerable variation in morphology, size and ecological adaptation, *Coffea* species share a common genome and cross readily with one another, producing fertile hybrids.

Recent studies by CIRAD demonstrated that *Coffea* taxa can be classified into 5 main phylogenetic groups, corresponding to geographic regions (fig B1.1). Analysis of the chloroplast DNA made it possible to visualize the maternal filiation of *C. arabica*, which appeared closely linked to species from East & Central Africa (*C. eugenioides* & *C. sp.* 'Moloundou'); on the other hand, ribosomal DNA studies revealed that paternal characters from *C. arabica* originate in the canephoroid group of Central & West Africa (*C. brevipes, C. canephora, C. congensis*). Thus, *C. arabica* resulted from a recent hybridization between 2 other *Coffea* species from different phylogenetic groups.

Arabica coffee represents 71 % of total Coffee consumption in the World. Its center of genetic diversity is located in the Abyssinian Plateau in Ethiopia. All existing varieties descend from the few trees that survived efforts to spread arabica coffee from Yemen (where it was introduced by the Arabs in the Middle Ages), into other regions of the World.

The Yemen trees derived into to two botanical types: *C. arabica* var. *'Typica'*, the earliest grown in Asia and Latin America; and *C. arabica* var. *'Bourbon'*, introduced to Latin America from the Reunion Island. Today's most cultivated varieties also derive from those basic types: 'Caturra' is a short-internode mutation of Bourbon, and 'Catuaí', is a hybrid of both (Caturra x (Typica x Bourbon)).

Adapted from Cros *et al.*, 1998; Lashermes *et al.*, 1996 a & b, 1997; Anthony *et al.*, 1993, 1997, 1999 a & b.

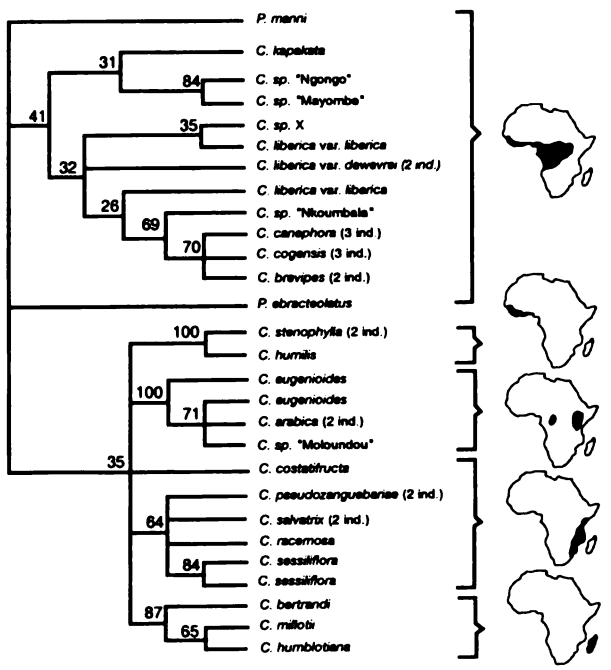


Figure B1.1 Phylogenetic classification of 25 *Coffea* taxa based on chloroplast DNA and geographic distribution of main classification branches.
(From Anthony et al., *1997a and reproduced with permission from* Plantations, Recherches, Développement*)*

More importantly, it was demonstrated that 41 % of those individuals were highly resistant to coffee rust (*Hemileia vastatrix*) – strain II, whereas 12 % presented moderate resistance and the remaining 47 % were susceptible. It was also found that the variability observed could be delineated as a function of the geographic origin of those materials within Ethiopia: Gimma-Gore and Tippi-Gore materials were located at the extremes of the variability range (Bertrand *et al.*, 1995b).

These results were encouraging for coffee breeding programs: the high variability found implied good possibilities for broadening the genetic base of Coffee through the use of wild materials. Also, the geographical structure of such variability should facilitate the selection of progenitors for breeding purposes (*e.g.*, Gimma-Gore x Tippi-Gore hybrids should show highest possible heterosis).

On the other hand, in order to establish a detailed database on the characteristics of CATIE's coffee collection, phenological data were recorded during six flowerings for a group of 1,567 genotypes. Fruit and bean characterizations were conducted on 483 samples of 200 grains each. And monthly observations of leaf rust resistance of 1,835 wild and cultivated genotypes were also made during 1997 (Anthony, 1998 a).

In addition, Catimor and Sarchimor lines have been known for presenting phenotypic differences among their progenies, according to the HT genitor from which they originate[7]. Twenty-seven different Catimor and Sarchimor lines were tested against Caturra and Catuaí for their productive and disease resistance characteristics. HT-derived varieties were, in general, more productive and vigorous than traditional varieties, and presented good levels of resistance to coffee rust and to some *Colletotrichum kahawae* (Coffee Berry Disease, CBD) strains. Resistance to the root-knot nematode (*Meloidogyne* spp.) was more prevalent in Colombian Catimors. No differences in sensitivity to the root lesion nematode (*Pratylenchus* spp.) or grain size were detected, but caracoli rate seemed a bit higher in Catimors and Sarchimors (Bertrand *et al.*, 1997; Aguilar *et al.*, 1996).

Finally, an important phenotypic trait was found in 5 Ethiopian individuals: **the absence of pollen production.** Cytological studies carried out on anthers from those trees, confirmed their male-sterile condition, as also did autopolinization and propagation tests under different soil and climatic conditions. Observed male sterility was classified as sporogeneous, with mutations occurring around meiosis stage, and having a nuclear and recessive genetic determinism. Male-sterile (MS) individuals were characterized by a total lack of microspores at flowering stage due to early destruction of the tapetum (Figure 1). Pollination carried out with pollen from Catimor and Sarchimor lines showed the cross-fertility of those plants. An evaluation of these hybrids and their genitors is under way, in order to determine MS inheritance as well as their value as parents for breeding purposes (Jiménez *et al.*, 1994; Eskes, 1995; Dufour *et al.*, 1997).

[7] All Catimor and Sarchimor lines originate from 3 individual HT plants: CIFC 832/1, whose cross with Caturra produced Brazilian Catimors; CIFC 1343, which produced Colombian Catimor when crossed with Caturra; and, CIFC 832/2, which gave rise to Sarchimors after being crossed with Villa Sarchi in Brazil (Bertrand *et al.*, 1997).

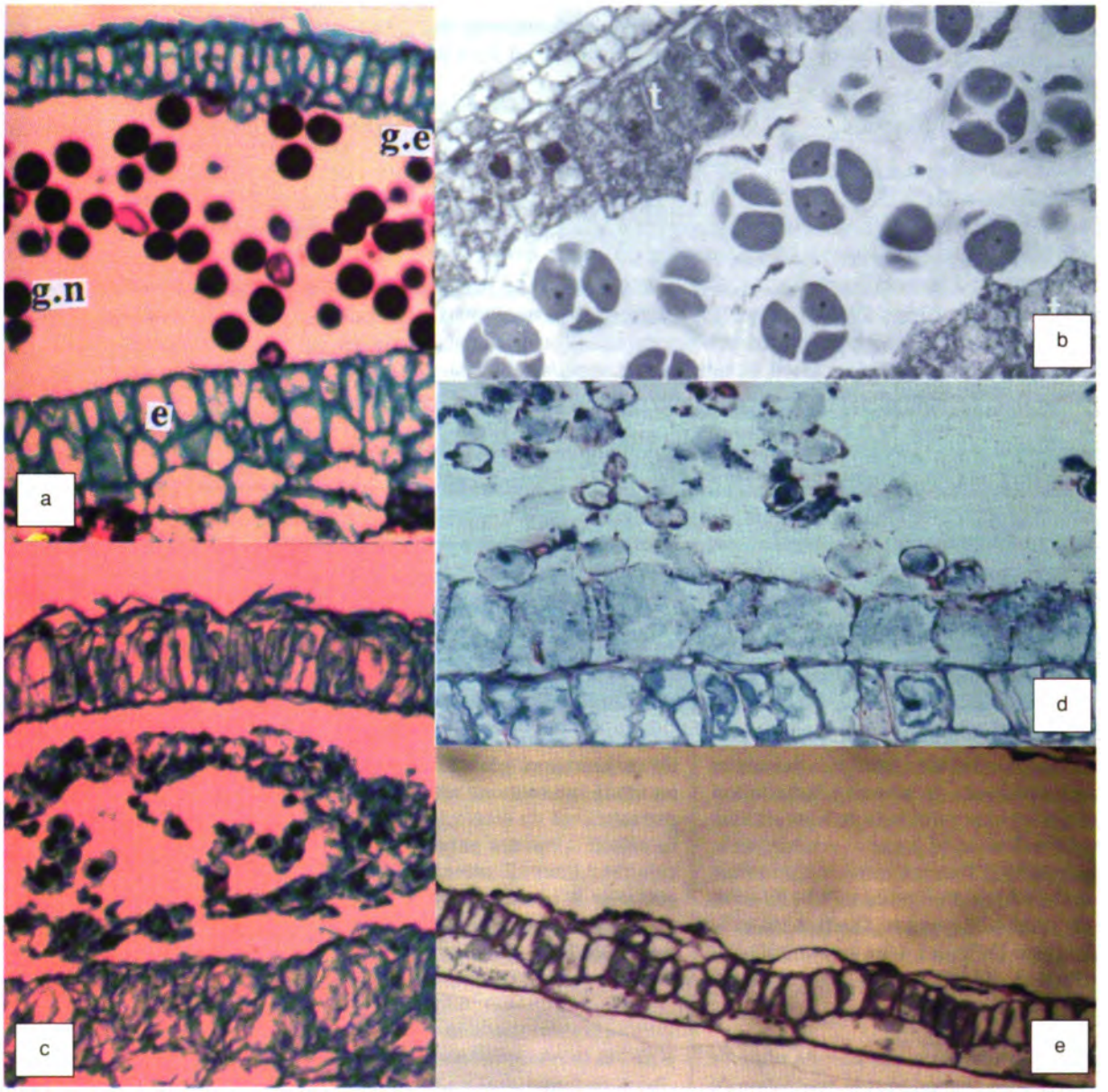


Figure 1. Micrographs showing male-sterile condition of some wild Arabica accessions at CATIE germplasm collection. a. Coffee anther with pollen grains (g. n. = normal grains; e = endothecium); b. Normal tetrades; c. Coffee anther with abnormal pollen grains in cultivar 4,905; d. Microspore degeneration; e. Empty pollen sacks at time of anthesis. *(Pictures b, d & e taken from Dufour* et al., *1997, and reproduced with permission of* Plantations Recherche, Développement*)*

Genotypic Evaluation

The expression of phenotypic markers is highly influenced by the environment. They are thus fundamental in the assessment of adaptive diversity of populations, whose structure changes as a function of the selective value of site environmental constituents. For the same reason, their value for structuring genetic diversity is reduced: they should only be considered if the same ecological conditions apply (Crochemore, 1998).

Genetic variability and taxonomic studies require markers that are not subject to environmental selection pressure, and whose diversity results from genetic drift, migration or mutation. Molecular markers present those characteristics (see Box 2), and have been utilized during the 1990's to assess and structure the genetic diversity of *Coffea arabica* germplasm preserved by CATIE (Anthony *et al.*, 1993).

Development of RAPD markers allows for the examination of genomic variation without prior knowledge of DNA sequences. RAPD's have also been preferred for diversity assessment as they require smaller amounts of crude genomic DNA, consume less time, and need no labelling probes (Anthony *et al.*, 1999b). Prior to the use of RAPD's, experiments were conducted to refine DNA isolation and amplification procedures (*e.g.* a protocol for obtaining reproducible DNA amplifications was established based on the work of Wilches (1995), and Anthony & Quirós (1996)). Also a very useful method for DNA extraction from coffee flushes was defined at CATIE by Nuñez *et al.*, (1993). This protocol is both low cost and low risk (does not use cesium chloride) and requires short centrifugation periods. It was also applicable to *C. canephora* and *C. liberica* materials.

Using RAPD markers on *C. arabica* accessions, Lashermes *et al.* (1996a) as well as Anthony *et al.* (1999 a & b) confirmed the narrow genetic base of commercial Coffee varieties and the larger genetic diversity within wild accessions. Sixteen primers out of 150 tested (10.7%), generated polymorphisms. In a sample comprised of 118 individuals which represented 88 wild *C. arabica* accessions plus 6 Ethiopian cultivars and the Bourbon and Typica varieties, a total of only 29 polymorphic markers were obtained. This confirmed earlier assertions about the low genetic variability of *C. arabica*, which results from long selection cycles and autogamy of this species, both forces favoring homogeneity (Lashermes *et al.*, 1995; Anthony *et al.*, 1999a) (see Figure 4).

Those 29 RAPD markers were utilized to structure the genetic diversity of analyzed individuals, utilizing a similarity index (generated after comparisons of pairs of individuals) and a dendrogram that shows the genetic distances among genotypes[8] (Anthony *et al.*, 1999b) (see Figure 5).

According to these studies which verify results from previous experiments (Anthony *et al.*, 1993; 1997a; Lashermes *et al.*, 1996a), coffee materials were organized in 3 genetic groups: Wild Ethiopian materials on the one hand and varieties derived from the 'Bourbon' and 'Typica' botanical groups on the other. But as shown in Figure 5, Anthony *et al.* (1999b) also found that Ethiopian materials were divided in 4 well-defined groups: one with accessions from southwest Ethiopia (Ethiopia 1, comprising 78 wild accessions and 2 cultivars) and 3 with materials from the southeast (Ethiopia 2, 3 & 4, including 7 wild accessions and 2 cultivars).

[8] This dendrogram was generated utilizing Dice's similarity index (Dice, L. R. 1945. Ecology 26: 297-302), after applying the unweighted pair-group method based on arithmetic averages –UPGMA- of the NTSYS-PC software.

BOX 2: What are Molecular Markers?

Genetic diversity in crop plants is analyzed by means of several characteristics: morphological, agronomic, ecogeographic, biochemical and DNA markers. *Lato sensu,* Molecular Markers are any measurable biochemical or molecular characteristic inherited according to a simple Mendelian model. It includes DNA markers as well as their direct biochemical products (proteins, mainly isoenzymes and seed proteins, though chlorogenic acids and caffeine contents have been used in coffee as well). Expression of these markers is neutral or unaffected by environmental selection pressure.

Stricto sensu, molecular markers refer to DNA segments which are considered to be reference marks for genome analysis and which can be generated and visualized through specific procedures. An unlimited variety of DNA sequences makes them the most utilized device for assessing genetic diversity. DNA markers present more polymorphisms than biochemical or any other markers. They show segregation and can be analyzed in young plants and even parts of plants through molecular biology techniques such as RFLP and RAPD.

Restriction Fragment Length Polymorphism analysis, RFLP, is a technique capable of detecting DNA polymorphisms due to punctual mutations, additions, deletions and recombinations. RFLP markers are highly polymorphic and codominant (*i.e.* both alleles in locus are visualized). RFLP analysis has 5 steps: a) DNA digestion by an endonuclease which recognizes specific DNA sequences or restriction sites; b) Separation of DNA fragments by electrophoresis; c) Transfer of fragments to a nylon or nitrocellulose membrane; d) Hybridization of fragments with sequence-specific probes; e) Visualization of probe/fragment hybrids with either radioactive, chemical dye or chemiluminiscence methods (see Figure 2). Polymorphisms are thus detected by the presence of those hybrids. Obtaining appropriate probes is key to this technique, whose main drawbacks include a painstaking process and the use of radioactive materials ("Cold" probes have been developed only very recently).

Random Amplified Polymorphic DNA markers, RAPD's, are derived from the Polymerase Chain Reaction technique (PCR). PCR consists of an *in vitro* enzymatic synthesis of DNA sequences using 2 oligonucleotide primers that hybridize to opposite strands and flank the region of interest in target DNA. RAPD markers derive from the amplification of DNA fragments from 100 to 2,000 pb using only one small size primer (8-10 nucleotides of random sequence) plus a DNA-polymerase of bacterial origin (Taq). RAPD's are even more polymorphic than RFLP's and their analysis includes 3 steps: a) DNA denaturation (separation of both strands); b) Annealing of a primer plus a complementary sequence in one of the threads; c) Synthesis of a complementary DNA fragment (departing from one extreme of the primer) by means of the Taq polymerase enzimatic action. This process is repeated 40 times to obtain an exponential amplification of synthesized fragments which are then separated by electrophoresis, colored with ethidum bromide and finally observed under ultraviolet rays (fig. 3). RAPD's are dominant markers: their main inconvenience is not revealing heterozygous loci.

Adapted from Gepts, 1995; Fritz *et al.*, 1995; Anthony *et al.*, 1997; Phillips-Mora, 1999.

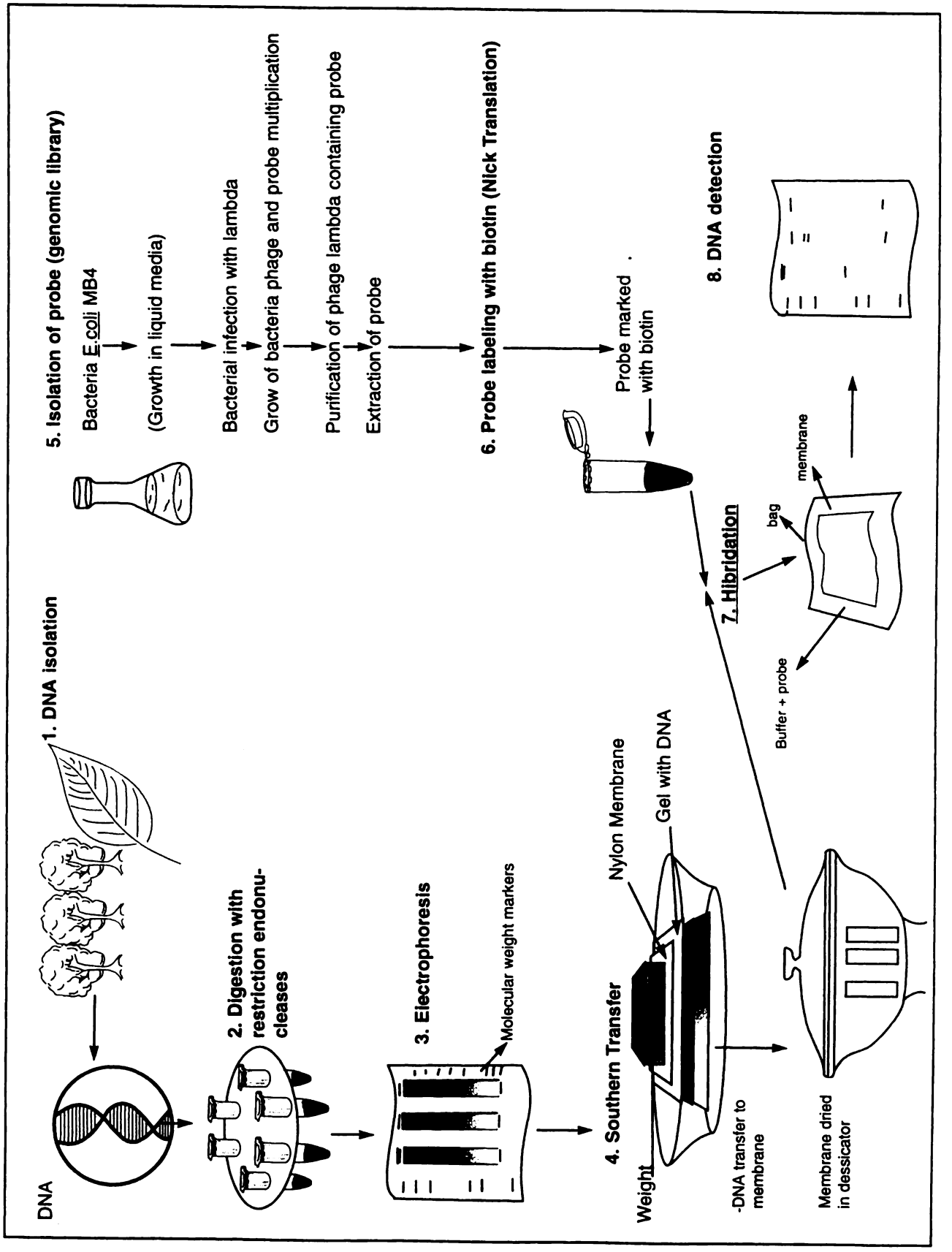


Figure2. RFLP Analysis (from Fritz *et al.*, 1995.).

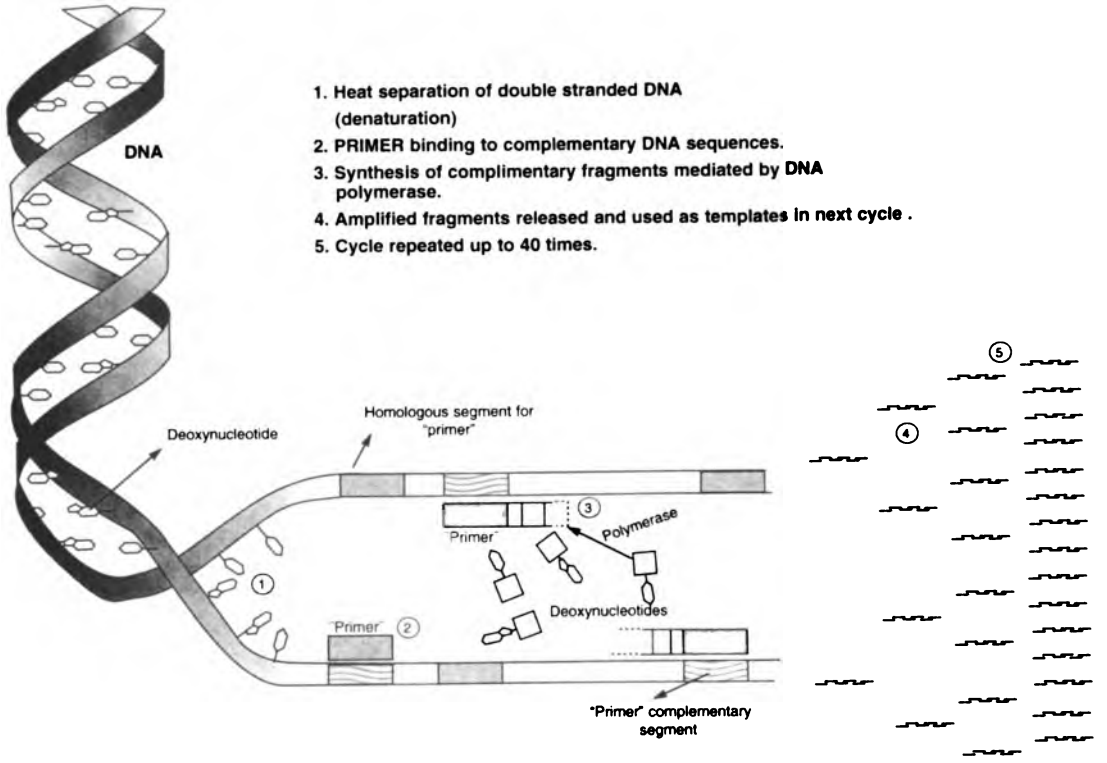


Figure 3. RAPD Analysis (From Fritz *et al.*, 1995).

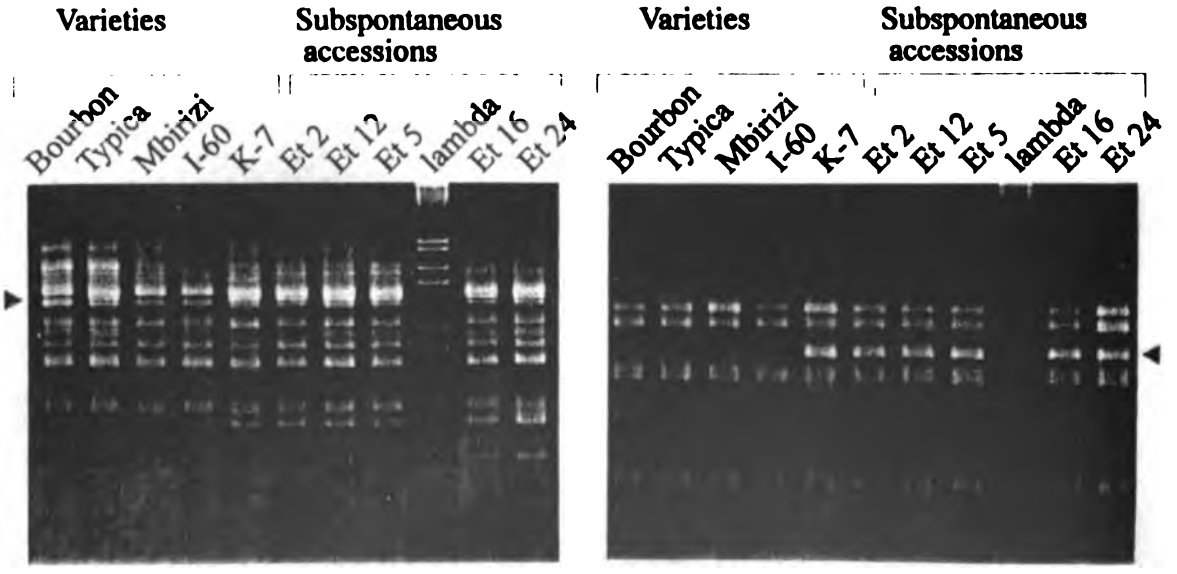


Figure 4. Examples of DNA polymorphisms detected among *C. arabica* accessions. Ethidium bromide stained agarose gel of amplification fragments produced with primers M4 (left) and N20 (right). (*From Lashermes* et al., *1996 a, and reproduced with permission of* Euphytica).

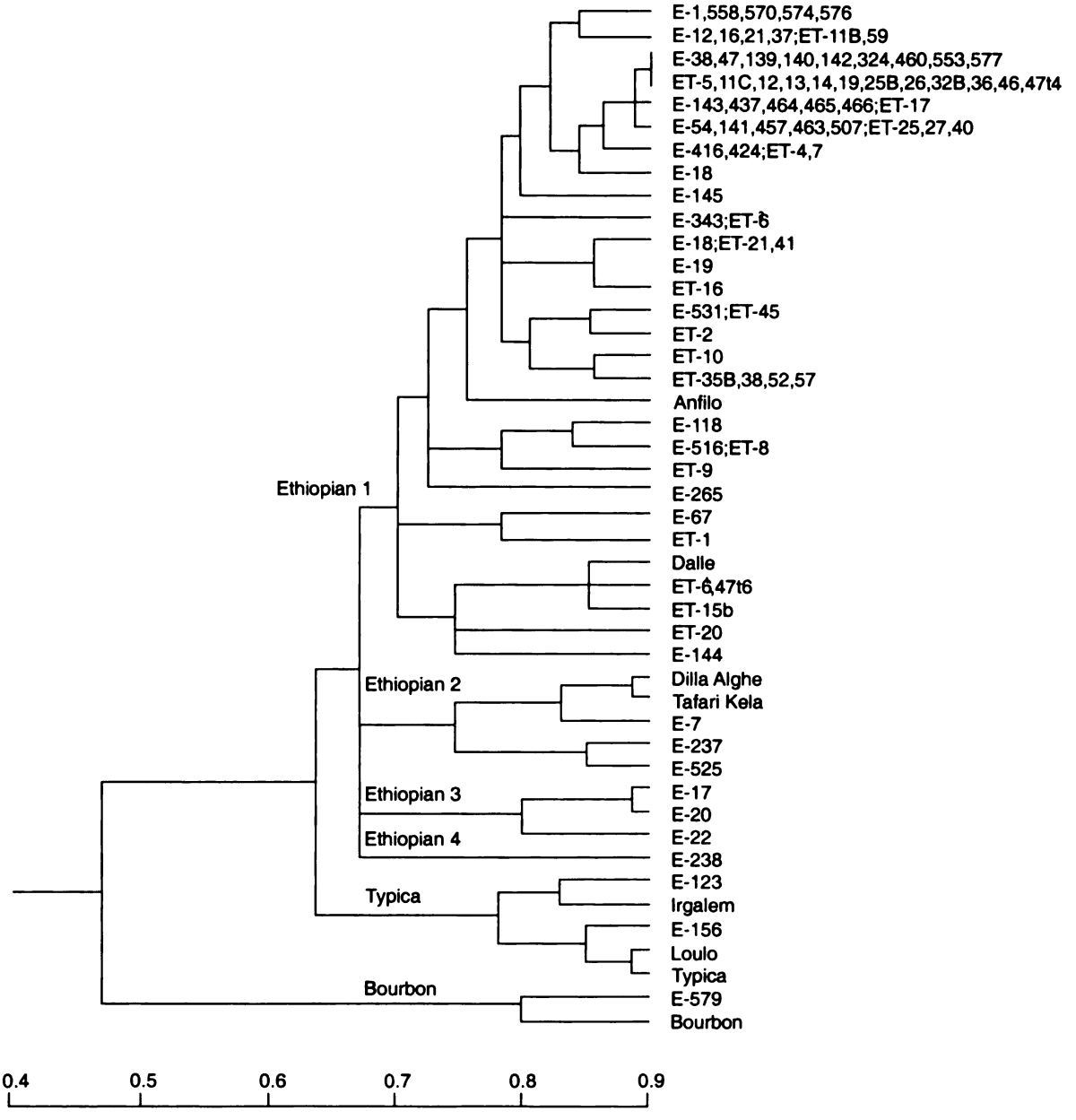


Figure 5. Dendrogram showing the structure of the genetic diversity of 118 individuals selected from CATIE's *C. arabica* germplasm collection and generated after UPGMA, based on Dice's similarity index for obtained RAPD data. Accessions with indexes higher than 0.9 were grouped. Individuals covered 88 wild Ethiopian accessions (E-#), 6 Ethiopian cultivars, and Typica and Bourbon varieties. (From Anthony *et al.*, 1999b).

It was also found that most of the diversity detected was generated by southwestern genotypes, which presented 28 of the 29 identified markers, whereas the other groups contained from 5 to 16 at most. Major genetic differentiation occurred between Bourbon and Ethiopian groups 2, 3 & 4, as well as between Typica and Ethiopian groups 3 & 4. Meanwhile, the genetic distance between Typica and Bourbon was relatively low, and Ethiopia 1 appeared little differentiated from all other groups. Thus, the genetic bases of cultivated materials are little differentiated from wild materials from southwest Ethiopia (Anthony *et al.*, 1999b).

As for the geographical (southeast/southwest) differentiation evidenced among Ethiopian materials, it has been suggested that such diversity was probably a result of the differential evolution that occurred in both sides of the Great Rift Valley since its formation 30 million years ago. This assertion is nonetheless contradicted by phylogenetic studies that provided evidence of a recent origin of this species (Cros *et al.*, 1998, and Lashermes *et al.*, 1996b), and by the fact that differentiation between materials on both sides of the Rift was not too marked in the study by Anthony *et al.* (1999b).

Also, previous studies argued that materials introduced by the Arabs into Yemen, which later gave rise to all modern varieties[9], would have originated on the eastern side of the Rift (Lashermes *et al.*, 1996b; Anthony *et al.*, 1993). Evidence summarized by Anthony *et al.*, (1999b) suggests instead, that commercial varieties are more closely linked to wild materials found in southwestern Ethiopia.

Germplasm Characterization

Results from phenotypic and genotypic evaluations have been used during the 1992-99 period to reveal the presence of duplicates and off-type accessions in CATIE's coffee collection. This characterization effort is fundamental in order to ensure the safe use of preserved materials in breeding programs, as well as to reduce conservation costs. Typica and Moka materials were reclassified according to their origins, whereas Typica and Bourbon accessions plus "Laurina" and "Murta" mutations not corresponding to their botanical description were identified (Anthony *et al.*, 1994; Anthony *et al.*, 1995; PROMECAFE, 1999).

RAPD markers were also effective in differentiating off-types within the ET-47 (trees # 4 & 6, which differed from other individuals of this wild accession by their abundant ramifications and narrow leaves) and ET-6 (trees T.16695 & T.17177) accessions. Those off-types might have resulted from identification errors or from cross-pollination in the field genebank in Cameroon, where CATIE accessions were obtained (Anthony *et al.*, 1999b).

9 Recent analyses of accessions collected by IPGRI in Yemen showed that they should be considered Typica materials (CATIE, 1998).

GENETIC IMPROVEMENT.

Developing and Evaluating Promising Materials

The pervasive susceptibility to pests and diseases among existing C. *arabica* varieties and cultivars was mentioned above. A genetic solution for this problem was envisaged, using preserved wild materials which showed resistance to some of those threats. Moreover, as all *Coffea* species share a common genome, desirable characteristics could also be transferred to *C. arabica* via interspecific crosses with *C. canephora* (rust & nematode resistance), *C. liberica* (nematode resistance) and *C. congensis* (rust resistance). Additionally, interesting traits such as drought resistance (*C. racemosa*) and low caffeine levels (*C. pseudozanguebarie*) could be tested (Anthony *et al.*, 1995).

However, classical breeding has not been capable of alleviating this situation because it takes approximately 30 years to generate and distribute a new variety, given that the seed-to-seed cycle of coffee trees can take three years; that descendants are evaluated over a minimum of 3 harvests; and that six back-cross cycles are necessary to achieve an acceptable homozygocity level.

To face those limitations, a Coffee Breeding Program aiming at generating varieties suited to actual producer needs within a short time span, was implemented by PROMECAFE, CATIE, IICA and the French Cooperation (CIRAD, IRD, MAE) in 1991. This Program was founded, in part, on classical methods, but also utilizes biotechnological and molecular biology tools. It comprises several modules, among which the following are highlighted.

Evaluation of resistance to pests and diseases

Resistance to coffee rust was the main coffee breeding objective in the 1980's. Resistance genes introgressed from either *C. canephora* or the Timor Hybrid, HT[10], into *C. arabica* varieties generated resistant materials such as Catimors and Sarchimors which were introduced into CATIE collections. Further selection of those materials gave rise to new, rust-resistant varieties in Nicaragua (Catrenic), Honduras (IHCAFE 90, Lempira 98), Costa Rica (ICAFE 90, Costa Rica 95) and Panama (MIDA 96) (PROMECAFE, 1999; Bertrand & Anthony, 1995).

In the early 1990's a growing concern for the widespread susceptibility to other aggressive pests and diseases (including the new rust-resistant materials too), led to a shift in the focus of breeding efforts. Since 1992, commercial and wild *C. arabica* materials were tested in order to identify sources of genetic resistance to the main sanitary problems (PROMECAFE, 1999; Bertrand *et al.*, 1995; Bertrand *et al.*, 1996; Bertrand *et al.*, 1997; Decazy *et al.*, 1997; Peña, 1994):

- ***Coffee Rust:*** Field observations and inoculation tests demonstrated partial resistance to *Hemileia vastatrix* in F1 and F2 progenies of crosses among Ethiopian accessions and commercial varieties. Genitors of those progenies responsible for resistance to coffee rust race II were identified.
- ***Leaf Spot:*** An inoculation methodology was developed to combat this disease, and resistance to "Ojo de Gallo", *Mycena citricolor,* has been tested since 1994 in more than 80 coffee lines, including Catimor, Sarchimor and Colombia variety lines plus 50 Ethiopian accessions. Results have yet to be confirmed.

10 A tetraploid hybrid derived from a natural cross between *C. arabica* and *C. canephora* found in Portuguese Timor (Carvalho, 1988).

- ***Coffee Berry Disease (CBD):*** Several samples have been sent to CIRAD to evaluate resistance to four different *Colletotrichum kahawae* strains. Only one Ethiopian accession (T16691) presented moderate resistance to this disease, while Catimor and Sarchimor lines were generally resistant to at least one of the strains.
- ***Nematodes:*** The same materials utilized for *Mycena* studies were initially tested for their resistance to diverse *Meloidogyne* and *Pratylenchus* species. Protocols for inoculation and for producing and preserving roots *in vitro* were developed (Dufour *et al.*, 1994a; Rosales & Marban, 1996). Also, dominant species per country were identified, providing the following results: *M. arenaria* in El Salvador; *M. exigua* in Honduras and Nicaragua; *M. exigua and M. arabicida* in Costa Rica; *M. incognita* and *M.* spp. in Guatemala (Decazy *et al.*, 1997) (see Figure 6).

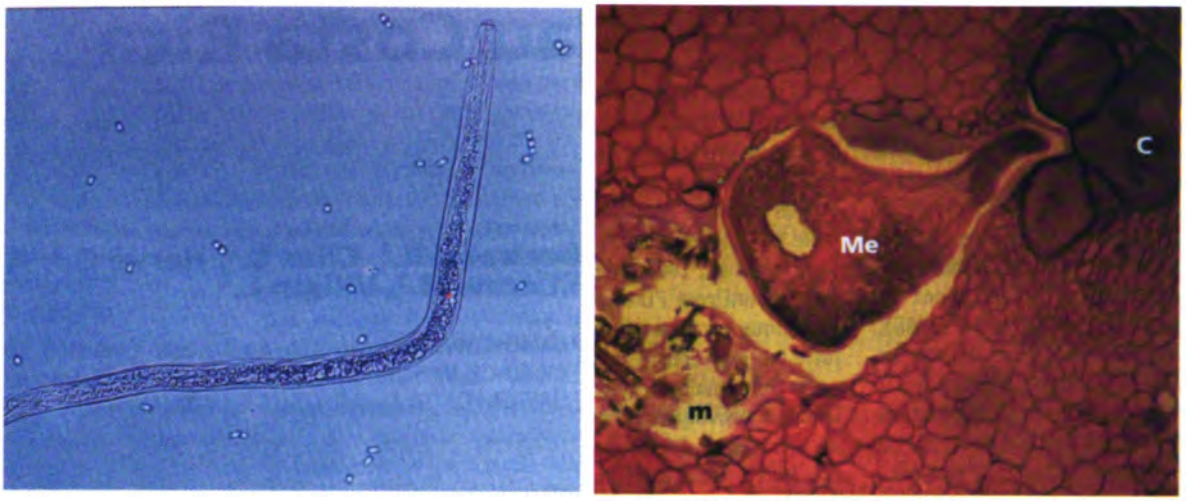


Figure 6. Micrographs of two coffee nematodes present in Costa Rica. a. *Pratylenchus* spp. b. *Meloidogyne exigua* (female –Me- with its egg mass –m- on nutritional site showing giant cells –c-). (*Taken from Bertrand* et al., *1996, and reproduced with permission of* Plantations, Recherche, Developpement)

Almost all Arabica materials proved susceptible to both nematode genera. Resistance to *M. incognita* was found on some wild materials and to *M. exigua* in one Ethiopian line (T4900-E531). On the other hand, some Catimor and Sarchimor lines were found to be resistant to *Meloidogyne* species. No resistance to *Pratylenchus* spp. was evidenced among Arabicas nor HT-derived varieties.

Complementary studies included Robusta (*C. canephora*) materials and provided more detailed information as illustrated in Table 3. Resistance to *Meloidogyne* species found in Catimors was obtained mainly on those lines produced in Colombia, on which resistance is inherited as a dominant gene (Aguilar *et al.*, 1996; Bertrand *et al.*, 1997).

Resistance to *Meloidogyne* spp. from Guatemala was tested among F1 hybrids from crosses between Catuaí, Catimor and Sarchimor lines with Ethiopian accessions. Catimor and HT lines appeared sensitive. Ethiopian genitors ET 59a2 and ET 52a2 conferred good resistance to their progenies. From tests on 26 Robusta hybrids, it was inferred that resistance traits in *C. canephora* are quite dependent on genotypes, as only two genitors transmitted good lev-

els of resistance to *Meloidogyne* and *Pratylenchus*: T3751 (1-2) and T3561 (1-2). These genitors were selected for obtaining and distributing new resistant materials, as described below.

TABLE 3. Results of coffee nematode resistance trials in CATIE collections.

	% of tested coffee materials showing resistance		
Resistance to:	Arabica	Robusta	Catimors & Sarchimors
"Corchosis"[11]	0%	50%	10-15%
M. exigua	0%	>90%	>30%
M. arabicida	0%	50%	5-15%*
M. incognita	No data	High	< 50%
Pratylenchus spp.	0%	High**	0%

* *No totally resistant lines found.*
** *Data available for only a few genitors tested.*

Figure 7. Robusta plant showing "Corchosis" sypmtoms with no gall formation.

Creation of new genetic materials

Since 1992 a series of efforts have been undertaken to generate new *Coffea* materials. Hybrids between commercial varieties and wild Arabica accessions have been produced; a new Robusta rootstock for micrografting experiments was developed; and attempts at generating haploid individuals have been made.

Arabica hybrids

A series of crosses between commercial varieties (Caturra, Catuaí, HT derivatives) and 30 wild accessions of *C. arabica* were achieved during the study period, generating about 5,500 F1 hybrids. Genetic and phenotypic evaluations of the materials obtained and their genitors made it possible

[11] A complex disease, recently reproduced under laboratory conditions, whose expression requires the presence of *M. arabicida*, *Fusarium* and *Pratylenchus* (Decazy *et al.*, 1997).

to select 42 **elite F1 families** which were vegetatively propagated. They are being distributed to other countries for acclimatization and agronomic tests. Also, some of those F1 hybrids have been self-pollinated to generate F2 materials. From those F2 progenies, 12 have been selected for further trials (PROMECAFE, 1999; Eskes, 1995).

As of 1999, eight F1 hybrids seem very promising, showing important improvements over commercial varieties (see Table 4): 30 to 40% higher productivity; superior vigor and architecture; better rust and nematode resistance; similar coffee quality levels and grain size. The only drawback observed is a slightly lower fertility rate (3-4% more caracoli + vain grains), which underlines the need for a more strict selection process based on that criterion (PROMECAFE, 1999; Bertrand *et al.*, 1997; Eskes, 1995).

TABLE 4. Performance of coffee commercial lines and experimental F1 hybrids developed by PROMECAFE-CIRAD-CATIE-IICA.

VARIABLE	LINES	HYBRIDS	% INCREASE
Production[1] (g)	2079	2724	31
Dry weight of 100 beans (g)	16.13	16.72	3.6
Stem diameter[2] (mm)	27.36	30.52	11.5
Height (cm)	102	111.7	9.5
No. of ramifications on branches	57.48	61	6.1
Average length of 2 largest 'bandolas'[3]	61.6	69	12
No. of berries on 6 more productive nodes	96.9	101.7	4.95
No. of peaberries on 6 more productive nodes	7.3	10.9	49
% vain grains	3.9	8	205

Source: Bertand *et al.*, 1997.

1. Cummulative production for years 3 & 4 in the field, in grams of berries per tree
2. Measured 5 cm above ground
3. 'Bandolas' = productive ramifications on main branches

"NEMAYA" rootstock

Given the importance of nematode damage in Central American coffee production (15-25 % losses in Guatemala and El Salvador) as well as the concomitant problems entailed by chemical control measures[12], selecting resistance to those parasites has been one of the main objectives of breeding efforts during the 1990's (Peña *et al.*, 1996; PROMECAFE, 1999).

However, as evidenced by data presented in Table 3, low levels of resistance were found on wild Arabicas as well as on HT-derived varieties (Catimors and Sarchimors), making the development of resistant Arabica varieties a slow process.

Grafting on resistant rootstocks represents an alternative solution to this problem. *C. canephora* has been traditionally used as a rootstock for propagating *C. arabica* scions in regions with high nematode problems such as Guatemala. Those rootstocks have high tolerance to *Pratylenchus* spp., which is the dominant nematode in that country. Nevertheless, as mentioned above, Robusta resistance to *Meloidogyne* species varies considerably according to genotype (Anzueto *et al.*, 1996; Anzueto *et al.*, 1995; Peña *et al.*, 1996).

A selection process was undertaken based on results of the resistance evaluation trials described above. In 1994, a hybrid resulting from the cross of two Robusta diploid lines preserved at CATIE, T3561 (2-1) and T3751 (1-2), showed the best resistance attributes among all materials tested (38 lines and their hybrids). It was developed as the new Robusta "variety" (hybrid) named NEMAYA (Anzueto, F. *et al.*, 1995).

NEMAYA shows moderate resistance levels (80%) to aggressive *Meloidogyne* species (*M. arenaria* from El Salvador, *M.* spp. from Guatemala), and total resistance to milder species (*M. exigua* and *M. arabicida* from Costa Rica, *M. incognita* from Nicaragua). It is also highly tolerant to *Pratylenchus* spp., and to root mealybugs, although its tolerance can be temporarily diminished on recently grafted young plants.

NEMAYA is currently being multiplied and distributed (Etienne *et al.*, 1997a; Etienne, 1998) (see Figure 8). Seed-producing lots where both genitors are present, are being established in Costa Rica, Honduras, Guatemala and El Salvador (Anzueto *et al.*, 1999; Etienne *et al.*, 1999a; Berthouly and Etienne, 1999). Improved seed distribution will begin in the year 2000 in Guatemala. Massive vegetative propagation techniques are being employed (see *infra*), since demand for these products is expected to grow steadily, even though coffee grafting is not yet a common practice in Central America. Recent studies by Bertrand *et al.* (1998) in Costa Rica demonstrated that *Pratylenchus* and *Meloidogyne* species coexist in coffee plantations under competitive equilibrium conditions, with dominant species and population proportions varying according to site. Thus, obtaining and utilizing *Meloidogyne*-resistant varieties is likely to cause an increase in *Pratylenchus* populations in the future. Under those circumstances, NEMAYA's tolerance to *Pratylenchus* reinforces its importance as a component of the *Meloidogyne* control strategy.

Figure 8. Nursery with Nemaya genitor vitroplants propagated through somatic embryogenesis in Guatemala (From Anzueto *et al.*, 1999)

[12] High-cost and high-toxicity products, punctual application efficiency, fast metabolization, re-colonization and residue problems (Anzueto *et al.*, 1995)

Haplomethods

The production of haploid plants[13] was another important objective of the Coffee Breeding Program, since after doubling the chromosome set of a haploid individual, the resultant homozygous plants would produce lines capable of accelerating pedigree selection programs and of producing homogeneous F1 when crossed. Also, detection of recessive characteristics and transfer of a single gene from wild to cultivated lines are facilitated after obtaining haploid plants. Because Arabica coffee is tetraploid, individuals resulting from haploidization efforts are, in reality, dihaploids (n = 2x = 22), though its anfidiploid nature ensures that they behave as real haploids (Neuenschwander and Dufour, 1993; Dufour *et al.*, 1995).

Production of haploid individuals is based on the fact that gametophytic cells (micro and macrospores) can be induced to abandon their ontogenetic pathway to follow a process conducive to the formation of a haploid sporophyte (Dufour and Neuenschwander, 1995). Biotechnological applications developed for this purpose, called haplomethods, basically consist of inducing androgenetic processes on *in vitro* cultured anthers or microspores (Dufour *et al.*, 1996a). Haplomethods have allowed a dramatic reduction in the time span required to obtain results while avoiding recombination problems (Escalant *et al.*, 1995).

Application of haplomethods to coffee at CATIE generated the first report published on successful androgenesis induction of isolated microspores in a tropical fruit tree (Neuenschwander and Baumann, 1995). They were successfully cultivated in a half concentration MS[14] medium, and the best responses were obtained under the following conditions (Dufour and Neuenschwander, 1995; Neuenschwander and Baumann, 1995; Vásquez *et al.*, 1995; Jiménez, 1995; Dufour, 1996; Dufour & Pereira, 1996; Dufour *et al.*, 1996a):

- ***Microspores developmental stage***: from mid-uninucleate to early binucleate. This stage corresponded to 13-15mm pale green flowerbuds, 2-3 days before anthesis.
- ***Microspores preparation***: anthers subject to cold pretreatment (4-5 °C for 48 hours) presented better callus formation and regeneration.
- ***Medium composition***: 50% MS + 6-12% sucrose + vitamins (tiamin + mesoinositol + piridoxin + nicotinic acid) + 16% coconut milk (as a source of nitrogen and vitamins) provided best results.
- ***Addition of growth regulators***: either the combination of Benzyl-amino-purine (BAP) + 2,4-Dichlorophenoxyacetic acid (2,4-D), or Naphthalenacetic acid (NAA) + 2,4-D + Kinetin, produced good results.

Initial efforts led to the formation of colonies of up to 64 cells within the microspore cell wall, which inhibited any further development (Neuenschwander and Dufour, 1993) (see Figure 9). In an attempt to overcome this limitation, the beneficial effect of nursing cells on further androgenetic development was tested, without success (Neuenschwander & Dufour, 1994; Dufour, 1996).

[13] Every individual has a number of chromosomes which is characteristic of the species to which this individual belongs. The basic number of chromosomes for each species has been arbitrarily named "n". If this number is present in one single set, individuals and species are known as HAPLOIDS. If the species or individuals present a duplicate set, they are called DIPLOIDS (2n). Following the same logic, the terms Triploid (3n), Tetraploid (4n), Pentaploid (5n) and Hexaploid (6n) are utilized in genetics.

[14] MS: classical nutrient solution utilized for in vitro culture, first proposed by Murashige & Skoog in 1962 (Murashige, T; Skoog, F. 1962. "A revised medium for rapid growth and bioassays with tobacco tissue culture". Physiologia Plantarum 15: 473-497)

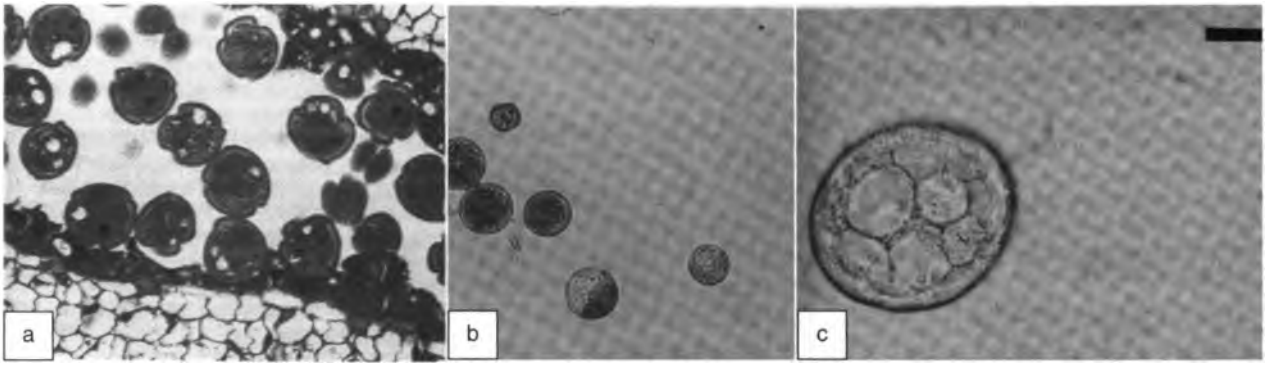


Figure 9. Micrographs showing diverse steps in the androgenetic process of isolated coffee microspores. a. Microspores in uni-nucleate stage. b. Isolated microspores 5 days after culture initiation, some of them showing a 3 to 5 fold volume increase in starch accumulation. c. Multicellular haploid structure resulting from microspore division.
(b and c were taken from Neuenschwander and Baumann, 1995, and reproduced with permission of Plant Cell Tissue & Organ Culture).

Finally, after numerous trials and more than 50,000 anthers cultured, 6 somatic embryos were obtained on a medium where sucrose was replaced by maltose. Only one of those embryos proved to be haploid, while the rest were dihaploid and hexaploid, probably as a result of spontaneous endo-duplication (Escalant *et al.*, 1995; Dufour & Pereira, 1996). Those results should be replicated in order to define a protocol, and before regenerating plants out of the embryos obtained.

PROPAGATION.

Enhancing Reproduction and Distribution of Strategic Genetic Materials.

CIRAD and CATIE have been conducting collaborative research under the umbrella of PROMECAFE for approximately 18 years. In recent years, the Coffee Breeding Program implemented by PROMECAFE, required the development of vegetative reproduction techniques capable of massive propagation of improved materials (*e.g.* F1 hybrids and rootstock varieties described above). CATIE's biotechnology laboratory was delegated the responsibility of research and development in this area, with French support. Emphasis was placed on state-of-the-art micropropagation techniques that made it possible to multiply and diffuse elite materials, and on developing innovative methodologies which could be transferred to national institutions in the region (Etienne *et al.*, 1999a).

Micrografting techniques

As mentioned in the preceding section, coffee grafting is a practice that is likely to become more and more utilized in the Americas once nematode resistant rootstocks are developed. A successful method for micrografting on butterfly-stage coffee plantlets was developed at CATIE. Micro-scions ranging from 0.8 to 2.5 cm long, and lateral incision techniques were recommended, given their high success rate (100%) as well as their unsurpassing growth (Dufour *et al.*, 1994b).

Microcutting multiplication and rooting

The microcutting culture technique has been used at CATIE during the 1990's to introduce new species of *Coffea*, to maintain collections of other materials of interest for genetic improvement, and to reproduce genitors of the Nemaya variety described above. In all cases, microcuttings are excised from regenerating zygotic and somatic embryos (Eskes, 1995; Decazy *et al.*, 1997; Etienne *et al.*, 1999a). This technique was successfully transferred to all participating PROMECAFE institutions (IHCAFE–Honduras, PROCAFE–El Salvador, ANACAFE–Guatemala, and ICAFE –Costa Rica).

The culture of coffee microcuttings involves the use of cytokinins to trigger axillary bud development on orthotropic nodes, followed by multiplication via subdivision of obtained orthotropic shoots. Initially, microcuttings had to be cultured on solid media, requiring a temporary placement in liquid medium to induce rooting. Later, a method was developed that obviated the need for induction treatments, because low auxin contents (1 mg/l IBA + 1 mg/l NAA) made direct rooting possible (Dufour *et al.*, 1994 b) (see Figure 10).

More recently, essays with liquid media and Temporary Immersion Systems (TIS) (see Box 3), demonstrated much higher multiplication rates as well as less manipulation of the materials (Decazy *et al.*, 1997). An average of 7.45 usable nodes was obtained with TIS, compared to the 2.33 nodes characteristic of solid media cultures (Dufour *et al.*, 1996b).

The multiplication of microcuttings using TIS showed better results when immersion was programmed for 15 minutes every 6 hours. Utilization of BAP (1 mg/l) increased the amount of activated axillary buds. Sprout enlargement occurred when BAP was replaced with GA3 in the last three weeks of the culture process. This, in turn, caused a substantial increase in the number of usable nodes produced. (Dufour *et al.*, 1996b).

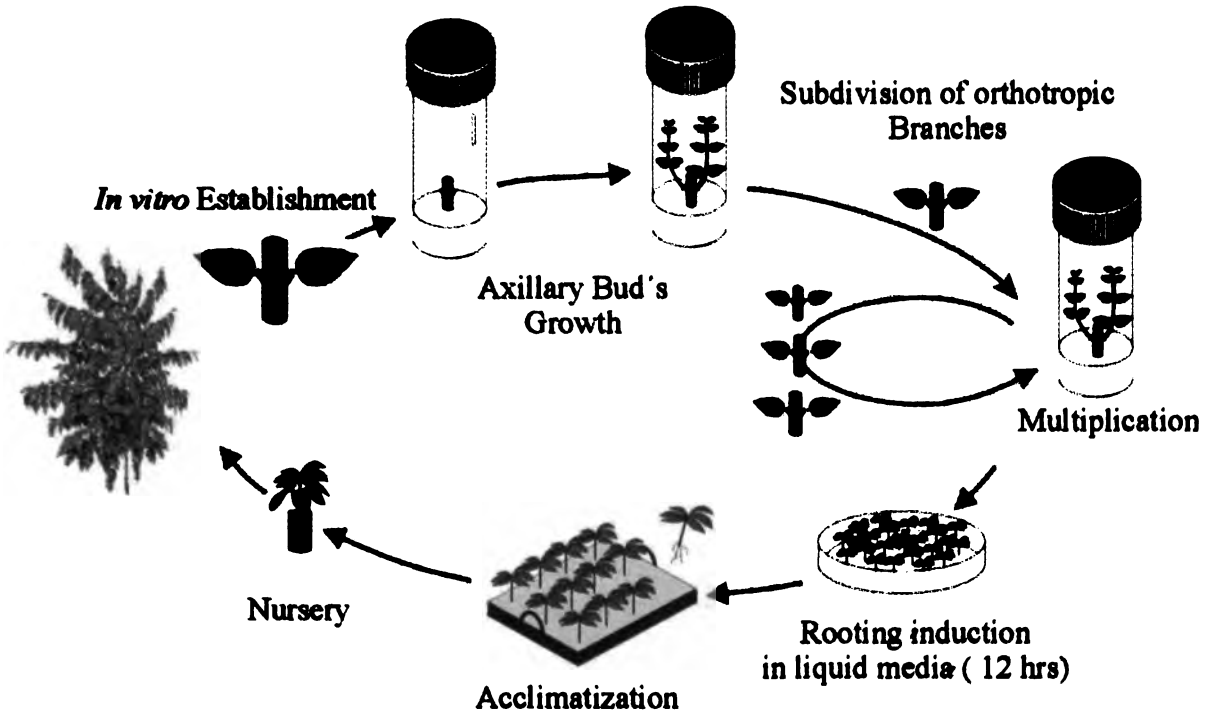


Figure 10. Multiplication process of coffee microcuttings at CATIE (*From Etienne* et al., *1999a*).

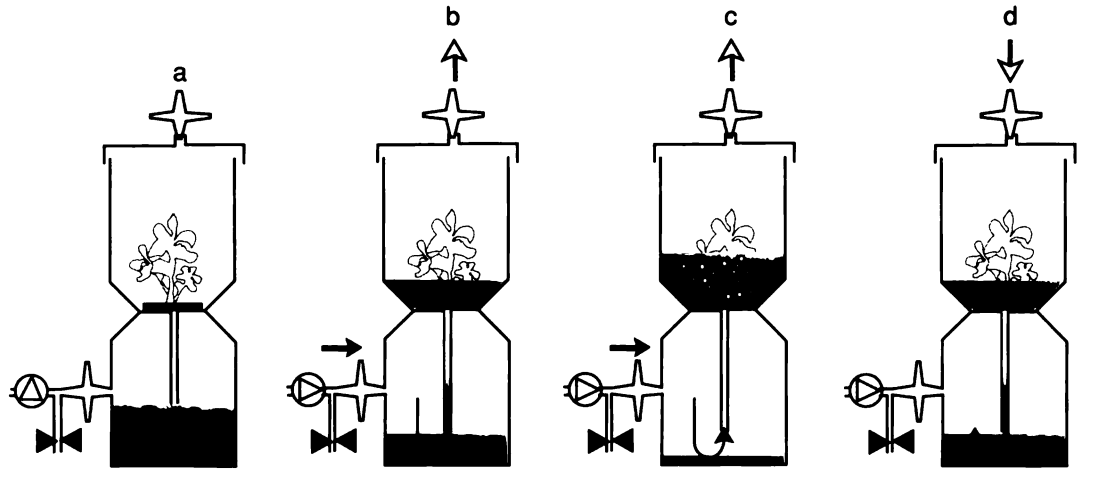
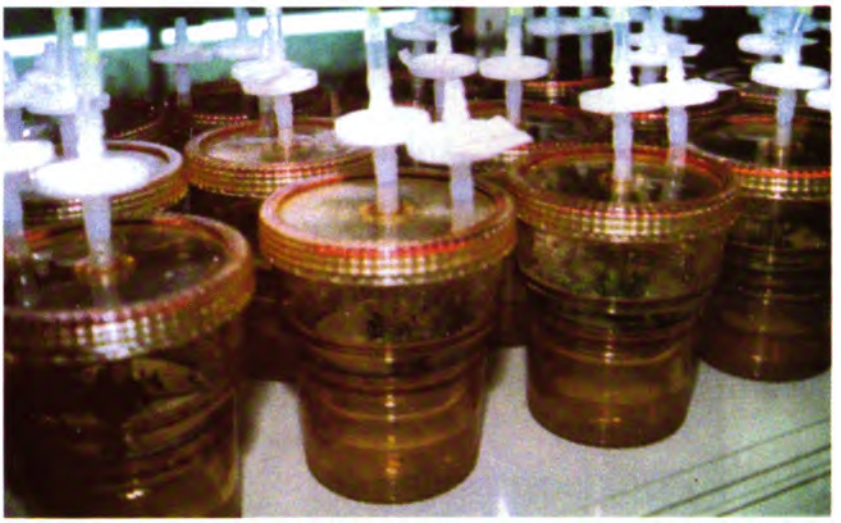


Figure 11. Principle of the temporary immersion liquid culture system. a). The culture medium is in the lower chamber. b) & c). Increasing the pressure in this chamber forces the solution into the upper chamber and the plantlets are immersed. d). When the pressure is released, the medium flows back into the lower chamber (*From Teisson* et al., *1995, and reproduced with permission from* Plantations, Recherche, Développement)



BOX 3: What is the Temporary Immersion System (TIS)?

Brief rather than continual immersion of explants is an effective way of utilizing liquid media for *in vitro* culture whilst avoiding its main drawback: excess water problems that affect normal plant growth.

To this end, the BIOTROP laboratory of CIRAD developed a small, flexible and economic device called RITA® (*Récipient pour Immersion Temporaire Automatisée*, or Automatized Temporary Immersion Recipient), which is a TIS that has been successfully applied to *Coffea* and *Musa* cultures at CATIE.

TIS is based on explant culture (*e.g.* microcuttings or somatic embryos) subject to short immersion periods in liquid media subsequently exposed to a humid and sterile atmosphere. Medium retention by capillarity on the explants is responsible for the success of the system. Explant nutrition is more efficient under these conditions and the medium does not degenerate as quickly as in other treatments.

The RITA device is a modified version of a simple autoclavable filtration unit, linking the upper and lower compartments with a glass tube. This system requires the use of a pressure pump and a timer to control the rhythm and length of immersion. Sterile conditions are provided by hydrophobic filters placed where air enters and exits the device.

The unit operates opposite from a filtration unit: plant materials are placed on the upper part and the liquid in the bottom. When the lower compartment is pressurized, the medium is forced to move up to the upper compartment through the glass tube. When pressure is released, the liquid flows back down again. Air blown into the container throughout the immersion period renews the internal pressure.

Because of its high multiplication rate and very low cost, TIS has no competitive alternative among biological multiplication procedures. TIS makes it possible for acclimatable plantlets to be regenerated directly from cellular suspension in the same recipient. This process also requires less manipulation. One RITA container can hold up to 1,000 plantlets, drastically reducing the space and energy required to obtain large numbers of plants. Use of liquid media eliminates the exorbitant cost of Agar.

Adapted from Teisson *et al.*, 1995; Dufour *et al.*, 1996b; Dufour & Escalant, 1996; Feyt & Bertrand, 1997.

®Trademark of CIRAD, France

Finally, a remarkable advancement was achieved by Etienne *et al.* (1997), who developed a much less expensive rooting procedure capable of 100% success rates. In this method, 2cm long cuttings are planted directly on a horticultural substrate after induction treatment (12 h in liquid media with low auxins) for rooting and acclimatization to *ex vitro* conditions (see below). The media utilized consists of a mixture of sterilized soil, sand and decomposed coffee pulp on a 3:1:2 proportion. This media can be inoculated with mycorrhizas for best results.

The main advantage of microcutting multiplication is guaranteed conformity to the original parent, a fact that has been widely confirmed by field trials (Berthouly *et al.*, 1987; Berthouly, 1989). Nevertheless, the technique is expensive because of the excessive manipulation required (Etienne *et al.*, 1999 a & b). In addition, the multiplication rate achieved is rather limited, varying from 7 to 9 every 3 months. On the other hand, it has been applied successfully at very low culture temperatures, thus representing a secure mechanism for the conservation and exchange of disease-free materials between countries (Etienne *et al.*, 1999 a & b).

Somatic embryogenesis

Somatic embryogenesis is the clearest expression of the totipotency phenomenon in plant cells. This process comprises the group of procedures that make it possible to clone embryos from a genotype and regenerate plantlets with identical characteristics out of them. The *in vitro* generation of somatic embryos out of diverse types of explants has been proved to be the best alternative for large scale propagation: multiplication rates are higher than in microcutting cultures, although the risk of somaclonal variation is also higher (Dufour *et al.*, 1996b; Etienne, 1998; Etienne *et al.*, 1999 a & b). Liquid media are preferred for mass propagation because larger quantities of embryos with higher reproduction and germination rates, and better morphology can be obtained, than with solid media (Carasco *et al.*, 1994; Teisson *et al.*, 1998).

Somatic embryos have been successfully produced since the beginning of the Coffee Breeding Program (CBP), but low vigor and lack of homogeneity after germination and during regeneration steps made it necessary to invest more effort into refining the technique (Girón, 1998). According to Carasco *et al.* (1994), SE takes only 60% the time required for its zygotic equivalent, to generate equally mature embryos. Thus, reserves are accumulated rapidly using SE. After several tests, it was recommended that a maturation phase, comprising the use of sucrose (40 g/l) and absicic acid (ABA) to induce dormancy and promote the accumulation of reserves, be included in the protocol, in order to prepare somatic embryos for germination (Girón, 1998).

According to Söndahl (cited by Carasco *et al.*, 1994), several SE pathways exist, the following being the two most important (see Figure 13):

- ***Low Frequency Somatic Embryogenesis (LFSE),*** utilizing only one media which gradually causes callus formation which later evolve into somatic embryos.

- ***High Frequency Somatic Embryogenesis (HFSE)***, where somatic embryos are organized in masses inside a very friable callus ideal for liquid media culture. It consists of two different stages: one aiming at generating embryogenic cells out of a primary callus produced on perivascular cells, and the other dedicated to the evolution of those embryogenic cells into somatic embryos.

LFSE, also called "Direct SE", was used to successfully multiply one of the genitors of the Neyama variety (T3751 (1-2)), using explants obtained from young leaves. However, the use of this pathway on other materials was limited, due to a strong genotype effect (Dufour *et al.*, 1996b).

HFSE, or "Indirect SE", is the most used developmental pathway in the collaborative research conducted by CATIE and CIRAD for the last 10 years. This is largely due to CBP's desire to produce and distribute massive amounts of elite materials. HFSE was required for the propagation of the other Nemaya genitor (T3561 (2-1)), which did not respond to LFSE. It was also successfully used to multiply the F1 hybrids derived from crosses between commercial and wild Arabica materials obtained from Ethiopia and Kenya.

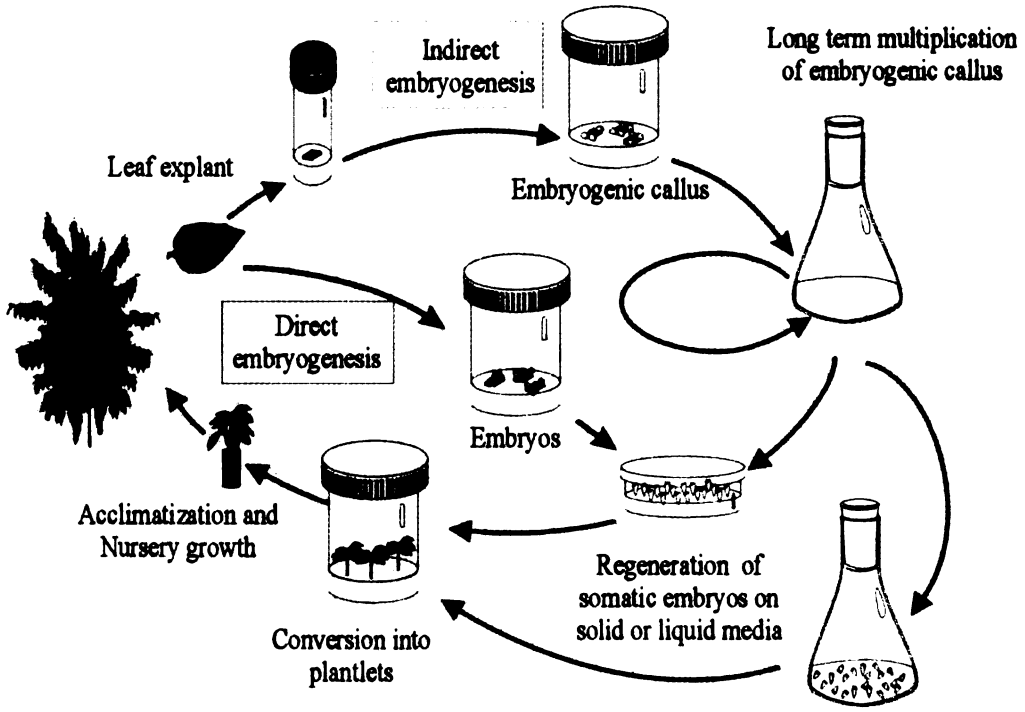


Figure 13. Outline of the two main pathways for coffee somatic embryogenesis: Direct or low frequency SE, and Indirect or high frequency SE (From Etienne *et al.*, 1999a).

To increase HFSE productivity, a method was designed in which all proliferation and regeneration stages were carried out in liquid media. HFSE implemented in this manner presented very high regenerative potential (up to 5,000 plants per gram of callus for the F1 hybrids evaluated) (Etienne *et al.*, 1997 a & c). Its utilization on coffee required several steps that can be summarized as follows (Etienne *et al.*, 1997c; Anthony *et al.*, 1997b; Girón, 1998):

- **Primary Callogenesis**: foliar explants are placed in the dark for 1 month, at 27 °C, on a media described by Van Boxtel & Berthouly (1996) until the primary callus is formed.
- **Embryogenic Callus Production**: the primary callus is transferred to an indirect light environment for 6 months, on an auxin-free media, specialized for production of high-density embryogenic calluses. Histologic and morphologic events during this phase were described in detail by Vásquez *et al.* (1998 a,b).

- **Cellular Suspension (Proliferation)**: the embryogenic callus is removed and placed on a genotype-specific media that induces proliferation, but requires constant agitation and subcultures every 10 weeks (Figure 14). This suspension ensures a permanent supply of large quantities of embryogenic material.

Figure 14. Cellular suspension stage in Coffee Somatic Embryogenesis.

- **Embryogenesis**: subsequent subcultures are moved to a direct light environment for 5 months on a media that induces development of somatic embryos. Liquid media are employed in this step for large-scale production.
- **Maturation**: somatic embryos are temporarily placed on a media containing ABA and sucrose, to induce dormancy and reserve accumulation.
- **Germination and Regeneration:** mature somatic embryos are transferred to a hormone-free media that induces germination and plantlet development. Also, non-sterile media such as that used for rooting microcuttings (*supra*), were successfully used to germinate somatic embryos and produce plantlets.
- **Acclimatization:** the materials obtained are finally transferred to the greenhouse to complete plantlet conversion and later to nurseries so they might be acclimatized before planting in the field (*see infra*). Figure 17 summarizes the mass propagation process through somatic embryogenesis utilizing the conventional and TIS systems.

When using TIS, optimal immersion frequencies during proliferation and embryogenesis phases were 1 minute per day. Embryogenesis required only 3 months using this method. Meanwhile, 1 minute every 12 hours produced the best results during regeneration, requiring only 6 months to obtain healthy plantlets (Anthony *et al.*, 1997b).

The adaptation of the TIS to coffee somatic embryogenesis brought a new era in coffee *in vitro* propagation. Large quantities of high quality and homogeneous somatic embryos can be obtained with this method. The RITA biorreactor greatly facilitated the process since, for the first time, the proliferation to germination stages could be accomplished in the same recipients. Manipulation was significantly reduced; labor was required for media adjustments only, with no transfer of materials. Also, the space required to produce large numbers of plants was minimized. These results were very encouraging, since labor and shelving space in the lab represent 70% and 10%, respectively, of the total vitroplant production costs (Etienne *et al.*, 1999a).

This somatic embryogenesis process, combining the use of TIS and direct planting of germinated embryos in the greenhouse is being used to diffuse the F1 improved varieties in order to establish a network of semi-commercial field tests in Costa Rica, Honduras, Guatemala and El Salvador

(figure 15). Definite selections of commercial varieties should be made by 2003. Agronomic tests are also being carried out at CATIE to evaluate the genetic conformity of materials multiplied in this way (Etienne, 1998).

Plantlet regeneration and acclimatization

Until recently, somatic embryos had to be previously germinated on solid media to obtain successful regeneration. After that, it was necessary to cut the *in vitro*-generated roots and to induce the formation of a new rooting system by cultivating embryos in a hormone solution (Etienne *et al.*, 1997b). This conventional protocol was too cumbersome and incompatible with the commercial use of embryogenesis.

In 1998, a new method was developed at CATIE to regenerate somatic embryos into plantlets by planting them directly into the soil (Etienne-Barry *et al.*, 1999; Etienne-Barry *et al.*, 1998; Etienne *et al.*, 1999b) (Figure 16).

A culture density of 1,600 embryos per biorreactor proved to generate best results in further embryo development. Applications of sucrose (234 mM) to the media, stimulated efficient subsequent conversion into plants (78 % success), as well as their vigorous growth.

Direct sowing reduced the time span necessary for vitroplant regeneration from 6 to only 3 months (see Table 5). It also drastically reduced handling time by 87%, and shelving area requirements to only 6.3% of the figures utilized by conventional acclimatization of plants developed on gel media: to obtain 9,000 plantlets, the required space was reduced from 22 to 1.4 m2, and handling time was reduced from 207 to 27 hours (Etienne-Barry *et al.*, 1999; Etienne *et al.*, 1999 a & b; CATIE, 1998). Additionally, somaplants obtained this way are morphologically the same as seed-propagated plants by the end of the nursery stage.

Thus, direct sowing of somatic embryos produced in a simplified bioreactor stands out as a new, highly competitive concept that could simplify *in vitro* technologies, such that they may be used commercially in the near future (CATIE, 1998).

Finally, a protocol for acclimatization of plantlets obtained by either the culture of microcuttings or somatic embryogenesis, was developed and described in detail by Etienne *et al.*, 1997b. It is an inexpensive and simple methodology that yields 93-98% acclimatization rates. This protocol was disseminated and is currently being applied by national coffee institutes to acclimatize genitors of the Nemaya variety. It is comprised of 3 stages (see Figure 17):

- **Plantlet Preparation**: plantlets are stripped, leaving only the top 2-3 pairs of leaves. Plantlet bases are immersed in rooting solution, and they are then transferred to a sterilized substrate (this was initially a 3:1:2 mixture of soil, sand and decomposed coffee pulp; more recently, however, coconut fiber alone has been successfully utilized).

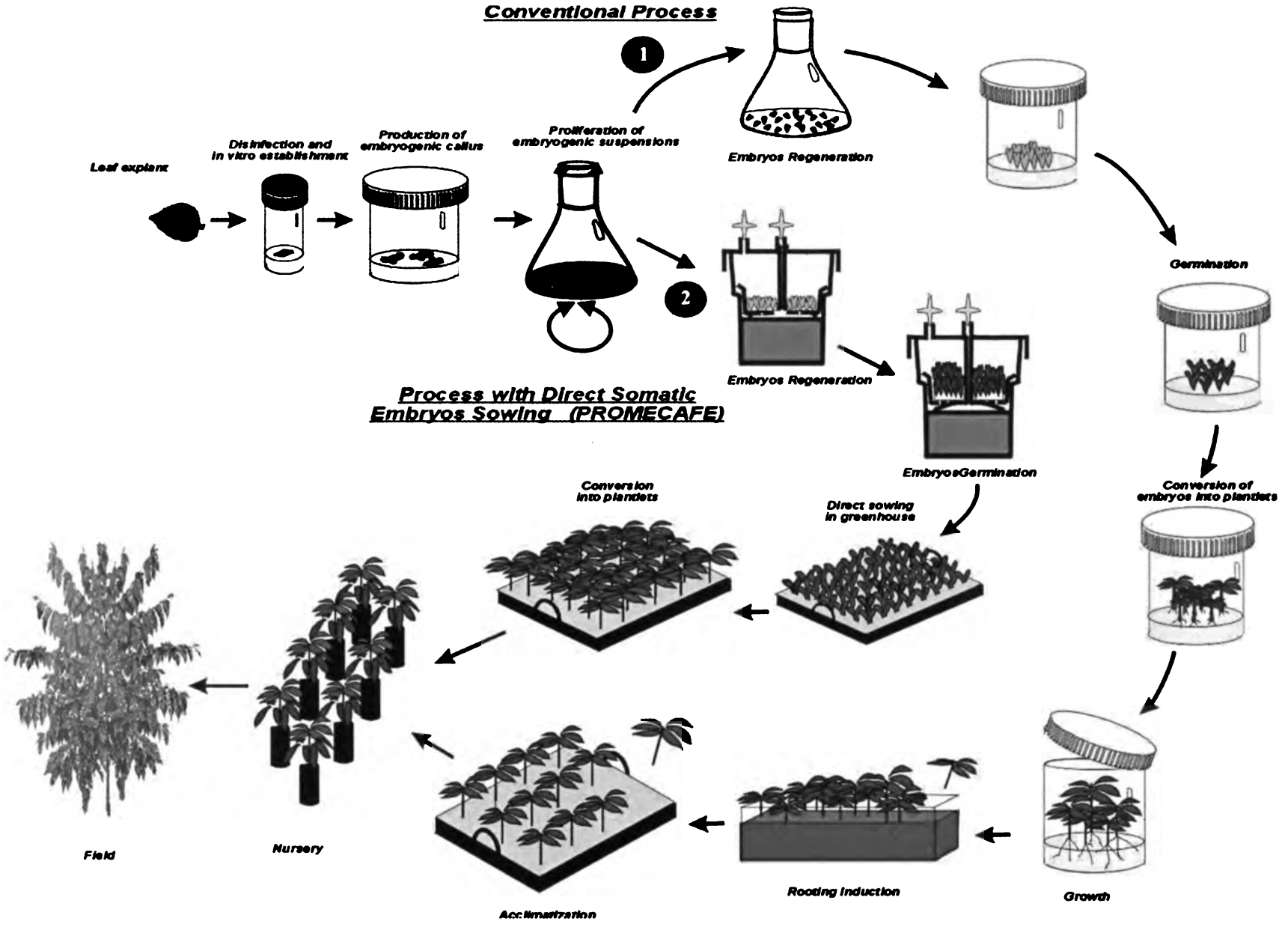


Figure 15. Steps in coffee mass reproduction through somatic embryogenesis utilizing a Temporary Immersion System. (From Etienne *et al.*, 1999a).

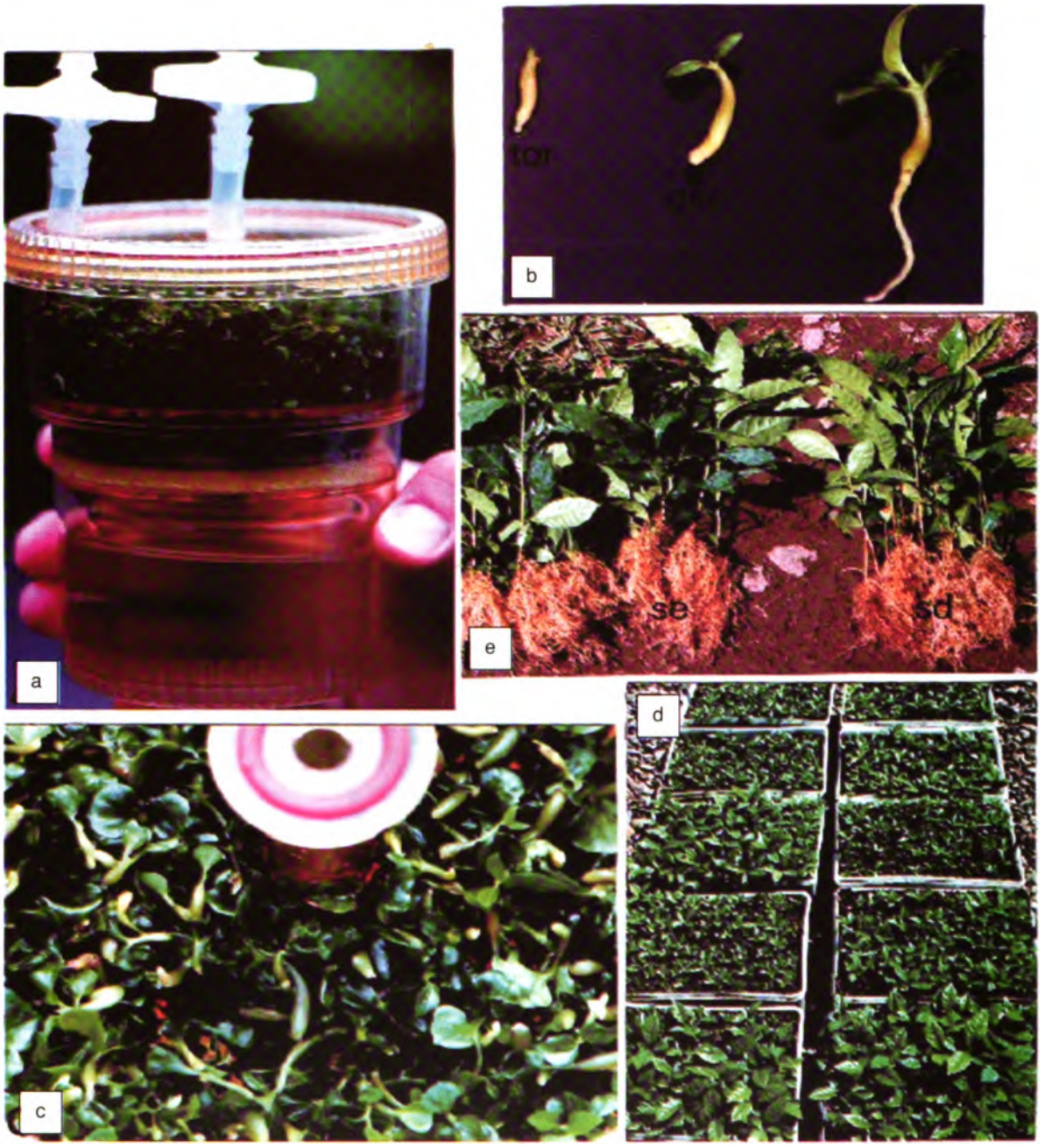


Figure 16. Stages of the new protocol developed at CATIE for direct sowing of somatic embryos produced in a bioreactor. a) Mass production of somatic embryos in a bioreactor, ready to be planted; b) Development stages of somatic embryos in a bioreactor (tor = torpedo, ger = germinated, pla = plantlet); c) Magnification of A) to real life scale; d) Nursery after 4 months in horticultural soil, established for mass production out of somatic embryos; e) Comparison of plantlet performance after 8 months in the nursery (se = originating from somatic embryos, sd = originating from zygotic embryos). (From Etienne-Barry *et al.*, 1999)

TABLE. 5. Comparison of efficiency between Conventional and PROMECAFE-CIRAD-CATIE-IICA methods for acclimatizing coffee vitroplants.

ACCLIMATIZING METHOD	Culture conditions used for germination and conversion into plants	Plant material used for sowing	Culture duration from germination to sowing (months)	*In vitro* culture area to obtain 9,000 plants (m^2)	Conversion into plants of selected embryos (%)	Handling to obtain 9,000 acclimatized plants (hours)	Plant size 5 months after sowing (cm)
1. CONVENTIONAL	Semi-solid media	Plantlets (2 to 4 pairs of leaves)	6 ± 1	22	50 ± 12	207	3.1 ± 1.0 a
2. CIRAD-CATIE-PROMECAFE	Bioreactor followed by direct embryo sowing	Germinated somatic embryos	3 ± 0.5	1,4	52 ± 9	27	3,0 ± 0,9 a

Data shown were estimated from a 9,000 plant batch, expressed in months, hours of work and m2 for culture duration (from germination to sowing), handling and culture areas. In vitro embryo conversion into plants was evaluated 3 months after germination for the conventional method, and 2 months after somatic embryo sowing for the new method. The values shown for plant sizes are means ± standard error (s.e.) of 3 replicates, each containing 60 plants. Plant sizes were compared by variance analyses followed by Duncan's test (at 0.05). (From Etienne-Barry *et al.*, 1999).

Photo 1. Preparing a microcutting for acclimatization.

Photo 2. Soaking the stem of a microcutting in an auxin solution to induce root system formation.

Photo 3. Planting an *in vitro* plantlet in a sterilized substrate.

Photo 4. View of the PVC structure of an *in vitro* plantlet acclimatization tunnel fitted with a micro-sprinkler system.

Photo 5. Trays of coffee *in vitro* plantlets being acclimatized in a tunnel fitted with a misting system.

Photo 6. Acclimatization tunnel under a black canvas shade

Photo 7. Acclimatized *in vitro* coffee plantlets (four months) removed from trays for transfer to an open soil nursery

Figure 17. Stages of the acclimatization protocol developed at CATIE for coffee somaplants produced either through microcuttings or somatic embryogenesis. (*From Etienne* et al., *1997b and reproduced with permission of* Plantations, Recherche, Developpement).

- **Acclimatization**: In this 3-month-long stage, the first 4 week period is critical. During this period, the plants must be protected from rain and bright light, temperature must be maintained constant and high relative humidity must be ensured. Any symptoms of chlorosis must be immediately treated. Automated misting devices under black canvas tunnels are recommended. A level of 30% of natural light is advisable during the critical period, after which light intensity must be gradually increased to ensure the plants' hardening by the end of this stage. Plantlets can be acclimatized in polybags or trays: polybags avoid root disruption and malformation provoked by transplanting, but require more sterilized media and tunnel/misting area.

- **Transfer to the nursery**: In the nursery, plants are reared either in polybags or directly in bare soil. Recommended planting density when transplanting to soil is 0.2 x 0.2 m, in 1m wide beds, providing 10-30-10 fertilizer and nematicide applications. Plants can be transferred to the field after approximately 8-10 months.

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CHAPTER TWO
CACAO

CONSERVATION.

Increasing Preserved Stocks and Improving Preservation Methods.

An important Cacao Genebank has been preserved at Turrialba since 1944. Since 1978, this genebank has been counted as one of only two in the world to be catalogued by FAO as an "International Collection". The collection is maintained on a 7-hectare field under the protection of leguminous and timber shade trees. Recent introductions to these collections as well as advancements made in the techniques used to preserve them, are reported in this chapter.

Field Collections

As of January 1999, CATIE's International Cacao Collection included a total of 771 *T. cacao* clones plus individuals from related species, as illustrated in Table 6.

TABLE 6. Number of accessions of Cacao genotypes and wild relatives preserved in CATIE's field collections as of January, 1999.

SPECIES	# OF ACCESSIONS
T. cacao	771
T. angustifolium, T. bicolor, T. gileri, T. grandiflorum, T. macrocarpa, T. mammosum, T. simiarum, T. speciosa, T. simiarum	9 *(1 of each* spp.*)*
Herrania albiflora, H. baloensis, H. cuatrecasana, H. nicterodendrum, H. nitida, H. purpurea, H. umbratica	7 *(1 of each* spp.*)*
TOTAL	**787**

These materials have been collected throughout Latin America (mainly Costa Rica, Brazil, Mexico, Ecuador and Guatemala) and the Caribbean (principally Trinidad), as well as from experimental stations in Florida, France and other countries[1]. A high percentage of the genotypes preserved in CATIE, such as the Central American criollos[2], cannot be found in any other cacao collection anywhere in the world (Morera y Mora, 1990; CATIE, 1995; Phillips-Mora, 1999a).

[1] Accessions re-introduced from Indonesia, Samoa and Cameroon are also included in CATIE's collection.

[2] CATIE preserves about 50 cultivars of these Central American criollos. These native materials have high quality grains. Deforestation and the introduction of commercial varieties may have caused a large reduction of Criollo diversity in Central America (Motamayor *et al.*, 1998).

BOX 4: The genus *Theobroma*

Cacao represents a very important cash crop in developing countries, second only to coffee in more than 40 countries located within 20 degrees of the Equator. It supplies chocolate flavor and cocoa butter, to all chocolate and foodstuffs manufacturers around the world.

The cacao tree, *Theobroma cacao*, is native to tropical America. There is no clear evidence about its specific center of origin, as wild populations have been identified from México to the Upper Amazon Basin in northern South America.

However, pre-Columbian culture of cacao was limited to Mesoamerica. Thus, words like Cacao and Chocolate derived from Mayan terms: "Kaj" (= bitter) + "Kab" (= juice), "Chacau" (= hot thing) + "Kaa" (= drink).

Theobroma belongs to the Sterculiaceae family. Dispersion by nomadic and commercial means through a wide variety of environments partially accounts for the large variability found worldwide. Phenomena like mutations and self-incompatibility (allogamy in this species is greater than 95%).

Through the 1940's, numerous species were identified and described. In recent years, these species have been re-organized into subgroups within the *T. cacao* species since there are no fertility barriers but there are intermediate individuals among the groups. Also, species from the genus, *Herrania,* can be crossed with *T. cacao* producing fertile hybrids. Therefore, these are also considered to be part of the genetic diversity of this ancient crop.

Great phenotypic variability can be observed in the shapes and colors of seeds, foliage and, above all, fruits. This provoked very complicated nomenclature describing cacao "varieties", including terms like 'Lagarto', 'Criollo', 'Calabacillo', 'Amelonado', 'Cundeamor', 'Angoleta', etc. Today, scientific literature classifies cacao variability into wide genetic groups (or subspecies) which comprise all varieties and cultivars. Those groups are: **"Criollos"** (Cylindrical fruit with narrow ends, rough skin, variable fruit color, and seeds varying in color from white to barely pigmented. Short trees with rounded canopies, small, oval, pale-green leaves, highly susceptible to disease), **"Forasteros"** (oval fruit with rounded ends and often with a bottleneck at the base, fruit generally green with purple seeds. Vigorous trees with large, bright green leaves, and higher disease tolerance), and **"Trinitarios"** (hybrids of the preceding two groups, with intermediate characteristics).

Information adapted from Osei *et al.*, 1995; Fritz *et al.*, 1995a; Enríquez, 1985; León, 2000.

These materials have been preserved in field collections in Turrialba (600 m.a.s.l., average daily temperature of 22 °C, and average annual precipitation of 2,640 mm), under leguminous shade trees such as *Inga* spp. and *Erythrina* spp., utilizing a 3x3m planting density.

During the 1990's, important introductions were added to the Collection. In 1996, a group of 87 genotypes from the Mayagüez Experimental Station of Puerto Rico were included. Also, all accessions (with an average of 10-15 plants each) were entirely rejuvenated during the last ten years, utilizing grafting techniques developed at CATIE (CATIE, 1996).

Cryopreservation

The possibility of preserving cacao germplasm via cryomethods was also studied at CATIE during the 1990's. The benefits of these methods are discussed above in the Coffee section.

The cryopreservation of **zygotic embryos** of Cacao was successfully carried out using embryos excised from 2-to-3-months-old pods. The effects of cryoprotective treatments (embryo incubation

in sucrose or dimethyl-sulphoxide, DMSO) and slow precooling before immersion in Liquid Nitrogen (LN) were tested. In all cases, embryos were desiccated by placing them under sterile air stream for two hours (Cisne, 1992; Abdelnour-Esquivel, 1993).

Best results were obtained for embryos incubated in sucrose (1 M) prior to direct immersion in LN. Recovery was not possible when the sucrose pretreatment was not performed.

Additionally, conditions were tested for recovery through secondary embryogenesis (the production of somatic embryos from cryopreserved zygotic embryos). Highest recovery rates (14%) were achieved by incubating zygotic embryos in either a 1 M sucrose + coconut milk + casein medium for 3 days before slow precooling (reducing 1°C/minute down to –40°C, before immersion in LN), or a liquid MS + 0.5 M sucrose medium before direct immersion in LN (Cisne, 1992; Abdelnour-Esquivel, 1993).

The same authors were also able to cryopreserve **somatic embryos** of *T. cacao,* which were derived from cotyledons, utilizing a method described by Aguilar *et al.* (1992). Cryoprotective treatments using sucrose, and slow pre-cooling down to -40 °C were tested. Desiccation with sterile air was utilized in all cases.

Best results (20% recovery) were obtained when incubating somatic embryos in a 0.5 M sucrose medium for 24 hours followed by slow precooling treatments before immersion in LN. Similar results (16% recovery) were obtained when embryos were incubated for 12 hours in 0.75 M sucrose before direct immersion in LN. In all cases, best results were achieved when the somatic embryos being cryopreserved were at the globular developmental stage (Cisne, 1992; Abdelnour-Esquivel, 1993).

GERMPLASM EVALUATION.

Broadening Knowledge of our Preserved Stock

Phenotypic analyses were conducted on the entire germplasm collection during the 1990's, using inoculation techniques developed at CATIE, in order to determine the reaction of clones to diseases such as *Phytophthora palmivora* and *Moniliophthora roreri* (Sánchez, 1982; Phillips-Mora & Galindo, 1989; Phillips-Mora *et al.*, 1996; Phillips-Mora & Castillo, 1999b).

An efficient testing method was developed to evaluate resistance to *P. palmivora*, based on artificial inoculations, and consisting of the following steps (Fritz *et al.*, 1995b; Phillips-Mora & Fritz, 1996):

- Impregnate a small paper disc with zoospores and place disc on a detached five-month-old cacao fruit
- After inoculation, cover fruit with plastic bag to maintain humidity
- Evaluate for incidence and size of lesion after five, and ten days.

A new evaluation of the cacao collection was initiated recently using this method. As of today, more than 50% of the genotypes have been analyzed. Of a total of 437 clones tested, 22% showed resistance or moderate resistance to *P. palmivora,* providing evidence that this trait is polygenic and dominant (Phillips-Mora and Castillo, 1999b).

Another methodology developed by Sánchez (1982) and improved by Phillips-Mora (1996b) for the artificial inoculation of *M. roreri* on cacao, made it possible to conduct resistance studies for this disease, which is the most destructive and economically important in Central America. As of today, 441 clones have been tested for their reaction to Moniliasis. Moderate resistance has been observed in only 2.3% of tested materials, including those listed in Table 7 (Phillips-Mora & Castillo, 1999 b; Morera *et al.*, 1997; Phillips-Mora, 1999a):

Nevertheless, finding such resistance, however rare, was a very important result, since it seems to be a very infrequent characteristic in cacao. In order to take advantage of this important finding, resistant materials have been reproduced and considered in recent breeding efforts (Phillips-Mora, 1999a).

TABLE 7. Clones showing some resistance to *Moniliophthora roreri* among those preserved in CATIE's Cacao Collection as of September, 1999.

Clone	Country of origin	Reaction to M. roreri
B-8	Brazil	MR
CC-240	Costa Rica	MR
CC-252	Costa Rica	R
EET-75	Ecuador	MR
EET-183	Ecuador	MS
ICS-43	Trinidad	R
ICS-95	Trinidad	MR
PA-169	Peru	MR
UF-273	Panama	R
UF-296	Costa Rica	R
UF-712	Costa Rica	R
ARF-14, ARF-22, ARF-37, B-2, CC-27, CC-124, CC-137, EET-59, ICS-46, ICS-100, Laranja, PMCT-13, PMCT-27, PMCT-87, UF-676	Various	MS

MR = Moderately Resistant; R = Resistant; MS = Moderately Susceptible

As a result of these efforts, a total of 328 trees from 19 different crosses between a Moniliasis-resistant clone (UF-273) and other cultivars, were recently tested for their reaction to *M. roreri.* A very large proportion (87%) showed susceptibility to the fungus, reinforcing the perception that Moniliasis resistance is a recessive polygenic characteristic. Nonetheless, low heritability of this trait from the UF-273 parent might also play a role (Phillips-Mora & Castillo, 1999b; Phillips-Mora, 1999a).

GENETIC IMPROVEMENT

Developing and Evaluating Promising Materials

T. cacao is an allogamic diploid (2N=20) species from the Sterculiaceae family. Commercial cacao varieties have a narrow genetic base, showing susceptibility to many diseases. It has been estimated that disease and pests are responsible for reducing cacao yields by 10 to 40% annually (Osei *et al.*, 1995; Crouzillat *et al.*, 1999a).

In tropical America, the main diseases affecting Cacao include Monilia Pod Rot or 'Moniliasis' (*Moniliophthora roreri*), Black Pod disease (*Phytophthora palmivora*) and 'Witches Broom' disease (*Crinipellis perniciosa*). Moniliasis is the single most important factor limiting the development of cacao in Central America. Losses reported since its appearance in 1978 rose to 80% in only a few years, causing former cacao producers like Costa Rica to become net importers of this commodity (Phillips-Mora & Castillo, 1999).

Thus, one of the major tasks of any breeding program, aimed at addressing the main problems faced by cacao producers in the Region, is to produce materials resistant to these fungi (Phillips-Mora & Castillo, 1999). CATIE's work in Cacao breeding during the 1990's is described in this section. It distinguishes between traditional and non-traditional improvement methods, and how each can best be used to achieve resistance to key diseases.

Traditional cacao breeding

Because of Regional needs, traditional cacao breeding conducted at CATIE during the 1992-1999 period, focused on selecting elite materials from among preserved clones to generate and evaluate crosses among them. Resistance to *M. roreri* and yield were the main selection criteria.

Clonal testing

Several testing trials were implemented during the 1990's aimed at selecting outstanding genotypes with potential use in further breeding efforts (Phillips-Mora, 1996).

In an early experiment, 12 cultivars were planted in Turrialba and given artificial inoculations continuously from 1985 to 1994. Of the 12 clones studied, 5 were selected because they showed some resistance to *M. roreri* (UF-273, UF-296, EET-183, CC-137 and EET-75). Cultivars CC-137 and EET-183 were also among those with the highest yields (812 and 685 kg dry beans/ha/year average respectively, after 5 years[3]). The other high-yield cultivars were Moniliasis-susceptible (EET-67, EET-59). In general, Black Pod incidence was low among all cultivars (ranging from 2.6 to 14.1 %), though EET-183 presented notable levels of resistance (Morera *et al.*, 1997; Phillips-Mora, 1996; Morera & Mora, 1996).

A second trial planted in 1991 under high natural inoculum pressure conditions (La Lola, Costa Rica) confirmed initial results. Cultivars EET-75, EET-183 and CC-137 proved again as highly resistant to disease, and their yield performance was more than acceptable (Phillips-Mora, 1996; Morera *et al.*, 1997). EET-183 showed early flowering and fruiting traits, producing approximate-

[3] These figures are rather low, but genitor clones were selected exclusively because of their high disease resistance levels.

ly 800 kg/ha after only one year in the field. On the other hand, CC-137 production was initially low but increased to 1,000 kg/ha after three years in the field and exceeded 1,600 kg/ha after the fifth year. Annual production for both clones (including data from all eight harvests) averaged 1,000 and 775 kg/ha respectively, compared to Pound-7 (a susceptible cultivar used as control) whose production reached only 94 kg/ha/year (Mora, 1999, *unpublished*).

More recently, 42 outstanding cultivars were selected, based on disease and productivity assessments of a large section of CATIE's Cacao germplasm. The materials selected were planted in La Lola, (an experimental farm owned by IICA in the Caribbean coast in Costa Rica), in 1998, to continue assessing their performance under more severe environmental conditions (Phillips-Mora & Castillo, 1999 b).

Interclonal cross testing

Twelve promising hybrids obtained from crosses between resistant clones and highly productive cultivars were planted in Turrialba in 1992, to continue the selection process. As of July, 1999, two early-producing crosses had been identified: UF-712 x CATIE 1000 and UF-712 x CCN-51. In addition, a tree from the latter cross showed resistance to both Moniliasis and Black Pod, which is very unusual. These highly promising crosses were rapidly reproduced and planted in La Lola to assess their performance under those conditions (Phillips-Mora & Castillo, 1999b).

Between 1990-1993, a group of 182 hybrids were planted in La Lola. Since that time, they have been tested for productivity and disease resistance, in order to select promising materials. Those hybrids were obtained by crossing compatible clones that presented both high yields and disease resistance. One genitor for 19 of these hybrid crosses was Moniliasis-resistant. The results are described in the germplasm evaluation sections above (Phillips-Mora, 1996).

In recent years, 84 new crosses were planted in La Lola with the goal of accumulating key genes for cacao resistance to Moniliasis. At least one of the genitors in each cross demonstrated resistance to *M. roreri,* while the other parent was characterized by either resistance to *C. perniciosa* or *P. palmivora*, high productivity, or auto-compatibility. This study will: assess resistance segregation; select superior germplasm; and generate improved genotypes which incorporate traits for both disease resistance and high productivity.

Even though this work is still under way, some interesting results have already been produced (Phillips-Mora & Castillo, 1999b; Phillips-Mora, 1999a):

- The UF-273 x CATIE-1000 cross showed more vigorous growth
- Crosses with UF-712 as one of the genitors presented earlier jorquetting
- 12 crosses have already been selected for further evaluation under commercial conditions in Talamanca, Costa Rica, and La Masica, Honduras

Non-traditional cacao breeding

Notwithstanding the promising achievements described in the preceding section, traditional breeding practices have typically made slow progress. These practices have relied mainly on heterosis between parents from different genetic groups[4]. This is probably a result of the long grow-

[4] Upper & Lower Amazon Forasteros, Trinitarios and Criollos.

ing period (3-5 years) and high heterozygosity that characterize this crop. Moreover, cacao breeding has focused on traits that are under polygenic control and have low heritability, such as yield and disease resistance. As a consequence, F1 hybrids from commercial clones have typically resulted in plantations with variable field performance and yields below those obtained with parent materials (Osei *et al.*, 1995; Ronning *et al.*, 1995).

The development of a genetic linkage map (Box 5) and the design of a reliable method for selecting adult characteristics at the seedling stage, became key inputs to facilitate the breeders' efforts (Osei *et al.*, 1995; Fritz *et al.*, 1995a). CATIE's contributions to these goals during the 1990's are described in the following section.

Cacao genetic linkage map

The genetic linkage map (GLM) for Cacao was constructed based on a genetically defined F1 cacao population initiated at CATIE in 1978 by crossing an upper Amazon clone (Pound 12) with a lower Amazon clone (Catongo). Pound 12 is one of the Nanay clones collected as budwood near the headwaters of the Amazon by F.J. Pound in 1943. It is a purple-seeded, purple-staminode, autoincompatible, upper-Amazon Forastero. Catongo is a highly homozygous, autocompatible, white-seeded and white-flowered, lower-Amazon Forastero, found near Urucuca, Bahía (Brazil) in 1939 (Figure 18) (Morera *et al.*, 1994).

As a result of this cross, 120 F1 trees were obtained[5]. In 1990, pollen from one of the hybrid tress (#33) was used to pollinate the Catongo parent in a backcross designed to produce a segregating population. One hundred thirty-seven (137) individuals from the backcross progeny, planted in 1991, were selected to create the linkage map (Fritz *et al.*, 1994 & 1995 a & b).

Two medium density GLM's were constructed in a joint effort between CATIE and FRANCERECO during the 1990's, based on the aforementioned backcross and F1 populations. The development of these maps, which has been reported in successive works by Crouzillat *et al.* (1995, 1996, and 1999 a & b), Fritz *et al.* (1994 a & b; 1995 a & b), Osei *et al.* (1993 & 1995), Phillips-Mora & Fritz (1995) and Rodríguez *et al.* (1993 & 1994), involved the following steps:

- Identification of primers revealing specific polymorphisms
- Evaluation of polymorphisms in a trial cross (F1 or backcross)
- Segregation analysis

The first map was obtained for the intraspecific cross between Catongo and Pound 12 clones. Mapping population consisted of 55 F1 hybrid trees planted in 1977. The map included 162 markers, 125 of them from RFLP plus 36 RAPD (23 RAPD + 13 AFLP) and one phenotypic marker[6], organized into 12 linkage groups and covering 772 cM in total. The number of linkage groups differed from the expected value of 10 (which corresponded to the number of chromosomes in *T. cacao)*, probably as a result of the small sample utilized, which reduced the significance threshold for the linkage between markers (Morera *et al.*, 1994; Crouzillat *et al.*, 1999a).

One hundred thirty-seven (137) trees from that backcross population progeny were utilized to construct the GLM. A total of 1,200 primers (consisting of random sequences of 10 base-pairs each) were tested in the PCR for polymorphisms between Pound 12 and Tree #33 (but not with Catongo).

[5] Later studies confirmed that only 55 of those genotypes were true hybrids (Morera *et al.*, 1995)
[6] Self-compatibility appeared as the only phenotypic segregating marker in the F1 population.

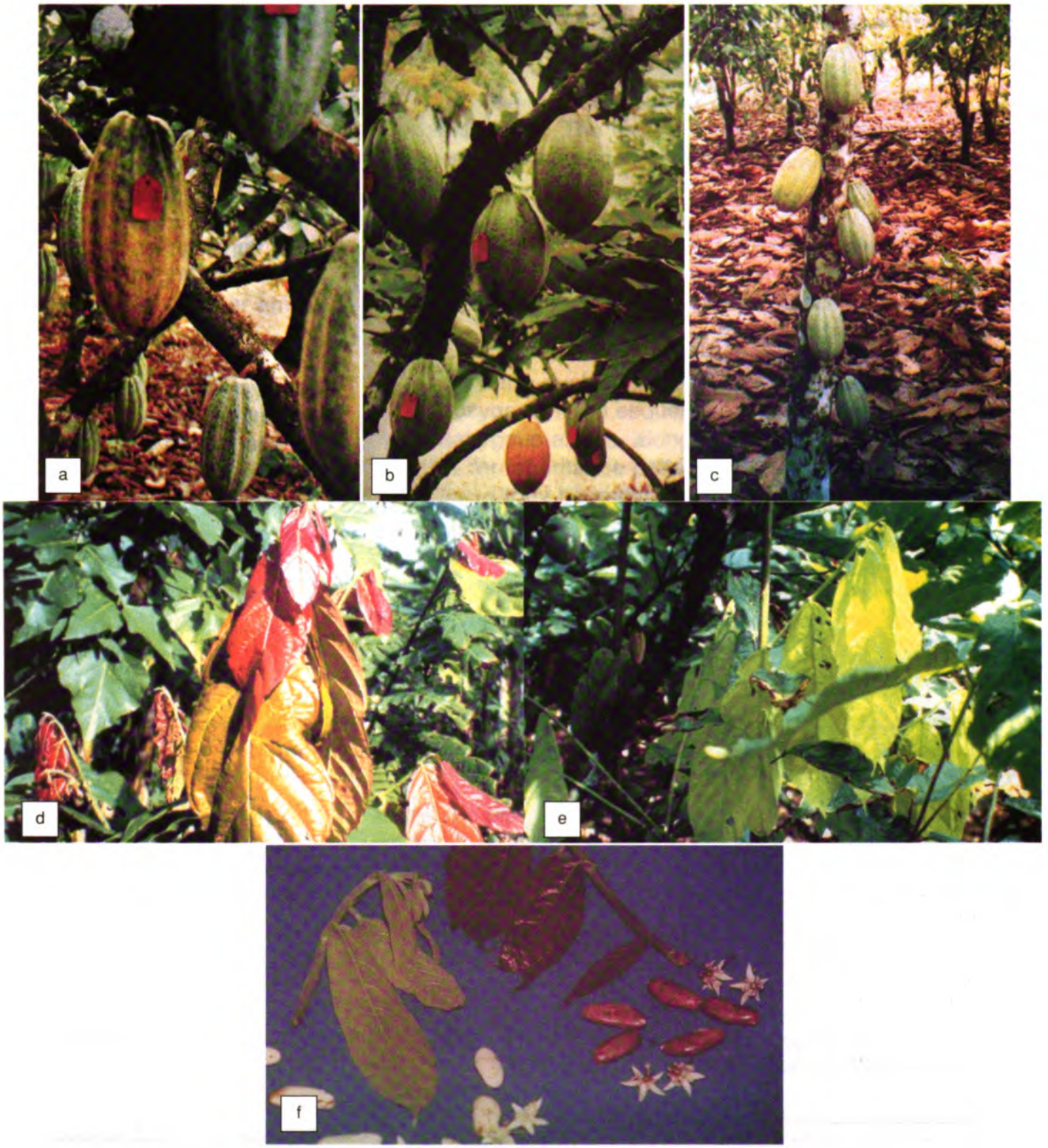

Figure 18. Genotypes utilized at CATIE to generate a backcross population for cacao genetic mapping. a) Pound; b) Catongo; c) F1 Hybrid (Catongo x Pound 12) tree # 33; d) Pound 12 flushes; e) Catongo flushes; f)Comparison of Catongo`s pale green flushes & white seeds and flowers (left) vs. Pound 12`s purple flushes, seeds and flowers (right).

BOX 5: What is a Genetic Linkage Map?

Genetic linkage is the term used in genetics to describe distances between loci within a chromosome. Genetic distances between two loci, which are generally expressed as map units or centimorgans (cM), are measured through recombination frequencies, as the probability of a crossover occurring between two loci is a function of the distance separating them. The greater the separation, the higher the frequency of recombinants appearing in the next generation.

Genetic Linkage Maps (GLM) are graphic representations of the chromosomes, detailing the relative positions of loci (genes or genetic markers) in them. Loci are located in the diagram according to the "distance" inferred from their recombination frequencies.

The establishment of a GLM is based on a segregating population in order to identify recombination frequencies. In the past, recombination was only observable on phenotypic, morphological traits. With the advent of molecular marker technology, it is now possible to identify recombination between loci without observing its phenotypic expression. The more loci the map identifies, the shorter the distances between them and the higher the density of the map.

Information adapted from Fritz *et al.*, 1995 b.

When those primers, each representing separate loci, were found, Tree #33 DNA was inferred to be heterozygous and the cross with the homozygous recessive Catongo locus should give a 1:1 segregation (Fritz *et al.*, 1995b).

This behavior was obtained in only 104 primers. Electrophoretic patterns of amplified DNA were tested for each of those primers in 137 backcross trees, and linkage was determined by comparing the number of amplified bands they had in common. Differences were attributed to recombination, and their appearance frequency represented the map distance between both markers (Figure 19) (Fritz *et al.*, 1995b; Osei *et al.*, 1993).

In addition to those 104 RAPD markers, the GLM generated for the backcross population also included 32 RFLP markers and two phenotypic loci[7]. These 138 markers were statistically organized into ten linkage groups[8], and covered 1,068 cM of the Cacao Genome (which according to diverse theoretical estimates, ranges between 1,078 and 1,112 cM). Average distance between adjacent markers was 8.3 cM. (Crouzillat *et al.*, 1996). Figure 20 shows the relative position of those markers in the different Cacao chromosomes.

Finally, only 6 of those markers showed a significant deviation from the expected 1:1 segregation ratio. Therefore, it can be concluded that 25% of the alleles in the backcross population had a paternal origin, while the remaining 75 % were due to the recurrent parent (Catongo) (Crouzillat *et al.*, 1996).

This kind of map made it possible to determine which loci are related to quantitative characteristics (QTL's), and to further identify, isolate and manipulate desirable genes.

[7] The backcross progeny apparently segregates for 2 simple-gene loci, both on chromosome #4: one controls Anthocyanin synthesis in seeds, leaves and flowers, and the other controls Self Compatibility. White color in Catongo would be recessive, while purple would be dominant and homozygous in Pound 12. Thus, all F1 hybrids were expected to be purple and heterozygous, while the backcross should produce a 1:1 segregation ratio. On the other hand, auto-compatibility in Catongo would be heterozygous, while auto-incompatibility would be recessive in Pound 12, so that the F1 and the backcross populations should present 1:1 and 3:1 segregation ratios respectively (Crouzillat *et al.*, 1995 & 1996; Fritz *et al.*, 1994b & 1995b; Morera *et al.*, 1993a).

[8] Corresponding, as expected, to the haploid number of chromosomes in Cacao (n=10).

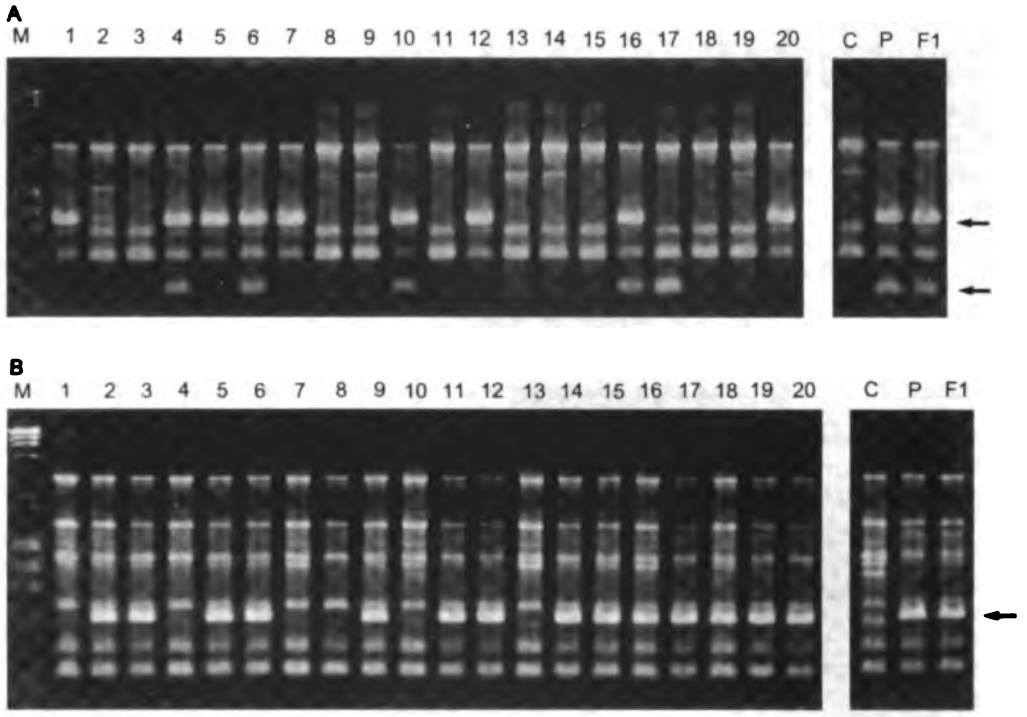

Figure 19. RAPD segregation patterns of backcross trees (1-20), Catongo (C), Pound 12 (p) and the F1 tree used for backcroos with Catongo for the primers AM10 (A) and L03 (B). Numbers 1-20 represent a sample of segregating backcroos trees. Scorable RAPD's are marked with an arrow. (*From Crouzillat* et al., *1996 and reproduced with permission* ofTheoretical and Applied Genetics).

Phenotypic Analyses

Once obtained, linkage maps are useful tools for locating certain chromosomal areas associated with morphological and agronomic traits. A complementary work for the GLM study described, was undertaken to identify the phenotypic characteristics of all populations involved (Catongo and Pound 12 parents, F1 tree #33, Backcross population). The study assessed the relationship between genetic markers and desirable agronomic characteristics: if any markers were found to cosegregate with genes affecting the traits of interest, DNA analysis could be used to screen materials, expediting cacao breeding (Fritz *et al.*, 1995b).

The list of monitored characteristics included the following traits (Fritz *et al.*, 1995 a, b & c; Morera *et al.*, 1993 a & b; 1995; Mora *et al.*, 1993):

- Early flowering and phenological patterns
- Flower, flush and seed color
- Self-compatibility
- Growth rate
- Yields
- Seed and Pod indexes

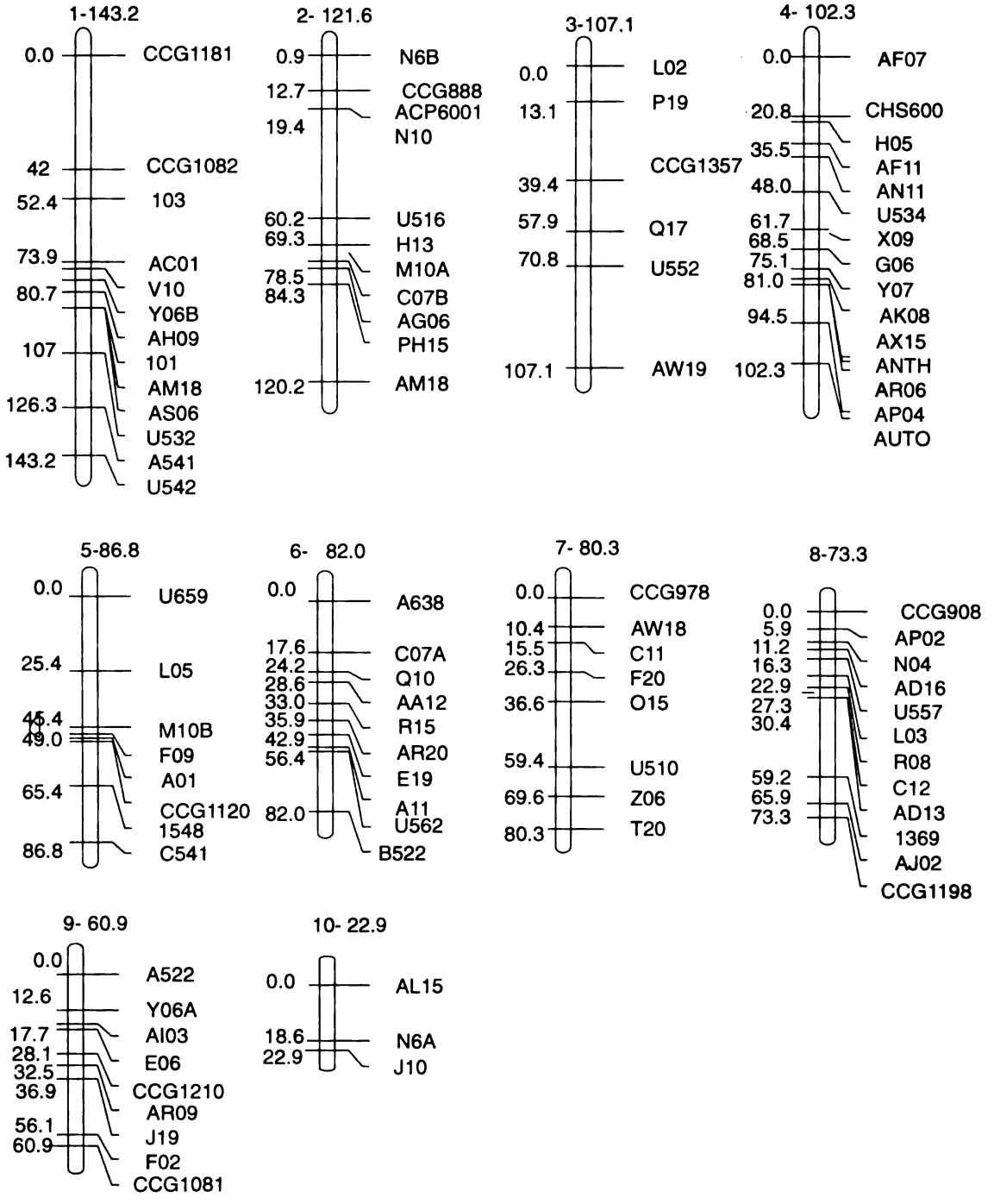

Figure 20. Genetic linkage map for a cacao backcroos population (Catongo x (Catongo x Pound 12)) obtained at CATIE.

- Biochemical composition of seeds
- Chocolate taste and aroma
- Resistance to pests and diseases

Results from the **phenotypic analysis of the F1 hybrids** were summarized by Morera *et al.* (1993, a & b; 1994), and by Mora *et al.* (1993). Incompatibility occurred in 50% of the F1 population. Self-compatibles had more pods and better dry weight yields. Yields were also highly variable, with averages ranging from 250 to 2,500 kg/ha/year over 15 years (Figure 21). The hybrids performed better than the parents in all yield characteristics except for the number of seeds per pod. Nevertheless, only 34% of the hybrids presented yields over 1.5 kg/tree/year. Variation in the species utilized as shade for cacao had no significant effects on yield. Other growth parameters such as tree height, and stem diameter were highly variable. Significant and negative correlation was found between pod index and dry bean weight per pod, making it possible to use the former for breeding plants with an acceptable fruiting display. Finally, florescence in the hybrids appeared as sensitive to rain distribution, while the same trait was very stable in the parental material.

A **phenotypic analysis of the backcross population** was reported by Fritz *et al.* (1994a). It showed that all but 7% of 137 backcross trees were flowering in the field by year 3 (Figure 22), though only 36.7% were bearing pods (Figure 23). Among those pod-bearing individuals, the amount of pods produced per tree, by year 3, ranged from 1 to 40. Eleven trees were judged to be early flowering and 13 to be early pod-bearing. Nevertheless, only six individuals were early in both flowering and pod bearing. Of these, only one tree produced high yields. Table 8 provides averages and ranges of vigor indicators obtained for all backcross trees after two years in the field (Figures 25-28).

TABLE 8. Average and ranges for diverse vigor indicators obtained from 137 cacao backcross trees (Catongo x (Catongo x Pound 12)) after 2 years in the field.

INDICATOR	AVERAGE	RANGE
Stem diameter (30 cm above ground) (cm)	6.0	3.0 - 9.2
Jorquette height (cm)	99.0	45.0 - 99.2
Leaf area (sq. cm)	438	165 - 750
Ovule number per flower (#)	49.2	43.9 - 55.4

In this study, a high correlation was found between yield and stem diameter, which accounted for 30% of the variability in yields. A 3:1 ratio was found regarding auto-compatibility and a 1:1 ratio for seed & flush color as expected from their single gene determinism (Fritz *et al.*, 1994a).

In addition, the reaction of the F1 and the backcross populations to Black Pod (*P. palmivora*) was assessed in order to determine segregation patterns of this trait, as the basis for further QTL analysis (discussed below).

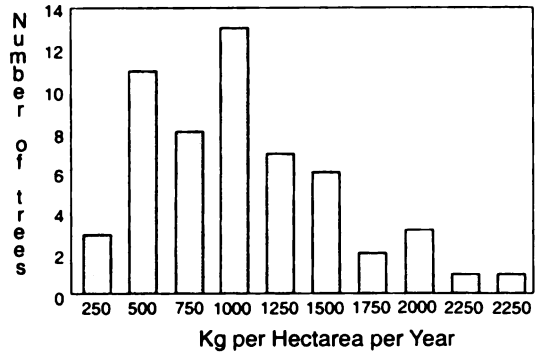

Figure 21. Frequency distribution for average production over 15 years of Catongo X Pound 12 hybrids under *Cordia* and *Erythrina* shade.

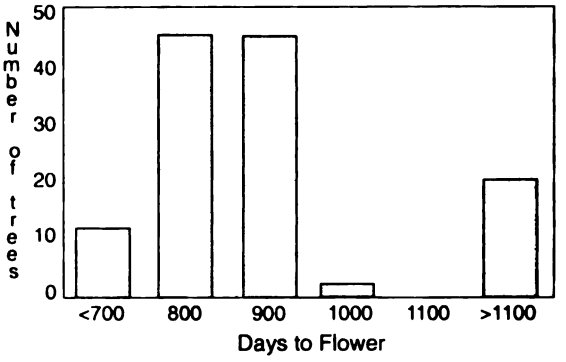

Figure 22. Frequency distribution for days-to-flower in cacao backcross trees (Catongo X (Catongo X Pound 12)).

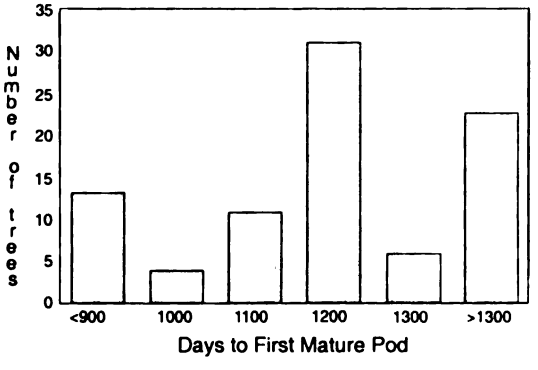

Figure 23. Frequency distribution for number of days from germination to first mature pod in cacao backcross trees (Catongo X (Catongo X Pound 12)).

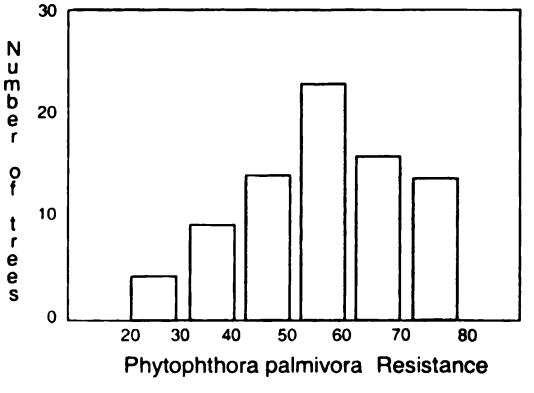

Figure 24. Frequency distribution for *P. palmivora* resistance in cacao backcross trees (Catongo X (Catongo X Pound 12)).

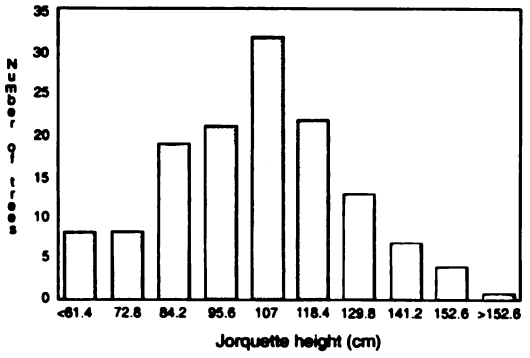

Figure 25. Frequency distribution for height at first jorquette in cacao backcross trees (Catongo X (Catongo X Pound 12)).

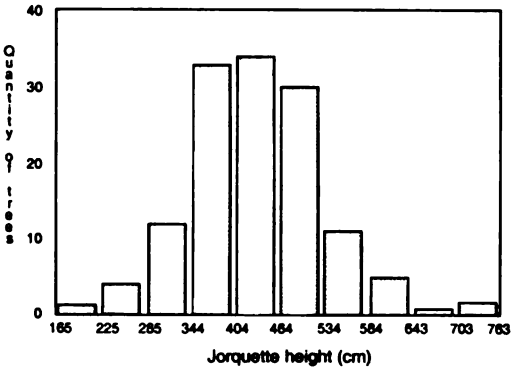

Figure 26. Frequency distribution for leaf area in 3-years-old cacao backcross trees (Catongo X (Catongo X Pound 12)).

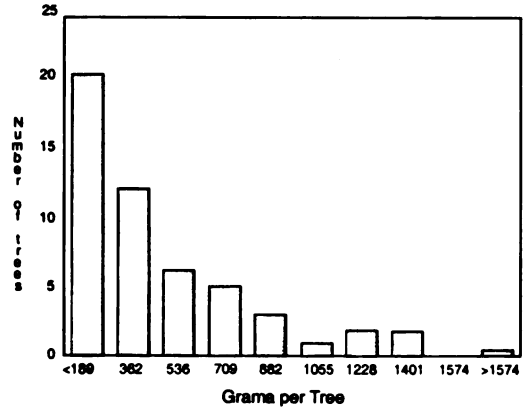

Figure 27. Frequency distribution for dry weight of cacao beans per tree in 3-years-old cacao backcross trees (Catongo X (Catongo X Pound 12)).

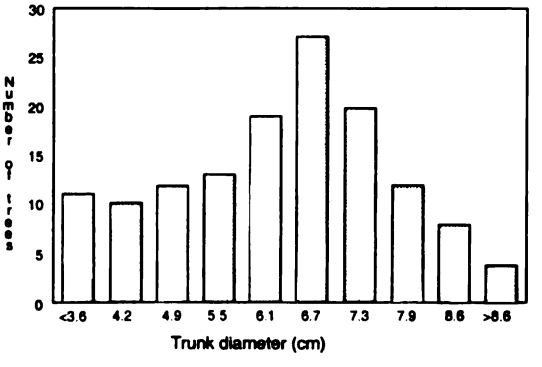

Figure 28. Frequency distribution for stem diameter in 3-years-old cacao backcross trees (Catongo X (Catongo X Pound 12)).

The same process was recently initiated to test for Moniliasis (*M. roreri*) resistance. To date, a total of 110 backcross trees (69% of the backcross population) have been tested for their resistance to *M. roreri*; only 1% showed moderate resistance and another 2% resulted moderately susceptible. Existence of segregation is a very positive finding, as it will make it possible to conduct QTL analysis for Moniliasis resistance[9] (Phillips-Mora & Castillo, 1999).

Determination of Quantitative Trait Loci (QTL)

Once numerous phenotypic and molecular marker data have been obtained, the correlation between them is established by analyzing variance, and by using unpaired T-tests with diverse statistical packages, in order to detect chromosomal areas associated with phenotypic traits (Phillips-Mora & Fritz, 1995). For example, in the case of cacao leaf area, DNA from 62 backcross trees was homozygous (band absent) when used in the PCR with primer O15, while 68 backcross trees were heterozygous (band present). Average leaf area for the 62 homozygous trees was 411.8 cm^2, and 460.7 cm^2 for the heterozygous ones. The difference of 48.9 cm^2 was statistically significant, allowing the conclusion that there is a site on chromosome 7, close to RAPD marker O15, that contains a gene which encodes a protein that plays a role in determining leaf area[10]. Four other loci related to leaf area were found in the backcross population (Fritz *et al.*, 1995 b).

With this information at hand, the previously discussed cacao GLM was utilized to localize QTL for traits like early flowering, stem diameter, jorquette height and ovule number in the backcross generation, using both single-step ANOVA and interval mapping (Crouzillat *et al.*, 1995).

BOX 6: What are QTL?

In nature, most observable traits that reveal genetic variation are determined by groups of genes functioning in concert to establish such characteristics as yield, vigor, height, etc. These features of polygenic inheritance are called quantitative traits, given the additive action of all loci related to each of those traits. The individual loci controlling a quantitative trait are referred to as polygenes or Quantitative Trait Loci (QTL). Their phenotypic expression is likely to be affected by the environment, showing a continuous rather than discrete variation, whose frequency distribution typically renders bell-shaped curves.

QTL analysis is founded on the idea that if phenotypic traits co-segregate with molecular markers, it is likely that genes associated to those traits will also be transmitted in association with markers. Once marker data have been assembled in a Genetic Linkage Map, comparisons between inheritance of markers and phenotypic traits of interest should allow detection of chromosómal areas related to the quantitative trait.

QTL's are useful in Plant Breeding because they can serve as markers for selection at the seedling stage of plant development, and also because they facilitate the identification of genes associated with a particular trait, thus providing a means for understanding biochemical processes responsible for yield, disease resistance and any other agronomic trait.

Adapted from Tanksley, 1993 and Fritz *et al.*, 1995 b.

[9] Also, a segregating population made up of 260 trees of the Pound-7 x UF-273 cross was established recently to study segregation of Moniliasis and black pod resistance, given the contrast between both genitors (Moniliasis resistance, black pod susceptibility, red fruits and self-compatibility in UF-273; moniliasis susceptibility, black pod resistance, green fruits and auto-incompatibility in Pound-7). Phenotypic data have already been collected for 3 semesters and are being processed (Phillips-Mora, 1999a).

[10] Nevertheless the role of this gene is not very determinant, since the percent of variance in leaf size due to this site was only 6.4.

Table 9 summarizes the different QTL's identified as related to each of the analyzed traits of the cacao backcross population, also indicating the amount of variance in that trait which was explained by the QTL. The relative position of those QTL's in the Cacao Genetic Linkage Map is presented in Figure 29.

A minimum number of 3 to 4 QTL ($P < 0.01$) involved in the genetic expression of the studied traits was detected. The combined estimated effects of the diverse QTL mapped explained from 11.2 % to 25.8% of the total phenotypic variance observed in the backcross population. Besides, coincident map locations for jorquette height and stem diameter suggested the possibility of pleiotropic effects for both traits (Crouzillat *et al.*, 1996).

TABLE 9. Summary of QTL for the cacao backcross population, Catongo x (Catongo x Pound 12) obtained at CATIE.

TRAIT	NUMBER OF QTL	QTL on Chromosomes	PERCENT PHENOTYPIC VARIANCE		
			MAXIMUM	MINIMUM	TOTAL
First flowering	6	1,2,4,5,6	9.2	3.1	40
Ovule number	3	3,6,7	8.6	4.7	29
Pods/tree/year	9	2,4,5,7,8,9	14.7	3.0	50
Seed dry weight/tree/year	7	2,4,5,6,7,8	7.8	4.0	40
Jorquette height	3	1,4	22.2	6.3	47
Stem diameter	6	1,2,3,4,9	7.0	2.8	25
Maximum growth rates	5	1,3,5,7	4.5	3.1	18
Leaf area	5	2,5,7,8	12.5	2.9	34
Caffeine/seed	4	3,7,8	14.1	7.3	38
Theobromine/seed	4	2,8,9	10.9	7.1	40
P. palmivora resistance	3	5,8	23.9	7.1	43
Seeds/pod	7	2,4,5,6,8,9	10.6	5.0	57

Source: Fritz *et al.*, 1995b; Crouzillat *et al.*, 1995.
Correlation of QTL with markers listed at a 99% confidence level.

QTL's were also obtained for the Catongo x Pound 12 interclonal cross. Some of them are reported in Table 10 (Fritz *et al.*, 1995 b).

TABLE 10. Summary of QTL for cacao the cacao F1 population (Catongo x Pound12).

TRAIT	NUMBER OF QTL	QTL on Chromosomes	PERCENT PHENOTYPIC VARIANCE		
			MAXIMUM	MINIMUM	TOTAL
Mean Yield	5	1,3,4,8,10	18.3	7.0	58.5
Stem Diameter	7	1,4,7,8,10	12.8	7.4	73.1
Leaf Area	4	1,4,10	11.2	7.2	35.9
No. Seeds per Pod	3	4,9,10	26.5	7.3	46.2
P. palmivora resistance	6	1,2,3,4,6	29.3	11.7	100
Jorquette height	2	4,7	16.4	13.9	30.3

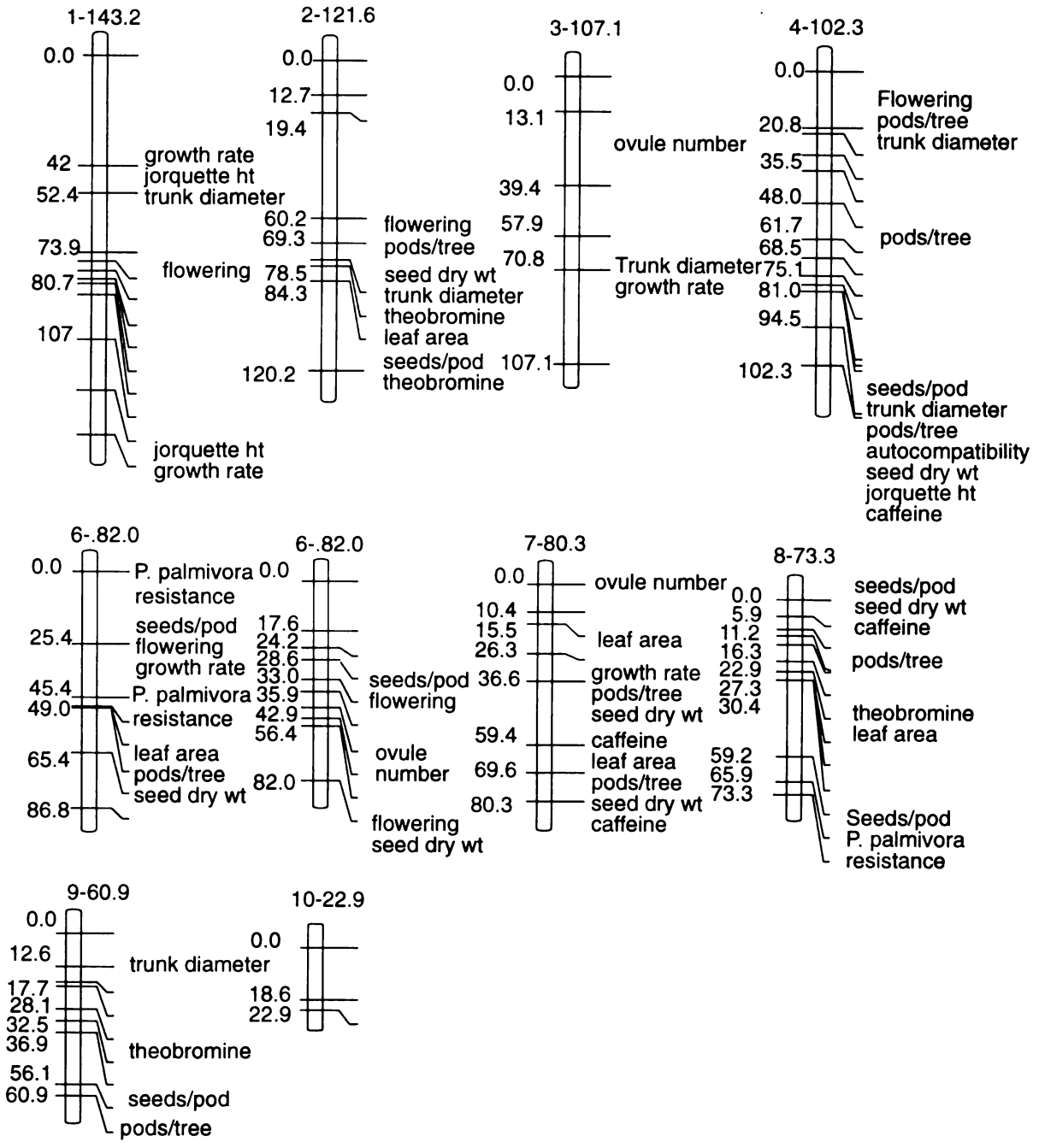

Figure 29. Linkage map for quantitative trait loci in a cacao backcross population (Catongo x (Catongo x Pound 12)).

QTL's for resistance to *Phytophthora palmivora*

The identification of resistance to Black Pod (*P. palmivora*) has been a major goal of cacao breeding efforts for more than a decade. This fungus is considered the most significant cacao disease worldwide, accounting for up to 30% of world harvest losses every year, *i.e.* more than US$ 1 billion/year. Resistance to this fungus appears to be a quantitative trait, since no complete resistance has been found to date. Additionally, no major resistance genes have been identified even when high resistance levels are present in some varieties. Most authors agree that resistance to *P. palmivora* in cacao is provided by various additive genes (Fritz *et al.*, 1995b; Phillips-Mora *et al.*, 1996; Phillips-Mora, 1999b; Crouzillat *et al.*, 1999a)

Slow cacao growth and the need for sufficient pods to evaluate resistance represent a heavy liability for evaluation programs, which require up to ten years to be completed. The use of molecular markers opened possibilities for the selection of resistant genotypes from the seedling stage.

A detailed assessment of the inheritance of resistance to Black Pod in cacao was undertaken by means of QTL in cooperation with FRANCERECO, in 1994. Working with the (Catongo x Pound 12) F1 and the (Catongo x (Catongo x Pound 12)) backcross populations and utilizing the GLM's described above, six loci related to *Phytophthora* resistance were identified on five different linkage groups. These results represented the first molecular-level confirmation of horizontal/polygenic resistance to *P. palmivora* in cacao (Fritz *et al.*, 1995b; Phillips-Mora & Fritz, 1996; Phillips-Mora *et al.*, 1996; Crouzillat *et al.*, 1999a).

Research for this study involved three stages (Phillips-Mora *et al.*, 1996; Phillips-Mora & Crouzillat, 1999):

- Molecular marker analysis of a sample comprising the 137 backcross trees plus their parental materials. Utilizing more than 1,000 RAPD primers from which 15% revealed polymorphisms, plus 21 RFLP probes, a medium-density GLM with 139 loci identified and covering 914 cM divided in 10 linkage groups was generated.
- Field testing of resistance to *P. palmivora* on 131 backcross trees, utilizing a methodology developed at CATIE, first described by Phillips-Mora & Galindo (1989) and improved by Phillips-Mora (1996b).
- Data integration, analysis of variance and unpaired T-testing to determine inheritance patterns for the diverse markers and for resistance characteristics, in order to define the presence of QTL.

Resistance was estimated using the aforementioned inoculation method on more than 1,600 pods from 79 backcross and 55 F1 trees. The trees were inoculated on more than 20 occasions. Initially, lesion size was estimated as the sum of lesion width and length, and was measured 5 and 10 days after inoculation. The "area under the curve" (AUC) when plotting time *vs.* lesion size was utilized at that time as a proxy for resistance. AUC ranged from 25.3 to 78.8 cm^2 in the backcross population. Averages for Catongo, Pound 12 and the F1 Tree #33 were 53.0, 60.8 and 51.9 cm^2 respectively (Fritz *et al.*, 1995b).

The total phenotypic variance accounted for by the five QTL obtained for resistance to Black Pod was about 63% (Fritz *et al.*, 1995b; Phillips-Mora *et al.*, 1996). Individual QTL explained from 7.4 to 47.9% of the total variance, indicating that genetic factors of major and minor effects were involved in the control of this trait.

More recently, lesion diameter 10 days after inoculation has been utilized to qualify cacao reaction to *P. palmivora*, with **resistant** clones ranging from 0 to 3 cm, **moderately resistant** from 3-6 cm, **moderately susceptible** from 6-9 cm, and **susceptible** over 9 cm. Both Catongo and Pound 12, as well as the F1 tree #33 were susceptible according to this scale (12.8, 14.0 and 13.6 cm respectively). The F1 individuals showed, on average, higher resistance levels than the backcross population (Crouzillat *et al.*, 1999 a).

It was evidenced in these studies that two of the alleles conferring resistance came from Catongo, while the other 4 were provided by Pound 12, despite their own susceptibility. Thus, these findings suggest the presence of transgressive traits and the possibilities of using susceptible plants as sources of variability for disease resistance selection (Crouzillat *et al.*, 1999a).

Additionally, only one of the QTL was found in both the F1 and the backcross progenies, and appeared to be a major component of disease resistance as it explained 48% of the phenotypic variation in the F1 cross (though only 11-12% in the backcross). These results support the idea that the genetic background could regulate the expression of a given QTL depending on gene combinations (Crouzillat *et al.*, 1999a).

QTL's for cacao yield

In a recent study aimed at determining the genetic components that control yield in the F1 cross, genetic maps for the two parents were developed. These maps detected 158 loci covering 772 cM for the heterozygous Pound 12 and only 4 loci representing 16.9 cM of a linkage group in the highly homozygous Catongo. Correlations with yield records over 15 years indicated 10 yield QTL in eight linkage groups. Two major QTL explained around 20% of total variance of average yield each, suggesting their usefulness in early yield selection. A very high correlation was obtained between pod number and yield, so that the former could be used to estimate yield. Finally, high correlation obtained between characteristics such as pod index and stem diameter, suggested that some areas of the genome might influence more than one yield component trait (Crouzillat *et al.*, 1999b).

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CHAPTER THREE
BANANAS & PLANTAINS

CONSERVATION.

Increasing Preserved Stocks and Improving Preservation Methods

Field Collections

A small but valuable sample of *Musa* spp. genotypes has been preserved by CATIE in its field collections for years. As of January, 1999, the collection encompassed a total of 48 introductions (represented by various numbers of individuals) and included the following *Musa* species: (Mora, A. 1999. *Pers. Comm.*).

TABLE 11. Number of accessions of *Musa* genotypes preserved in CATIE's field collections as of January, 1999.

Species	# Accessions
M. acuminata	1
M. balbisiana	2
M. beccarii	1
M. paradisiaca	3
M. velutina	2
M. spp.	39
TOTAL	**48**

In vitro Conservation

Bananas and Plantains are seriously affected by a wide range of insects, and viral and fungal diseases. This fact prompted interest in preserving *Musa* germplasm, using methods capable of reducing maintenance costs from those incurred by field collections, while ensuring a wide genetic base for future plant breeding (Abdelnour-Esquivel *et al.*, 1992).

BOX 7: The Genus *Musa*

Musa (family Musaceae) is a genus that includes the largest herbaceous plants known, with some wild species reaching up to 15 m in height. The family includes species utilized as ornamentals and as raw materials in the textile industry. Nevertheless, the genus is mostly known for its importance as fruit crops. Sweet and cooking bananas are the second largest fruit crop in the world after *Citrus*. Edible cultivars are primarily grown for local consumption and play an important role in human nutrition in developing countries. Most of those cultivars are grown by small farmers, and types of fruit differ widely from one region to another; 7.4 million tons of Cavendish bananas are exported to developed countries each year, yet this figure represents a scant 10% of total world production.

Table B7.1. Annual World Banana Production in 1998 (Based on FAOSTAT).

Type of fruit	**Genotype**	**Millions of tons**	**Production areas**
Highland bananas	AAA	7.0	East/Central Africa
Cavendish bananas	AAA	34.7	Latin America, Asia,
Pacific Islands Plantains	AAB	17.2	West/Central Africa and Latin America
Cooking bananas	ABB	12.1	Asia, Pacific Islands, East Africa
Other desert bananas	AA, AAB	14.3	Latin America, Asia, Pacific Islands

The genetic background of cultivated *Musa* is highly complex due to the involvement of different wild seminiferous species and subspecies, and also to the spontaneous occurrence of many triploids caused by anomalies during meiosis. Classification is further complicated by the large number of material exchanges that occurred during the early domestication of *Musa*, and by vegetative multiplication which allows immediate fixation of mutants of agronomic interest. The most frequent mutations concern height (dwarfism), plant color, and bunch and fruit characters.

Two wild diploid species (2n = 2x = 22) are the main components of cultivated bananas, namely *M. acuminata* and *M. balbisiana*. Both species are originally from Southern Asia. A taxonomic scoring system proposed by Simmonds & Shepherd (1955[1]) is utilized to classify all cultivated bananas. If an *acuminata* genome is designed A, and B is used for *balbisiana* genomes, all edible diploids are AA (*e.g.* 'Lady Finger'), whereas triploids, which make up the majority of cultivars, are mainly AAA (originally from Malaysia; *e.g.* 'Gros Michel', 'Cavendish', 'Grand Nain'), AAB (originally from India and diversified in Africa; *e.g.* 'Dominico', 'Currare') or ABB (originally from India and diversified in the Philippines; *e.g.* 'Bluggoe' & 'Pelipita'). There are also cultivated tetraploids and diploid hybrids (*e.g.* AB 'Ney Poovan', cultivated in India).

Edibility evolved with development of parthenocarpy in *M. acuminata*, together with sterility (a trait due to *Musa* genetic characteristics and reinforced by poliploidy). Both factors conferred *Musa* with the major commercial characteristic of producing seedless fruits which develop with no need for pollination. The nutritional value and physical characteristics of Bananas for consumption, as well as the existence of diverse *Musa* ecotypes adapted to very different environments, and their easy vegetative reproduction, paved the way for *Musa's* widespread production and consumption.

Adapted from Teisson & Côte, 1996; León, 2000.

[1] Simmonds, N.W.; Shepherd, K. 1955. *J. Linn. Soc. Lond. Bot.* 55: 302-312.

Studies undertaken at CATIE by Pocasangre (1992) demonstrated that *Musa* materials could be preserved *in vitro* for up to six months in a 15°C environment. Explants of *Musa* AAB cv 'Currare' and *Musa* AAA cv 'Grand Nain' were placed on a media containing diverse concentrations of sucrose, mannitol and tri-iodine-benzoic acid (TIBA). Best results were obtained with 30 g/l sucrose + 0.2 M mannitol + 5 mg/l TIBA. These results represented an important step toward developing practical methods for maintaining and mobilizing large quantities of *in-vitro* produced materials while maintaining their viability.

Taking advantage of these accomplishments, an agreement was established with INIBAP in 1998 to maintain a back-up *in vitro* collection of *Musa* germplasm at CATIE. A total of 162 accessions collected worldwide by INIBAP have been preserved in incubation chambers (15°C) and have undergone 11 multiplication cycles since December, 1998, utilizing conventional *in vitro* methods (meristem culture).

Cryopreservation

The long-term preservation of *Musa* germplasm, with the guarantee of its genetic stability, was effectively achieved at CATIE after using cryopreservation methods.

Cryopreservation of *Musa* **zygotic embryos** was successfully carried out with mature embryos excised from disinfected fruits of the fertile diploid species *M. acuminata* spp. *burmanicoides* and *M. balbisiana*. Dehydration of the embryos (2 hours under sterile air stream) was essential to ensure their tolerance of direct immersion in Liquid Nitrogen (LN) and to guarantee their survival afterward. Best recovery rates (around 85%) were obtained when embryos presented moisture contents between 14 and 20% (Abdelnour-Esquivel, 1993; Abdelnour-Esquivel *et al.*, 1992).

The same researchers were able to cryopreserve **somatic embryos** derived from male flowers of *Musa* AAA cv 'Grand Nain' utilizing a method described by Escalant *et al.* (1994 a). Desiccation with sterile air was utilized in all cases.

Best results (100% recovery) were obtained when somatic embryos received a cryoprotective treatment (incubation in a 0.5 M sucrose + 5% DMSO medium for 1-3 days), followed by a slow precooling treatment (-1°C/min down to –40°C) before immersion in LN. Sixty percent of those embryos germinated directly, after placing them in the recovery media (MS + 0.1 M sucrose + 0.5 g/l BAP + 2 mg/l IAA). The remaining 40% formed viable calluses which were recovered later (Abdelnour-Esquivel *et al.*, 1994).

It is important to note that when the precooling cryoprotection treatment was omitted, the survival rate decreased to 75%. Of the surviving embryos, only 50% germinated directly. Finally, using direct LN immersion (neither cryoprotection nor pre-cooling) reduced the survival rate to 40%, however, all embryos preserved in this manner germinated directly (Abdelnour-Esquivel *et al.*, 1994).

More recently, **embryogenic cellular suspensions** of *Musa* were successfully utilized in cryopreservation trials (Yah, 1998; Yah *et al.*, 1999). A cryopreservation protocol first defined by Panis *et al* (1990) for this type of materials was modified utilizing cellular suspensions obtained from immature flowers of the *Musa* AAB cv. 'Dominico' through methodologies generated at CATIE (Escalant *et al.*, 1994 a; Grapin *et al.*, 1998). Three of the four stages defined by the cited protocol were refined as follows:

- Crystallization induction was essential to cellular regeneration and embryo germination after cryotreatments (fig. 30)
- Optimum sucrose concentrations for pre-treatment conditions were found to be between 0.26 and 0.39 M (fig. 31)
- The pre-culture stage was more effective when utilizing sucrose (0.39 M) instead of lactose for seven days.
- Cellular recovery was more effective in solid media with high density plating

The modified protocol was then applied to embryogenic cellular suspensions of 5 different *Musa* cultivars (Gros Michel 3 (AAA), Dominico 1 and Currare 3 (AAB), SF265 and Col 49 2.8 (AA)). The last four withstood cryopreservation. Suspensions recovered after cryopreservation showed satisfactory viability, according to fluoresceine-diacetate (FDA) tests conducted 48 hours later (Yah, 1998; Yah *et al.*, 1999).

Regeneration of somatic embryos from suspensions recovered in this manner was also assessed. After seven weeks in embryo-inducing media, somatic embryos were observed (Figure 32). Regeneration efficiency in cryopreserved suspensions varied from 0 to 26% as compared to control tests, and differed according to genotypes (*e.g.* higher efficiency rates were obtained in cv. Col 49 2.8, which presented numerous proembryos in suspension). Regenerated embryos were successfully germinated, and germination was improved by crystallization treatments (fig. 33) (Yah, 1998; Yah *et al.*, 1999).

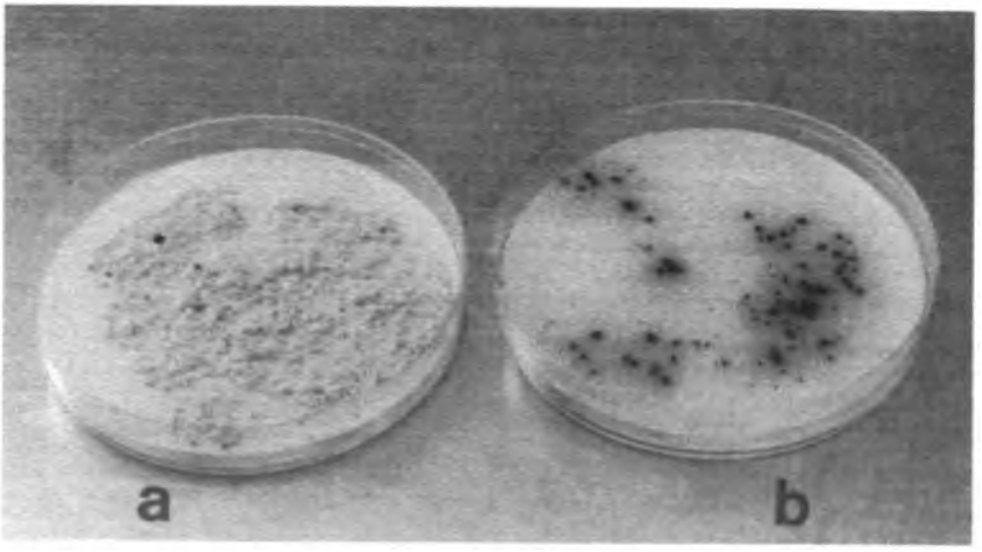

Figure 30. Somatic embryos obtained from cryopreserved cellular suspensions of *Musa* AAB cv 'Dominico'; a) with, and b) without crystallization induction.

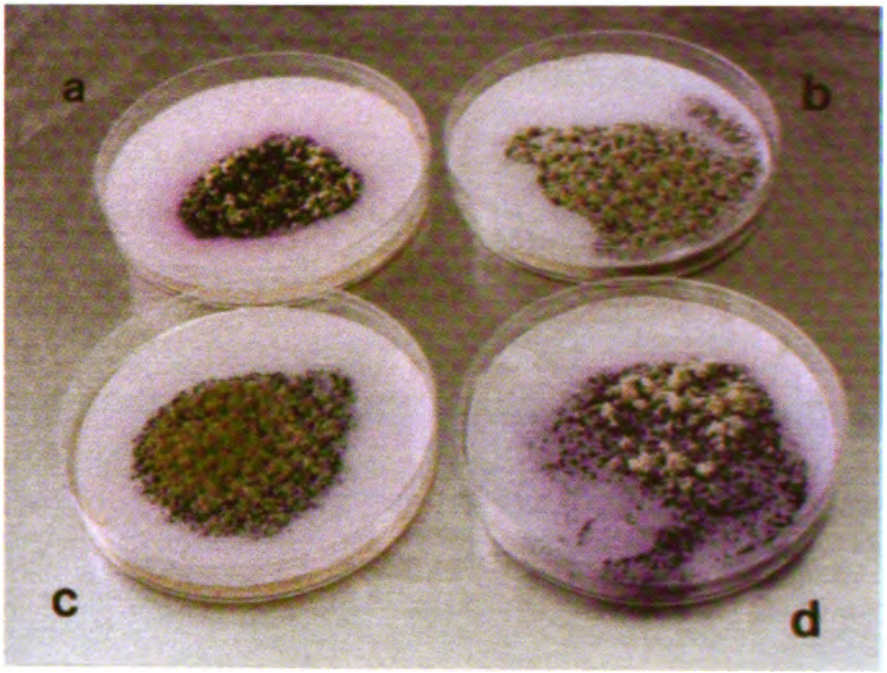

Figure 31. Somatic embryos obtained from cryopreserved cellular suspensions of *Musa* AAB cv. 'Dominico', pretreated with different sucrose concentrations. a) 0.13 M; b) 0.26 M; c) 0.39 M; d) 0.53 M

These results highlighted the importance of cryopreservation as a viable technique for the long-term conservation of *Musa* germplasm, mainly for preserving *in vitro* generated materials which are prone to show genetic variation and lose their regenerative potential.

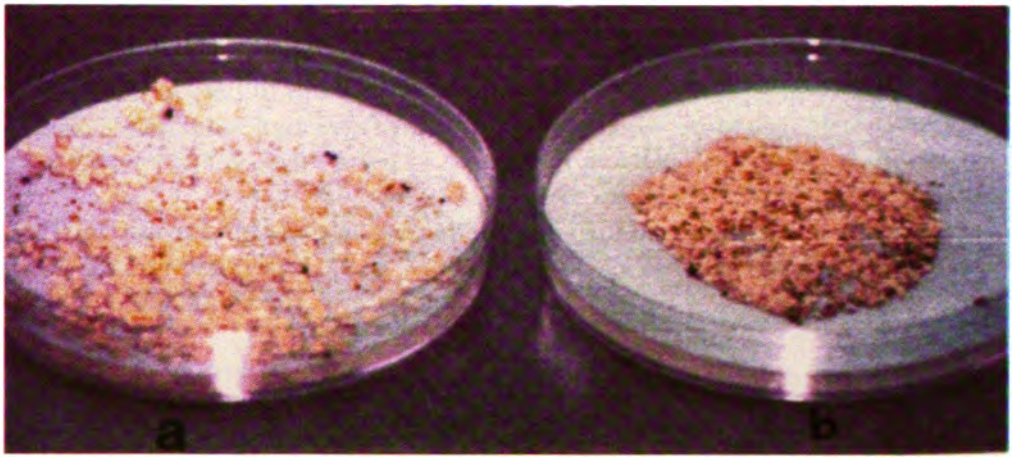

Figure 32. Somatic embryos obtained from cryopreserved cellular suspensions of *Musa acuminata* cv. Col '49 2.8. a) Control; b) Cryopreserved treatment

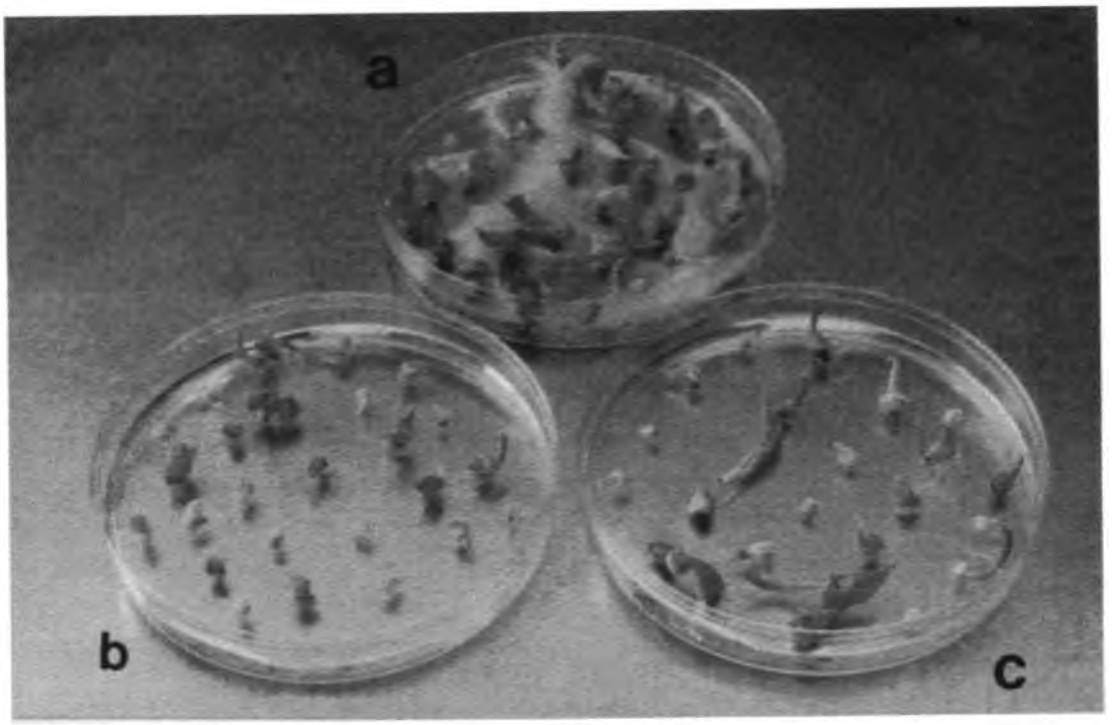

Figure 33. Germination of somatic embryos obtained from cryopreserved cellular suspensions of *Musa* AAB cv. 'Dominico'. A) Control; b) without crystallization induction; c) with crystallization induction.

GENETIC IMPROVEMENT.

Developing and Evaluating Promising Materials

Three major strategies to overcome the major constraints in banana production (*i.e.* pests, diseases and inferior agronomic characteristics) have been identified in the literature since the 1980's (Panis *et al.*, 1996):

- selection and introduction of superior, existing cultivars from germplasm collections
- introduction of new clones obtained through classical breeding
- introduction of genetically engineered plants.

Nevertheless, genetic resistance to problems like viruses and nematodes has not been confirmed in *Musa*, thus limiting application of the first two options. To further complicate matters, classical breeding has been hampered by long generation time, sterility and the polyploidy characteristics present in most edible cultivars. Finally, the lack of reliable *in vitro* methodologies for genetic transformation also limited development of the third option listed (*ibid*).

Thus, the most important advances in *Musa* breeding in the future will have to rely more on unconventional pathways. CATIE's efforts and achievements in the quest for innovative methods to improve *Musa* materials during the 1990's are reported in the following chapter.

Genetic Transformation

Genetic engineering tools were proposed during the early 1990's as valuable tools to further *Musa* breeding efforts at CATIE. Transformation techniques such as protoplast culture and electroporation were impeded by difficulties in protoplast regeneration, and *Agrobacterium*-mediated transformation was hindered at that time by low infection rates of *Musa* tissues. The availability of efficient callus induction and regeneration methods led to the use of biolistic[1] transformation (Box 7).

The transient expression of the *GUS* gene in banana cells as a result of genetic transformation experiments was first reported in the literature in 1993. A few months later, similar results were obtained at CATIE's Biotechnology Lab by utilizing biolistics on embryogenic cell suspensions and somatic embryos (Figures 34 & 35).

Four major research actions were implemented to develop a genetic transformation method of *Musa* (AAA cvs. 'Grand Nain' and 'Gros Michel'; AAB cv. 'French Sombre') at CATIE. They included (Escalant & Rabot, 1996):

- selection of target tissues suited to these techniques
- optimization of bombardment conditions
- selection of promoter sequences
- generation of effective agents to select transformed cells

[1] Biolistics, a generic term used in genetic engineering as synonym for particle bombardment, derived from "Biolistic", a trademark of E. I. du Pont de Nemours & Co. which describes a commercially distributed particle bombardment device.

BOX 8: What is DNA biolistic delivery?

Genetic Engineering comprises a whole set of biotechnological techniques aimed at inserting genes into individuals, in order to change their characteristics in a useful manner. In Agriculture, these techniques have already allowed the generation of transgenic crops, changing crop attributes faster and beyond the limits of classical breeding.

Gene addition to individuals is achieved through diverse methods. The use of *Agrobacterium tumefasciens* represents the best available procedure: this bacteria transfers and integrates the T-DNA region of its Ti plasmid into the recipient genome. However, its use is very specific to dicotyledoneous species and is severely limited in other crops. To overcome those restrictions, physical methods for gene transformation have been developed, including DNA uptake by protoplasts through electroporation, or mechanical introduction of DNA into the cell by microinjection or high-speed microprojectile bombardment.

Utilizing a particle gun, the biolistic method delivers DNA packages or Plasmids (comprising the desired gene plus a promoter, an enhancer, and a terminator) coated on a microcarrier –a metal particle, generally tungsten-, into the target tissue. Target tissues usually include cell suspensions or ruptured somatic embryos.

Particles are first dropped into the ruptured membranes of target tissues, and then accelerated by the use of helium pressure under a slight vacuum, allowing them to reach the cell nuclei. During this acceleration step, the membrane is stopped by a retaining pore-plate which allows the further forward movement of the plasmids. Once they reach the nuclei, the DNA coating of Plasmids may be unloaded for its incorporation into the plant genome.

Stable transformation has not yet been obtained with this methodology; all Biolistic assays have yielded only transient expression of marker genes (such as GUS), but particle-mediated transformation may still constitute an intermediate technology for Genetic Engineering.

Adapted from CATIE, 1995; Gray and Finer, 1993.

Selecting target tissues

According to Panis *et al.* (1996), the main characteristic of a good *in vitro* system for supporting genetic transformation is one by which normal true-to-type plants can be regenerated from explant material at a high frequency. *Musa* tissues with such a potential include meristems, embryogenic cell suspensions, somatic embryos, and protoplast cultures.

Genetic transformation trials performed at CATIE utilized cell suspensions and somatic embryos as target tissues. Homogeneous pro-embryogenic masses (PEM) obtained from cell suspensions in exponential growth, were preferred, since most cells were totipotent and also because their ulterior regeneration proceeded smoothly through somatic embryogenesis, thus reducing chimerism risk during the selection of transgenic plants. The main flaws associated with this material were the time-consuming initiation process necessary and its difficult maintenance (see section C-1 below) (Panis *et al.*, 1996).

On the other hand, adventitious somatic embryos obtained from embryogenic cultures of male flowers at an early step of the exponential growth stage, also yielded acceptable results (Escalant *et al.*, 1995 a & b; Escalant & Rabot, 1996).

Figure 34. Particle acceleration device utilized at CATIE for biolistics experiments.

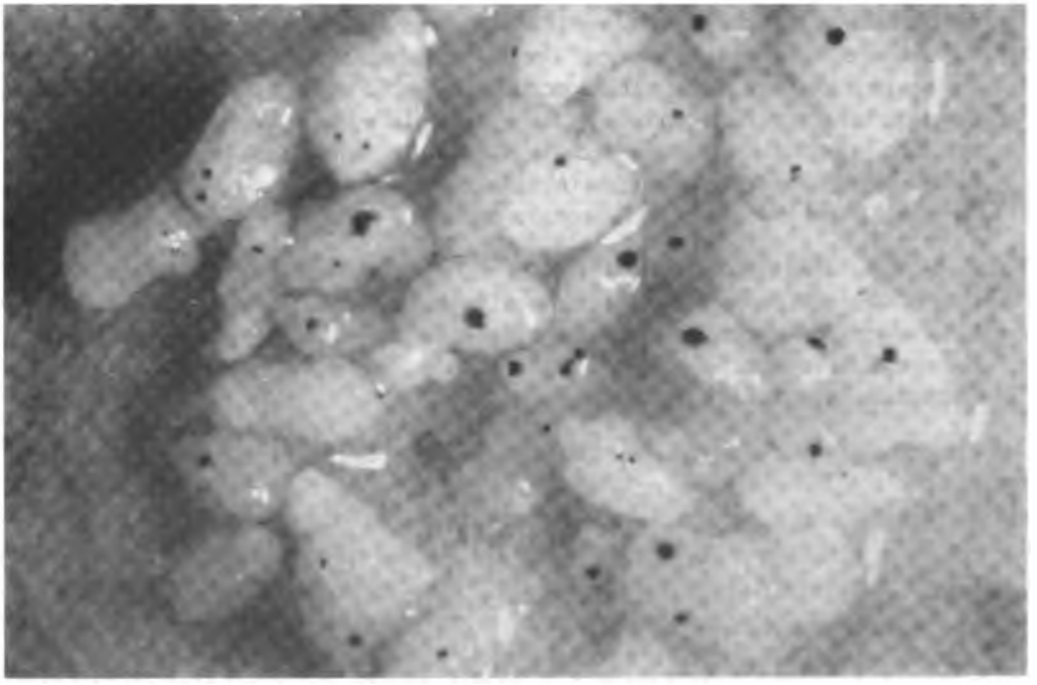

Figure 35. Stable GUS expression on somatic embryos of *Musa* AAA cv. 'Grand Nain', four weeks after bombardment with the enhanced 35 S promoter (pCaMV2).

Bombardment optimization

In order to improve biolistics results, a series of parameters including acceleration force, target distance, and vacuum conditions were refined in various experiments. The *gusA* gene (Box 1.3) was successfully utilized as a reporter to detect transient gene expression. Bombarded cells showed blue marks after X-Gluc staining, and embryogenic clusters turned blue two months after bombardment (Côte *et al.*, 1995 a; Panis *et al.*, 1996).

Best expression levels were obtained when target tissue growth was high and a high helium flow pressure was utilized. Less than 100 blue marks per shooting were obtained at 900 PSI while more than 1,200 marks appeared at 1,500 PSI. Expression levels of up to 4,000 marks per shooting were obtained, but it was found that an average of just over 1,000 was preferable (Côte *et al.*, 1995 a).

The result of bombardment on those tissues was a mixture of non-hit cells, lethally damaged cells, and cells where foreign DNA had entered. It was estimated that only a low percentage (0.5 – 1 %) of the transformed cells could become transgenic and maintain the introduced gene during cell division (Panis *et al.*, 1996).

Promoter sequences for Musa *transformation*

Plasmids introduced through biolistics generally contain the following sequences: i) a reporter gene for visual detection of transient expression; ii) a selectable marker coding for antibiotic or herbicide resistance; and iii) an agronomically useful gene. Those genes need to be preceded by a promoter sequence which regulates their *in vitro* expression (Panis *et al.*, 1996).

Experiments on transformation of **PEM** by Côte *et al.* (1995a) produced higher expression levels with plasmids pCa2GUS and pUGC1 utilizing the Ubiquitine promoter sequence.

However, Biolistics application at CATIE utilizing **somatic embryos** as target tissues, relied on the enhanced Cauliflower Mosaic Virus (CaMV) 35S promoter (or pCaMV2), and the Alfalfa Mosaic Virus (AMV) leader sequence to obtain the most effective expression of the *gusA* gene. Scores between 400 and 800 blue marks per shooting were recorded, with a stable expression obtained five weeks after transformation (Escalant *et al.*, 1995 a & b; Escalant & Rabot, 1996).

Selection of transformed cells

To select the transformed cells, a gene is introduced as part of the plasmid in order to make cells resistant to a selective agent (a herbicide or an antibiotic), so that only cells containing this gene could resist its action. As an example, in experiments with PEM, the most effective selective agents proved to be hygromycin (50 mg/l) and glufosinate (12 mg/l), requiring the introduction of the p*Bar* and the *hph* genes[2] as their respective selectable markers (Côte *et al.*, 1995 a; Panis *et al.*, 1996).

In CATIE experiments, where somatic embryos were utilized as target tissues, those embryos were placed in a media containing 5 mg/l glufosinate (BASTA®, a commercial herbicide) two

[2] PBar = a gene coding for phosphinotricin acetyltransferase; hph = gene coding for hygromycin phosphotransferase

weeks after bombardment, and were kept under the temporary immersion system for a period of two months. They were subsequently regenerated on a semisolid medium which also contained 5 mg/l BASTA. After this selection procedure, only 0.25% of the embryos were still capable of developing into plants (Escalant *et al.*, 1995 a & b; Escalant & Rabot, 1996).

Finally, regarding the agronomically useful genes to be included in plasmids, CATIE's objectives focused on increasing plant tolerance to fungal diseases and resistance to nematodes. It was suggested that in future research endeavors, the Chitinase gene (Chi2), a hydrolitic enzyme which digests fungal cell walls and occurs naturally in healthy plants, be introduced, as well as protease inhibitors, which inhibit gut proteinase of insects and nematodes.

Haplomethods

Obtaining homozygous plants from wild diploids is another alternative to overcome limitations faced by traditional improvement methods in *Musa* breeding. The utilization of haplomethods and tissue culture techniques should speed up this process (Escalant *et al.*, 1995 c).

During the 1990's, a protocol was defined at CATIE to obtain haploid plants from seeds fertilized by irradiated pollen in order to stimulate parthenogenetic development of the oosphere (Escalant *et al.*, 1994 b).

Gynogenesis induction was studied through interspecific crosses of irradiated pollen of *Musa balbisiana* type TANI and of ornamental species (*M. ornata, M becarii*) and ovules of *Musa acuminata* ssp. *burmannicoides* ('Calcutta 4') and *Musa acuminata* ssp. *malaccensis*. Pollen preserved more than 50% of its original germinative potential after irradiation with gamma rays (60Co), even when irradiation levels were as high as 1,000 gray. The germ tube was capable of growing and reaching the ovule at any of the irradiation rates used. Irradiated pollen presented unorganized nuclear structure. It was also found that the higher the irradiation rate, the higher the percentage of abnormal seeds formed (Escalant *et al.*, 1995 c; Moreno, 1993).

In vitro embryo rescue techniques applied from 90 to 120 days after fertilization to seeds lacking endosperm, allowed recovery of numerous plants. Biochemical analysis of 188 plants obtained this way generated no positive results in producing homozygous individuals. New induction rounds were proposed in order to continue refining the protocol (Escalant *et al.*, 1995 c; Moreno, 1993). Nevertheless, Teisson & Côte (1997) pointed out more recently, that gynogenesis trials have failed until now, while androgenesis has suceeded on *M. acuminata* ssp. *burmanica*. This suggests a new possible route for future research.

In vitro screening for Sigatoka resistance

To further the process of achieving genetic resistance in *Musa* cultivars, a methodology was designed to test the biological effect of raw *Mycosphaerella fijiensis* extract on *Musa* vitroplant leaves, both in the greenhouse and *in vitro*. Vegetative materials tested included 'Grand Nain', 'Calcutta 4', 'Curraré', 'Embrapa 403', 'Saba' and 'Yagambí'. The use of crude *M. fijiensis* extract allowed a rapid and efficient (48 hours in the greenhouse and 72 hours in callus crops) identification of the degree of resistance to the disease in diverse *Musa* materials. This represents a significant reduction in time and resources necessary in comparison to tests carried out in the field (Hernández, 1995).

PROPAGATION.

Enhancing Reproduction and Distribution of Strategic Genetic Materials

Because they are sterile, bananas and plantains have traditionally been propagated through buds, suckers and pieces of corm obtained from old plants. The scarcity of these planting materials led to the development of horticultural methods in the 1960's, which would increase the natural rate of sucker production. This was accomplished by stimulating the production of adventitious buds on corms (Teisson & Côte, 1997).

Those practices have been replaced by *in-vitro* techniques as the major *Musa* propagation methods since the late 1980's, given the much higher multiplication rates of the latter (100 to 1, compared to traditional propagation) and their capacity to produce clean planting materials. Additional advantages include the ease of transporting vitroplants because of their small size, the opportunities for increasing the homogeneity of plots after sorting plants in the nursery, and the increased yields regularly obtained at initial harvests with vitroplants (*op cit*).

According to Teisson & Côte (1997), the use of planting materials obtained by *in vitro* micropropagation was the most significant advancement in banana cultivation techniques in the past decade. Vitroplants are now widely used throughout the world, and CATIE has played an important role in developing, improving, and diffusing those techniques and materials in Latin America. A review of the most relevant progress in *Musa* micropropagation techniques achieved at CATIE since 1992 is presented in the following section.

Somatic Embryogenesis

Somatic embryogenesis, particularly through cell suspension, is considered to be the most promising micropropagation technique, especially given its high potential multiplication rate. Somatic embryogenesis enables the production of encapsulated and artificial seeds, facilitates cryopreservation or desiccation and, as a result, simplifies the management of *in vitro* populations (Teisson & Côte, 1997; Côte *et al.*, 1996).

In *Musa*, satisfactory callogenesis leading to somatic embryogenesis has long been considered difficult to achieve. Early experiments at CATIE and elsewhere tackled the problem by first mastering embryogenesis in wild seeded species using immature zygotic embryos, whose embryogenic capacity was well known in monocotyledons (Escalant & Teisson, 1988 & 1989; Marroquín *et al.*, 1993). However, the limitations of seminiferous, non-cultivated genotypes hampered the application of this method (Teisson & Côte, 1997; Grapin *et al.*, 1998).

Cell suspensions have been tried as an embryogenic material since the 1960's. However, the first successful regeneration of somatic embryos from embryogenic cell suspensions was not obtained until 1989 when Novak *et al.* used basal leaf sheaths and corm tissue of *in vitro* plantlets. These authors utilized a complex protocol calling for seven media, which was difficult to replicate (Côte *et al.*, 1996; Teisson & Côte, 1997).

Other researchers (Dhed'a *et al.*, 1991) utilized meristematic layers of highly proliferating shoot tips as explants. They were able to produce successful embryogenic cell suspensions and plant regeneration in a few cultivars of the ABB and AAB groups as well as in *M. balbisiana,* but they did not provide quantitative data on regeneration efficiency (Teisson & Côte, 1997; Grapin et al., 1998).

More recently, a method first developed by Ma (1991) to initiate cell suspensions and subsequently regenerate somatic embryos from immature male flowers, made the universal application of the method within the *Musa* genus possible. This method was adapted at CATIE in 1992 and, after diverse methodological improvements, it became possible to produce embryogenic callus and embryos in the majority of genomes tested, particularly on those of major agronomic interest, such as 'Grand Nain' (AAA), 'Gros Michel' (AAA) and 'French Sombre' (AAB), as well as in hybrid tetraploids (Escalant *et al.*, 1994; Pocasangre & Escalant, 1994; Côte *et al.*, 1996; Bieberach, 1995; Grapin, 1994).

But despite being successful, Ma's technique was limited to genotypes which had a persistent male bud. For that reason, the technique recently developed at CATIE to obtain embryogenic cell suspensions from immature female flowers, finally permitted the extension of this method to all *Musa* varieties, mainly to those of the AAB 'False Horn' group which show no male buds. It is also important to point out that the culture methodology was the same for either explant and for all genotypes, requiring no modifications to the basic protocol that has been successfully replicated in several laboratories (Grapin *et al.*, 1997 & 1998).

A brief description of the steps involved in these techniques, jointly developed by CIRAD and CATIE for somatic embryogenesis *via* cell suspensions of young *Musa* flowers, is provided below.

Initial explants

Two explant types have been utilized at CATIE to initiate cell suspensions: immature zygotic embryos (Escalant & Teisson, 1989; Marroquín *et al.*, 1993) and immature inflorescences (Escalant *et al.*, 1993; Grapin *et al.*, 1996; 1997; 1998). The former has been less utilized because sufficient immature zygotic embryos can only be obtained from select non-edible, diploid bananas. Of the latter, both male and female immature flowers have been successfully cultured.

To obtain male-flower explants, floral buds were collected after bunch development, and immature male flowers were removed under aseptic conditions utilizing a dissecting microscope. Within the bud, the male section is located in the tip portion of the inflorescence; consequently only groups from the floral rows located closest to the floral apex (rows 1 to 15) were cultured, with sizes below 3mm and containing 15 to 20 flowers each (Grapin *et al.*, 1996 & 1998; Escalant *et al.*, 1993; 1994 a & c; Côte *et al.*, 1996).

On the other hand obtaining female-flower explants required the *Musa* plant to be cut at its base when in transition from the vegetative to the floral stage. The pseudostems are opened lengthwise to extract the young bud, which contains very few differentiated hands at this point. Those hands formed after the sixth row contain only female flowers, making them ideal for this technique (Grapin *et al.*, 1997 & 1998).

Embryogenic Cultures

To obtain embryogenic cultures, both male and female immature flowers were inoculated on a 2,4-D (4mg/l), sucrose (30g/l) medium (M1). Results were similar with both explants: first, flowers smaller than 2 mm developed a yellow nodular callus after 1-2 months in culture. During the following 3 to 5 months, a very friable and translucent callus bearing numerous immature somatic embryos appeared at the globular stage in all cultivars tested (Grapin *et al.*, 1998; Panis *et al.*, 1995; Bieberach, 1995; Côte *et al.*, 1995 b).

The percentage of male buds forming embryogenic callus was related to genotype (40% in AAA 'Grand Nain'). While, for female flowers, 24% of the cultured buds formed embryogenic callus in cv. 'Curraré' (Grapin *et al.*, 1998).

Direct plant regeneration out of these immature somatic embryos was successful when they were removed from the embryogenic callus to a germination media. Also, when transferred to a temporary immersion system into a medium described by Bieberach and Escalant (1996), these embryos underwent a very intense secondary (also called 'adventitious' or 'amplified') somatic embryogenesis, with multiplication rates recorded between 40-100 in 2-3 months. Recovery of these embryos was very successful regardless of the genotypes utilized, with regeneration frequencies ranging between 60 and 85% (see Figure 36) (Escalant *et al.*, 1993; 1994 a, b & c; & 1995 d; Grapin, 1994; Côte *et al.*, 1996; Bieberach, 1995; Dufour & Escalant, 1996;).

Embryogenic cell suspensions

Cell suspensions were successfully established by transferring the embryogenic calli cultures described above to a liquid medium (M2) described by Côte *et al.* (1996). The development and composition of those cell suspensions was similar, whether they originated from female or male flowers, and whatever the genotype utilized (Grapin *et al.*, 1998). In this medium, the yellow calluses became necrotic whereas the friable embryogenic tissues released proembryos (which also became necrotic), isolated cells, and embryogenic aggregates. These aggregates multiplied soon after, forming many-lobed structures of varying sizes held in suspension (Côte *et al.*, 1996)

These suspensions multiplied with a proliferation rate of two to six, in terms of cellular volume, at each monthly culture cycle, and presented an embryogenic aggregate density of 3 x 10^5/ml of packed cell volume (PCV) (Teisson & Côte, 1997; Grapin *et al.*, 1998).

After several months of subculture, the suspensions contained mainly cell aggregates with a friable structure, and occasional formation of proembryos. Nonetheless, culture conditions prevented the completion of ontogenesis, permitting the long term preservation of suspensions. Some cell suspensions have been successfully subcultured for more than two years, maintaining their embryogenic and regenerative capacities (Côte *et al.*, 1996; Grapin *et al.*, 1998). Taking advantage of this fact, their management at CATIE was facilitated by adapting cryopreservation methodologies to this type of culture, as described above.

Embryogenic calli and cell suspensions were successfully established on many commercial cultivars (see Table 12).

Regeneration of cell suspensions

The embryogenic structures developed into embryos after plating the cell suspensions onto semisolid or into liquid media (M3), both devoid of 2,4-D and supplemented with other cytokinins plus auxins. Somatic embryos appeared 20 to 30 days after plating and remained in M3 until day 80 (see Figure 37) (Côte *et al.*, 1996). Histological studies confirmed the unicellular origin of these structures (Bieberach & Vásquez, 1996).

The embryos obtained germinated on a semisolid or in a liquid medium (M4), where they were maintained for 60 days. After root formation on a hormone-free medium (M5, 60 days), they developed into perfectly shaped individual plantlets (Côte *et al.*, 1996; Teisson & Côte, 1997; Panis *et al.*, 1995; Bieberach & Escalant, 1996).

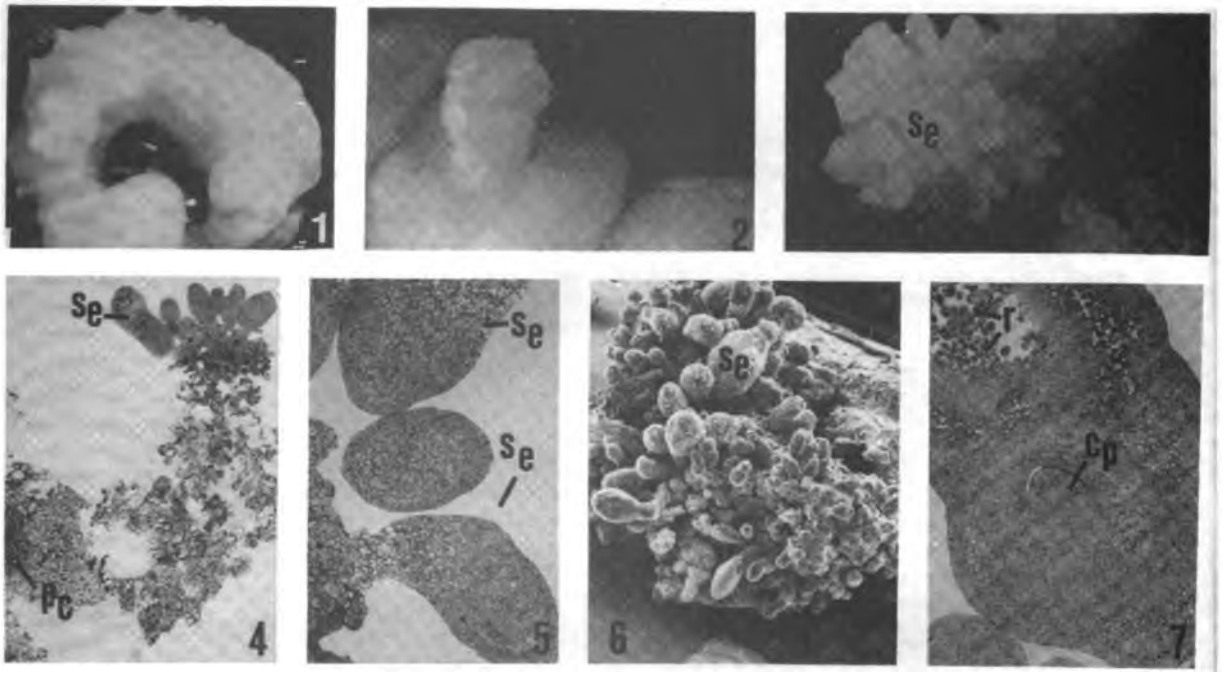

Figure 36. Stages in amplified somatic embryogenesis of *Musa* immature male flowers obtained at CATIE by placing embryogenic cultures in the temporary immersion system.
1. Young cluster of male flowers after 15 days of culture; 2. Small yellow callus derived from male flowers 2 months after culture; 3. White and translucent callus with somatic embryos (se); 4. Cross section of callus with compact tissue in the middle part; note the pseudocambial zone (pc) and the loose tissue carrying embryos on the surface; 5. Cross section of embryogenic tissue carrying somatic embryos (se); 6. Somatic embryos at various stages of development and derived from translucent and friable embryogenic tissue; 7. Transverse section of a somatic embryo; note the cauline pole (cp) and the haustorium-like structure with proteical reserve.
(*From Escalant* et al., *1994a, and reproduced with permission of* In Vitro Cellular and Developmental Biology.

TABLE 12. Generation of embryogenic calli and cell suspensions in research efforts undertaken at CATIE for somatic embryogenesis of cultivated *Musa* genotypes.

EXPLANTS	TYPE	GENOTYPE	Embryogenic callus formation	Subculture and regeneration of embryogenic cell suspensions
Male Flowers	AA	903	√	√
		Col. 49	√	√
		SF 265	√	√
	AAA	Grand Nain	√	√
		Gros Michel	√	√
		Yangambi	√	Not tested
	AAB	French Sombre	√	√
		Dominico	√	√
		Mysore	√	Not tested
		Silk	√	Not tested
	ABB	Pelipita	√	Not tested
		Bluggoe	√	Not tested
	AAAB	FHIA 1, 2	√	Initiation in progress
		FHIA 21, 23	√	Not tested
		Embrapa 403	√	Not tested
	AABB	FHIA 3	√	Not tested
Female Flowers	AAB	Curraré	√	√
		Curraré Enano	√	√

√ = Yes
Sources: Grapin *et al.*, 1998; Escalant *et al.*, 1995

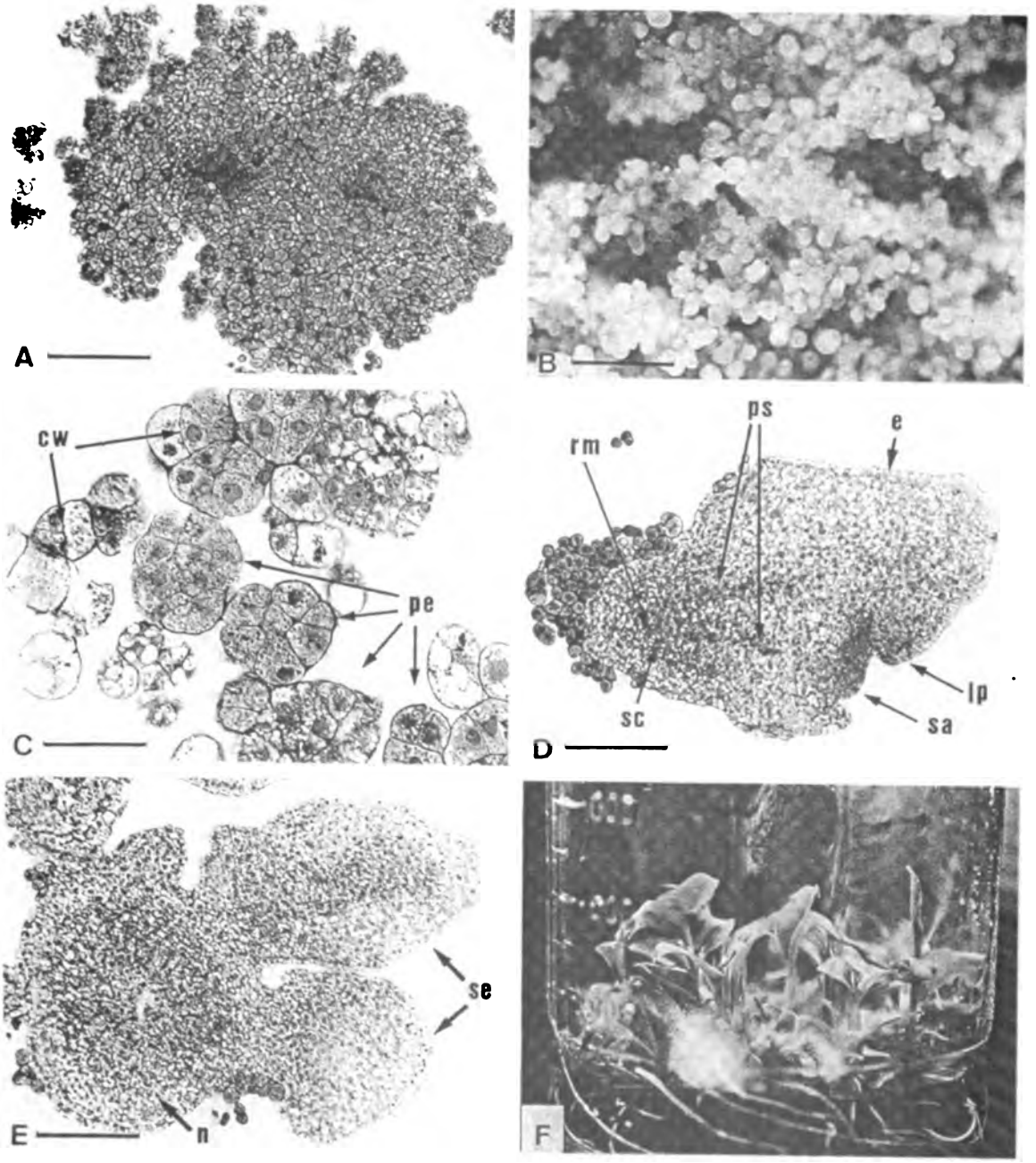

Figure 37. Somatic embryogenesis of cell suspensions obtained from immature male flowers in banana (*Musa* AAA cv. 'Grand Nain').
A) Section of a cell aggregate in suspension; B) Embryos regenerated on M3 medium 80 days after plating (cw = cell wall; pe = proembryo); C) Section through proembryos formed by internal cell divisions 20 days after plating (rm = root apical meristem; e = embryo; lp = leaf primordium; sa = shoot apical meristem; sc = shoot caulinary meristem); D) Longisection through a single embryo; E) Section through a group of embryos originating from a nodule (se = somatic embryo; n = nodule); F) Plantlets grown from somatic embryos.
(From Côte et al., *1996, and reproduced with permission from* Physiologia Plantarum.*)*

Regeneration efficiency varied according to genotype, with conversion rates ranging from 3 to 40% of the somatic embryos. For example, plating 1 ml of PCV of the cv. 'French Sombre' led to the formation of 10^5 embryos, of which 10-40% developed into plantlets. These figures changed to 3.7 x 10^5 and 5% in 'Grand Nain', and 1-5 x 10^5 in 'Curraré'. In addition, utilization of the Temporary Immersion System increased both somatic embryo formation, and regeneration rates (Grapin *et al.*, 1998; Côte *et al.*, 1994)

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CHAPTER FOUR
OTHER IMPORTANT CROPS

CONSERVATION.

Increasing Preserved Stocks and Improving Preservation Methods

As stated earlier, CATIE began establishing germplasm collections in 1942 when it was still IICA. In 1976, a Plant Genetic Resources Unit was established following the recommendations of the plant genetic resources specialists meetings held in Beltsville, Maryland in 1972, and at CATIE (CATIE/FAO) in 1973 (Morera, 1994; Morera *et al.*, 1995).

The Plant Genetic Resources Unit preserves the germplasm of more than 300 currently or potentially important species and comprising above 35,000 introductions. Accessions of crops producing orthodox seeds represent 86% of all preserved materials. These seeds are preserved in cold storage chambers: a short-term (active) collection, maintained at 5°C and 35% RH conditions; and a long-term (basic) collection preserved at –17 °C. In order to control the viability of stored seeds, germination potential is periodically monitored and those showing a low performance rate are regenerated immediately.

A total of 30,300 introductions are preserved in cold chambers, including 6,628 introductions of horticultural species, 245 introductions of tree species (representing around 80 species), and a duplicate set of CIAT's world collection of common beans (*Phaseolus vulgaris*), with 23,427 introductions. Apart from *P. vulgaris*, other species with a great number of accessions are: *Cucurbita* spp. (pumpkins); *Capsicum* spp. (sweet and hot peppers); *Lycopersicon* spp. (tomatoes); and *Zea* spp. (corn) (see Table 13a).

Another 4,860 introductions are preserved in field collections. These accessions, planted in a 49 ha field at CATIE, are systematically characterized in order to learn more about their agronomic potential. In addition to the aforementioned cacao, banana and coffee collections, the following species stand out because of the quantity of *in vivo* material preserved at CATIE:

- peach palm (*Bactris gasipaes*)
- sapote (*Pouteria* spp.)
- cassava (*Manihot esculenta*)
- sweet potato (*Ipomoea batatas*)
- annato (*Bixa orellana*)
- guava (*Psidium guajava*)
- yam (*Dioscorea* spp.)
- sapodilla (*Manilkara zapota*) (see Table 13b).

TABLE 13. Inventory of main species preserved at CATIE seed banks and field collections, as of November, 1999.

CROPS	SPECIES HELD	ACCESSIONS
A) Seed banks		
Grains		**697**
Amaranthus (Amaranth)	*A. caudatus, A. cruentus, A. hybridus, A. hypodondriacus*	267
Crotalaria (Crotalaria)	*C. longirostrata*	26
Zea (Corn)	*Z. mays, Z. mexicana*	404
Horticultural crops		**4,207**
Capsicum (Pepper)	*C. annuum, C. baccatum, C. chinense, C. frutescens, C. galapagoense, C. pubescens,*	1,530
Cucurbita (Pumpkins, Squash, Zucchini)	*C. ficifolia, C. foetidissima, C. lundelliana, C. martinezii, C. maxima, C. mixta, C. moschata, C. pepo, C. sororia*	1,882
Cucumis (Melon, Cucumber)	*C. anguria, C. melo, C. sativus*	15
Lagenaria (Bottle gourd, Calabash marrow)	*L. siceraria*	145
Lycopersicon (Tomato)	*L. esculentum, L. pimpinellifolium, P. peruvianum*	457
Physalis (Mexican tomato)	*P. ixocarpa, P.* spp.	84
Solanum (Black nightshades, Cocona, Naranjilla)	*S. americanum, S. hirsutissimum, S. mammosum, S. melongena, S. nigrescens, S. quitoense, S. radiata, S. topiro, S. umbellata*	94
Leguminous crops		**1,581**
Canavalia (Canavalia, Sword bean)	*C. ensiformis, C. gladiata*	21
Dolichos (Hyacinth bean)	*D. lablab*	34
Pachyrhizus (Yam beans)	*P. ahipa, P. erosus, P. ferrugineus, P. panamensis, P. tuberosus*	172
Phaseolus (American bean, Kidney bean, Lima bean, Teparies)	*P. acutifolius, P. aureus, P. coccineus, P. dumosus, P. lunatus, P. vulgaris*	1156
Psophocarpus (Winged bean)	*P. tetragonolobus*	19
Vigna (Cowpeas)	*V. radiata, V. umbellata, V. unguiculata, V. vexillata*	179
Duplicate of CIAT's *Phaseolus vulgaris* collection		**23,427**
Forest species (aprox. 80 spp)		**245**
Miscellaneous species		**143**
Total accessions preserved in seed banks		***30,300***

CROPS	SPECIES HELD	ACCESSIONS
B) Field Collections		
Roots & Tubers		**387**
Dioscorea (Yams)	*D. alata, D. bulbifera, D. cayenensis, D. chambon, D. dumetotum, D. esculenta, D. pentaphylla D. trifida, D.* spp.	73
Ipomoea (Sweet potato)	*I. batatas*	145
Manihot (Cassava)	*M. esculenta*	169
Tropical Fruits		**567**
Annona (Custard apples)	*A. glabra, A. muricata, A. pittieri, A. purpurea, A. reticulata, A.* spp.	62
Byrsonima (Golden spoon)	*B. crassifolia*	25
Chrysophyllum (Caimito, Star apple)	*C. cainito*	26
Diospyros (Black sapote)	*D. digyna*	11
Lecythis (Sapucaia nut)	*L. elyptica, L.* spp., *L. zabucajo*	13
Licania (Zonzapote)	*L. platypus*	23
Litchi (Lychee)	*L. chinense*	10
Mammea (Mammee apple)	*M. americana*	10
Manilkara (Sapodilla, Chicle tree, Nase berry, Beef apple)	*M. zapota*	70
Pouteria (Canistel, Sapote, Sapodilla, Caimo)	*P. caimito, P. campechiana, P. hypoglauca, P. sapota, P.* spp., *P. viridis*	242
Psidium (Common, Purple, & Costa Rican guavas)	*P. acutangulum, P. cattleianum, P. cajanillum, P. friedrichsthalianum, P. guajava, P. guineense, P.* spp.	75
Tropical crops		**3,435**
Bactris (Peach Palm)	*B. gasipaes*	648
Bixa (Annato)	*B. orellana*	132
Coffea (Coffee)	*C. arabica, C. brevipes, C. canephora, C. congensis, C. eugenioides, C. liberica, C. pseudozanguebarie, C. racemosa, C. salvatrix, C. sessiliflora, C. stenophylla*	1,782
Elaeis (Oil Palm)	*E. guineensis, E. oleifera*	13
Macadamia (Macadamia)	*M.* spp.	24
Musa (Banana & Plantain)	*M.* spp.	48
Theobroma (Cacao)	*T. angustifolium, T. bicolor, T. cacao, T. gileri, T. grandiflorum, T. macrocarpa, T. mammosum T. simiarum, T. speciosa, T. subincanum,*	788
INIBAP's *Musa* collection preserved *in vitro*		**162**
Miscellaneous species (aprox. 180 spp.)		**308**
Accessions preserved in Field Collections		***4,859***
TOTAL AMOUNT OF ACCESSIONS PRESERVED AT CATIE (seed banks + field collections)		**35,159**

TABLE 14. Level of importance of CATIE's germplasm collections for selected crops, according to IPGRI's worldwide Directory of Germplasm Collections in 1999.

GROUP	TAXON	NUMBER OF ACCESSIONS IN MAJOR WORLD GERMPLASM COLLECTIONS OF SELECTED CROPS					
		Largest collection	2nd largest	3rd largest	4th largest	5th largest	OTHER
Tropical Crops	*Coffea* (COFFEE)	CIRAD Cotê d'Ivoire 6,560	CIRAD France 3,800	**CATIE** **1,782**	Centre de Recherche Agronomique de Nkolbisson Cameroon 1,552	Jima Agricultural Research Station Ethiopia 1,284	-------
	Theobroma (CACAO)	University of West Indies - CRU Trinidad & Tobbago 2,325	CENARGEN Brazil 2,286	CENIAP Venezuela 1,058	INIAP-Estación Experimental Tropical de Pichilingue Ecuador 954	**CATIE** [1] **788**	-------
	Musa (BANANA & PLANTAIN)	INIBAP-Transit Center Belgium 1,046	CORBANA Costa Rica 769	CIRAD France 520	UFCO Honduras 490	DAL-Agricultural Rehabilitation Programme Papua-New Guinea 482	**CATIE**[2] **(#10)** **210**
	Bactris gasipaes (PEACH PALM)	UCR-Escuela de Biología Costa Rica 1,207	INPA Brazil 725	**CATIE** **648**	MAG-Estación Experimental Diamantes Costa Rica 635	CORPOICA-Centro de Investigaciones La Selva Colombia 254	-------
	Bixa orellana (ANNATO)	**CATIE** **132**	Universidad de Palmira Colombia 100	INIA-Estación Experimental El Porvenir Peru 26	INIA-Estación Experimental de Putumayo Peru 21	USAC-Facultad de Agronomía Guatemala 19	-------
Leguminous Crops	*Phaseolus* (KIDNEY & LIMA BEANS, TEPARIES)	CIAT Colombia 27,528	**CATIE**[3] **24,583**	USDA-West Regional Plant Station USA 13,781	INIFAP México 12,752	CNPAF-EMBRAPA Brazil 10,723	-------
	Pachyrhizus (YAM BEAN)	**CATIE** **179**	INIAP-Estación Experimental Santa Catalina Ecuador 70	INIFAP-Campo Experimental El Bajío México 49	NBPGR India 40	Philippine Root Crop Research & Training Center Philippines 29	-------
Horticultural Crops	*Capsicum* (PEPPER)	AVRDC Taiwan 5,177	INIFAP México 4,661	Institute of Vegetable Crops Maritsa Bulgary 4,089	USDA-Southern Regional Plant Introduction Station USA 3,877	INIFAP-Campo Experimental El Bajío México 3,590	**CATIE (#10)** **1530**
	Cucurbita (PUMPKIN, SQUASH, ZUCCHINI)	N.I. Vavilov Research Institute of Plant Industry Russia 2,000	**CATIE** **1,882**	CAAS China 1,831	INIFAP México 1,580	Institute of Agrobotany Hungary 1,500	-------

Table 14 (cont.)

	Dolichos lablab (HYACINTH BEAN)	National Dryland Farming Research Center Kenya 83	N.I. Vavilov Research Institute of Plant Industry Russia 70	Central Arid Zone Research Institute India 51	Universidad Nacional de la Loja, CATER Ecuador 41	INIAP-Estación Experimental Santa Catalina Ecuador 33	**CATIE (#6) 26**
	Lagenaria siceraria (CALABASH)	USDA-Southern Regional Plant Introduction Station USA 430	**CATIE 145**	UPLB-Institute of Plant Breeding Philippines 139	Cornell University-Dept.of Horticultural Sciences USA 55	IPK Genebank Germany 47	------
	Physalis spp. (HUSK TOMATO)	INIFAP-Campo Experimental El Bajío Mexico 220	INIFAP Mexico 190	University of Nijmegen-Botanical Garden The Netherlands 114	CORPOICA-Centro de Investigación La Selva Colombia 88	**CATIE 84**	------
	Solanum americanum (NIGHTSHADES)	**CATIE 24**	IPK Genebank Germany 11	University of Nijmegen-Botanical Garden The Netherlands 7	USDA-Southern Regional Plant Introduction Station USA 3	*	------
Grains	*Amaranthus* (AMARANTH)	USDA-North Central Regional Plant Introduction Station USA 2,570	Universidad San Antonio Abad del Cuzco Peru 1,600	Rodale Institute USA 1,200	NBPGR India 824	INIFAP México 700	**CATIE (#10) 267**
Tropical Fruits	*Annona* (CUSTARD APPLES)	University of Puerto Rico, Department of Horticulture Puerto Rico 250	Universidad Nacional de la Loja, CATER Ecuador 150	IPA Brazil 130	USDA-Subtropical Horticultural Research Station USA 90	UPLB-Institute of Plant Breeding Philippines 74	**CATIE (#7) 62**
	Byrsonima crassifolia (GOLDEN SPOON)	**CATIE 25**	CENTA El Salvador 10	*	------	------	------
	Chrysophyllum cainito (STAR APPLE, CAIMITO)	UPLB - Institute of Plant Breeding Philippines 31	**CATIE 26**	CENTA El Salvador 7	CIRAD-FLHOR, Station de la Guadeloupe France 5	*	------
	Diospyros digyna (BLACK SAPOTE)	**CATIE 11**	*	------	------	------	------
	Licania platypus (SUNZAPOTE, ZONZAPOTE)	**CATIE 23**	*	------	------	------	------
	Mammea americana (MAMMEE APPLE)	**CATIE 10**	-CENTA/El Salvador -USDA-Subtropical Horticultural Research Station/USA 5 each	INPA Brazil 3	*	------	------

Table 14 (cont.)

	Manilkara zapota (SAPODILLA, NASE BERRY, CHICLE TREE)	IPA Brazil 270	UPLB-Institute of Plant Breeding Philippines 77	**CATIE** **70**	USAC-Facultad de Agronomía Guatemala 43	-CENTA/El Salvador -Indian Institute of Horticultural Research/India 26 each	------
	Pouteria (SAPOTE, SAPODILLA, CANISTEL)	**CATIE** **242**	USAC-Facultad de Agronomía Guatemala 88	CUNSUROC Guatemala 60	USDA-Subtropical Horticultural Research Station USA 52	CENTA El Salvador 39	------
	Psidium guajava (GUAVA)	Njala University College Sierra Leone 200	Dirección de Investigación de Citros y otros frutales Cuba 142	IPA Brazil 140	Indian Institute of Horticultural Research India 119	IAC Brazil 115	**CATIE (#7)** **73**
	Solanum quitoense (NARANJILLA)	Universidad Nacional de Palmira Colombia 1127	INIAP-Estación Experimental Santa Catalina Ecuador **90**	University of Nijmegen-Botanical Garden The Netherlands 15	**CATIE** **14**	IPK Genebank Germany 4	-------
Timber Trees[4]	*Cordia alliodora* (LAUREL)	COHDEFOR/ CONSEFORH Honduras 122	**CATIE** **55**	•	-------	-------	-------
	Swietenia macrophylla (MAHOGANY)	**CATIE** **91**	USDA, Subtropical Horticultural Research Station USA 5	ESNACIFOR/Jardín Botánico Wilson Popenoe Honduras 3	•	-------	-------
	Vochysia guatemalensis (WHITE YEMERI)	Estación Biológica La Selva, OTS Costa Rica 45	**CATIE** **23**	•	-------	-------	-------
	Cedrela (SPANISH CEDAR)	**CATIE** **364**	COHDEFOR/ CONSEFORH Honduras 92	ESNACIFOR/Jardín Botánico Wilson Popenoe Honduras 4	ANAM, CEMARE-BSF Panamá 3	•	-------

[1] Together with CRU's collection in Trinidad, CATIE's field collection is one of the only two ranked as international cocoa collection by IPGRI

[2] In the case of *Musa*, CATIE preserves a small collection (48 accessions) and maintains a partial duplicate of INIBAP's *in vitro* collection under restricted access conditions.

[3] In the case of *Phaseolus*, CATIE holds a medium collection (1200 accessions) and maintains a partial duplicate of CIAT's *P. vulgaris* collection (23,427 accessions).

[4] In tree species, accessions correspond to "progenies" or "families".

• Remaining collections too small to be included (1-2 accessions).

TABLE 15: Centers of origin of the main cultivated plants and list of promising crops cultivated in the Americas in pre-Columbian times which became marginalized after 1492. (Staple crops shown in bold type).

MAIN WORLD CROPS NATIVE TO AMERICA	MARGINALIZED PRE-COLUMBIAN CROPS FROM THE AMERICAS ,	MAIN WORLD CROPS INTRODUCED INTO THE AMERICAS AFTER 1492 AND LISTED BY CENTER OF ORIGIN		
SOUTH AMERICAN REGION Cacao *(Theobroma cacao)* Cashew *(Anacardium occidentale)* **Cassava** *(Manihot esculenta)* Groundnut *(Arachis hypogaea)* Lima bean *(Phaseolus lunatus)* Papaya *(Carica papaya)* Passion fruit *(Passiflora edulis)* Pineapple *(Ananas comosus)* **Potato** *(Solanum tuberosum)* Squash family *(Cucurbita* spp.*)* **Sweet Potato** *(Ipomoea batatas)* Tomato *(Lycopersicon esculentum)* Vanilla *(Vanilla planifolia)* **CENTRAL AMERICAN AND MEXICAN REGION** Avocado *(Persea americana)* Blackberries *(Rubus glaucus)* Cacao (*Theobroma cacao*) Common bean *(Phaseolus vulgaris)* Cotton *(Gossypium hirsutum, G. barbadense)* Guava *(Psidium guajava)* Jamaican Pepper *(Pimenta dioica)* **Maize** *(Zea mays)* Mahogany (*Swietenia* spp.) Mexican sage *(Lippia graveolens)* Papaya *(Carica papaya)* Pepper *(Capsicum* spp.*)* **Potato** *(Solanum tuberosum)* Quinine *(Cinchona* spp.*)* Squash family *(Cucurbita* spp.*)* Tobacco *(Nicotiana tabacum)* Tropical Pine (*Pinus caribaea*) **NORTH AMERICAN REGION** Blueberry *(Vaccinium* spp.*)* Sunflower *(Helianthus annuus)* Radiata pine *(Pinus radiata)*	**Amaranth** *(Amaranthus* spp.*)* Annato *(Bixa orellana)* **Arrowroot** *(Maranta arundinacea)* Barbados cherry *(Malpighia glabra)* Bitter wood *(Quassia amara)* Black sapote *(Diospyros digyna)* Bottle gourd *(Lagenaria siceraria)* Brazil cherry *(Eugenia dombeyi)* Chayote *(Sechium edule)* Cheeseplant *(Monstera deliciosa)* Costa Rican Guava *(Psidium friedrichsthalianum)* Custard apples & Soursop *(Annona* spp.*)* Golden spoon *(Byrsonima crassifolia)* Guaba *(Inga* spp.*)* Guaraná *(Paullinia cupana)* Husk tomato *(Physalis philadelphica)* Kola nut *(Cola acuminata)* Jaboticaba *(Myrciaria cauliflora)* Jocote *(Spondias purpurea)* Madrone *(Rheedia acuminata, R. madruno)* Mammee apple *(Mammea americana)* Melon pear *(Solanum muricatum)* Naranjilla *(Solanum quitoense)* **'New Cocoyam'** *(Xanthosoma sagitifolium)* Peach Palm *(Bactris gasipaes)* Peach Tomato *(Solanum topiro)* Persimmon *(Diospyros virginiana)* **Peruvian parsnip** *(Arracacia xanthorrhiza)* Pitaya roja *(Hylocereus ocamponis)* Prickly pear *(Opuntia* spp.*)* **Quinoa** *(Chenopodium quinoa)* Sapodilla *(Manilkara zapota)* Sapote *(Pouteria sapota)* Star apple *(Chrysophyllum cainito)* Sword bean *(Canavalia ensiformis)* Tepary bean *(Phaseolus acutifolius)* White sapote *(Casimiroa edulis)* Wormseed *(Chenopodium ambrosioides)* Yam bean *(Pachyrhizus* spp.*)* Yerba mate *(Ilex paraguarensis)* **Yampi yam** *(Dioscorea trifida)* Zunzapote *(Licania platypus)*	**CHINESE-JAPANESE REGION** Bamboo *(Dendrocalamus asper)* **Millet** *(Panicum miliaceum)* Mustard *(Brassica nigra, B. juncea)* Oranges, Mandarins & other citrus fruits *(Citrus sinensis, Citrus* spp.*)* Peach *(Prunus persica)* Radish *(Raphanus sativus)* **Rice** *(Oryza sativa)* **Soybean** *(Glicine max)* Tea *(Camellia sinensis)* **INDOCHINESE-INDONESIAN REGION** Bamboo *(Dendrocalamus asper)* Banana *(Musa* spp.*)* Coconut *(Cocos nucifera)* Grapefruit *(Citrus paradisi)* Mango *(Mangifera indica)* **Rice** *(Oryza sativa)* Sugarcane *(Saccharum officinarum)* **Taro** (Dasheen, 'Old' Cocoyam) *(Colocasia esculenta)* Teak *(Tectona grandis)* **Yam** *(Dioscorea* spp.*)* **AUSTRALIA** Macadamia nut *(Macadamia* spp.*)* Eucalyptus (*Eucalyptus* spp.) **AFRICAN REGION** Castor *(Ricinus communis)* Coffee *(Coffea* spp.*)* Melon *(Cucumis melo)* **Millet** *(Panicum miliaceum)* Oil Palm *(Elaeis* spp.*)* Okra *(Lathyrus ochrus)* Sesame *(Sesamum orientale)* **Sorghum** *(Sorghum* spp.*)* **Teff** *(Eragrostis teff)* Watermelon *(Citrullus lanatus)* **Wheat** *(Triticum aestivum)* **Yam** *(Dioscorea* spp.*)*	**CENTRAL ASIAN REGION** Apple *(Malus domestica)* Apricot *(Prunus armeniaca)* Bean *(Vigna* spp.*)* Carrot *(Daucus carota)* Grape *(Vitis vinifera)* Melon *(Cucumis melo)* Onion *(Allium cepa)* Pea *(Pisum sativum)* Pear *(Pyrus communis)* Plum *(Prunus domestica)* **Rye** *(Secale cereale)* Spinach *(Spinacea oleracea)* Walnut *(Juglans* spp.*)* **Wheat** *(Triticum aestivum)* **NEAR EASTERN REGION** Almond *(Prunus dulcis)* **Barley** *(Hordeum vulgare)* Date Palm *(Phoenix dactylifera)* Fig *(Ficus carica)* Grape *(Vitis vinifera)* **Lentil** *(Lens culinaris)* Melon *(Cucumis melo)* Pea *(Pisum sativum)* Pistachio *(Pistacia vera)* **Rye** *(Secale cereale)* Safflower *(Carthamus tinctorius)* **Wheat** *(Triticum aestivum)* **EUROPEAN-SIBERIAN REGION** Apple *(Malus domestica)* Cherry *(Prunus avium)* Chicory *(Cichorium intybus)* Hops *(Humulus lupulus)* Lettuce *(Lactuca sativa)* Pear *(Pyrus communis)*	**MEDITERRANEAN REGION** Beetroot *(Beta vulgaris)* Cabbage (plus Cauliflower, Broccoli & Brussels sprouts) *(Brassica oleracea)* Celery *(Apium graveolens)* Faba bean *(Vicia faba)* Grape *(Vitis vinifera)* Lettuce *(Lactuca sativa)* **Oats** *(Avena sativa)* Olive *(Olea europaea)* Radish *(Raphanus sativus)* **Wheat** *(Triticum aestivum)* **HINDUSTANI REGION** Banana *(Musa* spp.*)* Bean *(Vigna* spp.*)* Chick-pea *(Cicer arietinum)* Citrus *(Citrus* spp.*)* Cucumber *(Cucumis sativus)* Eggplant *(Cucurbita melongena)* Gmelina (*Gmelina* spp.) Mango *(Mangifera indica)* Mustard *(Brassica nigra, B. juncea)* Pepper *(Piper nigrum)* Pigeon pea *(Cajanus cajan)* **Rice** *(Oryza sativa)*Sugarcane *(Saccharum officinarum)*

Adapted from: Tribe, 1994; Rehm & Espig, 1991; León, 2000; Hernández-Bermejo & León, 1994; and FAO, 1995.

As illustrated in Table 14, CATIE germplasm collections are among the largest included in IPGRI's worldwide directory of germplasm collections, for 28 taxa (genera or species). CATIE's collections represent the most important genebanks in the world for crops like Annato, Yam Bean, Nightshades, Golden Spoon, Sapote, Black Sapote, Sunzapote, Mammee Apple, Mahogany, and Spanish Cedar.

Table 15 shows a list of other species, that those in CATIE collections, as an illustration of the movement of plant genetic materials that occurred after America's discovery in 1492. As this table shows, the vast majority of species preserved at CATIE correspond to promising crops, native to either Mesoamerica or South America. These crops were domesticated and planted in pre-Columbian times, but became marginalized with the arrival of numerous competitor species during the colonial period. In preserving these materials, CATIE makes a remarkable contribution to the future diversification of global food production.

GERMPLASM EVALUATION.

Broadening our Knowledge of our Preserved Stocks.

Sapotaceae:

CATIE's collection of sapotaceae trees contains 340 accessions from the genera: *Chrysophyllum, Manilkara* and *Pouteria.* During the first half of the 1990's studies were conducted to evaluate the genetic diversity of Sapotaceae trees preserved in the CATIE germplasm collections. These studies were undertaken as part of the project entitled, "Diversity, Conservation and Sustainable Use of Native Fruit Germplasm of Tropical America"[1]. Agronomic, morphologic and quality characteristics of trees, leaves, fruits and seeds, were utilized to classify and characterize such diversity. Those traits were also utilized to undertake a preliminary selection of outstanding genotypes. As of today, the main species of this family, *i.e. Pouteria sapota, Manilkara zapota* and *Chrysophyllum cainito*, have been assessed (Mora *et al.*, 1997).

Star Apple or Caimito

Forty nine individual trees of the *Chrysophyllum cainito* collection, representing 20 accessions and 77% of all caimito accessions, were characterized utilizing a set of 29 quantitative plus 18 qualitative characteristics. These materials were collected in Costa Rica (39), Guatemala (8), and Mexico (2), and planted in CATIE fields between 1977 and 1980. Five of those genotypes had green fruit; the rest bore purple fruit. Besides this trait, few other characteristics are highly variable within the germplasm preserved, probably because it all originated from few specimens introduced from the Caribbean islands into Mesoamerica. In this study, higher variation coefficients were found for quantitative traits such as: pulp acidity, fruit weight and shell weight (Table 16) (Gazel *et al.*, 1995; Morera *et al.*, 1995)

Utilizing the F test for quantitative characteristics, a total of 21 traits produced significant differences, and rendered 6 groups on which the phenotypic variability of these individuals was classified, according to cluster analysis.

Also, 11 of those discriminating characteristics were easily measured in the field, while the rest required laboratory processing. Among the qualitative traits, only pulp color, leaf upper face color, and fruit juiciness presented significant differences among all 6 groups, according to Chi-square analysis (Gazel, 1995; Gazel *et al.*, 1995; Morera *et al.*, 1995; CATIE, 1997).

[1] This project was coordinated by IPGRI and implemented from 1992 to 1997, through the Mesoamerican Network on Plant Genetic Resources, REMERFI.

BOX 9: The Sapotacean fruits

The sapote, *Pouteria sapota*, originates in the lowlands of Mesoamerica. It is successfully planted in areas where the temperatures do not fall below 15°C and the rainfall exceeds 800 mm/year. Wild populations are still found from Mexico to Costa Rica under 1,000 m.a.s.l. Its name is derived from the Aztec "tzapotl", a collective name applied to several species of sweet and spherical fruits with large seeds. Sapote trees reach up to 30 m in height, with a symmetrical crown. Their ovate to lanceolate leaves concentrate on the apex of branches. The aromatic fruit ranges from elongated to spherical in shape, and weigh up to 3 kg. The skin is hard, rough and brittle, and the flesh varies from red to greyish in color and turns soft and sweet when ripe.

Sapote is an open-pollination species, generally multiplied by seed, whose fruit may be eaten raw, and the flesh is utilized to prepare jams, ice creams and sauces. Seeds are used in parts of Central America to make candy ("cajetas de sapoyol"), to prepare medicinal drinks to treat sinusitis, or ground and mixed with cocoa to give chocolate a bitter flavor and characteristic aroma. Oil from the seeds is utilized as skin tonic, to prevent baldness, to reduce muscular pain and to treat rheumatic ailments. Latex from the tree is used to remove skin funguses, and the wood is hard enough to be used to make furniture or fences. Cultivation of this species still cannot meet the external market demands (mainly from the Cuban community in Florida, where several cultivars are currently grown) and may play an important role as a source of revenue and as a complement to the diet for low-income populations.

The sapodilla, *Manilkara zapota*, is also originally from Central America, where it was domesticated and selected for taste and fruit size by the native peoples. Nevertheless, its commercial cultivation expanded mainly in South East Asia, and its recent selection occurred in Florida, India, Indonesia and the Philippines. It is also a highly variable species, with trees ranging from short and open-ramified to tall (up to 30 m) and compact crowns, and with fruits of diverse size (up to 9 cm long in Florida cultivars), shape, flesh color (pale yellow to reddish), and number of seeds (0-12). It is the most known and appreciated of the sapotacean fruits, and most commonly eaten fresh. It can be stored or transported for up to 5 weeks, if harvested before fully ripe. It is also utilized for its latex, as sapodilla trees produce chicle (formerly the chief ingredient in the chewing gum industry, but which is now synthetically produced). Chicle is collected locally from wild trees in the origin area, which are tapped at long-time intervals. The wood is particularly resistant to humidity.

The star apple or caimito, *Chrysophyllum cainito*, is native to the West Indies. The phenotypic diversity is lower in this species, as it probably originated from a reduced population which was distributed in northern South America in pre-Columbian times. The caimito tree grows up to 20 m, with few ramifications. Its foliage is very attractive, as it shows alternate elliptic leaves with a golden pubescence on the lower face and a dark green and shiny color on the upper face which becomes red with aging. Its attractive foliage has prompted its use outside the region as an ornamental tree. The fruit has a delicious flavor and is eaten fresh or processed as jam.

The sapotacean family also includes other species of great value, such as the canistel (*Pouteria campechiana*), pan de vida (*P. hypoglauca*), lúcuma (*P. obovata*), caimo (*P. caimito*) and green sapote (*P. viridis*).

Adapted from León, 2000; Pennington, 1990; Rehm & Espig, 1991; Hernández-Bermejo & León, 1994; IPGRI, 1998.

TABLE 16. Summary of variation registered among quantitative characteristics evaluated in CATIE's sapote, caimito and sapodilla germplasm collections.

Characteristic	SAPOTE				CAIMITO				SAPODILLA			
	Min.	Max.	Avg.	VC%	Min.	Max.	Avg.	VC%	Min.	Max.	Avg.	VC%
FRUIT												
Weight (g)	173.6	713.0	388.5	35.1	42.8	244.7	100.1	43.8	64.5	124.4	92.8	21.0
Length (mm)	64.6	135.7	98.7	16.0	40.4	74.3	56.1	14.5	43.6	67.0	53.0	12.6
Diameter (mm)	62.8	106.2	84.0	12.5	42.5	78.7	54.8	14.6	48.9	65.3	56.2	9.7
SHELL												
Weight (g)	24.4	96.2	53.1	31.9	14.9	81.0	33.5	41.5	14.5	23.4	18.1	13.8
Thickness (mm)	1.0	2.9	1.9	21.9	1.9	4.6	2.7	25.7	0.6	1.7	1.0	27.6
PULP												
Yield (%)	60.2	83.0	74.6	6.5	53.1	72.6	60.4	7.8	68.4	82.7	75.5	6.1
Total sugars (%)	13.3	32.0	21.2	18.3	5.9	11.9	9.6	13.6	7.9	18.4	14.2	23.4
Reducing sugars (%)	4.1	22.4	9.8	48.6	3.6	6.4	5.1	15.0	6.8	14.2	9.9	22.4
Brix	19.9	38.4	30.0	13.8	9.0	14.8	12.0	10.8	13.8	29.4	19.1	23.7
Proteins (%)	0.3	2.0	0.9	32.6	0.3	1.2	0.6	30.0	0.3	0.9	0.6	30.5
Carbohydrates (%)	23.6	43.6	34.6	14.6	10.6	22.3	14.8	14.3	19.3	34.6	23.7	17.1
Acidity	3.9	66.5	34.8	35.8	27.1	172.8	65.1	58.1	9.6	41.2	27.8	35.2
SEED												
Weight (g)	18.4	106.2	41.2	30.3	2.0	8.9	4.9	27.6	1.7	6.6	3.9	38.2
Number	1.0	3.9	1.4	33.9	3.3	8.7	6.2	21.0	2.1	7.0	4.2	35.2

Min. = minimal value, Max. = Maximal value, Avg.= Average, VC% = Variation coefficient.

Beef Apple, Sapodilla or Chicle tree

Thirteen *Manilkara zapota* trees collected in Mexico (5), Guatemala (5), Costa Rica (2), and Florida (1), representing 11 accessions (16% of all sapodilla materials), and which were introduced into CATIE collections between 1976 and 1981, were also characterized based on 31 quantitative and 18 qualitative variables (Morera *et al.*, 1995; Gazel, 1995; CATIE, 1997).

As can be observed in Table 16, pulp acidity and weight, and number of seeds per fruit were the most variable quantitative characteristics. According to the F test only 6 quantitative characteristics were significant and they permitted to group the analyzed genotypes into 3 clusters. Only 2 of those variables required laboratory processing. Among qualitative traits, only the fruit shape showed significant differences among all 3 groups, based on chi-square analysis (Figure 38) (*ibid*).

On the other hand, 21 Sapodilla accessions plus 15 Caimito introductions were recently evaluated for their resistance to diverse insect pests. Accessions 6663, 11384 and 8304 of Caimito, and 8023, 8241, 9538, 11328, 11600 and 14660 of Sapodilla collections were the most tolerant genotypes against *Conotrachelus* sp. (Col: Curculionidae), the most important pest for both crops in Central America (Carballo & Coto, 1999).

Sapote

For *Pouteria sapota,* 50 accessions preserved in CATIE genebanks (24% of all *Pouteria* germplasm), represented by a sample of 66 individuals, were characterized based on 30 quantitative and 18 qualitative variables. This collection includes materials collected from Mexico to Panama, which were introduced between 1977 and 1981 into CATIE fields (CATIE, 1997).

Average height of analyzed trees was 6.8 m with a stem diameter of 25 cm. High levels of heterogeneity were found for fruit, peel, flesh and seed characteristics, as shown in Table 16. This was an important finding, as it opens possibilities for selecting for better quality (CATIE, 1997).

The F test yielded a total of 25 quantitative variables producing significant differences between

genotypes, which after cluster analysis were separated into 6 groups. Thirteen of those variables required laboratory analysis to be measured. Branch architecture, fructification distribution, fruit production, flowering and fruiting season were the only qualitative, variables that produced significant variation among all 6 groups (Figure 39). Group composition was highly correlated with the geographical origin of accessions (Morera *et al.*, 1995; Gazel, 1995; Mora *et al.*, 1997; Gazel *et al.*, 1999).

In addition, as a result of these studies, several promising caimito, sapodilla and sapote genotypes were identified, which will be utilized in future breeding works (see Table 17). Also, characterization studies will facilitate selection of genotypes showing the highest variability, to create core collections as intended by IPGRI (1998) to reduce conservation costs.

TABLE 17. Promising sapotaceae genotypes identified in CATIE germplasm collections after characterization of three valuable species.

TAXON	Outstanding genotype identification
Chrysophyllum cainito	Purple fruits: 8353-1, 8263-2, 8617, 10818-2, 11320-1 y 2, 11536-3, 11914-2 Green fruits: 8263-3, 8332-1, 9605-1
Manilkara zapota	8562-1, 9175-1, 5909-3, 8241-2
Pouteria sapota	7730-1, 8303-2, 8754-1, 9001-2, 9004-2, 9555-1, 9767-3, 10617-1, 10640-3, 10641-1, 10686, 10702-1, 11129-1 y 2, 11200-1, 11252-1, 11301-1, 14747-1

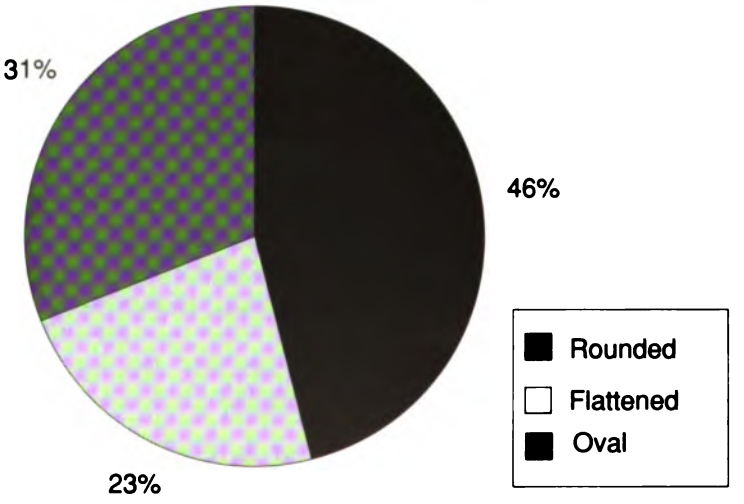


Figure 38. Percent distribution of fruit shapes in CATIE's Sapodilla germplasm collection, 1995.

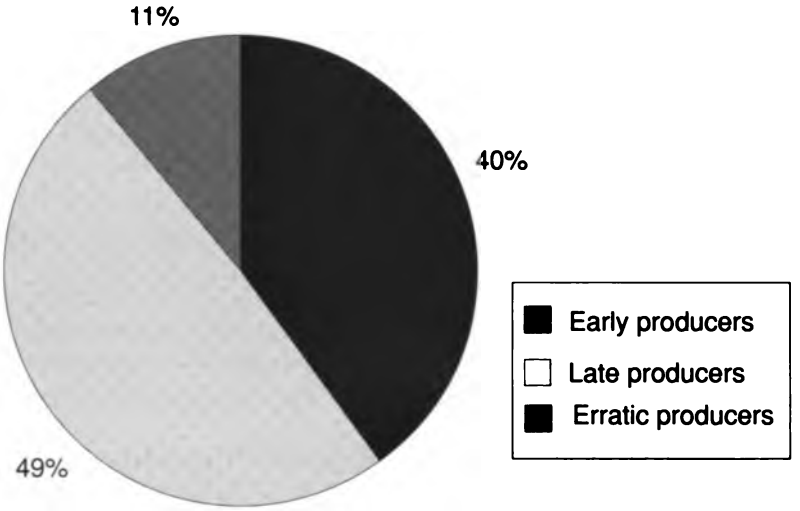


Figure 39. Percent distribution of fructification period in CATIE's Sapote germplasm collection, 1995.

Yam Beans

A research project on the Yam Bean (*Pachyrhizus* spp.) was established at CATIE in 1989. It was part of a cooperative effort financed by the EEC and headed by the Royal Veterinary and Agricultural University (RVAU) of Denmark, with counterpart institutions in México (INIFAP), Ecuador (INIAP), Tonga (Ministry of Agriculture & Forestry), Senegal (ISRA), and Benin (University of Benin) (Phillips-Mora *et al.*, 1993; Jiménez *et al.*, 1998). As part of this project, the genetic diversity of the yam bean germplasm preserved at CATIE was characterized and evaluated, in order to classify such diversity and thus facilitate further selection and breeding efforts.

Germplasm Characterization

The systematic characterization of 40 accessions of *P. erosus* was carried out in 1992. A matrix of distances between accessions for a total of 86 variables was created and then utilized with Ward's hierarchic grouping analysis[2]. The collection was classified into 3 mutually exclusive groups based on marked differences in 13 qualitative and 6 quantitative characteristics, which presented the highest discriminating power (Table 18). Among those variables, flower characteristics were the most important for classification. The first group presented the lowest internal variability, as a result of their common origin (Mexico) and their early domestication and intense cultivation. Group 2 presented higher variation given their more diverse geographical origin, although they also showed similarities attributed to a common ancestor introduced into several regions. Finally, group 3 encompassed all wild accessions, thus presenting the greatest phenotypic variability, mainly in flower, tuberous root and vegetative growth characteristics (Marquez, 1992).

In 1998, a morpho-agronomic and molecular characterization of 31 *P. tuberosus* accessions from CATIE germplasm collections was undertaken. For the morphologic analysis, a total of 70 qualitative and quantitative characteristics were included to design a distance matrix among samples. (Tapia, 1998)

[2] Ward, J.H. Jr. 1963. J. Am. Statistical Assoc. 58:236-244.

BOX 10: The genus *Pachyrhizus*

At least 25 species of the approximately 18,000 comprised by the Fabaceae (formerly Leguminosae) family are cultivated in the world for consumption of their tuberous roots. These species represent a very important resource, especially for resource-poor farmers, given their high productivity, nitrogen-fixing capacity and nutritional value. Several species from the *Pachyrhizus* genus, known as yam beans, occupy a privileged position among these promising resources, as they can also be exploited for extraction of natural insecticides (rotenone), starch and oil. Also, given its profuse vegetative growth, *Pachyrhizus* can also be utilized as green manure.

Yam beans are originally from the New World. The genus *Pachyrhizus* (derived from greek terms, *pachys* = thick, *rhiza* = root) includes 2 wild (*P. ferrugineus, P. panamensis*) and 3 cultivated species (*P. erosus, P. tuberosus, P. ahipa*). These species are largely polymorphic and hard to distinguish from each other. The large genetic variability has permitted this species to be adapted to very diverse environments of the tropics and subtropics, ranging from wet plains at the sea level to dry valleys over 2,000 masl in Mexico and the Andes. Very few natural enemies of yam beans have been identified.

P. erosus was apparently important to the ancient Mayas. This crop was more recently expanded to the Philippines and other islands in the Pacific Ocean, and it is currently cultivated from El Salvador to Mexico where it has commercial importance. In these countries it is consumed locally and exported to the US, where it is substituted for other ingredients in Chinese food. *P. erosus* is a climbing species with highly ramified stems reaching up to 5m long. Its trifoliate leaves are highly variable in terms of size and shape (lobate to dentate, seldom entire folioles). It presents pods measuring 3-15 cm long, whose seeds are flattened, and range from 4-12 mm long.

P. tuberosus was known in Peru some 5,500 years ago. This species still grows wild in the upper basin of the Amazon river. It is common from Ecuador to Argentina and the Amazonian lowlands, but despite its introduction into Asia and Africa in recent times, it is less known than *P. erosus*. *P. tuberosus* is also a vigorous climbing species, with darker and more abundant foliage than *P. erosus*. Its folioles are often whole, its pods are longer on average (3-30 cm long) and its seeds are more rounded and larger (10-15 mm long) than those *of P. erosus*. In the Chaco region (lowlands extending from Bolivia into nortwestern Argentina), shorter yam beans have been planted traditionally and they were assigned to a different taxon, *P. ahipa*, although few other characteristics separate this species from *P. tuberosus*.

Yam beans are generally propagated by seed, although asexual propagation is also very effective. Young tuberous roots are sweet and watery and are thus consumed raw (or with lemon and pepper, as in the Mexican tradition). Those over 1-year-old are preferred for starch production. Yields go over 100 tons/ha, depending upon local conditions and planting density. In Mexico, some cultivars have been selected for productivity and sugar content. Young pods are also utilized as vegetables, although they become poisonous when ripe. Their leaves are also poisonous, but rotenone contents are higher in seeds.

Adapted from León, 2000; Phillips-Mora et al., 1993; Rehm & Espig, 1991; Sørensen, 1994.

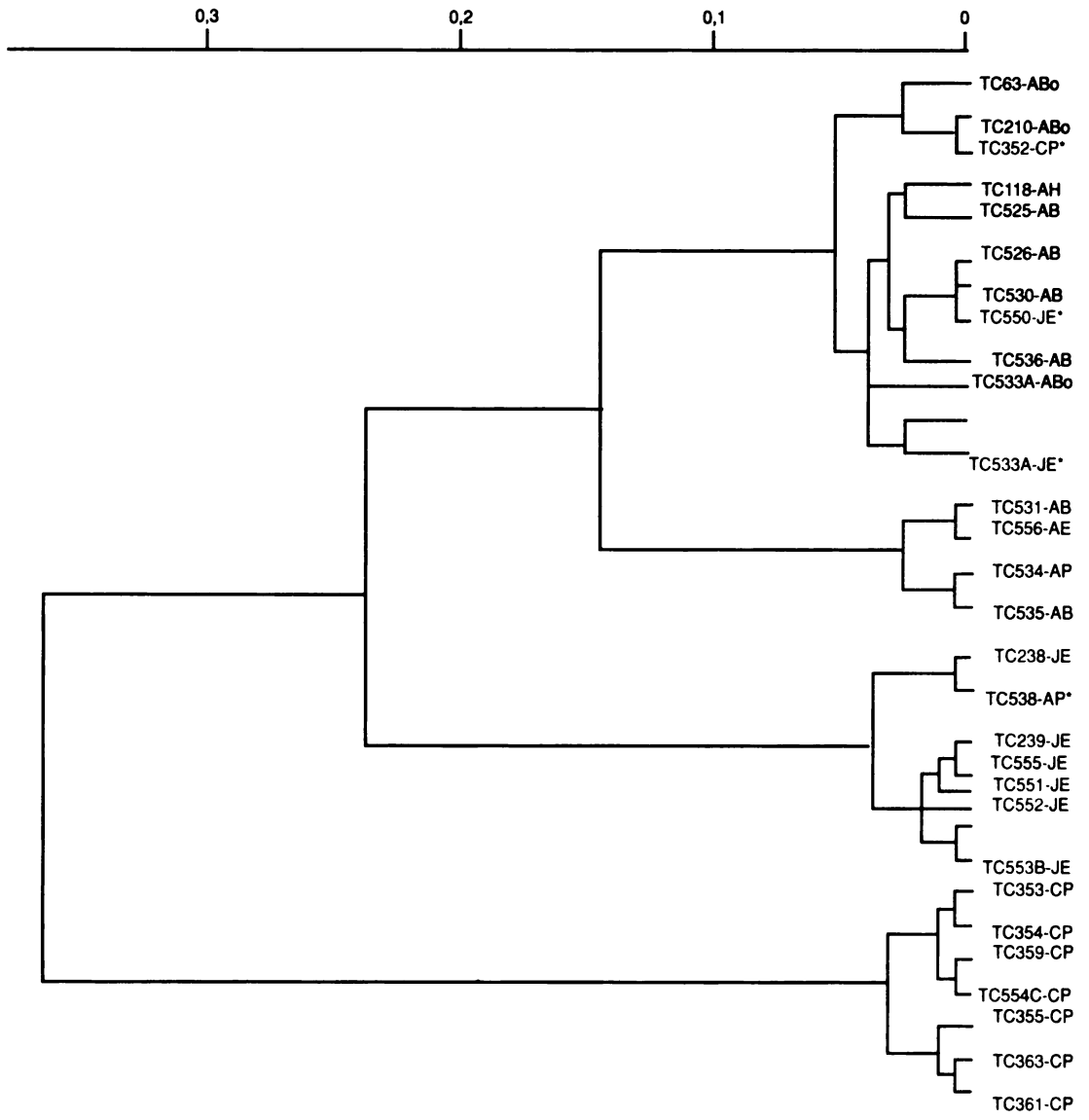
TABLE 18. Phenotypic characteristics in yam beans which present the most discriminating power to identify groups in germplasm collections, and to separate individuals within those groups.

Pachyrhizus erosus[1]		*Pachyrhizus tuberosus*[2]	
To separate groups in the collection	**To separate accessions within those groups**	**To separate groups in the collection**	**To separate accessions within those groups**
Growth habit	-Growth speed	-Growth habit	-# days to anthesis
-# days to anthesis	-# days to anthesis	-# days to anthesis	-Central foliole L/W ratio
	-Central foliole width and L/W ratio	-Central foliole width and L/W ratio	-Lobe type and shape in the central foliole of the leaf
	-Lobe shape in the central foliole of the leaf	-Lobe type and shape in the central foliole of the leaf	-Pod curvature
		-Pod curvature	-Immature pod color
		-Immature pod color	-# days to pod physiological maturity
		-# days to pod physiological maturity	
-Root shape	-Root shape		
-# flower buds per inflorescence	-# flower buds per inflorescence		
-Maximum number of floral buds	-Maximum number of flower buds		
-% of medium-size roots	-% of medium-size roots		
-Color of standard petal	-Color of standard petal		
-Seed size	-Seed length	-Seed shape	-Root L/W ratio
-Root flesh hardness	-Root length	-Root flesh color	-Stamen length
-Seed color	-Pod color at physiological maturity	-Sepal color	
-Flowering habit	-Stem pubescence	-Stem color	
-Flowering period length	-Sepal pubescence	-Pod width	
-% fertile plants	-Root surface defects	-Growth speed of main stem	
-% small roots	-Maximum number of pods per plant		
-% root dry matter	-Kg/ha of root dry matter		
-# branches per plant	-% crude protein (dry basis)		
-# branches with inflorescences			
-# of inflorescences per branch			
-# leaves per plant			

1 After Marquez, 1992.
2 After Tapia, 1998.

After Ward's hierarchic grouping analysis (Figure 40), the *P. tuberosus* collection was classified into four main groups: Ashipas (groups 1 & 4), Chuines (group 2) and Jíquimas (group 3). Ten qualitative and 7 quantitative variables demonstrated greater discriminating power to identify groups and samples within groups (see Table 18).

Among those, the form and type of the central foliole's lobe (figs. 41 & 42), root pulp color, growth habits, and the number of days to anthesis and to physiological maturity, were the most useful characters to be utilized in an initial description (Tapia, 1998; Tapia *et al.*, 1999).



* accessions whose morphology did not correspond to passport data.

Blue = group 1	A= Ashipas	Bo =Bolivia
Red = group 4	C = Chuin	P = Peru
Green = group 2	J = Jíquima	H =Haiti
Orange = group 3		B = Brazil
		E = Ecuador

Figure 40. Phenogram obtained after Ward's hierarchic grouping of 31 *P. tuberosus* accessions from CATIE germplasm collection. (Gower distances are shown horizontally)

Figure 41. Dominant shapes of the central foliole lobe in Jíquima, Ashipa and Chuin plants of the *P. tuberosus* germplasm collection

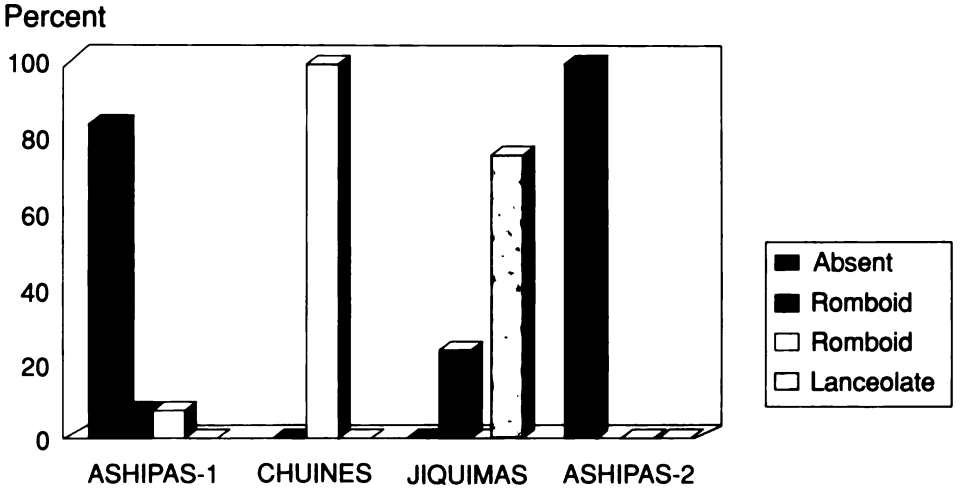


Figure 42. Percent distribution of shape of lobe in the central foliole of 4 *P. tuberosus* groups of accessions, CATIE, 1998.

Accessions from groups 1 & 4 ('Ashipas') were characterized by having the most diverse geographic origin (Brasil, Bolivia, Ecuador & Peru), as well as the largest phenotypic variability for leaves, roots and flowers. These 2 groups were morphologically similar, and showed small distances between them. 'Chuines' (group 2) contained introductions endemic to the Peruvian Amazon basin, presenting a very similar morphology. Root characteristics were useful in differentiating among those accessions. The 'Jiquimas' group (#3) comprised only early flowering materials with bushy growth, obtained from the Ecuadorian coast. These results confirmed a previous classification made by Sørensen (1996), based on ethnobotanic and phenotypic data (Tapia *et al.*, 1999; Tapia, 1998).

For molecular characterization, RAPD markers were obtained (Figure 43), and by means of diverse analyses (distance matrix, dendrograms, "bootstrap" and canonical discrimination), the variability of those *P. tuberosus* accessions was determined. Ten primers, out of 86 tested, generated 32 polymorphisms among accessions. Seven of those primers were most useful in differentiating between groups, finding duplicates and labeling mistakes, and characterizing individuals (*ibid*).

As in the phenotypical analysis, the molecular characterization also yielded 4 groups within the *P. tuberosus* germplasm. Three groups clearly corresponded to the 'Ashipas', 'Chuines' and 'Jíquimas' classification made by Sørensen (1996), and presented low genetic distances between them. The fourth group presented a much higher genetic distance from the rest of groups, and comprised two accessions previously classified as *P. tuberosus,* but now revealed as *P. tuberosus* x *P. erosus* hybrids (Tapia, 1998). The genetic distance between these accessions, measured according to Nei (1979), can be visualized in a dendrogram presented in Figure 44[3].

RAPD-based characterization detected several errors within the *P. tuberosus* collection. A total of 12 accessions appeared to be duplicates of other introductions. In addition, as can be observed in Figure 44, three accessions were mislabeled (passport data did not correspond) (*ibid*).

Finally, the congruence between both types of characterization in *P. tuberosus* was tested through correlation analyses based on the distance matrix. They provided evidence showing that the best correspondence was obtained between classifications based on molecular markers and qualitative characteristics. The positive correlation obtained made it clear that the methods were complementary (*ibid*).

[3] Nei & Li, 1979. Proc. Natl. Acad. Sci. 76: 5269-5273

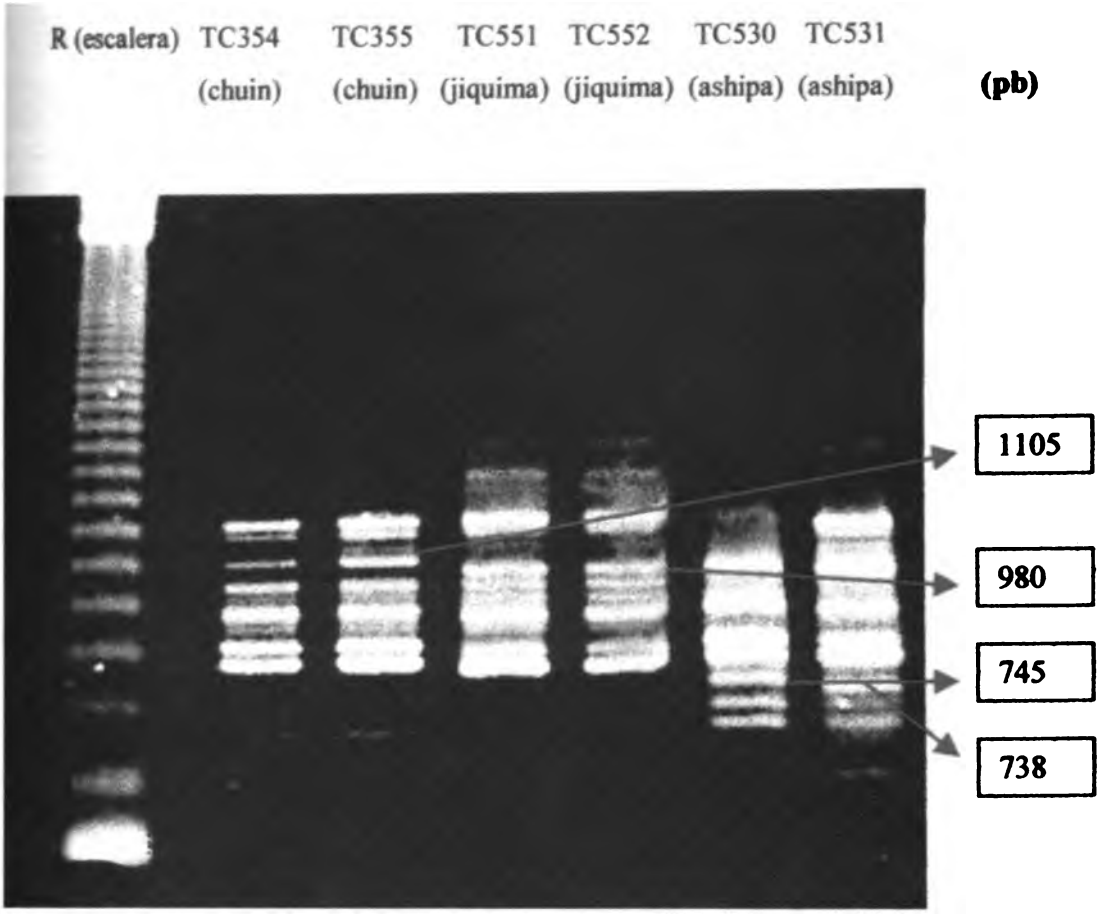


Figure 43. DNA amplification of *P. tuberosus* genotypes utilizing RAPDs and the UBC72 primer. Sample included accessions of the Chuin, Jiquima and Ashipa groups. (R = Reference DNA, with fragments revealed every 123 pb).

Germplasm evaluation

In order to further develop this marginalized crop, several experiments were implemented during the 1992-1999 period, which tested the adaptation of preserved genotypes to diverse ecological conditions, planting densities, and cropping systems. Those analyses will be useful for future genetic breeding of this crop, and their results can be summarized as follows:

Effect of the species considered and its origin

Morera (1994) reported that materials from *P. erosus* produced higher yields than that of other species. Also, materials from Mexico were better adapted to Turrialba conditions and generally performed much better than others in terms of growth and yield. Both conclusions were made after 8 trials comparing a total of 58 accessions (including 46 cultivated and 5 wild *P. erosus* genotypes, plus 3 *P. panamensis* –wild-, 3 *P. ahipa* –cultivated- and 1 *P. tuberosus* –cultivated- genotypes). The cultivated Mexican *P. erosus* accessions, EC-511, EC-532 and EC-542, appeared promising for cultivation in the Region. Other accessions producing remarkable yields were: EC-114, EC-502, EC-510, EC-536 & EC-557 (Mora & Morera, 1997b).

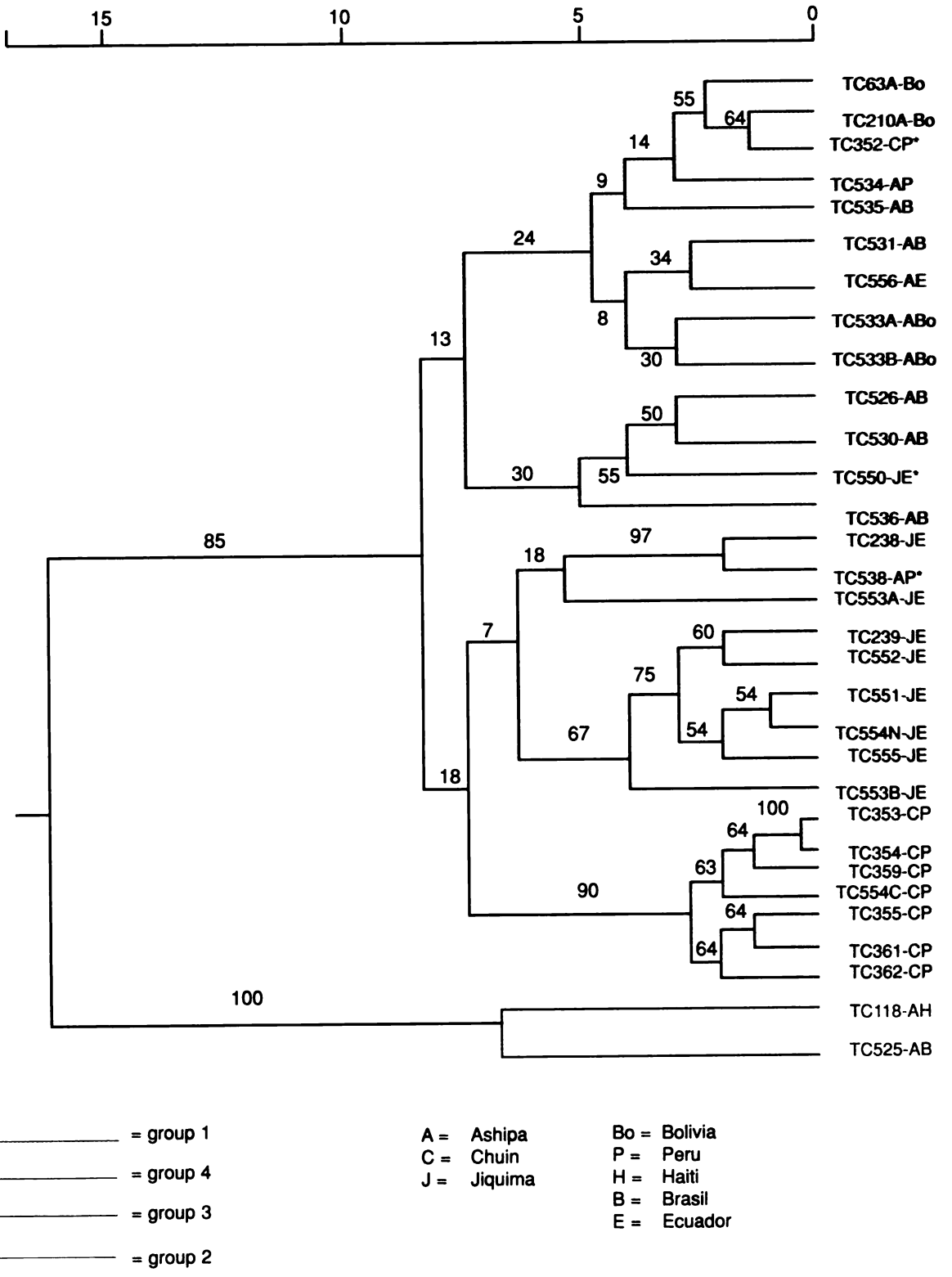


Figure 44. Dendrogram of 31 *P. tuberosus* accessions from CATIE germplasm collections, obtained after RAPD-based characterization and based on Nei's genetic distance analysis.

Effect of ecological conditions

At the beginning of the Yam Bean project, preliminary trials were carried out in four regions of Costa Rica, in order to test the performance of 58 *Pachyrhizus* accessions in diverse climates. Test sites included:

CATIE, Turrialba Valley (602 masl, 21.7°C, 2,630 mm of rain/yr, 88% RH, 130 hrs of solar bright/yr)

Estación Experimental Fabio Baudrit-UCR, Alajuela, Central Valley (840 masl, 22.4°C, 1,870 mm of rain/yr, 78% RH, 208 hrs of solar bright/yr)

EARTH, Guácimo, Atlantic Region (93 masl, 24.1°C, 4,400 mm of rain/yr, 89% RH, 107 hrs of solar bright/yr)

Río Claro, Southern Region (13 masl, 26.5°C, 3,700 mm of rain/yr, 87% RH, 158 hrs of solar bright/yr)

The following round of experiments were located in Turrialba and Alajuela. These two sites were selected because their climatic conditions appeared more suited to the needs of *Pachyrhizus* as determined in the previous tests. Furthermore, these trials concentrated on 10 *P. erosus* cultivated genotypes, in order to test their performance under more rigorous experimental conditions (CATIE, 1993; Mora & Morera, 1995; Mora *et al.*, 1994; CATIE, 1997).

Results from this analysis can be observed in Figure 45. There was a marked tendency with respect to location: Alajuela conditions appeared much more appropriate for all accessions tested, with average production for all genotypes almost double the yields achieved in Turrialba. Thus, Yam beans seem to prefer drier, sunnier zones (*ibid*).

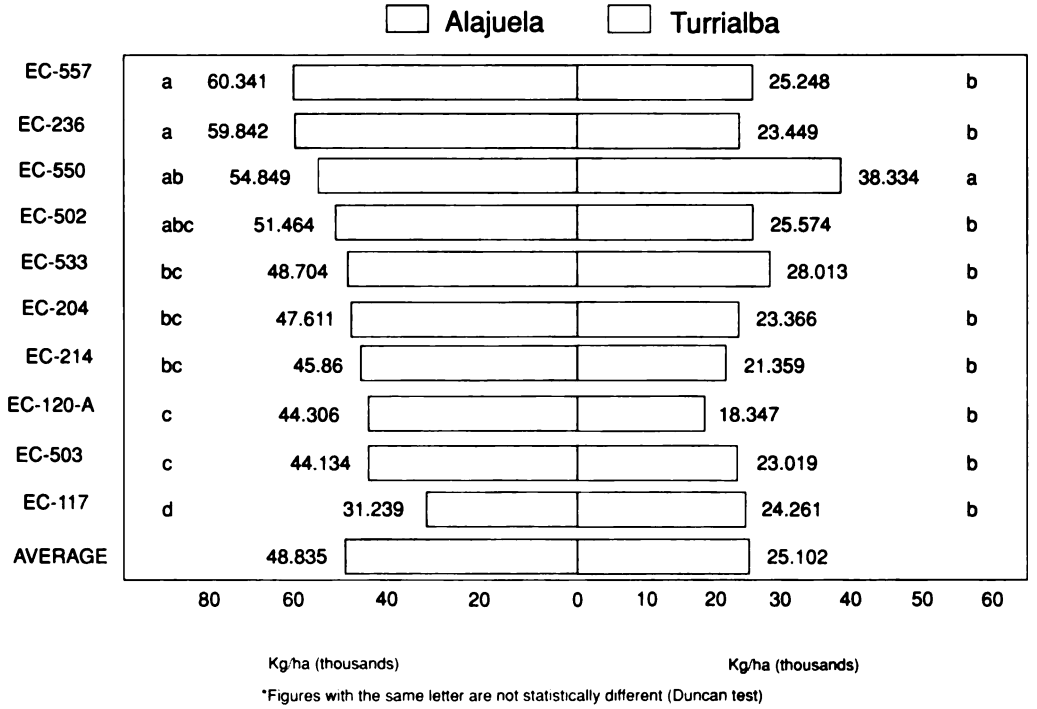


Figure 45. Average fresh weight of tuberous roots produced by 10 yam bean accessions evaluated in two different Costa Rican locations in 1993.

Effect of planting densities

Initial works with Yam bean at CATIE produced very low yields due to the low planting densities utilized (1-2 plants/ m^2). Because recommended planting distances vary considerably according to the length of the growth period, the genotype, the desired root size, and the length of days at planting time, several trials were conducted in order to approach the optimum planting densities for diverse accessions under Turrialba conditions (Mora *et al.*, 1996b; CATIE, 1997).

In a study evaluating three *P. erosus* accessions (EC-032, EC-509 & EC-534), a strong interaction was observed between genotype, planting density and the resulting number of small sized tubers. Planting distances of 0.1, 0.15 and 0.2 m between plants in a row (with 2 rows 0.25 m apart per ridge, and ridges separated 0.75 m), generated planting densities of 266,667; 177,778; and 133,333 plants/ha respectively. Accessions with similar fresh weight yields per hectare varied significantly in tuber size, a trait that will play an important role when selecting towards a specific ideotype (Figure 46) (*ibid*).

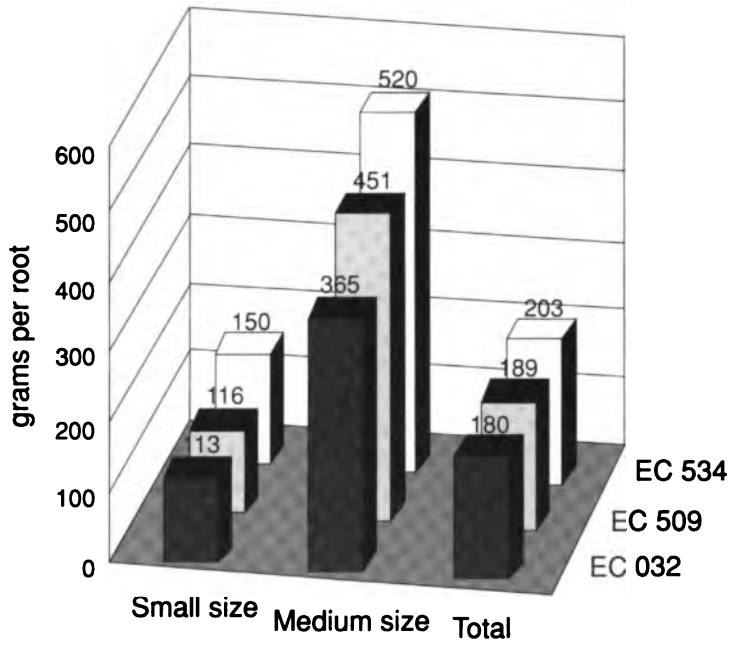


Figure 46. Average weight of tuberous roots produced by 3 *P. erosus* accessions of CATIE germplasm collection in 1995.

A planting distance of 0.1 m was not recommended for any of the three genotypes tested, as it increased the number of small-sized tubers obtained (no commercial value) (Figure 47). It must be noted that Yam beans are generally mono-tuberous, and thus with lower planting densities, less tubers were expected. But a compensatory effect occurred, which increased the size of the tubers produced at lower densities (Figure 48). Also, the lower the density, the higher the percentage of sown plants yielding tubers (92% at 0.2 m, 83% at 0.1 m), and therefore, the higher the total weight harvested. As a result of these variables, higher yields were obtained at a planting distance of 0.15 m, for 2 of the accessions tested (Mora & Morera, 1995; Mora *et al.*, 1996b; CATIE, 1997).

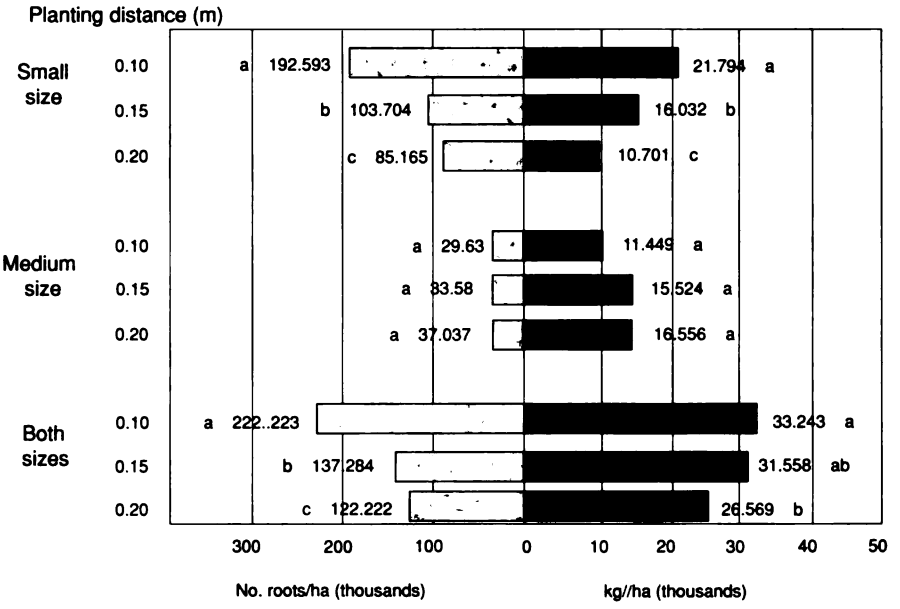


Figure 47. Average number and weight of total roots produced by 3 yam bean accessions sown in Turrialba at 3 different planting distances in 1993.

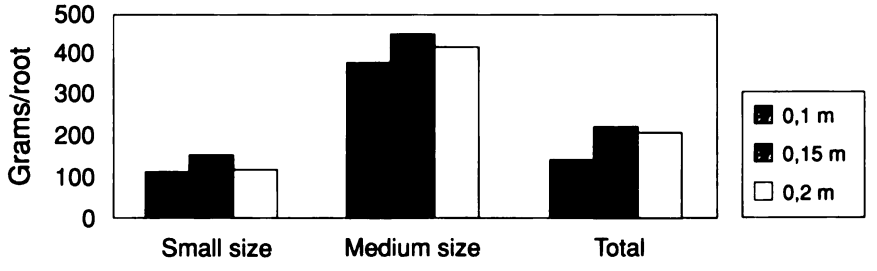


Figure 48. Average weight of roots produced by 3 *P. erosus* accessions planted at 3 different distances in 1995.

Effect of intercropping

In Latin America, cassava is planted as an intercrop 40% of the time, combined with crops such as beans and corn. Combination with leguminous crops is preferred in order to generate a more sustainable system, and thus, yam beans have been proposed as a good alternative for planting with manioc. Nevertheless, a detailed study undertaken at CATIE with 5 *P. erosus* accessions demonstrated a drastic reduction in yam bean yields occurring as a result of intercropping (Figure 49).

Reductions were more evident in the second yam bean cycle, and result from the increased shading provided by taller cassava plants, which diminished radiation received by the yam bean plants by 50%. Of the accessions tested, EC-509 was more affected by shade, and this fact suggested that more genotypes should be tested in order to ascertain whether accessions more suited to intercropping conditions exist or need to be developed. Also, despite reduction in yam bean yields, intercropping could be an interesting alternative system as combining both crops offers a good alternative to monoculture, adding a protein-rich source (Mora & Morera, 1997; Morera et al., 1998; Mora *et al.*, 1998; CATIE, 1997).

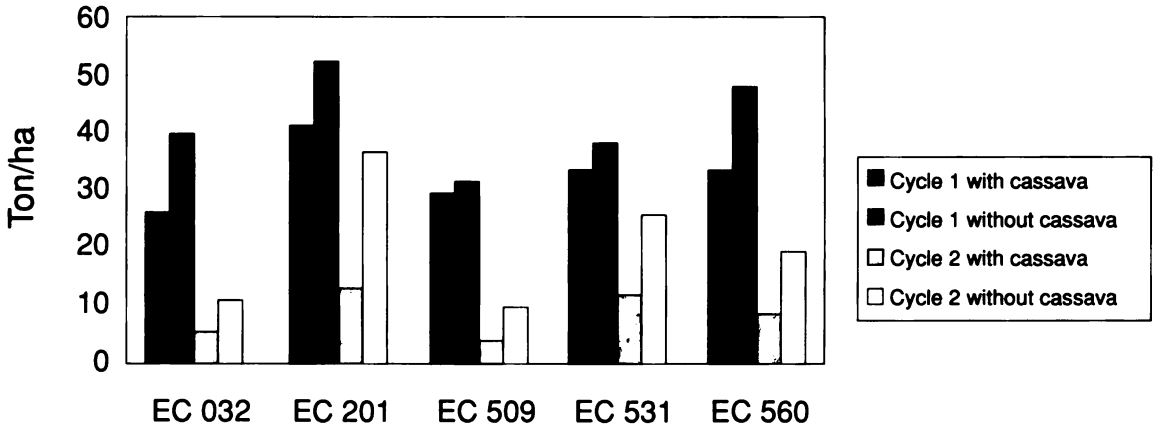


Figure 49. Cycle interaction per system per accession for yam bean total weight of roots in 1996

Effect of flower elimination

Since yam beans were first planted at CATIE, it became evident that tuber and reproductive growth (flowering and pod filling) compete in this crop. Thus, diverse trials were conducted at CATIE to assess the effects of removing flowers from yam bean plants. One of those analyses evaluated the behavior of 5 accessions (EC-114, 509, 511, 523 & 536), and revealed that after manual flower removal, production of tuberous roots increased by an average of 116%. However, results varied according to genotype: in the case of EC-536, production increase was 6-fold in response to flower pruning. Yield increases were higher in early-flowering accessions, as competition for assimilates between shoot and tuber was halted at an earlier growth stage (Mora *et al*, 1996a; CATIE, 1997).

In addition, utilization of the herbicide 2,4-D as a chemical method to eliminate flowers was also tested at CATIE during 1995-96. Plants treated with two concentrations of the herbicide (50 ppm and 100 ppm) increased production of small sized roots and foliage weight, but produced significantly inferior yields compared to those obtained after manual elimination of flowers. Yields were slightly higher in the lower concentration treatment, indicating that the higher the concentration of the herbicide, the worse the effect on yields. Nonetheless, other chemical products should be assessed as options to reduce pruning costs (Mora & Morera, 1998; CATIE, 1997).

Horticultural Crops

During the 1992-1999 period, effort was made to characterize the diversity of the germplasm collections preserved at CATIE for very important horticultural crops, specifically: Peppers, Tomatoes, Pumpkins and Yams. Results from those activities are reported below.

Tomato

CATIE's tomato collection preserves some 350 accessions of *Lycopersicon esculentum*, the majority of which were collected between 1976-1986 in Mexico, Guatemala, Panama and Peru; some materials were also obtained from collections in Taiwan and the USA (Phillips-Mora *et al.*, 1996). Characterization and evaluation tests run on those materials during the 1990's are described below.

Germplasm Characterization

In 1995, a sample of 96 accessions from this collection were analyzed with molecular markers to determine the extent of the genetic variability within the collection. The study was conducted in collaboration with the University of Wisconsin. Based on 103 RAPD bands, the genotypes of the CATIE collections were located on a MDS plot in which the genetic distances are shown as physical distances (Figure 50).

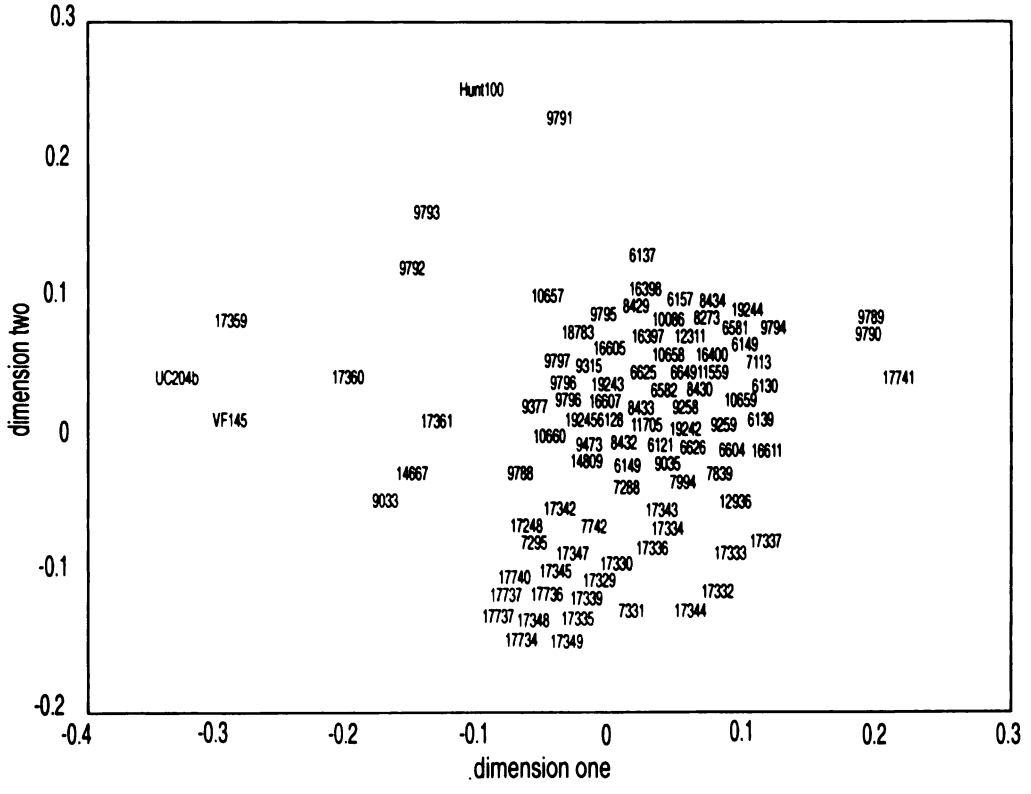


Figure 50. MDS plot of genotypes analyzed for 103 RAPD bands in CATIE *Lycopersicon esculentum* germplasm, 1995.

Mesoamerican genotypes were grouped into 2 clusters: one with accessions from Panama, and the other with accessions from Mexico and Guatemala. Some Taiwanese genotypes were grouped together with those from Panama, while the rest were dispersed in the plot. Genotypes from the USA were clearly different from the rest and corresponded mainly to commercial varieties. Knowledge of these genetic differences is very useful because it facilitates the efficient sampling and utilization of germplasm resources (*ibid*).

This work also aimed at comparing the genetic characteristics and variation between the CATIE and AVRDC collections. As evidenced by Figure 51, the AVRDC and CATIE collections appeared complementary, despite some overlap. This fact stressed the uniqueness of the materials preserved at CATIE, and the importance of conserving them (CATIE, 1995).

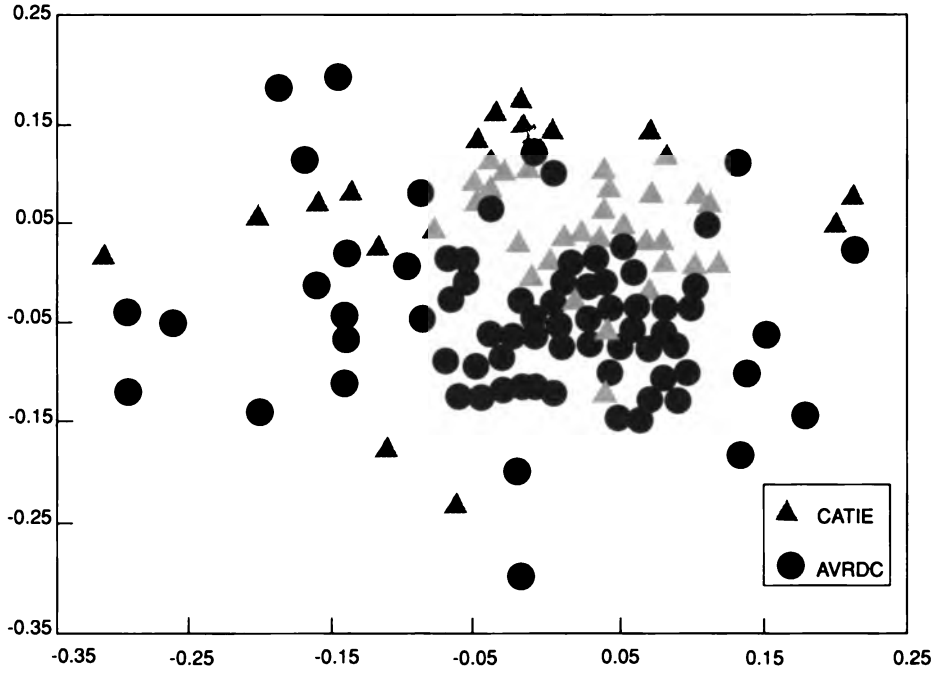


Figure 51. MDS plot of tomato genetic diversity among germplasm accessions from CATIE and AVRDC.

Germplasm Evaluation

Between 1992-93, a greenhouse evaluation of the resistance to tomato bacterial wilt (*Pseudomonas solanacearum*) was performed in a sample of CATIE's tomato germplasm which included 223 accessions plus 7 commercial cultivars. Resistance was evaluated on tomato seedlings against four *P. solanacearum* strains (UW-25, UW-256, UW-130 & UW-255) of economic importance in Central America. Evaluations were based on a disease index which proved to be a good criterion to select resistance as well as to evaluate large amounts of plant materials (González, 1993).

No immunity was found in any material, but some resistance was found for each of the strains tested: 5 accessions *vs* UW-25; 24 *vs* UW-256; 49 *vs* UW-130; and 32 *vs* UW-255. But

more importantly, some introductions showed resistance to more than one strain, and the following deserve specific mention:

- Accessions with some resistance against all 4 strains tested: 17334, 17740, Hawaii 7998 and Rotam 4.
- Accessions with some resistance against 3 of the strains tested: 17137, 17333, 17343, 17345, 17349, 17740 and 116-5.

These materials will surely play a strategic role in future breeding efforts against this important disease (*ibid*).

More recently, 27 *L. esculentum* accessions plus one commercial cultivar were screened for resistance to Tomato Yellow Mosaic Virus (ToYMV). Plants were inoculated with viruliferous whiteflies (*Bemisia tabaci*), the natural vector of this geminivirus. The flies acquired the virus after feeding for 24 hours on infected plants and were then transferred to the trial plants for 48 hours. Screened materials were later examined for the detection signal of viral DNA, utilizing a nucleic acid spot hybridization technique, and estimating the intensity of said signal based on a 0 to 4 scale (0 = no signal, 4 = positive and very strong). The development of symptoms was evaluated at 1 week intervals, and symptom severity was rated based on a scale suggested by Rom *et al.*, (1993)[4] (Rivas-Platero *et al.*, 1997; Rivas-Platero 1998; Rivas-Platero, 1999; CATIE, 1998).

The detection signal was positive in the vast majority of the materials analyzed. Only 2 accessions (7113 & 9035) presented neither signal nor symptoms. Also 44% of materials tested showed severity indexes below 1.5 (*i.e.* mild reduction in plant size and moderate chlorosis in apical leaves). The control cultivar (Hayslip) presented a severity index of 3.8. Two accessions also presented high susceptibility to ToYMV: 14667 and 6604, with severity indexes of 3.7 and 2.8 respectively (*ibid*).

The viral DNA hybridization test made it possible to detect the virus presence in every tomato line tested, and could therefore be incorporated into programs selecting germplasm for geminivirus resistance (*ibid*).

Pepper

Pepper is also a very important horticultural crop native from the Neotropics. It was the first spice found by the Spaniards in the New World, and was rapidly distributed to other regions. There are 5 cultivated species: *Capsicum annuum, C. chinense, C. frutescens, C. baccatum* and *C. pubescens*. CATIE germplasm collection contains more than 1,500 *Capsicum* accessions. The collection includes more individuals from the first three species listed above than for any other (Ferreira *et al.*, 1995). Efforts to characterize and evaluate a portion of these materials during the 1990's are summarized below.

Germplasm Characterization

Following the same methodology described for tomato, a large sample containing accessions from CATIE pepper collection plus US commercially grown varieties, were analyzed in 1995 utilizing molecular markers. This study, undertaken jointly with the University of Wisconsin, aimed at determining the extent of the genetic variability within this collection.

[4] Plant Disease 77: 253-257.

The analyzed genotypes were located on a MDS plot showing the genetic distances as physical ones (Figure 52). CATIE's collection demonstrated a rich genetic diversity, showing also evidence of related species that might be not appropriately classified yet.

On the other hand, a morpho-agronomic characterization of 1,070 accessions of CATIE's pepper germplasm, including materials from Costa Rica (257), Guatemala (229), Mexico (98), Peru (78), Ethiopia (69), Panama (44), Honduras (43), El Salvador (22), and some other countries, was implemented from 1993-1995, gathering data on 21 qualitative and 5 quantitative characteristics (*ibid*).

After performing a canonical discriminating analysis on the information gleaned, a total of 8 groups were identified which differed basically in the position of the flower during anthesis, the characteristics of the fruit (persistence, neck presence in the fruit base, fruit position and shape), and the number of days until physiological maturity of fruit. Those variations differ greatly depending on the species and the geographical origin of each accession (*ibid*).

Group 1 comprised basically all *C. frutescens* accessions (147), presenting erect flowers during anthesis with purple anthers and blue filaments, as well as erect fruits of high persistence. Groups 3 and 4 included almost all *C. annuum* accesions (175 and 112, respectively), presenting erect flowers during anthesis and low fruit persistence. Group 4 differed from group 3 in its conical fruit shape and erect growth habit. Group 5 was very similar to group 4 but did not show the same growth habit; it included the large majority of *C. baccatum* accessions plus the remaining *C. annuum* introductions (192 in total). Group 8 comprised almost all *C. chinense* introductions (226). All remaining groups comprised individuals of diverse origin, whose classification has been more difficult; they present few but marked differences from the other groups (Figure 52) (*ibid*).

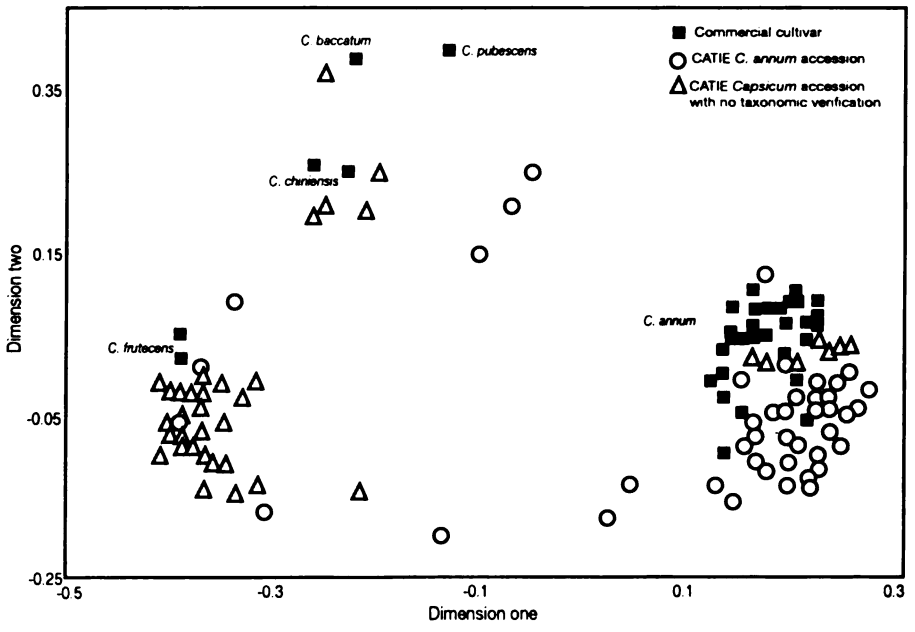


Figure 52. MDS plot of pepper genetic diversity among germplasm accessions from CATIE and commercially grown peppers in the US.

More recently, Mora *et al.* (1999a) conducted a limited study characterizing 100 *capsicum* accessions[5] of a more diverse geographical origin (14 countries), and based on more traits (70 used by IPGRI as descriptors for this genus). Ten groups were identified after hierarchic grouping analysis, whose conformation was determined by a set of 32 qualitative and 8 quantitative characteristics that presented the highest discriminating value. Among those, the following stand out as essential to simplify collection characterization (Table 19).

TABLE 19. Qualitative and quantitative traits presenting the highest discriminating value to classify *Capsicum* spp. germplasm.

Qualitative variables	Quantitative variables
Hypocotil pubescence	Fruit length/width ratio
Flower position	# days to fruiting
Corolla color	Days to flowering
Color of the corolla spot	Filament length
Filament color	Fruit length
Fruit shape	Stem diameter
Fruit base shape	# seeds per fruit
Pungency	Mature leaf width

According to this study, Costa Rican materials were mainly classified in groups 3, 5 and 6; Mexican accessions were categorized into groups 4, 7 and 8; Guatemalan introductions were grouped into sets 6 and 7; Peruvian materials made up the majority of groups 1, 9 and 10; and materials brought from Russia were also classified into group 3. Groups 2 and 3 comprised basically non-pungent *C. annuum* materials. While group 4 also contained *C. annuum* accessions, these showed very high pungency. Groups 5 and 6 included almost all *C. frutescens* materials. *C. chinense* included the majority of the highly pungent genotypes, but they were distributed among 5 groups, showing the high variability of this species. Groups 7 and 8 were the most numerous in this study, and encompassed materials from diverse species. In addition, several sub-accessions could not be classified down to the species level (Mora *et al.*, 1999a).

Results of this study were also analyzed through the genetic distance matrix. A dendrogram showing the degree of similarity among subaccessions, their distribution in 10 groups, and the variability within each group, was generated as a result and will be published shortly.

Dendrograms are useful to sample the variation present among all accessions tested, which could greatly facilitate the creation of a core collection focused on future research and breeding efforts (CATIE, 1999).

Germplasm Evaluation

The fungal wilt of red pepper caused by *Phytophthora capsici* is a severe disease which may produce losses between 10 to 100%, according to the resistance and management of planted cultivars. Cultural and chemical practices are not sufficient to control the pest in the majority of pepper fields, and genetic resistance has emerged as the most appropriate

[5] Given phenotypic variations within accessions, they were divided into 136 sub-accessions.

option to face this problem (Mercado & Bustamante, 1993). Saborío & Jiménez (1993) evaluated 40 genotypes of CATIE *Capsicum* germplasm, including accessions of *C. frutescens, C. annuum* and *C. chinense*, against four different isolations of *P. capsici*. Mortality rates resulted between 2.7 and 100%. No immunity was found, but major resistance was found in 'Santaka' and 'Pimentão Nacional', two commercial *C. annuum* cultivars developed in Japan & Brazil. At the same time, 'Panameño' cultivar (*C. chinense*) was highly susceptible to this disease. High variability was found among all other *C. annuum* and *C. frutescens* materials, but this work helped identify some promising genotypes for future breeding.

Figure 53 shows results from a preliminary study in which 9 *Capsicum* materials were tested against a set of 8 different organisms (pests and diseases). Low variability was found in the response of those accessions to aphids, whiteflies and *Cercospora* spp. For all other organisms tested, accessions varied from immune to highly susceptible, according to the severity indexes utilized. These results were only preliminary, and this study will have to be extended to a larger sample of genotypes, but a valuable variability was found, which could be exploited in future genetic improvement programs (CATIE, 1995).

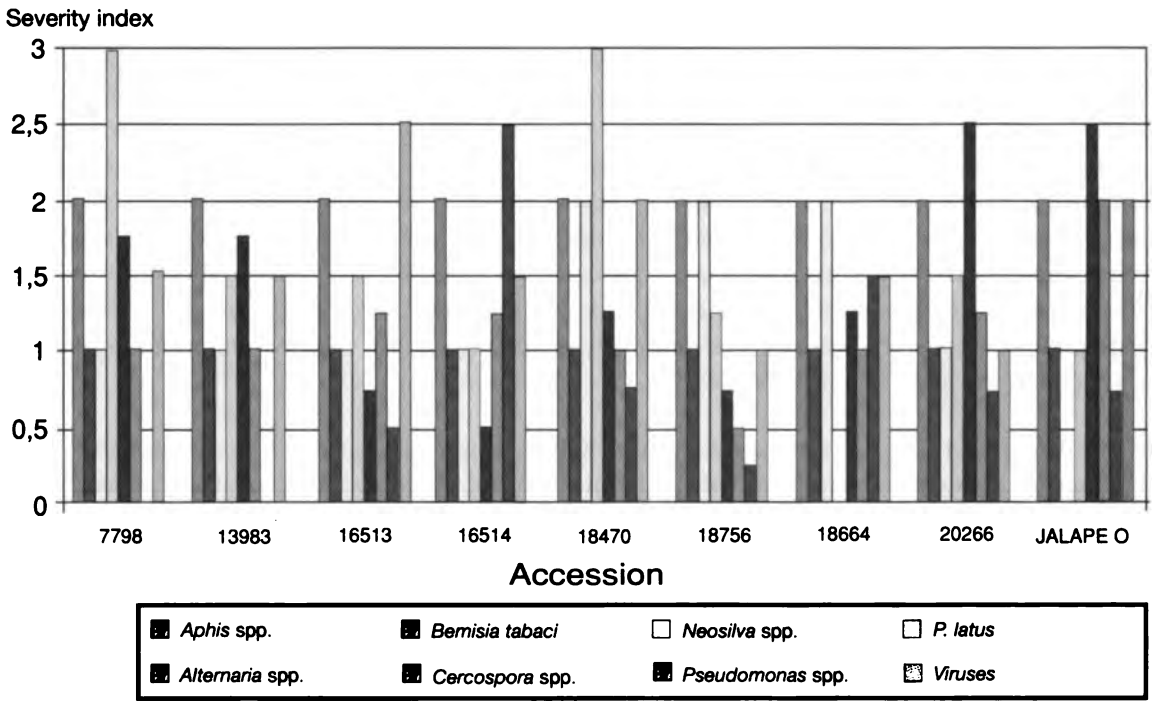


Figure 53. Reaction of *Capsicum* spp. accessions to some pests and diseases in 1995.

Seminole Pumpkin

Cucurbita moschata is the Cucurbitaceae species most widely extended in the American tropics, given its adaptability to diverse humidity and temperature patterns. It has been cultivated in Mexico since at least 1500 BC and it is found growing wild throughout Mesoamerica. It is a high-

ly variable species mainly utilized as a vegetable and in the preparation of preserves. Its immature fruits and stems, as well as its flowers, are also consumed (Mora *et al.*, 1999b).

Germplasm characterization

CATIE preserves more than 1,500 *C. moschata* introductions in its germplasm collection but they have had limited use in breeding programs because the materials have not been characterized. In 1999, 282 accessions were utilized to undertake the preliminary characterization of this species. High variability was found among individuals from the same accession, requiring the sample to be separated into subaccessions. Thus, a total sample of 322 genotypes was assessed for a set of 53 characteristics utilized by IPGRI as descriptors for this species (Mora *et al.*, 1999b).

Main traits utilized to classify materials in the field were fruit shape, size, and color. Table 20 summarizes the main statistics generated by this characterization effort, to date. Highest variation coefficients were found for fruit weight and length[6] (61 and 44.5%, respectively). This characterization process has yet to be completed, and data analysis will be undertaken soon to classify the diversity of *C. moschata* into groups, and to generate a dendrogram similar to that obtained for *Capsicum* spp. (*ibid*).

TABLE 20. Main statistics for quantitative traits measured in 322 *Cucurbita moschata* materials from CATIE germplasm collections.

Variable	Minimum value	Maximum value	Average	Variation coefficient (%)
Internode length (cm)	5.1	26.1	14.9	30.2
Days to flowering	41.0	112.0	69.8	18.6
Days to physiological maturity	71.0	202.0	117.4	17.3
% Dry matter	3.2	26.7	9.4	41.8
Fruit weight (g)	131.6	13,000.0	2,153.9	61.2
Fruit length (cm)	5.8	55.0	20.9	44.5
Fruit width (cm)	6.8	32.0	15.9	24.0
Peduncle length (cm)	2.0	14.5	5.5	32.8
Fruit shell width (mm)	0.4	6.4	2.5	40.7
Flesh width (cm)	0.5	6.6	2.4	37.2
Diameter of fruit cavity (cm)	5.1	21.7	10.2	24.0
Weight of 100 seeds	3.3	22.8	11.0	24.8
# seeds per fruit	56.0	974.0	454.1	34.5
Seed length (mm)	11.2	20.0	15.7	8.9
Seed width (mm)	5.6	11.5	8.2	10.2
Seed caliper (mm)	1.6	5.1	3.1	15.3
Fruit length/width ratio (cm)	0.5	4.3	1.4	55.0
Seed length/width ratio (mm)	1.5	2.5	1.9	8.6

Germplasm evaluation

A set of 27 accessions of *C. moschata* from CATIE germplasm collection was analyzed for their response to powdery mildew (*Oidium* spp.) and viruses (basically a viral complex which includes the Cucumber Mosaic Virus, or CMV, and the Watermelon Mosaic Virus race 2, or WMV-2). All accessions were susceptible to powdery mildew, with very high plant disease proportions (Pdp > 0.7) 40 days after inoculation. Line 8003 responded best in terms of severity. In the case of viruses, the Pdp was highly variable and accessions 5957, 7416,

[6] *E.g.*, averages of 10 fruits per subaccession ranged from 300 to 6000 g in weight, and individual extremes varied from 132 g to 13 kg.

9065, 9818, 9819, 9820, 10664, 10719 and 20122 presented intermediate resistance. These analyses will be extended in the future to a larger sample, but initial results are promising for breeding purposes (Rivas-Platero, 1999).

Yams

Diverse species of the *Dioscorea* genus represent a very important staple crop in the world, mainly in areas with low grain output. One of the main problems faced by yam producers is disease, particularly Anthracnose (*Colletotrichum gloeosporioides*) and the Yam Mosaic Virus (YMV). The management of those diseases has been very difficult, and the generation of genetically resistant varieties is seen as the most reliable and sustainable option available (Rivas-Platero & Chavarría, 1997).

In order to contribute to that goal, a study was undertaken at CATIE to evaluate tolerance to *Colletotrichum* spp. and to YMV in 27 *Dioscorea* spp. accessions of CATIE's germplasm. For Anthracnose, a severity index proposed by Simons & Greene (1994)[7] was utilized, while the severity of YMV was evaluated according to a scale proposed by Rivas-Platero as follows:

0 = No infection,
1 = Moderate mosaics,
2 = Strong mosaics without apparent effect on plant growth,
3-5 = Typical symptoms of wrinkled leaves with evident reduction in plant growth ranging from mild (3), to severe (5).

Low *Colletotrichum* infection levels were recorded on accessions: # 7390; 11684; and 11685 with indexes close to 2. Also, accession # 7390 presented interesting results because of its good recovery after anthracnose infection, a disease which is more devastating on mature leaves (CATIE, 1995 & 1997; Rivas-Platero & Chavarría, 1997; Rivas-Platero, 1999). Figure 54 shows results of anthracnose severity analysis on part of the tested accessions.

According to this study, diverse mechanisms to respond to this anthracnose may be present in different accessions, but in general it was found that potassium (K) content in leaves determines the severity of infection. This relationship was so strong that the following model was developed to explain this behavior: $Y = 6.3 - 0.98\ X$ ($r2 = 0.57$; $p < 0.01$), where Y represents the severity index, and X represents the K content. This equation might be useful as a selection parameter for future germplasm evaluation (Rivas-Platero & Chavarría, 1997; Rivas-Platero, 1999).

For YMV, least infected lines were: # 6328; 7390; and 12451 although the severity indexes found were low overall (average < 1.7) (Figure 55). For viruses, no correlation between K content in leaves and severity index was found (*ibid*). The combination of both diseases in susceptible lines reduced yields significantly (< 1 kg/plant). This study needs to be expanded to a larger sample, but already constitutes a good source of information by identifying promising materials for breeding programs (*ibid*).

[7] Tropical Science 34: 216-224

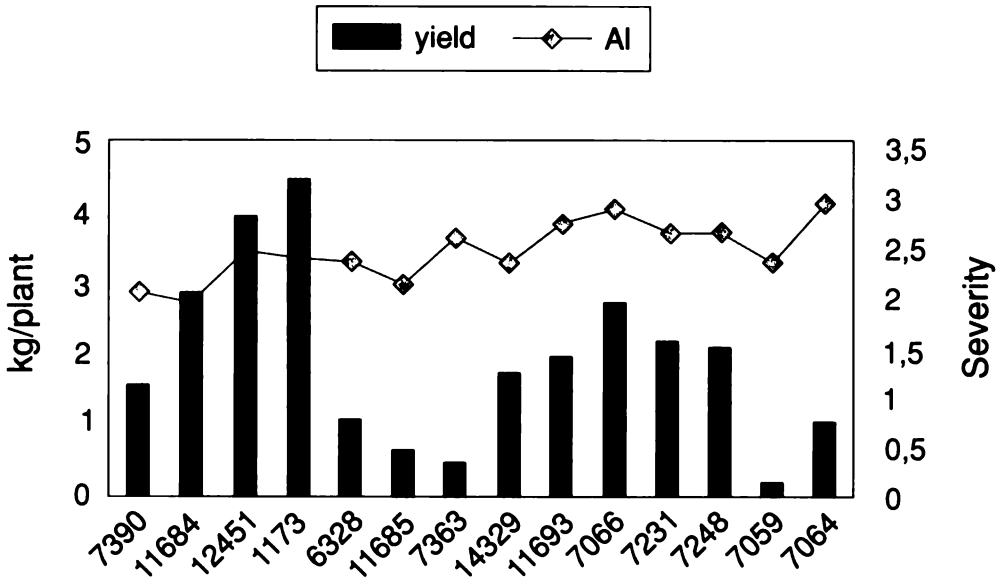


Figure 54. Anthracnose severity indexes (AI) and yield (kg/plant) for 14 *Dioscorea* spp. accessions from CATIE germplasm collections.

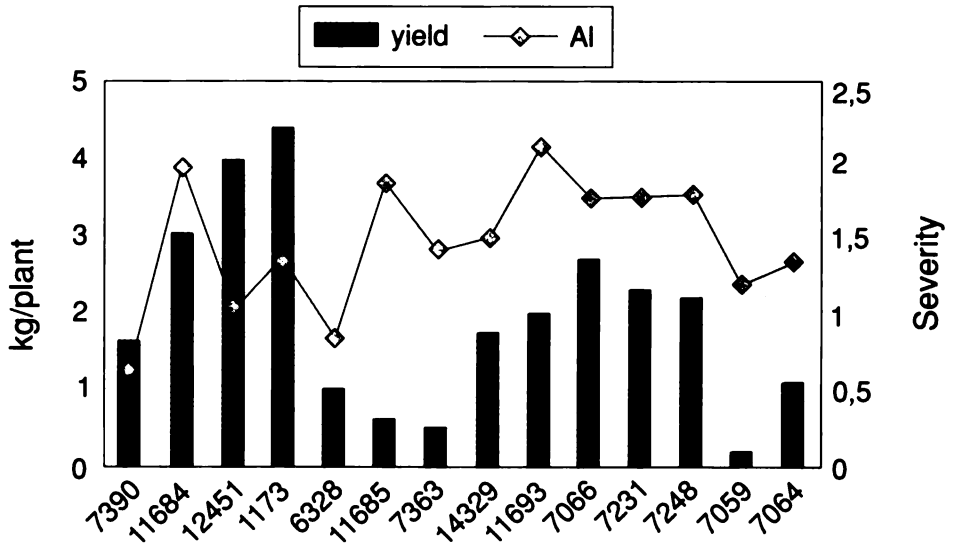


Figure 55. Yam Mosaic Virus severity indexes (VI) and yield (kg/plant) for 14 *Dioscorea* spp. accessions from CATIE germplasm collections.

PROPAGATION

Enhancing reproduction and distribution of strategic genetic materials.

Sapotaceae

Field collections present a lot of difficulties for conservation of tropical fruit trees such as those belonging to the sapotaceae family, as the large size of individual trees (up to 30 m) mean large space requirements and high maintenance costs. Moreover, according to IPGRI (1998), Turrialba conditions are not ideal for growing sapotacean fruit trees, and the vegetative propagation of these allogamous species was not very successful at the beginning of the 1990's. These factors imposed a huge threat to the long-term preservation of some of the world largest sapotaceae germplasm collections maintained at CATIE.

For those reasons, the Center began several studies to improve the propagation of sapotacean fruit trees as part of the Project entitled, "Diversity, Conservation and Sustainable Use of Native Fruit Germplasm of Tropical America". These included work on *in vitro* propagation, as well as enhancement of the grafting technique utilized for these species. These efforts are reported in the present section.

Vegetative macropropagation

Sapote trees have traditionally lacked an efficient propagation method for elite materials. Grafting and attempts at rooting stakes and cuttings at CATIE for the sake of maintaining the germplasm collection, customarily produced low levels of success (Lobato *et al.*, 1999).

In 1997, CATIE finally developed a successful grafting technique for this species, that was welcomed and immediately diffused by IPGRI in Mesoamerican countries (IPGRI, 1998). This methodology was designed by Umaña (1997), building upon previous procedures also developed at CATIE (Gutiérrez, 1986). Basic elements of this method include:

- Grafting technique: Side Veneer or Spliced Side Graft (Figure 56).

- Scion: twigs with 14-18 mm in diameter and 12-15 cm long, holding 10-20 lateral dormant buds. They must be grafted into scion as soon as possible in order to assure the highest attachment rates. Best scions are twigs obtained from defoliated trees that have swollen but closed apical buds (if the tree is at another physiological stage, twigs must be prepared beforehand, by defoliation or another procedure, but success rates will be lower) (Figure 57).

- Rootstock: healthy and well-shaped young trees, with the sufficient diameter to make the grafting at least 25 cm above ground. They must not be moved from the nursery for at least 90 days before grafting. Once the grafting has been achieved, the top 10 cm of the stock are cut back on the same grafting day. Half the remaining stock crown is cut back once the lateral sprout reaches 5 cm long, and the rest is eliminated back to the joint when the growing scion gets to be 10 cm long.

- Compatibility: not all genotypes present adequate compatibility for grafting: 10617-1 achieved a maximum of 50% success when grafted on diverse stocks, while 10669-3 and 12007-1 presented 100% on the same rootstocks.

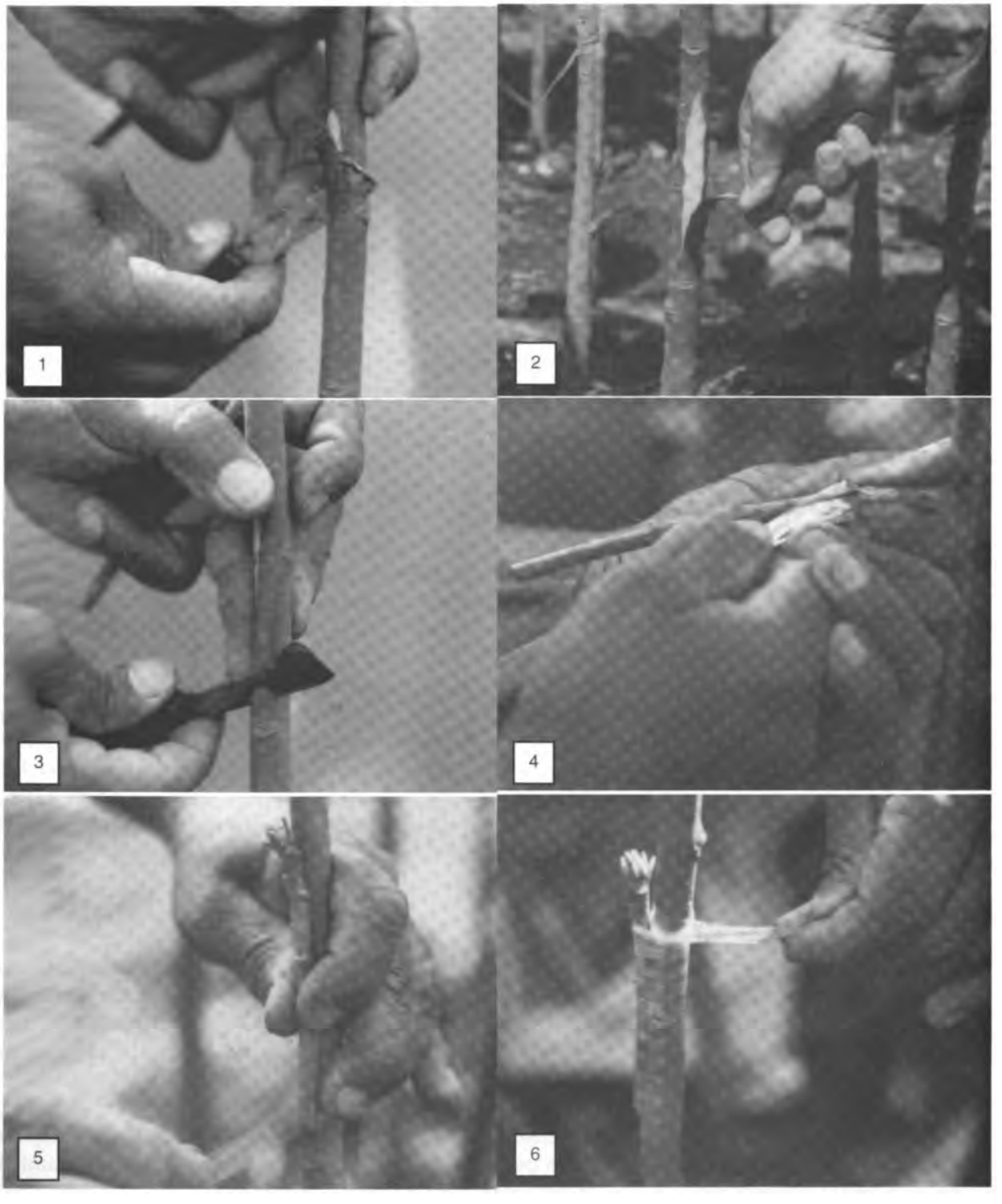


Figure 56. Steps for successfully grafting *Pouteria sapota* materials, according to a side-veneer method designed and tested at CATIE.

Figure 57. Twigs excised from *P. sapota* trees in the best physiological stage to be utilized as scions for grafting (defoliated, swollen but unopened buds).

Diverse trials were implemented at CATIE to test this methodology. Lobato *et al.* (1998) obtained in average a 50% success rate when grafting three different genotypes on accession 10641-1 as rootstock, utilizing the methodology described above. Meanwhile, those figures decreased to 25% when the technique used was cleft grafting. According to these studies, controlled greenhouse conditions favored grafting success (Lobato, 1998; Lobato *et al*,. 1998).

It was also found that diverse anatomic characteristics of *P. sapota* limit the success of grafting. On the one hand, fiber bands previous to the cambium in the stems, interfere in dedifferentiation processes which are fundamental for grafting (as well as for rooting stem cuttings, a technique that has not been very successful in this species[8]) (Figure 58). However, the presence of numerous resinous channels in the bark of some genotypes might also interfere with their response to grafting, because the presence of latex favors rot (Lobato *et al.*, 1999) (Figure 59).

In vitro *propagation*

In vitro techniques have also been developed for the vegetative reproduction of sapotacean species. Procedures were developed to successfully grow *P. sapota* and *M. zapota* plants from zygotic embryos, as well as to regenerate plantlets from those vitroplants by cultivating nodal sections with at least one bud[9]. It was also possible to obtain callus formation on cotylendon sections for both species (Astorga & Escalant, 1996b; IPGRI, 1998).

Lobato (1998) recently encountered serious difficulties in the *in vitro* propagation of *P. sapota*, although relevant progress was achieved in overcoming limitations imposed by culture contamination and oxidation in these species. Disinfection with sodium hypochlorite (0.53 or 1.06%) and treatment with activated carbon plus citric acid to prevent oxidation, were recommended for future trials.

[8] Stem cuttings of 12-15 cm long and 14-17 mm in diameter were tested in rooting trials, utilizing diverse concentrations of IBA to promote root formation, and diverse substrates (soil, sand, and a 1:1 mix) in sub-irrigation propagators, without obtaining satisfactory results, except for accession 10600-2 (Lobato, 1998).

[9] Endogenous bacterial contamination limited in vitro propagation trials with *C. cainito* (Astorga & Escalant, 1996a).

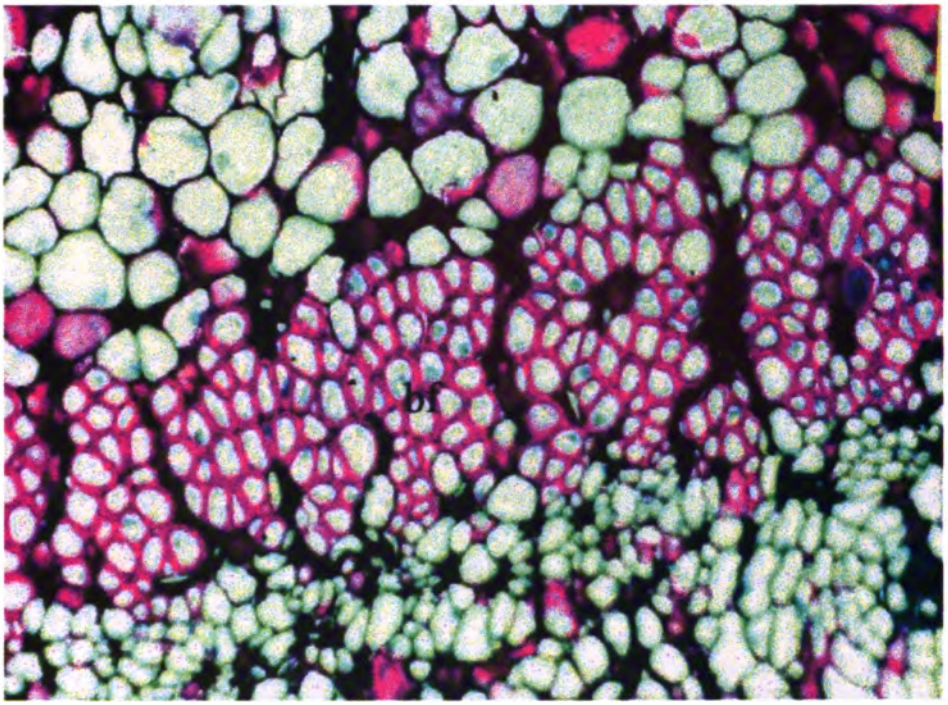


Figure 58. Transverse section of *P. sapota* stems showing a band of protophloic fibers (bf), CATIE.

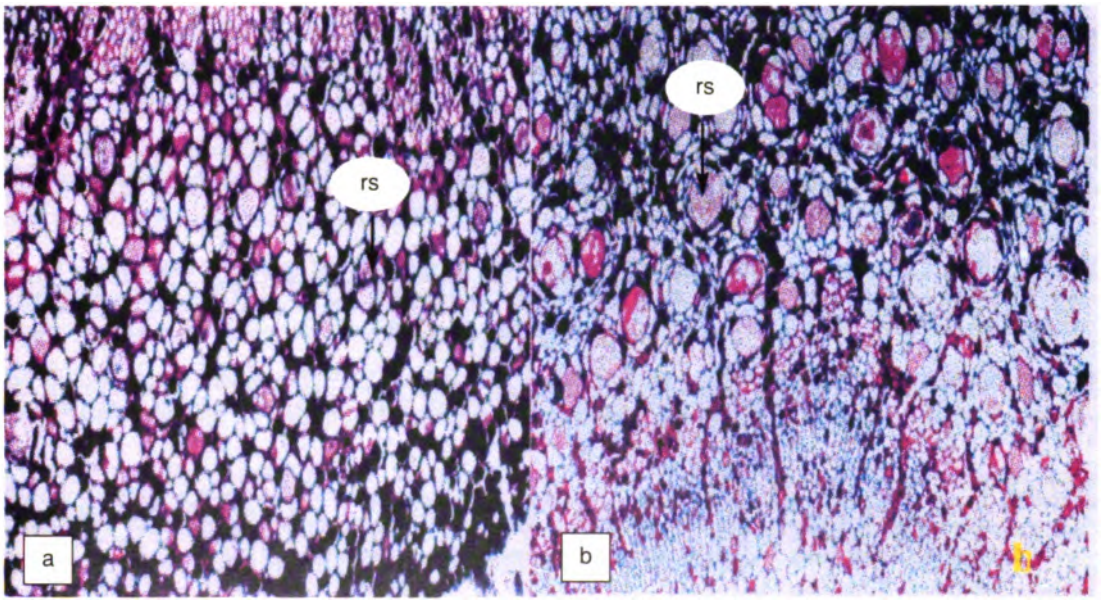


Figure 59. Transverse sections of *P. sapota* stems showing the cortex parenchyma, CATIE. a. Accession 11251-1, showing few and small resinous channels (rs). b. Accession 10800-2, presenting larger and more numerous rs.

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