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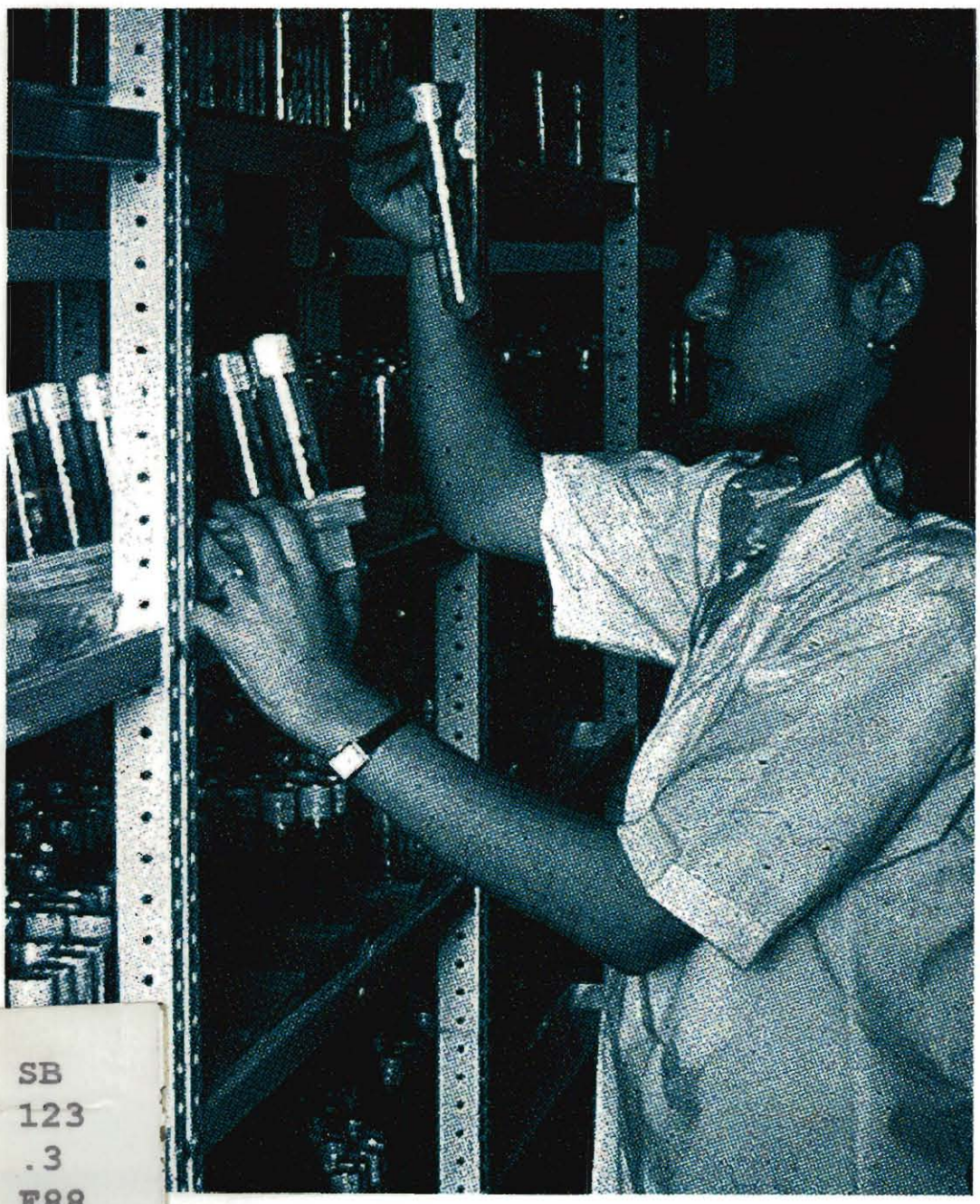
Biotechnology Research Unit and Cassava Program, CIAT, Cali, Colombia



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FOREWORD

This report describes the outcome of collaborative research between the Centro International de Agricultura Tropical (CIAT) and IBPGR. The need to explore various practical and biological aspects of managing an *in vitro* genebank was identified by the IBPGR Advisory Committee on *In Vitro* Storage at a special meeting in 1985. *In vitro* techniques were recognized at that time to hold great promise for the genetic conservation of problem crops that could not be stored as seed. However, although there were many collections of *in vitro* cultures in existence, most research had concentrated on the development of techniques *per se*, rather than seeking to explore rigorously the most appropriate procedures to follow and the inputs necessary to maximize the efficiency and security of this approach to genetic conservation. To address this, the Pilot *In Vitro* Active Genebank was conceived. In this endeavour, IBPGR was fortunate to establish an excellent collaboration with the staff of the CIAT Biotechnology Research Unit, under the leadership of Dr. William M. Roca, a member of the IBPGR Committee. The experiences of Dr. Roca and his colleagues as set out in this report will provide unique insights into the complexities of managing an *in vitro* genebank for cassava and will, undoubtedly, provide an extremely useful baseline to capitalize upon that experience in the management of collections of other crops.

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1. Scientific adviser to the project and IBPGR *In Vitro* Conservation Officer, 1988-1992. This post is now held by Florent Engelmann, whose assistance in preparing the report for publication is gratefully acknowledged.

1. BACKGROUND AND JUSTIFICATION

The potential of *in vitro* culture methods for the conservation of the genetic resources of vegetatively propagated crop species was recognized during the late 1970s. The International Board for Plant Genetic Resources (IBPGR) established, in the early eighties, a working group of scientists to look at all aspects of *in vitro* plant genetic resources conservation. As the outcome of the work of this group, a series of actions relevant to *in vitro* conservation were advanced (Withers 1980, IBPGR 1983): a) the basic conceptual framework for *in vitro* conservation was clarified; b) species requiring priority attention for *in vitro* conservation were identified; c) several critical areas of research in *in vitro* conservation were identified such as: i) the problem of genetic instability of cultures: ii) need for parameters for characterization of accessions entering *in vitro* storage; iii) need for disease indexing techniques at the *in vitro* level; iv) development of techniques for *in vitro* germplasm collection and exchange; and v) development of an efficient documentation and data management system for *in vitro* conservation.

Two types of *in vitro* genebank for conservation have been proposed (Withers and Williams, 1985): a) the *in vitro* active genebank (IVAG) where cultures are maintained under slow growth and b) the *in vitro* base genebank (IVBG) where cultures are cryopreserved. *In vitro* active collections have been developed for cassava, potatoes, sweet potatoes, banana, yams, coffee and sugar cane. These constitute working collections; their counterparts would be field collections and sexual seed collection under short-term storage.

An essential feature of the *in vitro* active genebank is that all material flows through a cyclical process, the essential components of which are: introduction, multiplication and subculturing into storage. The frequency of these processes will vary with the crop and will be related to the practical procedures agreed for maintenance of viability and genetic stability with minimal input. Thus, the IVAG is maintained by successive subculturing which automatically renews the conserved material and provides samples for use in monitoring and distribution. Determination of the time interval between each subculturing event for maintenance will rely on scientifically proven conditions which result in slow growth yet which neither depress viability unduly nor promote or cause genetic instability or selection. The type of work in an IVAG is therefore markedly different from that used in rapid mass propagation systems which do not, by their very nature, use intentionally growth limiting and therefore stressful or sub-optimal conditions.

Because IVAGs will maintain material for long periods under sub-optimal conditions, risks of somaclonal variation must be minimized by careful choice of initial explant and multiplication system. The IVAG will therefore avoid culture systems which are prone to somaclonal variation. For example protoplast, cell suspension and callus culture systems are unlikely to meet required standards for genetic conservation, whereas organized plant structures (embryos and shoots) will. Within the latter, non-adventitious systems will be preferable (Scowcroft,1984).

Adequate slow growth will be a natural feature of some culture systems but the majority will involve special culture conditions such as a reduction in the growth temperature and/or supplementation of the culture medium with growth retardants.

In contrast to the IVAG, the essential feature of the *in vitro* base genebank (IVBG) under cryopreservation is that material enters via freezing and remains in a static condition for very long periods. The only divergences from this are sampling at infrequent intervals to monitor stability and multiplication to replenish the IVAG when necessary, or regenerate the stock in the IVBG itself. The counterpart of the IVBG would be the sexual seed collection under long-term storage (IBPGR, 1986; Roca, *et al.* 1989).

In the last 10 years, efforts have been made to develop more efficient methods for germplasm conservation of vegetatively propagated species such as cassava, potato and sweet potato. Research has been focussed to develop *in vitro* germplasm storage systems. In 1986 the IBPGR Advisory Committee on *In Vitro* Storage recommended the development of basic approaches for the exchange of germplasm of high priority crops, especially primitive cultivars propagated vegetatively, giving particular attention to problems of disease indexing and movement through quarantine, where bottlenecks are often encountered. It was also recommended to link that strategy with genetic resources activities from collecting in the field to *in vitro* storage. The IBPGR Advisory Committee also recommended the identification of the extent to which *in vitro* techniques were being applied to genetic conservation of vegetatively propagated crops and to pinpoint gaps in the scientific knowledge and inadequacies in technologies, thereby providing data regarding research needs. So it was clear that a conceptual framework was lacking for *in vitro* plant genetic conservation (Withers and Williams, 1985), and that information and guidelines on the number of replicated cultures per clone to be maintained and on assessing the genetic stability of *in vitro* storage cultures for long-term conservation were inadequate.

In this area, IBPGR has been laying the groundwork for development of genetic conservation of certain crop species using *in vitro* techniques. Since many existing tissue culture collections do not necessarily constitute genebanks in the conservation sense, although many are vital adjuncts as breeder's collections, it was found necessary to test out the theoretical framework developed by IBPGR. In this context, this IBPGR-CIAT collaborative project was set to take advantage of the experience gained at CIAT in both tissue culture, and in the monitoring techniques for viability and genetic stability, as well as of the large cassava germplasm collection available at CIAT both in the field and *in vitro*.

IBPGR assigned to CIAT worldwide responsibility for cassava germplasm conservation. This includes: a field collection (nearly 6000 accessions) and an *in vitro* collection (5500 accessions), and 37 wild *Manihot* species which are maintained both in the form of sexual seeds and as *in vitro* cultures.

During the last ten years, *in vitro* culture techniques have been developed at CIAT for cassava to eliminate viruses, exchange and collect germplasm and to maintain a collection under minimal growth conditions (Roca, *et al.*, 1984, 1989). This work has been carried out with the collaboration of IBPGR. In addition, disease indexing techniques and biochemical methodologies have been implemented at CIAT to help establishing disease free stocks of cassava germplasm and to characterize cassava genotypes, respectively. Standard storage conditions of the CIAT *in vitro* collection involve reduced temperature and illumination, as well as changes in medium composition which allow lengthening of the transfer period to up to 18-20 months (Roca, 1985). Promising preliminary results in cryopreservation of cassava shoot-tips have been obtained in a collaborative project with IBPGR. Due to the above mentioned developments, cassava was considered as a suitable crop species for developing the pilot *in vitro* active genebank (P-IVAG).

2. MAJOR ISSUES IN *IN VITRO* CONSERVATION

A number of issues have long been a matter of concern in the potential application of *in vitro* techniques for germplasm conservation:

- Genetic stability
- Basic tissue culture competence
- Reproducibility of techniques
- Applicability to a wide range of genotypes
- Safety
- Management

The material retrieved from the *in vitro* genebank should genetically represent the material accessed. Any *in vitro* system will not be acceptable if it introduces risk of genetic instability and/or selection among heterogeneous genotypes (Withers, 1988).

It has been reported that genetic instability can take place during *in vitro* culture. As a consequence of instability there can be variation in ploidy and structural chromosome changes expressed in morphological and biochemical traits (Scowcroft, 1984). Somaclonal variation can be enhanced, particularly where regeneration is adventitious. It has also been suggested that certain *in vitro* storage conditions can put germplasm integrity at risk because of directional genetic change in response to selection. Since mutations and chromosomal rearrangements have been associated with callus cultures and plant regeneration from callus, meristems and shoot-tips were used in this P-IVAG project (IBPGR, 1983).

In vegetatively propagated crops morphological criteria have been used to confirm the maintenance of a genotype. However, such differences are difficult to detect in *in vitro* propagation. In addition, the selection pressure in an *in vitro* system is different from that for plants growing in soil. This could cause a shift to a variant type in *in vitro* propagation (Schilde and Roca, 1987). The use of axillary buds as a base for multiplication reduces the probability of genetic variation. Therefore, formation of callus and adventitious shoots should be avoided (Roca *et al.*, 1989). This project has given attention to this issue, by looking at the extent to which this variation can occur during *in vitro* storage of organized structures and how this variation can be detected by morphological and biochemical criteria.

Regeneration of whole plants from all culture systems is often the limiting step in applying *in vitro* culture techniques to crop improvement. The implementation of molecular and cellular genetic methods in breeding programs requires an efficient regeneration system from somatic tissues, including protoplast cultures. Despite the ability to initiate callus from a variety of tissues and organs in many crop species, reproducible regeneration of whole plants remains problematic. Adventitious regeneration via somatic embryogenesis is highly desirable as the process affords high multiplication rates and results in propagules that possess both root and shoot axes (Stamp and Henshaw, 1987).

The viability evaluation of *in vitro* cultures should be carried out systematically, depending on the crop species conserved and the conditions of storage. Under slow growth conditions in which the cycle term is extended for several months the evaluation frequency of cultures is reduced dramatically.

This final report on the P-IVAG involves three years of research work (January 1987 - December 1989), and mainly covers the principal steps from the selection and sampling of material from the field to the evaluation of genetic stability of retrieved material growing under slow growth conditions.

3. OBJECTIVES OF THE PROJECT

3.1 General

The overall objective of this project was to investigate the technical and logistical aspects of establishing and running an *in vitro* active genebank (IVAG), based on the standards agreed by IBPGR and CIAT, using cassava as a model. Special attention was paid to the issues of monitoring genetic stability, definition of replicates and development of a computer information system for *in vitro* conservation.

3.2 Specific

i. To select a condensed and representative sample of cassava genotypes from the world cassava germplasm collection assembled at CIAT, process these samples into *in vitro* storage under conditions of slow growth, and characterize those clones using morphological and biochemical traits.

ii. To monitor diseases, genetic stability and viability during slow growth in *in vitro* storage.

iii. To determine the needs for laboratory facilities, equipment, consumable items, and technical staffing involved throughout the operation of the *in vitro* genebank.

iv. To provide guidelines and testing parameters for establishing and running an IVAG on the basis of the experience gained with cassava.

4. GENERAL WORK PLAN

Using the experience gained at CIAT in the *in vitro* conservation of cassava germplasm and the recommendations of IBPGR on *in vitro* germplasm conservation of root crops, a working plan was made relating to the different projects. This plan allowed the evaluation of the technical and logistical aspects for the establishment

and operation of the *in vitro* active genebank. A flow scheme was designed to show the major steps of the P-IVAG for a period of three years (Fig. 1): selection, sampling and characterization of clones; introduction of clones into *in vitro* culture; and monitoring genetic stability; with the parallel development of an information system (see Section 5.15).

5. ACTIVITIES AND RESULTS

5.1 Selection of Material

A sample of 100 cassava genotypes were selected from the world cassava collection maintained by CIAT in the field (Table 1). The selection of the 100 clones was based on morphological variation and ecogeographic criteria. All the morphological descriptors known for the crop species were used in the selection of the clones. The range of morphological variability among the 100 cassava varieties provided the necessary morphological criteria for the characterization of the variability across the cassava "genepool" selected for this study. The distinct morphological traits in most of the varieties also served to monitor their stability throughout *in vitro* storage and field multiplication within particular clonal progeny.

In addition to morphological variation, other criteria were taken into account for selection, namely a) adaptation to each one of the six edaphoclimatic zones (ecosystems) of cultivated cassava (see Table 2); b) country of origin (15 countries); and c) a few native cultivars with outstanding breeding potential and three advanced clones were selected from the cassava breeding program. As a result, the material selected contained a broad spectrum of variation for morphology and other traits. To evaluate genetic stability, and following the recommendation of IBPGR, 5 replicates per accession were obtained.

5.2 Sampling of Material

Plants of the selected clones were taken and labelled in the field genebank. Four lignified stem cuttings (20-25 cm) were taken from each one of the five plants per clone. Out of twenty stakes per clone, five were planted in an Associated Field Genebank (AFG) assigned to the project, and 15 were planted in the glasshouse for indexing and electrophoretic isozyme characterization, and for thermotherapy and meristem-tip culture.

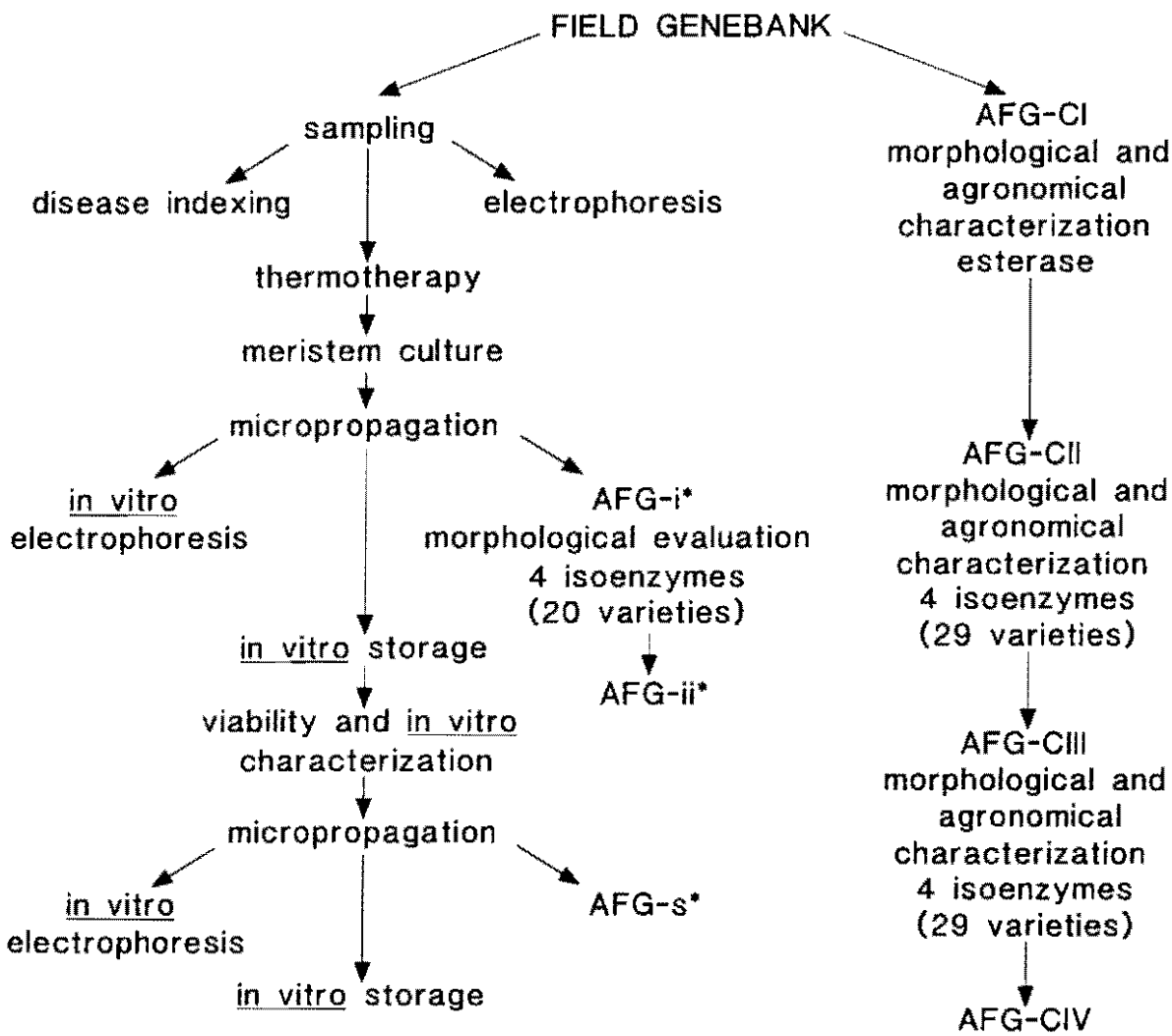

FIGURE 1
MAJOR STEPS IN ESTABLISHING AND MANAGING THE P-IVAG
(*see Section 5.13 for details of the Associated Field Genebank - AFG)

TABLE 1
ACCESSIONS IN CIAT'S CASSAVA GERMPLASM COLLECTION AND SELECTED FOR P-IVAG

COUNTRY OF ORIGIN	NUMBER IN FIELD GENEBANK	NUMBER SELECTED FOR P-IVAG	CLONES SELECTED FOR P-IVAG		
Colombia	1774	15	MCOL 22	MCOL 72	MCOL 226-A
			MCOL 304	MCOL 809-B	MCOL 890
			MCOL 955	MCOL 985	MCOL 1107
			MCOL 1185	MCOL 1357	MCOL 1413
			MCOL 1684	MCOL 2131	MCOL 2264
Brazil	778	13	MBRA 12	MBRA 110	MBRA 132
			MBRA 217	MBRA 309	MBRA 325
			MBRA 328	MBRA 329	MBRA 337
			MBRA 356	MBRA 403	MBRA 405
			MBRA 534		
Peru	376	10	MPER 206	MPER 213	MPER 229
			MPER 255	MPER 281	MPER 297
			MPER 328	MPER 353	MPER 370
			MPER 403		
Venezuela	242	9	MVEN 298	MVEN 36	MVEN 40-B
			MVEN 50	MVEN 82	MVEN 217
			MVEN 219	MVEN 331	MVEN 332
Paraguay	193	8	MPAR 25	MPAR 35	MPAR 38
			MPAR 69	MPAR 71	MPAR 75
			MPAR 98	MPAR 100	
Costa Rica	148	7	MCR 1	MCR 12	MCR 19
			MCR 32	MCR 79	MCR 126
			MCR 133		
Ecuador	117	6	MECU 10	MECU 72	MECU 117
			MECU 150	MECU 166	MECU 171
Guatemala	91	6	MGUA 41	MGUA 44	MGUA 58
			MGUA 62	MGUA 63	MGUA 78
Cuba	74	5	MCUB 5	MCUB 8	MCUB 16
			MCUB 53	MCUB 58	
Mexico	64	6	MMEX 6	MMEX 8	MMEX 43
			MMEX 55	MMEX 59	MMEX 71
Panama	41	2	MPAN 127	MPAN 137	
Argentina	15	4	MARG 2	MARG 9	MARG 12
			MARG 15		
Puerto Rico	15	2	MPTR 8	MPTR 102	
Dominican Republic	5	2	MDOM 3	MDOM 5	
Bolivia	3	2	MBOL 1	MBOL 2	
CIAT hybrids	188	3	CG 7-64	CM 507-37	SG 107-35
Old World	126	-	-		
Total	4250	100			

TABLE 2
ORIGIN OF P-IVAG GERMPLASM

ECOSYSTEM	GENERAL DESCRIPTION AND REPRESENTATIVE AREAS	MEAN TEMPERATURE	NUMBER OF CLONES USED IN P-IVAG
1	Lowland tropics long dry season; low to moderate annual rainfall; high year-round temperature	Above 25°C	8
2	Lowland tropics with moderate to high rainfall; savanna vegetation on infertile, acid soil; moderate to long dry season; low relative humidity during dry season	Above 25°C	23
3	Lowland tropics with no pronounced dry seasons; high rainfall; constant high relative humidity	Above 25°C	20
4	Medium-altitude tropics; moderate dry season and temperature	21 - 24°C	26
5	Cool highland areas; moderate to high rainfall	17 - 20°C	6
6	Sub-tropical areas; cool winters; fluctuating daylengths	Minimum 0°C	17

Due to the large work load and space required during the initial sampling and processing stages the material was divided into five batches each comprising 20 clones, with a four to five weeks interval per batch. Lineage identity of clones, plants and stakes were kept throughout the steps of sampling and introduction to *in vitro* culture (Table 3).

TABLE 3
MAJOR ACTIVITIES OF THE P-IVAG

ACTIVITIES	CLONAL BATCHES - EACH COMPRISING 20 VARIETIES					NUMBER OF TECHNICAL STAFF INVOLVED
	BATCH 1	BATCH 2	BATCH 3	BATCH 4	BATCH 5	
Sampling material in the field (5 replicates per variety; 4 cuttings per replicate)	March 17, 1987 20 varieties	April 30, 1987 20 varieties	Jun 15, 1987 20 varieties	Aug 3, 1987 20 varieties	Sept 21, 1987 20 varieties	2
Planting in the greenhouse	March 19, 1987	May 4, 1987	Jun 18, 1987	Aug 6, 1987	Sept 24, 1987	2
ELISA test (5 replicates per variety)	Apr 9-10, 1987	Jun 2-3, 1987	Jul 13-14, 1987	Aug 27-28, 1987	Oct 13-14, 1987	2
Electrophoresis: EST (5 replicates per variety)	Apr 20-30, 1987	Jun 8-17, 1987	Jul 23-30, 1987	Sep 7-15, 1987	Oct 26-30, 1987	2
Grafting (5 replicates per variety)	May 19-20, 1987	Jul 6-7, 1987	Aug 18-19, 1987	Oct 1-2, 1987	Nov 23-24, 1987	3
dsRNA (Test per variety)	May 20, 1987	Jul 7, 1987	Aug 19, 1987	Oct 2, 1987	Nov 24, 1987	1
Thermotherapy (5 replicates per variety)	May 19-Jun 3, 1987	Jul 6-21, 1987	Aug 18-Sep 2, 1987	Oct 1-19, 1987	Nov 23-Dec 9, 1987	1
Meristem isolation/ culture (2-4 meristems per replicate)	Jun 3-5, 1987	Jul 21-22, 1987	Sept 2-4, 1987	Oct 19-21, 1987	Dec 8-11, 1987	3
Subculturing	Jun 30-Jul 2, 1987	Aug 13-14, 1987	Sept 28-30, 1987	Nov 17-19, 1987	Jan 4-5, 1988	2
3-4 micropropagations to obtain at least 10 shoots per replicate	± 6 months	± 9 months	± 12 months	± 14 months	± 15 months	2
Introduction into slow growth storage	Jan-Feb 1988	Jun-Jul 1988	Sept-Oct 1988	Feb-Mar 1989	April-May 1989	1
Viability Tests	Every month	Every month	Every month	Every month	Every month	1
In vitro morphological evaluation	Aug 1988	Jan 1989	April 1989	Sept 1989	Nov 1989	1
Subculturing	April-Jun 1989	Aug-Oct 1989	Jan-Mar 1990	---	---	1
Storage re-introduction	July 1989	---	---	---	---	1
Electrophoresis of *in vitro* cultures (EST, DIAP, ACP, GOT)	Aug 1989	Nov-Dec 1989	---	---	---	1

5.3 Disease Indexing

The phytosanitary condition of mother plants was determined prior to thermotherapy and meristem-tip culture using a combination of symptomatology, graft inoculation, ELISA and dsRNA tests for the detection of cassava common mosaic virus (CCMV), cassava X virus (CsXV), frog skin disease (FSD) and cassava latent disease. These viral pathogens are the most significant in Latin America and in the CIAT field collection. Table 4 shows the summarized results from disease indexing of clones prior to *in vitro* culture and storage (full details of disease indexing and other original data are available from IPGRI).

TABLE 4
NUMBER OF POSITIVE ACCESSIONS FOR EACH VIRUS INDEXING TEST

	FSD	GRAFT INOCULATION	ELISA		dsRNA TEST
			CCMV	CsXV	
NUMBER POSITIVE	13	16	0	5	52

Symptomatology
Disease indexing for FSD was done by visual examination. Symptoms consist of deep lip-shaped, longitudinal fissures on the starchy roots with induction of scales on the epidermis. FSD evaluation was carried out in the field, at the same time as cutting stakes for the P-IVAG; 13 accessions were found to be positive for FSD.

Graft Inoculation
This test was carried out to index for mosaic diseases. Stake samples, 20-25 cm long, from each clone were planted in 15 cm. diameter plastic pots with a mixture of 1 organic soil: 1 fine sand. After nine weeks from planting, shoots were 30-40 cm in length, with 5-10 mm diameter; the shoots were cut at the base and the apical part discarded. The stems (15 cm long) were used as stocks in grafting tests with scions of the virus susceptible cultivar "secundina" previously cleaned up by meristem culture. Grafted stocks were planted in 7 cm diameter styrofoam containers with 1 volume of organic soil: 1 volume of sand. After 4-5 weeks virus symptom evaluation (by visual estimation of expanded young leaves in grafted scions) was carried out.

Sixteen clones were positive by graft inoculation to the susceptible clone "secundina". Not all positive accessions in all replicates showed disease symptoms; sometimes only one replicate was positive. It was not possible to evaluate some plants because they died due to insufficient stem lignification.

ELISA Test

This test was used specifically for Cassava Common Mosaic Virus (CCMV) and Cassava X Virus (CsXV). Stake samples from the field genebank were planted in plastic pots and grown in the glasshouse. Shoot-tips from young stems, 4-5 weeks old, were sampled in plastic bags and the sap was extracted for ELISA. Assessment of results in microplates was made visually and by absorbance in a spectrophotometer. A value twice the mean control value was considered positive.

CCMV was not detected in any clones, but the ELISA test detected CsXV in five clones. Although we assumed that introduction of the germplasm into the *in vitro* process (thermotherapy followed by meristem-tip culture) would clean up the clones from viruses, the five accessed clones which tested ELISA positive for CsXV were retested for this virus with ELISA after the *in vitro* process with the result that all cultures of these clones were negative, confirming successful virus elimination.

Indexing for dsRNA

A method for detection of double-stranded RNA was used for the diagnosis of latent infections caused by unidentified RNA virus. Young apical stems (10-15 cm long) from each replicated plant were planted free of insects and fungal disease, in trays containing 1 volume of organic soil: 2 volumes of sand, and grown for four weeks. Shoot-tip samples (2.0-3.0 g tissue) were frozen in liquid nitrogen and powdered in mortars and used for centrifuging with extracting buffer. The supernatant was used for obtaining purified dsRNA. Electrophoresis was run in 1% agarose gel stained with ethidium bromide solution. The presence of fluorescent dsRNA bands was checked using an ultraviolet light box and photographed using a polaroid camera. The presence of bands was considered as positive. dsRNA bands were detected in 52 clones of the 100 clones tested.

In previous research, Gabriel *et al.*, 1987 and Roa *et al.*, 1987, have provided evidence that thermotherapy and meristem culture are effective to clean most of the clones which were positive in the dsRNA test when small shoot-tips (0.1-0.2 mm) were cultured from terminal buds grown under thermotherapy (40°C day/35°C night). A similar technique was used in this project to eliminate viruses.

We have found that there is little correlation between the indexing results from these tests. For example, since ELISA is more sensitive than dsRNA analysis, dsRNA is often not detected in cassava infected with CsXV and CCMV when the virus titer is low in the tissues. Likewise, graft inoculation does not detect the latent infections revealed by dsRNA analysis. Thus, a combination of tests was required for detecting viruses in the cassava material.

5.4 Morphological Characterization

Morphological characterization was carried out both in the associated field genebank and in *in vitro* cultures. The main objectives of this characterization were to assess the range of variation of the germplasm used in the P-IVAG genebank, record qualitative and quantitative traits for clonal identification and to monitor genetic stability after retrieving material from *in vitro* slow growth storage.

Morphological characterization of field material
Characterization of the 100 cassava clones (with five replicates) growing in the Associated Field Genebank (AFG1), has been performed using the descriptors recommended by IBPGR and modified by the Cassava Program of CIAT (detailed in Appendix I). Most of the descriptors were evaluated on six month-old plants, but those which relate to harvest were evaluated on one year-old plants. In all, a total of 46 descriptors were recorded for each genotype, 10 of these being passport data and 36 being discrete qualitative and quantitative morphological traits as above.

The information was recorded in specially designed cards for use in the field and for transcription to the computer. Morphological evaluation was carried out in three generations of the AFG-Control (AFG-C) and in fifty varieties in the AFG from *in vitro* cultures (AFG-i). Results of these evaluations indicated a wide range of genetic variability for most of the descriptors among the clones selected for the P-IVAG.

Cluster analysis of the 100 cassava varieties was made using 25 morphological traits. Sixty four similar groups were clustered mostly at the 75% level of affinity, indicating that this material was highly heterogeneous for each one of the morphological descriptors measured. Only four groups were clustered at the 99% level (9 clones in total), suggesting that these clones might be genotypically similar or duplicates, as shown in Table 5. Moreover, it was not possible to distinguish between these varieties using four different isozyme systems (see below; Section 5.5).

TABLE 5
MORPHOLOGICALLY SIMILAR GROUPS AFTER CLUSTERING OF THE 100 CASSAVA CLONES FROM THE AFG-C ON THE BASIS OF 25 DESCRIPTORS

GROUPS	CLONES		
1	MCR 12	MGUA 63	
2	MMEX 43	MGUA 58	
3	MBRA 356	MCOL 2264	
4	MCR 1	MGUA 44	MGUA 78

Morphological characterization of in vitro material
Morphological characterization of *in vitro* cultures was carried out after six months of *in vitro* storage, using 25 replicates for each clone, under slow growth conditions. As a result of the preliminary observations during the first year of the project, the following morphological descriptors were used as criteria for characterization of *in vitro* cassava cultures:

Etiolation: refers to stem elongation between nodes *in vitro* plants. The average internode elongation was 1.0 - 1.5 cm. When the plants showed this range, the value for etiolation was rated as 0. From 1.5 - 3.0 cm, the value was 1, and values above this were rated as 2 (see Table 6).

Shoot number: average number of shoots per culture, per variety. Each replicate culture was initiated from a sample with only one shoot-tip explant.

Callus formation: although callus is not common in shoot-tip cultures of cassava, it was included because of its importance in terms of genetic stability. The value 0 indicates absence and 1 presence.

Rooting: it was evaluated in terms of relative amount; 1, 2, 3 correspond to poor, medium and high, respectively.

Aerial roots: refers to roots produced on nodes; aerial roots can reduce the potential of propagation. It was evaluated only as present or absent.

Special notes on in vitro *characterization:* they relate to descriptors or traits which are not common under *in vitro* conditions, such as pigmentation, different leaf shape, etc.

The results of *in vitro* characterization are summarized in Table 6. Intervarietal variation in etiolation was observed in the stored material. Over 50% of the clones had average internode lengths between 1.5 - 3.0 cm and about 1/3 of the clones showed a "normal" elongation; this value was chosen according with the experience gained with the large collection at CIAT. These results suggest that storage illumination or temperature requires adjustment; i.e. the illumination may have been too low for the actual storage temperature. In earlier work with cassava at CIAT (CIAT, 1986) it was found that lower *in vitro* storage temperatures should be accompanied by lower illumination, and *vice versa*.

TABLE 6
RESULTS OF *IN VITRO* CHARACTERIZATION OF CASSAVA AFTER SIX MONTHS OF CULTURE

	DEGREE OF ETIOLATION			NUMBER OF SHOOTS PER VARIETY				CALLUS		AERIAL ROOTS		ROOTS (RELATIVE AMOUNT)		
	0	1	2	1	2	3	4	ABSENT	PRESENT	ABSENT	PRESENT	1	2	3
NUMBER OF VARIETIES	36	59	2	52	33	9	3	88	9	71	26	44	38	15

The number of shoots varied among genotypes ranging from 1-4 stems per culture; the clones which showed 4 shoots per culture were: MBRA 328, MCUB 58, MDOM 3. Fifty percent of the clones had only one main shoot per culture and about 1/3 had two shoots. A high number of shoots per culture represents a high propagation potential, in the case of cassava. Aerial roots occurred in 26 clones, (nearly 1/3 of the clones) since this character may interfere with propagation potential of the cultures after retrieval from storage, conditions (i.e. high relative humidity inside the culture vessels) should be adjusted to reduce aerial rooting. The occurrence of callus was found in only 9 accessions.

Anthocyanin pigmentation in young stems and petioles was only found in three (MBRA 337, MBRA 356 and MBRA 2264) clones, in spite of the fact that this

character occurred in the field in 63 clones. Variegation (yellow and green tissue) was also observed in all replicated plants from two variegated varieties (MBRA 356 and MCOL 2264). Leaf shape variation was only found in two clones from Venezuela (MVEN 331, MVEN 332) which showed a single lobe and sessile leaves: these traits also occurred in the field.

5.5 Isozyme Characterization

Isozyme electrophoresis has been used for the characterization and discrimination of the 100 clones using the following systems:

Polyacrylamide gel electrophoresis (PAGE)
α- β-Esterase (EST)
Glutamate Oxaloacetate Transaminase (GOT)
α- β-Acid Phosphatase (ACP)
Diaphorase (DIA)

Starch Gel electrophoresis (SGE)
Malic Enzyme (ME)
Hexose Kinase (HK)
Superoxide Dismutase (SOD)
Phosphogluco Isomerase (PGI)
Phosphogluco Mutase (PGM)
Isocitrate Dehydrogenase (IDH)
Malate Dehydrogenase (MDH)
Glutamate Dehydrogenase (GDH)
Shikimic Dehydrogenase (SKDH)
6-Phosphogluconate Dehydrogenase (6PGDH)
Glucose-6-Phosphate Dehydrogenase (G6PDH)
Peroxidase (PRX)

Isozyme characterization of field material
Stakes of the 100 selected clones were sampled from the field genebank and planted in pots in 2:1 sand-soil mixture and allowed to grow in the greenhouse at 27°C ± 5°C. Root-tip and young shoot-tip samples were taken after four weeks and extracted using appropriate extraction buffers and appropriate substrates (Ramirez, *et al.*, 1987) and stained for each of the isozymes listed above. Vertical polyacrylamide gel electrophoresis (PAGE) and horizontal starch gel electrophoresis (SGE) were performed.

α- *and* β-*Esterases (EST):* Banding patterns of α- and β- esterases were analyzed in 10% polyacrylamide gels. A total of 23 bands were identified for all the selected material, permitting the genotyping of every accession (Fig. 2). Isozyme clustering

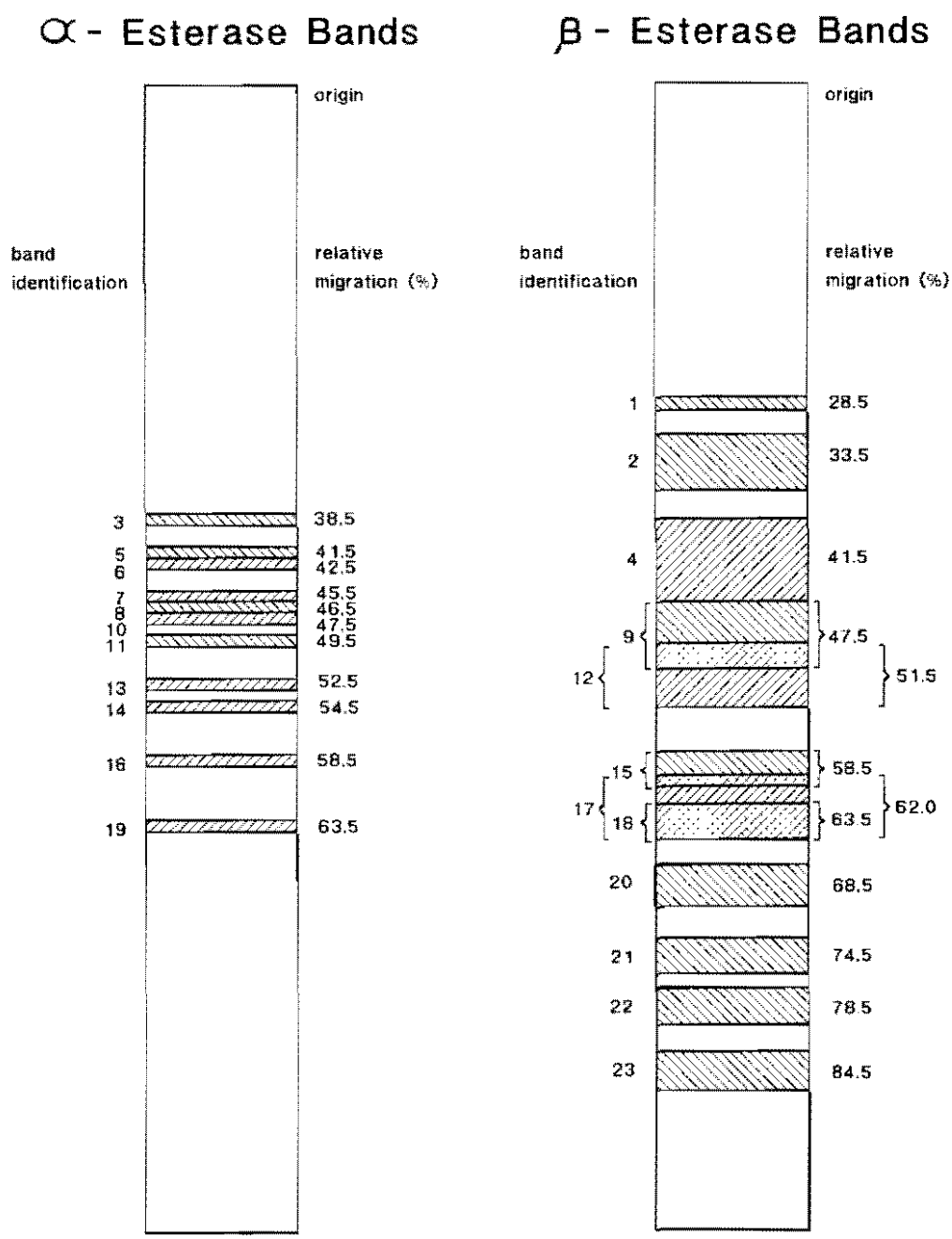

FIGURE 2
RELATIVE SIZE AND MIGRATION OF BANDS RESULTING FROM ELECTROPHORESIS ON POLYACRYLAMIDE GELS OF α- and β-ESTERASE ISOZYMES EXTRACTED FROM ROOT-TIP TISSUES OF CASSAVA

data of the 23 bands showed an association of the 100 clones in 84 clustered groups at 90% level of similarity index. From this, 8 groups comprising two clones and four groups of 3 clones were identified (Table 7). This cluster analysis enabled the identification of duplicates showed in Table 5. Zymograms (electrophoregrams) from the 100 clones were analyzed and, so far, 11 α- and 12 β-esterase bands have been identified to differentiate each genotype (Table 8). Twenty-nine clones showed consistent and well defined zymograms, from which the patterns of MBRA 309 and MMEX 55 were taken as a reference for comparison with all clones.

TABLE 7
ISOZYME CLUSTERING OF THE 100 CLONES OF THE P-IVAG

SYSTEM	LEVEL OF SIMILARITY	NUMBER OF GROUPS	COMPOSITION
EST	97%	12	8 groups with 2 varieties 4 groups with 3 varieties
GOT	98%	6	1 group with 48 varieties 2 groups with 6 varieties 1 group with 10 varieties 1 group with 4 varieties 1 group with 24 varieties
ACP	97%	22	2 groups with 5 varieties 5 groups with 4 varieties 7 groups with 3 varieties 8 groups with 2 varieties
DIAP	97%	4	1 group with 56 varieties 1 group with 2 varieties 1 group with 40 varieties 1 group with 2 varieties

Clustering data of α- and β- esterases were supplemented with data obtained from the LKB ultrascan XL laser densitometer, overcoming difficulties in accurately quantifying gels. The α-esterase band number 11, the most common to the germplasm in PAGEs of both root-tips and shoot-tip extracts, was used as a reference during the scanning of gels. High polymorphism in banding patterns for root tissue was found in esterases for fingerprinting clones. Genetic analysis of esterase isozyme loci carried out at CIAT allowed the identification of two loci, named EST.l, with five alleles controlling 11 electrophoretic phenotypes. These studies also permitted the interpretation that at least 8-10 alleles are present in the expression of the 23 α- and β- bands of cassava esterases.

TABLE 8
CODIFICATION OF α- and β-ESTERASE ISOZYME BANDING PATTERNS

BAND NUMBER	BAND TYPE	RELATIVE MIGRATION	IDEOTYPES P-IVAG
1	β	28.5	MBRA 132
2	β	33.5	MCUB 58
3	α	38.5	MVEN 217
4	β	41.5	MMEX 55
5	α	41.5	MBRA 325
6	α	43.5	MBOL 2
7	α	45.5	MBRA 329
8	α	46.5	MBRA 12
9	β	47.5	MCOL 1107
10	α	47.5	MVEN 219
11	α	49.5	MPER 353
12	β	51.5	MBRA 534
13	α	52.5	MCR 19
14	α	54.5	MVEN 36
15	β	58.5	MMEX 43
16	α	58.5	MPAR 38
17	β	62.0	MBRA 534
18	β	63.5	MCOL 2264
19	α	63.5	MGUA 63
20	β	68.5	MBRA 309
21	β	74.5	MBRA 328
22	β	78.5	MCOL 22
23	β	84.5	MPER 297
TOTAL	α: 11 bands β: 12 bands		

Glutamate Oxaloacetate Transaminase (GOT): A total of eleven electrophoretic bands were interpreted for GOT. Two polymorphic bands were found in the anodic region (slowest migration zone); this was interpreted as typical monomeric molecular structure with three electrophoretic phenotypes and the locus was named GOT 1. There are two other zones, intermediate and faster, that require further analysis to infer inheritance models.

Clustering of the 100 varieties with GOT banding pattern shows six big groups at the 98% level of similarity index (Table 7). This information was compared with clustering data from EST and morphological evaluation and the occurrence of duplicates shown in Table 5 was confirmed.

Acid Phosphatase (ACP): A total of 10 electrophoretic bands were characterized for ACP using extracts from root-tips. Six migration zones were analyzed and five displayed polymorphism. Further complementary genetic studies of offspring of parental clones allowed the structural interpretation of the two anodic regions of ACP. The fastest zone (the fastest anodic region) was named ACP.1 with two bands, three electrophoretic phenotypes and two alleles (ACP.1.1. and ACP.1.2). The second fastest anodic region (single banded) was coded ACP.2 with two electrophoretic phenotypes and two alleles, one active (ACP.2.1) and the other null or silent allele (ACP.2.0). Clustering of the 100 varieties with ACP zymograms showed 22 groups at 97% level of similarly index (Table 7). This information was compared with clustering data of EST, GOT and morphological discrete traits and the occurrence of duplicates was further confirmed.

Diaphorase (DIAP): All observed cassava clones exhibited only one polymorphic zone with four bands and another non-polymorphic distant region with three bands. PAGEs carried out with root-tips using 12% gels showed more resolution than with 10% polyacrylamide, and better results with the same banding patterns were obtained using young shoot-tips.

The interpretation of polymorphism among electrophoretic banding patterns in DIAP was very difficult because the frequent occurrence of artifacts and non definition of allozymes in PAGEs in terms of migration distance (or relative mobility), leading to the preliminary and erroneous interpretation of high polymorphism for every allozyme present among selected clones. Further studies permitted the interpretation of only one polymorphic zone probably in a monomeric manner.

Clustering of the 100 varieties with DIAP zymograms showed four groups at 97% level of similarity index (Table 7), and the duplicates showed in Table 5 were confirmed.

Starch Gel Electrophoresis (SGE): Isozyme survey using starch gel electrophoresis (SGE) was undertaken using 12 selected clones from the P-IVAG germplasm to increase the number of markers available for monitoring the genetic stability of *in vitro* material. Twelve isozyme systems were examined using SGE with two buffer systems, lithium borate and histidine, and extracts from root-tips (Table 9).

TABLE 9
ISOZYME SURVEY OF SELECTED CLONES USING STARCH ELECTROPHORESIS FOR MONITORING GENETIC STABILITY

ISOZYME	BUFFER	ROOT-TIPS			
		MIGRATION ZONES	NUMBER OF BANDS	POLYMORPHISM	RESOLUTION
ME	LB	1	1	0	3
	Hi	2	3	0	1
HK	LB	0	0	0	0
	Hi	0	0	0	0
SOD	LB	0	0	0	0
	Hi	0	0	0	0
PG1	LB	3	5	2	3
	Hi	0	0	0	0
PGM	LB	1	1	1	1
	Hi	2	3	0	1
PRX	LB	2	3	1	3
	Hi	3	4	1	3
IDH	LB	1	2	0	1
	Hi	1	1	1	1
MDH	LB	2	3	1	2
	Hi	1	4	2	2
GDH	LB	2	6	3	2
	Hi	-	-	-	-
SKDH	LB	1	1	1	1
	Hi	1	1	1	1
G6PDH	LB	2	2	2	2
	Hi	0	0	0	0
6GPDH	LB2	2	2	2	2
	Hi	2	2	1	2
DIAP	LB	2	4	2	2
	Hi	1	3	1	1
ACP	LB	2	3	1	2
	Hi	-	-	-	-
EST	LB	3	7	2	2
	Hi	-	-	-	-
GOT	LB	1	3	1	1
	Hi	-	-	-	-

LB = Lithium borate; Hi = histidine
Polymorphism: 0, monomorphic; 1, at one band; 2, at two bands; 3, at three or more bands
Resolution: 0, achromatic; 1, low; 2, fair; 3, high

TABLE 10
COMPARISON OF STARCH (SGE) AND POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) USING FOUR ISOZYMES

ISOZYME	GEL TYPE	ROOT-TIPS			
		MIGRATION ZONES	NUMBER OF BANDS	POLYMORPHISM	RESOLUTION
EST	PAGE	7	23	3	3
	SGE	3	7	2	2
ACP	PAGE	6	11	3	3
	SGE	2	3	1	2
GOT	PAGE	3	10	3	3
	SGE	1	3	1	1
DIAP	PAGE	3	7	1	2
	SGE	2	4	2	2

Polymorphism: 0, monomorphic; 1, at one band; 2, at two bands; 3, at three or more bands
Resolution: 0, achromatic; 1, low; 2, fair; 3, high

The isozymes, ME, SOD and HK showed monomorphic banding patterns. However polymorphism and good resolution have been observed with PGI, GDH, G6PHD and 6GPDH. Low polymorphism, usually restricted to one band was found with SGE of SKDH, MDH, IDH and PRX. PAGE banding patterns of EST, ACP, GOT were always superior than SGE banding patterns in both, polymorphism and resolution (Table 10).

Isozyme Characterization of In Vitro Material

PAGE analysis was made of *in vitro* culture of 29 selected clones (see Table 11) using EST, ACP, DIAP and GOT. The 29 clones were subcultured using shoot-tips in 4E culture medium. After four months, enough samples of root-tips were obtained (0.5 g per clone). EST-banding patterns of *in vitro* root-tips were mostly different from EST-banding patterns of root-tips from stakes; only a few common bands (a, b, d) out of the 23 coded for root-tips from stakes were found in *in vitro* material as well. For this reason it is not possible to compare varieties from *in vitro* and field. In addition, band resolution of *in vitro* material was lower than from stakes.

TABLE 11
SELECTED CLONES FOR ISOZYME CHARACTERIZATION OF *IN VITRO* MATERIAL

1 - MARG 15	9 - MGUA 78	17 - MGUA 58	25 - MVEN 298
2 - MBRA 132	10 - MCR 1	18 - MPAN 127	26 - MVEN 82
3 - MBRA 309	11 - MCR 126	19 - MMEX 55	27 - MVEN 217
4 - MBRA 329	12 - MCUB 5	20 - MPAR 100	28 - CG 7-64
5 - MCOL 226-A	13 - MCUB 58	21 - MPER 229	29 - CM 507-37
6 - MCOL 985	14 - MDOM 3	22 - MPER 328	
7 - MCOL 1684	15 - MECU 72	23 - MPER 370	
8 - MCOL 2264	16 - MECU 117	24 - MPTR 102	

5.6 Thermotherapy

Thermotherapy, allowed fast elongation of young sprouts from stakes planted in plastic pots. Thermotherapy of the 500 mother plants was carried out in a phytotron and consisted of a three-week treatment of 40°C/day and 35°C/night temperature and an illumination of 3000 - 5000 lux.

5.7 Meristem Culture

Meristem-tips were excised from surface sterilized apical buds of the shoots grown under thermotherapy and placed in culture in 4E medium developed at CIAT (Table 12).

5.8 Micropropagation

Meristem-tips were grown in 4E medium into plantlets in test tubes (No. 18) in the growth room at 28°C and 3000 lux. Shoot-tips were excised and cultured in 17N medium (Table 12) and grown into plantlets capable of establishment in the soil, first in the glasshouse and then in the associated field genebank (AFG). Intensive micropropagation (subculturing) was carried out using 4E medium to obtain 50 replicates per clone for electrophoresis and slow growth storage and for transplanting to the AFG-i. In *in vitro* storage, only one shoot-tip was used per test tube.

5.9 Slow Growth Storage

Experience at CIAT on *in vitro* conservation of cassava has provided the means to store cultures under minimal conditions of slow growth (Roca, 1984, 1985). It has been found that throughout 18-24 months of storage at 23°C, the rate of shoot elongation decreased to about one-fifth of that in cultures kept at 28-30°C. Storage temperatures lower than 18°C were detrimental to a number of cassava varieties if the illumination was kept high.

TABLE 12
COMPONENTS OF DIFFERENT CULTURE MEDIA USED FOR *IN VITRO* CULTURE AND MICROPROPAGATION IN THE P-IVAG (CONCENTRATIONS ARE GIVEN FOR A 100 ML SOLUTION).

CULTURE MEDIUM	MS SALTS (g)	AGAR (g)	SUCROSE (g)	THIAMINE 100 ppm (ml)	INOSITOL 8000 ppm (ml)	BAP 10 ppm (ml)	GA 10 ppm (ml)	NAA 10 ppm (ml)	PLANT. PROD. (mg)	pH
4E	4.3	8	20.0	10	12.5	4.0	5.0	2.0		5.8
8S	4.3	8	20.0	10	12.5	2.0	10.0	1.0		5.8
17N	1.43	8	20.0	10	12.5		10.0	10.0	25	5.8

MS = Murashige and Skoog salts; BAP = Benzylamino purine;
NAA = Napthaleneacetic acid; IBA = Indolebutyric acid;
Plant.Prod. = Plant promoter - commercial product

Shoot-tips were excised from the *in vitro* plantlets obtained by micropropagation on 4E medium and cultured in 8S medium using No. 25 test tubes, kept for elongation and rooting for about four weeks in the micropropagation room at 28°C, 3000 lux and 12 hour day length; then the cultures were placed under slow growth storage at 23°C, 1000 lux and 12 hour day length. The introduction of all material to *in vitro* storage was made in five batches with 20 clones each (Table 3). By the beginning of 1990, 48 clones had already been retrieved from *in vitro* storage and subcultured in order to continue another cycle of conservation.

Three varieties did not respond to the *in vitro* culture procedures as described in this report. Therefore they could not enter slow growth storage; hence, the total number of clones was reduced to 97.

Due to losses during micropropagation 27 varieties were represented by 1 to 4 replicates; the remaining 70 clones were represented by 5 replicates in slow growth. From this stage onwards no more losses of replicates occurred in the 97 varieties; i.e. not a single cassava variety was lost from *in vitro* storage. A total of 2,220 cultures were introduced into *in vitro* storage, considering that every replicate was in turn sub-replicated 5 times per variety (Table 13). For comparisons with losses in the field genebank, see Table 19.

TABLE 13
VARIETIES IN *IN VITRO* STORAGE,
WITH NUMBER OF REPLICATES AND SUBREPLICATES

NUMBER OF VARIETIES	NUMBER OF REPLICATES PER VARIETY	TOTAL NUMBER OF SUBREPLICATES PER VARIETY	TOTAL NUMBER OF CULTURES PER VARIETY
70	5	25	1,750
16	4	20	320
9	3	15	135
1	2	10	10
1	1	5	5
Total 97			2,220

5.10 Subculturing

Subculturing and recycling of cultures maintained under slow growth was performed with 48 clones from the first three groups introduced earlier. Cultures from *in vitro* storage were retrieved to the laminar flow cabinet, tips and nodes were cut from the shoots, and inoculated to fresh 4E medium, in test tubes (No. 18), and placed in the growth room for initial establishment under high light (1500 - 2000 lux) and a temperature of ca. 27°C for 4-5 weeks. Then, the apical buds were transferred to 8S medium, in randomized system using test tube No. 25, under a lower temperature and illumination.

Degree of defoliation, was a very important criterion for defining culture viability and recycling time. An average of 14 months of life span (viability) has been found

among the 43 clones. A range of variation in life span has been observed in this material. Some "early" varieties with 8 months (MBRA 309) and "late" varieties with 17 months (MMEX 55) were recorded (Table 14).

TABLE 14
SUB-CULTURE INTERVAL OF 48 CASSAVA CLONES DURING *IN VITRO* STORAGE

NUMBER OF VARIETIES PER SUBCULTURE INTERVAL	SUBCULTURE INTERVAL (MONTHS)
8*	17
9+	15
2+	14
11+	13
13+	12
7+	11
3+	9
3+	8

* not subcultured at time of recording
\+ subcultured once only at time of recording

Out of the 48 varieties, 50% had to be subcultured after one year of storage; about 1/3 of the clones however had a "life" span of 15 months or more; and 6 clones had to be subcultured only after 8 - 9 months. The subculture interval of the "early" varieties was compared with the interval of the same varieties in the large *in vitro* collection of CIAT and the average was the same, meaning that this could be a genotypic response.

5.11 Evaluation of Viability

The evaluation of viability of the cultures under slow growth storage was performed systematically every month using the information system developed for this purpose; the descriptors evaluated:

1. Contamination:	Absence	0
	Presence	1
2. Browning: (Phenolization)	Absence	0
	Poor	1
	Medium	2
	High	3
3. Defoliation:	Evaluated in terms of percentage; 100% is the maximum value.	
4. Bleaching:	Absence	0
	Poor	1
	Medium	2
	High	3
5. Notes	This space is for the identification of the contaminated sub-replicates and to describe the source of contamination, bacterial or fungal (BC or FC). Also it is used for special notes that affect viability like cracked stems, etiolation, etc.	
6. Dead cultures		
7. Date of death		

The data gathered on contamination are summarized in Table 15. Culture contamination was detected within the first five months of storage; only eight replicates showed contamination at the seventh month of storage. Contamination was much higher in some varieties than in others, for example MARG 12 showed the highest number of subreplicates with bacterial contamination and MVEN 332 with fungal contamination.

Results of viability evaluation using browning, defoliation and bleaching descriptors are summarized in Tables 16, 17 and 18. In the first evaluation of viability, none of the varieties showed browning. After six months, 69 varieties remained in the same condition but after 12 months of storage the number of browned varieties increased. On the other hand, 28 varieties showed a value of 1 after six months and which was maintained after twelve months, but decreased to 6 varieties in the last evaluation. Grade 2 of browning was reached at 12 months, with eight varieties showing it. By the time of reporting, the highest grade of browning has not been reached by any variety.

TABLE 15
MICROBIAL CONTAMINATION OF CULTURES DURING *IN VITRO* STORAGE

NUMBER OF VARIETIES	NUMBER OF SUBREPLICATES WITH BACTERIAL CONTAMINATION	NUMBER OF REPLICATES WITH FUNGAL CONTAMINATION
1	8	
2	4	
4	3	
8	2	
14	1	
1		5
8		2
14		1

TABLE 16
BROWNING OF CULTURES DURING *IN VITRO* STORAGE

DEGREE OF BROWNING	MONTHS OF STORAGE			
	1	6	12	17
0	97*	69	6	2
1	0	28	27	6
2	0	0	8	0
3	0	0	0	0
	97	97	41	8

* number of varieties.

All the varieties in *in vitro* storage showed defoliation of between 0-10% after the first month of storage. After six months, 30 varieties remained with a low level of defoliation while 20 varieties reached the highest percent (41-80%). Between 6 and 12 months of storage there was an increment of defoliation in most of the varieties. After twelve months of storage, none of the varieties showed the lowest degree of defoliation. A similar situation was observed after 17 months; at this time, only eight varieties remained in conservation with a percentage of defoliation between 41 and 80%. The remaining 89 varieties have been retrieved for subculturing.

TABLE 17
DEFOLIATION OF *IN VITRO* STORAGE VARIETIES.

% DEFOLIATION	MONTHS OF STORAGE			
	1	6	12	17
0-10	97*	30	0	0
11-20	0	20	0	0
21-40	0	27	10	0
41-80	0	20	31	8
	97	97	41	8

* number of varieties.

TABLE 18
LEAF BLEACHING OF CULTURES DURING *IN VITRO* STORAGE

DEGREE OF BLEACHING	MONTHS OF STORAGE			
	1	6	12	17
0	97*	57	20	2
1	0	26	12	6
2	0	14	9	0
3	0	0	0	0
	97	97	41	8

* number of varieties.

In the first evaluation, none of the varieties showed bleaching. At six months about 55% remained green, and the remaining 45% displayed 1 to 2 degrees of bleaching. Although some varieties had been subcultured at twelve months, the remainder were still in storage; 50% did not show any bleaching, i.e. 12 varieties showed 1 degree and 9 showed bleaching at level 2. At 17 months of storage, only 2 varieties remained without bleaching and 6 with the lowest value of bleaching. No variety showed the highest degree of bleaching (3).

Comparing browning, defoliation and bleaching in terms of subculture interval, it can be concluded that defoliation is the best indicator of the degree of deterioration

of the cultured material. For browning and bleaching, the material reached only a medium level at 12 months of storage; however more than 70% of the varieties showed the highest percentage of defoliation by the same storage time.

5.12 Monitoring Subculture Frequency

Three cassava genotypes, MCOL 22, MCOL 2264, MPAN 127, which exhibited striking morphological and isozyme patterns differences were selected from the P-IVAG clones and used for monitoring the effect of subculture frequency during *in vitro* storage on genetic stability, as shown in the flow diagram (Fig. 3). One stake was sampled from each clone and exposed to thermotherapy (40°C day/35°C night) for two weeks. Meristem-tips were excised from the most apical node (first = 1) and from a lower node (2) and cultured in 4E medium. Subculturing was carried out every 5 months. PAGE electrophoresis for two isozyme systems α- and β-EST and α- and β-ACP and morphological evaluation of the *in vitro* material were also carried out. The first micropropagation yielded four cultures, the second gave rise to eight cultures, the third to 16 cultures and the fourth to 32 cultures. Preliminary observations of the electrophoretic banding patterns after 5 months of storage and *in vitro* morphological characters of these clones shows no differences from the patterns of the control.

5.13 Associated Field Genebank (AFG)

The AFG comprised: a) The 100 clones without *in vitro* processing, i.e. the control (AFG-C); b) the same 100 clones micropropagated *in vitro*, but without storage (AFG-i); and c) the accessions after *in vitro* storage AFG-s (see Fig. 1). The AFG-C has been used for clonal evaluation and as a control for the P-IVAG. The AFG-i is the control for phenotypic changes of *in vitro* plants after storage (AFG-s). At the beginning, stake samples from each plant growing in the field genebank, were taken and planted in 28 x 60 m plot. Five plants per clone were planted at a distance of 1m between plants, 4m between rows and 3m between clones.

The cassava material maintained under field conditions in the AFG suffered from several problems: mite attack, salinity, etc. As a result, replicates have been lost; these were not replaced in the three generations of the AFG-C. Table 19 shows the number of cassava varieties and replicates in the AFGC-III at the time of reporting. One variety, MARG 15, was completely lost in the field. In the field only 38 varieties showed 5 replicates, while in *in vitro* storage 70 varieties showed 5 replicates (Table 13).

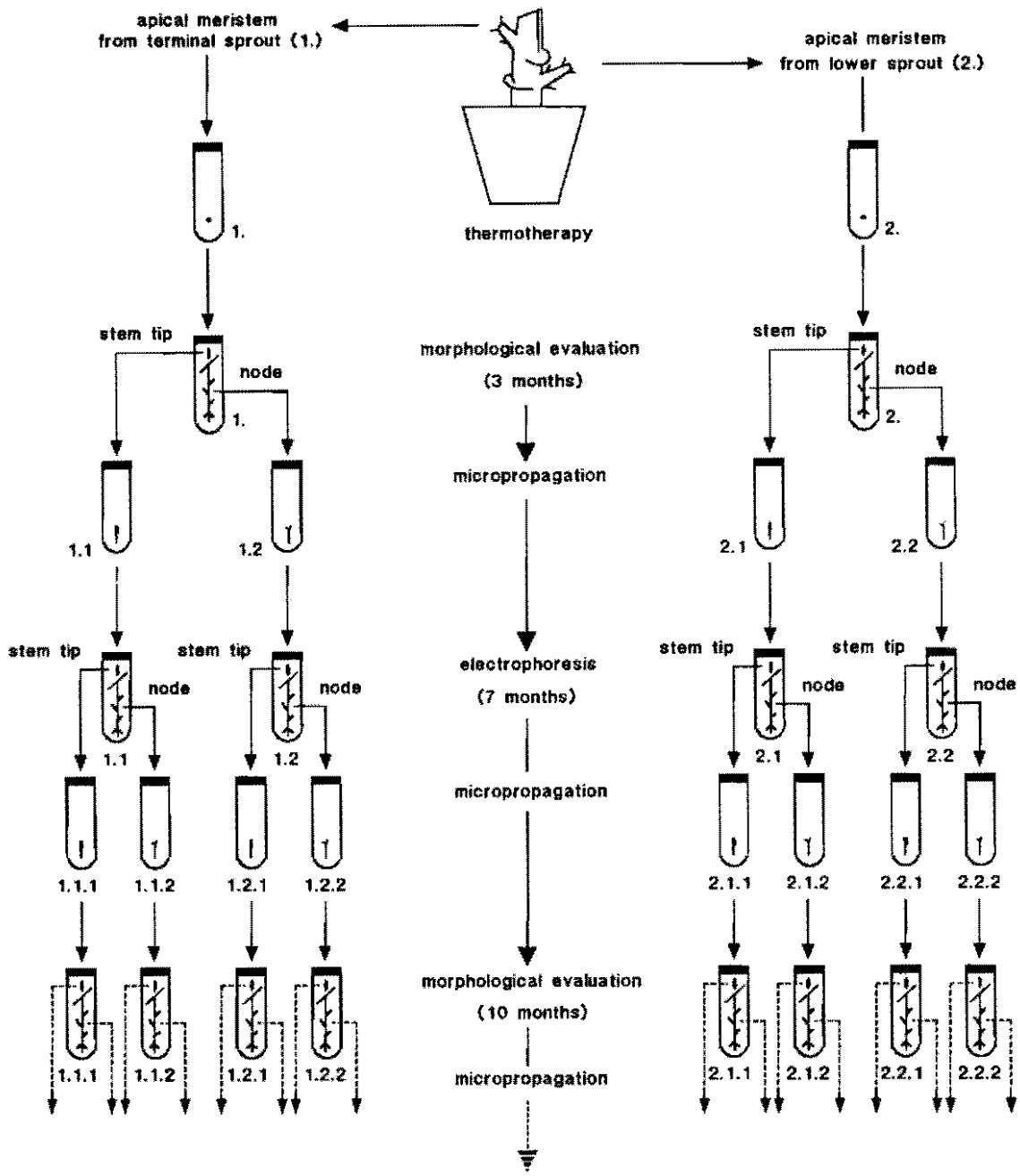

FIGURE 3
MONITORING SUBCULTURE FREQUENCY AND SOURCE
OF MERISTEMS IN THE P-IVAG

TABLE 19
CASSAVA VARIETIES AND REPLICATES IN THE AFGC-III

NUMBER OF VARIETIES	NUMBER OF REPLICATES
38	5
36	4
16	3
8	2
1	1
1	0

5.14 Monitoring genetic stability

In the field
A total of 36 discrete morphological traits were used as descriptors for the characterization of six month-old plants in the AFG-C, generations I, II and III (Appendix I). Data were compared among all generations and no changes were observed. In the AFGC-I, α- β-EST isozyme characterization of the replicates of each variety was carried out, in order to obtain the original patterns and detect any differences between replicates of each variety. No changes were found in EST patterns. For the AFGC-II, α- β-EST, α- β-ACP, GOT and DIAP were analyzed in 29 selected clones (Table 11), and comparisons were made between the AFGC-I and AFGC-II in α- β-EST patterns; no changes were observed.

Plants in the AFG-i (micropropagated, i.e. without storage) were maintained in the field for later evaluation with 4 isozymes and comparison with the AFGC-II. The AFG-i comprises the clones which have been processed through *in vitro* micropropagation; the material was moved to the field in batches of 20 clones. The first and second batches were evaluated morphologically and biochemically with four isozymes as the control for the material retrieved from *in vitro* storage. Minor differences in the general vigour of the plants and root yield were found between AFGC and AFGi material. These differences can be ascribed to the well known "seedling behavior" of *in vitro* plantlets which is evident in the first field cycle.

Two accessions out of the 100 tested, which had been found to be duplicates, showed some instability under field condition in the first generation. These clones, MBRA 356 and MCOL 2264 are variegated. In the first cycle of the AFG, morphologically different green shoots emerged from the variegated plants; these

were excised and grown in the AFG-II and AFG-III with the original variegated plants. Conspicuous morphological differences were found between variegated and green plants. Increase in root yield, plant size, vigour and fertility were recorded from the green plants, probably due to their higher photosynthetic activity. Electrophoresis of root-tip extracts using four isozyme systems did not show any differences in banding patterns. PAGE analysis of leaf tissue (green and yellow tissues separately) only showed differences in one DIAP band and one ACP band. Morphology and PAGE analyses suggest the occurrence of a periclinal chimera located in the L-I layer of the two variegated accessions. The same clones presented reversal to the green solid leaf in the large *in vitro* collection at CIAT.

In vitro

Two groups of plants, one comprising 10 and the other 7 clones from the 100 clones under *in vitro* storage, were selected for monitoring genetic stability with isozyme markers. The first group (A) was selected from the first 40 clones introduced into slow growth storage on the basis of contrasting morphology and electrophoretic patterns. The second group (B) comprised clones stored in slow growth for 10 years in the world cassava *in vitro* collection at CIAT, and micropropagated 8 times on average. They are: MVEN 82, MPER 132, MCOL 2264, MCOL 1684, MCOL 2131, MECU 72 and MBRA 12. This protracted *in vitro* storage could, as pointed out earlier, have affected genetic integrity. The 17 clones were retrieved from slow growth storage, micropropagated in 4E culture medium to obtain sufficient replicates analysis, transplanted to the glasshouse and then to the field for morphological evaluation and PAGE analysis.

Monitoring genetic stability of *in vitro* plantlets in slow growth storage has been undertaken using 16 isozyme systems and 20 morphological characters, comparing root extracts from *in vitro* plantlets with extracts from regenerated lignified plants growing in the field (AFG-i). The results will provide more solid data on stability; particular bands in control and stored clones can be monitored. The analysis will, however, only cover a small fraction of the cassava genome; as far as is known, other isozyme systems do not help fingerprinting *Manihot esculenta*. Moreover the isozyme patterns could be influenced by physiological/environmental conditions and enzymes coded by different genes may have similar mobilities, producing overlapping bands which could be interpreted erroneously.

DNA fingerprinting techniques are currently being used as a complement to morphological and isoenzyme characterization and to construct saturated genetic maps in many crops, like lentil (Havey *et al.*, 1989), soybean (Apuya *et al.*, 1988) and rice (McCough *et al.*, 1988), mainly because they can cover an extended portion of the genome. They can study polymorphism in expressed and non-expressed

genes and, furthermore, can be performed at any stage of plant development. DNA fingerprinting could be used to study stability after *in vitro* conservation as well as genotype characterization, and would complement morphological and isozyme analysis for assessing the stability of cassava clones maintained *in vitro*.

This combined approach has been taken to assess the genetic stability of the 7 cassava varieties maintained in slow growth for 10 years (see above). Two sets of controls were used for analysis of biochemical and molecular fingerprinting: (i) the same 7 varieties processed by meristem micropropagation and moved to the field; (ii) the same material obtained from the field collection. Analysis with α, β - esterase isozymes, with 16 single copy RFLP probes combined with 8 restriction enzymes (i.e. 128 enzyme probe combinations), with the M-13 probe, and with 20 RAPD primers, did not show any genetic fingerprinting differences between the cassava material stored for 10 years and the two controls.

5.15 Information System

A database was developed for managing the P-IVAG operations, e.g. recording morphological data, disease indexing, electrophoresis and monitoring genetic stability. The software package developed allows running all aspects of the P-IVAG and facilitates quick storage and retrieval of information. The information system was written in DBASE III PLUS and the version DOS 3.3 was used for the P-IVAG programs. An IBM-80 system-2 personal computer with 80 megabytes capacity has been operating with new software modified according to changes appearing during the development of the project and the final review. Those changes have been done with the cooperation of the CIAT Data Services Unit.

The database system includes the following characteristics: it is simple use; it has sufficient space to hold all the project information; it is self-contained so that it can operate independently on specific machines (either micro, mini or mainframe); the data within the system are easily extracted to update other systems; and this type of system will eventually be able to transfer the information to national programmes on floppy disks. The raw data collected during the three years of the project related to characterization, disease indexing, electrophoresis and all the technical and logistical aspects of the project have already been classified and fed into the database. The information has been updated systematically.

The database system of the P-IVAG was connected to CIAT's main frame in order to provide access to other cassava germplasm data at CIAT. The experience gained in this project allowed the development of a database for the operation of the

world cassava *in vitro* collection currently maintained at CIAT. The database for the IVAG is being adapted now, based on the database of the P-IVAG. It was written in DBASE III PLUS with same the operational specifications of the P-IVAG. Some options of the main menu (passport data, disease indexing, storage location and consumable products) were developed as in the P-IVAG. The database includes the following options in the main menu:

1. Entry, Updating and Viewing

Entry, updating and viewing data for every stage of the project:

1.1 Passport data: information on each accession was obtained from the CIAT cassava database. It refers to date of acquisition, collecting institution, origin, latitude, longitude, altitude, and local name. However, some data were not available for a few varieties.

1.2 Field characterization: the view/update menu (see Fig. 4) is organized to hold information on the Associated Field Genebank, for the AFG-i *in vitro* control) and two generations from *in vitro* storage under slow growth. A total of 36 field descriptors were evaluated for each genotype (see Appendix I and Section 5.4; Fig. 5a and 5b). Examples of screens for field characterization in the first generation are shown here but they are the same for every generation in the field.

```
                    IBPGR - CIAT
            Pilot In Vitro Information System          1.1.2
                  Field Characterization
                    View/Update Menu


        (1)  Associated Field Genebank I
        (2)  Associated Field Genebank II
        (3)  Associated Field Genebank III
        (4)  AFG From In Vitro Control
        (5)  AFG From First In Vitro Storage Gen.
        (6)  AFG From 2nd   In Vitro Storage Gen.


         ENTER DESIRED OPTION -

   Press (Esc) for Previous Menu, (Q) to Quit the Information System
```

FIGURE 4. SCREEN WITH FIELD CHARACTERIZATION MENU

CG7-64/1	ASSOCIATED FIELD GENEBANK I	GEN.1	Page 1

Colour Unexp. Apical Lv.:	7	Shape of Central Lobe:	5
Colour 1st Full Exp. Lv.:	3	Petiole Colour:	3
Petiole Length:	5	Pubescence of Young Lvs.:	3
Dist. Anthocy Pig Pet.:	1	Leaf Vein Colour:	3
Num. of Lv. Lobes.:	7	Dist. Anthocyan. Pig.Stem:	0
Stem Color:	1	Angle of Branch:	7
Num. Levels Branching:	3	Stor. Root Pulp Color:	2
Stor. Root Surf. Color:	2	Plant Form:	2
Grth. Hbt. of Yng. Stem:	1	Storage Root Form:	2
Stem Epidermal Color	1	Color of Sepals:	5
Color of Disc:	0	Color of Stigma:	1
Colour of Ovary:	1	Exocarp of Fruit:	1

Previous, Next, Update, Search, Exit --> Enter Choice:

FIGURE 5a. SCREEN FOR FIELD CHARACTERIZATION

CG7-64/1	Field Characterization Descriptors	GEN.1	Page 2

Storage Root Peduncle;	5	Clr. Out-rt Storage Context:	1
Storage Root Length;	5	Degree of Flowering:	0
Person Characteriz.	RCHAVEZ	Degree Fruit set:	0
Site of Eval.:	CIAT	Date to Field	04/12/87
Fresh Weight Score:	9		

	Repetition Number					
Pollen from Generation:	1	2	3	4	5	AVG.
Fresh Weight Obs:	0	0	0	0	0	0.0
Width of Central Lobe Obs:	0.0	0.0	0.0	0.0	0.0	0.0
Length of Central Lobe	3.5	3.7	3.4	3.6	3.4	3.5
Obs:	26.0	18.0	16.0	15.0	15.0	18.0
Height of Branch Obs:	45	35	40	35	30	37.0
Height of Plant Obs:	165	150	145	140	135	147.0

, , , and Enter keys to move the cursor on the screen
and the PgUp and PgDn keys to move between screens

FIGURE 5b. SCREEN FOR FIELD CHARACTERIZATION

CG7-641/1	Disease Indexing Elements		GEN.1
	Date	CCMV (Positive=1,	CSXV Negative=0)
ELISA:	08/10/87	0	0
		(Death=2, Positive 1, Negative=0)	
FSD:	07/09/87	0	
dsRNA:	10/10/87	0	
Grafting:	12/09/87	0	

Previous, Next, Update, Search, Exit --> Enter Choice:

FIGURE 6. SCREEN FOR DISEASE INDEXING

1.3 Disease indexing: contains the results of the initial virological tests using ELISA, symptomatology, dsRNA, and grafting for the 100 P-IVAG clones and their five replicates (Fig. 6). Also it includes the data for the second test with ELISA, done only on clones positive in the first test.

1.4 Electrophoresis: using the same system as in the field characterization, it is possible to store the results of electrophoresis at every step, e.g. initial control test of AFG-C I, AFG-C II, AFG-C III, also data from *in vitro* control test and *in vitro* test from the first and second generations from storage. In order to characterize the electrophoretic patterns, the presence or absence of bands in the gel, either in PAGE or starch gel electrophoresis was used (Fig. 7a and 7b). There is space for data on each replicate in every variety, but this is used only in the specific cases when the pattern of any variety shows differences between replicates.

1.5 Storage location details: holds information on the location of clones in the storage room, e.g. shelf, holder, number of replicates, date of entry into culture for storage, date of entry into storage room, and date of retrieval from storage.

```
CG7-641/1 |  INITIAL CONTROL TEST AFG I  | GEN. 1 |

                                        Enzyme Number
                      1              2              3              4

Date Elec. Perf. 09/19/87          / /            / /            / /
Enzyme Name:       EST             ACP            DIAP           GOT
Photo Number:
Holding LKB Resu
Loc.GEL for Enzy

     abcdefghijklmnop abcdefghijklmnop abcdefghijklmnop abcdefghijklmnop
Bands:1101010100000000 1100011111001001 1100111110000000 0110000000000000

Previous, Next, Update, Search, Starch Electrophoresis, Exit -> Choice:
```

FIGURE 7a. SCREEN FOR PAGE ANALYSIS

```
CG7-641/1 |  INITIAL CONTROL TEST AFG 1  | GEN. 1 | rt

                                                                      st
ME Gel No. 0              HK Gel No. 0              SOD Gel No. 0
Date  /  /                Date  /  /                Date  /  /
abcdefghijklmnop          abcdefghijklmnop          abcdefghijklmnop

PGI Gel No. 111           PGM Gel No. 0             PRX Gel No. 0
Date  /  /                Date  /  /                Date  /  /
abcdefghijklmnop          abcdefghijklmnop          abcdefghijklmnop

IDH Gel No. 0             MDH Gel No. 0             GDH Gel No. 111
Date  /  /                Date  /  /                Date  /  /
abcdefghijklmnop          abcdefghijklmnop          abcdefghijklmnop

SKDH Gel No. 0            G6PDH Gel No. 111         6GPDH Gel No. 0
Date  /  /                Date  /  /                Date  /  /
abcdefghijklmnop          abcdefghijklmnop          abcdefghijklmnop
```

FIGURE 7b. SCREEN FOR STARCH GEL ELECTROPHORESIS ANALYSIS

1.6 In vitro *characterization:* according to the experience in the BRU-CIAT, the cultures were evaluated with general qualitative descriptors. This evaluation was done after six months in culture. The characters evaluated were: presence or absence of roots, aerial roots, etiolation, callus formation and number of shoots.

1.7 Evaluation of viability: this evaluation was done every month with every variety in *in vitro* storage. It covered contamination, browning, defoliation, bleaching, death, date of death and special notes. It was designed to store information on five *in vitro* generations.

2. Report options

The Report Options generates the printed reports of stored data (as above, 1.1 - 1.7) in a general form for each stage.

3. Resource Tracking Option

The Resource Tracking Option covers data and reports on technical and logistical aspects of the project:

Staff name entry, listing and updating.
Task (job) entry, listing and updating.
Monthly staff entry, listing and updating.
Monthly staff and task reporting.
Equipment entry, listing and updating.
Monthly equipment use entry, listing and updating.
Monthly and basic equipment reporting.
Consumable products entry, listing and updating.
Consumable product and items reporting.

4. Query Option

The Query Option allows interrogation on specific aspects at different stages and comparison between *in vitro* generations at a specific stage. It is possible to obtain the information either on the screen or as a print-out. Questions are posed to respond to doubts or to select information on specific criteria, e.g.:

For field characterization, it is possible to compare one or more varieties in two or three generations in the AFG, for each descriptor.

For disease indexing, the program compiles the positive accessions with its replicates in each virological test in both the initial and the second test.

For storage and location details, four questions were asked: subculture interval in descending order, number of replicates per variety entered into storage, the shelf position, and test tube holder in the storage room. In any one of these it is possible to obtain the information per accession, for every *in vitro* culture generation. In the same way, for *in vitro* characterization, questions relate to each descriptor per accession and generation, and it is also possible to compare different generations.

Some activities can be planned ahead of time making it possible to produce a calendar that is useful for programming each step (Table 20). (Activities, that can not be planned depend on the behavior of the plant, e.g. micropropagation.)

TABLE 20
CALENDAR OF THE MOST IMPORTANT ACTIVITIES IN THE GREENHOUSE, THE FIELD AND *IN VITRO*

ACTIVITY	TIME
Planting stakes in greenhouse	0
ELISA	3 weeks
Electrophoresis	4 weeks
Grafting and dsRNA	9-10 weeks
Thermotherapy	10 weeks
Cutting meristems	13 weeks
Subculture	14 weeks
In vitro electrophoresis	At 4 months of growth in 4E medium

5. ***Back-up and Restore***

The main menu has the option to back-up and restore data files as a security menu.

5.16 Chemicals, Equipment and Facilities

Chemicals

About 60 kinds of chemical have been used during the three year project. They comprise reagents for PAGE and starch gel electrophoresis, for tissue culture, and for glasshouse and field work (herbicides, fungicides, fertilizers, insecticides).

Glassware
The glassware used in the P-IVAG mainly comprised test tubes, beakers, graduated cylinders, pipettes, micropipettes, Erlenmeyer flasks, trays for electrophoresis, etc.

Equipment
About 33 different types of laboratory apparatus were used from different CIAT laboratories in addition to the computer that was used in this project (Appendix II). Most of the equipment was already available at CIAT at the beginning of this project, such as shakers, centrifuges, autoclaves, electronic balances, etc. One power supply for electrophoresis, vertical tubes for electrophoresis, the personal computer and accessories were acquired for the P-IVAG. In addition, an LKB ultrascan laser densitometer with accessories and software was purchased by CIAT for the electrophoretic survey.

Facilities
The following CIAT laboratories and other facilities have been used for establishing and operating the P-IVAG.

Tissue culture preparation laboratory
Transfer room
Propagation room
In vitro storage room
Biochemistry (Electrophoresis) laboratory
Virology laboratory
Computer Center and Mainframe
Photographic, Graphic Arts and Library facilities
Glasshouses
Screen houses
Field plots

5.17 Costs

Cost estimates for laboratory and field activities are given in Tables 21a and 21b (1990 figures).

5.18 Staffing/Personnel

The CIAT staff, collaborators and advisors involved in the project are listed in Table 22.

TABLE 21a
GLOBAL EVALUATION FOR 3 YEARS IN THE LABORATORY

Item	US$
Culture medium (4E, 8S, 17N)	600
Test Tube No. 18 (3500)	1,500
Test Tube No. 25 (2800)	2,200
Glassware	1,700
sub-total	6,000
500 Acrylamide gels and stain	1,800
100 Starch gels and stain	1,500
Others	500
1 Power supply	2,500
1 Slab and tube vertical electrophoresis	1,000
2 Cells for starch	400
sub-total	7,700
Photography and others	2,000
Miscellaneous Equipment (Pipetman, syringes, etc.)	1,500
Total	17,200

TABLE 21b
GLOBAL COSTS FOR 3 YEARS IN THE FIELD (AFG)

Item	US$
Land Management	450
Irrigation	240
Workers	300
Equipment	340
Others	100
Chemicals	150
Total	1,580

TABLE 22
PROJECT STAFF, COLLABORATORS AND ADVISORS

Principal Staff	Time dedication (%)
R. Chavez	100
W. Roca	10
Research Assistance	
D.I. Arias	100
Technicians	
J.L. Claros	100
M. Quintero	100
R. Arias	100
Collaborators and Advisers	
C. Hershey (Cassava Program)	
B. Nolt/L. Calvert (CIAT Virology)	
L. A. Withers (IBPGR/IPGRI) Rome	
M. Perry (IBPGR/IPGRI) Rome	
H. Trejos (DSU - CIAT)	
H. Ramirez (BRU - CIAT)	
G. Lema (DSU - CIAT)	
B. Pineda (Virology - CIAT)	
F. Angel (Molecular Biology - BRU - CIAT)	
V. Barney (Thesis Student - BRU - CIAT)	

6. CONCLUSIONS

During the three-year period of the P-IVAG, important components of the establishment and operation of an *in vitro* active genebank were assessed; these included: the sampling and characterization of field material for entry into slow growth storage, micropropagation of clones for storage, and characterization of *in vitro* cultures. Other components of the P-IVAG such as the monitoring of genotypic stability and subculture frequency were evaluated only partially because the three-year period of the project did not allow full evaluation. An important management component, the information system for *in vitro* storage, was fully developed and tested. Logistical aspects of *in vitro* storage like equipment needs,

supplies and technical staff requirements were also determined in the three-year period of the P-IVAG. Hence, definite conclusions can be drawn from several aspects of the project, while others should be considered as preliminary:

1. The 100 cassava clones selected for the P-IVAG from the CIAT world collection represented a condensed sample of variability in terms of morphology and, as such, allowed monitoring of stability in the field.

2. The size sample of the material selected (100 clones, 25 replicates) was too large to handle throughout the project, especially in monitoring genetic stability in all replicates of every variety. Because of the large work load in the P-IVAG, the selected clones were divided into five batches for entry into slow growth storage. The actual timing of each batch, going through the various stages of preparation before entry into slow growth storage, can be illustrated by the case of the first batch (see Table 3):

From sampling of clones from the field to:		**Time in Months**
2.1.	Disease indexing (ELISA) and initial isozyme fingerprinting	1
2.2.	Second set of disease indexing and thermotherapy	2
2.3.	Meristem-tip isolation and culture	2.5
2.4.	Sub-culturing for further growth	3
2.5.	Micropropagation (3-4 times)	9
2.6.	Introduction into slow storage	10
2.7.	Stability tests (*in vitro*): based on morphology	18
2.8.	First subculturing (transfer to fresh medium)	24-26
2.9.	Stability tests: electrophoretic evaluation	27
2.10.	Re-entry into slow storage	28

3. Except for the dsRNA and the FSD tests, all other virus tests revealed a low incidence of virus diseases in the field collection. The high dsRNA readings should be seen cautiously because of the occurrence of non-specific reactions. There was little correlation between the different virus tests utilized.

4. A total of 46 qualitative and quantitative descriptors were recorded from the 100 clones of the associated field genebank and from 50 clones transplanted to the field from micropropagated control cultures (without storage). Clustering of the 100 clones, using 25 descriptors, yielded 64 groups with a 75% affinity level, indicating a high level of heterogeneity; only four groups of morphological duplicates were found at a 99% affinity level: 1) MCR12 and MGUA 63, 2) MMEX 43 and MGUA 58, 3) MBRA 356 and MCOL 2264 and 4) MCR 1, MGUA 44 and MGUA 7a. It was not possible to discriminate them with α- β-EST, α- β-ACP, GOT and DIAP.

5. Morphological characterization of cassava *in vitro* cultures could rely on five descriptors: etiolation, shoot number, callus formation, level of rooting and aerial roots; some of these depend on physiological conditions, e.g. etiolation. Morphological characterization of *in vitro* cultures was carried out after 6 months under slow growth storage and allowed determination of the range of response of the 100 clones to *in vitro* storage conditions. Over 50% of the clones showed etiolation above the normal internode elongation, suggesting the need for adjustment of illumination/temperature. About 50 of the clones grew into only one shoot; a few proliferated 4 shoots per explant. One third of the clones formed aerial roots indicating a high relative humidity within the test tube. Anthocyanin pigmentation was present only in three clones despite this character's being observed in 63 clones under field conditions. Leaf shape variation occurred in two clones which also showed a similar trait in the field.

6. Electrophoretic isozyme fingerprinting was carried out using 4 PAGE systems and 12 SGE systems, the most polymorphic being: EST, DIA, GOT and ACP.

6.1. With field material, EST, ACP, GOT and DIAP isozymes, (in that order), showed the highest polymorphism among the 100 clones. Clustering of the 100 clones was carried out into 84 groups with 97% similarity (EST), 6 groups with 90% similarity (GOT), 20 groups with 97% (ACP) and 4 groups with 97% similarity (DIAP). These clusters enabled the identification of 9 duplicates in the collection. Inheritance studies were also conducted for EST, GOT and ACP, which provided insight into the genetics of cassava isozymes, pattern of segregation and number of alleles present.

6.2. The electrophoretic banding patterns of *in vitro* material were different depending on the culture medium used. The 4E medium proved the best for electrophoresis. Working with *in vitro* material, isozyme electrophoresis of root-tip extracts was, to a large extent, different from stake material, except for three bands out of the 23 recoded on stakes. A set of 29 clones was selected for isozyme analysis of *in vitro* material; the 29 clones displayed well characterized, unique, patterns.

7. Thermotherapy, meristem-tip culture and micropropagation of 97 clones was carried out successfully using previously developed techniques for cassava. Three varieties did not respond to the protocol.

8. The initial number of replicates (25 per clone) was maintained without loss, during micropropagation of 70 clones; 27 clones lost some replicates for various reasons, and were represented by one to four cultures.

9. The sub-culture frequency for clones ranged from 8 to 17 months; this variability was attributed to genotypic effects. About 50% of the clones of the first and second batches and some of the third batch (4-8 varieties) had to be subcultured after one year of storage (see point 10 below).

10. Microbial contamination of cultures occurred mostly up to the 5th month of storage; there was a tendency for varietal effect on contamination; i.e. some clones tended to show higher contamination than others, but none of the clones was completely lost due to this problem (minimum cultures contaminated = 1, maximum = 8, out of 25 replicates). Out of the various characters evaluated (browning, bleaching, contamination, defoliation), percentage defoliation determined most accurately the degree of deterioration of the cultures during *in vitro* storage, indicating the need for sub-culturing.

11. Using 36 descriptors, field material did not show observable changes after three generations; nor were changes found in the EST patterns. Periclinal chimeras (variegation) of two cassava clones were broken in *in vitro* culture and in the field, yielding plants with solid green and variegated foliage.

12. A complete information system was developed for the P-IVAG. The system has been put into operation and is being used for most operations of the project. The data held include: passport data, field characterization, disease indexing data, electrophoretic data, entry into storage and location, *in vitro* characterization, viability evaluation and various logistical considerations of the project. Specific questions relating to the project can be posed.

13. About 60 kinds of chemical, were used in the P-IVAG in addition to a range of glassware and some 30 pieces of equipment.

14. Some 14 publications have been produced during the running of the project, ranging from newsletters and annual reports to refereed journal papers.

15. Increasing losses of replicates from the field collection after three generations (Table 13) fully justify the establishment of an *in vitro* collection. While one clone was completely lost from the field and 61 clones lost between 1 - 4 replicates, not a single clone was completely lost from the P-IVAG collection and only about one third of the clones experienced replicate losses of 1 to 3.

16. The minimum number of cassava cultures that can be maintained under slow growth storage is obviously one, but for security back-up, and for allowing faster build up of retrieved material for international distribution and field testing, 3-5 cultures per clone should be maintained.

17. Monitoring of the effects of sub-culture frequency on genotypic stability have continued.

18. Similarly, genotypic stability evaluations have continued using 7 clones from the CIAT *in vitro* active genebank after 10 years storage, plus 10 clones selected from the P-IVAG. Both morphological and isozyme electrophoretic analysis will be used for assessing stability.

19. DNA fingerprinting has also been developed as a complement to isozyme analysis.

20. Analysis of stability of 7 varieties after 10 years of slow growth conservation at CIAT, using one biochemical and three different molecular markers demonstrated complete genotypic stability of all material tested.

7. GENERAL RECOMMENDATIONS ON *IN VITRO* CONSERVATION BY SLOW GROWTH

1. Before embarking on *in vitro* conservation, the value of this approach should be balanced against other conservation strategies. An evaluation can be based on knowledge of the genepool, reproductive behavior, range of variability (geographical and genetic) and costs. The magnitude of *in vitro* storage should be

justified by the biological advantages of such an approach and complement other conservation strategies for the same crop species (seed, field genebank, etc.), including *in situ* conservation.

2. A thorough knowledge of the *in vitro* culture behavior (culture initiation, explant, micropropagation, etc.) and requirements of the species should be a pre-requisite for undertaking an *in vitro* conservation strategy.

3. Depending on the importance of the collection in terms of size, agronomic/economic importance, etc., two different levels of *in vitro* conservation approach could be envisaged: a fully implemented system versus a minimum input system. Once this has been defined, the necessary facilities, equipment, etc. should be defined, as well as the acquisition of adequate expertise.

4. The operational plan for the *in vitro* storage system should be designed taking into account all the steps, procedures and data needed throughout. The plan should include both the field and laboratory phases of the operation, timing, and requirements for technical help.

5. The rate of introduction of accessions into *in vitro* storage needs to be balanced against the risks of loss of accessions due to genetic erosion, pest or disease attack, or climatic hazards in the field on the one hand, and the possibility of introducing "non-cleaned" accessions into storage, on the other.

6. There is a phytopathological "bottleneck" due to the normally slow process of disease elimination versus the high multiplication rates needed after "cleaning" of pathogens. In any case, there is value in conducting good quality phytopathological processes at the beginning of the exercise (despite their slowness).

7. Genetic stability is an important condition of any *in vitro* conservation strategy. Monitoring techniques for stability will depend on the history of the crop species regarding this trait. Use of sophisticated, high-technology monitoring (isozymes, molecular markers such as RFLPs and RAPDs) has value when there is a sufficient basis to assume instability, either intrinsic or due to the culture system and sub-culture frequency. In other instances, visual observation of morphological changes may be sufficient in comparison with appropriate controls. This study has suggested caution regarding the value of isozymes for evaluating genetic stability, mainly due to the small coverage of the genome. In any case, if a thorough evaluation of stability is necessary, a combination of approaches (morphological, isozymes and molecular) may be the most appropriate.

8. Should variants appear in *in vitro* storage, it needs to be determined whether they are a consequence of mixtures or errors. In any case, one should go back to the original field collection to look for the "correct type" material. In this context, the Associated Field Genebank (AFG) could be the source of reference material. The establishment and running of the AFG is expensive and time consuming; its function can be replaced by the original field collection.

9. A field collection should exist for as long as the *in vitro* genebank has not been duplicated elsewhere for security reasons. Once duplication is done, only material for evaluation would be grown in the field.

10. Decisions regarding the number of replicates will depend on: size of collection, size of vessels, risk of losses during *in vitro* multiplication, subculturing and storage. Thus, in the case of cassava, a minimum of 1 and a maximum of 3 culture replicates were lost, hence, a replication of 3-5 per accession is recommended.

11. Depending on the size of the collection, a more or less sophisticated information system will be needed for the management of the collection. Simple cards or computer-aided systems can be adapted. Labelling of plants, cultures and replicates throughout the introduction, sub-culturing and retrieval of materials should be maintained.

8. P-IVAG PROJECT PUBLICATIONS

During the establishment and operation of the P-IVAG the following publications, relating to the project were produced:

Biotechnology Research Unit, CIAT. 1988. Annual Report.

Biotechnology Research Unit, CIAT. 1989. Annual Report.

Chavez, R. Arias, D.I. Roca, W.M. 1987. IBPGR-CIAT collaborative project for developing a Pilot *In Vitro* Active Genebank. Poster. *In:* International Congress of Plant Tissue Culture of Tropical Species - Abstracts. Bogota, Colombia, September 21-25, 1987.

Chavez, R. Roca, W.M. Arias, D.I. Withers, L. and Williams, T. 1988. Cooperative IBPGR and CIAT pilot project tests feasibility of *in vitro* Diversity 16: 8-10.

Chavez, R., Roca, W.M., Arias, D.I. Sarria, R., Ramirez, H. 1989. Genetic variability of isozyme loci among 100 cassava varieties and hybrid progenies. Poster. *In:* IX Congress Latinoamericano de Genetica. Lima, Peru, Oct. 1-5, 1989.

Chavez, R., Roca, W.M. and Williams, J.T. 1987. IBPGR-CIAT collaborative project on a pilot *in vitro* active genebank. FAO/IBPGR Plant Genetic Resources Newsletter 71, 11-13.

CIAT International. 1988.

IBPGR, 1987. Annual Report.

IBPGR, 1988. Annual Report.

IBPGR, 1989. Annual Report.

IBPGR, 1989. Joint project pioneers *in vitro* genebanks. Geneflow. p.12.

IBPGR, 1990. Annual Report.

Roca, W.M. Chavez, R. Marin, M.L. Arias, D.I. Mafla, G. and Reyes, R. 1989. *In vitro* methods of germplasm conservation. Genome 31, 813-817.

9. ADDITIONAL REFERENCES

Apuya, N.R., Frazier, B.L., Keim, P., Roth, Jill and Lark, K.C. 1988. Restriction fragment length polymorphisms as genetics markers in soybean, *Glycine max* (L). Merril. Theoretical and Applied Genetics 75, 889-901.

Bajaj, Y.P.S. 1977. Clonal multiplication and cryopreservation of cassava through tissue culture. Crop Improvement 4, 198-204.

CIAT, 1986. Biotechnology Research Unit, Annual Report.

Gabriel, C.J., Walsh, R. and Nolt, B.L. 1987. Evidence for a latent virus like agent in cassava. Phytopathology 77 (1), 92-95.

Havey, M.J. and Muehlbauer, F.J. 1989. Linkages between restriction fragment length, isozyme and morphological markers in lentil. Theoretical and Applied Genetics 77, 395-401.

IBPGR, 1983. IBPGR Advisory Committee on *In Vitro* Storage. Report of the first meeting. IBPGR, Rome.

IBPGR, 1986. Design, planning and operation of *in vitro* genebank. Report of a subcommittee of the IBPGR Advisory Committee on *In Vitro* Storage. IBPGR, Rome.

McCough, S.R., Kochett, G., Yu, Z.H., Wang, Z.Y., Khush, G.S., Coffman, W.R. and Tanksley, S.D. 1988. Molecular mapping of chromosomes. Theor. Appl. Genet. 76, 815-323.

Ramirez, H., Hussain, A., Roca, W.M. and Bushuk, W. 1987. Isozyme electrophoregrams of sixteen enzymes in five tissues of cassava (*Manihot esculenta* Crantz) varieties. Euphytica 86, 89-48.

Roa, J.C., Mafla, G., Roca, W.M., Pineda, B. and Nolt, B. L. 1987. Elimination of cassava (*Manihot esculenta*, Crantz) viruses by thermotherapy and meristem-tip culture. *In:* International Congress of Plant Tissue Culture of Tropical Species - Abstracts. Bogota, Colombia, September 21-25. 1987.

Roca, W.M. 1985. Cassava tissue culture. In Cock, J.H. and Reyes, J.A. (eds.). Cassava: Research, production and utilization. IJNDP-CIAT.

Roca, W.M., Rodriguez, J.A., Mafla, G. and Roa, J. 1984. Procedures for recovering cassava clones distributed *in vitro*. CIAT, 8 pp.

Stamp, H., Hussain, A., Roca, W.M. and Bushuk, W. 1987. Isozyme electrophoregrams of sixteen enzymes in five tissues of cassava (*Manihot esculenta* Crantz) varieties.

Schilde Rentschler, L. and Roca, W.M. 1987. Tissue culture for the international exchange of potato and cassava germplasm. *In*: Biotechnology in Agriculture and Forestry. Vol. 3. Bajaj, Y.P.S. (ed.). Springer-Verlag. pp. 453-465.

Scowcroft, W.R. 1984. Genetic variability in tissue culture: Impact on germplasm conservation and utilization. IBPGR Technical Report. 152, 41 pp.

Withers, L.A. 1980. Tissue culture storage for genetic conservation. IBPGR Technical Report. Rome

Withers, L.A. and Williams, J.T. 1985. *In vitro* conservation. IBPGR Research Highlights, 21 pp.

Withers, L.A. 1988. *In vitro* conservation and germplasm utilization. *In:* Brown, A.D.H., Marshall, D.R., Frankel, O.H., and Williams, J.T. (eds). The use of plant genetic resources. Cambridge University Press. pp. 309-334.

APPENDIX I

Descriptors for the morphological characterization of cassava in the Associated Field Genebank.

1. Colour of unexpanded apical leaves (COUNAL)

   3. Light green
   5. Dark green
   7. Green purple
   9. Purple

2. Colour of first fully expanded leaf (COFFEL)

   3. Light green
   5. Dark green
   7. Green purple
   9. Purple

3. Shape of central lobe (SHCELO)

   1. Elliptic
   2. Oblanceolate
   3. Elliptic undulate
   4. Pandurate
   5. Linear
   6. Linear pyramidal
   7. Linear hastatilobate
   8. Linear pandurate

4. Petiole length (PETLEN)

   0. Petiole absent
   3. Short (5-10 cm)
   5. Medium (15-20 cm)
   7. Long (25-30 cm)

5. Petiole colour (PETCOL)

   3. Light green
   5. Dark green
   7. Green purple
   9. Purple

6. Distribution of anthocyanin pigmentation in petiole (DAPPET)

   0. Absent
   1. Top part
   2. Central part
   3. Basal (insert on)
   4. Totally pigmented

7. Pubescence of young leaves (PVYLEA)

   0. Absent
   3. Little pubescence
   5. Moderate pubescence
   7. High pubescence

8. Number of leaf lobes (3, 5, 7, 9) (NVLELO)

9. Length of central lobe in cm (LEN.LF)

10. Width of central lobe in cm (WID.LF)

11. Leaf vein colour (LEAVCO)

   1. Green
   2. Purple

12. Stem colour (STEMCO)

   1. Pale silver green
   2. Dark silver green
   3. Orange
   4. Light brown
   5. Dark brown
   6. Yellow
   7. Purplish green

13. Stem epidermic colour (colenguima) (STEPCO)

   1. Pale green
   2. Dark green
   3. Yellow
   4. Purplish green

14. Distribution of anthocyanin pigmentation in stem (DAPSTE)

    0. Absent
    1. Top part
    2. Central and top part

15. Number of levels of branching (NULBRA)
    (actual number of levels)

16. Angle of branch (if branching) (ANGBRA)
    angle between vertical plane and first branches

    0. Not branching
    3. 15-30°
    5. 45-60°
    7. 75-90°

17. Height of first apical branch (if branching) (HGT.BRCH)
    in cm

18. Height of plant (to top of canopy) (HGT.PLANT)
    in cm

19. Storage root surface colour (SROSCO)

    1. cream
    2. Light brown
    3. Dark brown
    4. Orange
    5. Pink

20. Storage root pulp colour (SROPCO)
    immediately after being open

    1. White
    2. Cream
    3. Yellow
    4. Pink
    5. Orange

21. Growth habit of young stem (GHYSTE)

    1. Straight
    2 Zig-zag

22. Plant form (PLAFOM)

1. Compact
2. Open
3. Parasol
4. Cylindrical
5. Erect

23. Storage root peduncle (SROPED)

0. Absent
3. Short
5. Intermediate
7. Long

24. Storage root form (SROFOR)

1. Conical
2. Conical-cylindrical
3. Cylindrical
4. Fusiform
5. Irregular

25. Storage root length (SROLEN)

3. Short
5. Medium
7. Long

26. Total fresh weight of storage roots per plant (WGTSCL) measured in Kg

27. Colour of outer surface of storage root cortex (COSSRO)

1. White
2. Cream
3. Yellow
4. Pink
5. Purple

28. Degree of flowering (DEGFLO)

0. Absent
1. Low
2. Moderate
3. Abundant

29. Colour of sepals (COLSEP)

1. White or cream
2. Orange
3. Green
4. Red
5. Purple

30. Colour of disc (COLDIS)

1. White or cream
2. Orange
3. Green
4. Red
5. Purple

31. Colour of stigma (COLSTI)

1. White or cream
2. Orange
3. Green
4. Red
5. Purple

32. Colour of ovary (COLOVA)

1. White or cream
2. Orange
3. Green
4. Red
5. Purple

33. Degree of fruit set (DEGFRS)

0. Absent
1. Low
2. Moderate
3. Abundant

34. Exocarp of fruit (EXOFRU)

    3. Smooth
    7. Rough

35. Pollen fertility (Stainability) (POL.AVG)

36. Yield (FRSG.WGT)

    1. Very low
    3. Low
    5. Intermediate
    7. High
    9. Very high

APPENDIX II

Apparatus used in the P-IVAG project

Bacti-cinerator
Centrifuge
Stills
Sterilizer
Cooler for electrophoresis
Counter, manual, four digits
Photographic viewer
Electronic balance x 2
Electrophoresis constant supply
External disk drive - 5 1/4"
Filter - Milipore
Transiluminator
Growth chamber
Homogenizer
Incubator
IRMA Board for PS/2 model 80
Laminar flow cabinet
Light microscope
Microplate-titer
Mini-sub DNA
Orbital shaker
Personal computer
Power supply
Refrigerator/freezer
Slab and tube vertical electrophoresis tank
Soil pasteurizer
Stereomicroscope
Stirring hot plate
Transiluminator for gels
Scanning laser densitometer
Dispenser
Vacuum pump