IBPGR Research Highlights

IN VITRO CONSERVATION

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IN VITRO CONSERVATION

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The IBPGR is an autonomous international scientific organization under the aegis of the CGIAR. The basic function of the IBPGR is to promote and coordinate an international network of genetic resources centres to further the collection, conservation, documentation, evaluation and use of plant germplasm and thereby contribute to raising the standard of living and welfare of people throughout the world. Financial support is provided by the governments of:

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FOREWORD

Up to 1984, all IBPGR-supported research had been summarized in the Annual Reports. Starting in 1985, it was decided that, in addition to the summaries in the Annual Report, particular areas of research should be reported in greater depth. For 1984-85, IBPGR research on *in vitro* conservation forms the topic for the first of the IBPGR Research Highlights. It represents a consolidation and update on reports to a CGIAR Inter-center Workshop made in 1984 (Withers and Williams, 1985) and in 1985 to a 15th Anniversary Symposium for the Genebank at FAL, Braunschweig, Federal Republic of Germany.

ACRONYMS AND ABBREVIATIONS USED

AVRDC	Asian Vegetable Research and Development Center, Taiwan, China
CGIAR	Consultative Group on International Agricultural Research
CIAT	Centro Internacional de Agricultura Tropical, Cali, Colombia (CGIAR)
CIP	International Potato Center, Lima, Peru (CGIAR)
CRU	Cocoa Research Unit, University of the West Indies, Trinidad
FAO	Food and Agriculture Organization of the United Nations
GCGRA	Ghana Cocoa Growing Research Association
IBPGR	International Board for Plant Genetic Resources (CGIAR)
IITA	International Institute for Tropical Agriculture, Ibadan, Nigeria (CGIAR)
LN	Liquid nitrogen
ODA	Overseas Development Administration, UK
SERC	Science and Engineering Research Council, UK

Cover illustration (clockwise from top left): Shoot apices of banana suitable for storage by slow growth in an *in vitro* active genebank (N. Banerjee and E.A.L. de Langhe, Catholic University of Leuven, Belgium); liquid nitrogen refrigerator for storage of cryopreserved cultures in an *in vitro* base genebank (L.A. Withers, University of Nottingham, UK); excised coconut embryo developing in culture (C. Pannetier and colleagues, IRHO/CIRAD-ORSTOM, France); taro plants derived from rooted meristem cultures (M.S. Strauss, Northeastern University, Boston, USA).

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INTRODUCTION

Much of the world's germplasm is currently maintained as breeders' collections in plantations, orchards or even in so-called evolution gardens. Many of these are historical holdings, which in the past were used for development and improvement of plantation crops in the tropics or, for instance, for breeding of temperate fruits. They range from those which are grown from seed, such as rubber and coconut, to those which are vegetatively propagated such as citrus, cacao (in some cases), banana and many other fruits; they also include clonal collections of important staple food crops such as cassava, sweet potato, yam and aroids such as *Colocasia* and *Xanthosoma*.

IBPGR calls such collections 'field genebanks', a generic term to cover 'plantation', 'orchard', 'clonal repository' and the scientifically unsound expression 'living collection'.

From the point of view of genetic conservation, these field genebanks are, in most cases, totally unrepresentative of the range of genetic variability within the respective crop genepool and most of them do not constitute more than a fraction of the variability which should be conserved for the future.

When IBPGR started its field programme it perforce laid initial stress on seed crops such as the cereals and food legumes; later it moved to include fruits, vegetables and forages (Williams, 1984). Any strategy for collection and conservation of samples of crops that are normally propagated vegetatively, or that produce seeds which cannot be stored using normal procedures of seed drying and maintenance at low temperatures as used in seed genebanks, had to include a consideration of *in vitro* techniques. Problems of *in vitro* storage of such material when solved should also relate to cycling of the material through multiplication schemes, distribution of germplasm and also its characterization and evaluation (Figure 1). Hence the development of the full potential of *in vitro* culture storage and associated biochemical techniques would revolutionize the handling of germplasm that, for various reasons, is considered difficult to conserve.

IBPGR's Involvement in Research on *In Vitro* Conservation.

In 1979 IBPGR commissioned a scientific report to examine in detail the potential for developing *in vitro* methods of storage. The potential had been recognized from early research on the successful preservation of meristem/shoot-tip cultures at a low temperature which still permitted growth (Galzy, 1969) and cryopreservation (freeze-preservation) of embryogenic cell cultures (Nag and Street, 1973). Despite the results from such research, the application of the techniques to genetic conservation remained conjectural (Henshaw, 1975; Morel, 1975) because research had not been aimed at this specific objective.

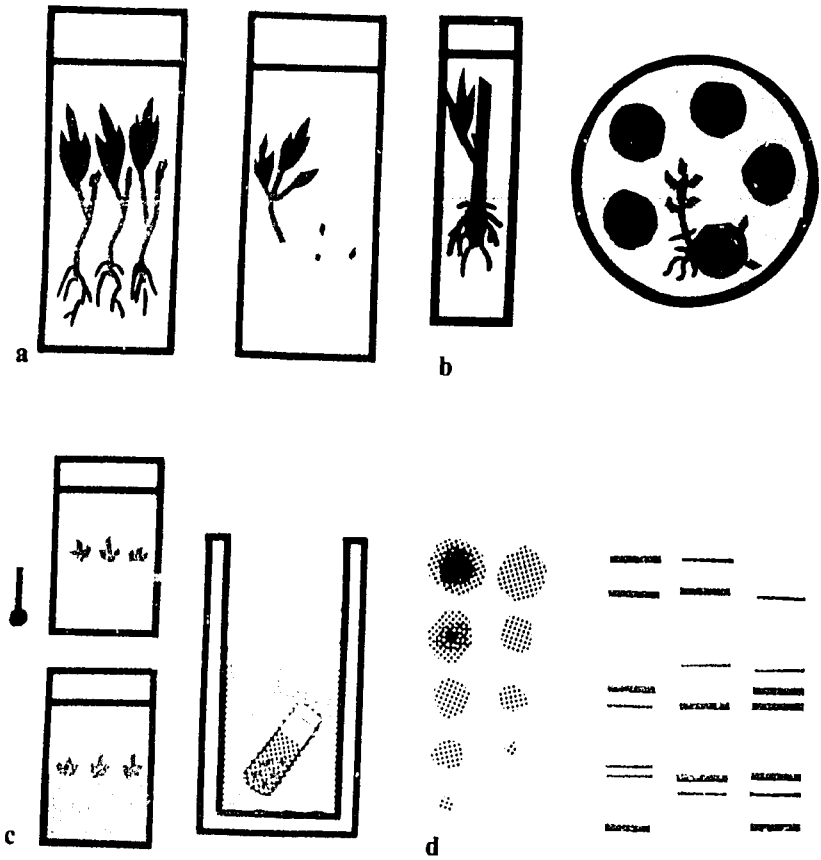

Figure 1. Novel techniques for handling and conserving plant germplasm include *in vitro* methods for (a) multiplication, (b) acquisition and distribution, and (c) storage by slow growth or cryopreservation, plus (d) biochemical/molecular methods for characterization, evaluation and disease indexing.

The report (Withers, 1980) stressed firstly the useful but limited rôle of slow growth of tissues *in vitro* and secondly the relatively unexplored potential of cryopreservation. These conclusions were extremely logical and had the advantage of being backed by more recent research on the storage of shoot cultures of crops such as potato and cassava. Unfortunately however, despite such scientific evidence, there had been no attempts seriously to apply the results to other species. The reasons for this were explored when a Workshop mutually sponsored by the International Union of Biological Sciences, the International Genetics

Federation and IBPGR was held in September 1980 to discuss the genetic conservation of 'difficult' material.

The difficulty resulted from two factors: (i) as mentioned above, materials were vegetatively propagated or possessed recalcitrant seeds and posed problems in long-term storage; or (ii) tissue cultures of many priority species for genetic conservation had not been worked on because they presented practical difficulties (such as 'browning' due to oxidation of phenolics); hence scientific research on tissue culture relied on species which were 'easy' to culture and these were largely irrelevant to genetic conservation because most of them could be conserved far more cheaply and easily as seed. Nonetheless ongoing research *had* made progress in other relevant areas including rapid multiplication of clones and cleaning-up plant materials from pathogens. Although the report of the Workshop was published two years later (Withers and Williams, 1982), IBPGR took speedy action immediately following the Workshop.

It was clear to IBPGR that a conceptual framework was lacking for *in vitro* genetic conservation, that there were widespread misunderstandings about maintenance of breeders' materials versus *in vitro* storage of cultures for long-term conservation, and such misunderstandings needed to be cleared up so that the largely unproductive lip-service paid to the potential of *in vitro* conservation in the prior decade could be translated into appropriately directed research.

IBPGR established an international expert working group, later established as a standing committee (see Appendix), to devise such a conceptual framework. This was chaired by Professor Edmond de Langhe, a Board member of IBPGR; members of the committee were drawn from all parts of the world and included a staff member of a sister CGIAR centre involved with *in vitro* research. All members were noteworthy for their contributions to scientific research. A back-up to the work of the committee was provided by an information gathering exercise which had been started by IBPGR to identify the extent to which *in vitro* techniques were being applied to genetic conservation, and to pinpoint gaps in the scientific knowledge and inadequacies in technologies, thereby providing data on research needs.

The committee has met twice in full and also through sub-committees to address specific research topics with the help of additional expert scientists. The reports are all available (IBPGR, 1983; 1984a; 1985; 1986). The deliberations of the committee and corresponding discussions by the Board of IBPGR led to the initiation of a global research programme with about 20 projects (see Table 1); the latter are summarized in this report. It is stressed that these research highlights stem from mission-oriented projects that have as their ultimate aim the provision of appropriate technologies to curators of germplasm holdings. The programme is still in its early stages but IBPGR is convinced of its speedy application in technology transfer.

CONCEPTS AND PRACTICES OF *IN VITRO* CONSERVATION

IBPGR recognizes a number of conservation techniques based on seed, the whole plant, and maintenance *in situ* or *in vitro*. Definitions of types of genebank, whether for *base* storage (long-term) or *active* (only for medium-term), are not necessary here. Nonetheless, it *is* to the point to emphasize that, in the main, *in vitro* methods will be adjuncts to other methods of conservation rather than the sole method of conservation. Accordingly *in vitro* methods should not be viewed as attractive biotechnological alternatives where more conventional approaches are adequate.

Germplasm Conservation Approaches

For instance, for cacao, *in situ* conservation in tropical S. and C. America, will be the only logical *major* way adequately to preserve the genepool since seeds cannot be stored and field genebanks will be limited by size, personnel and funding. The same will be true for many tropical fruits; *in vitro* conservation will play a supporting rôle as do the *ex situ* field genebanks. In other crops such as potato, cassava, sweet potato and temperate fruits, seed storage will satisfy most genetic conservation needs and *in vitro* storage will be supplementary. For root crops which rarely form seed, *in vitro* storage will, hand-in-hand with field genebanks, rate higher importance.

Breeders have needs for plant collections other than holding them for genetic conservation. As a result, they frequently require unique, adapted genotypes often as clones, which might represent only a portion of the spectrum of variability. Such derivatives from the crop genepool are often of interest only in the short term and hence long-term genetic conservation of the crop genepool cannot be based simply on these breeders' collections. When seed of vegetatively propagated crops are stored, they frequently are heterogenous; thus, their regeneration does not reproduce original clones. This state of affairs is of no direct relevance for genetic conservation; it is simply undesired by breeders.

In Vitro Genebanks

With respect to *in vitro* genebanks, two are recognized: (i) the *in vitro* active genebank (IVAG) where cultures are maintained under slow growth (such as the existing collections of cassava at CIAT and IITA, potato at CIP, and sweet potato at CIP, AVRDC and IITA); few exist at the moment; (ii) the *in vitro* base genebank (IVBG) where cultures are maintained under conditions of cryopreservation (see cover illustration); none exist yet.

IVAGs and IVBGs parallel the active and base seed genebanks. They will link with breeders' working collections, which are outside the scheme of representative genetic conservation; they will also link with the field genebank. The flow of material through the *in vitro* genebank and relationships to germplasm elsewhere are shown in Figure 2.

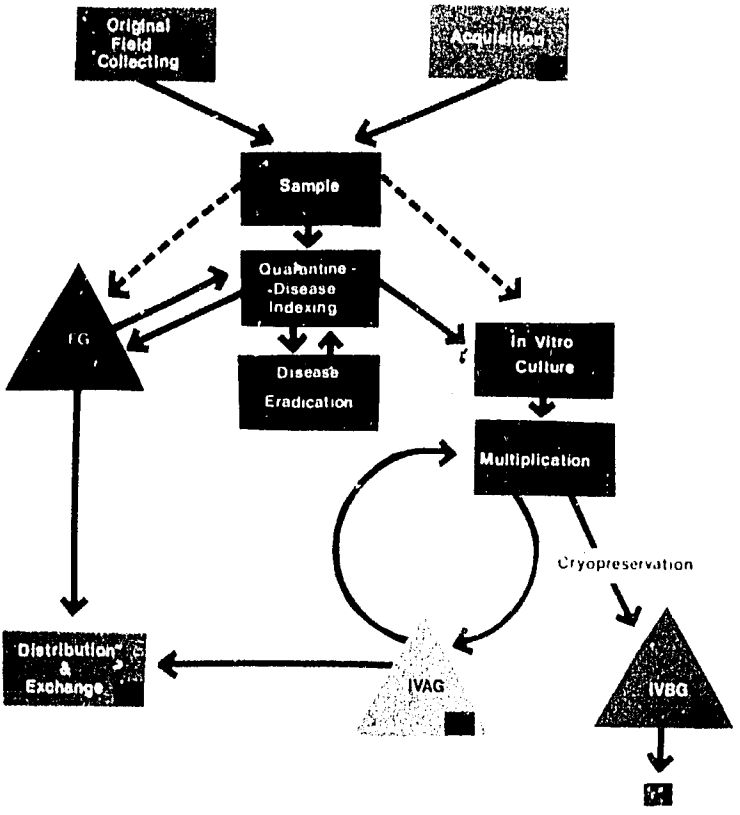

Figure 2. Relationships between accessed germplasm, the field genebank (FG), material for distribution and exchange, and the *in vitro* genebank. The latter is composed of the *in vitro* active genebank (IVAG) and the *in vitro* base genebank (IVBG). Arrows indicate the flow of material. Red rectangles indicate requirements for monitoring (for stability), characterization, evaluation, documentation and information management.

Operational Standards of an *In Vitro* Active Genebank

The design aspects and the tentative standards for an IVAG, based on existing scientific knowledge, were finalized in 1985 (see IBPGR, 1986). IBPGR intends, as soon as possible in 1986, to establish a working facility to generate data and to test and confirm standards.

The IVAG requires subculturing cycles of the material that has been accessed, inoculated into culture and multiplied. For example, an accession represented in the IVAG by five original cultures each replicated five times, will be maintained under conditions of slow growth and subcultured approximately every two years by a five-fold division.

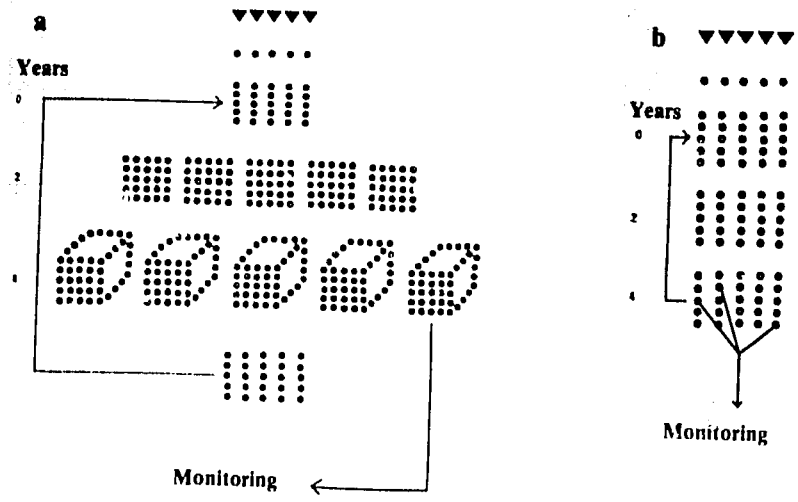

Figure 3. Subculturing routines for management of cultures in an *in vitro* active genebank. Two situations are considered: (a) Where stability is unknown; multiplication at two-yearly intervals of five replicate cultures of each of five representatives of an accession over a four-year cycle. Monitoring for stability is carried out after four years and 25 representative cultures are continued. (b) Where stability is known; the scheme can be simplified and the number of cultures maintained and monitored is reduced.

At the end of, say, four years, 25 representative cultures would be identified from the total of 625 for continuation into a second four-year cycle (Figure 3a). As there is evidence that cultures can generate mutants that might thrive under the stresses imposed by the slow growth conditions, extensive monitoring. of large numbers of the replicate cultures by biochemical or molecular techniques would be necessary to ensure that there is no unconscious selection for variants. Additionally, family lines need to be followed through the subculturing and strict stock control maintained in concert with monitoring.

For satisfactory genetic conservation, any instability in the cultures must be reduced to manageable and describable limits. As yet, scientific data on the genetic stability of cultures of most crops in slow growth — or even in normal growth — are highly fragmentary. Only when exact data are available and it is known that there is indeed a satisfactory level of genetic stability, can the numbers of cultures held in storage in the IVAG be reduced (Figure 3b). Monitoring will, nonetheless, remain important.

TABLE 1. CROPS AND AREAS OF IBPGR *IN VITRO* RESEARCH

	In vitro propagation	Slow growth	Cryopreservation	Disease indexing/ eradication	Characterization	Acquisition/ distribution	Other CGIAR Centres conducting research
Potato				■			CIP
Cassava						■	CIAT, IITA
Sweet Potato	■	▒	▒	■		■	CIP, IITA
Colocasia (taro)	■	▒	▒		■		—
Xanthosoma (cocoyam)	■	▒	▒				—
Musa (banana/plantain)	■	▒	▒	■	■		—
Coconut						■	—
Sugarcane	■	▒			■		—
Cacao	■	▒	▒		■	■	—
Citrus	■	▒	▒				—

The establishment of IVBGs lies further into the future and will depend upon satisfactory progress in the development of cryopreservation procedures. Maintenance and monitoring routines will differ from those in the IVAG; material will be subcultured in one cycle upon entry into the IVBG, frozen and stored in LN. No further multiplication cycles will be involved and monitoring will be to confirm the physical stability of the storage conditions.

Genetic Stability of Cultures

The principle of genetic conservation that materials retrieved should represent the materials accessed has caused concern about the use of *in vitro* systems because of the release of somaclonal variation; hence the emphasis, above, on adequate monitoring in the IVAG. IBPGR issued a status report, late in 1984, which summarized all existing scientific information (Scowcroft, 1984). This showed that the risk of genetic instability can range from minimal to considerable depending upon the culture systems used. IBPGR has to accelerate research in this area so that the causes of variation in culture may be elucidated, storage protocols modified and the levels of acceptable instability be circumscribed for each crop which will enter either IVAGs or IVBGs. Contract research will be initiated early in 1986 in a laboratory with suitable expertise and a range of techniques, biochemical, molecular and genetic, will be used.

It is interesting that the Workshop in 1980 stressed the need for closer work between plant physiologists and geneticists in the area of *in vitro* genetic conservation and yet the scientific community hardly heeded this need. IBPGR will rectify this short-coming.

Stability is a most interesting area of scientific research. There is evidence of the ubiquity of some instability in all biological systems, even in stored seeds and vegetatively propagated materials (Dourado and Roberts, 1984a; 1984b; Walbot and Cullis, 1985). This whole area of investigation, in which IBPGR is expected to precipitate rapid gains in the knowledge bases, is an exciting one because of recent advances in molecular biology and the emergence of new approaches to germplasm utilization and crop improvement. IBPGR research in this area has to be linked to developments such as efforts to store DNA sequences and the use of molecular probes to identify genes and index for diseases.

Disease Indexing

The identification of specific disease-causing organisms, especially viruses and viroids in tissues of vegetatively propagated species, and the subsequent cleaning-up of stocks (which largely rely on *in vitro* culture combined with thermotherapy) are essential for the rapid exchange and distribution of crop germplasm. IBPGR has stressed to its collaborators world-wide the need for this type of work and it is built into the *in vitro* genebank design (Figure 2).

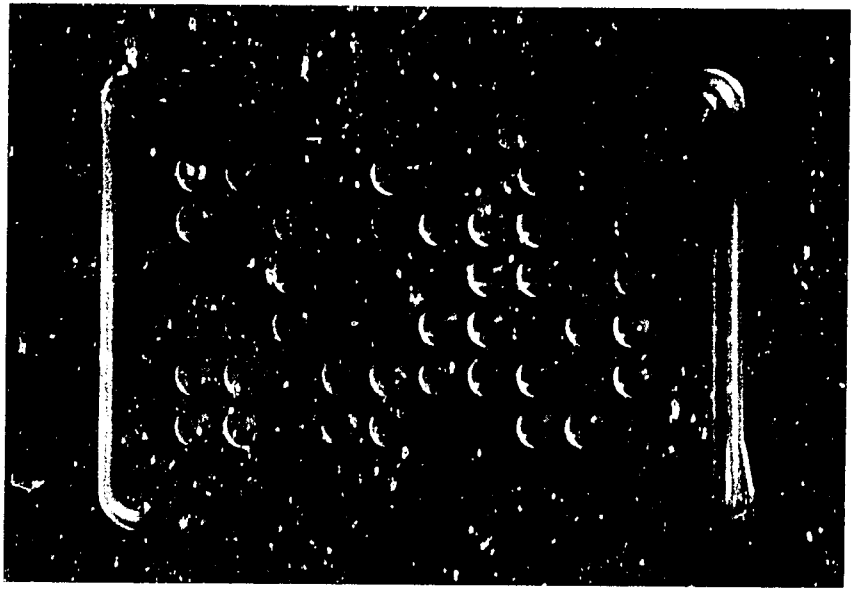

Figure 4. ELISA (enzyme linked immunosorbent assay) test plate for detecting viral infections.

So far, IBPGR's involvement has been small but it has incorporated disease indexing into its research on sweet potato and *Musa* and has links with a programme on potato. IBPGR has not, to date, used its limited funds for extensive work in this area because other centres, particularly other CGIAR centres and other institutes with funds for this purpose, have been active. Nonetheless, the time has come for an overview because the IBPGR International Database on *In Vitro* Conservation (see below) suggests that work in this field is not proceeding as fast as desirable, especially for crops requiring conservation *in vitro*.

The International Database

IBPGR maintains and continually updates a computerized database on information related to all aspects of *in vitro* conservation and associated areas of research, e.g. on propagation and disease indexing. A general outline of the work has been published (Wheelans and Withers, 1984). The database includes data on 28 named crops or groups of crop (plus others), with the facility to retrieve data in any one or a combination of 16 fields of information; searches are free to all *bona fide* enquirers.

The significant point of the database is that it includes a very large amount of unpublished facts and figures culled from scientists working

Figure 5. Coverage of the IBPGR International Database on *In Vitro* Conservation.

in over 70 countries spread throughout the world (Figure 5). In 1985 alone, some 1300 new sets of data were received. IBPGR therefore has its finger on the pulse of all significant *in vitro* research.

Characterization of Cultures

Germplasm in the form of *in vitro* cultures cannot be characterized by using the normal procedures of morphological measurement for those characteristics considered to be highly heritable. Electrophoretic examination using isozyme analysis (Figure 6) is a relatively simple and potentially widely-applicable technique. As it has received widespread coverage in the scientific literature, IBPGR has developed an assessment of published papers, incorporated the references into the database (see above) and published a report (Simpson and Withers, 1986). Isozyme analysis may also be used as a tool for assessing the stability of cultures. IBPGR research has used this technique for cacao, taro and *Musa*.

IBPGR RESEARCH ON CROPS

Table 1 lists the crop species for which the development *de novo* or the expansion of existing research on methods of *in vitro* conservation is a priority for the IBPGR programme (further details are available in Withers, 1984).

Other crops, not listed in the table, required thorough assessment of existing research results before action could be taken. These included *Allium* species and temperate fruits. Whereas seed storage is the conservation method of choice for *Allium*, there are important sterile materials

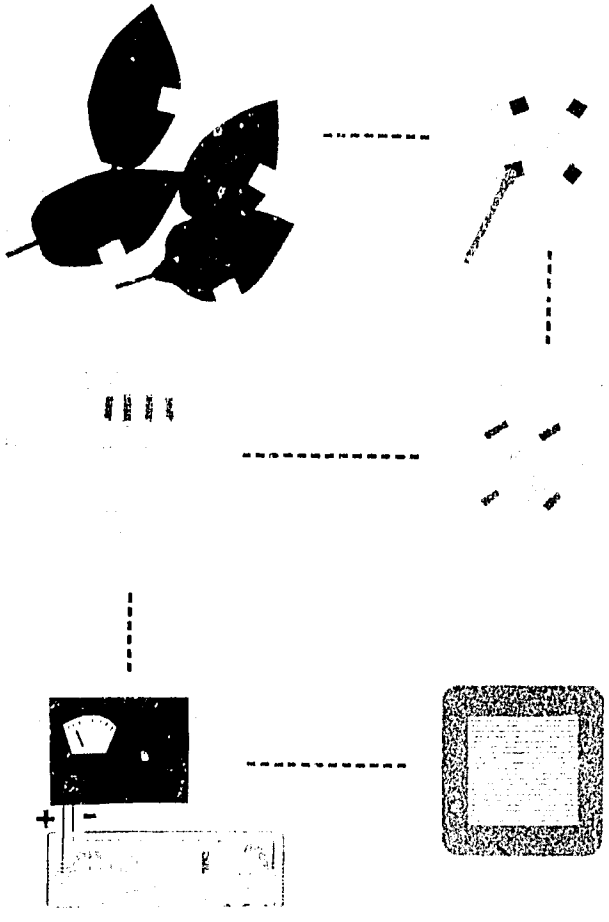

Figure 6. Outline of the procedure for carrying out isozyme analysis (top left to bottom right): Cut small pieces of tissue (e.g. leaf blade); grind tissue in buffer; collect extracts on wicks; load onto gel; subject to an electrical field (electrophoresis); stain to reveal bands. (From: L.A. Withers *et al.* (1986) Cocoa germplasm — some novel approaches to its conservation. In: *Cacao Biotechnology*, P.S. Dimick (ed.), Pennsylvania State University, pp. 97-110.)

and *in vitro* methods are also logical for materials freed of viruses. A report outlining the *in vitro* research needs was finalized recently by Dr. A. Altman and Dr. H.D. Rabinowitch of the Hebrew University of Jerusalem (Altman and Rabinowitch, 1985).

Dr. C. Stushnoff and Dr. C. Fear of the University of Saskatoon, Canada, provided a status report on *in vitro* culture of temperate fruits

and an extended summary of this has been published (Stushnoff and Fear, 1986). *In vitro* culture is most important in breeding and propagation. However, *in vitro* conservation does appear feasible, although research on cryopreservation for long-term storage has concentrated upon storage and subsequent culture or grafting of buds rather than true *in vitro* propagated systems. Earlier IBPGR research on *Vitis* developed techniques for slow growth (Barlass and Skene, 1983) and recent work at the Universities of Birmingham and Nottingham, UK, has developed techniques for *Pyrus* (Wanas, Callow and Withers, 1986). Cryopreservation of the latter provides hope for the development of long-term storage of a limited amount of material; most genetic conservation of most species of temperate fruit will be by seed storage along with limited *in situ* conservation of wild relatives.

The topics which form the basis of IBPGR research projects are itemized in Table 1. The research is not comprehensive because IBPGR consciously attempts to fill gaps and complement other ongoing work. Apparent omissions are filled by research in other centres, notably those of the CGIAR; their reports should be consulted for details.

Highlights of research on *in vitro* propagation, storage and characterization are provided below. Germplasm acquisition and distribution are covered in a later section. (In addition to the projects detailed below, IBPGR has also recently supported *in vitro* research on sugarcane and citrus.)

Potato and Cassava

Potato and cassava are mandate crops of CGIAR centres, and have also received much attention by other institutes. Most of the work has been directed towards the development and use of *in vitro* propagation methods and slow growth *in vitro*. IBPGR interest has concentrated on propagation and exchange *in vitro* of wild *Manihot* species (CIAT, Colombia), limited support for the development of molecular methods for disease indexing in potato, especially because of difficulties in movement of vegetative materials (Dr. M.J. Foxe, University College, Dublin, Republic of Ireland), and the enhancement of cryopreservation methods for cassava shoot-tips (Dr. K.K. Kartha, Plant Biotechnology Institute, Saskatoon, Canada). The latter is feasible but problems are experienced in achieving high quality regeneration after freezing.

Cassava shoot-tips frozen in droplets on a sheet of aluminium foil (Figure 7a) give the highest recovery but the quality of regenerants is seriously affected by the terminal temperature reached before thawing directly or after plunging into LN. Healthy plants *have* been recovered after storage in LN (Figure 7b) and material is now in its second year of field testing at CIAT. Results give cause for optimism concerning genetic stability (Dr. W.M. Roca, CIAT; personal communication to Dr. K.K. Kartha, Saskatoon, Canada).

a b

Figure 7. Cryopreservation of cassava shoot meristems: (a) Droplets containing meristems supported on aluminium foil and prepared for freezing; (b) plants developed from meristems cryopreserved in liquid nitrogen (K.K. Kartha, Plant Biotechnology Institute, Saskatoon, Canada).

Developments in the cryopreservation of cassava parallel those in potato where modifications at the freezing stage can improve survival (Henshaw *et al.*, 1985; UK-ODA-funded project). With potato, modification of the hormonal environment before and after freezing also influences the quality of regenerants (Dr. L.A. Withers and Dr. E.E. Benson, unpublished observations). This has yet to be evaluated for cassava. The impact of the results on performance and genetic stability of stored potato material is being tested at present as part of a UK-SERC funded project at the University of Nottingham, UK.

For other crops: sweet potato, taro, cocoyam, and banana and plantain, efforts have concentrated on the development of *in vitro* propagation and its adaption for slow growth storage.

Sweet Potato

In sweet potato, emphasis has been on shoot-tip/meristem culture to produce virus-free plants; procedures have been developed to give minimal contamination and the growth of meristems into plantlets capable of establishment in soil. A manual describing culture and disease-indexing procedures has been produced (Dr. B.B. Rhodes and colleagues. Clemson University, USA) and will be issued by IBPGR in 1986.

Taro and Cocoyam

In taro and cocoyam, procedures have been developed for the initiation of cultures and regeneration of plants (see cover illustration) from excised meristems of a range of genotypes and the storage of cultures under conditions of slow growth. Taro has been subjected successfully to a reduced growth temperature (3-4°C; Dr. M.C. Strauss and colleagues, Northeastern University, Boston, USA), and both taro and cocoyam (but with greater emphasis on the latter) have been used to initiate pest and pathogen-free cultures for propagation and storage under a range of growth limiting regimes at the Agricultural University, Wageningen, The Netherlands (Staritsky *et al.*, 1986). Genotypic differences in response to storage conditions are apparent but slow growth storage appears generally feasible. Of particular scientific interest are beneficial effects of combining low growth temperatures with osmotic stress by incorporation of mannitol in the medium, and interrupting extended periods of low temperature growth by brief exposures to normal growth temperatures.

Isozyme analysis in taro has involved a range of enzyme systems (Dr. M.S. Strauss and Mr. R. Warne, Northeastern University, Boston, USA). Six systems were identified as useful genetic markers in cultivar identification. As yet, no genetic instability has been detected in plants derived from *in vitro* cultures in either isozyme patterns or the phenotype of plants grown in the field.

Banana and Plantain

Banana and plantain cultivars of different genomic constitution have been propagated successfully *in vitro* by culturing shoot apices (see cover illustration); their performance in culture (to produce either a single plantlet or multiple shoots, and to promote rooting) can be manipulated by modification of the hormonal content of the culture medium. Multiple shoot cultures have been stored in slow growth at a range of sub-optimal temperatures. Below 15°C, cultures all suffer damage and/or die within three months. At 15°C, some genotypes will survive for up to 17 months with a viability of 92%; in others, viability is reduced to 50% within 13 months (Banerjee and de Langhe, 1985). Despite these variations, the foundation is now laid for the development of storage in IVAGs.

Electrophoretic examination has been used to evaluate genetic stability in plants derived from *in vitro* cultures (Dr. O. Reuveni and colleagues, Agricultural Research Organization, Bet-Dagan, Israel). Mutants in such plants occur at a higher rate than in normally propagated plants, although the mutants are those which also occur in the normal situation. *In vitro* propagated material shows more rapid growth than plants from suckers (but not corms); this is a physiological and not a genetic effect. Isozyme analysis has not proved useful in these studies for identifying

Figure 8. Banana plants raised from cultured shoot apices and established in the field.

cultivars or detecting mutants, although it does appear useful at the genomic level (Jarret, 1986).

In all of the above cases, progress has been satisfactory but research results underline the fact that attention to the crop species and even genotype is essential to success in the use of *in vitro* conservation. IBPGR is a long way from being able to recommend standard procedures across a spectrum of crops and techniques applicable to the widest range of genotypes within any particular crop. The majority of the above research is continuing and experimental work will examine techniques of cryopreservation.

Cacao

Cacao has been the subject of research at the University of Nottingham, UK, in which *in vitro* propagation, storage, characterization and germplasm distribution are components. Propagation is a fundamental requirement in order to implement all other aspects of *in vitro* conservation and, in cacao, major constraints in this area have been experienced. Propagation via nodal cuttings has had limited success but extensive investigations into the culture of leaf pieces with a view to inducing somatic embryogenesis (paralleling the performance of immature zygotic embryos) have been largely unsuccessful. Recent findings by Dr. R.E. Litz (University of Florida, Homestead, Florida, USA; personal communication) indicate genotypic specificity in the response of leaf material. Further details of the research on cacao are incorporated into a research report prepared by Dr. L.A. Withers for an

IBPGR Cacao Working Group. This evaluates the state of development of *in vitro* methodology for the crop and of ongoing research world-wide (IBPGR, 1984b).

Characterization of cacao germplasm using isozyme analysis has been investigated. Of a total of 24 enzyme systems screened, 17 are detectable in leaf tissue and 7 show reproducible variation in the limited number of genotypes examined to date. Additionally, peroxidases are detectable and vary in bark extracts. This work has been sponsored by IBPGR and GCGRA at the University of Nottingham, UK, and is continuing at the CRU in Trinidad through the IBPGR Intern scheme.

GERMPLASM ACQUISITION AND DISTRIBUTION

Problems exist in moving vegetative material and in some cases survival is low (cacao in particular). Other crops such as coconut involve the transport of bulky nuts. IBPGR addressed this problem and found that only the most preliminary investigations had been carried out on cassava (Dr. W.M. Roca, CIAT, personal communication) and cacao (Dr. L.A. Withers, University of Nottingham, UK). IBPGR agreed that there is great potential in developing a simple appropriate low-cost technology for *in vitro*-based collecting (IBPGR, 1984a).

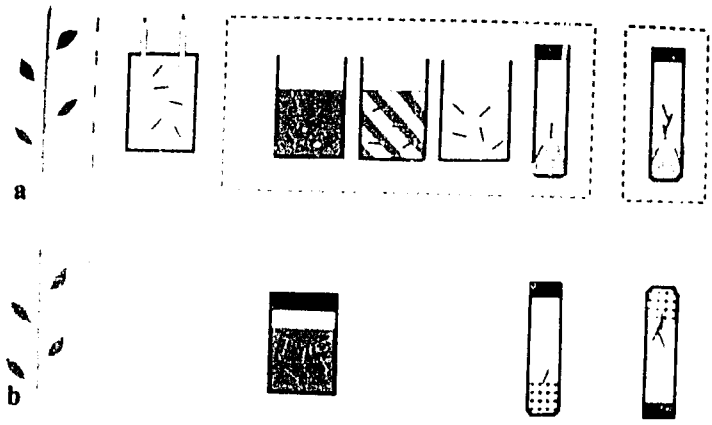

Figure 9. Modification of the *in vitro* propagation procedure to permit germplasm collection in the field: (a) Original method involving washing trimmed shoot cuttings in running water, transfer to a laminar air-flow cabinet, surface sterilization, washing, inoculation into culture on a filter paper bridge in contact with liquid medium and transfer to a controlled environment room; (b) simplified method involving surface sterilization of cuttings in a 'benign' sterilant and inoculation into culture on semi-solid medium containing anti-microbial agents; culture is under ambient conditions and explants can tolerate physical disturbance in transit. (From: L.A. Withers *et al.* (1986) Cocoa germplasm — some novel approaches to its conservation. In: *Cacao Biotechnology*, P.S. Dimick (ed.), Pennsylvania State University, pp. 97-110.)

Figure 10. *In vitro* germplasm collecting technique: (a) Removing stem pieces from the plant; (b) trimming and surface sterilizing nodal cuttings; (c) inoculation into tubes of culture medium; cuttings at (d) time of inoculation, and (e) six weeks later showing axillary bud development.

Efforts with cacao have continued, primarily as part of a project funded by the GCGRA. The standard *in vitro* propagation procedure forms the basis of the collecting method but is simplified to eliminate the need in the field for either a power supply, a sterile laminar air-flow inoculation bench or large quantities of sterile water (Figure 9). Explants consisting of nodal cuttings are surface sterilized in a mixture of fungicide and water purifying tablets dissolved in boiled water. They are then inoculated, without further washing, into a tube of sterile culture medium prepared in advance and containing antibiotics and fungicides (Figure 10). The method has been tested in the collecting programme of the London Cocoa Trade Amazon Project in the field in the Amazon, (Mr. J.B. Allen, personal communication). Efforts are now underway to refine sterilant and media formulations and to develop procedures to process the materials collected. Grafting onto aseptically germinated seedlings appears the most promising follow-up. Details of the collecting procedure have been published (Yidana *et al.*, 1986).

In principle, an *in vitro* collecting technique (or the use of it in distribution of germplasm) may be applied to any type of explant, and IBPGR advocated the testing of the approach with a crop for which embryo culture was relevant, i.e. a recalcitrant seed producer. Attention has thus recently been given to modifying the embryo culture procedure for coconut to render it feasible in the field for collecting germplasm. A technique had already been establised at IRHO, Bondy, France, for the conversion of both immature and mature embryos cultured *in vitro* to independently growing plants with a success rate of approximately 90% (Dr. C. Pannetier, Dr. J. Buffard-Morel and Ms. B. Assy Bah, personal communication; see cover illustration). This is being adapted for field collecting of germplasm in a collaborative IBPGR project with colleagues in the Côte d'Ivoire (Station La Mé, Bingerville). Efforts are being directed towards finding ways of holding excised embryos for a period of time in a sterile condition prior to their despatch to a laboratory where inoculation can be carried out under aseptic conditions. Embryos in a cylinder of albumin sterilized by a hypochlorite solution can be stored in sterile water for up to 6 days and yield 80% sterile cultures with 100% viability. Attempts are being made to extend the temporary storage period and monitor development of embryos once in culture.

Results from research on cacao and coconut should provide a basis for extending the *in vitro* collecting procedure to a wider range of crops and, at the same time, aid the development of simple, *in vitro*-based propagation techniques that will help in germplasm multiplication.

A further novel possibility resulting from the *in vitro* collecting research relates to developments in germplasm collecting policy, characterization, and techniques for surveying diversity in the field. A combination of various technological developments should permit accurate preliminary surveying of collecting sites in remote areas, particularly when genepools of long-lived perennials are involved. Preliminary surveying can be followed up by targets to collect in 'hot-spots' of genetic diversity.

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It is hoped that through the activities of the IBPGR *In Vitro* Programme, momentum may be maintained in the development of *in vitro* conservation. IBPGR support will continue for mission-oriented projects but it is recognised that IBPGR can only fund a fraction of the work necessary to realise the full benefits of novel conservation techniques. Thus, IBPGR's central role in crop genetic resource conservation should provide a theme and focus for research to be initiated and supported by other organizations.

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5. IBPGR collaborator for research reported in these Highlights
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