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INTERNATIONAL BOARD FOR PLANT GENETIC RESOURCES

IBPGR Advisory Committee on In Vitro Storage

Second Meeting
Leuven, Belgium
10-12 September 1984

REPORT

IBPGR Secretariat
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The International Board for Plant Genetic Resources (IBPGR) is an autonomous international scientific organization under the aegis of the Consultative Group on International Agricultural Research (CGIAR). The IBPGR was established by the CGIAR in 1974 and its Executive Secretariat is provided by the Food and Agriculture Organization of the United Nations. 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. The Consultative Group mobilizes financial support from its members to meet the budgetary requirements of the Board.

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INTRODUCTION

1. The Second Meeting of the IBPGR Advisory Committee on In Vitro Storage was held 9-11 September 1984 at the Catholic University of Leuven by kind invitation of the Committee's Chairman, Prof. E.A.L. De Langhe. In addition to the Chairman the following members attended: Dr. L.A. Withers (Technical Secretary) and Drs. B. Finkle, A.N. Rao, O. Reuveni, W.R. Scowcroft and J.T. Williams. Drs. W. Roca and Sakai were unable to attend. In addition the following participated: Prof. T. Akihama (Japan), Dr. J.H.W. Holden (Senior Adviser, IBPGR) and Dr. G. Staritsky (Netherlands). Addresses of participants are shown in Appendix 1.

REPORT

2. Dr. Williams provided a review of IBPGR activities, in the area of in vitro research, initiated as a result of decisions taken by the first meeting of the Committee held 16-20 August 1982. This included:

(i) The commissioning of status reports on genetic variability in tissue culture; on in vitro research in temperate fruits; and on the applicability of in vitro methods to the conservation of Allium germplasm. In addition, the IBPGR had requested a report on the potential for using isozyme analysis in characterization of germplasm both for plants and tissue cultures.

(ii) Initiation of research 1/ on development and/or experiences of in vitro techniques for Colocasia, Xanthosoma and sweet potato; on cryopreservation of cassava and Musa; on disease indexing in Musa and sweet potato; and on downstream checking of in vitro propagated Musa.

(iii) The continuation of previously initiated projects on in vitro conservation of cocoa and Musa.

(iv) The establishment by the IBPGR of an international computerized data base on in vitro conservation and allied techniques 1/.

(v) The meeting of a sub-committee in September 1983 to advise the IBPGR on the feasibility of developing new in vitro-based techniques for collecting samples of certain clonally propagated root and tuber tropical crops, aroids, coconut and cocoa 2/. In this context it should be noted that a pilot study carried out as part of a continuing research project on the in vitro conservation of cocoa is now at the field-testing stage in the Amazon in association with the London Cocoa Trade Amazon project in Ecuador.

Genetic variability in tissue culture

3. The first meeting of the Committee pointed out that data on genetic stability in cultures are lacking. In addressing this problem the above mentioned downstream checking in the field of in vitro propagated Musa was funded by the IBPGR. In addition, a status report was prepared (see para. 4). There has been, in general, a notable lack of any characterization procedures as proposed by this Committee although CIAT has started work in this field. These techniques include microtechniques based upon biochemical analyses linked to traditional methods of characterization. The Committee noted also that there have not been adequate assessments of stability and that there appears to be an apparent lack of concern about the integrity of the materials in in vitro collections and associated research. The dangers of relying on untested assumptions of stability cannot be emphasized too strongly.

1/ A resume is to be found in IBPGR Annual Report 1983

2/ See IBPGR (1984) Withers, L.A. The Potential for Using In Vitro Techniques for Germplasm Collection.

A report on this subject was tabled (for author's conclusions see Appendix II). The Committee noted:

(i) That some clonal variation occurs in most if not all in vitro propagation systems which have been examined in this respect. The greatest incidence of somaclonal variation appears in protoplast, cell and callus cultures. However, the assumptions that meristem/shoot tip and somatic embryo (embryoid) cultures do produce an adequately low level of somaclonal variants is based on inappropriate and inadequate experimentation.

(ii) Asexually propagated plants are more likely to retain somaclonal variations through successive generations than sexually propagated ones because the former lack a "meiotic serve".

(iii) Incidence of somaclonal variation may be correlated with genotypes, ploidy level and nature of explant.

(iv) Media constituents including growth regulations appear not to be directly mutagenic.

(v) Somaclonal variation increases with time in unorganized culture.

(vi) There is no current method to control absolutely somaclonal variation in cultures.

(vii) In addition to gross changes at the chromosome and genome levels a full assessment of the incidence of somaclonal variation requires a formal genetic analysis of progeny. It is recognized that this will not be possible for some cultivars or species of interest to this Committee.

5. The committee recognizes that any in vitro conservation system will include a number of stages at which somaclonal variations can occur. As a result the Committee recommends that the IBPGR initiates a study to assess the risks at each stage. This must examine the frequency of occurrence of somaclonal variation during culture initiation and propagation, slow growth in relation to time and cryopreservation and regeneration route after cryopreservation. Explants should be shoot tips/meristems because they are likely to be the materials of choice for in vitro conservation (para. 4). For this research it would be sensible to choose species with a short generation time and known genetic markers, e.g. pea, Arabidopsis, alfalfa, Brassica. There is an urgent need for this research to be completed in as short a time as possible (possibly by means of a project not to exceed 3 years) and it should be located in an already fully-equipped laboratory. IBPGR is asked to consider funding a post-doctoral scientist, at an estimated cost of US$25,000 p.a. The most expeditious way to organize such a project would be to advertise widely for invitations for research proposals.

Isozyme analysis in germplasm analysis

6. The report mentioned in para. 2 (i) was tabled (see Appendix III for authors' summary) and discussed. A large body of literature exists on the subject indicating that there is a value of isozyme analysis as a supplement to morphological and agronomic evaluation. However its inadequacy as a sole method of characterization is stressed. Isozyme analysis has utility in the characterization of materials in seed genebanks and field genebanks and in vitro collections. In the latter it will provide means of assessing stability.

7. The Committee looks forward to the exploitation of isozyme analysis and other biochemical techniques (iso-electric focussing, DNA restriction analysis and nucleic acid probes) as part of a more critical approach to the management of in vitro collection. The highest scientific standards must be adopted in this work.

In vitro research on temperate fruits

8. The report mentioned in para. 2 (i) was tabled (see Appendix IV for summary) and

discussed. During this discussion, the Committee recognized the extensive use of *in vitro* methods, but these are, with few exceptions, directed towards rapid propagation. The committee reaffirmed the principle that conservation should be of the genetic variability of the genepool and not individual adapted genotypes and that in the case of the temperate fruits this may be done most effectively and efficiently through storage of seed (especially of wild species). This point does not appear to be widely understood by breeders of temperate fruits. In consequence, widespread discussions on the establishment of field genebanks (clonal repositories) have rarely been based on scientific understanding of genetic conservation but rather on short-term utility. This has given rise to often unnecessary excursions into *in vitro* methods. The Committee does, however, point out that such methods should and will continue to be an integral part of new breeding strategies and propagation of material for planting.

9. The report provided a comprehensive compilation of current information and enabled the Committee to reach the following decisions upon the strategy for conservation of temperate fruits:

(i) In addition to the primary conservation method of this material by seed, there may be justification for supplemental methods whereby a limited number of clones need to be preserved. These clones will have to be chosen on the basis of specific attributes, e.g. parthenocarpy, seedlessness, or special attributes. In this respect it is noted that breeders will wish to maintain a larger number of clones and these will fall outside the framework of genetic conservation. The responsibility for their maintenance lies with the breeders. The Committee points out that the only way these collections should be established is through the widespread collecting of clones, followed by their description and rationalization (particularly reduction in numbers).

(ii) For the specially identified clones there may, in certain cases, be a justification for parallel field and *in vitro* genebanks. Such methods could greatly reduce the cost of duplication for safety. However it is noted that appropriate research to date has been inadequate.

(iii) The storage of pollen is likely to prove useful as part of a complementary conservation strategy (see paras. 11-13 below).

Pollen storage

10. Professor Akihama outlined to the Committee the current status of pollen storage techniques in Japan. These mainly relate to temperate fruits. This led the Committee to a discussion on the role of pollen storage in genetic conservation and utilization of genetic resources. Advantages of pollen storage noted were: (i) rapid utilization in breeding; (ii) compactness; (iii) low cost; (iv) ease of exchange; and (v) enhancement of the value of field genebanks and breeders' collections of clones. Disadvantages and information gaps noted were: (i) absence of cytoplasmic genes; (ii) possibility of pathogen transfer; (iii) unknown genetic stability; (iv) storage behaviour is not known for many taxa; and (v) in species where pollen storage is possible, information is lacking on duration of viability.

11. The magnitude of the task involved in the regeneration of seed from tree species is such that it is unlikely to be carried out on an adequate scale. Pollen storage offers a possibility of simplifying this process by permitting the pollination of existing mother trees. Larger seed samples must then be returned to storage in order to represent variability adequately. The use of this method is limited to one cycle of regeneration during the long-term storage of seeds.

Complementary storage methods

12. The Committee advises the IBPGR that it is now appropriate for decisions to be made on the relative inputs necessary into various aspects of conservation when these affect individual crops in various ways. In particular, the Committee points out the

widespread misunderstanding, particularly with vegetatively propagated species, that breeders' collections are within the scope of the Board's work. In this respect the Committee emphasizes that in some crops, especially where there is little or no active breeding, the IBPGR becomes by default the suppliers of breeding material. This should, however, only be a temporary measure and the IBPGR must continue to draw the attention of other organizations to assume responsibility. Clear examples are banana and coconut.

13. The priority crops of the Advisory Committee on In Vitro Storage were considered on an individual basis. Recommendations are made below:

Potato: The responsibility of the IBPGR is to see that seeds are conserved in base collections. In vitro cultures are the responsibility of breeders; with Centro Internacional de la Papa (CIP) and other institutions these are adequate for this work. For in vitro conservation this should be restricted to those varieties which are sterile and those which have been well-evaluated landmarks in breeding or known to have special assemblies of genes and these should be used as sources of somaclonal variants. Such material should be cryopreserved. Nonetheless it will only form a very small part of the conservation scheme.

Cassava: 75% of IBPGR effort should be on seed conservation, especially that of wild material. 25% of its effort should be on in vitro material. The current emphasis for genetic conservation is too heavily weighted to in vitro culture of cultivars.

Sweet potato: 50% of IBPGR effort should be on collection and storage of seed, especially wild material. 50% of effort should be on in vitro active genebanks and cryopreserved in vitro material. It is essential that emphasis by IBPGR is shifted to genetic diversity and away from cultivar diversity.

Taro: IBPGR emphasis should be largely on in vitro cultures (both active and base) and the development of in vitro collecting. Seeds, when available, must be put into long-term storage.

Musa: For the time being, IBPGR will have to address in vitro methods for cultivars and cryopreservation. The storability of seeds of wild species needs examination as a matter of urgency.

Coconut: It is urgent to develop reliable in vitro systems and in vitro collecting techniques.

Sugarcane: Emphasis should be on storage of seed, especially of wild relatives and cultivars should be cultured in vitro.

Cocoa: IBPGR attention should be directed to in situ conservation, in vitro collecting for limited ex situ field genebanks and development of good in vitro collections therefrom.

Coffee: No in vitro techniques are needed for conservation.

Citrus: Active in vitro collections are needed because of disease; disease indexing methods must be developed as priority; major emphasis must be on seed storage.

Temperate fruits (including Vitis): Major emphasis must be placed on seed storage. Only limited members of clones in field genebanks are necessary and these should enter in vitro systems (see potato). Storage of pollen is complementary (see para. 11).

Allium: Recommendations are delayed pending further information; they will be considered at the next meeting of the Committee.

In vitro base collections

14. Although progress is being made on the development of techniques, especially for cryopreservation, the Committee agreed that it is too early to start the designation of such collections. Nonetheless due consideration will have to be given to whether these should be crop-orientated or multi-crop and regional or national, largely for political considerations.

15. The Committee recommends that the IBPGR establishes a Sub-Committee to discuss this matter. It should include the Chairman and Technical Secretary of the Advisory Committee on In Vitro Storage, an expert on cryopreservation, a good tropical agronomist and an expert on the technological implications.

Other items

16. The Committee recognizes the need for continued provision of information from its in vitro data bases and that this is clearly a service function of the IBPGR for the foreseeable future. The IBPGR should continue to fund its information project. Efforts should be made to draw the attention of the scientific community to the existence and availability (at no cost) of this information and to urge contributions of up-to-date information.

17. In view of recent establishment of in vitro laboratories in many parts of the world, the Committee agreed that Professor Rao would visit laboratories in Southeast Asia and provide information in a report to the Committee on their capacity for work of interest to the Board.

18. The Committee welcomes the suggestion of IBPGR that a virologist be added. It requests that when the membership of the Committee is considered for the next three years that a suitable virologist with molecular interests becomes a member.

19. It is urgent that centres where scientists will be trained in use of disease indexing methods should be identified. This should be addressed by the proposed Sub-Committee (para. 15).

20. The Committee expressed its appreciation to its Chairman, Prof. E.A.L. De Langhe, for the arrangements made and for the opportunity to attend the celebrations in Leuven marking the 10 year anniversary of the IBPGR.

APPENDIX I

PARTICIPANTS OF THE SECOND COMMITTEE MEETING

Members of Committee who attended

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APPENDIX II

GENETIC VARIABILITY IN TISSUE CULTURE

Conclusions from a report by W.R. Scowcroft

1. *In vitro* culture methods are essential to conserve germplasm resources for species where it is impractical, difficult or impossible to conserve seed.

2. Tissue culture instability and somaclonal variation is ubiquitous. This variation has possible benefits as an adjunct to plant improvement but is a serious disadvantage for *in vitro* germplasm conservation and exchange.

3. Variation occurs at all tissue culture levels. Genetic instability and somaclonal variation has been recorded in callus, liquid suspensions, cultured protoplasts, anther culture, adventitious shoots, somatic embryos, in plants regenerated from tissue cultures and among seed and vegetative propagules of regenerants.

4. As a consequence of instability there is variation in ploidy levels and chromosome structure, morphological and biochemical traits and changes at the nucleotide level have been observed for both nuclear and cytoplasmic genomes.

5. Genetic analyses show that somaclonal mutants can be under simple or polygenic inheritance, may be recessive or dominant and mutants can be unstable.

6. Somaclonal variation is more likely to be enhanced, a) in asexually propagated species relative to seed propagated species; b) if callus formation is a significant phase in the regeneration cycle; c) with increased duration of culture; d) in certain genotypes; e) from specific explants.

7. *In vitro* plant culture systems may be ranked for instability (low to high) as meristem multiplication, adventitious shoots, somatic embryogenesis, organogenesis from callus and cultured protoplasts.

8. Instability and somaclonal variation can arise from but may not be causally related to chromosomal aberrations. DNA amplification or diminution and DNA sequence transposition are included in mechanisms which could cause somaclonal variation. The effect of virus elimination on phenotype may be wrongly interpreted as somaclonal variation. Tissue culture media and growth regulators appear not to be mutagenic.

9. *In vitro* germplasm storage currently uses slow growth techniques rather than cryopreservation. Minimal growth conditions put germplasm integrity at serious risk because of directional genetic change in response to selection, and somaclonal variation is possibly enhanced, particularly where regeneration is adventitious. Further work is necessary.

10. On current knowledge cryopreservation of meristems shoot tips provides the greatest security against genetic instability during *in vitro* germplasm conservation.

APPENDIX III

CHARACTERIZATION OF GERMPLASM USING ISOZYME ELECTROPHORESIS

Conclusions from a report by M.J.A. Simpson and L.A. Withers

Isozyme electrophoresis is quick, relatively inexpensive and provides a rich source of genetic markers which are not necessarily at the mercy of environmental conditions and which allow the discrimination of heterozygotes and homozygotes. It is not surprising, therefore, that the method has proved its value in both basic and applied genetics.

Clearly, there is a role for isozymes in the evaluation of plant genetic resources, as a valuable supplement to morphological and agronomic evaluation. Both approaches must be based on sound taxonomy. Where classifications are unsatisfactory, the techniques of numerical taxonomy can be applied to representative samples of accessions, to provide summaries of the overall pattern of variation within species. Most accessions can be assigned to the resultant groups on the basis of a preliminary morphological characterization, together with details of their geographical origin.

For more precise measurements of genetic variation within single accessions or related accession groups, isozyme electrophoresis may be used. Such data would facilitate identification of areas in which a particular species is most variable and suggest appropriate sampling strategies. Where morphological data and passport information indicate accession duplication, isozyme characterization could prove decisive, especially where intra-accession variation is at a low level. Repeated isozyme assays on specific samples would enable genetic integrity to be monitored, which would be of particular importance in material that requires frequent regeneration.

Routine use of isozyme electrophoresis would entail identification of suitable buffer systems and enzyme stains for particular species, as well as determination of isozyme genetics. As a wealth of isozyme literature demonstrates, such basic information is of considerable value to the genebank itself and to its clients, be they plant breeders, population geneticists, taxonomists or developmental biologists.

APPENDIX IV

IN VITRO RESEARCH ON TEMPERATE FRUITS

Conclusions from a report by C. Stushnoff and C. Fear

In vitro techniques have been developed for the following purposes: (a) clonal propagation; (b) elimination of viruses and maintenance of "virus-free" stock; (e) embryo culture to rescue immature embryos and those which otherwise abort; (d) conservation and preservation of genetic resources, and (e) generation of variability, as a biotechnological technique for fruit breeding.

Micropropagation. In vitro clonal propagation (often referred to as micropropagation) has received a great deal of research attention in recent years. The commercial attributes of micropropagation have been recognized as a means of rapid propagation and have been developed as procedures with some crops such as strawberries, apple rootstocks, blueberries and blackberries. In a few cases more research is needed to develop optimum methods for commercial application but it does appear that in almost all instances, where effort has been applied, successful methods have been developed. A logical extension of the concept of micropropagation, to capitalize on cost effectiveness, would be to produce asexually propagated cultivars for production on their own roots, thus eliminating the need for the budding and grafting operation. This presents a new dilemma for fruit breeders, in that the stock-scion interaction has been a key component to maximize production.

Elimination of viruses. In vitro technology, in conjunction with heat therapy has been used very successfully to eliminate virus infestation from standard cultivars and thus improve their productivity. The same methods have been used effectively in maintaining disease-free stocks and promise to ease quarantine restrictions and international transfer of propagation material. Furthermore, these methods applied to storage appear to be far superior to field trees as a means of maintaining virus free germplasm.

Embryo rescue in breeding. One of the earliest in vitro techniques used with fruit crops was to culture embryos from seedlings of early maturing Prunus crosses. Attempts to grow seedlings in the normal manner from such crosses failed because of abortion. This technique has also been used in difficult interspecific crosses.

Conservation of genetic resources. A number of research reports have been published extolling the virtues of in vitro techniques as a means of conserving crop genetic resources. Some of these reports have dealt with fruit species and represent significant advances in this area of research. In spite of numerous research reports on in vitro preservation and storage one must concur with the IBPGR Advisory Committee on In Vitro Storage. They concluded that there has been very little research appropriately directed in this area, and that a great deal of work needs to be done to develop the key factors which will indeed make in vitro technology a key strategy for germplasm conservation with vegetatively propagated temperate fruit crops. The question of genetic stability associated with storage and preservation is of utmost priority in this regard. In vitro techniques offer an opportunity to develop systems, which can facilitate rapid multiplication and exchange of germplasm and there are aspects to these systems which are amenable to germplasm conservation on this technology. This area of research must be addressed in much greater detail. It is insufficient to assume that because certain plant material can be propagated by in vitro techniques and that it can be stored for periods of time by either restricting growth or by lowering temperature even to the extent of cryopreservation, that the problem has been solved. Indeed, there are aspects of in vitro storage which may create instability through handling procedures and these must be thoroughly researched.

Storage of tissue cultured shoots under conditions of minimum growth, could provide a valuable technique for handling genetic resources of temperate fruits.

Short-term storage under conditions of minimum growth appears to provide a relatively safe, clean, low input system which is feasible for strawberry, grape and possibly apple. The disease free aspect, ease of exchanging germplasm, and ability to evaluate for viability are attractive features. However there are pitfalls: Susceptibility to losses from mechanical failure of environmental control systems, length of time to develop mature plants from culture and possibly screening for unknown selective genes as a consequence of mutation and handling procedures. These possible problems need research attention, as well as, determination of the ideal conditions for supporting minimum growth such as media constituents, holding temperature and gaseous environment.

The IBPGR Advisory Committee on In Vitro Storage has placed emphasis on cryopreservation long-term conservation. Attempts to cryopreserve shoot tips and meristems, have not yet been researched thoroughly enough to establish a predictable, high rate of success with a high level of confidence in survival and genetic stability. Despite some reports describing the preservation of shoot tips and meristems of apple and strawberry these are based upon one or a few cultivars and have not considered the question of genetic stability. More important, the integrity of meristematic apices, subsequent to cryopreservation must be studied in more detail. Shoot regeneration from callus tissues would clearly be an unacceptable method on which to base germplasm conservation, in that the intermediate step of callus has often been shown to generate instability. It may be possible to manipulate conditions so that regeneration will originate directly from an organized meristem but more work must be done to prove that this can be accomplished.

Although cryopreservation of suspension cultures has been accomplished with several plant species, there is little or no literature on regeneration of whole plants for fruit crops from such cultures. Furthermore, there would probably be an intermediate callus step which would need to be looked at very carefully in terms of the instability.

In cryopreservation studies of organized meristems and particularly callus or suspension cultures, a great deal of research must be conducted to determine microscopically and genetically the extent of damage arising from storage. The presence of repair mechanisms and the processes involved in reorganization of a new meristem, and implications on the level of stability or instability should also be studied.

Although not strictly an in vitro technique, cryopreservation of dormant apple buds (which can be retrieved from liquid nitrogen and repropagated to produce shoots for rapid use in breeding) appears to be a promising alternative for long-term conservation. Methodology is now available which can be used to manipulate buds of this material from a broad array of genetic background so that successful cryopreservation can be achieved. This method also has the advantage of providing rapid shoot growth in a relatively short period of time from preservation to utilization. However, research is needed to determine the impact of such treatment on long-term survival and genetic stability. It is also essential that virus free material be used in developing a genetic conservation system using this methodology. There is no evidence to indicate that development of shoots from buds stored in such a manner arise from any process other than normal morphological development, but this needs to be verified microscopically and through the presence of stable propagules following such manipulation. At present, cryopreservation of dormant buds from woody plants appears to be the most promising technique which provides an opportunity to preserve the genetic integrity of an individual genotype. Additional research is necessary to determine if a similar approach can be used for cryopreservation of dormant buds of other temperate fruits.

Cryopreservation of pollen can be successfully carried out and this method does provide some opportunities to preserve part of the diversity.

There is also a need for research to study the inadvertent shifts in population which may occur as a result of selection from a particular in vitro cryopreservation process, itself. It is conceivable that in the processes of manipulating meristems in terms of cold hardening, freeze dehydration, cryopreservative protection,

cryopreservative dehydration, the freezing process itself, deterioration in liquid nitrogen storage, and the processes associated with recovery from liquid nitrogen such as rehydration and thawing, inadvertent selection for particular genotypes might occur. Research on inadvertent genetic shifts should be well suited for asexually propagated species because identical genotypes can easily be maintained as control, comparative systems and such studies should be initiated to determine these critical possibilities.

Generation of variability for biotechnological breeding. A promising area for improving productivity with plant breeding research is the creation of variability associated with tissue culture technology. This is a new area of research and with fruit crops much basic work is needed even to regenerate whole plants from cell systems.

Other considerations. It is recognized that the technology associated with *in vitro* techniques will present special difficulties for scientists in developing nations, at least until the knowledge and equipment becomes available to them. Temperate fruit crop genetic resources are valuable on a worldwide basis and steps must be taken to avoid exclusive use by any one group. *In vitro* procedures should enhance exchange and availability if steps are taken to disseminate knowledge pertaining to the use of this technology.