IBPGR

In vitro culture and disease aspects

INTERNATIONAL BOARD FOR PLANT GENETIC RESOURCES

INTERNATIONAL BOARD FOR PLANT GENETIC RESOURCES

IBPGR ADVISORY COMMITTEE ON IN VITRO STORAGE

Conservation and Movement of Vegetatively Propagated Germplasm: In Vitro Culture and Disease Aspects

Report of a subcommittee meeting
held at
North Carolina State University, USA
17-19 August, 1987

IBPGR
Rome, 1988

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). IBPGR was established by CGIAR in 1974. The basic function of 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. It thereby contributes to raising the standard of living and welfare of people throughout the world. Financial support for IBPGR's core programme is provided by the Governments of Australia, Austria, Belgium, Canada, China, Denmark, France, FRG, India, Italy, Japan, the Netherlands, Norway, Spain, Sweden, Switzerland, the UK and the USA, as well as the World Bank. The Food and Agriculture Organization of the United Nations provides the Headquarters.

Citation:
IBPGR. 1988. IBPGR Advisory Committee on In Vitro Storage. Conservation and Movement of Vegetatively Propagated Germplasm: In Vitro Culture and Disease Aspects. International Board for Plant Genetic Resources, Rome

ISBN 92-9043-125-3

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INTRODUCTION

1. The IBPGR Advisory Committee on In Vitro Storage recommended, at its third meeting in 1986, that a subcommittee be organized to assess the status of disease indexing and associated procedures for priority crops. Special emphasis was to be given to those which are, primarily, propagated vegetatively. The specific objectives of that subcommittee were to:

(1) develop a basic philosophy for moving germplasm of high priority crops (especially primitive germplasm), giving particular attention to problems of disease indexing and movement through quarantine, where bottlenecks are often encountered;

(2) link that philosophy with genetic resources activities from collecting in the field to conservation (particularly in vitro storage);

(3) review priority crops and methods of disease indexing; and

(4) recommend areas which need research.

2. The subcommittee was convened and met at North Carolina State University, USA, 17-19 August 1987. The local arrangements were made by Prof. J.W. Moyer of that establishment, who also chaired the meeting. The membership of the subcommittee included Dr. J.L. Dale, Mr. E.A. Frison (IBPGR Pathology and Quarantine Officer), Mr. J.A. Foster, Dr. S.M. Garnsey, Dr. L. Navarro, Dr. L.F. Salazar, Dr. S. Spiegel, Prof. J.T. Williams (Director, IBPGR) and Dr. L.A. Withers (Chairman, IBPGR Advisory Committee on In Vitro Storage). The names and addresses of the participants are given in Appendix I. The membership of the subcommittee was determined so as to contain scientists with expertise on disease indexing, germplasm storage, in vitro techniques, quarantine and germplasm movement both in general and on priority crops. Crops represented included both those that are well advanced with respect to disease indexing and those about which little is known.

3. The participants were welcomed by Profs. Moyer and Williams who explained the task of the subcommittee in the context of IBPGR's Field and Research Programmes. The timeliness and importance of addressing problems in the safe movement of germplasm to ensure its conservation and efficient utilization were outlined. Mr. Frison further explained IBPGR's interest and needs for pathogen testing of germplasm.

4. To assist the task of the subcommittee, a survey was carried out among relevant curators of germplasm collections, quarantine specialists and plant pathologists to determine the current status of disease indexing procedures and their application, particularly for priority crops. The individuals surveyed were identified from lists of germplasm collections provided by IBPGR, USDA Crop Advisory Committees and members of the subcommittee. This information, together with status reports presented by members of the subcommittee on their assigned areas, provided a basis for the discussions and recommendations that follow. The status reports are listed in Appendix II and appear in Appendix III.

THE PROBLEM

5. The safe movement of germplasm from one country or continent to another for the purposes of genetic conservation, evaluation and utilization is highly dependent upon the ability to assure the host country that unwanted pests or diseases will not be released as a result of introduction with the germplasm. In many countries, a very conservative attitude is taken towards plant introduction. This has led to severe impediments in the movement of materials, particularly in vegetative form. This conservatism is often justified by the lack of adequate post-entry quarantine facilities and a frequently poor knowledge of the etiology and distribution of relevant pests and diseases.

6. The realization that the reservoir of plant genetic resources is vanishing rapidly places greater pressure upon germplasm movement procedures and their operations, as risks of irreversible loss become balanced against risks of disease through relaxation of standards. In order to make the systems more efficient, measures must be taken to enable informed decisions to be made in each instance to then match the action taken to the genuine level of risk.

PRESENT STATUS OF SOME MAJOR CROPS

7. The members of the subcommittee discussed the present status of disease indexing and germplasm movement for a number of crops whose handling ranged from the relatively well worked-out and unproblematic (potato) to the under-examined and/or highly problematic (Musa and temperate fruits). In vitro techniques and advanced indexing procedures make varying contributions to the treatment of the different crops.

Potato

8. Several biochemical and molecular tests are available for the detection of viruses and viroids in potato. These include nucleic acid spot hybridization (NASH), latex agglutination test (LAT) and enzyme-linked immunosorbent assay (ELISA). These techniques are backed up by inoculation of indicator plants (particularly for unknown viruses) and regular inspection of plants by experts. Eradication of virus and viroid infection is carried out by thermotherapy (heat or cold treatment) and meristem-tip culture. Reinfection is prevented by appropriate precautions. Distribution of potato germplasm in vitro is a routine procedure.

Sweet potato

9. Grafting to the indicator host plant Ipomoea setosa is the only technique routinely used for sweet potato virus indexing, although other methods are under development. Progress in indexing is hampered by poor knowledge of the etiology of the diseases and of the distribution of the virus in the plant. Meristem-tip culture is used to eradicate viruses from sweet potato.

Citrus

10. Whilst some citrus diseases are common in most growing areas, other serious ones are localized. The introduction elsewhere of the latter would be disastrous for the citrus industry. Viruses and virus-like pathogens present the greatest problem; their etiology is frequently poorly understood. Indexing methods include graft or mechanical inoculation, serological methods including ELISA, and electron and light microscopy. Nucleic acid hybridization tests are under development. Technical complexity, lack of development of specific test conditions and the absence of internationally accepted guidelines hinder efficient indexing.

11. Despite indexing problems, virus eradication in citrus can be carried out very effectively by thermotherapy and shoot tip grafting. Material introduced as budwood is inspected on receipt without opening the containers. Heavily contaminated or infested material is destroyed; acceptable material is surface sterilized and inoculated into culture on a simple medium. Shoot-tips are excised from the flushes produced in culture and grafted onto etiolated seedlings. Grafted seedlings are transplanted to the glasshouse/screenhouse, indexed and eventually released to germplasm collections or growers. Shoot-tip grafting is extremely effective in eradicating viruses in citrus and is technically feasible in a number of citrus relatives and other fruits (e.g. Prunus and Malus).

Musa

12. Like citrus, banana and plantain are susceptible to diseases that include both widespread and localized examples. In vitro tranfer is becoming increasingly attractive as an option to avoid disease introduction, particularly for non-viral pathogens. Some viral diseases are relatively well described, and detection methods, including molecular tests, are available or under development. However, other unrelated or undescribed viruses give cause for concern. Visual inspection by experienced pathologists and other non-specific tests, such as double-stranded RNA (dsRNA) analysis and extraction of viral DNA or general viroid extractions, are necessary for unknown diseases. Meristem-tip culture is unlikely to exclude viruses due to the size of usable explants, so avoidance of introduction remains a priority. However, in vitro culture methods are attractive for contained movement and storage of Musa germplasm.

Temperate fruits (particularly small fruits and grapevine)

13. Virus infections are common in temperate fruits. Latency and the production of obscure symptoms hinder the detection of infections. Combined infections and restricted host range of some viruses further complicate the accurate description of diseases and the characterization of their causal agents. Graft transmission, mechanical inoculation, ELISA, immunosorbent electron microscopy (ISEM) and dsRNA tests are used for detection of viral infections. The serological tests are used increasingly and monoclonal antibody techniques are likely to become available in the near future. The development of dsRNA tests has been hindered by technical difficulties relating to extraction procedures and by variability of sample material due to a number of factors. However, this area is viewed with optimism.

14. The combination of heat therapy and meristem-tip grafting is considered safest for virus eradication. High carbon dioxide treatment has shown promise. Generally, in vitro methods are not widely in use for the storage and distribution of temperate fruit germplasm, although the technologies are likely to be available and/or in need of little specific development. Concern over genetic instability may be discouraging the adoption of these methods despite the relatively safe culture systems likely to be used.

RISK ANALYSIS

15. The actual net risk involved in the movement of a given material has many contributing factors. Risk analysis has proved to be the only realistic way of tackling the problem of quarantine that is compatible with the safe yet expeditious movement of material. It should be recognized that the contributing causes to risk are factorial and that, therefore, the achievement of a very low or zero score in any one area significantly reduces or removes the risk. (For example, if a pathogen is already widely distributed in the recipient country, other component risk elements are irrelevant.) It should be recognized that it is not necessary to have full information on all of the factors to carry out a realistic analysis. Furthermore, risk analysis reveals areas requiring development and highlights new opportunities for reducing the net risk.

16. The contributing factors to a risk analysis will include the following:

1. Information on pests and diseases

   a) Geographical distribution (including both country of origin and recipient country)
   b) Etiology
   c) Epidemiology
   d) Potential economic hazard to recipient country

2. Information on plant material

   a) Type (see Fig. 1)
   b) Previous treatment (see Fig. 1)
   c) History (e.g. whether vegetative material originated from seedlings or from many generations of clonal propagation)

3. Destination and handling

   a) Recipient category (genebank; research establishment; commercial establishment)
   b) Degree of containment
   c) Potential for effective therapy

4. Efficacy and extent of quarantine and plant protection services in recipient country.

SCOPE FOR IMPROVEMENT

Recommendations to collectors

17. In order to increase the ease and efficiency of the movement of germplasm in vegetative form, collectors should:

1) meet with quarantine officials prior to starting the collecting mission to obtain information regarding risks involved with the material to be collected and discuss the possibilities of minimizing these risks. At that time, arrangements can also be made concerning shipments, port of entry, and advice given on packaging materials and labelling, etc;

2) collect seeds whenever possible; if no seed is available at the time of collection, consider the possibility of taking vegetatively propagated material to a greenhouse and later collecting seeds from it; and

3) favour material that has recently been through seed production or has been grafted onto seedling rootstocks rather than clonally propagated rootstocks.

In vitro transfer of germplasm

18. *In vitro* techniques offer the potential for the improvement of procedures in several areas including transfer, therapy, indexing and contained quarantine. The first of these is dealt with here; for other aspects, see paras 24 36.

19. In terms of risk, *in vitro* cultures have advantages in that the mass of material transferred is reduced, non obscure pests and pathogens are excluded as is soil, and the transferred material is contained. However, it must be emphasized that without appropriate therapy and indexing, *in vitro* culture alone cannot guarantee a pathogen-free status. Material in culture should be considered to be less risky than plants from the glasshouse, field or other less controlled origin but more risky than material that has passed through a quarantine station and/or has been certified free of pests and pathogens.

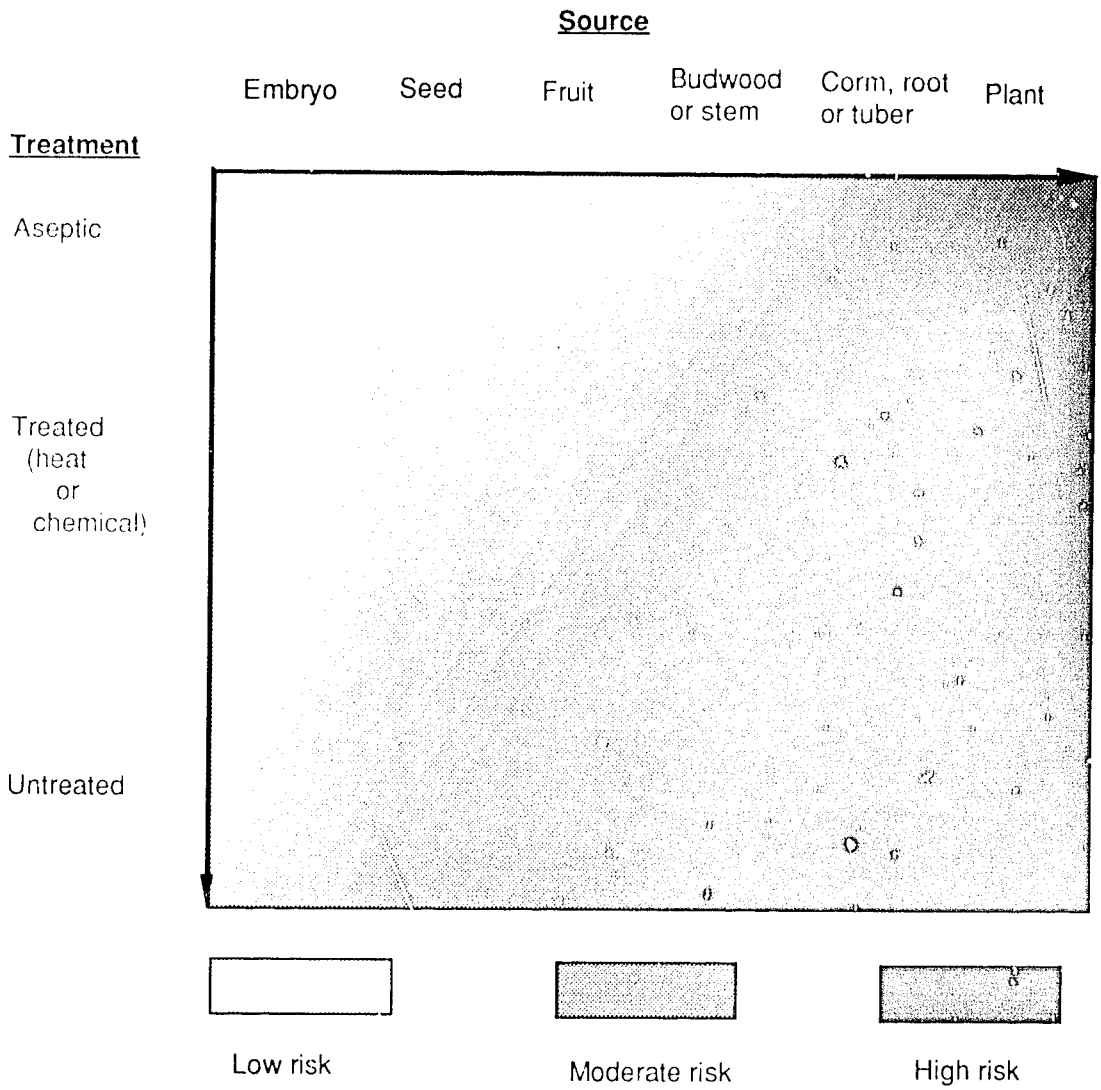

Fig. 1. Factors contributing to risk analysis.

20. In vitro transfer is of value at two stages in the germplasm handling process, namely collecting and exchange. In vitro collecting has been pioneered by IBPGR and collaborators and successful techniques are emerging for a number of crops (see Appendix III). The techniques range in decreasing levels of sophistication from those that utilize local laboratory facilties to those that involve simple hand-carried items. It should be noted, however, that in vitro collecting is likely to lead to the circulation of more primitive, untested material; it should not be confused with procedures that lead to pathogen elimination, and indexing and containment should be appropriately vigilant.

21. In vitro exchange has been used for a number of years by institutes, including several International Agricultural Research Centers (IARCs), to distribute germplasm efficiently and securely. Practical procedures for culture packaging, handling before and after transit, certification and in vivo transfer are well established and success rates are high (see Appendix III).

22. The IBPGR In Vitro Conservation Database contains a large number of entries detailing the wider use of in vitro exchange by scientists engaged in both research and crop improvement or production. Exchanges are generally very successful, with practical problems occurring only occasionally. There appears to be a low level of awareness of phytosanitary considerations among scientists engaged in exchanges except from IARCs and other centres of similar status.

23. For some crops, in vitro transfer will firstly necessitate the development of suitable in vitro propagation systems. This may involve the use of techniques such as shoot tip grafting (see para. 11), when shoot culture and proliferation are not possible.

Improved therapy techniques

24. Recent developments have shown promise for the improvement of therapy methods. Besides heat treatment and meristem tip culture, which are widely used, alternative techniques include shoot tip grafting, cold treatment, chemotherapy, other physiological/environmental host manipulations (e.g. high carbon dioxide levels). Their sequential or combined use should be investigated further. Therapy methods in vitro offer evident advantages in reducing space and equipment requirements. They are therefore very attractive.

25. The applicaton of appropriate therapy techniques also plays an important role in decreasing the risk involved with transfer of material. This is particularly important for material from wild origin where unknown diseases might occur. In this particular case, the systematic application of therapy techniques will minimize the risk of introducing unknown diseases. By decreasing the risk, the indexing procedure could, in some cases, be simplified. However, this should not be misunderstood; therapy can not simply replace indexing for diseases.

Improved indexing

Molecular and biochemical techniques

26. For many crop/disease combinations, indexing techniques are lacking, unreliable or very time-consuming. The past two decades have seen tremendous progress in serology. Techniques such as ELISA and serologically specific electron microscopy (SSEM) also called Immunosorbent electron microscopy (ISEM), have proven reliable as diagnostic tests for many diseases. The use of monoclonal antibodies is another step towards more specific and more reproducible results. Isolation of dsRNA, electrophoresis of extracted nucleic acid and, more recently, NASH techniques have opened new fields for pathogen detection. All of these techniques combined with developing amplification techniques will make it possible to detect extremely low concentrations of viruses and viroids. Such detection was not possible hitherto.

27. The development of broad spectrum tests, including detection of dsRNA, monoclonal antibodies to epitopes that are highly conserved between viruses of a given taxon and broad spectrum DNA probes for hybridization assays, are all potentially very useful for general disease indexing purposes.

In vitro techniques

28. Procedures for the detection of bacterial, fungal and viral pathogens are generally based on symptom detection, whole-plant bioassay, or biochemical or molecular techniques. Whilst these meet most requirements in terms of sensitivity, they do have drawbacks with respect to speed and containment. There is scope for the development of in vitro indexing; this general term covers both indexing by appropriate methods of material held in vitro and the use of in vitro bioassay systems (see Appendix III).

29. The indexing of material held in vitro will only be successful if (i) adequate quantities of tissue can be generated for testing, and (ii) adequate concentration of the disease-causing organism develops in vitro as a result of the operation of culture conditions. This is the opposite strategy to in vitro eradication. Sampling strategies will need to be determined both with respect to the explant selected for in vitro inoculation and the selection of material from culture for indexing. There is evidence for both greater and lesser titres of pathogen in culture than in the parent plant; some attenuation may occur in vitro and it should be determined whether this is due to loss of symptom expression or a genuine reduction in titre of pathogen.

30. Several seed-borne pathogens can be detected by in vitro procedures. Contained germination of suspect material and revelation of relatively obscure pathogens are practicable.

31. In vitro bioassay systems for vegetative material are not yet in general use but examples from the literature and unpublished examples illustrate the potential for developing this approach to disease detection.

32. In the case of citrus the presence of exocortis viroid can be detected by in vitro rooting and callusing reactions. Also in Citrus, virus and canker can be detected by inoculation of extracts on to in vitro germinated seedlings. A similar approach is available for Xanthomonas ampelina of Vitis. These are the only examples known that were developed specifically for indexing.

33. In addition to screening using infectious inocula, it may also be possible to utilize in vitro reactions to pathogen toxins, thus increasing the safety of indexing.

34. The development of in vitro indexing using either infectious or non-infectious inocula would require the establishment of adequately sensitive indicator cultures, themselves certified free of infection by complementary tests. A related point is that in vitro material could be used to hold reference collections of some pathogens to serve as positive controls in indexing by all procedures.

Contained quarantine

35. The themes of in vitro exchange, indexing and storage can be drawn together to develop a strategy of contained quarantine that has the potential to overcome many of the operational disadvantages of current procedures whilst maintaining a high level of phytosanitary control. It further increases the potential for utilizing third country quarantine, as climatic constraints in the maintenance of both material in quarantine and indicator plants are alleviated by the use of in vitro systems.

36. For a quarantine system based upon in vitro procedures to be successful, a number of requirements must be met:

1) the pathogen-tested status of the material should be clearly documented and understood. In vitro techniques must not be used just to circumvent quarantine;

2) quarantine authorities should be advised of the special requirements, advantages and disadvantages of in vitro material; and

3) strict control should be operated over the destination of in vitro material and its destruction in the case of gross infections or those which cannot be eradicated should be an acceptable option to recipients.

'Pathogen untested' and 'pathogen tested positive' in vitro collections

37. The unavailability of adequate indexing and/or therapy techniques for some vegetatively propagated crops creates problems in the safe transfer and maintenance of germplasm. After discussing the safeguards offered by in vitro movement and conservation, the subcommittee urges the wide acceptance of the concept of 'pathogen untested' and 'pathogen tested positive' collections. Their maintenance and movement would be for germplasm conservation and research, with safeguards that would prohibit distribution.

38. 'Pathogen untested' collections would contain plants which have not been pathogen tested and could be infected with one or more pathogens while 'pathogen tested positive' collections would contain material that had been pathogen tested, found to be positive for one or more pathogens, and had not received therapy nor subsequent certification of freedom from pathogens.

39. Maintenance of such collections provides the following opportunities:

(1) accommodation of anticipated improvements in indexing and therapy techniques;
(2) safe storage of material prior to therapy and indexing;
(3) selection of resistant or tolerant genotypes;
(4) rapid development of polycross nurseries under containment; and
(5) preservation of pathogens.

FRAMEWORK FOR THE MOVEMENT OF GERMPLASM

40. In an attempt to synthesize the subject of the movement of germplasm of vegetatively propagated crops, the subcommittee established a conceptual framework that is flexible enough to include most of the situations related to the movement and conservation of germplasm of crops that are usually multiplied vegetatively. This is represented in the form of a flow diagram (Fig. 2) which includes the important concepts of 'pathogen untested' and 'pathogen tested positive' in vitro collections and their movement.

RECOMMENDATIONS

Risk analysis

41. A careful risk analysis should be carried out before any collecting operation or germplasm exchange can be initiated. All available germplasm should be considered for collecting/exchange; the safest plant material should be selected. Whenever possible, this should be seed. The value and safety of in vitro material should be recognized without, however, underestimating its risks. Collectors should be fully briefed ahead of their work on the quarantine requirements of recipient countries. Appropriate documentation should be obtained before attempting movement of material.

Disease etiology and geographical distribution

42. Strategic research that would determine the etiology of important diseases should be encouraged in each priority crop.

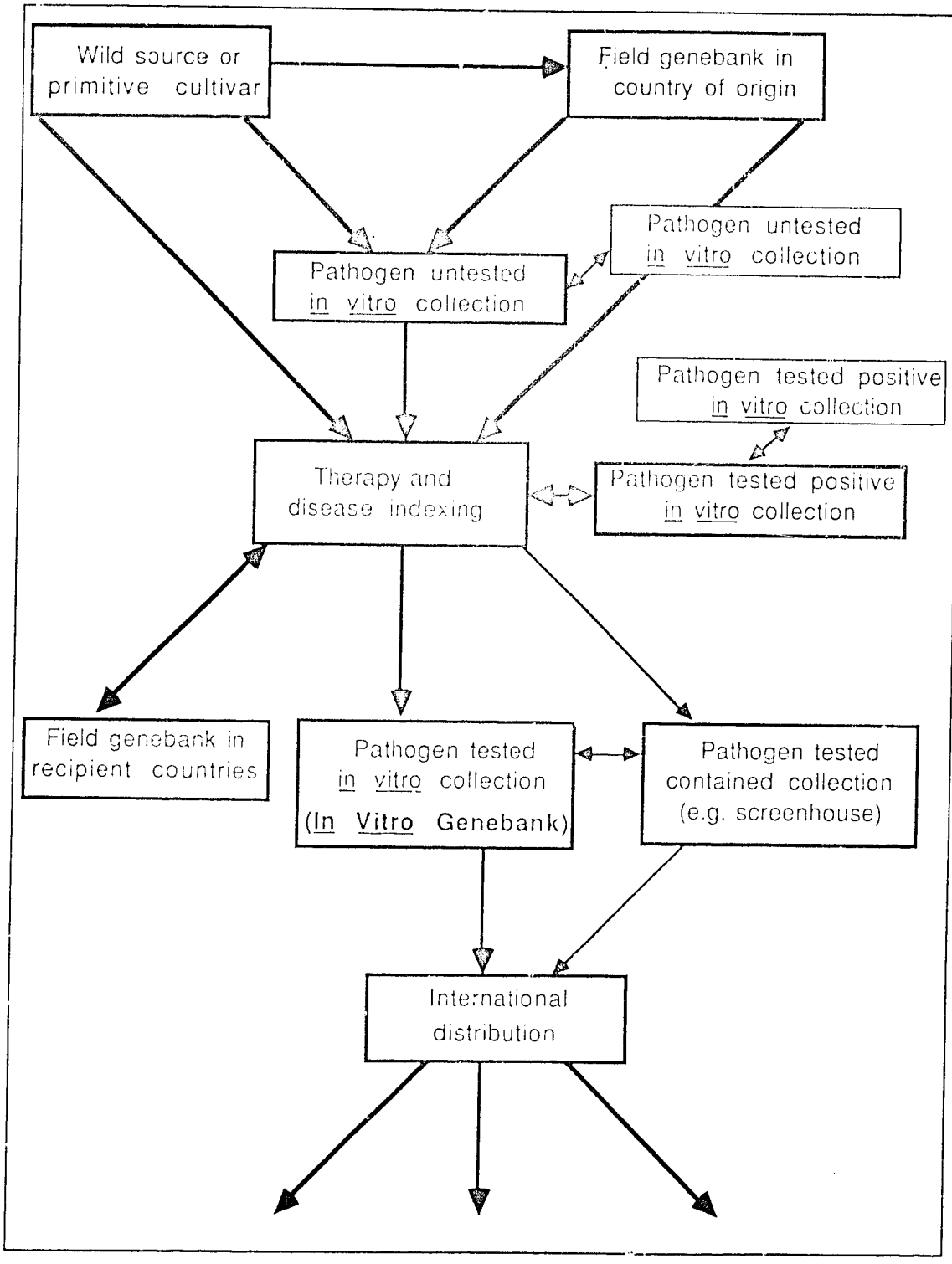

Fig. 2. Flow diagram of a framework for the movement of germplasm.

43. Information should be gathered on the geographical distribution of the diseases of priority crops. The current lack of this information represents a major gap in our knowledge.

Disease indexing

44. In order to develop internationally accepted procedures for disease indexing it is recommended that the international scientific community establishes, on a crop basis, standing technical advisory committees with relevant expertise. The members should be identified by international centres, professional societies, etc. These committees should:

(1) assemble acceptable and unacceptable assays;
(2) recommend documentation for validation;
(3) encourage the international use of standard positive controls (non infectious whenever possible);
(4) encourage identification of resource centres for indexing materials (e.g. biological indicators, antisera, hybridization probes, positive controls); and
(5) determine the relative importance of pathogens and consider geographical risk.

45. The development of broad spectrum and non specific biochemical and molecular assays for indexing should be encouraged.

46. The development of totally contained systems for pathogen testing is recommended. This includes the development of biological indexing methods using plants grown in vitro and of pathogen detection procedures directly from in vitro grown plants. A model system to both illustrate the value of contained quarantine and to explore attendant problems and research deficiencies would be of high priority to IBPGR.

47. Sampling techniques, taking into consideration the biological distribution of the pathogen, the host growth stage, the season, and the preparation of the host, have to be developed for the different host/pathogen associations.

48. Further research is needed to increase the sensitivity and reliability of tests. This should be encouraged.

49. The use of confirmatory tests based on alternative properties should be encouraged.

50. The maintenance of positive controls in vitro should be encouraged to reduce risks.

51. There is a need for 'hands on' training in disease indexing.

Therapy

52. Research should be carried out to increase the efficiency of therapy in order to facilitate indexing whilst maintaining sufficient levels of confidence.

53. The systematic use of therapy for crops of unknown disease background should be encouraged.

54. The development of alternative and combined therapy methods should be encouraged.

Contained quarantine

55. The wider scientific concept of a contained quarantine procedure based on in vitro techniques that could extend from collecting/sampling in the field through to genebank conservation (and would certainly include in vitro exchange and containment during indexing) should be promoted.

'Pathogen untested' and 'pathogen tested positive' in vitro collections

56. There is considerable value in the maintenance of 'pathogen untested' and 'pathogen tested postive' in vitro collections. Quarantine authorities should be advised of the value and safety of such collections; appropriate procedures should be developed for their maintenance consistent with research needs and phytosanitary regulations. Such collections should be overseen by a pathologist, not a horticulturalist or breeder.

APPENDIX I

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APPENDIX II

List of Status Reports by the Participants

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APPENDIX III

IBPGR's interests and needs for pathogen testing of germplasm

E.A. Frison 1/

The basic function of the International Board for Plant Genetic Resources (IBPGR) is to promote the collection, conservation, evaluation, utilization and exchange of the rapidly vanishing reservoir of plant genetic resources.

Incorporating resistance to biotic and abiotic stresses into crops is considered the only possible way to stabilize and increase food productivity in many areas, especially in developing countries. This requires the access to a wide range of genetic diversity which is not always available in the area where the breeding work is carried out and therefore breeders often express their desire, and need, for unrestricted exchange and movement of germplasm. This is, in fact, a prerequisite for further crop improvement through plant breeding.

It is widely recognized that the widespread distribution of plant materials has the inherent potential risk of also distributing plant pathogens and parasites in previously unaffected areas. Vegetatively propagated crops are deservedly considered the most problematic with regard to the propagation of pests and diseases. Indeed, fungal, bacterial and viral disease as well as insects, mites and nematodes are readily transmitted through vegetative propagules. For this reason, the movement of such materials has been restricted and sometimes forbidden by plant quarantine regulations. These regulations, to a large extent, have not been updated with the development of new techniques.

Modern in vitro culture techniques have considerably improved the phytosanitary aspects of plant introduction as they have a built in capability of detecting pests and pathogens. If the medium remains uncontaminated with fungi and bacteria the system must be free of insects, mites, nematodes and pathogens. The system however, does not detect obligate parasites such as viruses, viroids and certain fungi.

Viruses and viroids have, however been successfully eliminated from many different vegetatively propagated crops by meristem tip culture alone or a combination of thermotherapy and meristem tip culture.

This means that modern in vitro techniques would make it possible to transfer genetic stocks of vegetatively propagated crops safely, if an efficient method of virus and viroid detection were available.

IBPGR has recognized that lack of efficient indexing methods for these pathogens is actually the major bottleneck in the safe transfer of the germplasm of these crops.

The reasons for the unavailability of adequate indexing methods can be roughly divided into two categories:

1. In some cases reliable disease indexing methods are not available because little is known of the etiology of the diseases. Therefore studies on etiology of those diseases have to be encouraged for crops ranked as high priority by the IBPGR.

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2. Where sufficient knowledge is available of the etiology, and reliable virus-indexing methods exist, they are not always practical because they are too lengthy or labour intensive to be widely used for the transfer of large numbers of germplasm accessions. Recent developments in serology and molecular biology provide the basic technology to improve indexing methods of these crops.

At its third meeting in 1986, the IBPGR Advisory Committee on In Vitro Storage recommended that this subcommittee meeting be organized with the following terms of reference:

(i) to develop a basic philosophy for moving germplasm of high priority crops (especially primitive germplasm), giving particular attention to problems of disease indexing and movement through quarantine, where bottlenecks are often encountered;

(ii) to link that philosophy with genetic resources activities from collection in the field to conservation (particularly in vitro storage); and

(iii) to identify areas which need research (e.g. etiology, developmental work, neutralization of interfering host substances).

In the priorities set by the IBPGR Advisory Committee on In Vitro Storage, research on disease indexing was recommended on Musa, citrus, aroids, yams, sugarcane, cassava, temperate fruits and Artocarpus.

It is hoped that this meeting will review these crops and that for each crop the present state of knowledge and recommended areas of research and development will be outlined.

Disease Indexing and International Distribution of Potato Germplasm

L.F. Salazar 1/

The mandate of the International Potato Center (CIP) for distributing potato genetic resources throughout the world has obliged our adopting the most sensitive methods for pathogen identification. Though bacteria, fungi, nematodes and insects are significant in quarantine regulations, methods for their detection and elimination were known when distribution from CIP started in 1975.

Viruses and the viroid causing potato spindle tuber (PSTV) required the development of specific methods and overall procedures that could meet the extremely strict quarantine regulations in force in most countries.

The methods for pathogen detection and elimination were modified or replaced according to developments in this area. The methods and procedures now in use might even require further adjustments as basic research into methods of pathogen detection continues.

Testing materials for pathogens

A general scheme is shown in Fig. 1.

Viroid detection

Since PSTV is one of the major pathogenic agents of potato specifically addressed in quarantine regulations in most countries, CIP requires that materials should be viroid free. The tomato test (Fernow, 1967; Yang and Hooker, 1977) played an important role in viroid detection at the beginning though even at present the assay is useful for confirming doubtful results. An electrophoresis procedure modified from that described by Morris and Wright (1975) allowed us more rapid testing of a large number of samples. However, its reliability depended to a great extent on the concentration of viroid in plants. Plants grown at temperatures below 20-25°C in most cases did not contain enough viroid to be detected by electrophoresis (Lizárraga et al., 1980). The dependence of viroid replication on high temperatures was the basis for the development method of viroid elimination by cold treatment and tissue culture (Salazar et al., 1985).

The development of nucleic acid spot hybridization (NASH) for detecting PSTV was a tremendous improvement in our system (Salazar et al., 1983). Further studies (in press) showed that RNA probes were more sensitive (Table 1) and specific than double- or single-stranded DNA probes, allowing detection of as little as 0.3 pg of PSTV or detection of viroid from one infected true potato seed in a batch of 100. The procedure for viroid detection used at present is shown in Fig. 2.

As shown in the general pathogen-tested procedure, testing for PSTV is required before and during clonal multiplication.

Virus detection

Since CIP has to distribute materials from countries where the virus situation is incompletely known, two situations had to be met for detecting potato viruses:

(a) occurrence of known potato viruses; and

(b) probability of occurrence of previously unidentified and little known viruses.

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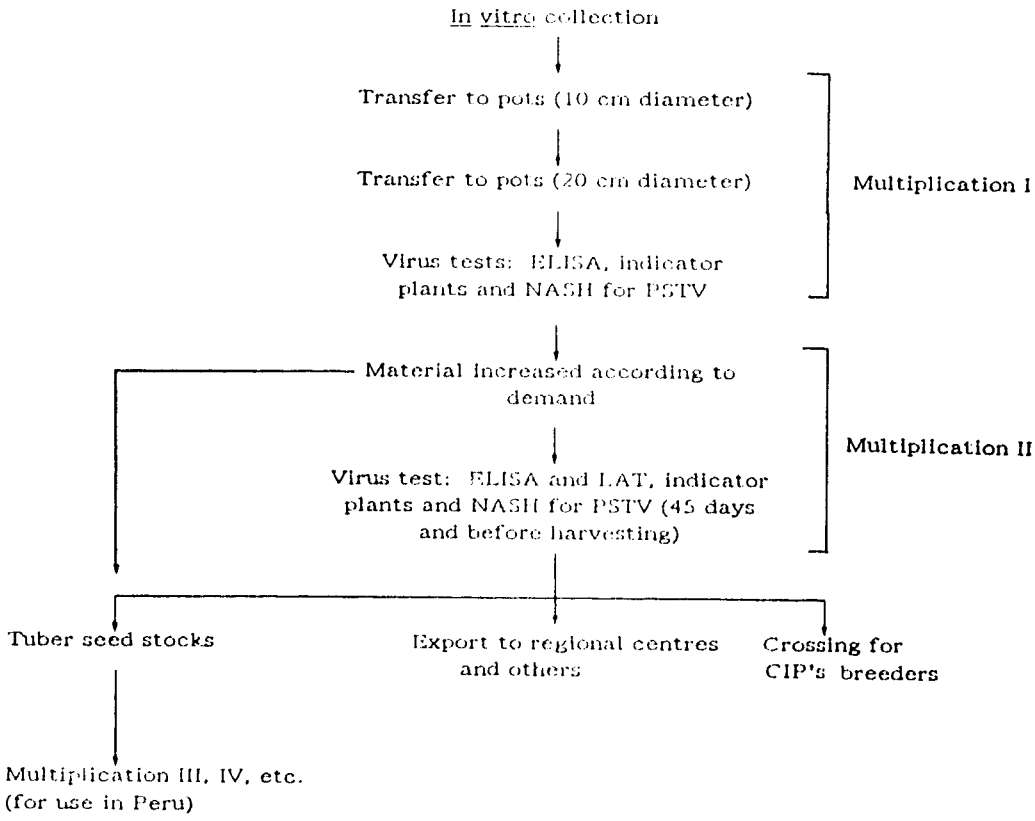

Fig. 1. Multiplication of pathogen-tested clones.

Detection of known viruses relies on serology. For this, the latex agglutination test (LAT) and the enzyme linked immunosorbent assay (ELISA) are in use at present. LAT is sufficiently sensitive to detect viruses occurring in relatively high concentrations in plant tissues. The standard procedure consists of mixing a drop of conveniently diluted antibody-sensitized latex with plant sap diluted 1/10 and 1/100 on to a squared plastic plate, and agitating for 30 min in a rotary shaker. Positive reactions are clearly distinguished by the flocculation that takes place, in contrast to the milky appearance of healthy controls.

Table 1. Reciprocal of maximum dilutions of infected extracts and minimum concentration of purified PSTV detected by three types of probe

Probe type	Tomato leaves	Potato leaves	Potato tuber sprout	True potato sprout	Pure PSTV (pg)
M13 ss PSTVcDNA	512	512	512	128	10-20
Nick-translation dsDNA	64-128	128-256	512	64	100
RNA transcription	1024	1024	1048	256	0.33

Homogenize tissue (tissue grinders, plastic bags)

↓

Formaldehyde/10 x SSC, 1:1 (2 ml/g tissue)
0.5 by vol. chloroform
0.5 by vol. saturated phenol
Centrifuge or let stand overnight at 4°C

Aqueous phase containing PSTV

↓

Spot 3 mul of aqueous phase on to nitrocellulose filter
Dry at room temperature
Bake 1.5 h at 80-100°C in oven

PSTV immobilized on membrane

↓

Hybridize 5-7.5 x 10^5 cpm/ml in 2 x SSC + 40% formamide
+ 10% dextran sulphate at 55°C, overnight

Formation of RNA-RNA (PSTV-^{32}P-labelled cPSTV) hybrids

↓

Wash 2 x 10 min at room temp. using 1 x SSC + 0.1% SDS
Wash 30 min at 65°C in 0.1 x SSC + 0.1% SDS
Wash 15 min at room temp. in 2 x SSC + RNAase A (2 mug/ml)
Wash 2 x 10 min at room temp. in 1 x SSC + 0.1% SDS
Wash 30 min at 50°C in 0.1 x SSC + 0.1% SDS

Autoradiography at -70°C

↓

Visualization of specific RNA-RNA hybrids

Fig. 2. Protocol for the detection of PSTV by NASH using RNA probes.
SSC, sodium saline citrate; SDS, sodium dodecyl sulphate.

ELISA, however, is more sensitive than LAT and so is used more often. The protocol of Clark and Adams (1977), with minor modifications, is used at CIP. PVX, PVY, PVS, PVM, PLRV, PVT, APLV and APMV are tested by serology. Detection of previously unidentified viruses is based on the reaction of already known potato viruses in a selected host range (Table 2). If all known potato viruses can be detected by inoculation to one or more of the hosts of Table 2, it is very possible that by using a similar host range, unknown potato viruses could also be detected. If the host range fails to detect an unknown virus, there is still some possibility that the virus could be detected in the infected potato plants which are carefully observed during growth in the screenhouse.

Other disease indexing

Since clonal propagation is initiated by tissue culture, several common organisms of the potato are likely to be eliminated. In addition, hygienic precautions and preventive treatments during multiplication and before shipment guarantee pathogen and pest control. During handling of plants in insect-proof screenhouses, a bactericide treatment (Dimanin), soap washing and rinsing with a 10% solution of calcium hypochlorite is used for disinfecting operators' hands and tools.

Regular inspection by entomologists, pathologists and nematologists is conducted during plant growth. Disease contamination so far has not occurred.

Table 2. Local (L) and systemic (S) symptoms induced by potato viruses in a selected host range

Reactions with diagnostic value only have been included. The best host for each virus host combination is shown with an asterisk.

	PVX	PVY	PVA	PLRV	PVS	PVM	PVT	APMV	APLV	PMTV	TRV	PSTV
Nicotiana tabacum	L,S	S	S	-		-	-	S	S	-	L,S	-
N. clevelandii	S	S	S	-			-	S*	S	-	S	-
N. glutinosa	S	S	S	-	-		-	S	S	-	S	-
N. debneyii	S	S	S	-	S*	L*	S	S	S	S	S	-
Physalis floridana	L,S	L,S	S	S*	-	-	-	-	-	-	-	-
Lycopersicon esculentum	S	S	S	-	-	S	-	-	-	-	-	S*
Datura stramonium	S	-	-	S*	-		-	S	-	-	-	-
D. metel	S	S	S	-	-	-	-	-	-	-	-	-
Gomphrena globosa	L*	-		-	L	L	-	-		-	L	-
Chenopodium amaranticolor	L	L/-	-	-	L/-	L/-	S*	-	L*	L*	L*	-
C. quinoa	L	L/-		-	L/S	-	S	-	L	-	L	-
Phaseolus vulgaris		-	-	-	-	L	L/-	-	-	-	L	-
Clone A6	L	L*	L*	-	L/-	-	-	-	-	-	-	-

Testing of tuber families

CIP also distributes first-generation tubers from selected crosses. Seedlings for tuber family production are also produced in screenhouses similar to those mentioned above. Special care is taken to test for PSTV and PVT the only two pathogenic agents transmitted by true potato seed. In addition, our regulations require that mother plants be free of those agents before crosses are made.

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Virus Indexing and Distribution of Sweet Potato Germplasm

J.W. Moyer 1/

Sweet potatoes (Ipomea batatas (Lam.) L.) harbouring graft-transmissible agents which cause diseases can be found in nearly every commercial sweet potato planting not produced from disease-indexed planting material. This phenomenon results from the propagation of sweet potatoes with vegetative organs in order to preserve varietal purity. Vegetative propagation provides an excellent vehicle to perpetuate these agents from one planting to the next. It is generally assumed that viruses or viroids are the cause of these diseases. However, the etiology of many of these sweet potato diseases remains to be determined (Table 1).

Early in the development of information on sweet potato viruses, each new disease thought to be caused by a virus was frequently given a new name. The name was usually indicative of the symptoms expressed in the genotype in which the disease was described. It has seldom been possible to compare biological or biochemical characteristics of the causal agent(s). This practice has led to the problem of synonymy similar to that which occurred in the early studies of virus diseases of other crops. Our knowledge of the viruses which cause these diseases has developed slowly because the sweet potato-virus pathosystems have not been adequately developed. The viruses are difficult to transmit mechanically to and from sweet potato, virus-indexed sweet potato is not always available for completion of Koch's Postulates, and high quality antiserum for virus comparison and indexing purposes is not readily available because of the difficulty in purifying these viruses. For these reasons there have been no internationally agreed-upon criteria for sweet potato virus identification.

These sweet potato diseases interfere both directly and indirectly with the farmers' ability to realize the full potential of sweet potatoes. Viruses have been implicated in acute and chronic sweet potato diseases. Acute diseases, such as sweet potato virus disease complex found in Africa, have a dramatic impact on the production of edible roots. Chronic diseases, such as those caused by various strains of sweet potato feathery mottle virus (SPFMV), have a less severe effect on individual plants but still may significantly reduce yield when considered on a regional basis. Grower efficiency is indirectly reduced by the justified impediment that viruses cause to the international exchange of germplasm. This slows the introduction of desirable traits into national breeding programs to resist stresses and generally improve yield potential. This should not be interpreted as implying that quarantine regulations should be relaxed. In general, quarantine standards are based on available biological information. Our lack of understanding of sweet potato viruses has greatly contributed to the conservative establishment of standards to reduce the probability of introducing new pathogens. Improved understanding of this group of pathogens will significantly increase the confidence with which these standards are upheld, as well as providing more efficient technologies that can be used to document the status of plant health.

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Table I. A list of viruses and selected virus like-diseases reported from sweet potatoes

	Vector	Geographic distribution 1/
Viruses		
SPFMV 2/	Aphid	World-wide
SPVMV 3/	Aphid	Argentina
SPLV 4/	?	Taiwan
SPMMV 5/	Whitefly	East Africa
SPCV 6/	?	Puerto Rico
SNYDV 7/	Whitefly	Taiwan
CMV 8/	Aphid	Africa, other
Virus-like diseases		
Sweet potato virus disease	Aphid and whitefly	Africa, ?
Sweet potato leaf curl	Whitefly	Taiwan, Japan
Sweet potato mosaic	?	Taiwan
Unnamed	?	World-wide

1/ Based on published reports and personal communications. 2/ Sweet potato feathery mottle virus. 3/ Sweet potato vein mosaic virus. 4/ Sweet potato latent virus. 5/ Sweet potato mild mottle virus. 6/ Sweet potato caulimo-like virus. 7/ Sweet potato yellow dwarf virus. 8/ Cucumber mosaic virus.

Indexing standards for the purpose of international exchange of sweet potato germplasm has relied almost exclusively on a graft assay into the indicator host Ipomoea setosa. All of the known viruses cause varying degrees of symptom expression on this host. The weaknesses of this scheme are generally recognized and serological assays are being incorporated as they become available. At the present time the only other biological assay is the use of SPFMV-infected Tib-8 (developed at the International Institute for Tropical Agriculture) for the detection of the whitefly component of the sweet potato disease complex found in Africa.

Significant improvement of indexing procedures for sweet potato viruses awaits additional information on the etiology of the virus-like diseases and the elucidation of factors which influences the distribution of these viruses in sweet potato plants.

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Recommendations For Improvements in the International Exchange of Temperate Fruit Tree Germplasm

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The governments of many countries with significant fruit production regulate the importation of fruit tree plant material for propagation to prevent the entry of hazardous foreign pests. The pests of temperate fruit trees commonly listed in quarantine regulations include insects, nematodes, fungi, true bacteria, fastidious bacteria, mycoplasma-like organisms, viruses and pathogens of unknown etiology. In some cases, pests are not named, or general categories of pests, such as virus diseases, are used as justification for the regulation of temperate fruit tree material. Seed is usually restricted less than vegetatively propagated plant material, because seed has been reported to carry fewer hazardous pests than clonal material. Plant material imported as germplasm for scientific research may be handled differently than commercial shipments. In any case, each country regulates the importation of temperate fruit tree plant material in a different manner, because each country has different environmental conditions, specific commercial cultivars, an unique range of pests and often a different regulatory philosophy.

Regulatory officials may require certification, inspections, treatments, or specific pathogen tests as a condition of entry. A visual inspection at a port of entry would detect most arthropods and pathogens that are large enough to be seen under a light microscope or that cause obvious symptoms in the imported plant material at the time of importation. Treatments are most effective in eliminating larger pests which occur on the surface of plant material. However, in order to detect developing fungal or bacterial infections or hatching arthropods, regulatory officials may require that imported plant material must be grown in isolation for a time after entry and inspected regularly for pests. The plant pathogens that are smaller than bacteria cannot be seen under a conventional light microscope, they occur within plant parts, and do not consistently cause obvious symptoms in every genotype of a host under all environmental conditions. Consequently, specific tests to detect these small pathogens must be done in the exporting or importing country to prevent the introduction of these pathogens with imported plant material.

The accuracy of pathogen tests depends on the sensitivity and reliability of the testing procedures as well as the competence of the individuals performing the tests. However, in the case of temperate fruit tree pathogens, there is also a direct correlation between the extent of our knowledge of a particular pathogen and the speed and accuracy of the tests used to detect that pathogen. Viruses which have been isolated and more thoroughly characterized can be detected within days by sophisticated serological, nucleic acid or electron microscopic techniques. Bacteria can be detected by serological tests or culturing on specific media. Poorly characterized pathogens or pathogens of unknown etiology can be detected within weeks using grafting tests in the greenhouse if the pathogen consistently causes leaf symptoms in a particular indicator plant under specific controlled conditions. Tests for pathogens that can be detected only by fruit, bark or branch symptoms must be carried out by grafting on to sensitive indicator trees in the field. In general, detection tests that depend on the pathogen to produce a symptom are more time-consuming and more variable in accuracy than tests that actually detect some part of the pathogen.

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Regardless of whether inspections, treatments or pathogen tests are required during international exchange of temperate fruit tree germplasm, there are some general recommendations that can be made to facilitate such exchanges. These recommendations can be characterized as either improvements in the efficiency of present procedures for germplasm exchange or as projects for the development of techniques and procedures that would improve future germplasm exchanges.

Importers of temperate fruit tree germplasm should seriously consider the value to their programs of importing seed or pollen as opposed to importing clonally propagated materials, especially in the case of wild germplasm. Quarantine regulations on imported temperate fruit tree seed or pollen are usually not as severe as on clonally propagated materials, because none of the small pathogens of pome fruits and only a few viruses in Prunus are reported to be transmitted in seed; whereas all known pathogens of host trees can be carried in or on vegetatively propagated plant material. In many countries, seed or pollen of temperate fruit trees is allowed to enter immediately after inspection at a port of entry, but clonal material of the same species must be tested for exotic pathogens for years before entry. Even if Prunus seed or pollen is regulated because of seed-borne or pollen-borne viruses, the testing necessary to detect these viruses is easier and less time-consuming than testing clonal material for all exotic small pathogens that may be present. If specific crosses will eventually be made with imported germplasm, perhaps arrangements could be made with quarantine officials to grow imported clonal material at a quarantine facility and then, after appropriate tests for seed-borne viruses in the case of Prunus, use the pollen or ovaries produced by the clone for crosses to obtain seed for importation without actually importing the clonal material. In essence, scientists interested in foreign germplasm should consider whether their program would progress better by immediately evaluating and breeding seedlings for a particular character or by waiting years to obtain clonal material of precisely described genetic composition.

When clonal fruit tree germplasm must be imported, the history of a particular genotype will significantly affect the time and effort necessary to process it through a pathogen-testing program required as a condition of entry. Obviously, the plant material for export should be harvested in a dormant condition from a vigorous tree to maximize the probability of germplasm survival during shipment. The probability of infection can be reduced from that expected in nature if the trees which are sources for exported material are maintained in a greenhouse, screenhouse or in an isolated field plot, where pathogens which spread naturally in that area can be avoided. Germplasm previously budded on clonally propagated rootstocks or interstocks, or top-worked on established trees, should be avoided in favour of similar germplasm which has always been propagated on seedling rootstocks or on its own roots. Vegetatively propagated rootstocks, interstocks and trees usually contain all of the internal pathogens present in every piece of scionwood successfully propagated on them and their ancestors, and therefore accumulate pathogens over time. Propagation from seed consistently filters out all but a few viruses in Prunus and all small pathogens of pome fruits.

In every instance possible, germplasm which appears to be healthy, has a history of minimal exposure to infection or originates from a certification program, should be selected for international exchange, because this germplasm is more likely to test negative for pathogens and so be introduced into the importing country sooner than infected germplasm which must be tested, exposed to therapy treatments and retested for a few years to prevent the introduction of a hazardous pathogen with the germplasm.

Scientists who wish to import temperate fruit tree germplasm must be familiar with the quarantine regulations and procedures of the importing country and make the proper arrangements for the foreign plant material before importation. Before arranging importation, the importer should determine if the desired germplasm is available from an approved certification program in the country of origin or another country, because certification acceptable to the importing country will reduce or eliminate the quarantine period required by the importing country.

Importers that neglect their responsibility to obtain the necessary permits and clearances before shipment will usually encounter delays during shipment and therefore jeopardize the health and viability of their germplasm. Once the germplasm has been selected for shipment, it is critical that (1) the plant material is properly identified, (2) the germplasm is not mixed with other more severely regulated plant materials or soil, (3) acceptable packing material and packaging are used, and (4) proper documents including phytosanitary certificates are included with the germplasm. The discovery of unidentified plant material, hazardous plant or plant parts, soil, unacceptable packing or packaging materials, or incomplete or inaccurate documents leaves regulatory officals no choice but to prohibit entry of the entire shipment. Delays, lost shipments or damage to valuable germplasm may also result when small quantities of germplasm are inadvertently mixed in with commercial or manufacturing shipments instead of being imported by procedures specially devised by the quarantine service for germplasm.

In the future, germplasm exchanges would benefit from the development of a computerized information system containing a list of available germplasm in each country as well as the important known characteristics of each genotype. For this objective, more international cooperation and perhaps some coordination by international agencies will be necessary. Such a system could be used to identify the germplasm of potential value to a particular breeding program and improve the chances of selecting and utilizing desirable genetic material. In addition, importation of useless germplasm or of germplasm similar to previously imported germplasm could be minimized, and then available resources could be concentrated more on truly valuable genotypes. The most valuable germplasm could then be distributed among cooperating plant pathologists for pathogen testing and therapy so that the tested germplasm could meet the entry requirements of most countries. The development of such a system would also identify gaps in our knowledge of the important characteristics of certain germplasm and guide plant breeders or horticulturalists toward valuable areas of research.

Tissue culture and in vitro preservation will certainly play an increasingly important role in germplasm exchange. At the present time, very little temperate fruit tree germplasm is shipped as tissue culture, but in the future, this method of exchanging germplasm will be used more often. For quarantine programs, techniques may be developed for pathogen testing in tissue culture. Therapy procedures that now involve heat treatment and tip grafting may be more successful using meristem culture after heat treatment. After germplasm has tested negative for submicroscopic pathogens, maintenance in tissue culture will ensure that the germplasm does not become reinfected. Preservation and shipment of in vitro cultures is also less expensive and requires less labour than conventional methods using trees and budwood.

Future research to develop more rapid and reliable detection methods for submicroscopic pathogens could decrease the quarantine period for temperate fruit tree germplasm and therefore facilitate germplasm exchanges. At the present time, pathogen testing of uncertified clonal germplasm as a condition of entry requires years of observations on leaves, branches and fruit of budded indicator trees in the field, because these tests are the most reliable methods known to detect many of the poorly characterized pathogens. The use of recently developed biotechnology as well as new approaches developed in other sciences may reveal the identity of the unknown pathogens. Once the type of pathogen is known, detection techniques developed for similar pathogens can be adapted to solve the problem. However, progress with these pathogens may be slow and not always assured, so incentives may have to be offered for the best scientists to explore such problems. Importers of temperate fruit tree germplasm who are frustrated with present quarantine requirements can and should be an effective lobby to encourage an increased emphasis on research into more rapid and reliable detection techniques for these pathogens.

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Disease Indexing and the International Movement of Temperate Small Fruits and Grapevine Germplasm

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Small fruits (strawberry, raspberry and other Rubus plants) and grapevine are almost always propagated vegetatively. These crops, like other vegetatively propagated crops, are frequently infected with viruses and other graft-transmissible diseases (hereafter called virus diseases for convenience). Virus diseases are major causes of reduction in yield and fruit quality in all three crops discussed in this paper. A few viruses express symptoms in commercial cultivars and are even lethal to plants carrying them. Most viruses are latent in commercial cultivars and occasionally produce obscure symptoms in their natural hosts. Thus, plants become symptomless carriers of viruses from one generation to the next, spreading viruses with infected symptomless propagation material. Combinations of two or more viruses in infected plants are very common in small fruits and grapevine and usually result in a drastic reduction in yield and vigour. However, even in such cases it would be impossible to determine by inspecting them what causal agent/s are present. Many small fruit and grapevine viruses are restricted to one genus and are not transmitted naturally to other plant genera. Therefore, many viruses have not yet been purified and characterized. The literature on virus diseases of small fruits and grapevine has been reviewed (Bovey et al., 1980; Converse, 1966; Converse, 1987; Frazier, 1970, Goheen, 1977; Maas, 1984; Martelli, 1986). This paper will focus on the current situation in indexing of virus diseases of small fruits and grapevine and implications to the international transfer of germplasm of these crops.

Virus detection methods

Detection and identification of diseases where the disease agent is unknown or is not inciting obvious symptoms is still a difficult task. Specific indexing procedures are based on bioassays, serological methods (where antisera are available) and on biochemical methods in which virus-related nucleic acids are detected in the infected tissue.

Transmission to indicators

Graft transmission to sensitive indicators and sap inoculation onto herbaceous test plants are standard bioassay procedures to determine the presence of latent viruses.

Most viruses of small fruits and grapevine are still diagnosed by grafting leaves or buds from infected plants on to sensitive indicators which will develop symptoms characteristic of the virus. Suitable indicators recommended for diagnosing viruses of these crops are selected after considerable efforts over many years (Converse, 1979; Frazier, 1974). Graft analysis is the only method employed at present for diagnosis of the very important small fruit and grapevine viruses - aphid-borne in strawberry and Rubus and viruses with unknown vectors in grapevine. Grafting procedures are laborious and expensive, require greenhouse space for growing indicators in good physiological condition and maintaining them in moderate temperatures and light intensity over the long observation time for possible symptom appearance.

Sap transmission is achieved with several viruses of small fruits and grapevine, especially with nematode-borne (nepoviruses) and pollen-borne viruses. Infected plants are usually good sap-inoculation sources when in the early stages of infection and with the onset of new growth after breaking dormancy. Chronically infected plants are poor virus sources for sap inoculation.

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Serological methods

Recent advances in virus purification procedures and production of antisera have lead to the development of various serological tests. The adaptation of enzyme-linked immunosorbent assay (ELISA) for plant virus detection (Clark and Adams, 1977) and the introduction of immunosorbent electron microscopy (ISEM) methodology (Milne and Luisoni, 1977) into this field were breakthroughs in the rapid diagnosis of plant viruses for which antisera are available.

ELISA and ISEM are being used increasingly for detection of nepoviruses, pollen-borne viruses and tobacco necrosis virus in infected small fruit and grapevine plants (Russo et al., 1980; Spiegel and Cohen, 1985; Jones and Mitchell, 1986). Many of these viruses occur naturally in a wide range of crops and weeds. Serological tests are usually performed with antisera prepared against these viruses in hosts other than small fruits and grapevine. Reliable detection by ELISA is achieved with the isolates from small fruits and grapevine following sap transmission to herbaceous indicator plants and with some viruses directly in the infected tissue. Nepoviruses which infect grapevines comprise a large and most important group in this crop. The contribution of serological detection assays to rapid diagnosis of grapevine viruses in single and mixed infections is a major one. Recently, progress has been made with serological detection assays of mycoplasma-like organisms (MLOs) in plants (Clark et al., 1983; Weivaert and Samyn, 1985). However, further developmental work is needed before the applicability of this method can be evaluated. Problems like non-specific reactions, uneven distribution of some pathogens in host tissue, strain specificity and variability between batches of antisera should yet be overcome. Stace-Smith (1984) recommended that ELISA indexing be used in conjunction with other diagnostic procedures, such as sap transmission, to check for possible unusual isolates.

The advantage of ISEM is its sensitivity and lack of strain specificity (Van Regenmortel et al., 1980). This method requires minute amounts of antiserum and plant tissue and is very useful when diagnosing viruses in mixed infections - a very common case with small fruit and grapevine viruses. ISEM can be done easily on a small scale and is therefore a helpful tool in the preparation of virus-tested plants.

Monoclonal antibodies are a recent addition to serological detection of plant viruses. Monoclonal antibodies are not yet available for most small fruit and grapevine viruses, except for raspberry bushy dwarf virus (RBDV) (Martin, 1984). It is expected that in the future this technique will provide a very effective assay for diagnosing viruses and distinguishing between virus strains.

Double-stranded RNA analysis

The isolation of disease-specific dsRNA from virus-infected tissue offers a valuable diagnostic tool for detection of uncharacterized viruses. Because of the general difficulty in purifying viruses from these hosts, the potential of this method is obvious. Attempts to use dsRNA analysis for small fruits and grapevine have been made in a few laboratories. Modifications of existing procedures were required as the high content of polysaccharides and phenolic compounds interferes with extraction of nucleic acids.

Virus-related specific dsRNA bands have been detected in strawberry plants infected with strawberry mild yellow-edge virus (SMYEV) (Spiegel, 1987), in raspberry infected with RBDV (Jones et al., 1986: Kurppa and Martin, 1986; Murant et al., 1986), cucumber mosaic virus, (CMV), black raspberry necrosis virus (BRNV) (Jones et al., 1986), raspberry leafspot virus (RLSV) and tomato ringspot virus (TomRSV) (Kurppa and Martin, 1986) and in grapevine affected by leafroll disease (Cameron and Walter, 1985; Mossop et al., 1985).

The presence of high molecular weight dsRNA bands of unknown origin in virus-tested strawberry (Spiegel, 1987) and Rubus plants (Kurppa and Martin, 1986; Murant et al. 1986) as well as in other plants (Wakarchuk and Hamilton, 1985) calls for the use of appropriate controls in each test. The low concentration of viruses and/or their unavailability to the extraction medium requires large samples of plant tissue per test and retesting of negatives before being taken as healthy. Double-stranded RNA analysis is still in its early stages for small fruits and grapevine. In my opinion, increasing the sensitivity of this method will allow including it reliably in virus-indexing procedures, at least for quick elimination of infected plant material. Purified dsRNA has the potential, using present technology, to be the starting point for making complementary DNA (cDNA) probes for uncharacterized viruses. Further development is needed to make this technique applicable to these viruses.

Attempts to apply hybridization to disease specific probes are being made for several strawberry viruses (Morris, 1985; Stenger et al., 1986) and for a virus associated with leafroll disease in grapes (Tanne, unpublished). However, further work is needed to overcome technical problems and make this method applicable.

The suitability and reliability of any chosen detection method need to be evaluated for each virus in each laboratory. Differences in climatic conditions, sampling time, physiological status of indicator plants, etc. can influence detection, especially when dealing with viruses present in the host tissue in very low concentration.

Elimination of viruses

Elimination of many small fruit and grapevine viruses can be achieved by heat therapy of by tissue-culture methods of tip meristems. Some viruses and MLOs are heat stable and resist heat therapy. In several cases, plants resulting from tip meristems were not free of viruses (Mori, 1977). Therefore, the combination of obtaining tip meristems from heat-treated plants, followed by growing meristems to plantlets in tissue culture, is a 'safer' route to follow in developing clones free of viruses. Plants fail sometimes to produce new growth and die within a few weeks when grown at 38°C with ambient carbon dioxide. Artificial elevation of the carbon dioxide level during thermotherapy was found to increase markedly the ability of grapevine to withstand prolonged high temperature (Kriedeman et al., 1976). Recently, Converse and George (1987) reported on the elimination of MLOs in highbush blueberry with high carbon dioxide thermotherapy. An alternative to heat therapy of infected parent plants is the incubation of infected plant material in the in vitro stage at the desired high temperature. Many test tubes with infected plant material can be heat treated in a simple growth chamber, and even when the survival rate is low, chances are better for survival of several healthy clones. This method was employed for several viruses (Walkey, 1980; Snir and Stein, 1985) and could most likely be useful for other virus-plant combinations.

Following the tissue-culture step, plants are allowed to develop and when ready are potted and grown in insect proof screenhouse. At this stage, plants are indexed for diseases and evaluated for trueness to horticultural type. Plants found to be free of viruses serve as a source of virus-free propagation material and for collections of germplasm.

Maintaining and exchanging germplasm

International exchange of small fruits and grapevine plant material involves a large risk of spreading a great number of viruses. Quarantine regulations vary among countries but most require that a certificate of health accompany imported plants. Sometimes, quarantine regulations prevent importation of rooted material. In many cases a health certificate may simply declare that the material has been inspected in the country of origin and has no evidence of infestation by pest and disease (Posnette, 1977; Bovey and Pelet, 1977).

With most virus diseases of small fruits and grapevine this certificate is of very little value. Plants must be carefully indexed before being released to the importer. With the techniques available presently this is a long process (Converse, 1979) during which the virologist usually becomes the enemy of the importer, especially if the new plant introduction is virus-infected.

The advantage of meristem or bud in vitro cultures and cold storage for germplasm conservation and exchange is very obvious, as long as no assumptions of freedom from viruses are being made. Methods for in vitro maintenance and propagation of strawberry (Mullin and Schlegel, 1976); Rubus (Donnelly et al., 1980; Pyot and Converse, 1981) and grapevine (Barlass et al., 1982) have been established. However, the possibility of genetic instability and the lack of rapid virus-indexing methods are still drawbacks which discourage virologists and horticulturalists from keeping small fruit and grapevine germplasm in tissue culture. This situation may change when more studies examining the variability of a large number of different cultivars resulting from tissue culture will be performed and rapid virus-indexing procedures will be developed.

Future objectives

Establishing rapid disease-indexing procedures ia a critical need in today's small fruit and grapevine virology. Applying these procedures to in vitro cultures will contribute to the efficient maintenance and transfer of virus-free germplasm. Two independent approaches should be encouraged:

1. Improving virus-purification procedures to the point where viruses will be purified at least in minute quantities, and antisera (most likely monoclonal antibodies) will be prepared.

2. Developing nonserological biochemical detection procedures, such as for dsRNA, which are independent of successful virus purification. Attempts to make cDNA probes from dsRNA should also be made.

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Collection and Propagation of Citrus Germplasm for International Distribution

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Germplasm from polyembryonic varieties of citrus can be safely transferred by seed, although nucellar seedlings have undesirable juvenile characters. Monoembryonic varieties do not reproduce true-to-type plants when propagated by seed. As a consequence, vegetative material is the most appropriate for international exchange of citrus germplasm.

Tissue culture techniques specially developed for exchange of citrus vegetative material could solve the problems of transfer of diseases (Navarro et al., 1984). These include the following.

Budwood collection

Vigorous pencil-size budwood is collected, washed with detergent and water and packaged in transparent polyethylene bags. Budwood should be kept refrigerated until shipped. If refrigeration is not possible, collection should be done during the dormant period (winter in subtropical areas and the dry season in tropical areas).

Budwood reception

Upon arrival at the reception country, the budwood is inspected without opening the bags. The parcel should be destroyed if it is heavily contaminated or infested with a living pest. Otherwise, budwood is disinfected by immersion for 20 min in a 2% sodium hypochlorite solution containing 0.1% Tween 20 wetting agent, and then rinsed three times with sterile distilled water.

Budwood culture in vitro

Budsticks about 15 cm long are individually cultured in 38 x 200 mm culture tubes containing the plant cell culture salt solution of Murashige and Skoog (1962) solidified with 1% Bacto agar. The cultures are kept in a growth chamber at 32°C, exposed 16 h daily to 10,000 lux illumination. 10-15 days later the buds of the budstick produce vegetative flushes.

Shoot-tip grafting in vitro

Flushes produced in vitro are excised from the budsticks under aseptic conditions, disinfected by immersion for 5 min in a sodium hypochlorite solution containing 0.1% Tween 20 wetting agent and rinsed three times with sterile distilled water. Then shoot tips containing the apical meristem and three leaf primordia, measuring 0.1-0.2 mm, are excised. Shoot tips are micrografted onto young rootstock seedlings growing in vitro, according to the standard method of Navarro et al. (1975).

Transplanting to soil and indexing

Plants recovered by shoot-tip grafting in vitro are transplanted to greenhouses where they are grown for observation and indexing by biological or laboratory methods. Healthy plants may then be established in the field and maintained in insect-proof screenhouses to avoid reinfection.

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By this method the only material actually introduced into the country is a very small shoot tip that is free of pest and diseases, even if the original trees were affected.

This method seems to be very cumbersome, but in practice it is very simple. Only a regular tissue culture laboratory and a good but small greenhouse are needed. In addition, an operator is obviously necessary with good manual dexterity. In Spain the method has been successfully used to introduce over 40 citrus species.

The method is very well developed for Citrus species, but it has only been applied to the citrus relatives Poncirus sp. and Fortunella sp. We are investigating its application to other citrus relatives, where the main problem to be solved is shoot-tip grafting in vitro. An alternative method may be in vitro rooting of shoots produced by the budsticks. Additional research should be done to establish adequate in vitro methods for citrus relatives.

Once healthy plants are established in insect-proof screenhouses, it could be very safe to move budwood from these plants to other citrus areas.

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Detection of Citrus Diseases and Prevention of Their Spread During International Exchange of Citrus Germplasm

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Citrus is attacked by many pests and diseases, some of which are extremely damaging. International movement of scion varieties of citrus is commonly done by shipping budwood which may be contaminated or infected with various pathogens. In some instances, intact plants are moved with a correspondingly greater hazard of transferring unwanted pests and/or pathogens.

Some citrus diseases are now common in nearly every major citrus-growing region and often it is too late to restrict their movement. Other pests and diseases, however, are localized in specific areas. All major citrus areas are threatened by one or more diseases exotic to them. Accidental introductions of tristeza, greening, stubborn and citrus canker into new areas have all caused serious losses. A noteworthy example is the recent destruction of profitable citrus industries in several southeastern Asian countries by greening disease.

Many citrus-growing areas and countries have stringent quarantine regulations governing movement of citrus germplasm. In the United States, for example, both federal and state quarantines are in effect. Movement of citrus germplasm into Florida and California is allowed for commercial and research purposes, but only under the strictest guidelines.

Accidental introduction of cryptic pathogens (virus and virus-like agents) is more difficult to prevent than introduction of fungal and bacterial diseases which produce visible symptoms and can be eliminated by appropriate sanitation procedures. The detection of cryptic citrus pathogens and the means to avoid their dissemination is emphasized here.

A large number of virus and virus-like pathogens of citrus have been described (Table 1). These include known viruses, presumed viruses, viroids and several procaryote-induced diseases, such as stubborn and greening. Some of the diseases affect specific grafted combinations. Virus-induced bud-union disorders may not be expressed in young trees.

In addition, several serious decline diseases of undetermined etiology have been described. The most significant one is blight (also called declinio in Brazil).

Historically, these virus and virus-like pathogens have been identified by graft inoculation to specific citrus cultivars which are reactive to one or more of the pathogens listed. This approach is frequently labour intensive, time consuming and not highly accurate. Other methods have been sought to improve detection. Some citrus viruses can be detected by mechanical inoculation to herbaceous hosts. Antisera, including some monoclonals, are now available for tristeza, satsuma dwarf (and related diseases), citrus variegation, citrus leaf rugose, stubborn and tatterleaf.

Various test procedures can be used, but labelled-antibody procedures such as ELISA are usually the serological method of choice. Serology can be combined with light microscopy by using fluorescent-labelled antibodies, and with electron microscopy (immunospecific EM). Proper selection of tissue for assay is highly important for obtaining reliable results by serological testing.

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Nucleic and hybridization probes have been used experimentally to detect tristeza virus and several citrus viroids in plant extracts, but conditions for routine application have not been defined. Viroids have also been detected by polyacrylamide gel electrophoresis. Analysis of dsRNA patterns has also been used for citrus virus detection. Strain differentiation of citrus tristeza virus based on subgenomic RNA species has been proposed.

Electron microscopy has been used for routine diagnosis of tristeza infection. Immunospecific EM is a sensitive modification for viruses to which antisera have been prepared. Examination of sectioned tissues by EM has been used to confirm diagnosis of greening disease based on the unique wall structure of the causal agent. EM procedures have also been used for stubborn, but since Spiroplasma citri is easily cultured and has a very characteristic helical structure, diagnosis can be done more easily by phase or dark-field microscopy of liquid cultures.

Light microscopy of sectioned and stained fresh tissue has been used to observe the large inclusions formed by tristeza virus and provides a very simple and rapid assay.

There are a number of major problems in detection of cryptic pathogens of citrus. No internationally accepted guidelines are available for known citrus pathogens, and appropriate conditions and controls still need to be established for most techniques. There is evidence for the presence of pathogens which are not readily detectable by current procedures, and procedures are needed for these latent infections. There are major decline problems of unknown etiology for which no indexing tests are available. Some existing protocols are not practical at many locations because of time and/or technical requirements.

Despite the obvious problems in detecting citrus pathogens, most disease hazards associated with the movement of citrus germplasm can be eliminated or greatly reduced by a combination of relatively simple procedures. Shoot tip grafting plus moderate thermotherapy can eliminate all know cryptic pathogens. Rapid monitoring procedures are available for some of the agents most difficult to eliminate and screening for selected pathogens should predict total health. It may well be more effective to emphasize improved therapy procedures combined with selective indexing than to rely only on indexing and to continually expand and modify detection procedures. Use of thermotherapy and/or chemotherapy in combination with shoot-tip grafting should improve elimination rates and facilitate the procedure.

Careful initial selection, appropriate decontamination procedures and care in isolation of imported materials until their disease status is known are the other important components in safe germplasm exchange.

The pathogen content of citrus relatives is not well documented. A number of citrus pathogens do infect closely related citrus relatives such as Poncirus and Fortunella under cultivation, but the status of wild plants of these genera is unknown. Several citrus viruses including tristeza can infect one or more citrus relatives in a number of genera including Aegle, Aeglopsis, Afraegle, Citropsis, Eremocitrus, Microcitrus and Severinia under experimental conditions. This suggests that a number of species in the subfamily Aurantioideae of the Rutaceae may have some potential as hosts of citrus viruses.

Citrus relatives which are graft compatible to citrus can be indexed by all standard procedures for citrus virus and virus like pathogens. Those which are not graft compatible can only be indexed by those procedures which do not require graft inoculation to citrus indicators.

Table I. Detection and elimination of virus and virus-like pathogens of citrus

Abbreviations: MI/HH = mechanical inoculation to herbaceous hosts, ELISA = enzyme-linked immunosorbent assay. PAGE = polyacrylamide gel electrophoresis, EM = electron microscopy, FM = fluorescent microscopy, NAH = nucleic acid hybridization assay, STG = shoot tip grafting, NI = no information. The asterisk signifies an economically important disease.

Disease	Causal 1/ agent	Indexing 2/			
		Citrus	Alternatives	Potential	STG
Algerian navel	Virus?	None	MI/HH	EM	NI
Brittle twig yellows	?	Sweet orange	-	-	NI
*Cachexia	Viroid	Parson's Sp. mand.	-	Page, NAH	Yes
Citrus leaf rugose	Virus	Lemon	NI/HH, ELISA	NAH	NI
*Citrus variegation	Virus	Lemon	NI/HH, ELISA	NAH	Yes
Citrus yellow mottle	Virus?	Sweet orange	-	EM	NI
*Concave gum	Virus?	Sweet orange	-	?	Yes 3/
*Cristacortis	Virus?	Orl. tangerine	-	?	Yes
*Exocortis 4/	Viroid	Etrog citron	PAGE, NAH		Yes
Fatal yellows	?	Alemow	-	-	NI
Fovea	Viroid?	Murcott	-	?	NI
*Greening	Procaryote	Mandarin	EM	ELISA, FM	Yes
Gummy bark	?	Sweet orange	-	?	NI
Gum pocket	Viroid?	Trifoliate orange	-	?	NI
*Impietratura	Virus?	Sweet orange	-	?	Yes
Leaf curl	Virus?	Sweet orange	-	-	NI
Leathery leaf	Virus	Sweet orange	MI/HH	ELISA	NI
*Leprosis	Virus?	Sweet orange	EM	?	NI
Multiple sprouting	?	Sweet orange	-	-	NI
Nagami kumquat dis.	Virus?	Kumquat	-	?	NI
*Psorosis	Virus?	Sweet orange	-	?	Yes 3/
*Ringspot	Virus	Grapefruit	MI/HH	ELISA	Yes
Rubbery wood	?	Mexican lime	-	?	NI
*Satsuma dwarf 5/	Virus	Satsuma	MI/HH, ELISA	NAH	NI
*Stubborn	Spiroplasma	Sweet orange	Culturing, FM	ELISA, NAH	Yes
Tatterleaf	Virus	Rusk citrange	MI/HH	ELISA	Yes 3/
*Tristeza	Virus	Mex. lime	ELISA, SSEM	NAH	Yes
Yellow vein	Virus?	Mex. lime	-	-	Yes

1/ Virus? indicates causal agent is probably a virus; Viroid? indicates that the causal agent is probably a viroid; and ? indicates that there is insufficient information to speculate on the probable identity.
2/ Only the primary citrus indicator plant is listed. Other indicators can also be used for many of the diseases listed.
3/ Difficult without partial heat therapy.
4/ Includes other citron viroids recently described.
5/ Includes citrus mosaic, navel orange infectious mottling and Natsudaidai dwarf viruses.

The highest risk to commercial citrus from importation of noncompatible citrus relatives in vegetative form would be if they contained diseases which are vectored and which are detectable only by graft inoculation to citrus. The major virus like disease in this category is greening. Noncitrus hosts in the Rutaceae for greening have been suspected, but are unproven. If plants of genera which are good hosts of the citrus psyllid vectors of greening (Clausena, Vepris, Murraya, Fagara and Microcitrus) are obtained from areas where greening is endemic they may pose some threat. This is difficult to quantify with present information.

Therapy procedures such as thermotherapy and shoot-tip grafting have not been evaluated for citrus relatives, except perhaps for Fortunella or Poncirus. Data obtained from citrus should be applicable to those relatives that are fully graft compatible with citrus.

The Status of Disease Indexing and the International Distribution of Banana Germplasm

James L. Dale 1/

Introduction

Bananas are monocots in the family Musaceae and are grown as food crops in practically all tropical and sub-tropical countries. The two major types, dessert bananas (Musa acuminata or M. acuminata x M. balbisiana) and cooking bananas or plantains (M. acuminata x M. balbisiana) together constitute the world's most important fruit crop. In some countries, the banana is the primary source of carbohydrate while in others it is the major export earner. In 1980, over 60 million tonnes were produced, of which about 10% were exported (virtually all bananas exported are the dessert type). Even though the banana originated in the southeast Asian region, the major producers are outside this region; Central and South America produce about 42% of the crop but account for a massive 85% of the export trade. The only large exporter outside the Americas is the Philippines. The African continent produces about 30%, Asia 26% and Oceania only 2%.

Bananas are propagated vegetatively not only to retain cultivar characteristics but also because the commercial and other useful cultivars do not produce viable seed. Grafting is not successful and so propagation has been traditionally based on the planting of 'bits' (pieces of corm) or suckers. The major problems with this method are that vascular pathogens are easily transmitted in such material and often considerable amounts of soil adhere to the planting material, a mechanism of disseminating soil-borne pathogens. Recently, there has been a worldwide trend towards the use of tissue cultured plants. This approach looks promising but there are a number of question marks including the percentage of 'off-types' and possible disease transmission which shall be discussed later.

Attempts to breed new cultivars of bananas, particularly with improved disease resistance, have been singularly unsuccessful. To date, no new cultivars have been released from any of the programs. The current approaches to generating new cultivars include programs based on traditional breeding techniques, somaclonal mutation and mutation induced chemically or by irradiation.

The disease problems

The important diseases in bananas are Panama, black Sigatoka, moko, bunchy top and burrowing nematode. While most of these diseases are present in most of the producing regions, there are some significant exceptions (Table 1). The soil-borne disease Panama, caused by Fusarium oxysporum f.sp. cubense, has two important races, 1 and 4. Race 1 is widespread in virtually all banana-growing regions but does not cause wilt in Cavendish cultivars, which have generally replaced Gros Michel as the banana grown for world commerce. Race 4, on the other hand, causes Panama disease in Cavendish and is a major threat to production worldwide. Fortunately, race 4 is confined to Australia, the Philippines, southern Africa, Taiwan, China and the Canary Islands. As the disease is soil-borne, further spread of race 4 can be avoided by strict quarantine control of planting material.

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Table I. Distribution of important banana diseases

	Africa	America	Asia	Oceania
Panama race 1	+	+	+	+
race 4	+		+	+
Black Sigatoka	+	+	+	+
Moko		+	+	
Bunchy top	+		+	+
Burrowing nematode				

Moko, caused by Pseudomonas solanacearum, is another serious soil-borne disease which was initially restricted to the American tropics but which has now spread to Asia. Black sigatoka, however, is present in all producing regions but not all countries within those regions. It is caused by the airborne fungus Mycosphaerella fijiensis var. difformis and for this reason it is likely to continue to spread, although at a slower rate, if quarantine control measures are strictly adhered to. The nematode Radopholus similis appears to be present in all-banana producing countries for which there are adequate records. The nematode is soil-borne and should not pose an international quarantine problem.

Avoiding the non-viral diseases

There are a number of diseases of bananas which have limited distribution and are of sufficient severity or potential severity that their introduction into an uninfected region or country could prove disastrous. It is, therefore, essential that germplasm moved internationally must be disease free. This poses some particular problems in the case of bananas. Most vegetatively propagated crops can be moved with some degree of safety by means of budwood or cuttings thus avoiding possible soil contamination. Neither option is available for bananas. Movement of bits or suckers should be avoided because of soil contamination and the possibility of incipient infections. The only viable alternative is to put all germplasm for movement into tissue culture. This overcomes nearly all the problems associated with the non-viral pathogens. Freedom from these pathogens can be verified as follows:

(a) Mycosphaerella fijiensis var. difformis - infected material should be removed when preparing the initial explant; if, in the unlikely event, the pathogen is not excluded, symptoms of the disease should become evident.

(b) Fusarium oxysporum f.sp. cubense - any contaminated soil is removed while preparing the explant and further presence of the pathogen can be detected by destructive sampling and plating out onto culture media.

(c) Pseudomonas solanacearum - this pathogen will grow out of infected explants into the tissue culture medium and therefore becomes self-evident.

(d) Radopholus similis - infested tissue should be removed during preparation of the explant; it is very likely that if nematodes were introduced into the tissue culture with the explant, those present would become evident from the growth of associated saprophytic organisms.

The movement of banana germplasm as tissue cultures together with visual inspection should ensure such material is free of non-viral pathogens. This is not so for the virus diseases of bananas.

The virus diseases

There are a number of virus or virus-like diseases recorded in bananas. Most of these have been insufficiently described to determine whether they are actually transmissible diseases. Five, however, are transmissible diseases and do cause significant loss. The best known and most important is banana bunchy top virus (BBTV). This virus devastated the Australian banana industry early this century and is at the present time causing considerable concern in the producing regions of Africa, Asia and Oceania (Table 2). Within these regions, however, the virus is not present in all countries. Fortunately, it has not spread to Central or South America. The virus has yet to be purified but from all information available, including aphid transmission, it is classified as a probable member of the luteovirus group. Identification and detection is currently based on symptoms and transmission as no antisera or probes are available. This is highly unsatisfactory.

Table 2. Banana virus distribution and importance

	Importance	Distribution
Banana bunchy top	++++	Africa, Asia, Oceania
Infections chlorosis	++	Probably worldwide
Banana streak	+	Morocco
Abaca bunchy top	+	Philippines
Abaca mosaic	+	Philippines

Infectious chlorosis, caused by cucumber mosaic virus (CMV), appears to be present in all banana-producing countries and is particularly common where other crops such as cucurbits are grown between the bananas. The virus can be easily detected using any of the sensitive techniques available for CMV. Banana streak virus has only been recently described and has only been recorded in Morocco. The other two virus diseases have been recorded only in the Philippines and only on Musa textalis or abaca, a Musa species grown as a fibre crop. Abaca mosaic is caused by sugarcane mosaic virus and abaca bunchy top appears to be related to banana bunchy top. Neither are considered to be a threat to banana production.

Avoiding the viral diseases

The adoption of tissue culturing as a mechanism of ridding bananas of their pathogens and thus allowing easier geographic distribution of planting material has been very successful with regard to fungi, bacteria and nematodes but not to viruses. The explant in bananas is usually a relatively large meristem tip and it is extremely unlikely that any virus would be eliminated from this tissue. Experiments currently underway in our department have shown that explants from BBTV-infected plants can be tissue cultured without difficulty. The more worrying result is that while plantlets established 9 months after the initial tissue culturing all showed BBTV symptoms, only 66% of plantlets established after 14 months showed symptoms. We are currently trying to determine whether the remaining symptomless plantlets are BBTV infected or not. If this is symptomless infection or delayed symptoms expression, then considerably more care must be taken in moving banana germplasm in tissue culture.

Collection -- with banana pathologist

Tissue culture in - country of origin
- non-banana-growing country
- non-banana-growing area

Indexing in - exporting country
- importing country
- developing country

Field planting - visual inspection

Maintain in
tissue culture

Fig 1. Strategy for collection and maintenance of Musa spp.

Essentially, highly sensitive and accurate detection techniques are required that will ensure freedom from virus diseases. These are already available for CMV and could be developed for banana streak virus relatively quickly. However, for the most important virus, BBTV, these are not available. This must, therefore, be the research priority in relation to international movement of banana germplasm. This research is not being neglected. We are currently working towards a recombinant DNA probe (funded by IBPGR) and three other groups are following lines of research directed towards producing an antiserum.

While progress is being made in developing detection techniques for the known virus diseases, perhaps the greatest danger is from the unknown ones. The detection methods mentioned here are highly specific, and have no ability to detect unrelated or undescribed viruses. As mentioned previously, the centre of diversity of the banana is in the southeast Asian region. The study of plant viruses in this region is confined to the problems in the major food crops and there is no time available for the more academic pursuit of investigating viruses of primitive or wild relatives of food crops. It is very possible, though, that these plants will harbour viruses hitherto unknown. It is these viruses that may pose the greatest threat and any strategy for collecting such wild material should be designed to minimize the risk of introducing 'new' viruses into cultivated banana crops. Because these viruses are undescribed, the detection techniques employed must be non-specific. The most non-specific of all is visual inspection by a pathologist with field experience of banana diseases. Other non-specific virus techniques that could be used include dsRNA analysis, viral DNA extractions and general viroid extractions.

A possible strategy for the collection and international distribution of banana germplasm

By necessity, a strategy for collecting and distributing banana germplasm must be based on tissue culturing the material (Fig. 1). Where possible, indexing procedures should be implemented to ensure pathogen freedom using specific detection techniques. Where such techniques are not available, every effort should be made, using non-specific techniques, to exclude unknown pathogens.

The Current Status of In Vitro Culture for the International Movement of Plant Germplasm

Lyndsey A. Withers [1/]

INTRODUCTION

In vitro methods can be used to move plant germplasm at various stages in the conservation/utilization process. Material can be collected at source using simplified culture inoculation procedures appropriate for field use. Genebank accessions can be exchanged with other collections or distributed to users in the form of cultures. In the latter two cases, the material may be introduced into culture either as the storage mode or specially for exchange.

There are two main considerations that determine the success of in vitro exchange, namely practical points relating to the culture and transportation processes, and phytopathological points relating to the disease status of the cultured material and quarantine requirements. It should be stated from the outset that the author believes that in vitro movement of plant germplasm has enormous advantages but that it could carry serious disadvantages through engendering a false sense of security in the apparent cleanliness of cultures. IBPGR's examination of this topic is timely as there is an increasing interest in the use of in vitro movement, particularly by scientists who may be unfamiliar with quarantine regulations or even unfamiliar with the plant pathology of the material with which they are working.

PRACTICAL ASPECTS

In vitro collecting

The possibility of using collecting techniques based upon in vitro methodology has been explored in recent years for both vegetative material and seeds, particularly recalcitrant seeds. A range of techniques with different levels of sophistication have been developed:

1. The use of a fully equipped local laboratory. Material is initially collected conventionally but transferred as soon as possible to culture.

2. The use of an improvized laboratory in a nearby building. Simple laboratory facilities are provided but these do not include a laminar air-flow cabinet, for example.

3. The establishment of facilities in the field that attempt to achieve the high level of cleanliness provided in the laboratory. Inoculations are carried out in a portable glove box.

4. The use of an outdoor working area. Inoculations are carried out on a clean table and under the protection of a simple box cover. Explants are either inoculated into culture in the field or held in a sterile salt solution for later inoculation in the laboratory.

5. The use of minimal facilities. Explants are sterilized using non-toxic agents and are inoculated into culture without further aseptic precautions. The culture medium is supplemented with fungicides and antibiotics.

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Further details of these techniques can be found in the literature (Assy Bah et al., 1987; Sossou et al., 1987; Withers, 1987). The most successful attempt to implement simple collecting techniques has been the use of an outdoor working area (5, above) to collect zygotic embryos of coconut (Assy Bah et al., 1987). This work is supported by IBPGR and will, hopefully, interface with in vitro methods for clonally propagating and conserving coconut germplasm.

The potential for using in vitro collecting techniques for the germplasm of problem crops is vast. However, there may be important consequences in relation to quarantine. More material is likely to enter into circulation from truly wild sources. This material may have greater 'phytosanitary credibility' being in vitro but is more likely to carry pests and pathogens than material that has passed through rigorous sterilization and inoculation procedures.

In vitro exchange

This operation has been carried out successfully for several years by some of the International Agricultural Research Centers (IARCs) and other organizations. Details are given below of IARC activities. In addition to this systematic, routine use of in vitro culture for germplasm exchange, there has been a growing casual use of the technique in the scientific community for the exchange of planting and research material. The IBPGR In Vitro Conservation Databases (Withers and Wheelans, 1986) compiled from a survey carried out between 1980 and 1985 show a steady increase in reports of the distribution of cultures both nationally and internationally. In a later section, an analysis is presented of entries in relation to crops, participating countries, transport methods, success rates and problems.

IARCs

International Potato Center (CIP): The distribution of potato germplasm in the form of in vitro cultures is a well established, routine procedure (Anon, 1980; CIP, 1986; Roca et al., 1979; Schilde Rentschler and Schmiedichc, 1984). Shoot cultures are inoculated into small tubes containing semi-solid medium. They are cultured for 2-3 weeks to induce rooting and to reveal any microbial contamination. For transit, the tubes are packed into polystyrene boxes and despatched by air mail, air courier, or hand carried along with the necessary documentation. Typical transit times are 10-12 days and recovery levels after receipt range from 10-93%. Inoculation into a culture passage before potting appears to enhance survival. Training of recipient personnel is essential to the successful transfer of material in vitro. In 1984, CIP exported 2500 culture tubes to more than 30 countries world wide (Tovar et al., 1985). In the following year, 88% of native material certified pathogen free was distributed as cultures rather than field grown tubers (Huaman, 1986). The possibility of using in vitro-produced tubers for distribution has been explored; in 1985, tubers were sent to 9 National Programmes to test the feasibility of the method (CIP, 1986). Over 50 potato genotypes have been induced to form in vitro tubers (Espinoza et al., 1984).

Tubers are attractive for distribution as they are more robust than plantlets. Whereas the latter are relatively easily disturbed by careless handling and will die if kept in the dark for more than 3 weeks, tubers will withstand more physical disturbance and will survive at least 4 months of storage in the dark (Tovar et al., 1985).

Table 1. Attempts at germplasm exchange *in vitro* recorded in the IBPGR *In Vitro* Conservation Databases by crop, year of survey and culture system

C = cell/callus; P = meristem/shoot/plantlet; E = embryo/embryoid; T = *in vitro* tuber; ? = unspecified.

Crop	Phase 1 1980/81	Phase 2 1983	Phase 3 1985
Acer pseudoplatanus	?		C
Actinidia spp.		P ?	P
Adiantum capillus-veneris			P
Agave spp.			P
Allium spp.			P
Alnus spp.		P	P
Aistonia junnanensis			C
Alstroemeria spp.			C
Ananas comosus	P ?	P	P
Anthurium andraeanum			P
Apium graveolens			C
Araucaria heterophylla			P
Asparagus officinalis			C ?
Atropa belladonna		?	
Azalea spp.			P
Bambusa glaucescens			?
Beta vulgaris		C	C P
Brassica napus			C
Bupleurum falcatum			C
Caladium sp.	?		P
Camellia sinensis		?	
Carica papaya	P	P E	
Castanea crenata			P
Casuarina spp.			?
Catharanthus roseus			C
Ceratonia siliqua	C		
Chrysanthemum cinerariaefolium	?		
Cicer arietinum	?		
Cinchona spp.		C P	C P
Cirtomium falcatum			P
Citrus spp.		C P	C P
Clerodendron trichotomum			C
Coffea spp.	P	?	C P
Colocasia esculenta	C	P	P
Coptis japonica		C	
Cotinus obovatus		?	
Cucumis spp.	?		?
Cucurbita pepo			C
Curcuma longa			?
Cynara scolymus			P
Cyphomandra betacea		P	
Dahlia sp.			?
Dalbergia latifolia			?
Daucus spp.			C ?
Dianthus caryophyllus	?		
Dieffenbachia spp.	?		P
Digitalis lanata			C
Discorea sp.	C P	C P	P
Elaeis guineensis	C P	?	C P E
Emmenopteris henri		?	
Eucalyptus spp.		P	P
Ficus spp.	?		
Fragaria spp.	?	P	P

Table 1. Continued

Crop	Phase 1 1980/81	Phase 2 1983	Phase 3 1985
Freesia spp.	P		?
Gerbera spp.		P	P
Gladiolus hortulanum			P
Glycine max	C		
Gossypium sp.			C
Hamamelis spp.			P
Hevea brasiliensis	C		P E
Hibiscus syriacus			P
Hordeum spp.			C
Hordeum x Secale hybrids			C
Hyacinthus spp.			?
Ipomoea spp.	P	P	P
Juglans sinensis			P
Kalanchoe blossfeldiana		?	
Lachenalia spp.			P
Lilium spp.			?
Lithospermum erythrorhizon			C
Lycopersicon spp.		P	P
Malus spp. scions and rootstocks		P	P
Mangifera indica		C	
Manihot spp.	P	P	P
Medicago spp.		C P	C P
Medicinal species (misc.)		C	
Melaleuca alternifolia			P
Musa spp.	P	P	P
Nephrolepis sp.			P
Nicotiana spp.		C	C P
Olea europaea			C
Orchids			?
Ornamentals (misc.)			?
Ornithogalum spp.			P
Oryza spp.			C
Panax ginseng		C	C
Papaver spp.			C ?
Parthenium argentatum		?	
Pelargonium spp.			P
Persea americana		P	
Petunia hybrida	C		
Phlox paniculata			P
Phoenix dactylifera	E ?	C P	?
Physcomitrella patens		P	
Phytolacca americana			C
Picea sitchensis			P
Pinus spp.			P
Pistacia vera		P	P
Pisum sativum			C
Populus spp.	P	P	P
Powlownia tomentosa			P
Prunus spp.		P	C P
Pseudotsuga menziesii			C
Pteris cretica			P
Punica granatum			P
Pyrus spp.		P	P
Quercus spp.			P
Rauwolfia serpentina		C	
Rhododendron spp.			P

Table 1. Continued

Crop	Phase 1 1980/81	Phase 2 1983	Phase 3 1985
Ribes sp.			P
Robinia pseudoacacia			P
Rosa spp.			C P ?
Rubus spp.		P	P
Saccharum spp.	C P E	C P	C P
Saintpaulia ionantha			P
Salix spp.			P
Sanguinaria canadensis			C
Santalum spp.			C P
Simmondsia chinensis			P
Solanum spp.	P	C P	C P T ?
Sorghum bicolor			C
Sphaeropteris cooperi			P
Stachys sieboldii			T
Tabernaemontana spp.			C
Tectona grandis		P	P
Theobroma cacao	?		P
Tilia amurensis			P
Trifolium spp.			C P
Ulmus sp.			C
Vaccinium spp.		P	P
Vanilla spp.			P
Vitis spp.	C P	P	C P
Woody species (misc.)	?	P	C ?
Xanthosoma sagittifolium	C P		P
Zea mays			C

A further advantage is that tubers can easily be transferred directly to pots or the nursery bed, thereby being more suitable for recipients who are unable or prefer not to handle cultures. Tubers are shipped either packed in moist paper or after inoculation into culture tubes whereupon shoots form (Schilde-Rentschler and Schmiedichc, 1984). The latter method would appear to negate one advantage of the use of tubers but it still allows for the increased flexibility at the production stage offered by the use of tubers.

Centro Internacional de Agricultura Tropical (CIAT): In 1985 and 1986, CIAT distributed over 3,500 culture tubes of nearly 100 cassava clones to 15 countries word-wide (CIAT, 1986 and 1987). CIAT recognizes the importance of handling upon receipt of *in vitro* material and provides training and detailed instructions for recipients on immediate handling, further micropropagation, potting and field transfer (Roca *et al.*, 1984). In collaboration with IBPGR and National Programs, over 500 clones were introduced during these years into CIAT in the form of *in vitro* cultures from Costa Rica, Guatemala, Panama, Peru and South East Asia (The Philippines, Thailand, Indonesia and Malaysia). For the first time, clonal material from IITA has been transfered to CIAT using *in vitro* techniques, following indexing at the Scottish Crop Research Institute and micropropagation at the University of Bath, U.K. Including this material, cassava germplasm introduced to CIAT *in vitro* in the last 6 years amounts to over 2,000 clones.

International Institute of Tropical Agriculture (IITA): Data published in 1985 indicate that IITA had distributed sweet potato germplasm in the form of in vitro cultures to 47 countries throughout the world (Ng and Hahn, 1985). Cassava cultures had been distributed to 31 countries in Africa. The exchanges were carried out with the approval of the Inter-African Phytosanitary Council and in collaboration with the Nigerian Plant Quarantine Service (PQS). The active participation of the Instituut voor Plantenziektenkundig Onderkoek (IPO) in the Netherlands was involved for sweet potato and the Kenya Agricultural Research Institute (KARI) for cassava. IITA also imports sweet potato, yam and cocoyam in the form of in vitro cultures, with the approval of FQS. Sweet potato is imported via IPO where disease indexing and elimination are carried out. IPO will accept tubers and other vegetative explants and transfer them as cultures to IITA after appropriate treatments. Recently, scientists at IITA have been testing in vitro tubers as a more convenient form in which to exchange germplasm of yam (Ng, 1986). The method appears to be successful, as in the case of potato (see above).

IBPGR in vitro conservation database entries

Questions on the use of in vitro techniques to exchange plant germplasm have been included in the questionnaire used for the IBPGR Survey since its inception, although an increased amount of information has been requested in the more recent issues of the questionnaire. Three phases of the survey have been completed: 1980/81, 1983 and 1985. Phase 4 is underway at present. The first 3 phases attracted 569, 545 and 1341 entries of which 67, 117 and 310 recorded attempts to use in vitro exchange. These represent 12, 21 and 23% of the entries. This increasing use is reflected in an increased coverage of crops (29, 49 and 116 genera; see Table 1). Both unorganized (cell, callus) and organized (meristem/shoot/plantlet) cultures have been exchanged, the latter representing up to 62% of cases. The unorganized culture examples are likely to involve research material rather than germplasm for further conservation or crop production. A low number of exchanges have involved embryos/embryoids throughout the survey and, in Phase 3, the use of in vitro tubers was recorded.

Over the 3 phases, at least 55 countries have been involved in exchanges. The breakdown into national and international exchanges is detailed in Table 2 where the mode of transport is also indicated. Numbers of records are more or less equally divided between national and international exchange but more countries have undertaken international exchanges. Looking at the mode of transport, most national exchanges have involved some form of surface transport (car/bus/train/surface mail) and most international ones air mail/freight or, in a few cases, hand carriage by air. Journey durations (when specified) for surface transport range between 1 hour and 10 days (average: 32 hours; one record of a journey's taking 6 weeks was not used in the calculations). Air transport took between 1 hour and 4 weeks, with an average of 5 days. Hand carriage by air involved journey durations of between 10 hours and 3 weeks (average 6 days).

In a few cases, both countries participating in international exchanges were specified. Details are given in Table 3. In terms of duration of journey, these are typical but in success rate they are, at 75%, below average for all international exchanges (see below). However, the data are too sparse to allow firm conclusions to be drawn.

Table 2. Analysis of IBPGR *In Vitro* Conservation Database entries recording attempts at germplasm exchange

Data from Phases 1, 2 and 3 are combined.

Country	National					International					Unspecified			
	Total	Surface: car etc.	Mail	Air	?	Total	Air	Air/ hand	Other	?	Total	Air	Other	?
Australia	22	10		10	2	3	3							
Austria						1			1					
Barbados						6	5	1						
Belgium						2	1	1						
Brazil	5	1		4		2	2							
Cameroon						1	1				3			3
Canada	13	5	1	4	3	13	9	1		3	3		1	2
Chile	2	1			1	1	1				2	1		1
Colombia	2	1		1		5	4	1						
Costa Rica	1				1	4	1			3				
Denmark	4	2		1	1	2	1			1				
Ecuador						2	1	1						
Egypt											1	1		
Fiji	1	1				2	1	1						
Finland	7	6	1			2	1		1		2	1		1
France	21	6		5	10	17	10			7	2	1		1
FRG	3	1		1	1	1				1	1			1
Ghana						1	1							
Greece	1	1												
Guadeloupe	1	1				1	1							
Guyana	1			1		1	1							
Honduras						3	3							
India	9	2		4	3	5	4		1		3	3		
Ireland	5	2	1		2	4	3			1	1	1		
Israel	1	1				5	4	1			2	2		
Italy	14		1	6	7	7	7							
Japan	7	4		1	2	3	3				2	1		1
Kenya						7	4			3				
Korea (Rep. of)	11	11				20	20							
Malaysia						6	5	1						
Mexico	7	5		2		2	2				1			1
Nepal						1	1							
Netherlands	7	5	1		1	12	7	1	4		1			1
New Zealand	8	1		7		10	9	1						
Nigeria						7	1	4		2				
Norway						1	1							
Papua New Guinea						1				1				
Peru	2	1		1		6	6							
Philippines	2			2		3	2	1						
Portugal	1				1	2				2				
Poland	3				3	2		1		1				
Reunion	1			1		3	3							
Solomon Islands						2	2							
South Africa (Rep. of)						1				1	3	1	1	1
Spain	1				1									
Sweden	1			1		2	1		1					
Switzerland	4	2			2	6	3		1	2	1			1
Taipei	8	2	2		4	3	3							
Tanzania	1	1									1			1
Turkey						2	2							
UK	33	7	16	5	5	25	17	7		1	1			1
USA	36	9	9	15	3	20	15	1		4	8			8
USA (Hawaii)	3	1		2		1	1							
USA (Puerto Rico)						2	2							
USSR											8	1	4	3
Venezuela	10	8		2										
Yugoslavia	1	1				1	1							

Table 3. Details of international exchanges specifying both countries recorded in the IBPGR *In Vitro* Conservation Databases

Countries		Crop	Culture system	Mode of transport	Duration	Success
From	To					
Colombia	Philippines	*Manihot esculenta*	Shoots	Air/hand	2-3 weeks	+
Ecuador	UK	*Theobroma cacao*	Shoots	Air/hand	3 weeks	-
France	China	*Stachys sieboldii*	Tubers	Air	?	-
France	Reunion	*Saccharum* spp.	Callus, shoots	Air	?	+
Malaysia	Belgium	*Hevea brasiliensis*	Embryo	Air	3 days	+
Nigeria	Philippines	*Ipomoea batatas*	Plantlets	Air/hand	7 days	+
Papua New Guinea	UK	*Elaeis guineensis*	Callus	?	?	?
UK	Mexico	*Malus* sp. rootstocks	Shoots	Air/hand	40 hours	+
UK	Mexico	*Malus* sp. scions	Shoots	Air/hand	40 hours	+
UK	Mexico	*Prunus avium*	Shoots	Air/hand	40 hours	+
UK	Mexico	*Punica granatum*	Shoots	Air/hand	40 hours	+
USA	Nigeria	*Elaeis guineensis*	Callus	Air	1 day	+

Generally, exchanges have been very successful. Database records reported attempts as 'unsuccessful' (partially or completely) in 7, 10 and 12 cases, representing 10, 9 and 4% of all attempts in Phases 1, 2 and 3 respectively. Judgement on success was unspecified in 18 and 6% of the records in Phases 2 and 3. (The Phase 1 questionnaire did not give this option.) Over the 3 phases, international exchanges have been considered 'successful' in 82% of all attempts and national exchanges in 88%. Table 4 details problems reported. These fall into the categories of culture procedure/materials, handling/environment in transit, administrative interventions, and post-arrival deterioration. The first category predominates throughout, which is an interesting reflection upon the other factors, particularly the adequacy of transport facilities in terms of speed and the provision of a reasonable environment. A frequent problem relates to a need to take into account the potential for disturbance in transit and the consequent need to use denser agar to support cultures and tighter enclosures to prevent leakage and exclude contaminants. Problems that might relate to vigilance by quarantine authorities occur only twice in Phase 1 and once each in Phases 2 and 3 of the survey. Throughout all survey entries, clear indication of an awareness of phytosanitary considerations occurs only once in relation to the inter-state movement of grapevine material within Australia. Even accepting that in addition to this record and the example in Table 4, the IARCs who have entries in the Databases will certainly have taken account of quarantine requirements, it is still interesting that there appears to have been little official impedance of germplasm movement *in vitro*.

PHYTOPATHOLOGICAL ASPECTS

Risk assessment for *in vitro* culture

In vitro cultures are normally free of all surface contaminating pests and pathogens and free of non-cryptic systemic bacteria and fungi. Many bacteria and fungi will grow, their presence being very obvious, on the culture media routinely used for *in vitro* culture of higher plants. The culture of meristem-tips, used alone or in combination with chemotherapy or thermotherapy, can free plants of many viral infections.

These points taken together could lead to a misconception among the scientific public that in vitro culture is a 'safe' way of moving germplasm across borders protected by quarantine regulations. However, it should be noted, firstly, that few cultures are initiated under the stringent conditions that apply to meristem tip culture for virus elimination (which, in any case, is not always successful), secondly, that not all bacteria and fungi will be visible on higher plant culture medium, and thirdly, that in vitro plantlets may be sufficiently changed in morphology as a result of culture procedures to render any meaningful visual assessment for the symptoms of disease impossible. It is safest, therefore, to consider the advantages of in vitro culture to be in relation to:

1. a reduction in the mass of plant material involved in germplasm exchange;
2. the exclusion of soil and other extraneous material;
3. the exclusion of non obscure pests and pathogens; and
4. containment of exchanged material impeding contact with or release of pests or pathogens.

In all other respects, the imported material must be considered to have the same pathological status as the mother plant and must be treated accordingly. Kahn (1979) has placed in vitro cultures very appropriately as third in the order of decreasing risk of source material:

1. passage through quarantine station;
2. certified to test negatively for pests and pathogens;
3. tissue cultures;
4. plants from greenhouses;
5. plants from field plots;
6. plants from commercial nurseries;
7. plants from farms or gardens;
8. tubers or roots from the marketplace (= dormant when collected); and
9. plants collected in the wild.

Applications of in vitro culture

Several applications of in vitro culture that have the potential to help overcome phytosanitary, quarantine and related problems can be specified. It should be noted that the advantages of using cultures relate to increasing the speed and efficiency of necessary procedures rather than circumventing them.

1. The safe storage of clean material free of the risk of reinfection by local pests and pathogens. This, along with the compactness and convenience of in vitro maintenance, is the logical basis for the germplasm collections at CIP, CIAT and other centres.

2. The exchange of vegetative material free of the risk of reinfection by local pests and pathogens. The first record of in vitro exchange is probably the transfer of asparagus to Kenya. Quarantine regulations were changed in 1972 to admit in vitro cultures. Previously, regulations had prevented all imports of vegetative material in order to exclude nematodes and insects. In vitro exchange is now used to aid the passage through quarantine of potato material in a number of national programmes (Miller Jones and Howell, 1986).

3. The multiplication of imported clean material to delay reinfection once transferred to the field. This used for potato, for example, (George, 1986; Matthews et al., 1986; Miller Jones and Howell, 1986; Uyen, 1984).

4. The transfer of material of unknown disease status for post entry quarantine, possibly using in vitro indexing techniques (see 7. below). Harries (1977) proposed this approach for handling coconut germplasm. Material would be imported as in vitro embryo cultures and, after indexing for disease, released as micropropagated clones.

Table 4. Details of problems encountered in carrying out exchanges recorded in the IBPGR *In Vitro* Conservation Databases

Crop	Country	Mode of transport	Nat'l/ Int'l	Duration	Success	Problem	
						Description	Classification
Phase 1 (1980/81)							
Ipomoea batatas	USA (PR)	Air	I	?	-	Delay; no further detail	?
Manihot esculenta	USA (PR)	Air	I	?	-	Delay; no further detail	?
Phoenix dactylifera	USA	Air	N	?	-	Agar shaken up	Culture
Saccharum officinarum	USA	Air	I	?	-	Agar shaken up; shoots damaged	Culture
Saccharum officinarum	USA (HI)	Air	N and I	?	-	Loss of totipotency	Culture
Saccharum spp.	USA	Air	N	?	-	Agar shaken up; leakage of medium	Culture
Vitis vinifera	USA	Air	I	?	-	Agar shaken up; shoots damaged	Culture
Phase 2 (1983)							
Colocasia esculenta	USA	Air	N	?	+/-	Held up by USDA for several months	Administrative
Dioscorea deltoidea	USA	?	?	24 hours	+/-	Contamination	Culture
Ipomoea batatas	Nigeria	Air/hand	I	3-20 days	+/-	Water management of transplants, rats	Post-arrival
Ipomoea spp.	Costa Rica	?	I	?	-	No further details	?
Malus spp. (2 entries)	India	Air/hand	?	?	+/-	Browning of immersed tissue	Culture
Manihot esculenta	Nigeria	Air/hand	I	3-20 days	+/-	Water management of transplants; rats	Post-arrival
Parthenium argentatum	USSR	?	?	5 hours	?	Contamination	Culture
Saccharum officinarum	USA	Air	N and I	?	+/-	Leakage and disturbance of culture	Culture
Theobroma cacao	Nigeria	?	I	?	-	No further details	?
Phase 3 (1985)							
Dioscorea spp.	Netherlands	Air	I	3 days	+/-	Smearing of agar (high temperature?)	Handling
Elaeis guineensis	Malaysia	Air	I	6 days	-	Contamination	Culture
Ipomoea batatas	Netherlands	Air	I	3 days	+/-	Smearing of agar (high temperature?)	Handling
Malus domestica	UK	Air	I	<7 days	+/-	Contamination	Culture
Medicago falcata	Canada	Air	I	2 days	+/-	Callus 'lost'	?
Pisum sativum	Colombia	Air/road	N	1-2 days	-	'Restrictions'	Administrative
Populus spp.	New Zealand	Air	I	3-7 days	+/-	Agar shaken up	Culture
Prunus avium	Chile	Air/road	N and I	2-3 days	+/-	Desiccation	Culture
Prunus spp.	UK	Air/road	N and I	<7 days	+/-	Contamination	Culture
Solanum tuberosum	Netherlands	Air	I	3 days	+/-	Smearing of agar (high temperature?)	Handling
Stachys sinboldii	France	Air	I	?	-	No details given	?
Theobroma cacao	Ecuador	Air/hand	I	3 weeks	-	Contamination/temperature change	Culture; handling

5. The exchange of vegetative material that is infected but could be cleaned up using in vitro and other techniques. The material must be securely contained pending cleaning up. This approach was used to handle germplasm of sugarcane imported into Kenya through quarantine but reinfected with local strains of sugarcane mosaic virus. The infected clones were sent as cultures to Glenn Dale, USA, cleaned up using thermotherapy and re-imported to Kenya without passing through quarantine, which would have taken a further 18 months (Kahn, 1977).

6. The safe exchange of obligate parasites that could only survive on living tissue. Material required for research purposes can safely be imported as cultures. Corn mildew fungus has been imported thus into the USDA Plant Disease Laboratory in Frederick, Maryland.

7. In vitro indexing. For seed material: Embryo culture can be used to produce a plantlet that provides tissues for microbiological assays and virus indexing. This has been used to improve quarantine procedures for legumes in Kenya (Kahn, 1979). A second example involves the detection of obligate seed-borne pathogens. Tobacco suspected of carrying tobacco blue mould fungus has been germinated aseptically, shoots then being transferred to a fresh culture. The culture vessel is used as a 'dew cabinet' by subjecting it to alternating cold and warm temperatures, thereby inducing conditions conducive to the revelation of infection. Standard microbiological or other culture methods would not detect infection (Kahn, 1979). For vegetative material: Indexing for pests and pathogens could, given adaequate development, be carried out under similarly contained and controlled conditions (see next section).

Disease indexing in vitro

The development of in vitro methods for disease indexing, to interface with in vitro exchange could provide a complete, contained quarantine system. As far as the author is aware, in vitro indexing is not in use on a routine basis by any plant introduction stations or quarantine authorities, although examples can be found in the literature of plant-pathogen or plant-pest reactions that would provide the basis for indexing in this way.

Navarro (1981) has described a means of detecting citrus exocortis viroid infection by the callusing and rooting reaction of stem pieces introduced into culture. Citrus tristeza virus can be detected by inoculation of aseptically germinated seedlings (Navarro, personal communication). Lopez and colleagues have developed an in vitro method to detect citrus canker (Lopez and Navarro, 1981) and Xanthomonas ampelina infection of grapevine (Lopez et al., 1983).

The following examples originated, not from the viewpoint of plant pathology as such but were developed to aid screening for disease resistance in genotypes created or selected by in vitro procedures. Such tests for disease resistance or susceptibility may be turned to advantage for disease indexing by inoculating in vitro explants or plantlets of known response with extracts of the tissues to be indexed. Reactions that would lend themselves to this approach include the following.

Apple genotypes that are susceptible to scab caused by Venturia inaequalis become overgrown by mycelium in culture (Yepes and Aldwinckle, 1986). Larch genotypes have been screened for susceptibility to Gremmeniella abietina by exposure of in vitro shoots to the pathogen (Abdul Rahman et al., 1986). The pathogen's life cycle is completed in 4 weeks, compared with 1.5 years in the field, indicating the great advantage of speed that could be obtained by in vitro screening. Susceptibility to crown rot in apple and peach has been detected in culture by measurement of lesions on stem pieces of the host (Jeffers and Aldwinckle, 1986; Ukhede, 1986). The latter example has the advantage that seasonal variations in response of field-grown material and seasonal restrictions on carrying out indexing are avoided.

Susceptibility or resistance to Xanthomonas campestris pv. pruni has been evaluated in peach using a method in which a wick dipped in bacterial suspension is inserted into a cut in an in vitro propagated stem. After removal of the inoculum and transfer of the stem to fresh medium, a section of the stem is excised, crushed and inoculated onto bacteriological medium. A colony count reveals the extent of infection. Again, the test can be carried out all year round rather than just March to August as in the field (Hammerschlag, 1986). Resistance to root knot nematode can also be assessed in vitro for peach. Symptoms of infection develop after 3 weeks in culture compared with 6 months in the glasshouse or 1 year in the field (Hammerschlag, personal communication; Huettel and Hammerschlag, 1986).

All of the above cases utilize infectious material. However, it is possible in some cases to induce disease symptoms using non infectious pathogen extracts. One example is the reaction between hop cultures and a culture filtrate of Verticillium albo-atrum. Resistant genotypes have been isolated by selective killing of sensitive genotypes within a mixed callus population (Connell and Heale, 1986). The advantages of using non infectious extracts are obvious.

The value of the above suggestions for developing in vitro disease indexing can only be judged by those familiar with currently used techniques and their drawbacks. Also, of course, the in vitro approaches need to be considered alongside biochemical and molecular techniques that utilize immunological reactions, protein analysis and nucleic acid technology.

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