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A Status Report

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THE POTENTIAL USE OF *IN VITRO* STORAGE FOR TEMPERATE FRUIT GERMPLASM:

A Status Report

by

C. Stushnoff and C. Fear
Department of Horticulture
University of Saskatchewan
Saskatoon, Saskatchewan
Canada

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IBPGR Executive Secretariat
Crop Genetic Resources Centre
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Via delle Terme di Caracalla, 00100 Rome, Italy

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PREFACE

There are several potential advantages of conserving temperate fruit germplasm in vitro, either using minimum growth methods for short- to medium-term storage, or cryopreservation for long-term storage, but many unanswered questions on genetic stability, cooling/freezing regimes, optimum storage conditions, thawing techniques and maximum safe storage times remain. Propagation methods have to be developed for many fruit species, especially when cultures other than shoot tips and meristems are used. In vitro methods may be feasible for some, but not all species.

This report was commissioned by the IBPGR Advisory Committee on In Vitro Storage in order that decisions could be made by the committee on work that needs to be done. At the same time it was felt that such a report would be informative to the many curators of temperate fruit germplasm.

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INTRODUCTION

1. The conservation strategy as defined by Frankel (1970) depends on the nature of the plant material, and on the objective and scope of conservation. The length of the life cycle, the mode of reproduction, the size of individuals and their ecological status - whether wild, weedy or domesticated - must all be taken into account. The needs of genetic conservation are not necessarily served by the preservation of unique genotypes, although they are likely to be of the most immediate value for plant breeders. A number of methods of germplasm storage can be considered for most temperate fruit crops. They are the storage of seeds, the maintenance of field genebanks and natural stands, the storage of vegetative material, and in vitro storage over short-, medium- or long-term periods. In practice it may be appropriate to use a combination of some or all of these methods for particular crops. Pollen storage, although feasible at both low (-20°C) and ultra-low (-196°C) temperatures (Omura and Akihama, 1980; Parfitt and Almehdi, 1984), does not conserve the whole genotype of a plant, and only has a complementary role in the long-term conservation strategy.

2. Seed storage is generally the best means for the long-term conservation of genetic variation in temperate fruit crops (IBPGR, 1985a and 1985b). Some fruit crops, such as seedless grapes, do not form viable seeds, while in others, mainly tropical fruit crops, the seeds are recalcitrant. Many fruit trees have long juvenile periods, which may present a problem when fresh supplies of viable seeds are needed. With many fruit crops, however, it is possible to graft young seedlings onto established trees, and by this means to obtain earlier flowering, and fruit and seed production.

3. Field genebanks or orchards are costly to maintain, and are vulnerable to floods, drought, frost, pests and diseases, and it may be necessary to maintain virus-free plants in screenhouses. Lamb (1974) estimated that 16 ha would be required for a collection of apple germplasm which includes cultivars, rootstocks, species and ecotypes. Natural stands of wild trees can be maintained, as reported in several countries, including the USSR (Zagaja, 1983), but for various reasons, including the possibility of epidemics, they can only form part of the conservation strategy.

4. Storage of vegetative material preserves, in principle, the unique genetic constitution of an accession. Several studies have demonstrated the feasibility of storing dormant scions at low temperatures. Omura et al. (1978) stored scions of several cultivars of grape and pear for up to 4 years at 1°C to -5°C. The viability of some declined considerably after 1 to 2.5 years. Shoots of apple, pear, gooseberry, currant and raspberry survived storage in liquid nitrogen, apple buds being still viable after 23 mo (Sakai and Nishiyama, 1978). Methods of storing apple buds have been developed (Stushnoff and Tyler, unpublished data, 1984).

5. In vitro techniques have been applied to temperate fruit crops for the following purposes: clonal propagation; elimination of viruses and maintenance of virus-free stock; culture of immature embryos and those which otherwise abort; and generation of variability as a biotechnological tool for plant breeding. This report summarizes the present status of in vitro technology for the conservation of temperate fruit crop genetic resources, which were rated a third priority by the IBPGR Advisory Committee on In Vitro Storage (IBPGR, 1983). Information in Appendix I covers some related areas of research and application. Morel (1975), Henshaw (1975), Wilkins and Dodds (1983), and Withers (1980) have discussed the advantages and disadvantages of using in vitro techniques in germplasm conservation.

6. The advantages include:

(a) In vitro cultures occupy very little space, so large numbers of genotypes can be stored;

(b) The unique genetic constitution of a plant should be conserved;

(c) Cultures can be freed of pathogens relatively easily, and maintained thus;

(d) They are often amenable to rapid in vitro multiplication, by placing them in conditions suitable for rapid growth;

(e) They are easily transported, and by virtue of their aseptic condition, are less liable to problems of quarantine; and

(f) Less maintenance is required, and at less cost than with other methods.

7. Potential disadvantages include:

(a) Genetic instability due to somaclonal variation;

(b) Lack of effective methods of storage, as some species or cultivars are more difficult to store than others, and little, if any work has been done with wild species;

(c) The difficulty of assessing the viability of germplasm, especially if frozen;

(d) The possible loss of germplasm because of mechanical failure of equipment; and

(e) The unusual characteristics occasionally exhibited by plants after regeneration, such as increased vigour or branching, or the need for special acclimatization.

METHODS OF IN VITRO CONSERVATION

8. The aim in long-term in vitro storage is to reduce cell division and metabolism so that there is both increased longevity of cultures and minimal genetic change. Techniques include the use of minimal growth media, growth inhibitors in media, mineral oil overlays and low temperatures. Media suitable for tissue culture growth may be unsuitable for germplasm storage (Withers, 1980; Wilkins and Dodds, 1983).

9. Westcott (1981a) studied factors prolonging storage life of 'nodal' and multiple shoot cultures in potato. Storage life was increased by 1 to 12 mo or longer by such factors as low temperatures, temperature cycling (between 12°C/6°C 8 h night), high sucrose levels, unsealed culture vessels and increased volumes of media. Westcott (1981b) also studied effects of growth retardants on potato shoot-tip cultures. Addition of ABA or mannitol to the medium increased survival after 12 mo storage from 15% to 60%, and mannitol and ABA at reduced temperature further increased survival to 73%. These levels of survival are much too low for long-term genetic conservation.

10. A promising method of in vitro storage is the use of low temperature. Galzy (1969) demonstrated that shoot-tip cultures of Vitis rupestris survived and resumed

normal growth after 290 d of storage in the dark at 9°C, 7°C or 2°C. Barlass and Skene (1983) compared methods of in vitro storage of several Vitis species and cultivars for periods of 6 to 12 mo. Cultures were maintained either as proliferating cultures, or as single rooted shoots at 9.5°C. Recovery after storage varied with genotype, although 1 specific type of storage was satisfactory for most genotypes. Plants were produced more easily from single shoots than from proliferating shoot cultures.

11. Mullin and Schlegel (1976) kept strawberry meristem plantlets viable for 6 years in vitro at 4°C in the dark with only occasional addition of fresh media. Westcott (1981a) also demonstrated an effect of temperature in that 61% of nodal potato cultures survived after 12 mo at 6°C, compared with only 14% at 22°C. Damiano (1979) reported results of storage of 19 strawberry cultivars at 2°C. Survival of 1 cultivar was 100% after 13 mo, and 50% and higher viability was reported for 2 cultivars after storage for 27 mo.

12. In vitro storage of apple shoots, produced by shoot-tip culture in the dark at -17°C, 1°C, 4°C, or 26°C in the light was investigated by Lundergan and Janick (1979). Shoots died at -17°C, but no cryoprotectant or slow freezing regime was used. There were high losses due to contamination, desiccation and nutrient depletion at 26°C. All of the shoots survived storage for 12 mo at 1°C, whereas 70% of them survived for 12 mo at 4°C.

13. Cryopreservation, here defined as storage in liquid nitrogen at -196°C, at which temperature most or all metabolic and cell activities cease, is the most promising method of in vitro germplasm storage. Theoretically, storage at this temperature could be continued indefinitely. Disadvantages of cryopreservation are the difficulty of quickly assessing tissue viability, and sometimes the lag phase before new growth starts. Techniques used have been discussed by Withers (1980), Kartha (1981) and Bajaj and Reinert (1977).

CRYOPRESERVATION OF IN VITRO CULTURES

Callus, cell suspensions and protoplasts

14. Plants of most fruit species cannot currently be regenerated from callus or single cell cultures, and such cultures may not be genetically stable. Torrey (1967) reported a progressive loss of organ-forming capacity, and an increase in chromosomal abnormalities, such as polyploidy and aneuploidy, during prolonged subculturing of callus of garden pea. Similar observations have been reported for several other species. We agree with Henshaw et al. (1980) that non-organized cultures should not be used for germplasm conservation.

15. Callus, cell suspensions and protoplasts of several species have survived and regenerated plants after freezing. Tumanov et al. (1968) reported cold-hardening of cherry callus to -30°C after exposure to low temperature in a strong sucrose solution. Steponkus and Bannier (1971) were able to harden Chrysanthemum morifolium and Hedera helix callus to survive -17°C and -13°C, respectively after keeping them at 4.5°C for 6 weeks (C. morifolium) or 2 weeks (H. helix). The resistance to frost of sour cherry and apple callus was increased by a period at 2°C on a medium containing 15% sucrose, to as low as -50°C in the apple cv. Sibirka (Ogolevec, 1976).

16. Poplar callus survived immersion in liquid nitrogen (with pre-freezing to -20°C or -30°C) after a 60 d pre-conditioning period of 15°C day/0°C night with 8 h day-length

followed by 20 d at 0°C (Sakai and Sugawara, 1973). Sugawara and Sakai (1974) investigated the effects of pre-freezing sycamore cell suspension to temperatures between -23°C and -70°C before immersion in liquid nitrogen; pre-freezing to -30°C to -50°C resulted in the highest survival. Sakai (1960, 1965) has also shown an optimum pre-freezing temperature for twigs prior to immersion in liquid nitrogen, the temperature varying with species. Latta (1971) showed that carrot cell suspensions survived for 2 mo in liquid nitrogen, and Nag and Street (1975a) reported that a high percentage of carrot cells stored for 10 mo at -196°C survived, and maintained their level of biomass production, and embryogenic potential. Plants were grown from cells stored at -196°C of carrot and tobacco (Nag and Street, 1975b; Bajaj, 1976a; Bajaj, 1976b). Callus of date palm (Phoenix dactylifera L.) has been induced to form plantlets after 3 mo storage in liquid nitrogen (Tisserat et al., 1981).

17. The ability of cells to survive freezing depends on a number of interacting factors, including: the age, nature and density of cells; cryoprotectants used; cooling rate; storage temperature; and thawing rate (Bajaj and Reinert, 1977). Effects of such factors are indicated in Table 1.

Embryogenic callus, early embryos and small plantlets

18. By modifying the methods used with carrot cell suspensions, Withers (1979) was able to recover early somatic embryos and plantlets which resumed growth after freezing in liquid nitrogen. This was done by using dimethyl sulphoxide (DMSO) at 2.5% to 20%, with partial dehydration, and freezing at 5°C min^{-1} or less, and transfer of tissues to liquid nitrogen at -100°C.

19. Bajaj (1976a) reported the survival of pollen embryos from cultured anthers of Nicotiana tabacum and Atropa belladonna, mixed with 5% DMSO and either directly immersed in liquid nitrogen or frozen at 2°C min^{-1} followed by thawing at 37°C in a water bath. Survival of early and late globular embryos was higher than that of early heart-shaped embryos. Storage of haploids, which tend to revert to higher ploidy levels in culture, may be possible by this means.

Meristems and shoot tips

20. Meristems or shoot tips have several advantages over suspension or callus cultures, including: their greater genetic stability; the possibility of producing virus-free plants; and the relative ease of in vitro propagation of many species. Meristematic cells are small, thin-walled, highly cytoplasmic and non-vacuolated, and so survive freezing well. The optimum conditions for cryopreservation of meristems differ from those of suspensions or callus cultures, due to the presence of different cell types, including differentiated cells. Factors influencing recovery of plants after cryopreservation are shown in Table 2.

21. Sakai et al. (1978) reported survival and growth in vitro after freezing meristems of the strawberry cv. Hokowase in liquid nitrogen. Shoots developed in 60-80% of apices. Apices were frozen slowly to -20°C or -30°C in 16% DMSO, followed by rapid freezing in liquid nitrogen and rapid thawing in liquid Murashige and Skoog medium at -40°C. Kartha et al. (1980) demonstrated survival in liquid nitrogen of meristems of the strawberry cv. Redcoat, taken from in vitro cultures. They achieved 95% survival in plant regeneration from meristems pre-cultured for 2 d in 5% DMSO followed by freezing at 0.84°C min^{-1} to -40°C followed by storage for 1 week in liquid nitrogen. By comparison there was only 35% survival with glycerol as a cryoprotectant (after pre-culturing for 3 d on a media containing 5% glycerol and treating with 5% glycerol for 30 min prior to freezing) and there was only 5% regeneration following pre-culturing without cryoprotectant.

Table 1. Factors influencing success of cryopreservation of callus, cell suspensions, and protoplasts

Factor	Species	Reference
Treatment preceding freezing		
Culture under hardening conditions	Populus euramericana	Sakai and Sugawara, 1973
Pre-freezing to intermediate temperature prior to liquid nitrogen (LN)	Phoenix dactylifera Acer pseudo-platanus	Tisserat et al., 1981 Sugawara and Sakai, 1974
Cell type and sampling time		
Higher survival of small, highly cytoplasmic cells	Daucus carota	Bajaj, 1976b
Cells in late lag and early exponential phase of cell cycle survive better	Daucus carota Acer pseudo-platanus	Withers and Street, 1977 Sugawara and Sakai, 1974, 1978
Cells in G1 phase of cell cycle had better survival	Acer pseudo-platanus	Withers, 1978
Cell density		
Recovery after freezing dependent upon cell density in freezing ampoule	Datura stramonium Daucus carota Glycine max Nicotiana tabacum	Bajaj, 1976a
Beneficial use of cryoprotectants		
	Acer pseudo-platanus Atropa belladonna Daucus carota	Nag and Street, 1975a, 1975b
	Catharanthus roseus	Kartha, Leung, Gaudet-LaPrairie and Constabel, 1982
Freezing rate		
1°C to 2°C min^{-1} optimum	Atropa belladonna Daucus carota	Nag and Street, 1975b
1°C min^{-1} optimum	Acer pseudo-platanus	Nag and Street, 1975b
	Glycine max	Takeuchi et al., 1982
1.3°C min^{-1} optimum	Triticum aestivum Hordeum vulgare	Takeuchi et al., 1982
Thawing rate		
Rapid rate optimum	Atropa belladonna Daucus carota	Nag and Street, 1975b

Table 2. Factors influencing success of cryopreservation of shoot tips and meristems

Factor	Species	Reference
Beneficial effect of explant hardening prior to freezing	Dianthus caryophyllus	Seibert and Wetherbee, 1977
Beneficial effect of pre-culture period prior to freezing	Solanum etuberosum	Towill, 1981
Beneficial effect of freezing to intermediate temperatures prior to transfer to LN	Solanum etuberosum	Towill, 1981
	Manihot esculenta	Kartha, Leung and Mroginski, 1982
	Fragaria x ananassa	Kartha et al., 1980
	Malus domestica	Katano et al., 1983
Use of various freezing rates		
Rapid: Direct exposure to LN	Dianthus caryophyllus	Seibert and Wetherbee, 1977
	Solanum goniocalyx	Grout and Henshaw, 1978
Intermediate: 20°C to 55°C min^{-1} from 0°C to -120°C, then immersion in LN	Lycopersicon esculentum	Grout et al., 1978
Slow: 0.3°C min^{-1} to -40°C to LN	Solanum etuberosum	Towill, 1981
0.5°C min^{-1} to -25°C, -30°C, or -40°C to LN	Manihot esculenta	Kartha, Leung and Mroginski, 1982
0.6°C min^{-1} to -40°C to LN	Pisum sativum	Kartha et al., 1979
0.84°C min^{-1} to -40°C to LN	Fragaria x ananassa	Kartha et al., 1980
0.46°C min^{-1} to -40°C to LN	Malus domestica	Katano et al., 1983
Interaction of pre-freezing temperature with thawing rate after LN	Malus domestica	Katano et al., 1983
	Dianthus caryophyllus	Uemura and Sakai, 1980
Effects of types and concentration of cryoprotectants	Pisum sativum	Kartha et al., 1979
	Fragaria x ananassa	Kartha et al., 1980
	Manihot esculenta	Kartha, Leung and Mroginski, 1982
	Solanum goniocalyx	Grout and Henshaw, 1978
	Dianthus caryophyllus	Seibert and Wetherbee, 1977
	Solanum etuberosum	Towill, 1981
Addition of gibberellic acid to media necessary for organized shoot growth after freezing	Lycopersicon esculentum	Grout et al., 1978

22. Katano et al. (1983), working with apples, reported survival and subsequent growth in vitro of excised dormant shoot tips of several cultivars, rootstocks, and species when slowly frozen to -40°C without cryoprotectants. Shoot tips of the cv. Fuji survived liquid nitrogen without cryoprotectant when given adequate pre-freezing. Their study demonstrated the importance of thawing rate and its interaction with pre-freezing temperature. Shoot tips from dormant buds pre-frozen to -15°C survived liquid nitrogen regardless of re-warming method, but survival of shoot tips pre-frozen to -_0°C depended on the recovery method. When shoot tips pre-frozen to -10°C were re-warmed slowly in air or at 0°C, none survived, compared to high survival of shoot tips re-warmed in 38°C or 0°C water. It was suggested that ice crystals were formed during cooling to -196°C in shoot tips pre-frozen to -10°C, and that these grew, causing injury during slow re-warming. Apparently, with pre-freezing to -15°C, dehydration of cells occurs, preventing lethal intracellular ice crystal growth during slow re-warming.

23. Apple shoot tips derived from in vitro cultures have shown high survival in liquid nitrogen when previously given 4 weeks of cool temperature (10°C) and relatively short days (10 h) followed by pre-freezing to -20°C, -30°C, or -40°C in 10% DMSO + 10% glucose (Katano and Ishihara, pers. comm., 1984).

24. There have been no long-term studies with liquid nitrogen storage. Bajaj (1981) reported regeneration from potato meristems after cryopreservation for 24 mo, but survival data were not given. The capacity for regeneration of pea meristems stored in liquid nitrogen declined after 26 weeks from 68% to 61% (Kartha et al., 1979). Preliminary results with carnation shoot apices indicated no decrease in viability after 6 mo storage at -196°C (Seibert and Wetherbee, 1977). Strawberry meristems retained a high level of viability after over 3 years of storage in liquid nitrogen (Kartha, pers. comm., 1984).

25. The genetic stability of plants grown from meristem cultures presumably depends on their continued organized growth after storage. Grout and Henshaw (1980) studied the structure of shoot tips of Solanum goniocalyx after immersion in liquid nitrogen and rapid thawing at 40°C in a warm bath. Examination of the explants 24 h after thawing revealed areas of extensive damage, often associated with rupture of the epidermis. Transmission microscopy of surviving explants showed cells with withdrawn plasmalemma and nuclear membranes as well as plasmalemma rupture. Many of the shoot tips produced plantlets despite the damage. The intact meristem is apparently not necessary for normal, organized shoot growth, and callus may be produced where cell damage is too extensive. In cassava, frozen meristems failing to produce shoots produced callus and leaves, probably because only the leaf primordia survived (Kartha, Leung and Mroginski, 1982).

26. Anatomical studies of cryopreserved meristems have also shown extensive damage to the dome area of the meristem. Haskins and Kartha (1980) studied pea meristems before and after storage in liquid nitrogen. No damage resulted from the isolation technique, after culture for 48 h on a medium containing 5% DMSO, or culture for 7 d on differentiation medium. However, after meristems pre-cultured with DMSO were stored for 1 h in liquid nitrogen, cells in the meristems seemed to be dead. Live cells appeared in primordial leaf tissue and at sites lateral on the shoot apical meristem and laterally on the meristematic dome, and presumably new shoot meristems developed from them.

27. Other studies indicate that serious damage to the meristem does not occur and organized growth may resume after thawing. In carnation shoot apices, Seibert and Wetherbee (1977) reported that the meristematic region of the shoot apex survived, as indicated by cell division and growth, while leaf primordia were damaged after exposure to liquid nitrogen. Towill (1981) reported that outer leaf primordia died and greening

and proliferation occurred from a small region of frozen Solanum etuberosum shoot tips. The apical meristem and leaf primordia were more resistant to freezing than subapical tissue in carnation (Uemura and Sakai, 1980) and strawberry (Sakai and Nishiyama, 1978) shoot apices. Microscopic examination of strawberry meristems after exposure to liquid nitrogen indicated that they did not sustain large amounts of damage (Kartha, pers. comm., 1984).

28. Genetic stability is a prerequisite for germplasm storage. Meristem or shoot-tip culture is the only in vitro method which satisfies this requirement. There have been relatively few studies examining variability of plants from shoot-tip and meristem cultures. Swartz et al. (1981) showed the presence of a few discrete variants and morphological differences between tissue culture and conventionally propagated plants, although the plants were generally uniform. Blackberry plants propagated in vitro were uniform (Swartz et al. 1983). Denton et al. (1977) regenerated potato plants from shoot-tip culture and compared them with plants grown from tubers. Morphological differences were attributed to environmental and developmental factors, and there were no qualitative differences in electrophoretic patterns.

29. Only background radiation can presumably cause genetic change at -196°C. The effect of radiation on mouse embryos was studied by Whittingham et al. (1977), who concluded that background radiation during storage was a negligible hazard. Kartha, K.K., Leung, N.L., Gaudet-LaPrairie P. and Constabel, F. (1982) examined the frequency of mitosis and ploidy levels in periwinkle (Catharanthus roseus L.) cell suspensions before and after 1 h of storage at -196°C. No differences in the frequency of mitoses and of ploidy levels were observed, but cryopreserved cells had a large vacuole rather than numerous small ones.

30. If genetic damage does occur during cryopreservation, then DNA repair may occur upon thawing and rehydration. Work by Villiers (1975) indicating that repair mechanisms are active in imbibed seeds but inactive in dry-stored seeds may be relevant here. The usual procedure in cryopreservation has been to thaw material rapidly and place it in optimum conditions for growth before repair mechanisms would have an opportunity to operate. Although recovery might be improved, repair would not necessarily ensure maintenance of genetic integrity.

31. One disturbing aspect of genetic stability in long-term storage is the possibility of selection and genetic drift. Variation for tolerance to long-term storage may occur among species, cultivars, and individual tissues and cells. Selection for other characteristics, such as ability to grow on specific media, to withstand freezing and thawing, and to resume growth could also occur.

APPENDIX I

RELATED AREAS OF RESEARCH AND APPLICATION

Variability of in vitro cultures

32. Variation between plants which have been regenerated from tissue culture may be of genetic, epigenetic or physiological origin. Apart from its use in plant breeding, such variation is usually unwanted. Well-known examples have been noted in plants derived from potato protoplasts (Shepard et al., 1980; Thomas et al., 1982), in dihaploid tobacco plants from anther culture (Burk and Matzinger, 1976), in sugarcane plants from callus (Heinz and Mee, 1971; Scowcroft and Larkin, 1982), and in plants of scented geranium grown from callus (Skirvin and Janick, 1976).

33. 'Somaclonal' variation, which itself may have several causes, describes heritable differences of a genetic nature (Larkin and Scowcroft, 1981; Scowcroft, 1984). Polyploid cells can originate from endopolyploid or endoreduplicated chromosomes (Bayliss, 1980), which are quite common in many plant species (D'Amato, 1975), or chimaeral cells (D'Amato, 1977). Culture conditions may themselves sometimes be mutagenic (Liu and Chen, 1978). Chromosomal rearrangements, such as multicentric bridges, translocations, deletions, inversions and ring chromosomes can also lead to phenotypic variation.

34. An example of epigenetic variation, which is transmissible by mitotic but not by meiotic cell division, is habituation, in which cultures progressively lose their requirement for auxin or cytokinin (Meins, 1983). Kester (1983) suggested that the juvenile growth characteristics of grape (Mullins et al., 1979) and blueberry plants (Lyrene, 1981) regenerated from culture, may represent epigenetic changes.

35. Physiological variation, such as tricotyly in tobacco (Chaleff and Keil, 1981), is even less persistent, lasting only as long as cells or tissues are grown in their unique culture environment. Other phenotypic changes can be due to the elimination of viruses (Krul and Myerson, 1980). Meristem culture can be used to produce virus-free plants of a number of fruit crops, including gooseberry, strawberry and raspberry (Kartha, 1981).

36. The amount and nature of variation from culture depends upon the type and age of culture, culture conditions and the cultivar and species grown. Meristems or shoot tips are the most genetically stable (D'Amato, 1977), and if phenotypic uniformity is required the use of totipotent single cells such as callus and protoplasts should be avoided (Skirvin, 1981). Changes in ploidy are frequent, as reported for several species grown from anther culture (Michelion et al., 1974; Rosati et al., 1975; Zhukov and Shchekotova, 1982), but whether plants originated from gametic or somatic tissue is in some cases not known. There are many examples of increasing ploidy levels in callus cultures, for example in almond (Mehra and Mehra, 1974), grape (Vashakidze, 1978) and apple (Ravkin and Popov, 1974); in the latter case the ploidy level of cells increased to 19x by the fifteenth sub-culture. The chromosome numbers of cells within a single callus may vary widely. Kursakov et al. (1984) found that aneuploid cells and haploid, diploid, tetraploid, hexaploid and higher ploidy cells were all found in callus from plum x apricot hybrids, while Hu and Liu (1978) observed chromosome numbers in cells from endosperm-derived callus of apple from 17 to over 200.

37. Strawberry plants regenerated from tissue cultured shoot tips, although generally uniform, consistently produced more runners and had higher yields than conventionally-propagated strawberry plants (Damiano, 1980; Swartz et al., 1981),

effects which may have been due to the physiological conditions in culture, especially levels of gibberellins and cytokinins. Tissue culture-propagated strawberry plants have, however, shown increased susceptibility to Verticillium (D'Ercole and Nipoti, 1981) and Rhizoctonia and Fusarium (Sansavini and Gherardi, 1981). Differences between sub-clones from a common cultivar have also been observed in strawberry (Swartz et al., 1981; Sansavini and Gherardi, 1981) and apple rootstocks (Ishihara and Katano, 1982; James and Thurbon, 1981).

38 Swartz et al. (1983) observed rather little variation in thornless blackberry plants grown from tissue culture, and any differences may have been related to their greater vigour compared with conventionally-propagated plants. Some variant types of Prunus avium were obtained by Ancora et al. (1982) by culturing shoot tips, but the original explants were chimaeral. Although some fruit species, such as apple, may exhibit juvenile morphology when grown from shoot tip culture, other features associated with juvenility, such as delayed flowering, do not necessarily occur (Ishihara and Katano, 1982; Zimmerman, 1982). Grape plants regenerated from culture became temporarily juvenile as indicted by the disappearance of tendrils, spiral phyllotaxy, and in some cases altered leaf indentations (Grenan, 1982; Chee and Pool, 1982). By contrast, grape plants produced by somatic embryogenesis, were quite different from their parents, being more vigorous and with red instead of green stems, a different cluster shape and spicier fruits (Krul and Myerson, 1980).

Micropropagation

39. The commercial propagation of fruit crops by in vitro methods is an alternative form of vegetative propagation to those conventionally used, such as layering and grafting. Vegetative propagation is necessary because seedlings of outcrossing cultivars do not grow true-to-type. Whether in vitro methods are used or not depends on how economically plants can be propagated conventionally. Even when not routinely practiced, in vitro methods may be valuable when a new cultivar is released, to speed up the testing of selections by plant breeders, or to produce virus-free clones.

40. Methods based on in vitro culture of shoot tips, which as defined by Styer and Chin (1983) are meristematic regions between 0.5 and 5.0 mm long (while meristems are less than 0.5 mm long), are sometimes used for the commercial propagation of strawberry (Scott and Zanzi, 1981), apple rootstocks, blueberry and blackberry (Zimmerman and Broome, 1980a and 1980b). Techniques have also been developed for several other crops, including kiwi fruit (Wang et al., 1983), various Prunus species such as sweet cherry (Snir, 1982), and pear (Singha, 1980).

41. Callus growth can be induced by growing explants from various parts of many plant species on appropriate media. It is often possible to induce differentiated growth of embryoids, or of roots, shoots or buds, by subculturing the callus to new media containing essential growth hormones, and extremely rapid multiplication rates may be achieved by means of manipulations of this kind. Explants may be taken from the stem and leaf in kiwi fruit (Gui and Xu, 1983), fruit receptacle tissue in strawberry (Asahira and Kano, 1978), young embryos in peach (Hammerschlag and Bauchan, 1983), and sepals, stamens and petals in pears (Furukawa et al., 1975). Diploid callus has been produced from strawberry (Oosawa and Takayanagi, 1982) and grape (Hirabayashi and Akihama, 1982) anthers, while haploid callus has been produced from strawberry (Shen, 1982), and apple anthers (Bruniste and Popescu, 1983) from which plants have successfully been regenerated. Diploid plants from this source may in principle be di-haploids derived from pollen mother cells, but are more often likely to be clones of the mother plant.

APPENDIX I (Continued)

42. There has also been research on the culture of plant organs, cells or protoplasts in liquid media. Tripathi and Saussay (1980) were able to produce kiwi fruit plants by culturing staminal filaments in liquid medium. Strawberry plants have been regenerated from cultured protoplasts (Binding et al., 1982). Studies with apple cell suspensions have also been reported (Pech et al., 1976).

43. It is clearly necessary to be able to regenerate plants rapidly and reliably after in vitro storage. The results which have been briefly considered above indicate that successful techniques can often be developed for the crops being considered in a variety of different ways.

Fruit breeding

44. In vitro techniques may be a valuable adjunct in fruit crop breeding for the very reasons which limit progress using conventional methods: heterozygosity, polyploidy, long generation cycle etc., but they are expected to play a special role in the selection of minor variants from existing well-known cultivars (Vasil et al., 1982; Ramming, 1983; Zimmerman, 1983).

45. The culture of immature embryos, which would normally have aborted, has been invaluable in the development of peach and nectarine cultivars (Layne, 1983), and has also been used with cherries (Fogle, 1975), plums (Weinberger, 1975), apricots (Hiley and Hough, 1975) and in the development of seedless grapes (Cain et al., 1983). The use of interspecific or intergeneric crosses is a method by which genes for disease resistance or cold-hardiness may be introduced into cultivars from wild species (Galletta and Puryear, 1983), but the resultant seeds may be of low vigour. Plants can often be reliably grown from such seeds by culturing their embryos in vitro (Raghaven, 1977; Brettell and Ingram, 1979; Uhlinger, 1982).

46. Pollination and fertilization can be carried out in vitro in certain plants (Rangaswamy, 1977). Although its practical use has not been reported with fruit crops, work by Stosser (1982) indicated that in vitro fertilization should be possible with plums. The technique can be used to overcome self-incompatibility in some plants, such as Petunia spp., and is a means by which haploid plants of Mimulus luteus can be produced (Hess and Wagner, 1974).

47. Plants can in principle grow from single totipotent cells; thus plants grown from single cells which have undergone ploidy changes should be genetically uniform throughout (D'Amato, 1977). Polyploidy may be induced in culture in various ways with colchicine. Lyrene and Perry (1982) treated shoot tips of blueberries with colchicine in culture and obtained polyploid shoots. Alternatively, colchicine can be incorporated in the culture medium, a technique which has been successfully used with sugarcane (Heinz and Mee, 1970).

48. Triploid plants can be produced by culturing seed endosperm (Johri and Bhowjani, 1977), which is a possible way of developing apple cultivars, several of which are triploid. In addition to anther culture, haploid plants of some species can be produced by culturing embryos in which chromosome elimination occurs. Homozygous diploids produced from such plants save many years of inbreeding, and are useful in studies of induced mutations.

APPENDIX I (Continued)

49. Work with plant protoplasts opens new possibilities for the genetic improvement of crops, including the following: the production of hybrid cells in interspecific, intergeneric and interfamilial combinations; the combination of cytoplasms; and cell transformation by introducing specific organelles and genetic elements, such as parts or all of chromosomes, mitochondria and chloroplasts (Bajaj, 1977; Flavell, 1981; Gamborg, 1981). Hybrid plants have been regenerated from somatic hybrids of potato and tomato (Melchers et al., 1978), and Chaleff (1981) and Gamborg (1981) list several other examples. There have been no cases reported to date with fruit crops.

50. Any mutations arising in culture, particularly single gene changes, may in principle make a significant improvement to otherwise quite acceptable cultivars. Tissue culture may allow the screening of thousands of cells for desirable mutants (Chaleff, 1981), but their recovery is not always simple. Lane et al. (1982) were able to selectively favour the growth of compact apple cultivars compared with standard ones by adjusting the benzyladenine concentration in the culture media, a procedure which could form the basis for a selection method. Work by Barlass and Skene (1981) indicated that selection of grape cultivars for salt tolerance should be possible in vitro, while differences in the reaction of apple cultivars to fireblight in culture were reported by Wanstreet and Lineberger (1982).

51. The successful use of the above methods in fruit crop improvement are contingent on there also being methods of rapid in vitro propagation. In vitro germplasm conservation might also aid fruit crop improvement by ensuring adequate and readily available sources of fruit germplasm diversity for fruit breeders.

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