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# *Vitro* Conservation of Temperate Tree Fruit and Nut Crops

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## 10.1 Introduction

Many germplasm facilities for the preservation and distribution of fruit and nut germplasm are now instituting slow-growth and cryopreservation strategies (Ashmore, 1997; Brettencourt and Konopka, 1989). Some genera have well-defined methods, while the techniques for others are still under investigation. Primary collections of plant germplasm are often in field plantings that are vulnerable to disease, insect, and environmental stresses. Slow-growth techniques provide a secondary storage method for clonal field collections (see Lynch, Chapter 4, this volume). Alternative germplasm storage technologies also provide storage modes for experimental material, allow for staging of commercial tissue culture crops, and provide a reserve of germplasm for plant distribution. Cryopreservation in liquid nitrogen (LN) provides a low-input method for storing a base collection (long-term backup) of clonal materials. Recent improvements in cryopreservation methods make these long-term collections of clonal germplasm feasible. Both *in vitro* and cryopreserved collections provide insurance against the loss of valuable genetic resources and may provide alternative distribution methods.

Medium-term storage of clonal plants involves slow-growth strategies such as temperature reduction, environmental manipulation, or chemical additions in the culture medium. Storage techniques developed thus far provide several options so it is now possible to match improved techniques with a facility's needs and resources for the best possible plant preservation.

New techniques and improvements in cryopreservation research have greatly increased the number of cryopreserved species. Suspension or callus cultures, dormant buds, *in vitro* grown apical meristems, isolated embryonic axes, seeds, somatic embryos, and pollen are now stored in LN. Cryopreserved collections of temperate plants of economic importance are now established in several countries. The storage of most temperate horticultural crops as base collections in liquid nitrogen is now feasible.

## Literature review of progress

### *10.2.1 Medium-term storage at above freezing temperatures*

*In vitro* collections play an important role in storing and distributing germplasm throughout the world. Certification programmes often incorporate *in vitro* culture as a standard technique for producing virus-negative plants from stock collections. *In vitro* culture systems are available for most temperate fruit and nut crops, but information on medium-term storage is limited for many genera. Most studies involve temperatures near freezing, but some tests of room temperature storage and chemical inhibition are also available (see Lynch, Chapter 4, this volume). Published research is available for *Malus, Morus, Prunus, Punica* and *Pyrus*; however most studies are restricted to a few genotypes and storage conditions. Published reports of *in vitro* storage systems for temperate nut trees are very limited; however, a species by species report of conservation methods currently applied to temperate tree fruit and nut crops is given below.

#### *Corylus*

More than 80 genotypes of hazelnut (*Corylus* sp.) *in vitro* cultures are stored at 4°C in low light (5 $\mu$mol $m^{-2}$ $s^{-1}$) at NCGR–Corvallis, Oregon, USA (Reed, unpublished). Storage was also successful in total darkness where the mean storage duration for accessions held at 4°C in the dark is 1.26 years with a range of 8 months to 2.5 years (Reed and Chang, 1997).

#### *Malus*

Lundergan and Janick (1979) first suggested the feasibility of *in vitro* germplasm storage after successfully storing *Malus domestica* Borkh. cv. Golden Delicious *in vitro* for 12 months at 1°C and 4°C. Success with other *Malus* species and cultivars included storage ranging from 9 months to 3.5 yr (Druart, 1985; Orlikowska, 1991, 1992; Wilkins *et al.*, 1988). Wilkins *et al.* (1988) studied cultures of five *Malus domestica* cultivars, *M. prunifolia* (Willd.) Borkh., and *M. baccata* (L.) Borkh. and successfully stored them for 12 to 28 months at 4°C with a 16 hour photoperiod on an agar-based multiplication medium. Some rootstock cultivars were successfully stored on liquid medium. Charcoal in the medium (see Lynch, Chapter 4, this volume) increased storage time, but eliminated proliferation during storage. Two apple rootstock cultivars kept at 4°C in the dark stored better on medium with 6-benzylaminopurine (BAP) than on medium lacking the growth regulator (Orlikowska, 1991, 1992). Storing plants immediately after subculture was important for the survival of multiple-shoot tufts, but shoot tips and nodal segments survived whether stored at 0, 10, or 20 days after subculture. Eckhard (1989) stored apple-shoot cultures at 2 to 4°C under low light for 1.5 years on a reduced sucrose, low BAP medium. Druart (1985) stored topped, partially submerged shoots of three *Malus* rootstocks and 'Golden Delicious' on hormone-free medium in the dark at 2°C for 1.5 to 3.5 years with 100 per cent survival. Apple species and cultivars (150 genotypes) are stored at Changli Institute of Pomology (China) on modified MS medium at 2 to 4°C in the light (10 $\mu$E $m^{-2}$ $s^{-1}$, 15 hour photoperiod) with yearly transfer (Reed and Chang, 1997).

## *Morus*

*Morus nigra* L. shoot tips survived for only six months on multiplication medium at 4°C with a 16 hour photoperiod, but with activated charcoal in the medium (see Lynch, Chapter 4, this volume), survival could be increased to 42 per cent after nine months at 25°C (Wilkins *et al.*, 1988). Sharma and Thorpe (1990) stored 15 genotypes of *Morus alba* L. for six months at 4°C in the dark on shoot proliferation medium (80 per cent viability).

## *Prunus*

Marino *et al.* (1985) stored shoot cultures of three *Prunus* (peach, cherry) genotypes at 8°C, 4°C or –3°C for up to 10 months on multiplication medium. A 16 hour photoperiod was important for successful 4°C and 8°C storage for 90 days, but ten-month, dark storage at –3°C was better than under lights for some genotypes. Cultures stored 14 days after subculture survived better than those stored immediately, and lower temperatures increased storage times. Druart (1985) stored 12 *Prunus* species and cultivars on basal medium at 2°C in the dark for up to four years; dimethyl sulphoxide or glycerol in the medium was toxic to the cultures. Survival of topped and partially submerged shoots was genotype dependent. Wilkins *et al.* (1988) stored five *Prunus* genotypes on multiplication medium at 4°C with a 16 hour photoperiod for nine to 18 months.

## *Punica*

Pomegranate, *Punica granatum* L., shoot cultures died at 4°C, but survived for 18 months at 10°C with a 16 hour photoperiod (Wilkins *et al.*, 1988).

## *Pyrus*

*Pyrus communis* L. ~~var. Caucasica~~ subsp. caucasica (Fed.) Browicz shoot tips grown on basal medium at 4°C, 8°C, and 12°C with a 16 hour photoperiod exhibited depressed growth for 12 to 18 months, with highest survival at 4°C. Adding mannitol or increasing sucrose in the medium were not successful for storage at either 4°C or 28°C (Wanas *et al.*, 1986). *Pyrus pashia* D. Don had 100 per cent survival on multiplication medium at 4°C or 10°C with a 16 hour photoperiod after 12 months (Wilkins *et al.*, 1988). *P. communis* cultivars La France, Bartlett, and La France × Bartlett had good survival at 5°C with a 16 hour photoperiod and at 1°C in darkness for 20 months, but 10°C and 15°C storage with light produced poor survival. Japanese pears (*P. pyrifolia* (Burm.) Nakai. CVs. 'Shinsui', 'Nijisseiki', 'Shinchu', 'Kosui' 'Hosui' and 'Hakatado' were killed when stored at 5°C, 10°C, or 15°C with a photoperiod, but had 100 per cent survival when stored at 1°C in the dark for 12 months. ABA did not improve the survival of stored Japanese pears (Moriguchi, 1995; Moriguchi *et al.*, 1990). Pears stored at NCGR–Corvallis in 1984 were kept in 20 × 100 mm tubes at 4°C in darkness, but tubes were replaced with tissue-culture bags in 1989. *Pyrus* accessions (169) were stored for eight months to 4.6 years with a mean storage time of 2.7 years in tissue-culture bags in the dark at 4°C (Reed and Chang, 1997). Storage of 46 genotypes with three treatments (4°C upright plants, 4°C three-quarters submerged, 1°C upright) showed genotype differences for the length of storage, but few differences were noted among the three storage treatments (Reed *et al.*, 1998).

### *10.2.2* Long-term storage in liquid nitrogen

Cryopreservation (see Benson, Chapter 6, this volume) of temperate fruit trees began in the 1970s when dormant bud freezing was successfully applied to apple, pear, peach, plum, and cherry; now additional techniques are available. Many temperate nut seeds are dehydration sensitive, liquid nitrogen sensitive, or survive for a year or less in 4°C storage. In this respect, they are similar to recalcitrant tropical seeds as demonstrated by Marzalina and Krishnapillay (Chapter 17, this volume). Excised embryonic axes are excellent material for cryopreservation of wild populations, but cultivars require methods similar to those developed for fruit trees. The three major cryopreservation techniques – slow freezing, vitrification, and encapsulation–dehydration – are useful for these plant materials. Slow-freezing techniques developed in the 1970s by several investigators are used on many different species (Kartha, 1985). Sakai (1993) developed several plant vitrification solutions with highly concentrated cryoprotectants that allow cells to dehydrate quickly and cellular liquids to form glasses at low temperatures. Vitrification solution components, the duration of exposure, the size of plant material, the cryoprotectant toxicity, and the temperature of application are all important to plant survival. Dereuddre *et al.* (1990a, 1990b) devised a new cryopreservation system involving encapsulation of shoot tips in alginate beads followed by dehydration and direct exposure to LN. In addition, combinations of these techniques are also used in certain situations.

#### *Carya*

Pence (1990) found most *Carya* embryonic axes dried to 5–10 per cent moisture content before being exposed to LN germinated or partially germinated, with some callus following thawing. Subsequent *in vitro* growth and development was best for fresh seed and declined from shoots to callus to no growth as the seed aged.

#### *Castanea*

*Castanea* axes dried to about 8 per cent moisture before freezing produced callus upon recovery in initial testing (Pence, 1990). Chestnut embryonic axes desiccated to 20–30 per cent moisture before LN exposure had improved survival and some shoot formation (Pence, 1992).

#### *Corylus*

Early studies by Pence (1990) found that cryopreserved embryonic axes and control axes of *Corylus* seeds produced only callus. Embryonic axes from fresh seed of *Corylus avellana* L. 'Morell' exhibited maximum recovery (85 per cent) when axes were frozen at 12 per cent moisture content, while 'Butler' required 11 per cent moisture to obtain 50 per cent recovery (Gonzalez-Benito and Perez, 1994). Whole seeds of *C. avellana* 'Barcelona' did not survive LN exposure following desiccation pretreatment, but embryonic axes were excised from the thawed seed and regrown in culture (Normah *et al.*, 1994). Axes from stratified 'Barcelona' seed had improved shoot growth for both control and LN exposed treatments. Axes from stored, stratified seed dried to 8 per cent moisture were cryopreserved with 85 per cent viability and 70 per cent shoot growth, while only 30 per cent of unstratified axes produced shoots (Reed *et al.*, 1994). Embryonic

axes from seeds of *Corylus colurna* L., *C. americana* Marsh., and *C. sieboldiana* var. mandshurica (Maxim.) C. Schneider were stored in LN using this technique at NCGR–Corvallis and the National Seed Storage Laboratory, Ft Collins, CO. Regrowth of the thawed axes was 75–80 per cent for all three species (Reed, unpublished).

## *Juglans*

Dried axes of *Juglans* seeds (5 per cent moisture) germinated or partially germinated producing shoots and/or roots *in vitro* following cryopreservation (Pence, 1990). Cryoprotectant with 5 M 1,2-propanediol and 20 per cent sucrose produced 75–91 per cent survival and regrowth of *Juglans* embryonic axes (de Boucaud *et al.*, 1991). Slow freezing *in vitro* grown shoot tips of walnut was also successful (de Boucaud and Brison, 1995). Modified PVS2 cryoprotectant treatment combined with slow freezing (0.5°C/min) of shoot tips produced 34 per cent survival (Brison *et al.*, 1991). Encapsulation–dehydration and slow freezing methods were successful with isolated walnut somatic embryos (de Boucaud *et al.*, 1994).

## *Malus*

Apple and pear winter buds exposed to subzero temperatures at slow freezing rates retained their viability after immersion in liquid nitrogen and apple buds taken from the shoots grew after being grafted onto rootstocks in the greenhouse (77 per cent regrowth) (Sakai and Nishiyama, 1978). Dormant vegetative *Malus* buds cryopreserved using a combined dehydration–encapsulation technique had 80–100 per cent viability (Stushnoff, 1987; Stushnoff and Seufferheld, 1995; Tyler and Stushnoff, 1988a, 1988b; Tyler *et al.*, 1988). In related studies winter-dormant buds had moisture contents ranging from 48 to 60 per cent. They required desiccation to 20–30 per cent moisture to survive LN exposure. Very few tolerant species, however, could be desiccated below 10 per cent. At maximum hardiness most buds were tolerant of desiccation (Stushnoff, 1987, 1991; Tyler and Stushnoff, 1988a, 1988b). Genotypes that naturally tolerate desiccation and freezing to –30°C or colder at maximum hardiness would survive this procedure best. Cryopreserved dormant buds were either thawed slowly in room temperature air or rapidly in 40°C water (Sakai, 1985; Sakai and Nishiyama, 1978; Tyler and Stushnoff, 1988a; Tyler *et al.*, 1988). Pretreating dormant apple buds with sugars and other cryoprotectants enhanced the survival of less cold hardy taxa or those that do not sufficiently acclimate (Seufferheld *et al.*, 1991). Slow freezing below –10°C and immersion in LN without a cryoprotectant was successful with good regrowth *in vitro* after thawing for dormant-shoot tips from winter apple buds (Katano *et al.*, 1983). Dormant buds of 500 apple genotypes are stored in the vapour phase of LN at the National Seed Storage Laboratory (NSSL) in Ft Collins, Colorado (Forsline *et al.*, 1993). Single-bud sections from cold-hardened, dormant apple shoots are dried to 30 per cent moisture, cooled at 1°C/h to –30°C, held for 24 hours, and then stored in the LN vapour phase.

The first tests of cryopreservation on *in vitro* grown shoot tips of apple recovered only callus (Kuo and Lineberger, 1985). Caswell *et al.* (1986) found that high sucrose concentrations in the culture medium improved the hardiness of *in vitro* grown apple shoots, but that the cold hardening response was genotype dependent. The effects of sucrose *in vitro* were applied to *Malus* cryopreservation by Niino and Sakai (1992) who used the encapsulation–dehydration method to cryopreserve *in vitro* grown apple shoot tips and

obtained about 80 per cent regrowth after thawing. Decreases in moisture content of *in vitro* grown plants were also obtained through extended culture duration (Chang *et al.*, 1992). Plants cultured for 70 days without transfer before cold acclimatization (CA) had more shoot formation following slow freezing and LN exposure than those cultured for 35 days. These results are attributed to lower meristem moisture contents and slowed shoot growth. A freezing rate of 0.1 to 0.2°C/min was suitable for *in vitro* grown apple shoot tips (Chang *et al.*, 1992). Meristems of more than 70 cultivars are stored in LN at Changli Institute of Pomology, Hebei Academy of Agricultural and Forestry Sciences. Samples removed from LN after one month and one, two, and three years were recultured with no change in survival or plantlet regrowth (Chen *et al.*, 1994; Reed and Chang, 1997).

Vitrification is also a successful technique for *Malus* shoot tips. Apple shoot tips dehydrated with PVS2 (30 per cent glycerol, 15 per cent ethylene glycol and 15 per cent DMSO in MS medium containing 0.4 M sucrose) at 25°C for 80 minutes produced 80 per cent shoot formation following vitrification (Niino *et al.*, 1992c). Zhao *et al.* (1995) studied the effects of plant vitrification solutions PVS1 to PVS5 on apple meristems; plants treated in PVS3 (50 per cent sucrose, 50 per cent glycerol) for 80 minutes before exposure to liquid nitrogen had the best regrowth.

## *Morus*

Shoot tips of prefrozen winter buds of *Morus bombycis* Koidz. cv. Kenmochi survived immersion in liquid nitrogen, but grafts and cuttings did not survive (Yakuwa and Oka, 1988; Yokoyama and Oka, 1983). Wang *et al.* (1988) regenerated plants of *M. multicaulis* Loud. Cv. Lusang through shoot tip culture from frozen winter buds using a similar method. Niino *et al.* (1992b) demonstrated that excised shoot tips from winter buds of *M. bombycis* cv. Kenmochi, prefrozen to –20°C at 5°C/day produced more shoots than buds prefrozen at 10°C/day. Partially dehydrating the buds to about 38.5 per cent moisture content at 25°C prior to prefreezing to –20°C, improved the recovery rates. Alginate-coated, winter-hardened shoot tips of several *Morus* species had maximum shoot formation (81 per cent) when dehydrated to 22–25 per cent water content before freezing (Niino *et al.*, 1992b). Thirteen mulberry cultivars tested for cryopreservation as *in vitro* grown shoot tips produced survival ranging from 40 to 81.3 per cent with all methods tested: slow freezing (0.5°C/min to –42°C), vitrification (PVS2, 90 minutes), air-drying (24 per cent water content), and encapsulation–dehydration (33 per cent water content) (Niino, 1990, 1995; Niino *et al.*, 1992a).

## *Prunus*

Survival (70 to 80 per cent) of axillary apices excised from *Prunus persica* cv. GF 305 *in vitro*-cultured plants required pretreatment on a 5 per cent DMSO and 5 per cent proline culture medium before vitrification (Paulus *et al.*, 1993). This pretreatment medium was also effective and produced 69 per cent and 74 per cent shoot formation in two *Prunus* rootstock cultivars (Brison *et al.*, 1995). Dormant buds of ten *Prunus* species were cryopreserved with up to 100 per cent recovery by grafting (Stushnoff, 1985). *In vitro*-grown *Prunus* shoots survived cryopreservation with 75 per cent regrowth when held at –30°C for 24 hours before being transferred to LN. Culturing on 14 per cent sucrose medium and chilling at 4°C enhanced the low-temperature tolerance of cryopreserved plantlets (Stushnoff, 1985).

### *Pyrus*

Dormant hardy *Pyrus* shoots were able to survive LN after prefreezing to −40°C or −50°C (Sakai and Nishiyama, 1978). Moriguchi *et al.* (1985) found that shoot tips from dormant buds of Japanese pear required prefreezing to −40 to −70°C before being exposed to LN. Oka *et al.* (1991) and Mi and Sanada (1992, 1994) recovered whole plants from cryopreserved buds.

*In vitro* grown pear-shoot meristems were first successfully cryopreserved in 1990 (Dereuddre *et al.*, 1990a, 1990b; Reed, 1990). A slow-freezing method for *in vitro* grown pear meristems which incorporated cold acclimatization and slow cooling produced 55 to 95 per cent regrowth in cryopreserved shoot tips of four *Pyrus* species including a subtropical species, *P. koehnei* (Reed, 1990). Encapsulation–dehydration was applied to pear by Dereuddre *et al.* (1990a, 1990b). A 0.75 M sucrose preculture and four hour dehydration (20 per cent residual water) produced 80 per cent recovery (Scottez *et al.*, 1992). A modified encapsulation–dehydration method developed by Niino and Sakai (1992) produced 70 per cent shoot formation for three pear cultivars. They applied the vitrification method to pears and obtained 40 to 72.5 per cent regrowth (Niino *et al.*, 1992c; Suzuki *et al.*, 1997).

A comparison of slow freezing and vitrification methods using 28 *Pyrus* genotypes found that 61 per cent had better than 50 per cent regrowth following slow freezing (0.1°C/min), while only 43 per cent of the genotypes responded this well to the vitrification technique (Luo *et al.*, 1995).

## 10.3 Germplasm storage

Fruit and nut trees in field genebanks are at risk from severe weather, insect and animal pests, and diseases. Quarantine laws designed to prevent the spread of diseases or insects also restrict global exchange of field germplasm. Although *in vitro* cultures are not necessarily disease free, and may be virus infected or have bacterial contaminants, some countries allow *in vitro* cultures to satisfy quarantine restrictions. This makes *in vitro* collections valuable as complementary or secondary collections. Cryopreserved storage is the ultimate base storage; available for use in case of emergency but requiring little input of care or money.

### *10.3.1* In vitro *stored collections*

Collections of temperate tree fruit and nut crops are held at various experiment stations and plant breeding centres throughout the world (Ashmore, 1997; Brettencourt and Konopka, 1989). *In vitro* stored collections are sometimes used as secondary collections but may also be the primary collection. Complete listings of *in vitro* stored germplasm collections are difficult to find, but germplasm workers in many countries use *in vitro* culture for other purposes including virus elimination (Table 10.1).

### *10.3.2 Cryopreserved collections*

A few countries have initiated clonal-germplasm storage is liquid nitrogen. Research is underway in many more countries. For temperate tree fruit and nut crops, cryopreserved

**Table 10.1** Some of the world-wide germplasm related *in vitro* culture work and culture collections of temperate tree fruits and nuts

| Country | Collections[a] | Culture[b] |
|---|---|---|
| Belgium | *Prunus* | |
| Germany | *Prunus, Pyrus* | |
| Hungary | *Prunus* | |
| India | | *Prunus* |
| France | | *Prunus, Pyrus, Castanea, Juglans, Mauls* |
| Japan[c] | *Cydonia, Diospyros, Malus, Morus, Prunus, Pyrus* | |
| Portugal | *Prunus* | |
| United States | *Corylus, Cydonia, Pyrus* | *Malus, Prunus* |
| Uruguay | | *Malus, Pyrus, Prunus, Cydonia* |

[a] Brettencourt and Konopka (1989).
[b] Ashmore (1997).
[c] Personal communication (1998).

collections are held in China (*Malus in vitro* grown meristems), Japan (*Cydonia, Malus* pollen, *Malus, Morus* dormant buds) and the USA (*Corylus* embryonic axes, *Malus* dormant buds, *Pyrus in vitro* grown meristems, *Corylus, Pyrus* pollen).

### 10.3.3 Discussion: the role of storage technologies

#### In vitro *storage*

*In vitro* culture is an important tool for the international germplasm community (see Lynch, Chapter 4 this volume), but much remains to be learned about optimal *in vitro* storage conditions. Many of the factors mentioned in individual research reports require more investigation. Important data are available on the size and type of propagule stored. Barlass and Skene (1983) found that single-rooted *Vitis* shoots respond differently from proliferating cultures. For apple, single-shoot tips, nodal segments, and cultures with multiple shoots respond differently to various storage conditions (Orlikowska, 1991). Optimum age, size, and physiology must all be taken into account before cultures are stored.

Light quality and intensity are important for culture growth both before and during storage. In most cases experimentation into light effects is limited by lack of growth room availability in a facility. Data on culture storage in light versus darkness are available, but extensive information on the effects of light quality, duration, and intensity is not available for tree fruit and nut crops.

Culture conditions before storage, and culture time after subculture have important effects on storage time, but little has been done to study these conditions. The pre-storage culture period affected both storage length and the proliferation of *Prunus* rootstock cultivars following storage; 14 days was optimum (Marino *et al.*, 1985). In some apple genotypes, proliferation was better following cold storage than in non-stored plants, but this also varied with genotype (Orlikowska, 1992). Multiplication and storage media

greatly affect the survival of cultures after storage. Storage time may be improved or limited by the growth regulators in storage media. For the most part this is a genotype dependent phenomenon (Orlikowska, 1992; Reed, 1993). Research on the effects of growth regulators in storage media and the genetic stability of stored cultures is still limited (Wilkins *et al.*, 1988). Genetic analysis of *in vitro* grown and stored plants is not a standard practice; genetic instability appears to be genotype dependent and it is difficult to generalize on its causes or probabilities (Moore, 1991). Field and molecular analyses are needed to determine genetic stability (Harding, 1994; Kumar, 1995). Adventitious shoot production may be a cause of genotype variation during *in vitro* storage. Improvements in multiplication and storage media should reduce the likelihood of adventitious shoot production. Additional research is badly needed to develop standard techniques for genetic stability testing.

Contaminants are a major difficulty for any *in vitro* system. Slow-growing contaminants may persist without being noticed for long periods, then suddenly become evident during or after *in vitro* storage (Gunning and Lagerstedt, 1985). Stored cultures may die from the debilitating effects of latent infections (Wanas *et al.*, 1986). Indexing cultures for latent bacterial and fungal infections should be a standard step in germplasm storage procedures (Reed and Tanprasert, 1995). Bacteriological media used to detect cultivable contaminants are more effective than simply examining the cultures visually (Reed *et al.*, 1995; Tanprasert and Reed, 1997). Special methods are still needed to detect non-cultivable contaminants, such as obligate parasites. Healthier cultures, longer storage times, and safer materials for distribution can be assured through improved detection of bacterial contaminants. Germplasm storage in heat-sealed tissue culture bags can nearly eliminate fungal, bacterial, or insect contamination during the storage period (Reed, 1991, 1992, 1993).

## Cryopreservation

Cryopreservation techniques are now available for many forms of fruit and nut tree germplasm storage (cell suspensions, callus, shoot tips, somatic embryos, and embryonic axes). Some of these techniques are now used for long-term storage of germplasm (Reed and Chang, 1997). Future improvements in cryopreservation will require attention to several research topics. The choice of plant material is one important consideration since both growth stage and genotype affect survival following LN exposure. Response to cryopreservation techniques varies greatly with genotype; even related genotypes may have very different survival following LN exposure (Niino, 1995; Reed and Yu, 1995). The physiological status of mother plants directly impacts survival following cryopreservation (Chang *et al.*, 1992; Reed, 1988). More emphasis is needed on research into the physiological condition of plants prior to cryopreservation. Research into the physiology of cold acclimatization (CA) of cultures will be useful, especially since CA pretreatments are necessary for the success of many cryopreservation techniques. Comparison of cryopreservation techniques remains difficult due to wide variations in the temperature, light conditions, and duration of CA used in different laboratories.

Freezing rates and cryoprotectants have received much attention in the past but still require further study. Slow freezing, the first technique developed, remains an important protocol; a very slow freezing rate (0.1°C/min to −40°C) produces the best survival for *in vitro* grown shoot tips from many species including *Pyrus* and *Malus* (Chang *et al.*, 1992; Reed, 1990). Most *in vitro* systems require cryoprotectants, and highly concentrated solutions (such as PVS2) formerly used only with direct LN exposure are now used for some slow freezing and encapsulation methods (Brison *et al.*, 1995; Niino, 1995).

Combined methods are successful for cryopreserving some difficult genotypes. In a combination of the slow-freezing technique and the encapsulation–dehydration technique, encapsulated grape axillary-shoot tips were slowly cooled before being exposed to LN, significantly increasing survival and shoot formation over encapsulation alone (Plessis *et al.*, 1993). Dehydration of encapsulated dormant apple buds with a vitrification solution, followed by LN exposure was also successful (Seufferheld *et al.*, 1991). Advances in cryopreservation of difficult genotypes may result from further exploration of combined techniques. No phenotypic changes have been observed in meristem derived plants of cryopreserved plant material (Harding and Benson, 1994; Reed and Hummer, 1995). Genetic abnormalities due to cryopreservation are expected to be rare; however, more studies are needed to confirm the genetic stability of plants held in LN.

## Impact on the storage and distribution of germplasm

### *10.4.1* In vitro *storage*

The use of *in vitro* stored plants as primary or secondary collections of clonal crops reduces the land area required for field genebanks. Three replicates per genotype are considered ideal for germplasm storage and using an *in vitro* culture for one or two of these replicates greatly decreases field space and labour costs. *In vitro* cultures are not guaranteed to be pathogen free; however virus-indexed materials can be stored *in vitro* to keep them in virus-free condition. Bacterial and fungal indexing can detect cultivable contaminants and provide propagules in which requesters can have a high degree of confidence (Reed and Tanprasert, 1995; Reed *et al.*, 1995). *In vitro* cultures obtained from virus-elimination programmes and indexed for cultivable bacterial and fungal contaminants often meet phytosanitary requirements for import and export. Plants distributed as *in vitro* cultures are useful for many requesters and in many cases cultures survive international shipment better than traditionally propagated plants (Bartlett, personal communication). A large percentage of the NCGR plant material is distributed as *in vitro* plantlets. Acclimatization of these plantlets requires the same care as other *in vitro* grown materials. Each plant shipment should include information on *in vitro* growth and acclimatization procedures.

#### *Cryopreservation*

Cryopreservation is best suited for base (long-term) storage of clonal collections, (see Benson, Chapter 6, this volume for an appraisal of methodology). The greatest cost of cryopreservation is in the initial storage of an accession, but very little input is needed for many years after storage. Collections in LN require little storage space: a 40 l dewar can hold as many as 3000 sample vials (i.e., five vials for each of 600 accessions). A clonal collection with representatives in the field, *in vitro*, and in LN would provide active, backup, and base storage for an accession for less cost and greater security over the long term than three field plants. Although plants can be distributed as cryopreserved samples, they are best kept as insurance in case of loss of actively growing accessions. Plants cryopreserved at one location can be shipped to a second location in a specially designed travel dewar. *Pyrus*, *Ribes*, and *Rubus* meristems and *Corylus* embryonic axes cryopreserved in Oregon were shipped to Colorado by air freight in a travel dewar for

base storage (Reed *et al.*, 1997, 1998). For recovery the cryopreserved samples should be thawed by the recommended procedures, regrown *in vitro* into plantlets, and acclimatized to the greenhouse by techniques used for the specific plant type.

## 10.5 Conclusions

Plant conservation and germplasm exchange using *in vitro* methods have increased over the past decade, mirroring perhaps the advances in research in this field. Improved global transportation and communication have lead to a wider exchange of ideas as well as to the exchange of plant materials. More institutions are now taking advantage of improved techniques to provide *in vitro* base, primary, or secondary collections to protect their germplasm collections. The advantages of *in vitro* conservation of important plant collections are the same as in the past, both in terms of phytosanitary considerations and plant security, but the willingness of curators to provide alternative storage for crops has increased.

Further improvements to these techniques are of course always needed; research is needed to improve *in vitro* culture, storage and cryopreservation, including a myriad of aspects in each of these fields. Fortunately, *in vitro* culture and cryopreservation have progressed to the point where they can be used routinely in many laboratories. *In vitro* stored plantlets are used as primary or duplicate collections in several facilities. Cryopreserved samples for long-term (base) storage of important collections are now a reality as well. They provide an important, previously missing, form of germplasm storage (base storage) for vegetatively propagated plants.

## References

ASHMORE, S.E., 1997, *Status Report on the Development and Application of In Vitro Techniques for the Conservation and Use of Plant Genetic Resources*, p. 67, Rome: International Plant Genetic Resources Institute.

BARLASS, M. and SKENE, K.G.M., 1983, Long-term storage of grape *in vitro*, *FAO/IBPGR Plant Genetic Resources Newsletter*, **53**, 19–21.

BRETTENCOURT, E.J. and KONOPKA, J., 1989, *Directory of Germplasm Collections. 6. II. Temperate Fruits and Tree Nuts*, p. 296, Rome: International Board for Plant Genetic Resources.

BRISON, M., PAULUS, V. and DE BOUCAUD, M.T., 1991, Cryopreservation of walnut and plum shoot tips, *Cryobiology*, **28**, 738.

BRISON, M., DE BOUCAUD, M.T. and DOSBA, F., 1995, Cryopreservation of *in vitro* grown shoot tips of two interspecific *Prunus* rootstocks, *Plant Science*, **105**(2), 235–242.

CASWELL, K.L., TYLER, N.J. and STUSHNOFF, C., 1986, Cold hardening of *in vitro* apple and Saskatoon shoot cultures, *HortScience*, **21**, 1207–1209.

CHANG, Y., CHEN, S., ZHAO, Y. and ZHANG, D., 1992, Studies of cryopreservation of apple shoot tips, in *China Association for Science and Technology First Academic Annual Meeting of Youths Proceedings (Agricultural Sciences)*, pp. 461–464, Beijing: Chinese Science and Technology Press.

CHEN, S., CHANG, Y., ZHAO, Y. and ZHANG, D., 1994, Studies on the cryopreservation of *in vitro*-grown shoot tips, *Abstracts VIIIth International Congress of Plant Tissue and Cell Culture*, M-37, 274.

DE BOUCAUD, M.T. and BRISON, M., 1995, Cryopreservation of germplasm of walnut (Juglans species), in *Biotechnology in Agriculture and Forestry*, Vol. 32, edited by Y.P.S. BAJAJ, pp. 129–147, Berlin, Heidelberg: Springer-Verlag.

DE BOUCAUD, M., BRISON, M., LEDOUX, C., GERMAIN, E. and LUTZ, A., 1991, Cryopreservation of embryonic axes of recalcitrant seed: *Juglans regia* L. cv. Franquette, *Cryo-Letters*, **12**, 163–166.

DE BOUCAUD, M.T., BRISON, M. and NEGRIER, P., 1994, Cryopreservation of walnut somatic embryos, *Cryo-Letters*, **15**, 151–160.

DEREUDDRE, J., SCOTTEZ, C., ARNAUD, Y. and DURON, M., 1990a, Effects of cold hardening on cryopreservation of axillary pear (*Pyrus communis* L. cv Beurre Hardy) shoot tips of *in vitro* plantlets, *C. R. Acad. Sci. Paris*, **310**, 265–272.

DEREUDDRE, J., SCOTTEZ, C., ARNAUD, Y. and DURON, M., 1990b, Resistance of alginate-coated axillary shoot tips of pear tree (*Pyrus communis* L. cv Beurre Hardy) *in vitro* plantlets to dehydration and subsequent freezing in liquid nitrogen, *C. R. Acad. Sci. Paris*, **310**, 317–323.

DRUART, P., 1985, *In vitro* germplasm preservation techniques for fruit trees, in *In Vitro Techniques. Propagation and Long Term Storage*, edited by A. Schafer-Menuhr, pp. 167–171, Dordrecht: Martinus Nijhoff.

ECKHARD, A., 1989, Untersuchungen zur Entwicklung einer rationaellen Mehtode der in vitro-Depothaltung von Kern und Steinobst unter Kuhlbedingungen, *Archives Gartenbau*, **2**, 131–140.

FORSLINE, P.L., STUSHNOFF, C., TOWILL, L.E., WADDELL, J. and LAMBOY, W., 1993, Pilot project to cryopreserve dormant apple (*Malus* sp) buds, *HortScience*, **28**, 118.

GONZALEZ-BENITO, M.E. and PEREZ, C., 1994, Cryopreservation of embryonic axes of two cultivars of hazelnut (*Corylus avellana* L.), *Cryo-Letters*, **15**, 41–46.

GUNNING, J. and LAGERSTEDT, H.B., 1985, Long-term storage techniques for *in vitro* plant germplasm, *Proceedings International Plant Propagators Society*, **35**, 199–205.

HARDING, K., 1994, The methylation status of DNA derived from potato plants recovered from slow growth, *Plant Cell Tissue and Organ Culture*, **37**, 31–38.

HARDING, K. and BENSON, E.E., 1994, A study of growth, flowering, and tuberisation in plants derived from cryopreserved potato shoot-tips: implications for *in vitro* germplasm collections, *Cryo-Letters*, **15**, 59–66.

KARTHA, K.K., 1985, *Cryopreservation of Plant Cells and Tissues*, edited by K.K. KARTHA, p. 276, Boca Raton, Florida: CRC Press.

KATANO, M., ISHIHARA, A. and SAKAI, A., 1983, Survival of dormant apple shoot tips after immersion in liquid nitrogen, *HortScience*, **18**(5), 707–708.

KUMAR, M.B., 1995, Genetic stability of micropropagated and cold stored strawberries, MS Thesis, Oregon State University, Corvallis.

KUO, C.C. and LINEBERGER, R.D., 1985, Survival of *in vitro* cultured tissue of 'Jonathan' apples exposed to −196°C, *HortScience*, **20**, 764–767.

LUNDERGAN, C. and JANICK, J., 1979, Low temperature storage of *in vitro* apple shoots, *HortScience*, **14**, 514.

LUO, J., DENOMA, J. and REED, B.M., 1995, Cryopreservation screening of *Pyrus* germplasm, *Cryobiology*, **32**, 558.

MARINO, G., ROSATI, P. and SAGRATI, F., 1985, Storage of *in vitro* cultures of *Prunus* root-stocks, *Plant Cell Tissue and Organ Culture*, **5**, 73–78.

MI, W. and SANADA, T., 1992, Cryopreservation of pear winter buds and shoot tips, *China Fruits*, 20–22 and 38.

MI, W. and SANADA, T., 1994, Cryopreservation of pear shoot tips *in vitro*, *Advances in Horticulture*, 86–88.

MOORE, P., 1991, Comparison of micropropagated and runner propagated strawberry, *Fruit Varieties Journal*, **45**, 119.

MORIGUCHI, T., 1995, Cryopreservation and minimum growth storage of pear (*Pyrus* species), in *Biotechnology in Agriculture and Forestry*, Vol. 32, edited by Y.P.S. BAJAJ, pp. 114–128, Berlin, Heidelberg: Springer-Verlag.

MORIGUCHI, T., AKIHAMA, T. and KOZAKI, I., 1985, Freeze-preservation of dormant pear shoot apices, *Japanese Journal of Breeding*, **35**, 196–199.

MORIGUCHI, T., KOZAKI, I., YAMAKI, S. and SANADA, T., 1990, Low temperature storage of pear shoots *in vitro*, *Bulletin of the Fruit Tree Research Station*, **17**, 11–18.

NIINO, T., 1990, Establishment of the cryopreservation of mulberry shoot tips from in-vitro grown shoot, *J. Seric. Sci. Jpn.*, **59**, 135–142.

NIINO, T., 1995, Cryopreservation of germplasm of mulberry (*Morus* species), in *Biotechnology in Agriculture and Forestry*, Vol. 32, edited by Y.P.S. BAJAJ, pp. 102–113, Berlin, Heidelberg: Springer-Verlag.

NIINO, T. and SAKAI, A., 1992, Cryopreservation of alginate-coated *in vitro*-grown shoot tips of apple, pear and mulberry, *Plant Science*, **87**, 199–206.

NIINO, T., SAKAI, A., ENOMOTO, S., MAGOSI, J. and KATO, S., 1992a, Cryopreservation of *in vitro*-grown shoot tips of mulberry by vitrification, *Cryo-Letters*, **13**, 303–312.

NIINO, T., SAKAI, A. and YAKUWA, H., 1992b, Cryopreservation of dried shoot tips of mulberry winter buds and subsequent plant regeneration, *Cryo-Letters*, **13**, 51–58.

NIINO, T., SAKAI, A., YAKUWA, H. and NOJIRI, K., 1992c, Cryopreservation of *in vitro*-grown shoot tips of apple and pear by vitrification, *Plant Cell Tissue and Organ Culture*, **28**, 261–266.

NORMAH, M.N., REED, B.M. and YU, X., 1994, Seed storage and cryoexposure behavior in hazelnut (*Corylus avellana* L. cv. Barcelona), *Cryo-Letters*, **15**, 315–322.

OKA, S., YAKUWA, H., SATO, K. and NIINO, T., 1991, Survival and shoot formation *in vitro* of pear winter buds cryopreserved in liquid nitrogen, *HortScience*, **26**, 65–66.

ORLIKOWSKA, T., 1991, Effect of *in vitro* storage at 4°C on surviving and proliferation of apple rootstocks, *Acta Horticulturae*, **289**, 251–253.

ORLIKOWSKA, T., 1992, Effect of *in vitro* storage at 4°C on survival and proliferation of two apple rootstocks, *Plant Cell Tissue and Organ Culture*, **31**, 1–7.

PAULUS, V., BRISON, M., DOSBA, F. and DE BOUCAUD, M.T., 1993, Preliminary studies on cryopreservation of peach shoot tips by vitrification. *Comptes-Rendus-de-l'Academie-d'Agriculture-de-France*, **79**(7), 93–102.

PENCE, V.C., 1990, Cryostorage of embryo axes of several large-seeded temperate tree species, *Cryobiology*, **27**, 212–218.

PENCE, V.C., 1992, Desiccation and the survival of *Aesculus*, *Castanea* and *Quercus* embryo axes through cryopreservation, *Cryobiology*, **29**, 391–399.

PLESSIS, P., LEDDET, C., COLLAS, A., and DEREUDDRE, J., 1993, Cryopreservation of *Vitis vinifera* L. cv. Chardonnay shoot tips by encapsulation-dehydration: effects of pretreatment, cooling and postculture conditions, *Cryo-Letters*, **14**, 308–320.

REED, B.M., 1988, Cold acclimation as a method to improve survival of cryopreserved *Rubus* meristems, *Cryo-Letters*, **9**, 166–171.

REED, B.M., 1990, Survival of *in vitro*-grown apical meristems of *Pyrus* following cryopreservation, *HortScience*, **25**, 111–113.

REED, B.M., 1991, Application of gas-permeable bags for *in vitro* cold storage of strawberry germplasm, *Plant Cell Reports*, **10**, 431–434.

REED, B.M., 1992, Cold storage of strawberries *in vitro*: a comparison of three storage systems, *Fruit Varieties Journal*, **46**, 98–102.

REED, B.M., 1993, Improved survival of *in vitro*-stored *Rubus* germplasm, *Journal of the American Society of Horticultural Science*, **118**, 890–895.

REED, B.M. and CHANG, Y., 1997, Medium- and long-term storage of *in vitro* cultures of temperate fruit and nut crops, in *Conservation of Plant Genetic Resources In Vitro*, Vol. 1, edited by M.K. RAZDAN and E.C. COCKING, pp. 67–105, Enfield, NH, USA: Science Publishers, Inc.

REED, B.M. and HUMMER, K., 1995, Conservation of germplasm of strawberry (*Fragaria* species), in *Biotechnology in Agriculture and Forestry, Cryopreservation of Plant Germplasm I*, Vol. 32, edited by Y.P.S. BAJAJ, pp. 354–370, Berlin: Springer-Verlag.

REED, B.M. and TANPRASERT, P., 1995, Detection and control of bacterial contaminants of plant tissue cultures. A review of recent literature, *Plant Tissue Culture and Biotechnology*, **1**, 137–142.

REED, B.M. and YU, X., 1995, Cryopreservation of *in vitro*-grown gooseberry and currant meristems, *Cryo-Letters*, **16**, 131–136.

REED, B.M., NORMAH, M.N. and YU, X., 1994, Stratification is necessary for successful cryopreservation of axes from stored hazelnut seed, *Cryo-Letters*, **15**, 377–384.

REED, B.M., BUCKLEY, P.M. and DEWILDE, T.N., 1995, Detection and eradication of endophytic bacteria from micropropagated mint plants, *In Vitro – Plant*, **31P**, 53–57.

REED, B.M., DENOMA, J., LUO, J., CHANG, Y. and TOWILL, L., 1997, Cryopreserved storage of a world pear collection, *In Vitro Cellular and Developmental Biology*, **33**, 51A.

REED, B.M., PAYNTER, C.L., DENOMA, J., and CHANG, Y., 1998, Techniques for medium and long-term storage of pear (*Pyrus*) genetic resources, *FAO/IPGRI Plant Genetic Resources Newsletter*, International Plant Genetic Resources Institute, Rome.

SAKAI, A., 1985, Cryopreservation of shoot-tips of fruit trees and herbaceous plants, in *Cryopreservation of Plant Cells and Tissues*, edited by K.K. KARTHA, pp. 135–170, Boca Raton, Florida: CRC Press.

SAKAI, A., 1993, Cryogenic strategies for survival of plant cultured cells and meristems cooled to −196°C, in *Cryopreservation of Plant Genetic Resources*, Vol. 6, pp. 5–26, Tokyo: Japan International Cooperation Agency.

SAKAI, A. and NISHIYAMA, Y., 1978, Cryopreservation of winter vegetative buds of hardy fruit trees in liquid nitrogen, *HortScience*, **13**, 225–227.

SCOTTEZ, C., CHEVREAU, E., GODARD, N., ARNAUD, Y., DURON, M. and DEREUDDRE, J., 1992, Cryopreservation of cold acclimated shoot tips of pear *in vitro* cultures after encapsulation-dehydration, *Cryobiology*, **29**, 691–700.

SEUFFERHELD, M.J., FITZPATRICK, J., WALSH, T. and STUSHNOFF, C., 1991, Cryopreservation of dormant buds from cold tender taxa using a modified vitrification procedure, *Cryobiology*, **28**, 576.

SHARMA, K.K. and THORPE, T.A., 1990, *In vitro* propagation of mulberry, *Scientia Horticulturae*, **42**, 307–320.

STUSHNOFF, C., 1985, Cryopreservation of *in vitro* shoots from *Prunus pennsylvanica* and *Prunus fruticosa*, *FAO/IBPGR Plant Genetic Resources Newsletter*, **51**, 48.

STUSHNOFF, C., 1987, Cryopreservation of apple genetic resources, *Canadian Journal of Plant Science*, **67**, 1151–1154.

STUSHNOFF, C., 1991, Strategies for cryopreservation based on tissue hydration, *Cryobiology*, **28**, 745–746.

STUSHNOFF, C. and SEUFFERHELD, M., 1995, Cryopreservation of apple (*Malus* species) genetic resources, in *Biotechnology in Agriculture and Forestry*, Vol. 32, edited by Y.P.S. BAJAJ, pp. 87–101, Berlin, Heidelberg: Springer-Verlag.

SUZUKI, M., NIINO, T., AKIHAMA, T. and OKA, S., 1997, Shoot formation and plant regeneration of vegetative pear buds cryopreserved at −150 degrees C, *Journal of the Japanese Society of Horticultural Science*, **66**, 29–34.

TANPRASERT, P. and REED, B.M., 1997, Detection and identification of bacterial contaminants from strawberry runner explants, *In Vitro Cellular and Developmental Biology – Plant*, **33**, 221–226.

TYLER, N. and STUSHNOFF, C., 1988a, Dehydration of dormant apple buds at different stages of cold acclimation to induce cryopreservability in different cultivars, *Canadian Journal of Plant Science*, **68**, 1169–1176.

TYLER, N.J. and STUSHNOFF, C., 1988b, The effects of prefreezing and controlled dehydration on cryopreservation of dormant vegetative apple buds, *Canadian Journal of Plant Science*, **68**, 1163–1167.

TYLER, N., STUSHNOFF, C. and GUSTA, L.V., 1988, Freezing of water in dormant vegetative apple buds in relation to cryopreservation, *Plant Physiology*, **87**, 201–205.

WANAS, W.H., CALLOW, J.A. and WITHERS, L.A., 1986, Growth limitations for the conservation of pear genotypes, in *Plant Tissue Culture and its Agricultural Applications*, edited by L.A. WITHERS and P.G. ALDERSON, pp. 285–290, London: Butterworths.

WANG, L., ZHANG, X. and YU, Z., 1988, Cryopreservation of winter mulberry buds, *Acta Agriculturae Boreali-Sinica*, **3**, 103–106.

WILKINS, C.P., NEWBURY, H.J. and DODDS, J.H., 1988, Tissue culture conservation of fruit trees, *FAO/IBPGR Plant Genetic Resources Newsletter*, **73/74**, 9–20.

YAKUWA, H. and OKA, S., 1988, Plant regeneration through meristem culture from vegetative buds of mulberry (*Morus bombycis* Koidz) stored in liquid nitrogen, *Annals of Botany*, **62**, 79–82.

YOKOYAMA, T. and OKA, S., 1983, Survival of mulberry winter buds at super low temperatures, *J. Seric. Sci. Jpn.*, **52**, 263–264.

ZHAO, Y., CHEN, S., WU, Y., CHANG, Y. and ZHANG, D., 1995, Cryopreservation of *in vitro* shoot tips of apple by vitrification, in *China Association for Science and Technology Second Academic Annual Meeting of Youths Proceedings (Horticultural Sciences)*, pp. 406–409, Beijing: Beijing Horticultural University Press.