IN VITRO PRESERVATION OF TEMPERATE FRUIT CROPS
- A REVIEW

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ABSTRACT

Using cold hardening pretreatments has enhanced successful temperate crop plant cryopreservation methods. The potential of plant cryopreservation can only be fully exploited by effective technology transfer to genebanks and culture collection. While some cryopreservation methods are dependent upon the use of relatively expensive and specialist cryogenic facilities, others involve simplified methods, which are suitable, for use in less well resourced laboratories. Cryopreservation is now an accessible conservation option for a wide range of users.

The vast germplasm of horticultural crops has to be conserved using improved method of tissue culture. In vitro preservation has several advantages over conventional conservation such as less demand for land and manpower and low exposure of axenic culture to crop borne diseases and pests. The maintenance of plants in standard culture conditions provide a ready source of germplasm for propagation. However, the rapid growth rate achieved through in vitro culture, leading to labor-intensive tasks such as sub culturing makes this an expensive technique for maintenance of endangered species on a long-term basis. Further more repeated sub culturing may lead to loss of cultures due to handling errors, contamination or undesirable somaclonal variations.

The most common method of reducing growth of in vitro cultures is lowering the temperature. The desired temperature depends upon the growth rate of cultures and their tolerace to low temperature. This technique is useful for short to medium term storage. However, the phenomenon of somaclonal variation resulting in chromosomal changes may threaten the success of conservation.

Cryostorage is the storage of biological material at super low temperature (-130 to -196°C). Liquid nitrogen is the most common medium for cryostorage as it is relatively inexpensive and readily available. At these temperatures all metabolic activities in tissues have ceased and tissues can be stored in liquid nitrogen for longtime without loss of viability or genetic fidelity. This technique minimized the genetic drift and labor intensive tasks such as sub culturing. Successful cryostorage technique have been developed for a number of horticultural crops (Wu et al., 1999; Reed et al., 1998; Bajaj, 1981; Schafer et al., 1994; Engelmann et al., 1997).

Successful cryoconservation methods have been developed for temperate fruits viz, apple, pear, peach, kiwifruit, strawberry, currents and temperate nuts using cold hardening pretreatments (Benson et al., 1998).

Apple: The low-temperature storage of apple shoots in vitro has been investigated by Lundergan and Janick (1979), Malus domestica Borkh. cv. Golden Delicious shoot tips have been cultivated on MS medium supplemented with 0.3 μM thiamin-HCl, 2.4 μM pyridoxine, 4.0 μM nicotinic acid, 0.3 mM glycine, 22 μM BA, 0.087 M sucrose and 10 g/1 agar. After 12 months storage, there was a survival of 100% at 1°C, 70% at 4°C. At the low temperatures the average number of shoot per culture tube increased with storage time. In vitro-grown shoot tips of apples (Malus domestica Borkh. cv. Fuji) were successfully cryopreserved by vitrification. After rapid
warming, the shoot tips were expelled into 2 ml. of MS medium containing 12-M sucrose and plated on agar MS medium. The shoot elongation and shoot formation (80%) was observed in 3 weeks by Niino et al. (1992). Mi (1993) developed protocol for super low temperature cryopreservation of apple pear, Prunus avium. Mi et al. (1995) reported survival rates of apple shoots of several cultivar cultured on medium containing 1.5, 3 and 6% sucrose were 80, 85.7 and 46.2% respectively. When shoots were transferred to MS medium containing 2.5% dimethyl sulfoxide for 4 days after hardening at low temperature, the survival rate was 90%. Shoot tips of Malus domestica and M. robusta were cold hardened at 5°C for 3 weeks (Wu and Wu, 1997). They also observed that after preculturing, shoot-tips were encapsulated with 3% sodium alginate and 100 mm CaCl₂. The beads of 4-5 mm diameter were dehydrated in airflow for 4 h and preserved in liquid nitrogen. Regeneration rate after thawing in a water bath at 25°C and culture on MS media was more than 70% (Wu and Wu, 1997). Dereuddre et al. (1994) found high recovery rates of frozen apple (Malus pumila cv. Golden Delicious) shoot tips obtained after cold hardening of in vitro donor plants. In the encapsulation-dehydration procedure, shoot-tips were encapsulated in alginate beads, cooled in liquid nitrogen and warmed slowly in air.

Dormant shoot tips of apple cv. Golden Delicious were successfully cryopreserved by using 3 different methods of vitrification, encapsulation dehydration and 2 step freezing, with the regeneration rate reaching upto 83%. After pretreatment in vitrifcation solution (PVS3: 50: glycerol+50% sucrose) for 80 minute at 25°C, by using the encapsulation-dehydration method, the shoot tips could be immersed in liquid nitrogen directly for cryopreservation (Wu et al. 1999). Forsline et al. (1998) reported cryopreservation of 64 apple and other Malus accessions in liquid nitrogen using a dormant vegetative bud method. These buds were grafted on to rootstock to determine survival. Mean recovery was 76% for 40 cold-hardy accessions, 66% for 20 moderately cold-hardy accessions, and 24% for cold-tender accessions.

Pear: In the cryopreservation of pear, shoot tips were frozen in liquid nitrogen, survival was 100% in Flemish beauty, 92% in Mishirazes, 75% in Chojuro and 17% in Bartlett were observed by Suzuki et al. (1998). Auxiliary shoot-tips of pear (cv. Beurre Hardy) were encapsulated in alginate beads, cultured in a medium enriched with sucrose dried in sterile air flow and cooled in liquid nitrogen cold acclimation in in vitro culture for 8-12 weeks at 0°C greatly improved shoot recovery from cryopreserved shoot tips (Scottez et al., 1992). Mi et al. (1994) reported cryopreservation of pear varieties, with a survival rate of 100% and regeneration rate of 25%. Dereuddre et al. (1994) found high recovery rates of frozen pear (Pyrus communis cv. Beurre Hardy) shoot tips obtained after cold hardening of in vitro donor plants. In the encapsulation-dehydration procedure, shoot tips were encapsulated in alginate beads, cooled in liquid nitrogen and warmed slowly in air. Koh et al. (1995) stored pollen of Pyrus pyrifolia cv. Hosi for one year at -20°C in air (72%) or the organic solvent benzene and xylene (both 76%) at the same temperature. The pear germplasm may now be cryopreserved for long periods (> 100 years) utilizing slow freezing or vitrification of in vitro-grown shoot tips (Reed et al., 1998). They also observed that in vitro cultures of pear were stored under refrigerated temperatures for 8 months to 4.7 years, with mean storage time of 2.75 years. Shoot apices of more than 50 accessions were stored in liquid nitrogen (Reed et al., 1998 a).

Plum: Turmanov et al. (1968) investigated freezing survival of plum callus on white or MS medium. They cultivated callus tissues at
26°C and varied the sucrose content for 10 days from 2 - 15%. Calluses were tested at different temperature regime (-4 to -50°C). At low sugar concentrations callus survived at -25°C, at the high concentrations callus survived at -35 to -40°C.

Peach: The best survival rate (80-95%) of cryopreserved virus free peach (cv. GF305) shoot tips (alginate bead) was obtained with cryoprotectant PVS2 and by supplementing the preculture medium with 5% DMSO and 5% proline followed by rapid freezing (Paulus et al., 1993; Boucaud et al., 1994 and 1996). Helliot et al. (1997) reported cryopreservation of Prunus root stock by slow pre-freezing method. In vitro grown plantlets of Prunus root stock cv. Ferlenain-pluming were acclimated at 4°C combined with a 24 h apex preculture at 4°C in MS medium containing 5% dimethyl sulfoxide and 20% proline. Cryoprotection took place for 45 minutes at 4°C in modified PVS2 medium containing 5% proline, followed by a pre-freezing temperature of -40°C and a cooling rate of 5°C/min. The high rate of survival (50-60%) of Prunus root stock. ‘Ferlenin’ was achieved when shoot tips were pre cultured on solid medium supplemented with 5% DMSO and 20% proline at 4°C for 24 h, shoot tips were treated with 1 ml of modified PVS2 cryoprotective solution for 45 min at 4°C. Cryotubes were frozen at 5°C/min down -40°C and immersed in liquid nitrogen. After rapid thawing at 40°C, shoot tips were rinsed with 1.2 M sucrose solution, plated on standard solid medium, maintained for 2-3 days in the dark (Helliot et al., 1997a; Brison et al., 1997 and 1997 a).

Walnut: The cryopreservation of pollens of Juglans regia (walnut) was satisfactory for cultivars Adames and Chandles (with germination percentage virtually unchanged after 12 months) but not for Hartley and Jehma whose pollens were frozen in liquid nitrogen. All pollens with moisture content between 4-7.5% survived (Luza and Polito, 1985 and 1988). Reed et al. (1994) suggested that stratification of stored hazelnut (Corylus avellana) seed improved shoot production from both control and cryopreserved isolated embryonic axes. Boucaud et al. (1994 a) reported that cryopreservation of 1-2 mm isolated somatic embryos of Juglans regia x J. nigra was achieved after preculture on DKW medium supplemented with 5% DMSO and 0.5% proline. Gonzale and Perou (1994) reported that embryonic axes of two-hazelnut cultivar (Morell and Butles) were cryopreserved in liquid nitrogen. Highest recovery was achieved at 12.1% moisture content in Morell and at 11% moisture content in Butles.

Strawberry: Sterile plantlets of Fragaria annanasis were grown on filter paper with 2.5 ml cryoprotectant solution in test tube and stored in refrigerator at 1 and 4°C. The material was checked every 3 months and 1-2 drops of the solution were added to those cultures showing evidence of desiccation. The genotypes could be stored up to 6 years. Storage at 1°C yielded better results than at 4°C (Mullin and Schlegel, 1976). Strawberry meristems were isolated from plantlets propagated in vitro and precultured on MS with 10 μMBA, supplementing the cryoprotectants (5% DMSO or 5% glycerol). Stepwise freezing was done to -40°C with additional storage in liquid nitrogen or rapid freezing by plunging into liquid nitrogen. Prefreezing treatment with glycerol resulted in a lower survival rate than cells with 5% DMSO as a cryoprotectant. After 1 week storage in liquid nitrogen, the plant survival and regeneration was 95%, but after 2-8 weeks survival was reduced to 50-60% (Kartha et al., 1980). Reed (1992) reported strawberry plantlets which were cold hardened and stored at 4°C in Gelrite medium in polyethylene bags showed a greater average longevity. Navatél et al. (1997) developed efficient method of strawberry (cv. Chandles, Elsanta and Gento) cryopreservation. In vitro cultured plants grown on a multiplication medium and hardened for
2 months in cold storage. Axillary buds were encased in sodium alginate, precultured for 18 h in 0.75 M sucrose, dried on silicagel for 6-7 h and put into liquid nitrogen. After rewarming, rehydration and regrowth were stimulated by replacing the buds on a DKW medium supplemented with 0.1 mg BAP+0.01 mg IBA/litre. The regeneration rates were 65-73% for Chandles, 48-86% for Elsanta and 26-68% for Gento. Hirans et al. (1988) found that alginate coated meristems from in vitro-grown strawberry (Fragaria ananassa) cultivars were successfully cryopreserved and found encapsulated meristems remained green and than developed shoots within 1 week after planting without intermediary callus formation. Vysoetskaya et al. (1999) suggested that the use of glucose over sucrose in cryopreservation of strawberry meristem. Shoot apices were placed into ampulses filled with DMSO and sugar solutions with ice crystals and frozen, after storage in liquid nitrogen for 1 h -1.5 years, apices were thawed at 40°C and than recultured at 20°C on media supplemented with cytokinin (BA 0.5-1 mg/l) and sucrose or glucose (3%), plants were regenerated in these cultures.

Kiwi: Cryopreservation method of kiwi fruit (Actinidia deliciosa) cv. Hayward has been developed by Hakozaki et al. (1996), which involved culturing calluses in medium containing 24% or 41% sucrose for 2 days followed by dehydration twice. First for 22 minutes with 60% PVS2 solution (30% glycerol), 15% DMSO, 15% ethylene glycol and 13.7% sucrose and then for 23 minutes with 100% PVS2 solution. Following dehydration calluses were stored in liquid nitrogen. Suzuki et al. (1997) suggested that abscisic acid increase the viability of shoot tips of kiwi fruit seedling during cryopreservation. Non hardened shoot tips were pretreated on media containing ABA for 5 or 10 days or proline for 1.5 of 10 days. Shoot tips were encapsulated into calcium alginate and immersed into liquid nitrogen for 1 h. The highest survival rate (30-83.5%) was obtained at various growth stages, cold hardened for 4-6 weeks at 5°C and precultured on agar media containing high sucrose and using an alginate encapsulation dehydration method (Suzuki et al., 1996). Lateral buds excised from in vitro culture of Actinidia arguta were subjected to pretreatments in freezing in liquid nitrogen. A high survival rate was obtained after freezing and thawing in buds cold acclimated at 5°C for 4 weeks followed by 0°C for 2 weeks (Liu et al., 1998).

Cherry: Reed and Lagested (19° C) observed that shoot tips of Rubus frozen slowly to -40°C and then rapidly to -196°C in presence of cryoprotectant (mixture of polyethylene glycol, glucose and DMSO). Blackberry were precultured for 24 h with 1% Bovine serum albumin (BSA) in Heller’s liquid medium before adding 15% DMSO and cooling to 0°C. The suspension was cooled to -40°C and placed in liquid nitrogen for 5 months. TTC test showed 80% survival one day after thawing (Lett and Schmitt, 1992). Suzuki (1993) reported cryopreservation of Japanese flowering cherry (Prunus jedoensis Matsum) shoot tips in liquid nitrogen. In vitro-grown shoot tips of Prunus jamasakura cv. Sendaijies were successfully cryopreserved by one step vitrification. After preculturing of 3 mm shoot tips on MS medium and transferred to a 2 ml plastic cryotube. After treatment with PVS2 at 25°C for 105 minutes, the shoot tips were directly plunged into liquid nitrogen. After rapid thawing, the shoot tips were plated on agar medium. The survival rates was about 80% regardless of the length of storage in liquid nitrogen (Nino et al., 1997).

Currants: Apical meristem of Ribes aureum, R. diacantha and R. rubrum were successfully cryopreserved. Slow freezing at 0.3 or 0.5°C/min following pregrowth on 5% DMSO and cryoprotection with PGD produced moderate to high survival vitrification in PVS2 following pregrowth on sorbital and 20 mm
pretreatment resulted in low to moderate survival, while pre-growth on 5% DMSO improved survival of few genotype. Shoot tips of *Ribes nigrum* (Currents) were capable of surviving and regenerating new shoots following cryopreservation. Recovery responses were significantly enhanced when vitrification methods of cryopreservation were used (Benson et al., 1996). Suzuki et al. (1997a) reported that vegetative winter buds of p<ar cv. Beurred' Amanlis were successfully cryopreserved vegetative buds on young seedling also induced normal shoot growth.

REFERENCES