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CRYOPRESERVATION OF APPLE DORMANT BUDS AND SHOOT TIPS

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Summary

Shoot tips excised from apple (*Malus domestica* Borkh. cv. Golden Delicious) dormant buds immediately before freezing were successfully cryopreserved using three different methods (vitrification, encapsulation-dehydration and two-step freezing). Two-step freezing produced the highest *in vitro* growth recovery (83%) of cryopreserved shoot tips, followed by vitrification (60%) and encapsulation-dehydration (33%). By contrast, when dormant buds were cryopreserved using the two-step freezing technique, shoot tips excised from buds after rewarming and cultured *in vitro* displayed only 16% recovery.

Keywords: *Malus domestica*; dormant buds; shoot tips; cryopreservation.

INTRODUCTION

There is an increasing number of cases where cryopreservation is being used routinely for plant genetic resources conservation in the genebank context. Examples can be found notably with various temperate fruit trees including apple (2), mulberry (4) and pear (8).

In the case of apple, both dormant buds sampled from trees in field collections and shoot tips sampled from *in vitro* plantlets have been used for cryopreservation (11). Dormant buds of taxa which naturally tolerate desiccation and freezing to -30°C or colder at maximum hardiness are cryopreserved by prefreezing at a slow rate (1-2°C/h) to -20 or -30°C and holding at this temperature for 12-24 hours, during which freeze-induced desiccation of meristematic tissues occurs, followed by immersion in liquid nitrogen. With taxa which are less cold-hardy or do not acclimate sufficiently, survival of dormant buds has been achieved using both a modified vitrification procedure (10) and an encapsulation-dehydration procedure (11). In the case of *in vitro* shoot tips, various cryopreservation techniques have been investigated, including two-step freezing, vitrification, encapsulation-dehydration (see 7 for a review) and, more recently, encapsulation-vitrification (5).

The Changli Institute of Pomology holds a collection of temperate fruit tree germplasm which includes 1,092 accessions from 12 different species. This collection is maintained as whole plants in the field. Around 250 accessions are also duplicated *in vitro* and stored under

slow growth conditions. Research on the development of cryopreservation techniques has been initiated several years ago in the Institute, using as test material shoot tips sampled on apple *in vitro* cultures. As a result, various techniques, including two-step freezing, encapsulation-dehydration, vitrification and droplet freezing have been adapted to shoot tips sampled on *in vitro* plantlets (14, 15, 16) and applied to 11 apple cultivars. The studies performed in the Institute have also highlighted the importance of the physiological state of the mother plants to improve survival after cryopreservation.

To move forward with the utilization of cryopreservation for long-term conservation of apple germplasm, the Changli Institute of Pomology has decided to test the possibility of using dormant buds in addition to *in vitro* shoot tips. This paper reports on the preliminary cryopreservation experiments performed with dormant buds of apple cultivar Golden Delicious. The cold hardiness of this cultivar is very low, thus making it difficult to cryopreserve (1). Two different strategies were compared: 1) cryopreservation of shoot tips excised from sterilized dormant buds immediately before freezing using different techniques, followed by *in vitro* growth recovery of shoot tips; 2) cryopreservation of whole buds followed by sterilization of whole buds after rewarming, excision of shoot tips and *in vitro* growth recovery.

MATERIALS AND METHODS

Plant material

One-year old twigs with mature dormant buds were collected in late February 1999 from 20-years old apple (*Malus domestica* Borkh. cv. Golden delicious) trees growing in field plots of the Changli Institute of Pomology. The average temperature in Changli was -7.7°C in January and -3°C in February. Twigs, which had an average moisture content of around 50% (fresh weight basis) were cut into approximately 15 cm-long pieces which were enclosed in polyethylene bags and stored in a refrigerator at 5°C for no longer than one week, until they were used for freezing experiments.

Twigs were cut into smaller pieces (about 5-8 mm long) each with one bud, and sterilized as follows. After removing one or two layers of external scales on the buds, these smaller pieces were washed with tap water, immersed in 75% ethanol for 30 s, rinsed once with sterile water, immersed in a 0.1% (w/v) $HgCl_2$ solution containing 0.1% Tween 20 for 10 min and rinsed several times in sterile water. Shoot tips (approximately 2 mm long) were excised from the sterilized buds.

Cryopreservation

Two-step freezing: shoot tips were placed in 2 ml cryotubes (20 shoot tips/tube) with 1.5 ml cryoprotective solution A (liquid culture medium + 5% DMSO + 5% sucrose), B (liquid culture medium + 10% DMSO + 10% sucrose) or C (liquid culture medium + 50% glycerol + 50% sucrose) and kept for 1 h at 0°C. All cryoprotectant concentrations are expressed on a w/v basis. Solution C corresponds to the PVS3 vitrification solution (5). Samples were frozen in a programmable freezer (HIT-4, China) at 0.1°C/min from 0°C to -10°C, held at this temperature for 15 min, then cooled at the same rate to prefreezing temperatures ranging between -10 and -40°C before immersion in liquid nitrogen.

For cryopreservation of buds, small twig pieces with one bud (about 5-8 mm long) were placed in 2 ml cryotubes (4-5 twigs/tube) with 1.0 ml cryoprotective solution C, kept at 0°C for 1 h, prefrozen at 1°C/min to -40°C and immersed in liquid nitrogen.

Vitrification: shoot tips were placed in 2 ml cryotubes (20 shoot tips/tube) with 1.5 ml cryoprotective solution C (liquid culture medium + 50% glycerol + 50% sucrose), pretreated

for 80 min at 25°C, then immersed rapidly in liquid nitrogen. During pretreatment, the cryoprotective solution was renewed every 20 min.

Encapsulation-dehydration: shoot tips were precultured at 5°C in liquid media with daily increasing sucrose concentrations (0.1/0.3/0.7M). They were then encapsulated in 3% calcium alginate beads containing 0.5 M sucrose. Beads (about 5 mm in diameter) containing one shoot tip were cultured for 24 h at 5°C in liquid medium with 1.0 M sucrose, dehydrated under the laminar air flow cabinet for 4 h (down to around 30% moisture content, fresh weight basis) and immersed directly in liquid nitrogen.

Rewarming and growth recovery: after storage in liquid nitrogen for at least 1 day, shoot tips were thawed in a water-bath thermostated at 25°C and transferred on MS medium (3) containing 0.5mg/l benzylaminopurine (BAP), 0.05mg/l naphthaleneacetic acid (NAA), 5 g/l agar and 20 g/l sucrose. Cultures were placed at 25±2°C, under a 16 h light/8 h dark photoperiod with white fluorescent light (52 μmol m^{-2} s^{-1}). Twig pieces were thawed in a water-bath thermostated at 25°C and sterilized as described previously. Apices were then extracted from the buds and cultured under the conditions described above.

Recovery of shoot tips was measured after 40 days. Shoot tips that had formed green leaves were considered surviving. All surviving shoot tips gave rise to whole *in vitro* plantlets. The results presented in this paper are the average of two or three independent experiments performed with 20 shoot tips per treatment. Contaminated cultures (which reached up to 50% in several experiments) are not included in the results.

RESULTS

Effect of cryoprotective solution employed during slow freezing of shoot tips

Table 1. Effect of cryoprotective solution employed during slow freezing (0.1°C/min to -40°C) on recovery (mean % ± SD) of pretreated, prefrozen (-LN) and cryopreserved (+LN) apple (*Malus domestica* Borkh. cv. Golden Delicious) shoot tips. Cryoprotective solutions consisted of standard liquid medium (Control) + (A) 5% DMSO + 5% sucrose; (B) 10% DMSO + 10% sucrose; (C) 50% glycerol + 50% sucrose.

	Recovery (%)		
	Pretreatment	-LN	+LN
Control	100	0	0
A	100	0	0
B	98±2	16±12	16±2
C	100	85±3	83±6

Shoot tips did not withstand freezing to -40 or -196°C without cryoprotective treatment (Table 1). When a cryoprotective treatment was applied, no recovery was achieved with cryoprotective solution A and only limited recovery (16%) was noted after prefreezing and cryopreservation with cryoprotective solution B. Recovery increased dramatically after prefreezing (85%) and cryopreservation (83%) with cryoprotective solution C.

Effect of prefreezing temperature on shoot tip recovery

In all cases, no recovery was achieved when shoot tips were frozen immediately after pretreatment (0°C, Table 2). After pretreatment with cryoprotective solution B, recovery of prefrozen shoot tips decreased in line with decreasing prefreezing temperatures and after cryopreservation, limited recovery (16%) was obtained only after prefreezing to -40°C. When shoot tips were cryoprotected with solution C, survival remained very high after prefreezing and it increased progressively after cryopreservation from 5% (-10°C) to 75% (-40°C).

Table 2. Effect of prefreezing temperature and cryoprotective solution on recovery (mean % ± SD) of prefrozen (-LN) and cryopreserved (+LN) apple (*Malus domestica* Borkh. cv. Golden Delicious) shoot tips. Shoot tips were cryopreserved using the two-step freezing method (freezing rate 0.1°C/min). Cryoprotective solutions consisted of standard liquid medium + (B) 10% DMSO + 10% sucrose; (C) 50% glycerol + 50% sucrose.

	Prefreezing temperature							
	0°C		-10°C		-20°C		-40°C	
Cryoprotective solution	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
B	100	0	100	0	85±5	0	40±14	16±12
C	100	0	85±3	5±2	80±4	38±5	83±2	75±3

Effect of cryopreservation method on shoot tip recovery

Shoot tips were successfully cryopreserved using the three methods investigated, vitrification, encapsulation-dehydration and two-step freezing (Table 3). Two-step freezing was the most efficient, ensuring 83% recovery, followed by vitrification (60%) and encapsulation-dehydration (33%).

Table 3. Effect of cryopreservation technique on recovery (mean % ± SD) of pretreated (-LN) and cryopreserved (+LN) apple (*Malus domestica* Borkh. cv. Golden Delicious) shoot tips. For two-step freezing, the cryoprotective solution C (standard liquid medium + 50% glycerol + 50% sucrose) was employed.

Cryopreservation technique	Recovery (%)	
	-LN	+LN
Vitrification	100	60±11
Encapsulation-dehydration	100	33±5
Two-step freezing	100	83±3

Cryopreservation of dormant buds and shoot tips

Recovery was achieved after prefreezing and cryopreservation of both shoot tips and dormant buds (Table 4). However, a dramatic drop in recovery percentage of shoot tips excised from dormant buds was noted after prefreezing, with only 29% recovery. Recovery of shoot tips sampled from dormant buds decreased to 16% after cryopreservation. By contrast, high recovery was obtained with shoot tips after prefreezing (86%) and cryopreservation (70%).

Table 4. Recovery (mean % ± SD) of control, prefrozen (-LN) and cryopreserved (+LN) shoot tips frozen directly (shoot tips) or excised from dormant buds after freezing (buds) of apple (*Malus domestica* Borkh. cv. Golden Delicious). Shoot tips and dormant buds were cryoprotected with solution C (standard medium + 50% glycerol + 50% sucrose), then cryopreserved using the two-step freezing method (freezing rate 0.1°C/min to -40°C).

Material	Recovery (%)		
	Control	-LN	+LN
Shoot tips	93±7	86±5	70±5
Buds	100	29±13	16±3

DISCUSSION

In the present work, regrowth of shoot tips excised from dormant buds before freezing could be achieved using the three different techniques tested, two-step freezing, vitrification and encapsulation-dehydration. Regrowth of shoot tips was highest after two-step freezing, whereas in experiments performed with apices excised from *in vitro* material, even though regrowth reached comparable levels, the best results were achieved using the encapsulation-dehydration technique (12). This might be due to differences in physiological and physical characteristics of the plant materials *in vivo* and *in vitro*, including initial water content, intracellular concentration of cryoprotective compounds such as sugars, size and structure of explants, etc.

If regrowth of shoot tips sampled from cryopreserved dormant buds was successful, the highest regrowth percentage achieved was much lower than with cryopreserved shoot tips, reaching only 16% with buds, against 83% with shoot tips. This might be due to the too high water content of the buds employed in this study. Indeed, the average water content of the twigs collected from the field was around 50%, whereas Tyler *et al.* (13) have indicated that optimal moisture contents for cryopreservation of apple dormant buds are generally between 20 to 30% (fresh weight basis). These authors suggest different procedures to dehydrate apple buds including exposure of twigs to 0 to -4°C in a temperature-controlled freezer, using a fan to circulate the cold air to remove moisture while avoiding dehardening, or to low relative humidity over various saturated salt solutions in hermetic containers at 0 to -4°C. Another possibility could be to increase the duration of bud pretreatment with solution C. New experiments should be performed to test the efficiency of such procedures.

Freezing of shoot tips excised from dormant buds before cryopreservation was very efficient since high regrowth percentages were consistently achieved throughout the experiments. Lower survival was achieved using the encapsulation-technique, possibly because some detrimental ice crystal formation still took place during freezing in apices with a 30% moisture content. In the two-step freezing protocol experimented, the PVS3 solution was used as cryoprotective solution. Therefore, formation of extracellular ice could not take place during prefreezing, and slow freezing in presence of the PVS3 vitrification solution corresponds in fact to a vitrification procedure.

High contamination rates were obtained when freezing shoot tips, which led to discarding up to 50% of the cultures in some experiments. Such a high contamination level is not acceptable in a genebanking context. The sterilization protocol should thus be drastically improved or it might be preferable to use whole buds desiccated to the optimal moisture content for cryopreservation, to avoid the difficulties linked with *in vitro* culture of shoot tips. These buds should be grafted on rootstocks after rewarming, following the methodology employed by various researchers (8, 11).

In conclusion, additional experiments should be performed to optimize the water content of buds before freezing and to improve regeneration procedures after rewarming, before cryopreservation of dormant buds can be routinely employed for long-term conservation of apple germplasm at the Changli Institute of Pomology. The cryopreserved apple germplasm collection which will be established in a near future will include both dormant buds sampled from *in vivo* material and shoot tips sampled on *in vitro* material. The interest of storing other materials such as seeds and pollen for gene conservation will also be explored.

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