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CRYOPRESERVATION OF APPLE *IN VITRO* SHOOT TIPS BY THE DROPLET FREEZING METHOD

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Summary: Shoot tips sampled on *in vitro* plantlets of 5 apple cultivars were successfully cryopreserved using the droplet freezing method. After 3 weeks of cold-hardening at 5°C, the shoot tips were precultured in liquid medium with 1.0% DMSO for 24 h at 5°C, treated for 75 min. at room temperature with 0.3 M sucrose and 15% DMSO, placed in 5µl droplets of cryoprotectant medium containing 0.3 M sucrose and 15% DMSO on aluminium foil and cooled to –40°C at 0.2°C/min before immersion in liquid nitrogen. Recovery rates of cryopreserved shoot tips ranged between 70 and 92%, depending on the cultivar. Regrowth of frozen shoot tips was direct, without callus formation.

Key words: apple; shoot tips; cryopreservation; droplet freezing method.

INTRODUCTION

Several new cryopreservation techniques have been developed during the last 10 years and applied to a large number of plant species (2). These include vitrification, encapsulation-dehydration, encapsulation-vitrification, desiccation, pregrowth, pregrowth-desiccation and droplet freezing

Until now, the droplet freezing method established by Kartha *et al.* for cryopreserving cassava apices (3) has only been applied elsewhere to potato apices (5). In the case of potato, this method has proven very efficient and practical for routine utilization. It has been applied to 221 different genotypes, with an average plant recovery rate of 40%. Various cryopreservation techniques have been applied to apple shoot tips, including controlled slow freezing, encapsulation-dehydration and vitrifrication (1, 6, 7).

In this work, we experimented the droplet freezing technique with apices of one apple variety. The optimized protocol was then tested with four additional varieties.

MATERIALS AND METHODS

Plant material

In vitro cultured plantlets originating from 6 year-old plants of *Malus domestica* cv. Golden Delicious were used to develop the cryopreservation technique. The other four cultivars employed were Jinbiao, Red World, Jonwro and Ba Leng Haitang.

In vitro culture

Plantlets were cultivated on Murashige and Skoog (4) semi-solid medium containing 1.0 mg/L benzyl adenine (BA), 0.05 mg/L naphtalene acetic acid (NAA) and 30 g/L sucrose. They were grown on 50 ml medium in 100 ml glass flasks under white fluorescent light (52 $\mu mol/m^2/s$) under a 12 h light/12 h dark photoperiod at 25 ± 1°C. After 40, 70 or 100 d following the last subculture, plantlets were cold-hardened for 21 d at 5°C before apices were explanted for cryopreservation.

Cryopreservation

After excision, shoot tips (about 1.0-1.5 mm in size) were precultured in liquid medium containing 1.0% dimethylsulfoxide (DMSO) for 24 h at 5°C. They were then transferred at room temperature in liquid medium with 3% sucrose, to which DMSO was added over a period of 30 min, until a final DMSO concentration of 15% was reached. Apices were left in this solution for an additional 45 min. Liquid medium with 0.3 M sucrose and 15% DMSO was distributed in 5 µl droplets on sterile pieces of aluminium foil (3 x 0.7 cm; 5 droplets/foil). One apex was placed in each droplet and the foils were transferred in sterile 2 ml cryotubes (2 foils/cryotube). For freezing, the cryotubes were either immersed directly in liquid nitrogen (LN), or cooled at 0.2°C/min from 0 to –40°C before immersion in LN. After storage in LN for 24 h, cryotubes were rewarmed in a water-bath thermostated at 25°C until complete melting of the cryoprotectant medium was achieved. Thereafter, apices were transferred on standard culture medium for regrowth.

Assessment of regrowth

The percentage of shoot tips which produced normal shoots was assessed 40 d after transfer to standard medium. Results, presented with their standard deviation correspond to the average of 3 replicates of 20 apices per experimental condition.

RESULTS

Prefreezing apices to different temperatures had little effect on their regrowth level, which ranged between 80 and 90% (Table 1). No regrowth was obtained after rapid freezing. After slow freezing, regrowth of apices increased in line with decreasing prefreezing temperatures, from 30% regrowth for a prefreezing temperature of –10°C to 82% for a prefreezing temperature of –40°C.

Table 1: Effect of freezing procedure (rapid freezing: RF; slow freezing: SF) and of prefreezing temperature (-10 to –40°C) on the survival (%) of non-cryopreserved (-LN) and cryopreserved (+LN) shoot tips of apple variety Golden Delicious. Fourty d after the last subculture, mother-plants were cold-hardened for 21 d at 5°C before apices were used for cryopreservation.

	RF		SF/-10°C		SF/-20°C		SF/-30°C		SF/-40°C	
	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
Survival (%)	100	0	89 ± 3	30 ± 2	80 ± 1	50 ± 3	90 ± 1	60 ± 2	90 ± 1	82 ± 3

Table 2: Effect of duration between the last subculture of mother-plants and the time of cryopreservation on the regrowth (%) of cryopreserved apices of apple variety Golden Delicious. Forty, 70 or 100 d after the last subculture, mother-plants were cold-hardened for 21 d at 5°C before apices were used for cryopreservation.

Time since last subculture (d)	Regrowth (%)
61	44 ± 1
91	60 ± 1
121	80 ± 1

Table 3. Regrowth (%) of apices of different apple (*M. domestica*) cultivars after cryopreservation using the droplet freezing technique. One hundred days after the last subculture, mother-plants were cold-hardened for 21 d at 5°C before the apices were excised and used for cryopreservation.

Cultivar	Regrowth (%)
Jinbiao	87 ± 4
Red World	70 ± 1
Jonwro	76 ± 3
Ba Lang Haitang	88 ± 1
Golden Delicious	92 ± 1

Extending the duration between the last subculture of mother-plants and the cryopreservation of apices led to a progressive increase in regrowth percentage, from 40% after 61 d to 80% after 121 d (Table 2).

The droplet freezing technique established with apple cv. Golden Delicious was successfully applied to apices of 4 other cultivars (Table 3). The regrowth levels achieved ranged between 70 and 92%.

DISCUSSION/CONCLUSION

This work demonstrated that it is possible to cryopreserve apple shoot tips using the droplet freezing method. The highest recovery rates were obtained for apices isolated from plantlets which had been cold-acclimated for 21 d, 100 d after their last subculture. When these apices were precultured with 1.0% DMSO for 24 h at 5°C, treated with 0.3M sucrose and 15% DMSO for 75 min, then frozen to -40°C at 0.2°C/min in droplets of the same cryoprotectant medium before immersion in liquid nitrogen, more than 70% survival was achieved.

Contrary to what was observed with potato (5), regrowth of apices was achieved after slow freezing only, which indicates that freeze-induced dehydration of apices during the prefreezing step was needed.

Interestingly, regrowth of cryopreserved shoot tips increased in line with increasing durations between the last subculture of the mother-plants and the time when apices were used for cryopreservation. This underlines the importance of the physiological state of the explants on their response to cryopreservation. It is hypothesized that this might be due to the lower water content of the apices sampled on mother-plants which have not been subcultured for 100 days.

From a practical point of view, several efficient techniques are now available for cryopreserving apple apices (1, 6, 7). Large scale experiments should now be conducted so that the most suitable cryopreservation technique(s) for routine application in genebanks can be established.

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