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CRYOPRESERVATION OF APPLE SHOOT TIPS: IMPORTANCE OF CRYOPRESERVATION TECHNIQUE AND OF CONDITIONING OF DONOR PLANTS

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Summary: In this paper, the efficiency of three techniques (two-step freezing, vitrification, encapsulation-dehydration) for freezing *in vitro* shoot tips was compared and the effect of conditioning of mother-plants was investigated. Cold-hardening mother plants for 3 weeks at 5°C improved regrowth rate and pattern of shoot tips whatever the cryopreservation technique employed. Increasing the time during which mother-plants were maintained on standard medium without subculture before sampling of apices decreased the water content of shoot tips from about 85-88% to 63-66% (fresh weight basis) and increased regrowth rate and pattern whatever the cryopreservation technique employed. The best results (up to 86% regrowth after cryopreservation without any callusing) were obtained with the encapsulation-dehydration technique. Using apices sampled on mother-plants which had not been subcultured for 26 weeks allowed to reduce the duration of the sucrose pregrowth treatment of encapsulated apices, thus simplifying the protocol. This simplified encapsulation-dehydration protocol was successfully applied to 11 out of the 12 cultivars tested.

Keywords: apple (*Malus* spp.); cryopreservation; shoot tips; two-step freezing; vitrification; encapsulation-dehydration; cold-hardening; shoot tip water content.

INTRODUCTION

Various cryopreservation techniques which differ in the physical mechanisms employed for dehydrating explants are available for the long-term storage of plant material in liquid nitrogen. Classical procedures are based on freeze-induced dehydration of samples during cooling, whereas new protocols are based on dehydration of samples before freezing followed by vitrification of internal solutes during cooling. These new cryopreservation techniques which include vitrification, encapsulation-dehydration, encapsulation-vitrification, desiccation, pregrowth,

pregrowth-desiccation and droplet freezing have been developed during the last 10 years and applied to a large number of plant species, from temperate as well as tropical climates (3, 4, 16).

A key element to the successful development of cryopreservation protocols is the selection of the optimal physiological stage at which explants should be used for freezing. It is generally recommended to sample explants from rapidly growing cultures since actively dividing cells present characteristics, such as their little developed vacuolar system and dense cytoplasm which make them more likely to withstand cryopreservation (4, 7, 20). Conditioning treatments of mother-plants or of explants, including cold-acclimation for cold-tolerant species and/or culture on medium supplemented with osmoticums or cryoprotectants generally lead to improvements in the recovery rates after cryopreservation (5, 16).

Before routine application of cryopreservation for the long-term conservation of a given crop can be envisaged, it is necessary to compare the different cryopreservation techniques available for their quantitative and qualitative results and to perform experiments with a large number of genotypes to evaluate their applicability and operational practicality.

Cryopreservation of shoot tips of apple *in vitro* cultures has been performed by numerous authors using various techniques, including two-step freezing (1, 2, 8) encapsulation-dehydration (12, 15, 22), vitrification (13) and droplet-freezing (21).

The aims of the current work were to compare the results obtained with cryopreservation of apple shoot tips using three different techniques, i.e. two-step freezing, vitrification and encapsulation; to study the effect of conditioning treatments of mother-plants on the regrowth of apices after cryopreservation; and to assess the applicability of the technique selected by experimenting it with twelve different apple genotypes.

MATERIALS and METHODS

Materials:

In vitro plantlets originating from 6-year old trees of the following 18 *Malus* spp. cultivars were employed in the experiments: Jonagold, Fuji, Jinbiao, Baleng Haitang, Golden Delicious, new Jonagold, HAC-9, 278, Fuji-12, GQFuji, S-29, Yatac, CG-80, Fuji-1, Fuji-2, Tsugaru, Jonagold, Xianghong and Red Star were also used.

Methods:

In vitro culture

Mother plants were cultured on Murashige and Skoog (11) medium containing 1.0 mg/L BA, 0.05 mg/L NAA, 30 g/L sucrose and 5g/L agar. They were grown on 50 ml medium in 100 ml glass flasks under white fluorescent light (52 $\mu mol.s^{-1}.m^{-2}$) with a

12 h light/12 h dark photoperiod, at 25±1°C. Cultures were transferred to new medium every 9 weeks.

Cold-hardening

Nine weeks after the last subculture, *in vitro* plantlets were cold-hardened at 5°C for three weeks, under a 8 h light/16 h dark photoperiod. Shoot tips were then dissected and used for cryopreservation experiments. In some experiments, *in vitro* plantlets were submitted to the cold-hardening treatment 3, 9 or 26 weeks after the last subculture on standard medium.

Cryopreservation

Two-step freezing:
Shoot tips (about 0.5 to 1.0 mm long) were placed in 4 ml cryovials (20 shoot tips per vial) with 1.5 ml MS basic liquid medium at 0°C. The same volume of cryoprotective medium containing 5% glycerol (w/v) and 5% dimethylsulfoxide (DMSO - w/v) was added progressively over a period of 30 min and the samples were kept for an additional 30 min under these conditions. For prefreezing, the volume in the cryotubes was adjusted to 2.5 ml and the cryovials were transferred into the programmeble freezer (HIT-4, China), cooled at 0.2°C/min from 0 to –10°C, held at this temperature for 15 min, then cooled at the same rate to –40°C and immersed in liquid nitrogen (LN) where they were kept for 24 h. For rewarming, the cryotubes were immersed for 2–3 min in a water-bath thermostated at 25 °C.

Vitrification:
Apices were precultured for 24 h at 5°C on solid medium with 0.7M sucrose. Shoot tips (about 1.5 to 2.0 mm long; 20 shoot tips per vial) were pretreated in 4 ml cryovials with 1.5 ml of PVS3 vitrification solution consisting of 50% glycerol (w/v) + 50% sucrose (w/v) (14) for 80 min. During pretreatment, the PVS3 solution was renewed every 20 min. The volume in the cryotubes was adjusted to 2.5 ml and the cryovials were then plunged directly in LN and kept at this temperature for 24h. For rewarming, the cryotubes were immerged for 2–3 min in a water bath at 25 °C..

Encapsulation-dehydration:
Three different pregrowth treatments were experimented:

1. Shoot tips (about 1.5 to 2.0 mm long) were precultured at 5 °C in liquid medium with daily increasing sucrose concentration (0.1M; 0.3M; 0.7M), then encapsulated in 3% alginate beads. The beads were cultured at 5 °C in liquid medium with 1.0M sucrose for 1d.

2. Shoot tips were encapsulated in alginate beads without previous sucrose preculture. The apices were then cultured at 5 °C in medium with 1.0M sucrose for 1 d before dehydration.

3. Shoot tips were encapsulated in alginate beads without previous sucrose preculture. The encapsulated apices were then dehydrated immediately without sucrose preculture.

After the pregrowth treatment, the beads were dehydrated under the air current of the laminar flow cabinet or with silica gel to 20-30% water content (fresh weight basis). The beads were then placed in 2 ml cryotubes (10 beads/cryotube) and plunged directly in LN, where they were stored for 24h. For rewarming, the cryotubes were immerged for 2 –3 min in a water bath at 25 °C.

Regrowth conditions

Whatever the cryopreservation technique employed, shoot tips were transferred under standard culture conditions immediately after rewarming.

Assessment of regrowth

Assessment of regrowth was performed 40 d after cryopreservation. The total regrowth rate (TR) corresponded to the percentage of regrowing apices, either through direct development into shoots or through callusing. The direct regrowth rate (DR) corresponded to the percentage of apices which had developed directly into shoots and the callusing rate (CR) to the percentage of apices which had produced callus only. In some experiments, the time to observe regrowth (TS), which corresponded to the minimum time requested to observe the first sign of regrowth of apices after treatment, either through the development of leaf primordia or through callusing was also noted. Twenty apices were employed for each experimental condition.

Measurement of shoot tip and bead water content

For measurement of water content, shoot tips and beads were placed in oven at 85°C for 24 h. Water content was expressed on a fresh weight basis. Four replicates of 20 shoot tips or beads were employed to measure the water content under each experimental condition.

RESULTS

With all four cultivars tested and whatever the cryopreservation technique employed, cold-hardening of mother-plants ensured higher regrowth rates of shoot tips and to reduce the amount of callusing (Table 1). Based on total regrowth measurement, vitrification was more efficient than two-step freezing and encapsulation-dehydration, with an average regrowth rate of 81% against 66 and 62% for two-step freezing and encapsulation-dehydration, respectively. However, this difference was reduced when direct regrowth was considered, since no callusing was ever observed with encapsulation-dehydration. The average direct regrowth rate was 71.5% for vitrification against 62 and 60% for two-step freezing and encapsulation-dehydration, respectively.

Table 1: Effect of cold-hardening treatment and cryopreservation technique on the regrowth rate (%) and pattern of control (-LN) and cryopreserved (+LN) shoot tips of four apple cultivars. TR: total regrowth; DR: direct regrowth; CR: regrowth through callusing. Two-step freezing controls correspond to apices pretreated and prefrozen to –40°C, vitrification controls to apices submitted to vitrification treatment; encapsulation controls to encapsulated apices after desiccation. The shoot tips, cryopreserved 12 weeks after the last subculture, had a water content of 78% (fresh weight basis).

			Regrowth rate (%)					
			Two-step		Vitrification		Encapsulation	
Cultivar	Cold-hardening		-LN	+LN	-LN	+LN	-LN	+LN
Jonagold		TR	64	40	82	75	100	36
	+	DR	32	33	82	53	100	36
		CR	32	7	0	22	0	0
		TR	25	20	40	0	100	50
	-	DR	0	0	40	0	100	50
		CR	25	20	0	0	0	0
Baleng Haitang		TR	100	71	100	94	100	69
	+	DR	100	62	100	83	100	69
		CR	0	9	0	11	0	0
		TR	30	20	67	27	83	56
	-	DR	20	16	67	22	83	46
		CR	10	4	0	5	0	10
Fuji		TR	75	75	100	67	100	67
	+	DR	75	66	100	62	100	67
		CR	0	9	0	5	0	0
		TR	25	30	25	0	33	0
	-	DR	0	24	25	0	33	0
		CR	25	6	0	0	0	0
Jinbiao		TR	89	78	100	88	100	75
	+	DR	69	78	100	88	100	75
		CR	20	0	0	0	0	0
		TR	25	20	40	0	100	50
	-	DR	0	0	40	0	100	50
		CR	25	20	0	0	0	0

With all four cultivars tested and whatever the cryopreservation technique employed, the time requested to observe survival was shorter with apices sampled on cold-acclimated plantlets (Table 2). The first sign of regrowth was observed slightly more rapidly for apices cryopreserved using vitrification and encapsulation-dehydration.

Table 2: Effect of cold-hardening treatment (-CA: without; +: with cold-hardening) and of cryopreservation technique on the time requested to observe survival (%) of cryopreserved shoot tips of four apple cultivars. The shoot tips, cryopreserved 12 weeks after the last subculture, had a water content of 78% (fresh weight basis).

	Time to observe survival (d)					
	Two step		Vitrification		Encapsulation	
Cultivar	-CA	+CA	-CA	+CA	-CA	+CA
Jonagold	10	6	7	4	7	5
Baleng Haitang	6	5	7	4	6	4
Fuji	7	6	6	3	7	4
Jinbiao	8	7	6	5	9	5

The water content of shoot apices decreased from 85-88% to 64-66% when mother plants were kept without transfer to new medium for 6 and 29 weeks, respectively.

Table 3: Effect of time since last subculture (including 3-week cold-hardening period) on the water content (%, fresh weight basis) of shoot tips of three apple cultivars.

	Time since last subculture (weeks)		
Cultivar	6	12	29
Golden Delicious	85.3±4.1	77.0±5.0	64.6±5.2
New Jonagold	88.7±2.1	74.8±2.3	63.4±3.1
Baleng Haitang	85.9±2.9	77.4±3.0	66.3±4.1

When shoot tips with 68% water content were employed, both the total and direct regrowth rates were drastically improved in all cases (Table 4). Encapsulation-dehydration ensured the best results since 86% of shoot tips withstood cryopreservation and all of them regrew directly, without callus formation. The time required to observe regrowth was equivalent with apices at both water contents, and regrowth of both control and cryopreserved apices was slightly slower after two-step freezing (6-8 d) than after vitrification and encapsulation-dehydration (4-5 d).

Table 4: Effect of water content of shoot tips at the time of dissection (%, fresh weight basis) and of cryopreservation technique on the regrowth rate and pattern of control (-LN) and cryopreserved (+LN) shoot tips of apple cultivar Jonagold. TR: total regrowth; DR: direct regrowth; CR: regrowth through callusing; TS: time to observe survival (d). Two-step freezing controls correspond to apices pretreated and prefrozen to –40°C, vitrification controls to apices submitted to vitrification treatment; encapsulation controls to encapsulated apices after desiccation. Shoot tips with 78 and 86% water content were sampled on plantlets which had not been subcultured for 12 and 6 weeks (including 3-week cold-hardening period), respectively.

		Regrowth rate (%)					
		Two step		Vitrification		Encapsulation	
Water content of shoot tips (%)		-LN	+LN	-LN	+LN	-LN	+LN
78	TR	63	29	71	7	56	32
	DR	0	0	71	0	56	27
	CR	63	29	0	7	0	5
	TS	6	8	4	4	3	5
68	TR	100	46	100	72	100	86
	DR	33	23	100	50	100	86
	CR	67	23	0	22	0	0
	TS	7	8	4	5	4	4

When shoot tips with high water content were employed (i.e. sampled on plantlets 6 weeks after the last subculture), regrowth of cryopreserved apices was observed after the longest pregrowth treatment (Table 5). However, when apices with lower water content (i.e. sampled on mother-plants 29 weeks after the last subculture) were cryopreserved, regrowth was achieved after pregrowth directly with 1.0M sucrose, and even without any pregrowth treatment.

Table 5: Effect of water content of shoot tips of two apple cultivars at the time of dissection (%, fresh weight basis) and of pregrowth treatment on their direct regrowth rate (%) after cryopreservation using the encapsulation-dehydration technique. Shoot tips with 69 and 86% water content were sampled on plantlets which had not been subcultured for 29 and 6 weeks (including 3-week cold-hardening period), respectively.

		Regrowth rate (%)	
		Water content of shoot tips (%)	
Cultivar	Pregrowth treatment	69	86
HAC-9	1	65	59
	2	80	0
	3	28	0
Baleng Haitang	1	83	75
	2	50	0
	3	21	0

The regrowth rate of shoot tips with higher water content was lower under all conditions experimented (Table 6). Cold-hardening allowed to increase the regrowth rate, especially when apices with high water content were employed.

Table 6: Effect of cold-hardening treatment (+: with; -: without cold-hardening) of mother-plants and of water content of shoot tips at the time of dissection (%, fresh weight basis) of two apple cultivars on their direct regrowth rate (%) after cryopreservation using the encapsulation-dehydration technique. Shoot tips with 69 and 86% water content were sampled on plantlets which had not been subcultured for 29 and 6 weeks (including 3-week cold-hardening period), respectively.

Cultivar	Water content of shoot tips (%)	Regrowth rate (%)	
		Cold-hardening	
		+	-
HAC-9	69	85	72
	86	60	40
Baleng	69	65	64
Haitang	86	59	25

Apices of eleven out of the twelve cultivars employed could withstand cryopreservation after pretreatment 2, with direct regrowth rates ranging between 0 and 80% (average regrowth rate 53%) (Table 7). Apices of eight cultivars could even be cryopreserved immediately after encapsulation, without any pretreatment, but this procedure generally produced lower regrowth rates.

Table 7: Effect of pregrowth treatment on the direct regrowth rate (%) of shoot tips of twelve apple cultivars cryopreserved using the encapsulation-dehydration technique. Pregrowth treatment 2: after dissection and encapsulation, apices were pregrown for 24 h in medium with 1.0M sucrose, and desiccated to about 30% water content before cryopreservation. Pregrowth treatment 3: after dissection and encapsulation, apices were desiccated immediately to about 30% water content before cryopreservation. Shoot tips were sampled on plantlets which had not been subcultured for 29 weeks (including 3-week cold-hardening period).

Cultivar	Water content of shoot tips (%)	Regrowth rate (%)	
		Pregrowth treatment	
		2	3
278	68.1±2.8	42	0
Fuji-12	63.4±4.2	57	0
GQ Fuji	72.5±3.3	59	0
S-29	65.6±3.2	0	0
Yatac	64.8±5.2	75	40
CG-80	63.6±1.2	73	11
Fuji-1	68.6±1.4	33	8
Fuji-2	63.6±1.8	54	60
Tsugaru	59.6±3.2	41	10
Jonagold	61.2±2.5	79	21
Xianghong	60.6±4.2	45	50
Red Star	61.7±3.5	80	33

DISCUSSION/CONCLUSION

The choice of a cryopreservation technique for routine application to the conservation of a germplasm collection should be based on quantitative and qualitative results such as the regrowth rate and the regrowth pattern of cryopreserved explants, but also on the applicability of the technique to the broadest genetic diversity possible, and on its operational practicality.

In the present work, encapsulation-dehydration was selected as the method of choice for cryopreserving apple germplasm, mainly based on quantitative and qualitative observations. Under the optimal conditions determined, encapsulation-dehydration produced higher regrowth rates than the other two techniques experimented, and, most importantly, regrowth of cryopreserved apices was always direct, without callusing.By contrast, callusing was often observed during regrowth of shoot tips cryopreserved with the two-step and vitrification techniques. From a practical point of view, the requirement for an expensive programmable freezer would make the costs of using the two-step freezing technique prohibitive for many laboratories. However, additional experiments could be performed to try developing a simplified freezing procedure whereby slow prefreezing is performed in a domestic or laboratory freezer, as achieved with various materials such as sugarcane and citrus embryogenic calluses and carrot and coffee somatic embryós (6, 9, 10, 19). The same comments apply to the droplet freezing technique which has been recently developed for apple shoot tips by the authors of the present paper (21).

This study emphasized the importance of a cold-acclimation period of the mother-plants, which is a parameter of critical importance for the successful cryopreservation of most temperate species (4). Cold-acclimation of mother-plants has been applied to most cryopreservation experiments performed with apple *in vitro* shoot tips (18). One set of conditions only (5°C for three weeks, under a 8 h light/16 h dark photoperiod) has been experimented in the current study for the cold treatment. It is possible that modifications in the conditions, including the duration, temperature and photoperiod employed during the cold treatment could further improve the results, as demonstrated notably for the temperature and duration with pear shoot tips (17).

The physiological state of explants is also of paramount importance for the success of cryopreservation (4). This study demonstrated that apices sampled on plantlets which have not been subcultured for a long period underwent a reduction in their water content and could withstand cryopreservation better with all three techniques experimented than apices sampled on plantlets subcultured more regularly. This is an original finding which somehow contradicts the general assumption that actively growing material should be employed for cryopreservation (4, 7, 20). In the present case, the combination of a long period without subculture and of the cold-acclimation treatment may induce in the *in vitro* shoot tips a physiological state comparable to the dormancy observed in winter buds (16, 18). Additional studies should be performed with the same material to observe, notably at the biochemical and histo-cytological level, the modifications induced in the apices by the period without subculture and the cold treatment. The effect of an extension of the period without subculture of mother-plants on the survival of apices to cryopreservation should be experimented with

additional species, including sub-tropical and tropical ones. If successful, this would be a very simple procedure to increase recovery rates of apices after cryopreservation.

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