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CRYOPRESERVATION OF APPLE SHOOT TIPS BY ENCAPSULATION-DEHYDRATION: EFFECT OF PRECULTURE, DEHYDRATION AND FREEZING PROCEDURE ON SHOOT REGENERATION

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Summary: Shoot tips sampled on *in vitro* cultured apple plantlets of 6 accessions of *M. domestica* and one accession of *M. robusta* were successfully cryopreserved using the encapsulation-dehydration technique. Shoot tips were excised from plantlets which had been submitted to 3 weeks of cold-acclimation at 5°C, 70 d after their last subculture. After preculture at 5°C in media with progressively increased sucrose concentration (0.1 M,0.3 M and 0.7 M), shoot tips were encapsulated and pregrown in medium with 1.0 M sucrose for 1 d, dehydrated for 4 h under the air current of the laminar flow cabinet, thus reaching a moisture content of around 30% (fresh weight basis) and directly immersed in liquid nitrogen. The regeneration rate of cryopreserved apices varied between 70 and 90%, depending on the accession. Using apices sampled on plantlets which had been maintained on standard medium without subculture for 6 months, sucrose preculture became unnecessary to achieve regrowth after cryopreservation and the dehydration period was shortened. These experiments showed that the physiological state of the plant material directly affects the results and procedures for cryopreservation of apple shoot tips.

Key words: apple; cryopreservation; encapsulation-dehydration; shoot tips; sucrose preculture; dehydration; freezing procedure; physiological state.

Introduction

Cryopreservation is the only current method which allows safe and cost-effective long-term conservation of germplasm of vegetatively propagated and non-orthodox seed species. Various cryopreservation techniques have been developed which differ in the physical mechanisms employed for dehydrating samples. 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. Encapsulation-dehydration is one of the new techniques which have been successfully applied to apices of a wide range of tropical and temperate species (1, 2, 3).

Cryopreservation of apple shoot tips has been performed by several authors using various techniques, including encapsulation-dehydration (6).

This study investigated the importance of some of the factors which can affect the survival of apple shoot tips cryopreserved using the encapsulation-dehydration technique, with the aim of facilitating the utilization of this method for the long-term conservation of apple germplasm. The improved protocol was applied to 7 different accessions from the *in vitro* collection of the Changli Institute of Pomology.

Materials and Methods

Plant material

Tissue-cultured plantlets of *Malus domestica.* cv. Tsugaru were used to observe the effect of all parameters studied. The improved protocol was then tested with 6 additional accessions: *M. domestica* cv.Orin, *M. domestica* cv. red Fuji, *M. domestica* cv. Jonagold, *M. domestica* cv. HAC-9, *M. domestica* cv. Senshu and *M. robusta.*

In vitro culture

Plantlets were cultured on Murashige and Skoog (MS) medium (4) containing 1.0 mg/L benzyladenine (BA), 0.05 mg/L naphtalene acetic acid (NAA), 30g/L sucrose and 5 g/L agar. Plantlets were grown on 50 ml medium in 100 ml glass flasks at 25 ± 1°C, under white fluorescent light (52 $\mu mol/m^2/s$) with a 12 h light/12 h dark photoperiod. Subcultures were performed evry 9 weeks.

Plantlets were cold-hardened at 5°C for 3 weeks, 70 d after their last subculture. Shoot tips (about 2 mm long) were excised from cold-hardened plantlets. In one experiment (Table 3), plantlets which had been kept on the same medium without subculture for 6 months before cold-hardening were employed.

Cryopreservation

For pregrowth, apices were transferred on media with increasing sucrose concentration (0.1 M/0.3 M/0.7 M) at daily intervals. Shoot tips were then encapsulated in 3% alginate beads containing 0.5 M sucrose which were placed in medium with 1.0 M sucrose for 1 d before dehydration.

Beads were desiccated in the air current of the laminar flow cabinet for up to 4 h (to about 30% moisture content, fresh weight basis). Dried beads were placed in 2 ml cryotubes and cryopreserved by direct immersion of the cryotubes in liquid nitrogen. In one experiment (Table 4), rapid cooling of apices was compared with a two-step cooling by precooling the samples to -10, -20 or -40°C at 0.2°C/min before immersion in liquid nitrogen. Samples were rewarmed in a water-bath at 25°C and transferred onto solid medium under standard conditions for regrowth.

Assessment of regrowth

The regrowth rate was defined as the percentage of shoot tips which produced normal shoots 40 d after rewarming. Three replicates of 20 shoot tips were used for each experimental condition. Results are presented with their standard deviation.

Results

Without cold-hardening, only limited survival (30%) of cryopreserved apices was achieved after pregrowth treatment and this corresponded to the highest sucrose concentration used (Table 1). Survival of cold-hardened apices increased in line with increasing sucrose concentrations employed during preculture, from 0% with 0.1 M sucrose to 70% with 1.0 M.

Table 1. Effect of cold-hardening of mother-plants (3 weeks at 5°C) and apex preculture treatment on the recovery rate (%) of cryopreserved apices of *M. domestica* cv. Tsugaru. Encapsulated apices were pregrown for 24 h in medium with 1.0 M sucrose, then dehydrated to 30% MC before freezing.

	recovery rate (%)	
preculture treatment (sucrose molarity and time)	no cold-hardening	cold-hardening
0.1 M/1 d	0	0
0.1 M/1 d; 0.3 M/1 d	0	18.2 ± 3.0
0.1 M/1 d; 0.7 M/1 d	0	35.7 ± 2.2
0.1 M/1 d; 0.3 M/1 d; 0.7 M/1 d	0	50.0 ± 5.0
0.1 M/1 d; 0.3 M/1 d; 0.7 M/1 d; 1.0 M/1 d	30.0±12.0	70.0 ± 3.0

Without dehydration, the regrowth rate of frozen apices was only 10% (Table 2). Regrowth after cryopreservation increased progressively up to 77.8% when beads were pre-dried to a MC of 30%. Further pre-drying to 22% MC reduced survival.

Table 2. Effect of desiccation period on the bead moisture content (%, fresh weight basis) and recovery rate (%) of cryopreserved apices of *M. domestica* cv. Tsugaru. Mother-plants were cold-hardened for 3 weeks at 5°C, and the isolated apices precultured on media with progressively increasing sucrose concentration, according the following sequence: 0.1 M/1 d; 0.3 M/1 d; 0.7 M/1 d. Apices were then encapsulated and pregrown for 24 h in medium with 1.0 M sucrose before desiccation and freezing.

desiccation period (h)	bead MC (%)	recovery rate (%)
0	65.9 ± 0.2	10.0 ± 2.0
1	54.0 ± 0.7	17.6 ± 6.5
2	43.5 ± 0.4	44.0 ± 3.0
3	36.2 ± 1.3	50.0 ± 3.0
4	30.7 ± 0.8	77.8 ± 5.8
5	22.4 ± 1.2	27.2 ± 13.5

When apices sampled on plantlets which had not been subcultured for 6 months were employed for freezing experiments, the freezing procedure could be simplified and the optimal desiccation period shortened (Table 3). After the standard preculture on media with increasing sucrose concentrations and encapsulation (Procedure A), very high survival rates (90.0 to 98.5%) could be achieved without any desiccation or after 1 h of desiccation only. Survival of apices could also be achieved without preculture in progressively increasing sucrose concentrations (Procedures B and C), however at lower rates.

Table 3. Effects of preculture procedure and desiccation duration (h) on the survival of cryopreserved apices of *M. domestica* cv. Tsugaru excised from *in vitro* plantlets which had been maintained on standard medium for 6 months without subculture and cold-acclimated at 5°C for 3 weeks.

		0	1	2	3	4
Bead moisture content (%)		64.8 ± 1.3	55.8 ± 0.2	46.4 ± 0.5	38.7 ± 1.5	30.0 ± 2.3
preculture treatment	A	98.5 ± 2.0	90.0 ± 2.0	33.3 ± 3.2	10.0 ± 5.6	16.6 ± 5.0
	B	10.0 ± 12.6	72.2 ± 4.3	18.2 ± 8.5	41.6 ± 4.2	8.0 ± 5.0
	C	40.0 ± 3.0	60.0 ± 3.0	27.2 ± 10.0	36.3 ± 3.0	11.0 ± 2.3

Procedure A: apices were precultured in media with increasing sucrose concentration (0.1 M; 0.3 M; 0.7 M) at 5°C and encapsulated in beads containing 0.5M sucrose. Beads were cultured in medium with 1.0M sucrose for 1 d at 5°C. Procedure B: apices were encapsulated in alginate beads without sucrose preculture before dehydration. Procedure C: apices were encapsulated in alginate beads and precultured in medium with 1.0 M sucrose for 1 d at 5°C before dehydration.

Cooling procedure (slow cooling to various precooling temperatures or direct immersion in LN) had no effect on the survival rate of cryopreserved apices (Table 4).

Table 4. Effect of cryopreservation procedure employed (cooling at 0.2°C/min to various precooling temperatures or direct immersion in LN) on the survival of apices of *M. domestica* cv. Tsugaru. Apices were precultured in media with increasing sucrose concentration (0.1 M; 0.3 M; 0.7 M) at 5°C and encapsulated in beads containing 0.5 M sucrose. Beads were cultured in medium with 1.0 M sucrose for 1 d at 5°C and desiccated to 30% MC (fresh weight basis) before cryopreservation.

Cooling procedure	regeneration rate (%)
0.2°C/min to -10°C + LN	61.0 ± 2.0
0.2°C/min to -20 °C + LN	63.0 ± 2.1
0.2°C/min to -40 °C + LN	70.0 ± 3.2
Direct immersion in LN	62.0 ± 4.0

When the optimal cryopreservation protocol was experimented with additional accessions, the survival rates achieved ranged between 70.0 and 90.5% (Table 5).

Table 5. Regeneration rate (%) of apices of various apple accessions after cryopreservation. After 3 weeks of cold-acclimation of mother-plants at 5°C, apices were precultured in media with increasing sucrose concentration (0.1 M; 0.3 M; 0.7 M) at 5°C and encapsulated in beads containing 0.5 M sucrose. Beads were cultured in medium with 1.0 M sucrose for 1 d at 5°C, desiccated to 30% MC (fresh weight basis) and cryopreserved by direct immersion in LN.

Accession	regeneration rate (%)
M. domestica cv.Tsugaru	70.0 ± 12.0
M. domestica cv. Orin	90.5 ± 2.0
M. domestica cv. Red Fuji	88.0 ± 3.0
M. domestica cv. Jonagold	78.0 ± 1.3
M. domestica cv. HAC-9	90.5 ± 2.0
M. domestica cv. Senshu	77.8 ± 2.0
M. robusta	90.0 ± 2.3

Discussion

Apices of 7 apple accessions were successfully cryopreserved after daily transfers on media with progressively increased sucrose concentration (from 0.1 to 0.7M), encapsulation, preculture for 1 d in medium with 1.0M sucrose followed by desiccation to around 30% MC and rapid freezing. Regeneration rates of cryopreserved apices of all accessions were very high, ranging from 70 to 90%.

This study underlined the critical importance of cold-acclimation of mother-plants to achieve successful regrowth of apices after cryopreservation. Cold-acclimation has been identified as a key factor for success in cryopreserving apices of various cold-tolerant species using different techniques (2, 5). The cooling rate employed had no effect on the regrowth of cryopreserved apices, as observed with apices of most species cryopreserved using the encapsulation-dehydration technique (2, 3).

An original finding of this study is the positive effect of an increased duration between the last subculture of mother-plants and the cryopreservation of apices on their regeneration rate. It is hypothesized that this was due to the lower water content of the apices sampled on mother plants which had not been subcultured for 6 months, compared with the standard 9-week subculture intervals. Additional experiments will be performed to confirm this hypothesis and to assess more precisely the effect of the subculture interval on the recovery of cryopreserved apple apices.

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