Cryopreservation of citrus apices using the encapsulation-dehydration technique

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Introduction

Seeds of many citrus species display recalcitrant or intermediate storage behaviour, and therefore cannot be stored dry at low temperature. In addition, the seeds produced are heterozygous and particular gene combinations cannot be conserved through seed storage. Some cultivars are seedless and are thus propagated vegetatively. Citrus genetic resources are thus currently conserved as whole plants in field genebanks where they remain exposed to pests, diseases and other natural hazards such as drought, weather damage, human error and vandalism (Withers and Engels 1990).

The only current alternative for long-term conservation of problem crops is cryopreservation. Cryopreservation of citrus germplasm has been performed using seeds (Mumford and Grout 1979), ovules (Bajaj 1984), embryonic axes (Radhamani and Chandel 1992), somatic embryos (Marin and Duran–Vila 1988), embryogenic calluses and cell suspensions ((Sakai *et al.* 1990, 1991; Niino and Sakai 1992; Engelmann *et al.* 1994). However, these protocols were applied to juvenile material only, which requires several years before flowering and fruit production. Apical meristems represent the material of choice for citrus germplasm conservation, since plants regenerated from apices of adult cultivars will not present juvenility characteristics and will be true to type, in contrast to plants produced from any other type of material (Navarro *et al.* 1985).

The encapsulation-dehydration technique has led to successful results with apices of numerous temperate and tropical plant species (Engelmann 1997). In this paper we report the successful application of cryopreservation to citrus apices using the encapsulation-dehydration technique.

Materials and methods

Plant material

The plant material consisted of apices sampled on *in vitro* plantlets of citrus rootstocks: *Poncirus trifoliata* (L.) Raf., Troyer and Carrizo citranges obtained from seeds germinated *in vitro*. Plantlets were cultivated as described by González–Arnao *et al.* (1998) and subcultured every 45 d.

Cryopreservation experiments

For cryopreservation experiments, apices (size: 0.5–1 mm) were excised 20 d after the last subculture and left overnight on standard medium for recovery. Apices were encapsulated in alginate (3%) beads and precultured in liquid medium with various sucrose concentrations (0.3–1M) for different durations (1–10 d). A progressive pregrowth by daily transfer of apices to medium with higher sucrose concentration from 0.3M up to 1M also was tested.

Encapsulated apices were then dehydrated at room temperature down to 20–25% moisture content (MC, fresh weight basis) under the sterile air current of the laminar flow cabinet and transferred to sterile 2-ml polypropylene cryotubes. Samples submitted to preculture with progressive increase in sucrose concentration were desiccated under the same conditions down to a series of different MCs, ranging from 36 to 15%.

Freezing was performed either rapidly by direct immersion of the cryotubes in liquid nitrogen or slowly, by cooling at 0.5°C/min from +20 to –40°C before immersion in liquid nitrogen, using a programmable freezer (Bio-Cool, FTS Systems, USA). Samples were kept for at least 1 h at –196°C. For thawing, the cryotubes were placed under the air current of the laminar flow cabinet for 2–3 min. Beads were then transferred in Petri dishes on standard semi-solid medium. Apices were cultured for the first week in the dark, then transferred under standard lighting conditions. Survival was evaluated after 1 month by counting the number of apices which had developed into shoots.

Fifteen to 20 apices were employed per experimental condition, and experiments were replicated 2 to 3 times. The cryopreservation protocol was set up using apices of *Poncirus trifoliata* and the most efficient procedure was then applied to other rootstock species.

Results

The survival rate of encapsulated apices after pregrowth varied depending on the sucrose content in the preculture medium. Preculture for 1 d with 0.75 and 1M sucrose was detrimental to survival. By contrast, apices could withstand extended preculture durations (up to 10 d) in media with lower sucrose concentrations (0.3 and 0.5M). Preculture durations longer than 2 d resulted in survival rates around 80% after desiccation. After slow freezing, survival was achieved for all pregrowth durations tested, ranging between 10 and 50%, whereas after rapid freezing, survival was noted for 3 and 4 d of pregrowth only. The highest survival rate with slow and rapid freezing was achieved after 3 and 4 d of preculture, with 50 and 40% survival, respectively.

Preculture involving a progressive increase in sucrose concentration improved the tolerance to high sucrose levels in comparison with direct preculture in medium with the same final sucrose concentration. A 1M final sucrose concentration in the preculture medium was detrimental to survival. Survival of cryopreserved apices was higher after slow freezing than after rapid freezing.

Table 1. Effect of bead moisture content on the survival (%) of apices after dehydration, rapid or slow freezing. Apices were submitted to a progressive increase in sucrose concentration during preculture (0.3M/24 h + 0.5M/24 h + 0.75M/24 h).

	Bead moisture content (%)				
Treatment	**36**	**28**	**23**	**17**	**15**
Dehydration	95±2.6	90±6.2	88±4.6	50±5.6	20±4.6
Rapid freezing	0	15±5.3	20±2.7	0	0
Slow freezing	0	40±6.2	40±4.6	20±4.0	0

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Survival of desiccated apices decreased in line with decreasing bead moisture content (Table 1). After cryopreservation, the highest survival was obtained after slow freezing, with beads dehydrated down to 23 or 28% MC.

For recovery, control and cryopreserved apices were cultured on standard semi-solid medium. It was not necessary to extract the apices from the beads since they broke the alginate capsule without difficulty and developed into new plants without even transitory callus formation. Apices which did not survive became totally black or remained white. The optimal conditions established for freezing apices of *Poncirus trifoliata* achieved survival with apices of two additional citrus rootstocks (Table 2).

Table 2. Effect of cryopreservation protocol on the survival (%) of apices from three different citrus species after dehydration, rapid or slow freezing. Apices were submitted to a progressive increase in sucrose concentration during preculture (0.3M/24 h + 0.5M/24 h + 0.75M/24 h), then desiccated to 20–25% MC before rapid freezing.

	Survival (%)		
Species	**Pregrowth**	**Dehydration**	**Slow freezing**
Poncirus trifoliata	100	90±5	44±4
Troyer citrange	100	83±4	36±3
Carrizo citrange	100	95±5	55±5

Discussion

Up to now, all previous attempts to cryopreserve citrus apices had been unsuccessful (Pérez 1995). The cryopreservation protocol established for apices of *Poncirus trifoliata* rootstock comprised a preculture for 3 d in liquid medium with 0.5M sucrose or in medium with progressively increasing sucrose concentration (up to 0.75M), desiccation to 20–25% MC followed by slow freezing. This procedure allowed survival with apices of two additional rootstocks.

Citrus apices displayed high sensitivity to sucrose since exposure to a concentration of 0.75M was tolerated only after a progressive increase in sucrose concentration. Slow freezing produced better results than rapid freezing, suggesting that not all freezable water had been extracted from beads/apices during desiccation to 20–25%, and that further freeze-induced dehydration during slow prefreezing was necessary to achieve optimal survival.

Growth recovery of cryopreserved citrus apices occurred directly without callus formation. This allows us to assume that most cells of the apical region were only slightly or not at all damaged during the cryopreservation process, as observed by histocytological examination with sugarcane apices cryopreserved using the encapsulation-dehydration technique (González–Arnao *et al.* 1993).

This protocol, established for apices of juvenile plants, proved unsuccessful when tested with apices sampled on adult plants. However, preliminary positive results have been obtained recently with adult material using encapsulation-vitrification. Indeed, apices of three species (Nules clementine, Fortune mandarin and Pineapple sweet orange), when pretreated with a loading solution containing 0.7M sucrose and 2M glycerol, encapsulated and treated with the PVS3 vitrification solution, remained green after cryopreservation but did not regrow. Additional experiments are under way to optimize different steps of the protocol.

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