CRYOPRESERVATION OF CITRUS APICES USING THE ENCAPSULATION-DEHYDRATION TECHNIQUE

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Abstract
Cryopreservation of apices sampled on in vitro plantlets of Poncirus trifoliata (L.) Raf. was achieved using the encapsulation-dehydration technique. The highest survival rates after cryopreservation (up to 50 %) were obtained when encapsulated apices were pregrown for 3 d either directly in liquid medium containing 0.5M sucrose or in media with progressively increasing sucrose concentration from 0.3 to 0.75M, desiccated to 20-25% moisture content and frozen slowly (0.5°C.min-1) from +20°C to -40°C before immersion in liquid nitrogen. Recovery of whole plantlets from cryopreserved apices took place directly, without transitory callus formation.

Key words: Cryopreservation, citrus, apices, encapsulation-dehydration.

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. 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 (20). Nor are they in a condition that is readily conducive to germplasm exchange. Field genebanks are costly to maintain and, as a consequence, are prone to economic decisions that may limit the level of replication of accessions, the quality of maintenance and even their very survival in times of economic stringency. Even under the best circumstances, field genebanks require considerable inputs in the form of land (often needing multiple sites to allow for rotation), labour, management and materials.

The only current alternative for long-term conservation of problem crops is cryopreservation. Cryopreservation techniques have been developed for more than 100 species cultivated under various forms, including cell suspensions, calluses, apices, somatic and zygotic embryos (2).
Cryopreservation of citrus germplasm has been performed using seeds (8), ovules (1), embryogenic axes (15), somatic embryos (7), embryogenic calluses and cell suspensions (3, 4, 11, 16, 17, 18). However, juvenile material only can be conserved, 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 (10). The encapsulation-dehydration technique has led to successful results with apices of numerous temperate and tropical plant species (2).

In this paper we report the first 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 Poncirus trifoliata (L.) Raf. which were obtained from seeds germinated in vitro, which were kindly provided by IIC.

In vitro culture
Plantlets were cultivated on semi-solid MS medium (9) supplemented with 50 g.L⁻¹ sucrose, 0.5 mg.L⁻¹ benzylaminopurine (BAP), 0.25 mg.L⁻¹ indole butyric acid (IBA), 40 mg.L⁻¹ adenine, 750 mg.L⁻¹ malt extract and 10 g.L⁻¹ agar. They were maintained at 26°C under 8 h light/16 h dark photoperiod under a light intensity of 40 μmol.m⁻².s⁻¹ and subcultured every 45 d.

Cryopreservation experiments
For cryopreservation experiments, apices (size: 0.5-1 mm) were excised from in vitro plants 20 d after the last subculture and left overnight on standard medium for recovery. Apices were then encapsulated in alginate (3%) beads and precultured in liquid medium with various sucrose concentrations (0.3 to 1M) for different durations (1 to 10 d). In some cases, the sucrose concentration in the preculture medium was increased progressively by daily transfer of apices to medium with higher sucrose concentration, from 0.3 up to 1M. 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 2ml polypropylene cryotubes. Samples submitted to preculture with progressive increase in sucrose concentration were desiccated under the same conditions with the same MCs but increased at room temperature.
Fifteen to 20 apices were employed per experimental condition, and experiments were replicated 2 to 3 times. The results presented correspond to the average of these replications ± standard deviation. Survival was evaluated after 1 month by counting the number of apices which had developed into shoots.

**Results**

The survival rate of encapsulated apices after pregrowth varied depending on the sucrose content in the preculture medium (Table 1). 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).

**Table 1.** Effect of preculture duration and sucrose concentration on the survival (%) of encapsulated citrus apices.

<table>
<thead>
<tr>
<th>Preculture duration (days)</th>
<th>Sucrose concentration (M)</th>
<th>0.3</th>
<th>0.5</th>
<th>0.75</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>88±2.0</td>
<td>80±3.6</td>
<td>30±2.6</td>
<td>10±2.6</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>-</td>
<td>82±2.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>90±4.4</td>
<td>85±2.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>-</td>
<td>82±3.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>100</td>
<td>90±7.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>-</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>100</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>-</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

-: not tested

Preculture durations longer than 2 d resulted in survival rates of around 80% after desiccation (Table 2). After slow freezing, survival was achieved for all pregrowth durations experimented, 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, respectively.

**Table 2.** Effect of preculture duration in medium with 0.5M sucrose on the survival (%) of apices after preculture (P), dehydration down to 20-25% MC (D), rapid (RF) or slow (SF) freezing.

<table>
<thead>
<tr>
<th>Preculture duration</th>
<th>Survival (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Preculture involving progressive increase in sucrose concentration increased 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 (Table 3). Survival of cryopreserved apices was higher after slow freezing than after rapid freezing.

Table 3. Effect of progressive increase in sucrose concentration during preculture on the survival (%) of apices after preculture (P), dehydration (D), rapid (RF) or slow (SF) freezing.

<table>
<thead>
<tr>
<th>Preculture conditions</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
</tr>
<tr>
<td>0.5M (24 h) + 0.75M (24 h)</td>
<td>90±4.6</td>
</tr>
<tr>
<td>0.3M (24 h) + 0.5M (24 h) + 0.75M</td>
<td>100</td>
</tr>
<tr>
<td>(24 h)</td>
<td>30±4.0</td>
</tr>
<tr>
<td>0.5M (24 h) + 0.75M (24 h) + 1M</td>
<td>30±4.0</td>
</tr>
<tr>
<td>(24 h)</td>
<td></td>
</tr>
</tbody>
</table>

Survival of desiccated apices decreased in line with decreasing bead MC (Table 4). After cryopreservation, the highest survival was obtained after slow freezing, with beads dehydrated down to 23 or 28% MC.

Table 4. 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).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bead moisture content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>36</td>
</tr>
<tr>
<td>Dehydration</td>
<td>95±2.6</td>
</tr>
<tr>
<td>Rapid freezing</td>
<td>0</td>
</tr>
<tr>
<td>Slow freezing</td>
<td>0</td>
</tr>
</tbody>
</table>

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 transitory callus formation. Apices which did not survive became totally black or remained white.

Discussion

The cryopreservation protocol established for anices of *Poncirus trifoliata* rootstock...
also require progressive sucrose increase during preculture whereas other species such as sugarcane (12) can withstand direct pregrowth in high sucrose concentrations.

Slow freezing achieved better results than rapid freezing, suggesting that not all freezable water had been extracted from beads/apices during desiccation down to 20-25%, and that further freeze-induced dehydration during slow prefreezing was necessary to achieve optimal survival. Similar results were noted with encapsulated grape apices for which slow freezing was necessary to achieve optimal survival (12).

Growth recovery of cryopreserved citrus apices occurred directly without callus formation. It allows us to assume that most cells of the apical region were only slightly or not at all damaged during the cryopreservation process, as was observed by histocytological examination with sugarcane apices cryopreserved using the encapsulation-dehydration technique (5).

Up to now, all previous attempts to cryopreserve citrus apices had been unsuccessful (13). However, although further research is needed to improve the protocol established, these preliminary results demonstrate that it is possible to freeze apices from citrus juvenile plants. Cryopreservation of citrus apices from adult cultivars is currently under investigation using the same experimental approach,

Acknowledgements
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181
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Contents