Cryopreservation of Papaya Germplasm

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Abstract
Shoot tips and seeds of papaya (Carica papaya L.) were successfully cryopreserved. Shoot tips were incubated for 1 to 6 days before vitrification and optimum treatment time was 1-4 days. Duration of exposure to vitrification solution was varied and 70% recovery was obtained from the shoot tips which had been exposed to 100% PVS2 for 20 minutes at 0°C. Treatments for less than 20 minutes or more than 40 minutes resulted in no regeneration after liquid nitrogen (LN) treatment. Shoot tips closer to the apex had almost a two-fold rate of recovery compared with tips excised from the basal part of the shoot. Seeds of a southeast Queensland genotype were desiccated to moisture contents of 40%, 20%, 15%, 10%, and 5% (wet weight basis), followed by freezing in liquid nitrogen (LN). Germination rate of seeds that had been desiccated but not cryopreserved were consistently high (between 70% and 90%) across the range of moisture contents down to 5%. For seeds frozen in LN, the germination rate of seeds at 100%, 40%, 20% and 15% moisture content was 0%, 8%, 6%, and 8% respectively. A substantial increase in germination rate to 48% was achieved at 10% moisture content, declining to 20% at 5% moisture content for cryopreserved seeds. Within 10 weeks of transplanting to the field, growth rates of plants re-grown from cryopreserved seeds were significantly reduced (25%) when compared with the control plants. Substantially retarded growth was observed in one plant re-grown from a cryopreserved shoot tip, after 2.5 years in the field.

INTRODUCTION
Papaya (Carica papaya L.) is a popular tropical fruit crop. However, because of extensive monoculture and a narrow gene-pool, it is susceptible to numerous diseases including Papaya Ring Spot Virus (PRSV-P), which has caused widespread damage in many countries. Conservation of the germplasm of the crop gene-pool is important to provide a source of genes for the future development of resistant varieties, either via genetic manipulation or through conventional plant breeding. Papaya seeds have generally been classified as having intermediate seed storage capacities and storage for periods greater than 5 years is difficult (Ellis et al., 1991, Wood et al., 2000). Loss of viability has been reported at moisture contents below 8 to 10% (Ellis et al., 1991), although viability has been reported when seed was desiccated to 5% (Magill et al., 1994). More recently, Wood et al. (2000) have reported that dormancy results from desiccation of papaya seeds and that desiccation–induced dormancy can be reversed by heat shock. However, no protocols for long-term conservation of papaya seeds are currently available and work reported here aims to develop a method. An alternative conservation approach is to cryopreserve shoot tips. By this method clonal material from superior genotypes can be preserved. Cryopreservation of shoot tips has been shown for three papaya genotypes using a vitrification-based procedure, and >65% of tips were successfully thawed and regenerated for all three genotypes (Ashmore et al., 2001).

This paper reports on the continued development of cryopreservation methods for long-term storage of papaya germplasm. Two procedures, desiccation and cryopreservation of seeds and vitrification-based cryopreservation of shoot tips, have been employed, and growth of regenerated plants has been studied.
MATERIALS AND METHODS

Cryopreservation of Shoot Tips: Standard Protocol

Axillary buds from 8-weeks-old in vitro papaya plants were used as a source of shoot tips. The shoot tips used ranged from 1-3 mm in length. Excised shoot tips were incubated at 25°C for 1 day in a liquid medium containing high DeFossard minerals and vitamins (DeFossard et al., 1974) and supplemented with 0.5 μM BAP, 3 μM IAA, 0.6 μM GA₃ and 3% sucrose. After incubation treatment, the shoot tips were pretreated with a 20% plant vitrification solution - PVS2, as defined by Sakai et al. (1991) - for 1hr at room temperature. Shoot tips were then transferred to 100% PVS2 and incubated for 20 minutes at 0°C. After vitrification, the shoot tips were individually placed on aluminum foil strips with a drop of 100% PVS2. The strips were plunged into liquid nitrogen (LN).

When removed from LN, tips were plunged into thawing medium (45°C), which was made of the same components as the incubation medium, plus 1.4 M sucrose. The tips were kept in this medium for 10 minutes after which they were cultured on regeneration medium as described by Ashmore et al. (2001). The cultured tips were kept for 48 hrs in total darkness, before transfer to a 16hr daily photoperiod with a light irradiance of 25 μM m⁻² s⁻¹. The temperature of the growth room was maintained at 25°C ± 1°C. The rate of recovery was monitored. The recovered tips were grown into plants and acclimated using protocols developed by Drew (1992). Their subsequent growth rate was recorded. Modifications of this method were tested as described below.

Control treatments for all experiments consisted of dissected shoots tips grown initially on high DeFossard minerals and vitamins (DeFossard et al., 1974) and supplemented with 0.5 μM BAP, 3 μM IAA, 0.6 μM GA₃ and 3% sucrose, before being grown into plantlets using the protocols of Drew (1992).

Modifications to Pre-cryopreservation Incubation of Excised Shoot Tips

Excised shoot tips were incubated prior to cryopreservation for 1, 2, 4 or 6 days in the standard incubation medium described above. 10 shoot tips per treatment were used.

Modifications to Duration of Exposure to 100% PVS2

After initial exposure of shoot tips to 20% PVS2 for 1hr, 10 shoot tips per treatment were exposed to 100% PVS2 for 10, 20, 40, or 60 minutes at 0°C.

Effect of Position of Shoot Tips on Stems on Recovery post Cryopreservation

Shoot tips were dissected from above the mid-point (designated upper) and from below the mid-point (designated lower) of the stem of an in vitro grown shoot, to examine the effect of the position of shoot tips on the rate of recovery. Excised shoot tips were put through the standard cryopreservation protocol, plus or minus liquid nitrogen. Ten shoot tips per treatment were included in this experiment.

Seed Desiccation, Cryopreservation and Germination

Seeds were extracted from freshly harvested mature fruit on a commercial plantation in southeast Queensland. Fruit was harvested in January (mid-summer) and 10 batches of 50 were weighed to obtain the mean weight of fresh seed. Desiccation of seeds was achieved by incubation over silica gel in a sealed container. Seeds were desiccated to 40%, 20%, 15%, 10%, and 5% of the fresh moisture content by incubation for a range of times over silica gel. Seeds were either directly germinated flowing desiccation or packed in plastic coated aluminum packages for freezing in liquid nitrogen. Packages of seeds exposed to liquid nitrogen were thawed in water at 45°C. Seeds for germination testing were sown in germination trays using a germination mix consisting of 70% peat moss and 30% perlite. The trays were placed in a glasshouse, with a day temperatures of 30°C and night temperature of 25°C. The number of germinated seeds was recorded during a three-week period post sowing and 10 seedlings from each treatment were measured at 2-weekly intervals to determine their growth rates.
RESULTS AND DISCUSSION
All control treatments yielded 100% recovery of shoot tips that grew into plantlets.

The Effect of Duration of Pre-cryopreservation Incubation of Excised Shoot Tips

Previously we had reported the use of an overnight preconditioning treatment prior to vitrification treatment (Ashmore et al., 2001). In the current experiment it was found that the rate of recovery of shoot tips post cryopreservation increased when excised shoot tips were incubated for between 1 and 4 days. Extending incubation duration beyond 4 days proved to be detrimental to the shoot tips and no tips recovered from post LN exposure with a 6-day incubation treatment. These results are consistent with Towill (1981) who reported that shoot tips of Solanum tuberosum had a greater survival rate if the freshly isolated shoot tips were pre-cultured on the regeneration medium for a period of 2 or 3 days.

The Effect of Duration of Exposure to 100% PVS2 on Cryopreservation of Shoot Tips

Vitrification solution containing glycerol, ethylene glycol, and diethylsulfoxide (DMSO), in high concentration, is highly toxic to plant tissue. Therefore, it is important for successful vitrification to identify minimum exposure time to the solution to obtain sufficient dehydration but to avoid the toxic effects. This has been achieved previously by Yamada et al. (1991), who obtained 80% regeneration when the apical meristem exposed to PVS2 for 5 min. at 25 °C or for 15 min. at 0°C. When shoot tips of papaya were exposed to 100% PVS2 for different durations of time, there were differences observed in the recovery rates of the shoot tips. The best rate of recovery was 70% for the tips that had been exposed to 100% PVS2 for 20 minutes at 0°C. No recovery occurred post cryopreservation with exposure to PVS2 for 10 minutes or 60 minutes, suggesting that time of exposure to PVS2 was critical to cryopreservation success.

The Effect of Position of Tips on Rate of Recovery of Cryopreserved Shoot Tips

This study revealed a relationship between the recovery of axillary shoot tips following cryopreservation and their distance from the apex (Table 1). The shoot tips were divided into those from the upper part of the shoot and those from the lower. Rate of recovery of the tips from the upper part of the shoot (48%) was twofold that of those from the lower part (27%) of the plant. This is consistent with Dereudder et al. (1988) who reported that resistance to LN decreased with distance from the shoot apex in carnation (Dianthus caryophyllus L.).

The Effect of Moisture Content on the Rate of Germination of Papaya Seeds

Germination rate of fresh and desiccated seeds were consistently high (80-90%) across the range of moisture contents down to 5%. This confirms the published report (Wood et al, 2000) that papaya seed is desiccation tolerant. Percentage germination for seeds that were cryopreserved at 100%, 40%, 20%, and 15% moisture content was 0%, 8%, 6%, and 8% respectively. A substantial increase in germination rate to 48% was achieved at 10% moisture content, declining to 20% at 5% moisture content for cryopreserved seeds (Fig. 1). These preliminary experimental results suggest that Queensland varieties may exhibit orthodox seed storage characteristics and it may be possible to store them using standard techniques. In addition, these early results suggest that if seeds are desiccated to about 10% moisture content, it may be possible to cryopreserve seeds for long-term storage. Becwar et al. (1983) also cryopreserved papaya seeds at 9-10% moisture content and obtained no loss of viability.

Plants re-grown in a glasshouse from cryopreserved seeds showed significantly reduced growth (plants were 25% shorter) when compared with control plants (Fig. 2). Similarly, the rate of growth in terms of stem diameter was also reduced by 25% in cryopreserved plants when compared with control plants (Fig. 3). A similar observation was made on one male plant regenerated from a cryopreserved shoot tip. After 2.5 years,
this plant is significantly retarded in growth and is shorter than 1m in height. Future molecular marker analyses may reveal whether such differences are caused by DNA modification occurring as a result of cryopreservation treatments. When pollen from the male plant was used to pollinate a female papaya that had not been cryopreserved, resultant seedlings demonstrated normal growth. Thus this dwarf character trait did not persist into the next generation.

CONCLUSIONS
Cryopreservation of shoot tips and seeds of papaya can be considered for maintenance of papaya germplasm. Work is being undertaken to further optimize these techniques. Early results indicate that cryopreserved plants can have a reduced rate of growth immediately post cryopreservation. Significant reduction in height has also been demonstrated in one plant grown through to the field. Investigation of the effects of LN on growth of explants and seed of other species is warranted.

Literature Cited
Table 1. The effect of the position of the shoot tips of papaya on the rate of recovery in terms of % regeneration of shoot tips at 21 days post thawing. The standard cryopreservation protocol (±LN) was used on both treatments. Figures are means of 10 replicates.

<table>
<thead>
<tr>
<th>Position of Shoot Tips on the Stem</th>
<th>Recovery rate from upper- half</th>
<th>Recovery rate from lower- half</th>
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<tr>
<td>- LN</td>
<td>100%</td>
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<td>+ LN</td>
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Fig. 1. Effect of seed moisture content and cryopreservation on germination of papaya seeds. Data are means of 100 replicate seeds.
Fig. 2. Effect of liquid nitrogen on seeds and subsequent growth of papaya seeds. Data are means of 100 replicate seeds.
Fig. 3. Effect of liquid nitrogen on seeds and subsequent growth of papaya seedlings. Data are means of 100 replicate plants.