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The cryopreservation of embryonic axes of two wild and endangered *Citrus* species

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Abstract

The cryopreservation of embryonic axes of two wild and endangered species, *Citrus macroptera* Mont. and *C. latipes* Tanaka, was attempted using air desiccation–freezing, vitrification and encapsulation–dehydration. Successful cryopreservation was achieved in both the species using these three methods. However, the two species responded differently to: the rate of drying and the degree of tolerance to desiccation following air desiccation–freezing; the response to loading duration following vitrification; and to the sucrose concentration during pre-culture following encapsulation–dehydration. *C. macroptera* was more tolerant to desiccation and freezing than *C. latipes* with recovery rates of, respectively, 87% and 64%. Recovery from encapsulation–dehydration was 62% for *C. macroptera* and 45% for *C. latipes*. In both species, the vitrification protocol gave a significant improvement in recovery rates: 92% and 77% for *C. macroptera* and *C. latipes*, respectively. The air desiccation–freezing protocol being a simple and practical technique is recommended for the cryopreservation of these two species.

Keywords: air desiccation; *Citrus latipes*; *Citrus macroptera*; cryopreservation; embryonic axes; encapsulation; pre-culture; vitrification

Introduction

Northeastern India is thought to be the area of origin of several citrus species (Tanaka, 1958), and diversity is abundant in the Northeastern region and the foothills of the central and western Himalayan tracts. However, genetic erosion in its natural habitats is accelerating (Ahuja, 1996), so that actions to promote species protection and conservation are warranted. *Citrus macroptera* Mont. and *C. latipes* Tanaka are among the seven species categorized as endangered (Singh and Singh, 2003). The former is of local economic relevance as a source of fresh fruit, juice and dried fruit peel (Malik *et al.*, in press). The latter produces large inedible fruits, but because it is adapted to high altitudes (900 m a.s.l. and above), it is important as a source of genes for cold tolerance, and thus is of interest to citrus improvement. *In situ* conservation is costly and risks the loss of valuable germplasm due to biotic and abiotic stresses (Singh and Chadha, 1993), and so long-term conservation using cryotechnology is desirable as a complement.

Wide inter-specific variation has been reported in seed storage behaviour in 20 *Citrus* species (Hong and Ellis, 1995; Hong *et al.*, 1996). Preliminary studies on seeds of *C. macroptera* and *C. latipes* have demonstrated sensitivity to desiccation and freezing temperatures and hence these were categorized as intermediate (Malik *et al.*, 2003). The design of suitable cryopreservation protocols requires a prior determination of the desiccation and freezing tolerance of the target species. Successful cryopreservation has been achieved in some *Citrus* species and the allied genus *Poncirus trifoliata* using a range of explants such as zygotic embryos/embryonic axes (Radhamani and Chandel, 1992; Cho *et al.*, 2001a, b, 2002), shoot apices (Gonzalez-Arnao *et al.*, 1998)

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and somatic embryos (Marin *et al*., 1993). Both the present subject species bear relatively large seeds, with average length × breadth, 14 × 5 mm (*C. macroptera*) and 16 × 7 mm (*C. latipes*). This large seed size necessitates the need for cryopreservation of embryonic axes.

We report here the cryopreservation of *C. macroptera* and *C. latipes*, using embryonic axes. This has been attempted using three different approaches: air desiccation–freezing, vitrification and encapsulation–dehydration. The results obtained have been compared and factors responsible are discussed.

Materials and methods

Fruits of *C. macroptera* and *C. latipes* were collected from Meghalaya and Mizoram States of Northeastern India. The seeds were extracted and stored at 15°C. Before initiating the cryopreservation process, the seed coat was removed and the embryonic axes were excised aseptically. Fresh, desiccated and cryopreserved embryonic axes, both naked and enclosed in beads, were cultured in two media, modified from Murashige and Skoog (1962). Medium 'A' contained macro- and micro-nutrients, vitamins, iron, 1 g/l activated charcoal, 0.17 g/l NaH_2PO_4 and 1 mg/l each of 6-benzylaminopurine (BAP) and α-naphthaleneacetic acid (NAA). Medium 'B' lacked charcoal and contained only 0.1 mg/l each of BAP and NAA. Cultures were maintained at 25 ± 2°C with a 16 h photoperiod under a light intensity of 35 μE/m^2/s. Emergence of root and shoot from an embryonic axis was considered indicative of viability.

To obtain embryonic axes, seeds were surface sterilized with 0.1% mercuric chloride for 10 min, followed by three washes with sterilized deionized water. Excised embryonic axes were spread on sterile filter paper discs in batches of 20–30, and dried in a sterile laminar flow cabinet for 1–6.5 h. The moisture content of the material was determined every 30 min to 1 h by oven drying at 103 ± 2°C for 17 h (ISTA, 1976) in triplicate (each sample consisting of 10–15 axes) and expressed on fresh weight basis. Desiccated embryonic axes were sealed in sterile 1.2 ml polypropylene cryovials and fast frozen in liquid nitrogen. After a minimum of 24 h storage, the cryovials were thawed in a water bath at 38 ± 1°C for 5 min, and the axes were immediately cultured *in vitro*. Experiments were repeated three times with 15–20 embryonic axes per treatment.

For vitrification experiments, aseptically excised embryonic axes were pre-cultured on basal MS medium supplemented with 0.3 M sucrose and 2 M glycerol for 16–24 h. Embryonic axes in batches of 15–25 were then transferred to 1.2 ml sterile cryovials and treated with loading solution (0.4 M sucrose, 2 M glycerol in basal MS medium) for either 20 or 40 min at 25°C. The loading solution was replaced by Plant Vitrification Solution 2 [PVS2; 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) dimethyl sulphoxide] for 30 min at 25°C. Axes were then frozen by fast freezing in liquid nitrogen. In control treatments, the PVS2 solution was removed, and the axes were treated with unloading solution (1.2 M sucrose in basal medium) for 20 min before culturing on semi-solid media. Frozen cryovials were thawed after a minimum of 24 h storage by 5 min immersion in a water bath at 38 ± 1°C, following which the loading solution was replaced with unloading solution for 20 min. The axes were then germinated *in vitro*. Experiments were repeated three times with 15–20 embryonic axes per treatment.

For the encapsulation–dehydration process, aseptically excised embryonic axes were encapsulated in alginate beads by first suspending in calcium-free MS basal liquid medium containing 3% (w/v) Na-alginate. Drops of this solution, each containing one axis, were dispensed with a pipette into MS basal liquid medium supplemented with 100 mM $CaCl_2$. Beads were solidified by 60 min incubation at 25°C with occasional stirring, and then pre-cultured on a rotary shaker in liquid MS medium supplemented with sucrose (0.3, 0.5 or 0.75 M) at 100 rpm for 20, 30 or 40 h. The beads were removed from liquid medium and dehydrated at room temperature for 6 h in a laminar flow cabinet. The moisture content of the beads was determined by drying at 103 ± 2°C for 17 h. Beads were finally enclosed in 1.2 ml cryovials and fast frozen in liquid nitrogen. The cryovials were thawed in a 38 ± 1°C water bath for 5 min, and the beads cultured *in vitro*. Experiments were repeated three times with 10–20 axes per treatment.

Data for viability after vitrification and encapsulation–dehydration were subjected to univariate analysis of variance using SPSS 10.0 software. Means were compared by Scheffé's test.

Results

Embryonic axes of *C. macroptera* showed higher initial moisture content (40.9%) than that of *C. latipes* (35.7%) (Figs 1 and 2). With increasing duration of desiccation, the moisture content of the axes declined steadily (Figs 1A and 2A). *C. latipes* had a higher drying rate (Fig. 2A) than *C. macroptera* (Fig. 1A), especially during the initial 2 h of desiccation. Later the rate of moisture loss was comparable until 6.5 h of desiccation. The recovery growth of fresh, desiccated and frozen axes was rapid and normal on culture media 'A' in comparison to media 'B', so observations using only the former are reported. Initial viability values of embryonic axes were between 95.0 and 96.6% in the two species. The viability

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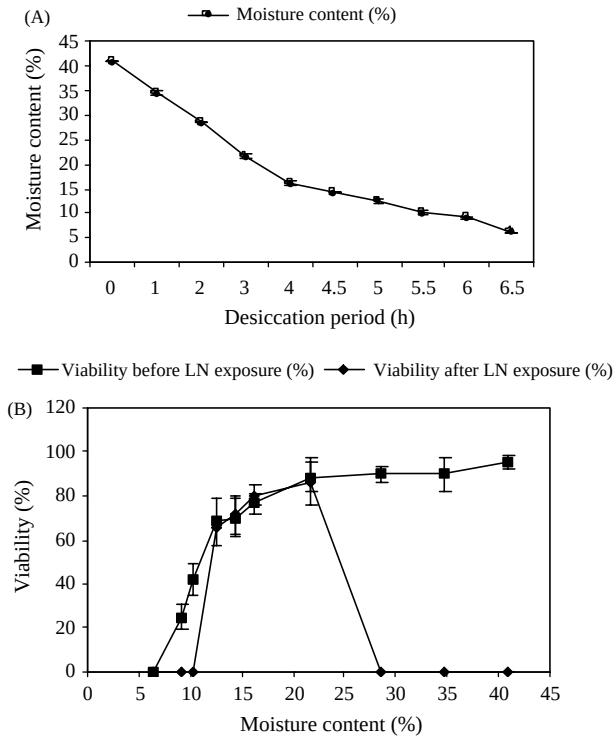

Fig. 1. (A) Decline in moisture content of embryonic axes in *Citrus macroptera* with increase in desiccation period; (B) effect of moisture content on viability of embryonic axes before and after liquid nitrogen exposure in *C. macroptera*.

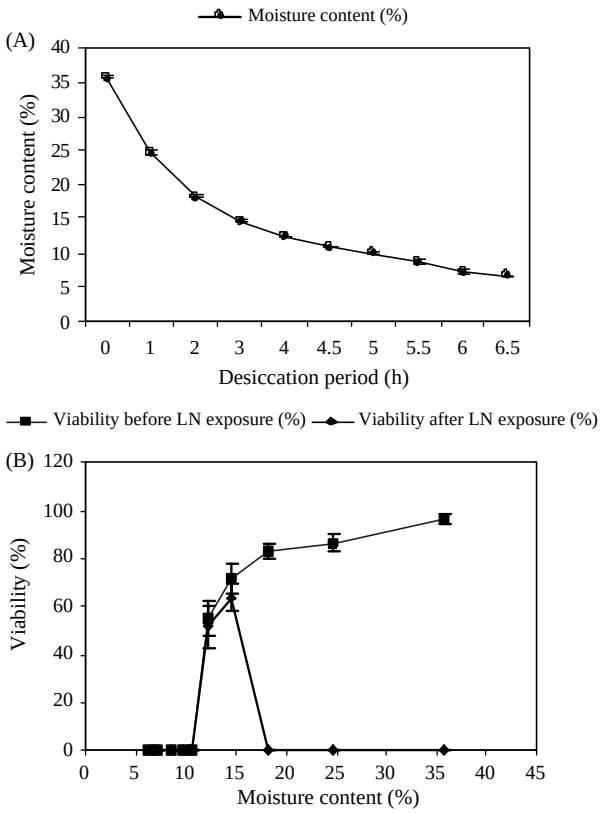

Fig. 2. (A) Decline in moisture content of embryonic axes in *Citrus latipes* with increase in desiccation period; (B) effect of moisture content on viability of embryonic axes before and after liquid nitrogen exposure in *C. latipes*.

of axes declined steadily with increasing duration of desiccation (Figs 1B and 2B). In *C. macroptera* viability remained high (about 90%) until 21.7% moisture content and declined to between 70 and 77% at a moisture content between 14 and 16%. Below 12.5% moisture viability fell rapidly, declining to 25% at 9.1% moisture content. Viability in *C. latipes* was 86.7% at 24.6% moisture, and declined rapidly to 55% at 12.3%. Viability was lost below this moisture level.

When the embryonic axes of both species at various moisture contents were fast frozen in liquid nitrogen (Figs 1B and 2B), those of *C. macroptera* frozen at moisture contents between 12.5 and 21.0% retained between 65.0 and 86.7% viability (Fig. 1B). Above 28.6% and below 10.2% moisture, the axes lost viability. In *C. latipes* the axes desiccated to 18% and higher moisture did not survive liquid nitrogen exposure (Fig. 2B), but those pre-desiccated to 12.3 and 14.5% survived cryostorage (about 51 and 64% viability, respectively). The viability of successfully cryopreserved axes were comparable to those of their respective desiccated controls in both species. In *C. latipes* the moisture content window at which success in cryopreservation was achieved was narrower (2.2%) than that for *C. macroptera* (9.2%).

Following vitrification after 16 and 24 h pre-culture, the axes of both species retained 90–100% viability. The 24 h pre-culture duration gave higher viability values, and thus this treatment was followed in further experiments (Table 1). Viability remained unaltered after loading treatments of both 20 and 40 min. Exposure to PVS2 for 30 min was optimal in terms of axis survival after liquid nitrogen exposure. PVS2 treatment had no deleterious effect on the viability of *C. macroptera*, but caused a decline in viability for *C. latipes* by about 11% and 17% in loading treatments of 20 and 40 min, respectively. After vitrification, the highest recovery percentages (92% in *C. macroptera* and 77% in *C. latipes*) were obtained using 20 min loading treatments. Axes processed for vitrification without any loading treatment had a very low survival rate of 5–7%.

Fresh encapsulated axes of the two species had excellent viability (95% and 82%, respectively; Table 2). Encapsulated axes following 40 h of pre-culture in various sucrose concentrations had moisture contents of 72–78%. A further 6 h of air desiccation reduced the moisture contents to under 20%, but was accompanied by a decline in viability. Pre-culture periods of 20 and 30 h followed by 6 h desiccation resulted in higher moisture contents (above 35%) and no subsequent axis survival.

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Table 1. Viability of embryonic axes after vitrification in *Citrus* species

Species	Viability after 24 h pre-culture (%)	Loading duration (min)	Viability after loading (%)	Viability after PVS2 treatment (%)	Viability after vitrification (%)
C. macroptera	96.7 (±1.6)	0	–	100.0 (±0)	7.5^{c} (±0.3)
		20	100.0	100.0 (±0)	92.0^{a} (±5.0)
		40	98.8	97.8 (±1.1)	75.2^{b} (±1.1)
C. latipes	95.0 (±2.8)	0	–	86.7 (±3.3)	5.2^{c} (±1.1)
		20	94.4	83.3 (±7.2)	76.7^{a} (±4.4)
		40	95.3	78.5 (±1.2)	55.2^{b} (±1.1)

Values in parentheses indicate the standard error of means. Means followed by the same superscript letter in the viability column after vitrification do not differ significantly ($P = 0.05$) based on Scheffé's test.

Using beads, the highest viability achieved for *C. macroptera* was 65.5% at 17.3% moisture content, and the lowest 40% at about 15% moisture content (Table 2). Lower viability was achieved for *C. latipes* (43, 32 and 13% at, respectively, moisture contents of about 18, 17 and 15%). *C. macroptera* and *C. latipes* beads desiccated to similar moisture contents of about 15% differed substantially in viability (40% versus 13%). Successful cryopreservation was achieved for all the desiccated beads with levels of viability ranging from 30 to 62% for *C. macroptera* and from 20 to 45% for *C. latipes*. Overall, *C. latipes* was found to be more sensitive to dehydration (air desiccation, vitrification and encapsulation) than *C. macroptera*. The axes of *C. latipes* were more sensitive to liquid nitrogen exposure, and exhibited a lower level of recovery.

The control, desiccated and cryopreserved axes of both species subjected to air desiccation–freezing initiated growth within 5–7 days of culture, and well-formed shoots and roots were evident within 20 days. Normal plantlets with 2–3 cm shoots and two to five pairs of leaves and 3–5 cm long roots were formed within about 80 days. Embryonic axes subjected to vitrification and encapsulation behaved in a similar way. Using the three methods, normal regrowth of axes without any intervening callus stage was observed for both species. The initiation of axis growth from beads was, however, delayed by 6–8 days compared to outcomes of the other two methods.

Overall among the three methods, vitrification was superior to air desiccation–freezing in terms of higher recovery, while encapsulation resulted in lower viability for both species.

Discussion

Cryopreservation techniques have found application in the long-term conservation of citrus germplasm using seeds, embryonic axes and somatic embryos. Viability percentages of 50–100% have been achieved using air desiccation (Normah and Hamidah, 1992; Cho *et al.*, 2002), pregrowth–desiccation (Cho *et al.*, 2002) and vitrification (Cho *et al.*, 2001a, b) in various species. Encapsulation–dehydration has not been attempted so far. Also until the present report, there has been no systematic study of the two wild and endangered species, *C. macroptera* and *C. latipes*.

Table 2. Viability of embryonic axes after encapsulation–dehydration in *Citrus* species

Species	Viability of encapsulated axes in controls (%)	Sucrose concentration in pre-culture media (M)	Moisture content after pre-culture for 40 h (%)	Moisture content after further 6 h air desiccation (%)	Viability of desiccated beads (%)	Viability after liquid nitrogen exposure (%)
C. macroptera	95.0 (±5.00)	0.30	78.64 (±0.35)	17.3 (±0.81)	65.5^{a} (±7.97)	61.7^{a} (±7.26)
		0.50	74.81 (±0.47)	15.7 (±0.62)	40.0^{ab} (±3.81)	33.4^{b} (±4.40)
		0.75	72.55 (±0.44)	14.9 (±0.85)	40.0^{ab} (±7.73)	30.0^{b} (±8.66)
C. latipes	82.0 (±3.15)	0.30	77.75 (±0.62)	17.9 (±1.02)	43.4^{a} (±6.66)	45.0^{a} (±5.00)
		0.50	74.84 (±0.25)	16.6 (±0.61)	32.5^{ab} (±5.89)	30.5^{b} (±6.63)
		0.75	72.52 (±0.69)	15.4 (±0.59)	13.4^{b} (±3.83)	20.0^{b} (±5.00)

Values in parentheses indicate the standard error of means. Means followed by the same superscript letter in the columns showing viability after desiccation and liquid nitrogen exposure do not differ significantly ($P = 0.05$) based on Scheffé's test.

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The desiccation sensitivity of an explant is the degree of its tolerance to lose free water without associated damage and a decline in viability. We have demonstrated differences between the two species in terms of their sensitivity to desiccation, reflecting both distinct rates of drying when subjected to similar drying conditions, and their degree of tolerance to desiccation. A higher sensitivity to air desiccation and freezing characterized *C. latipes*, and a combined effect of desiccation and freezing led to differential viability. The embryonic axes of the two species had a high initial moisture content, a relatively high critical moisture content (level below which significant reduction in viability is recorded), a significant decline in viability with the reduction in moisture content, and a sensitivity to freezing, all indicative of intermediate storage category. Seeds of both species have previously been shown to have intermediate seed storage behaviour (Malik *et al.*, 2003).

Successful cryopreservation using air desiccation–freezing was achieved in the embryonic axes of both species. A vitrification technique, used previously to freeze apices of a range of species, is being used with variable success to freeze embryonic axes of some intermediate and recalcitrant species (neem, Chaudhury and Malik, 1999; rubber, Sam and Hor, 1999; jackfruit, Thammasiri, 1999). Using this technique in *Citrus* species, a recovery rate of 62.5% was reported in *C. sinensis* (Sudarmonowati, 2000) and 82.5% in *C. madurensis* (Cho *et al.*, 2001b). In the present study, vitrification was successfully applied to the two new species *C. macroptera* and *C. latipes*, leading to high viability.

During vitrification, the pre-culture of explants on sucrose- or sorbitol-enriched medium for 1 or 2 days has been shown to induce dehydration tolerance, thereby improving the viability of cryopreserved cells, meristems and embryonic axes (Thammasiri, 1999; Sakai, 2000; Cho *et al.*, 2001b). We suggest that the beneficial effect of pre-culture may be attributed to two factors. Firstly, during 24 h pre-culture any difference in moisture content within the tissues of a given axis, and between axes (a characteristic of non-orthodox seed species), was probably equalized, so that tissue could be optimally desiccated; and secondly, it may have brought any immature axes present to physiological maturity.

The use of loading solution reportedly induces dehydration tolerance to freeze-dehydration or to PVS2, and imparts protection against injury to membranes. The beneficial effect of loading has been reported for several tropical monocots (Sakai, 2000), and was effective in our experiments to enhance the recovery of vitrified axes in both *Citrus* species.

Chemical dehydration with PVS2 before freezing affords better protection than simple air dehydration, as shown by the high percentage viability achieved after vitrification. The duration of PVS2 treatment is a further variable that can be manipulated to enhance the success of cryopreservation (Sakai, 2000). In similar studies of the desiccation-sensitive seed species *Citrus jhambiri*, we have found that vitrification proved better than air desiccation–freezing in improving recovery of plantlets (unpublished results). Unlike shoot apices that are known to be sensitive to osmotic dehydration and/or toxic chemicals, especially those used in vitrification solution, embryonic axes are hardier and hence can more easily survive the various steps of vitrification.

The encapsulation–dehydration technique, although successful, resulted in slower and less recovery than using the other two methods. To date, the technique has only been applied to embryonic axes of one other non-orthodox species, *Hevea brasiliensis*, where encapsulated axes pre-cultured with 0.3 M sucrose and desiccated to 10–26% moisture showed a survival rate of 60% (Yap *et al.*, 1999). The air desiccation–freezing method adopted by Normah *et al.* (1986) yielded better results.

In summary, we have demonstrated the utility of three cryopreservation techniques for the first time on the embryonic axes of two wild and endangered *Citrus* species from Northeastern India, and have developed systems which allow rapid recovery growth of healthy plantlets without an intervening callus stage. Both air desiccation–freezing and vitrification are adequate, but the former is recommended for the routine cryostorage of germplasm, particularly as it represents a simple and practical method which eliminates the need for cytotoxic chemicals.

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