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ORIGINAL ARTICLE

In vitro response of encapsulated and non-encapsulated somatic embryos of Kinnow mandarin (*Citrus nobilis* Lour × *C. deliciosa* Tenora)

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Abstract The aim of present investigation was to study the effect of storage conditions on percentage germination of encapsulated and non-encapsulated somatic embryos of Kinnow mandarin (*Citrus nobilis* Lour × *C. deliciosa* Tenora). Different batches of encapsulated and non-encapsulated embryos were preserved at room temperature, 4°C, in liquid nitrogen as such and by embedding in liquid paraffin. In the encapsulated somatic embryos stored at room temperature in sealed Petri plates, percentage of germination was 24.99%, but 5.55% in non-encapsulated embryos after 3 days of storage. Encapsulated embryos stored in vials containing liquid paraffin at room temperature were germinated at 18.05% after 60 days of storage, while it was 13.88% in non-encapsulated embryos after 45 days of storage. Encapsulated somatic embryos stored at 4°C in sealed Petri plates remained viable for up to 75 days with 6.94% germination, whereas non-encapsulated embryos remained viable for up to 45 days with 24.99% germination. Encapsulated embryos stored at 4°C in vials filled with paraffin germinated at 11.11% after 120 days of storage, but 5.55% in non-encapsulated embryos after 90 days of storage. Encapsulated and non-encapsulated embryos stored in liquid nitrogen showed 58.33 and 51.38% survival, respectively, after 7 months of storage. The plantlets developed from these embryos were transplanted after acclimatization and are growing normal.

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Introduction

Kinnow mandarin (*Citrus nobilis* × *C. deliciosa*) is one of the most economically important fruit crops of India. In recent years, Kinnow production in Punjab has been greatly affected due to Indian citrus ringspot virus infection, which has resulted in tremendous loss in yield and quality of this fruit crop. This has necessitated the development of strategies for controlling viral diseases and production of healthy virus free Kinnow orchards in the country, especially in Punjab. Recently, we demonstrated somatic embryogenesis from unfertilized ovules of Kinnow to produce virus free plants (Singh et al. 2005). Somatic embryogenesis is a powerful tool and has a potential application for clonal propagation of virus free plants. However, retention of embryogenic potential of embryogenic cells during long-term maintenance is difficult and the risk of occurrence of somaclonal variation increases with increasing culture durations (Wang et al. 2004). Frequent subculture of embryogenic cultures which has been found beneficial in preventing browning (Bornhoff and Harst 2000) and promoting embryo development (Coutos-Thevenot et al. 1992) is costly and laborious. The maintenance of virus free material (sanitorized lines) by in vivo or active in vitro conditions can be very expensive and not cost optimal. The risk of losing plants due to diseases, climate conditions or human errors is high (Brison et al. 1995). Therefore, in order to make the system economically viable, many workers have attempted to prepare synthetic seeds by encapsulation of somatic embryos in an

alginate matrix (Kitto and Janick 1985; Redenbaugh et al. 1986). Synthetic seed technology is an important application of micropropagation that offers several advantages, e.g. easy handling, storability, reduced size of propagules, transportability, conversion to plantlets, etc. (Gosh and Sen 1994; Onishi et al. 1994; Guerra et al. 1999). One of the main objectives of this technology is to develop a clonal propagation system that will enable the vegetative propagule to be stored for long periods of time and at the same time enable multiplication of plant (Janeiro et al. 1997).

It has been reported that both encapsulated and non-encapsulated somatic embryos of interior and black spruce survived 1 month of storage at 4°C with no loss in germination capacity (Lulsdorf et al. 1993). Similarly, Rao and Bapat (1993) reported that encapsulated embryos of *Santalum album* retained their germinability (18%) after storage at 4°C for 45 days. Cold storage of encapsulated embryos is important for post storage survival and germination. Cryopreservation has been considered as an ideal tool for long-term storage of germplasm during which all cellular divisions and metabolic processes of stored cells are stopped. Theoretically, the plant materials can thus be stored without any changes for an indefinite period of time (Engelmann 1997), and the procedure guarantees long-term storage of germplasm in an unaltered stage. Synthetic seed research has been carried out in a number of species including carrot (Timbert et al. 1996), alfalfa (Redenbaugh et al. 1986), conifers (Fowke et al. 1994), high value vegetable crops like celery, lettuce (Sanada et al. 1993) and pistachio (Onay et al. 1996), and a few woody species (Gray 1987; Bapat and Rao 1988; Rao and Bapat 1993).

This communication describes conservation strategies for somatic embryos of Kinnow mandarin by comparing the germination potential of encapsulated and non-encapsulated somatic embryos under different storage conditions.

Materials and methods

Production of somatic embryos

Somatic embryos of Kinnow (*C. nobilis* × *C deliciosa*) were obtained from unfertilized ovules as previously described (Singh et al. 2005). The ovules were cultured on MS medium (Murashige and Skoog 1962) supplemented with kinetin (KN) (9.29 μM) containing 3% (w/v) sucrose and 0.8% (w/v) agar (SRL, Mumbai) with pH adjusted to 5.6 and autoclaved at 121°C and 15 lb in^{-2} pressure for 20 min. The somatic embryos were allowed to mature on the same medium and embryos at heart-shaped stage to torpedo stage were used for encapsulation.

Encapsulation

Freshly developed embryos, mixed with sodium alginate (4%) prepared in liquid MS medium with 3% sucrose at pH 5.6, were dropped into 75 mM $CaCl_2 \cdot 2H_2O$ using a wide glass dropper. The drops (beads), each containing single embryo, were kept in $CaCl_2 \cdot 2H_2O$ solution for 45 min on a rotary shaker (70 rpm). Beads were recovered by decanting $CaCl_2$ solution. Beads containing no or more than one somatic embryos were eliminated. They were then thoroughly washed with distilled water and surface dried by spreading on sterile filter paper for 30 min in laminar cabinet. All the steps involved in the preparation of synthetic seeds were carried out aseptically in the laminar flow cabinet.

Storage conditions

Different batches of encapsulated and non-encapsulated embryos were preserved at room temperature, 4°C, and in liquid nitrogen as such, and by embedding in liquid paraffin. Autoclaved airtight vials filled with liquid paraffin and sterilized Petri plates were used for storage at room temperature and at 4°C. For storage in liquid nitrogen, encapsulated and non-encapsulated embryos (10/vial) were transferred to autoclaved cryovials (10 ml) containing 10% (v/v) DMSO and 0.4 M sucrose in liquid MS medium. These vials were kept at 4°C for 30 min, 0°C for 24 h, frozen slowly in an ultra cooling bath (Yorko) to –20°C and then transferred to liquid nitrogen.

Germination

The encapsulated and non-encapsulated embryos were allowed to germinate on simple MS medium or MS medium with different concentrations of KN (4.64, 9.29, 13.94 and 18.59 μM) to find out the best suitable medium for germination. The encapsulated and non-encapsulated embryos stored at room temperature in sealed Petri plates were cultured on medium after every 24 h to check their germination. The encapsulated and non-encapsulated embryos without storage were cultured along with each experiment as control. The encapsulated and non-encapsulated embryos stored in sealed Petri plates at 4°C and in vials filled with liquid paraffin stored at room temperature or at 4°C were checked for their germination after every 15 days until they responded. The encapsulated and non-encapsulated embryos stored in liquid nitrogen were checked for their viability after 1 h of storage and after every 1 month from date of storage and percent germination was calculated. Cryotubes removed from LN were rapidly warmed in a water bath at 40°C for 4 min and encapsulated and non-encapsulated embryos were washed in autoclaved distilled

water. All cultures were maintained at 26 ± 1°C with a luminous intensity of 40 μmol m^{-2} s^{-1} and 16:8 h photoperiod. The germination frequency of all encapsulated and non-encapsulated embryos was recorded after 15 days of inoculation.

Statistical analysis

For evaluation of viability of encapsulated and non-encapsulated embryos stored at each condition, 24 tubes were inoculated along with control and the experiments were repeated thrice. The data obtained from above experiments were subjected to one-way analysis of variance test, and the differences among means were compared by high-range statistical domain using Tukey's test (Meyers and Grossen 1974).

Results

Encapsulated and non-encapsulated somatic embryos cultured on MS medium supplemented with different concentrations of kinetin showed signs of germination after 6–8 days of inoculation (Fig. 1a) when roots of the embryo pierced the alginate matrix. Similarly, roots of non-encapsulated embryos started penetrating into the medium after 5–6 days of inoculation and established contact with the medium; subsequently, shoots also emerged. The successive stages of synthetic seed germination are shown in Fig. 1a. The somatic embryos, both encapsulated and non-encapsulated, failed to germinate on MS medium without kinetin (control). Among the various concentrations of kinetin tested, kinetin at 9.29 μM was found to be most effective with 81.94% germination for encapsulated and 86.11% germination for non-encapsulated somatic embryos (Table 1). The same medium was used for further studies on germination frequency of preserved synthetic seeds. Table 2 shows the effect of storage at room temperature in Petri plates on percentage germination of encapsulated and non-encapsulated somatic embryos. The percentage germination of encapsulated embryos decreased with increase in duration of storage with 80.55% for 24 h, 62.49% for 48 h, 24.99% for 72 h and very low or almost negligible at 96 h (1.38%). Similarly, percentage survival of non-encapsulated somatic embryos also decreased with increase in duration of storage up to 72 h. Further increase in storage duration at room temperature resulted in 0% survival of both encapsulated and non-encapsulated somatic embryos. Table 3 shows the effect of storage of somatic embryos at 4°C in Petri dishes on percentage germination. As seen in this table, percent germination of non-encapsulated and encapsulated somatic embryos decreased with increase in storage duration from 0–75 days.

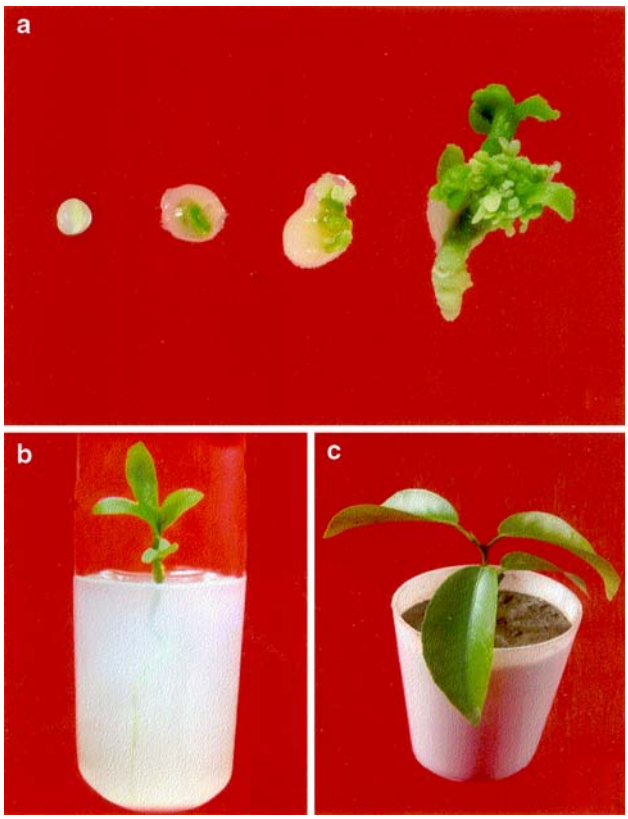

Fig. 1 **a** Successive stages of synthetic seed germination of Kinnow mandarin (*Citrus nobilis* Lour × *C. deliciosa* Tenora) on MS medium supplemented with kinetin (9.29 μM), **b** plantlet developed from synthetic seed on MS medium supplemented with kinetin (9.29 μM), **c** transplanted plantlet in plastic pot

Table 1 Effect of MS medium supplemented with different concentrations of kinetin on percentage germination of unstored encapsulated and non-encapsulated somatic embryos of Kinnow mandarin (*Citrus nobilis* Lour × *C. deliciosa* Tenora)

Kinetin (μM)	% Survival of encapsulated embryos[1]	% Survival of non-encapsulated embryos[2]
4.64	61.11 ± 1.40[b]	65.27 ± 1.39[b]
9.29	81.94 ± 1.38[a]	86.11 ± 1.40[a]
13.94	65.27 ± 1.39[b]	68.05 ± 1.38[b]
18.59	56.94 ± 1.39[b]	63.88 ± 1.39[b]

Data shown are mean ± SE of three experiments, each experiment consisted of 24 replicates

Means followed by the same letter are not significantly different ($P \leq 0.05$) using HSD multiple comparison test

[1] Data recorded after 10 days of inoculation

[2] Data recorded after 8 days of inoculation

Encapsulated embryos could be stored at 4°C for maximum of 75 days with 6.94% germination. Further increase in storage duration showed no response for germination of both encapsulated and non-encapsulated somatic embryos.

Table 2 Effect of storage at room temperature in Petri plates on percentage germination of encapsulated and non-encapsulated somatic embryos of Kinnow mandarin inoculated on MS medium supplemented with 9.29 μM kinetin

Storage duration (h)	% Germination of encapsulated embryos[1]	% Germination of non-encapsulated embryos[2]
0	81.94 ± 1.39[a]	86.11 ± 1.39[a]
24	80.55 ± 1.39[a]	79.16 ± 2.41[b]
48	62.49 ± 2.41[b]	30.55 ± 1.38[c]
72	24.99 ± 2.40[c]	5.55 ± 2.40[d]
96	1.38 ± 1.38[d]	–

Data shown are mean ± SE of three experiments, each experiment consisted of 24 replicates

Means followed by the same letter are not significantly different ($P \leq 0.05$) using HSD multiple comparison test

[1] Data recorded after 10 days of inoculation

[2] Data recorded after 8 days of inoculation

Table 3 Effect of storage at 4°C in Petri dishes on percentage germination of encapsulated and non-encapsulated somatic embryos of Kinnow mandarin inoculated on MS medium supplemented with 9.29 μM kinetin

Storage duration (days)	% Germination of encapsulated embryos[1]	% Germination of non-encapsulated embryos[2]
Control	81.94 ± 2.79[a]	86.10 ± 2.78[a]
15	76.38 ± 1.38[a]	69.44 ± 2.79[b]
30	70.83 ± 2.40[a]	43.05 ± 1.38[c]
45	65.27 ± 2.79[a]	24.99 ± 1.40[d]
60	22.22 ± 1.39[b]	2.77 ± 1.38[e]
75	6.94 ± 1.38[c]	–

Data shown are mean ± SE of three experiments, each experiment consisted of 24 replicates

Means followed by the same letter are not significantly different ($P \leq 0.05$) using HSD multiple comparison test

[1] Data recorded after 10 days of inoculation

[2] Data recorded after 8 days of inoculation

Effect of storage at room temperature in vials filled with paraffin liquid on percentage germination of encapsulated and non-encapsulated somatic embryos is shown in Table 4. Encapsulated embryos could be stored in liquid paraffin at room temperature for a maximum of 75 days with 2.77% germination frequency, while non-encapsulated embryos showed germination frequency of 13.88% after 45 days of storage in liquid paraffin. As seen in Table 5, both encapsulated and non-encapsulated somatic embryos could be stored in liquid paraffin for longer duration at 4°C. Encapsulated somatic embryos could be stored in liquid paraffin at 4°C for 120 days as compared to 75 days for room temperature. Effect of storage in liquid nitrogen on percentage germination of encapsulated and non-encapsulated somatic embryos is shown in Table 6. The percentage germination of 68.05% for encapsulated and 62.49% for non-encapsulated somatic embryos was observed after 1 h of preservation in liquid nitrogen. A slight decrease in percentage germination of encapsulated (66.66%) and non-encapsulated (61.11%) somatic embryos was observed after 1 month of storage which further decreased to 58.33 and 51.38%, respectively, after 7 months of storage. This decrease in percentage germination observed between 1 and 7 months of storage was found to be

Table 4 Effect of storage at room temperature in vials filled with paraffin liquid on percentage germination of encapsulated and non-encapsulated somatic embryos of Kinnow mandarin inoculated on MS medium supplemented with 9.29 μM kinetin

Storage duration (days)	% Germination of encapsulated embryos[1]	% Germination of non-encapsulated embryos[2]
Control	80.55 ± 1.38[a]	87.49 ± 2.39[a]
15	79.16 ± 2.41[a]	63.88 ± 1.38[b]
30	63.88 ± 1.38[b]	40.27 ± 1.39[c]
45	49.99 ± 2.41[c]	13.88 ± 1.40[d]
60	18.05 ± 1.39[d]	0.00 ± 0.00
75	2.77 ± 2.40[e]	–

Data shown are mean ± SE of three experiments, each experiment consisted of 24 replicates

Means followed by the same letter are not significantly different ($P \leq 0.05$) using HSD multiple comparison test

[1] Data recorded after 10 days of inoculation

[2] Data recorded after 8 days of inoculation

Table 5 Effect of storage at 4°C in vials containing paraffin liquid on percentage germination of encapsulated and non-encapsulated somatic embryos of Kinnow mandarin inoculated on MS medium supplemented with 9.29 μM kinetin

Storage duration (days)	% Germination of encapsulated embryos[1]	% Germination of non-encapsulated embryos[2]
Control	81.94 ± 1.38[a]	86.11 ± 1.39[a]
15	79.16 ± 2.40[a]	68.05 ± 1.39[b]
30	72.22 ± 1.39[a]	54.16 ± 2.40[c]
45	68.05 ± 1.40[a]	46.10 ± 2.72[c]
60	56.94 ± 1.39[b]	22.22 ± 1.38[d]
75	49.99 ± 2.38[c]	13.88 ± 1.39[e]
90	41.66 ± 2.40[d]	5.55 ± 1.38[f]
105	23.61 ± 1.39[e]	–
120	11.11 ± 1.39[f]	–

Data shown are mean ± SE of three experiments, each experiment consisted of 24 replicates

Means followed by the same letter are not significantly different ($P \leq 0.05$) using HSD multiple comparison test

[1] Data recorded after 10 days of inoculation

[2] Data recorded after 8 days of inoculation

Table 6 Effect of storage in liquid nitrogen on percentage germination of encapsulated and non-encapsulated somatic embryos of Kinnow mandarin inoculated on MS medium supplemented with 9.29 μM kinetin

Storage duration	% Germination of encapsulated somatic embryos[1]	% Germination of non-encapsulated somatic embryos[2]
Control	81.94 ± 1.39[a]	86.10 ± 2.78[a]
1 h	68.05 ± 1.38[b]	62.49 ± 2.39[b]
1 month	66.66 ± 2.39[b]	61.11 ± 1.38[b]
2 month	65.27 ± 2.79[b]	59.72 ± 1.38[b]
3 month	63.88 ± 2.79[b]	56.94 ± 1.40[b]
4 month	62.49 ± 2.40[b]	55.55 ± 1.39[b]
5 month	61.10 ± 2.39[b]	54.16 ± 2.40[b]
6 month	59.72 ± 1.38[b]	52.77 ± 2.79[b]
7 month	58.33 ± 2.38[b]	51.38 ± 2.78[b]

Data shown are mean ± SE of three experiments, each experiment consisted of 24 replicates

Means followed by the same letter are not significantly different ($P \leq 0.05$) using HSD multiple comparison test

[1] Data recorded after 10 days of inoculation

[2] Data recorded after 8 days of inoculation

statistically insignificant. The plantlets developed from these embryos were transplanted after acclimatization and are growing normal.

Discussion

The present results demonstrate the feasibility of preservation techniques to store virus free somatic embryos. Considering the importance of virus free propagation by somatic embryogenesis, developing a method of preservation is essential. Storage is not only beneficial for long-term usage but also prevents any somaclonal variation that may ensue the long-term maintenance of the embryogenic tissue (Ford et al. 2000). Synthetic seeds (encapsulated embryos) germinated without storage showed good germination percentage as alginate matrix, which facilitates regular nutrient supply and protects the somatic embryos from any mechanical injury during handling and from desiccation. This observation is in accordance with previous results (Sharma et al. 1992; Timbert et al. 1996; Naik and Chikkagouda 1997; Rodriguez et al. 2001; Malabadi and Nataraja 2002; Ipekci and Gozukirmizi 2003; Singh and Krishnasamy 2004). The encapsulated and non-encapsulated somatic embryos stored at room temperature in sealed Petri plates remained viable for only 3–4 days. Encapsulated embryos stored in vials containing liquid paraffin remained viable up to 75 days and non-encapsulated for 45 days. These findings are in accordance with some of the earlier findings reported in other plants, e.g. Schultheis et al. (1990) stored encapsulated somatic embryos of *Ipomoea batatas* (Ipomoea) at room temperature for 6 days and observed 40% viability in potassium starch acrylamide, 100% in Hydroxyethylcellulose and 0% in potassium acrylate. Encapsulated somatic embryos stored at 4°C in sealed Petri plates and in vials filled with paraffin germinated for only up to 75 and 120 days of storage, respectively. Bapat and Rao (1988) successfully germinated somatic embryos of *S. album* (sandalwood) after 45 days of storage at 4°C and observed that germination rate was reduced to 10%. Sharma et al. (1992) observed that percentage conversion of encapsulated protocorm like bodies of *Dendrocalamus wardianum* declined with increase in duration of storage at 4°C with a maximum of 100% up to 45 days and a minimum of 10% after 180 days of storage. Gill et al. (1994) observed 70% viability of encapsulated somatic embryos of geranium after 45 days of storage at 4°C. Sunilkumar (2000) observed 75% viability after 4 weeks of storage of excised embryos of *Hopea parviflora.* Soneji et al. (2002) observed that encapsulated shoots of pineapple (*Ananas comosus*) when stored at 4°C for 15, 30, 45 to 60 days show 75, 66.6, 29.17 and 8.33% viability, respectively. Ipekci and Gozukirmizi (2003) observed germination frequencies of 43.2 and 32.4% after 30 and 60 days of storage at 4°C. Singh and Krishnasamy (2004) stored encapsulated and non-encapsulated somatic embryos at 4°C for 60 days and observed viability of 6% for encapsulated and 0% for non-encapsulated embryos. Tsvetkov and Hausman (2005) observed 95% viability of encapsulated apical microcutting of *Quercus robur* (Turkey oak) after 42 days (6 weeks) of storage at 4°C. In the present study, the cryopreserved encapsulated and non-encapsulated embryos immediately after preservation (1 h) showed germination percentage of 68.05 and 62.49%, respectively, as compared to 81.94 and 86.11% for unstored encapsulated and non-encapsulated somatic embryos, respectively. Further storage of these somatic embryos resulted in only a slight decrease in percentage germination of encapsulated (66.66%) and non-encapsulated (61.11%) somatic embryos after 1 month of storage, which further decreased to 58.33 and 51.38%, respectively, after 7 months of storage. The encapsulated embryos subjected to cryopreservation without slow freezing and recovered without fast thawing did not show any regrowth even when cryoprotectant was used. The treatment most conducive for survival of encapsulated embryos in liquid nitrogen was observed to be 10% DMSO in Liquid MS medium with 3% sucrose. DMSO has been widely used as cryoprotectant in different plants, such as *Citrus sinensis* (Kobayashi et al. 1990; Sakai et al. 1990), *Anigozanthos viridis* (Turner et al. 2001), *Dioscorea floribunda* (Ahuja et al. 2002), *Porphyra yezoensis* (Liu et al.

2004), and *Musa* sp. (Panis et al. 2005). Wang et al. (2002) cryopreserved embryogenic cell suspension of *Vitis vinifera* (grapevine) by encapsulation–dehydration and observed 78% survival immediately after preservation. Fang et al. (2004) immersed encapsulated somatic embryos of cacao (*Theobroma cacao*) in LN and observed 63% survival. Panis et al. (2005) cryopreserved shoot tips from in vitro raised banana plants using 30% glycerol, 15% ethylene glycol and 15% DMSO as cryoprotectant solution and observed viability of 52.9%. Cryopreservation was found most suitable for preservation of virus free cultures for longer duration and storage at 4°C was found to be most suitable for short-term storage. The synthetic seeds germinate to produce well-developed plantlets when cultured on germination medium after 4–5 weeks of culture (Fig. 1b). The plantlets developed from these embryos were transplanted (Fig. 1c) after acclimatization and are growing normal.

This study showed that cryopreservation of encapsulated somatic embryos can be used for conservation of desired disease free stocks of Kinnow mandarin.

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