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Protocol for plant regeneration from encapsulated somatic embryos of *Citrus sinensis* L.

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Abstract

Background Citrus is an important fruit crop worldwide; artificial seeds are through encapsulation techniques of somatic embryos having many applications such as large-scale propagation and germplasm conservation. The aim of this study is the investigation of the viability of encapsulated citrus somatic embryos after different storage periods and to convert them into plantlets.

Results Cotyledonary-stage somatic embryos (5–7-mm size) regenerated from stigma explants of Washington navel orange (*Citrus sinensis* L.) were encapsulated individually in 3% sodium alginate. After different preservation periods (1, 2 or 3 months) at 10 °C, the encapsulated somatic embryos were cultured on Murashige and Skooge (MS) medium solidified with 7 g/l agar and supplemented with 50 g/l sucrose for germination. Percentage of regrowth, germination percentage, number of plantlets, plantlets height and number of leaves/shoot were recorded after different recovery periods. The germination percentages of encapsulated embryos were 90, 62.5 and 40% with storage for one, two and three months, respectively. Encapsulated somatic embryos preserved for 1 month developed the highest number of plantlets, while those preserved for 2 months developed the highest length of plantlets and the highest number of leaves/shoot after a recovery period of 10 months. Molecular analysis was performed of plantlets recovered from somatic embryos after preservation by encapsulation, and the results showed that the percentages of polymorphism were 7.7% with the two primers in all treatments.

Conclusions It could be concluded that callus developed from stigma explants was able to regenerate indirect somatic embryogenesis after 3 months. Cotyledonary-stage somatic embryos of citrus were successfully preserved by encapsulation using 3% sodium alginate. Frequencies of germination of encapsulated somatic embryos increased with increasing the recovery period on the germination medium. A maximum recovery frequency of 60.8% was obtained from encapsulated somatic embryos cultured on germination medium for up to 10 months. Also, recovery frequency of 62.5% was noticed from encapsulated somatic embryos preserved for 2 months at 10 °C. In addition, results indicated that recovered somatic embryos obtained from encapsulated somatic embryos were able to convert to normal plantlets.

Keywords Citrus, Encapsulation, RAPD, Somatic embryos

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Background

Citrus is one of the most popular and widely grown fruit crops in the world (grown in more than 140 countries around the world). Among the most commonly cultivated types, *Citrus sinensis* L. account for more than half

of world citrus production Oranges world production is the most significant within citrus, reaching 75.57 million tons (46.7% of citrus fruit production) in a harvested area of 9.93 million hectares (FAO 2023). Citrus cultivars are of major economic fruits in Egypt as local consumption or exportation. Washington Navel is the key cultivar of navel orange grown in Egypt and the best-known naval orange exported, primarily to Saudi Arabia, UAE, Russia and the Netherlands. Navel oranges represent 20 percent of total volume of oranges exported.

Studies on synthetic seed production using somatic embryos have been reported in a few forest species, such as *Paulownia elongata* (Ipekci and Gozukirmizi 2003), *Eucalyptus citriodora* (Muralidharan and Mascarenhas 1995) and *Chamaecyparis pisifera* (Maruyama et al. 2003). Artificial seeds are produced by mixing somatic embryos with 2% sodium alginate. This has a number of applications such as large-scale propagation, germplasm conservation and uniformity. (Reddy et al. 2020).

Hydrated and desiccated forms of encapsulation technology were employed in synthetic seed production (Sharma et al. 2013). In most cases, somatic embryos could be stored, providing the possibility of creating a gene bank for plants (Sunandar and Supena 2017).

Nirala et al. (2010) reported that cotyledonary-stage somatic embryos of grape (*Vitis vinifera* L.) were encapsulated individually in 2% alginate gel. Also, Koochani et al. (2020) used somatic embryos at torpedo stage and apical buds of Cucumber (*Cucumis sativus* L.) Arias-Pérez et al. (2021) found that somatic embryos of *Coffea arabica* L., encapsulated in the globular stage in sodium alginate, remained 100% viable after a month and a half of cold storage at 4 °C. Pintos et al. (2008) reported that Cork oak *(Quercus suber)* somatic embryos were coated with alginate for the production of synthetic seeds. Singh and Chand (2010) reported that cotyledonary-stage somatic embryos of *Dalbergia sissoo* were encapsulated using sodium alginate (2.5%) and calcium chloride (75 mM). The highest frequency (43.3%) for conversion of encapsulated somatic embryos into plantlets was achieved on ½MS medium with 2% sucrose. Prewein and Wilhelm (2012) mentioned that Cotyledonary *Quercus robur* L. somatic embryos were encapsulated in 4% (w/v) sodium alginate, Fifty-six well-developed plantlets regenerated 12 wk after germination, and 29 plants were transferred to the greenhouse. Raju et al. (2016) reported that the highest germination percentage of synthetic seeds of mango ginger (*Curcuma amada* Roxb.) (91.66%) was achieved on 1/2 MS solid medium supplemented with 3% (w/v) sucrose and 0.25 mg·L^{-1} gibberellic acid (GA_3).

Concerning the storage and recovery of encapsulated somatic embryos, Pandey and Chand (2005) reported that somatic embryos of *Hyoscyamus muticus* L. were encapsulated 3% sodium alginate and 75-mM $CaCl_2$ as gel matrix. The encapsulated embryos could be stored for up to 60 days at 4 °C. Also, Baskaran et al. 2015 found that combination of 3% SA and 100 mM $CaCl_2{\cdot}2H_2O$ provided higher survival (95.7%) and germination (73%) frequencies of synthetic seeds of *Mondia whitei*. Ali et al. (2018) reported that somatic embryos of *Coriandrum sativum* encapsulated in 3% sodium alginate + 100 mM $CaCl_2$ gave the maximum plant regeneration and noted that the 4 °C was the optimum temperature for synthetic seed conservation and plantlet regeneration compared to − 20 °C and 25 °C temperature conditions. Abbasi et al. (2020) reported that encapsulated embryos and buds from Asparagus plant maintained germination energy and viability with increasing storage time after 90 days of storage at 4 °C while un-capsulated embryos and buds completely lost viability after 60 days of storage at 4 °C. This study investigated the viability of encapsulated citrus somatic embryos after storage and to convert them into plantlets.

Methods

Obtaining somatic embryogenesis

Plant materials

Closed flower buds at balloon stage, buds are usually elliptic ovate in shape, length of corolla tube being approximately 2 cm (three times the calyx cup) just before opening as shown in Fig. 1 were from uninfected mature trees of Washington navel orange grown in the field of Horticulture research station in Kanater, Kalubia governorate, Egypt.

Preparation of explants

Closed flower buds harvested from uninfected mother trees as tested previously for CPsV by RT-PCR (El-Sawy et al. 2013) were washed carefully with running water and surface sterilized by immersion in 70-mL L^{-1}ethanol

Fig. 1 Complete developed flower bud of citrus at balloon stage (just before opening) used as a source of explants (stigma)

for 1 min, and then soaked in a 40% Clorox [commercial bleach (2% sodium hypochlorite)] for 15 min, followed by three 5-min rinses with sterile distilled water as described by Carimi et al. (1995) and D'Onghia et al. (2001) as they used the same method for explant sterilization. Stigma explants were excised from sterilized flower buds with a scalpel under aseptic conditions and placed vertically onto the culture medium.

Culture medium for embryogenic callus induction

Stigma explants were cultured on MS basal medium (Murashige and Skoog 1962) solidified with 7 g L^{-1} Difco Bacto agar and supplemented with sucrose 50 g L^{-1}, malt extract (ME) 500 mg L^{-1} and 6-benzylamino purine (BAP) 3 mg L^{-1} as reported by Carimi et al. (1995) and D'Onghia et al. (2001) as they used the same culture medium for embryogenic callus induction. The pH of the medium was adjusted to 5.8 with 0.5 M potassium hydroxide (KOH), then autoclaved at 1.5 PSI at 121 °C for 25 min. Explants were cultured in glass culture jars (250 ml capped with polypropylene closure) containing 25 ml of the culture medium. Each treatment consists of three replicates, three jars each, containing 5 explants/jar.

Culture conditions

Culture jars were incubated at 25 ± 2 °C under a 16 h day length with illumination of 45 µmol/m^{-2}/s^{-1} Osram cool white 18 W fluorescent lamps. All culture explants were subcultured at 4-week intervals into the fresh culture medium containing the same composition.

Encapsulation of somatic embryos

Somatic embryos derived from embryogenic callus of stigma explants at cotyledonary stage (5–7-mm size) were preserved by encapsulation according to Nirala et al. (2010. Somatic embryos were dried using filter paper under aseptic conditions and then mixed well with 3% (w/v) sodium alginate prepared in a half-strength MS salt solution for 5 min. Somatic embryos were then picked up and dropped one by one into calcium chloride ($CaCl_2$) solution (2.5%) and mixed continuously and kept for 40–45 min for hardening of the beads. Encapsulated somatic embryos were washed with sterile water to remove the excited $CaCl_2$. Then, the encapsulated beads were transferred to sterilized glass vessel for storage at 10 °C for 1, 2 or 3 months. Percentage of regrowth and germination of somatic embryos to plantlets was recorded monthly.

After different preservation periods (1, 2 and 3 months), the encapsulated somatic embryos were washed using sterile water. Then, the somatic embryo beads were cultured on MS medium solidified with 7 g L^{-1} agar and supplemented with 50 g L^{-1} sucrose for germination. All cultures jars (250-ml glass jars capped with polypropylene closure containing 25 ml of the culture medium) were incubated at 25 ± 2 °C under 16/8-h light cycle provided by white fluorescent lamps with light intensity of 45 µmol m^{-2} s^{-1} Osram cool white 18 W fluorescent lamps. Each treatment consists of five replicates; each replicate consists of two culture jars containing two explants (somatic embryos)/jar.

The results were recorded as follows: The number of germinated embryos was counted and expressed as percentage of somatic embryo germination every month. Also, the average number of shoots, shoot length (cm), number of leaves per shoot and rooting percentage of shoots generated from somatic embryos were recorded monthly during different recovery periods (through 12 months—one month for each).

Statistical analysis

All the data were subjected to analysis of variances (ANOVA) and were analyzed using CoHort program (Duncun's multiple range test at 5% level) according to Snedecor and Corchan (1972) to verify the differences between means of treatments. All experiments were designed in a completely randomized design; each treatment was the average of three replicates.

Molecular analysis of regenerated plantlets from somatic embryos after encapsulation

RAPD-PCR technique was used to test genetic stability of plantlets regenerated from encapsulated somatic embryos after preservation periods compared with somatic embryogenesis—derived plantlets from stigma explants before preservation.

Genomic DNA was isolated as mentioned by Murray and Thompson (1980).

DNA amplification

Nine random 10-mer primers as shown in Table 1 (Operon technologies Inc., Alameda, California, USA) were designed for use in RAPD analysis. The PCR reaction was performed in 25-µl mix containing 10-mM Tris–HCl pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP, dTTP, 0.5 µg of the random primer, 0.5 unit of Taq DNA polymerase (Promega) and 20 ng of DNA. The mixture was incubated at 94 °C/5 min; DNA amplification was performed for 45 cycles. Each cycle was at 94 °C/30 s. (Denaturation), 36 °C/30 s (annealing) and 72 °C/2 min (extension). Reactions were finally incubated at 72 °C for 7 min.

RAPD electrophoresis in agarose gel

The RAPD products were analyzed by electrophoresis in 1% agarose in 100 ml (1x) TBE buffer (containing 89 mM

Table 1 Random oligonucleotide primers used for RAPD analysis

Code	Seq. (5′–3′)	Length(bp)
Operon A-01	CAGGCCCTTC	10
Operon A-02	TGCCGAGCTG	10
Operon A-03	TTGGCACGGG	10
Operon B-02	TGATCCCTGG	10
Operon G-02	GGCACTGAGG	10
Operon K-05	CACCTTTCCC	10
Operon K-06	CCGTGACTCA	10
Operon M-07	CCTACGGGGA	10
Operon Z-02	GAGGGAAGAG	10

Tris-Base, 89 mM EDTA and 2.5 mM Boric acid for 10×buffer) and were stained with 3-µl ethidium bromide and photographed under ultraviolet light.

Preparation of samples

Two µl from loading buffer (0.25 g L^{-1} bromophenol blue, 0.25 g L^{-1} Xylene cyanol and 30 mL L^{-1} glycerol in 100-ml sterile distilled water) were added to 5–10 µl of the DNA sample and then loaded into the agarose gel. Running was performed at 70 V for 1 h. The DNA was detected under UV transilluminator and photographed by Polaroid camera.

Data analysis

Bands on agarose gels were scored as present (+) or absent (−), and a pair-wise similarity matrix was constructed using the Dice similarity (SD) index (Sneath and Sokal 1973). SD values were calculated as the doubled number of shared bands between two patterns divided by the sum of all bands in the same patterns. An UPGMA cluster based on SD values was generated using the NTSYS (Numerical Taxonomy system, Applied Biostatistics, Setauket, New York) computer program.

Results

In vitro *regeneration through indirect somatic embryogenesis from stigma explants*

Callus production

Friable-yellow callus, Fig. 2, was initiated from stigma explants taken from uninfected mother trees of Washington navel orange. Callus began to initiate after two weeks.

Somatic embryogenesis production

Callus developed from stigma explants and was able to regenerate indirect somatic embryogenesis after 3 months (Fig. 3).

Fig. 2 Callusing stage from stigma explants after 1 month of culture

Fig. 3 Stigma-derived embryogenic callus of Washington navel orange (after 3 months of culture)

Fig. 4 Encapsulated stigma-derived somatic embryos at cotyledonary stage in sodium alginate beads

Encapsulation of somatic embryos

Single embryos were used in encapsulation experiment at cotyledonary stage (Fig. 4). In these conditions, beads of uniform size and shape were obtained. Otherwise, the lack of any visible necrosis on these somatic embryos suggests that the increased resistance from

bead, rather than $CaCl_2$ toxicity, was responsible for the depressed germination rate could be occurred.

Regrowth percentage of encapsulated somatic embryos

The results in Table 2 show the effect of preservation periods (1, 2 and 3 months) of encapsulated somatic embryos on percentage of their ability to regrowth after transferred and re-cultured on recovery medium during different recovery periods (12 months—one month for each).

Concerning the effect of preservation period, results indicated that increasing the preservation period from 1, 2 to 3 months decreased significantly the percentage of encapsulated somatic embryos which were able to regrow after being transferred and re-cultured on recovery medium (70.0%, 55.2% to 27.5%, respectively) with significant differences among them.

Concerning the effect of recovery period, the results showed that increasing recovery period from 1 to 12 months increased significantly the regrowth as the same highest significant percentage of regrowth was observed after recovery for 10, 11 and 12 months (64.2%). This percentage reduced significantly to 60.8% after recovery for 9 months, 57.5% after recovery for 8, 7 and 6 months, 54.2% after recovery for 5 months, 50.8% after recovery for 4 months, and 40.0% after recovery for 3 months, 29.2% after recovery for 2 months and 10.8% after recovery for one month with significant differences among them.

The effect of interaction between preservation period and recovery period showed that preservation of encapsulated somatic embryos for one month increased significantly the percentage of regrowth to90.0% when the recovery period increased to 9 months until 12 months as shown in Fig. 5. This percentage reduced gradually when recovery period decreased. All encapsulated somatic embryos preserved for 3 months and recovered for one month cannot able to regrowth (00.0%).

Germination percentage of encapsulated somatic embryos

The results in Table 3 show the effect of preservation period (1, 2 and 3 months) of encapsulated somatic embryos on percentage of complete germination during different recovery periods (12 months—one month for each)

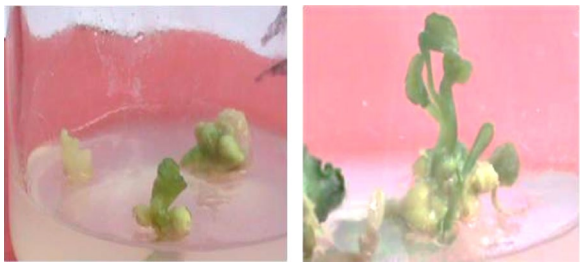

Fig. 5 Regrowth of encapsulated somatic embryos after one month of preservation and recovery for 3 months

Table 2 Effect of different preservation periods (month) on regrowth percentage of encapsulated somatic embryos during different recovery periods (12 months—one month for each)

Recovery period (month)	Preservation period (month)			Mean
	1	2	3	
1	20.0 j	12.5 k	0.0 m	10.8 H
2	40.0 g	37.5 h	10.0 l	29.2 G
3	50.0 f	50.0 f	20.0 j	40.0 F
4	60.0 e	62.5 d	30.0 i	50.8 E
5	70.0 c	62.5 d	30.0 i	54.2 D
6	80.0 b	62.5 d	30.0 i	57.5 C
7	80.0 b	62.5 d	30.0 i	57.5 C
8	80.0 b	62.5 d	30.0 i	57.5 C
9	90.0 a	62.5 d	30.0 i	60.8 B
10	90.0 a	62.5 d	40.0 g	64.2 A
11	90.0 a	62.5 d	40.0 g	64.2 A
12	90.0 a	62.5 d	40.0 g	64.2 A
Mean	70.0 A	55.2 B	27.5 C	

Values followed by the same letter (s) were not significantly different by Duncan's test at 0.05 level

Table 3 Effect of different preservation periods (month) on germination percentage of encapsulated somatic embryos during different recovery periods (12 months—one month for each)

Recovery period (month)	Preservation period (month)			Mean
	1	2	3	
1	0.0 m	0.0 m	0.0 m	0.0 G
2	20.0 j	12.5 k	10.0 l	14.2 F
3	30.0 h	25.0 i	10.0 l	21.7 E
4	40.0 f	37.5 g	20.0 j	32.5 D
5	60.0 d	50.0 e	20.0 j	43.3 C
6	70.0 b	62.5 c	30.0 h	54.2 B
7	70.0 b	62.5 c	30.0 h	54.2 B
8	70.0 b	62.5 c	30.0 h	54.2 B
9	70.0 b	62.5 c	30.0 h	54.2 B
10	80.0 a	62.5 c	40.0 f	60.8 A
11	80.0 a	62.5 c	40.0 f	60.8 A
12	80.0 a	62.5 c	40.0 f	60.8 A
Mean	55.8 A	46.9 B	21.7 C	

Values followed by the same letter (s) were not significantly different by Duncan's test at 0.05 level

Concerning the effect of the preservation period, the results indicated that increasing the preservation period from 1, 2 to 3 months decreased significantly the complete germination of encapsulated somatic embryos from 55.8%, 46.9% to 21.7% with significant differences among them.

According to the effect of recovery period, the results revealed that increasing recovery period to 10, 11 and 12 months increased significantly the percentage of complete germination as showed the highest significant percentage (60.8%). Decreasing the recovery period from 10 to 9, 8, 7 or 6 months decreased significantly the percentage of complete germination as showed the same percentage (54.2%), decreasing the recovery period gradually from 5, 4, 3 to 2 months decreased significantly the percentage of complete germination gradually from 43.3%, 32.5%, 21.7% to 14.2%, respectively. All encapsulated somatic embryos recovering for one month and cannot be able completely to germinate (0.0%).

According to the effect of interaction between the preservation period and recovery period of encapsulated somatic embryos, the results indicated that preservation of encapsulated somatic embryos for one month and increasing the recovery period to 10, 11 and 12 months increased significantly the complete germination percentage as showed the same percentage to 80.0% as shown in Fig. 6.

This percentage was reduced significantly to 70% by reducing the recovery period to 9, 8, 7 and 6 months. This percentage was followed significantly by percentage of encapsulated somatic embryos preserved for 2 months and increasing recovery period gradually from 6 to 12 months as showed the same percentage (62.5%). All encapsulated somatic embryos were preserved for 1, 2 and 3 months and recovered for one month failing to germinate (0.0%).

Number of germinated plantlets from encapsulated somatic embryos

The results in Table 4 show the effect of preservation period of encapsulated somatic embryos for 1, 2 and 3 months on the number of plantlets germinated from encapsulated somatic embryos during different recovery periods (12 months—one month for each).

Fig. 6 Germination of encapsulated somatic embryos after one month of preservation and recovery for 12 months

Table 4 Effect of different preservation periods (months) on the number of plantlets germinated from encapsulated somatic embryos during different recovery periods (12 months—one month for each)

Recovery period (month)	Preservation period (month)			Mean
	1	2	3	
1	0.0 h	0.0 h	0.0 h	0.0 E
2	2.0 fg	1.0 gh	1.0 gh	1.3 DE
3	3.0 ef	2.0 fg	1.0 gh	2.0 CD
4	4.0 de	3.0 ef	2.0 fg	3.0 BC
5	6.0 bc	4.0 de	2.0 fg	4.0 AB
6	7.0 ab	5.0 cd	3.0 ef	5.0 A
7	7.0 ab	5.0 cd	3.0 ef	5.0 A
8	7.0 ab	5.0 cd	3.0 ef	5.0 A
9	7.0 ab	5.0 cd	3.0 ef	5.0 A
10	8.0 a	5.0 cd	4.0 de	5.7 A
11	8.0 a	5.0 cd	4.0 de	5.7 A
12	8.0 a	5.0 cd	4.0 de	5.7 A
Mean	5.6 A	3.7 AB	2.5 B	

Values followed by the same letter (s) were not significantly different by Duncan's test at 0.05 level

According to the effect of preservation period, the results indicated that preserved encapsulated somatic embryos for one or two months showed the highest significant number of plantlets (5.6 and 3.7 plantlets, respectively) without significant differences inbetween. This number was reduced to 2.5 plantlets when encapsulated somatic embryos were preserved for three months. No significant differences were noticed between the number of plantlets developed from encapsulated somatic embryos preserved for two or three months (3.7 and 2.5 plantlets, respectively).

Regarding the effect of recovery period, the results showed that the highest significant number of plantlets was observed when encapsulated somatic embryos were recovered on germination medium for 10, 11 and 12 months (5.7 plantlets), for 9, 8, 7 and 6 months (5.0 plantlets) and 5 months (4.0 plantlets) without significant differences among them. All encapsulated somatic embryos recovered for one month failed completely to produce any plantlets. Number of plantlets developed from encapsulated somatic embryos recovery for 2, 3 and 4 months came inbetween (1.3, 2.0 and 3.0 plantlets).

The effect of interaction between the different preservation periods of encapsulated somatic embryos and different recovery periods on number of plantlets revealed that encapsulated somatic embryos preserved for one month showed the highest significant number of plantlets

when recovery for 10, 11 and 12 months as showed the same number of plantlets (8.0 plantlets) followed without significant differences by number of plantlets developed from the same encapsulated somatic embryos recovery for 9, 8, 7 and 6 months (7.0 plantlet). Preserved encapsulated somatic embryos for 2 months then recovered for 2 months and preserved encapsulated somatic embryos for 3 months then recovered for 2 and 3 months showed the lowest number of plantlets (1.0 plantlet). All encapsulated somatic embryos preserved for 1, 2 and 3 months and recovered for one month failed to develop plantlets. Number of plantlets developed from encapsulated somatic embryos preserved for 1, 2 and 3 months and recovery for other recovery periods under investigation came inbetween.

Average length (cm) of plantlets

The results in Table 5 show the effect of preservation period of encapsulated somatic embryos for 1, 2 and 3 months on the length (cm) of plantlets germinated from encapsulated somatic embryos during different recovery periods (12 months—one month for each).

Concerning the effect of preservation period, the results showed insignificant differences between the length of plantlets developed from encapsulated somatic embryos preserved for 1, 2 or 3 months (1.7, 1.6 or 1.2, respectively).

Regarding the effect of different recovery periods, results cleared that the highest significant length of plantlets was observed when encapsulated somatic embryos were recovering for 11 and 12 months (2.6 cm) followed without significant differences by length of plantlets developed from encapsulated somatic embryos recovery for 10, 9 and 8 months (2.4, 1.9 and 1.6 cm, respectively). Plantlets developed from encapsulated somatic embryos recovery for 3 months showed the lowest length (0.5 cm). The length of plantlets developed from encapsulated somatic embryos recovery for 4, 5, 6 and 7 months came inbetween (0.6, 0.8, 1.0 and 1.2 cm, respectively).

The effect of interaction between the preservation period of encapsulated somatic embryos and different recovery periods on length of plantlets showed that plantlets developed from encapsulated somatic embryos preserved for 2 months and recovery for 10, 11 and 12 months showed the highest significant length (3.0 cm) followed without significant differences by length of plantlets developed from encapsulated somatic embryos preserved for 1 month and recovery for 9, 10, 11 and 12 months (2.8 cm), preserved for 1 month and recovery for 8 months and preserved for 2 months and recovery for 9 months (2.5 cm), preserved for 1 month and recovery for 7 months, preserved for 2 months and recovery for 8 months, preserved for 3 months and recovery for 9, 10 11 and 12 months (2.0 cm) than those preserved for 1 month and recovery for 6 months and preserved for 2 months and recovery for 7 months (1.5 cm).

Table 5 Effect of different preservation periods (months) on plantlet length (cm) germinated from encapsulated somatic embryos during different recovery periods (12 months—one month for each)

Recovery period (month)	Preservation period (month)			Mean
	1	2	3	
1	0.0 e	0.0 e	0.0 e	0.0 E
2	0.5 de	0.5 de	0.5 de	0.0 E
3	0.8 de	0.5 de	0.5 de	0.5 DE
4	1.0 cde	0.8 de	0.5 de	0.6 CDE
5	1.0 cde	1.0 cde	1.0 cde	0.8 CDE
6	1.5 abcde	1.0 cde	1.0 cde	1.0 CDE
7	2.0 abcd	1.5 abcde	1.3 bcde	1.2 BCDE
8	2.5 abc	2.0 abcd	1.3 bcde	1.6 ABCD
9	2.8 ab	2.5 abc	2.0 abcd	1.9 ABC
10	2.8 ab	3.0 a	2.0 abcd	2.4 AB
11	2.8 ab	3.0 a	2.0 abcd	2.6 A
12	2.8 ab	3.0 a	2.0 abcd	2.6 A
Mean	1.7 A	1.6 A	1.2 A	2.6 A

Values followed by the same letter (s) were not significantly different by Duncan's test at 0.05 level

Number of leaves/plantlet

The results in Table 6 revealed the effect of preservation period of encapsulated somatic embryos for 1, 2 and 3 months on the number of leaves/plantlet germinated from encapsulated somatic embryos during different recovery periods (12 months—one month for each).

Regarding the effect of preservation period, the results indicated that increasing the preservation period from 1, 2 to 3 months did not affect significantly the number of leaves/ plantlet (6.1, 6.3 and 4.3 leaves/plantlet).

Concerning the effect of recovery period, the results indicated that plantlets developed from encapsulated somatic embryos recovery for 9, 10, 11 and 12 months showed the highest significant number of leaves/plantlet (8.8, 9.5, 9.5 and 9.9 leaves/plantlet, respectively).

These values were followed with significant differences by number of leaves/plantlet developed from encapsulated somatic embryos recovery for 8, 7 and 6 months (7.3, 6.0 and 4.7 leaves/plantlet, respectively). No significant differences could be observed between numbers of leaves /plantlet developed from encapsulated somatic embryos recovery for 6 and 5 months (4.7 and 4.0 leaves/plantlet, respectively). Also, no significant differences were observed between number of leaves/plantlet

Table 6 Effect of different preservation periods (month) on number of leaves/ plantlet germinated from encapsulated somatic embryos during different recovery periods (12 months—one month for each)

Recovery period (month)	Preservation period (month)			Mean
	1	2	3	
1	0.0 j	0.0 j	0.0 j	0.0 F
2	2.0 i	2.0 i	2.0 i	2.0 E
3	3.0 hi	2.0 i	2.0 i	2.3 E
4	3.0 hi	3.0 hi	2.0 i	2.7 E
5	4.0 gh	4.0 gh	4.0 gh	4.0 D
6	6.0 ef	4.0 gh	4.0 gh	4.7 D
7	7.0 de	6.0 ef	5.0 fg	6.0 C
8	9.0 bc	8.0 cd	5.0 fg	7.3 B
9	9.5 bc	10.0 b	7.0 de	8.8 A
10	9.5 bc	12.0 a	7.0 de	9.5 A
11	9.5 bc	12.0 a	7.0 de	9.5 A
12	10.7 ab	12.0 a	7.0 de	9.9 A
Mean	6.1 A	6.3 A	4.3 A	

Values followed by the same letter (s) were not significantly different by Duncan's test at 0.05 level

developed from encapsulated somatic embryos recovery for 4, 3 and 2 months (2.7, 2.3 and 2.0 leaves/plantlet, respectively).

The effect of interaction between the preservation periods of encapsulated somatic embryos and different recovery periods showed that plantlets developed from encapsulated somatic embryos preserved for 2 months and recovery for 10, 11 and 12 months produced the same highest significant number of leaves/plantlet (12.0 leaves/plantlet) followed without significant differences by the number of leaves/plantlet developed from encapsulated somatic embryos preserved for 1 month and recovery for 12 months (10.7 leaves/plantlet). No significant differences were noticed between number of leaves/plantlet developed from encapsulated somatic embryos preserved for 1 month and recovered for 12 months (10.7 leaves/plantlet), those preserved for 2 months and recovered for 9 months (10.0 leaves/plantlet), those preserved for 1 month and recovered for 9, 10 and 11 months as shown the same number of leaves (9.5 leaves/plantlet). The lowest number of leaves/plantlet (2.0 leaves/plantlet) was noticed when encapsulated somatic embryos were preserved for one month and recovered for 2 months, preserved for 2 months and recovery for 2 and 3 months and preserved for 3 months and then recovered for 2, 3 and 4 months. The number of leaves/plantlet developed from other treatments under this investigation came in between.

Molecular analysis of somatic embryos-derived plantlets after preservation by encapsulation

Genomic DNAs of plantlets generated from preserved somatic embryos by encapsulation (samples 1, 2 and 3) were extracted and compared with in vitro somatic embryo-derived plantlets before preservation by RAPD-PCR using random oligonucleotide primers. Out of nine primers, two (OPK5 and OPA3) successfully yielded 153 bands across the treatments. The other primers were not as efficient as the two mentioned primers in generating successive PCR products. Size ranged from 50 to 700 bp depending on the primer and treatments. For complete analysis to detect the pair-wise difference among the six treatments, intensive bands were considered as present (1), while weak or absent bands were considered as absent (0). Table 7 and Fig. 7 show the reaction of primers OPA3 and OPK5 with different treatments. The two primers yielded 14 different bands with sizes ranging from 75 to 700 bp. Two bands out of them were polymorphic. Their sizes were 75 bp for OPA3 and 600 bp for OPK5.The percentage of polymorphism was 7.7% on average across the two primers. The percentage of polymorphism was 7.7% in the two primers in most of treatments except for the last treatment (lane 6), and the

Table7 Effect of arbitrary sequence primers tested in RAPD analysis of stigma-derived plantlets of Washington navel orange after encapsulation and cryopreservation of somatic embryos

Treatment	Primer	Total no. of produced bands	Size range (bp)	No. of polymorphic bands	Size of polymorphic bands (bp)	% Polymorphism
Encapsulation (1 month)	OPA3	13	50–350	1	75	7.7
	OPK5	13	200–700	1	600	7.7
Encapsulation (2 months)	OPA3	13	50–350	1	75	7.7
	OPK5	13	200–700	1	600	7.7
Encapsulation (3 months)	OPA3	13	50–350	1	75	7.7
	OPK5	13	200–700	1	600	7.7

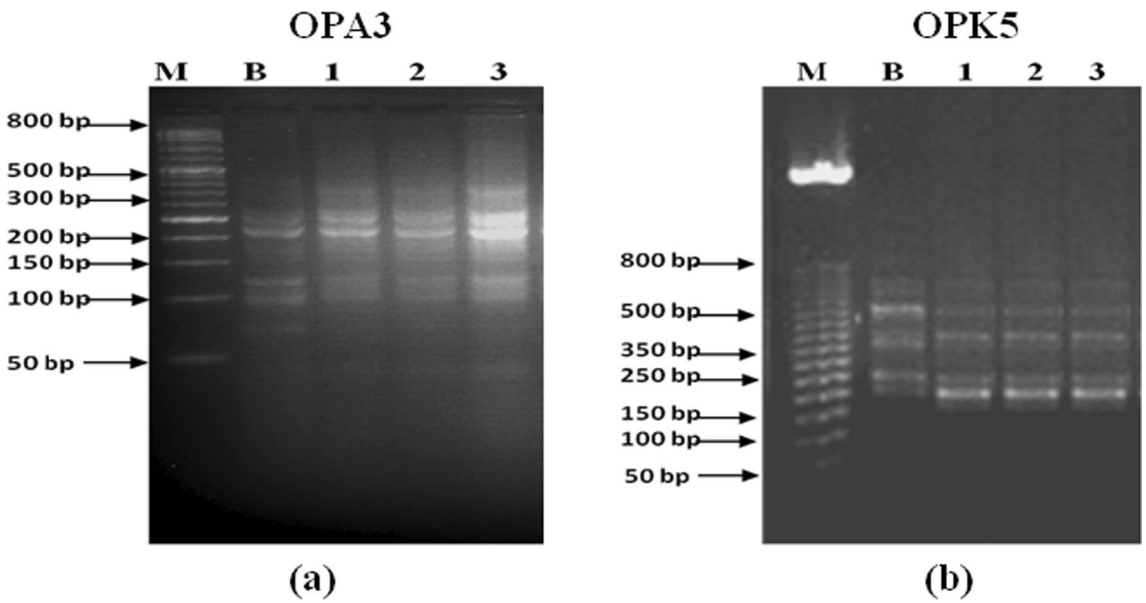

Fig. 7 Gel electrophoresis of RAPD fingerprints of Washington navel orange plantlets obtained, [**a** with random primer OPA3; **b** with random primer OPK5.] M: DNA marker. B: Stigma-derived plantlet before preservation treatments; 1, 2, 3: plantlets germinated from encapsulated somatic embryos preserved for 1, 2 and 3 months, respectively

percentage of polymorphism was 30%. It means that the RAPD fingerprints produced with different primers were almost identical.

Discussion

This study has shown that embryogenic callus can be induced successfully from and stigma of the sweet orange after 3 months. These results are similar to those obtained by Carimi et al. (1998) who mentioned that Somatic embryogenesis occurred 1–3 months after culture initiation from undeveloped ovule and stigma/style cultures of all the genotypes tested.

For successful encapsulation, the texture of beads was influenced by different concentrations of the gelling matrix. Concentrations of sodium alginate below 3% resulted in the formation of soft and fragile beads that were difficult to handle, whereas concentrations above 3%, produced isodiametric beads that were hard enough to cause considerable delays in germination as mentioned by Genhai et al. (2015). So in our experiment, 3% sodium alginate was used and gave a good bead structure.

In general, the genotype of the somatic embryo, the encapsulating agent used, and the matrix determined the success of synthetic seed technology in woody plants (Gantait et al. 2015).

From the results of preservation of somatic embryos, it could be concluded that frequencies of germination of encapsulated somatic embryos increased with increasing the recovery period on germination medium. Maximum recovery frequencies of 60.8% were obtained from encapsulated somatic embryos cultured on germination medium for up to 10 months. Also, recovery frequencies of 62.5% were noticed from encapsulated somatic embryos preserved for 2 months at 10 °C when transferred and cultured on germination medium without growth regulators (MS basal medium + 7 g L^{-1} agar + 50 g L^{-1} sucrose) for up to 6 months. In addition, results indicated that recovered somatic embryos obtained from encapsulated somatic embryos were able to convert to normal plantlets. In this concern, Pandey and Chand (2005) stated that a maximum conversion frequency of 69% was noted from encapsulated somatic embryos cultured on MS medium without growth regulators. The encapsulated embryos could be stored for up to 60 days at 4 °C or remained 100% viable after 6 weeks of cold storage at 4 °C (Arias-Pérez et al. 2021). Nirala et al. (2010) reported that of the germinated encapsulated somatic embryos, 36% developed into plantlets. Singh and Chand (2010) recorded that the highest frequency (43.3%) for conversion of encapsulated somatic embryos into plantlets of *Dalbergia sissoo* Roxb.(family: Fabaceae) was achieved on ½MS medium with 2% sucrose and 72.3% for non-encapsulated somatic embryos. Prewein and Wilhelm (2012) reported that the highest conversion rates (26%) were obtained with encapsulated somatic embryos of oak as well as artificial endosperm-coated somatic embryo encapsulation improved the regeneration into oak plantlets.

Ghosh and Sen (1994) achieved a maximum conversion of 34% for *Asparagus cooperi* after individual somatic embryo beads encapsulated in 3.5% sodium alginate were held in $CaCl_2$ solution for 40 min. Higher or lower levels of sodium alginate reduced the conversion frequency. Redenbaugh et al. (1987) and Redenbaugh, (1993) noted that variables related to encapsulation method, including alginate type and concentration, medium, and methods used to produce the synthetic seed, were responsible for significant variations in conversion percentages for alfalfa carrot and celery. Sodium alginate between 0.5 and 5.0 g L^{-1} was found to produce a sufficiently hard capsule while still maintaining embryo integrity; however, the authors determined that a 20-to-30-min exposure to a complexing agent was required to achieve complete gelation. In contrast, a shorter (10 min) exposure to $CaCl_2$ and only 2.5% alginate for encapsulated papaya SEs resulted in well-formed beads (Castillo et al. 1998).

Conclusions

It could be concluded that callus developed from stigma explants was able to regenerate indirect somatic embryogenesis after 3 months. Cotyledonary-stage somatic embryos of citrus were successfully preserved by encapsulation using 3% sodium alginate. Frequencies of germination of encapsulated somatic embryos increased with increasing the recovery period on the germination medium. A maximum recovery frequency of 60.8% was obtained from encapsulated somatic embryos cultured on germination medium for up to 10 months. Also, recovery frequency of 62.5% was noticed from encapsulated somatic embryos preserved for 2 months at 10 °C. In addition, results indicated that recovered somatic embryos obtained from encapsulated somatic embryos were able to convert to normal plantlets.

Abbreviations

MS	Murashige and Skooge
RAPD	Random amplification polymorphic DNA
RT-PCR	Reverse transcription polymerase chain reaction
CPsV	Citrus Psorosis virus
BAP	Benzylamino purin
ME	Malt extract
EDTA	Ethylenediaminetetraacetic acid

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Author contributions

AE designed the study, organized and supervised the experimental work and wrote the article. AH organized and supervised the work and reviewed the writing. MH and AA supervised the work. ND performed the experiments, collected data, performed the analysis, drafted the manuscript and wrote the article. All authors have read and approved the manuscript.

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Declarations

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Consent for publication

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Competing interests

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