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In vitro Trials of Manfaloty Pomegranate Germplasm Conservation Through Encapsulation Technique

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Future Science Association

Available online free at
www.futurejournals.org

Print ISSN: 2692-5826

Online ISSN: 2692-5834

DOI:
10.37229/fsa.fjh.2023.09.30

Received: 23 July 2023
Accepted: 2 September 2023
Published: 30 September 2023

Publisher's Note: FA stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Abstract: Germplasm conservation is considered the most valuable way to preserve endangered genotypes and commercial species. Besides, it is an important method for improving plant breeding programs through either in situ or ex situ preservation techniques. The most suitable ex situ conservation technique is in vitro culture under controlled conditions. This investigation is to evaluate the response of the Egyptian cultivar "Manfaloty" pomegranate germplasm to preservation through encapsulation technique using nodal segments for short and medium-term storage periods. Encapsulation was carried out by using sodium alginate at 2% and 3%. Both 2% and 3% Na-alginate treatments were cultured on MS media supplemented with Abscisic acid at 0.1, 0.2 and 0.3 mg/l and mannitol at 1.0, 2.0 and 3.0 g/l, in addition to free MS as a control treatment. Regrowth was evaluated as the percentage of capsules viability, growth and size increasing. Encapsulation using sodium alginate at 3 % gave the best results compared with 2%. Sodium alginate with free MS medium was the superior treatment for surviving and regrowth rates of preserved beads (100%), in compared to abscisic acid and mannitol. capsules that cultured with ABA recorded the highest survival percentage compared with the other capsules that cultured with Mannitol, the survival percentages were (83%, 66.6% and 50%) with ABA at 0.1, 0.2 and 0.3 mg/L, respectively and (50%, 25% and 16.6%) with Mannitol at 1.0, 2.0 and 3.0 mg/L, respectively. Adding ABA and mannitol to medium slowing and keeping the encapsulated nodal segments at low rates of growth led to prolonging the preservation periods at low concentrations. However, it suppressed the regrowth percent at high concentrations, which might be due to their inhibitory and osmotic influences. Moreover, increasing of storage duration at 7°C, the regrowth and survival percentages gradually declined; the survival percentages of encapsulated nodal segments and regrowth to survival

of were 40.0 and 45.0%, respectively, after 12 weeks. This decline in growth rate of encapsulated nodal segments stored at low temperatures may be due to the inhibited respiration of plant tissues caused by the alginate cover.

Key words: Pomegranate, Pollen grains, encapsulation, germplasm conservation.

INTRODUCTION

The pomegranate (*Punica granatum* L.) is one of the first known edible fruits that is mentioned in the Holy Bible and Quran. For various reasons, being one of the most significant commercial fruit crops. Firstly, it has great adaptability to be distributed and grown in many climatic regions worldwide, including the Mediterranean, tropical, and subtropical areas. Second, it is drought resistance, which allows it to grow and thrive on a wide range of soil types in arid and semi-arid zones. Moreover, Pomegranate fruit is desirable and preferred by consumers due to its appealing colors, juicy, sweetly acidic, and refreshing arils (**Ahmed *et al.*, 2022**). It also possesses medicinal and therapeutic effects, as well as a high nutritional value. In addition, various parts of the pomegranate tree such as leaves, bark skin and fruits, have long been utilized for their therapeutic benefits and other purposes such as tanning (**Mars 2000**).

Generally, Fruit tree genotypes are mainly preserved in field gene banks. Recently conservation was successful in various species in which encapsulation techniques were used to develop Syn-seeds using different types of tissues such as shoot tips, axillary buds, nodal segments etc., for different storage times (**Nower *et al*., 2007; Rai *et al*., 2009; Benelli *et al*., 2013; Benelli 2016; Abd Alhady 2017; Micheli *et al.* 2017; Gholami and Kaviani 2018). In pomegranate, Naik and Chand (2006**) developed a protocol for encapsulating axenic nodal segments in calcium alginate hydrogel. Syn-seeds technology is an auspicious tool for plant breeding programs and plant lines that are hard to propagate using traditional methods, as well as to overcome breeding problems in seedless plants. It combines the benefits of micropropagation and encapsulation techniques (**Sharma *et al*. 2013; Qahtan *et al*., 2019**). The texture, shape, and size of the beads are primarily affected by the impact of different sodium alginate and calcium chloride concentrations. Such encapsulation protects the micro shoots and allows for the exact handling of the natural seeds; it can also transfers germplasm between laboratories and reduce phytosanitary and quarantine issues (**Verma *et al*., 2010**).

Mannitol, as an alcohol sugar, has various advantages over those that only translocate sugars. One advantage is a high salt and osmotic stress tolerance because mannitol acts as a compatible solute. Another advantage is that mannitol metabolism might effect on plant responses to all biotic and abiotic stresses (**Stoop *et al*., 1996**). Mannitol also has an inhabitation effect on plant growth at high concentrations (**Pliego-Alfaro *et al.*, 1996**).

Abscisic acid (ABA) controls a number of physiological processes in plants, including seed dormancy and adaptive responses to abiotic and biotic stressors. Furthermore, ABA has a crucial role in growth and development. ABA can promote or hinder growth depending on concentrations, tissues, and environmental factors (**Brookbank *et al*., 2021**). Moreover, multiple combinations of cytokinins and auxins are efficient for *in vitro* culture initiation and shoot proliferation in pomegranate (**Teixeira da Silva *et al*., 2013**).

Reports on the usage of encapsulation technique with abscisic acid or mannitol for pomegranate conservation are rare; therefore, the current study was aimed to evaluate the response of the Egyptian cultivar "Manfaloty" pomegranate germplasm to preservation through encapsulation technique using nodal segments for short and medium-term storage periods.

MATERIAL AND METHODS

Plant materials

This study used anthers and nodal segments excised from Mature Manfaloty pomegranate trees grown at Horticulture Research Institute Experimental Farm, Giza. All *in vitro* procedures were done in the tissue culture Laboratory of Breeding Research Department of Fruits, Ornamentals and Woody Plants, Horticulture Research Institute (HRI).

1- Stem nodal segments sterilization and in vitro culture procedures

Shoots were excised from healthy juvenile branches of mature "Manfaloty" Trees. The explants rinsed with tap water to remove the superficial dust. Then, explants were surface sterilized by dipping in 70% ethanol for 2 minutes, followed by immersion in 25% Sodium hypochlorite (NaOCl) solution of commercial bleach (Clorox) for 25 minutes, and subsequently were rinsed three times with sterile distilled water. The sterilized shoots were cut into several stem nodes. For *in vitro* culture and multiplication stage, nodal segments were cultured on MS basal medium (Murashige and Skoog1962) at full strength supplemented with 30g/L sucrose, 7 g/L agar, BA at 1 mg/L and 1 mg/L NAA. The subculture was done every 4 weeks intervals for proliferation and multiplication issues. The cultures were incubated at 25°C ±2°C for a 16/8 h light/dark photoperiod regime with a light intensity of 2400 lux at an excellent level (standard culture conditions).

All media's pH was adjusted to 5.7using KOH and HCl before being autoclaved. All media were autoclaved at 100 K.pa (15 P.S.I) and 121°c for 20 min; then left to cool and stored at 25°C ±2 for 2 days before being used.

2- Encapsulation and in vitro storage procedures

For encapsulation, sodium alginate solution was prepared at 2.0% or 3.0% (w/v) in concentrations, whereas calcium chloride ($CaCl_2.2H_2O$) solution was prepared at 2% (w/v). Nodal segments of Manfaloty pomegranate were excised from *in vitro* cultures and used for encapsulation. Under sterile conditions, the explants were individually dipped in 2% and 3% sodium alginate solutions, then taken up with a sterile spatula and gently dropped into 100 ml $CaCl_2.2H_2O$ solution. The droplets containing explants were held for 30 minutes in the $CaCl_2.2H_2O$ solution to achieve sodium alginate polymerization. These resulting hydrogel beads (capsules) were collected and rinsed three times with sterilized distilled water to remove $CaCl_2.2H_2O$ traces. These hydrogel beads or capsules had an average diameter of 4-5 mm (Fig. 1 A). They prevented explant dehydration and could be easily handled under sterile conditions.

- For studying the effects of Abscisic acid and Mannitol, encapsulated segments were cultured on seven different MS media (30 g/L sucrose & 0.7% w/v agar) (Murashige and Skoog, 1962) as follows:
    - MS with ABA at 0.1, 0.2 and 0.3 mg/L.

- MS with Mannitol at 1, 2 and 3 g/L.
- MS without any plant growth regulator.

- For the storage experiment, encapsulated segments were stored for 0, 2, 4, 6, 8, 10 and 12 weeks at 7°C (in the dark) on Free full-strength MS medium (30 g/L sucrose & 0.7% w/v agar) (Fig. 2 B). After storage periods, encapsulated segments were subcultured on full-strength MS medium supplemented with 30g/L sucrose, 7 g/L agar, BA at 1 mg/L and 1 mg/L NAA (as proliferation medium).
- All cultures were incubated at 25°C ±2°C for 16 hrs. photoperiod a day with light intensity of 2400 lux at excellent level (standard culture conditions).
- Each treatment consists of 3 replicates; each replicate consists of 4 nodal segments. Encapsulated nodes were cultured on free MS medium for regrowth of plants. After six weeks, viability growth was recorded.
- The capsules were considered alive and viable if the explants were still green.
- Also, regrowth percentage was evaluated as a percentage of capsules that had shown visual growth besides those increases in plant size.

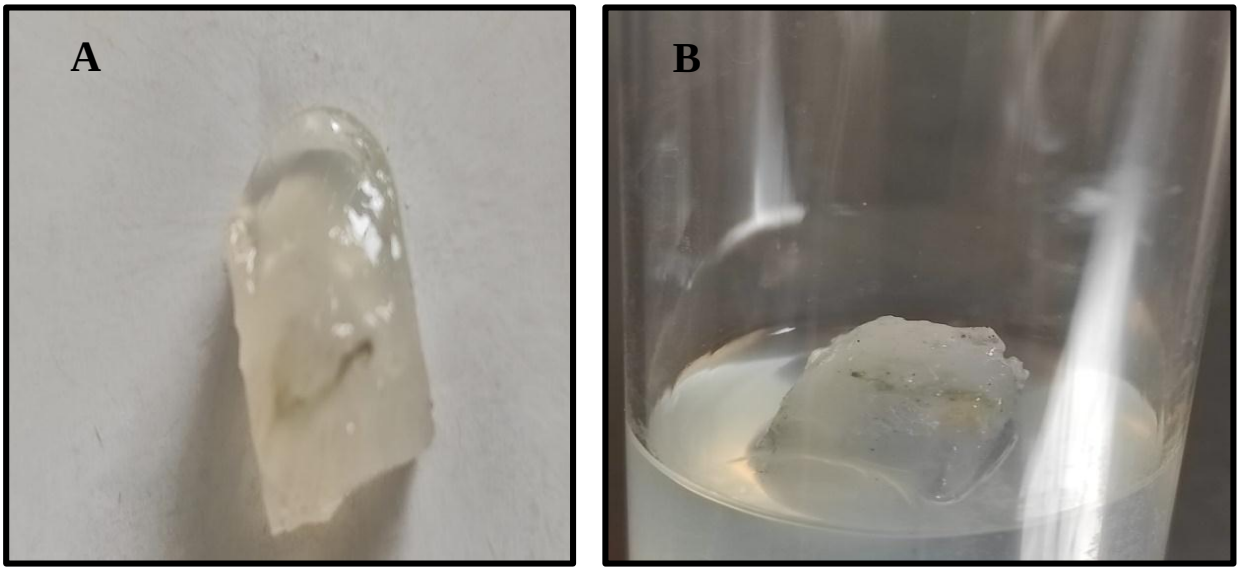

Fig. (1). Nodal segments after encapsulation by sodium alginate A), Encapsulated nodal segments after culture on media B).

Experimental Design and statistical analysis

A completely randomised design with three repetitions was used to set up the experiments. The statistical analysis was conducted using the CoStat package program (Version 6.303; CoHort Software, USA). Analysis of variance (ANOVA) was used to analyze the data. The significant differences between the means of the treatments were compared using Duncan's multiple range test at the 5% level of probability (**Waller and Duncan, 1969**).

RESULTS AND DISCUSSION

Survival percentage of Manfaloty encapsulated nodal segments

Sodium-alginate was used at 2% and 3% with 100 mM of $CaCl_2.2H_2O$ to determine the optimum concentrations to form ideal beads (Table 1). Sodium-alginate at 3% and 100 mM $CaCl_2.2H_2O$ formed the best shape of beads compared with 2% sodium-alginate, which gave soft beads to be handled. Moreover, the results showed that the components of culture media significantly affected the survival percentage of the encapsulated nodal segments (beads) after 4 weeks from being cultured on different MS media. The 3% sodium-alginate beads cultured on free MS medium had the highest survival percentage (100%), followed by the 3% sodium-alginate beads cultured on MS medium supplemented with 0.1 mg/L ABA (83.33%). The same pattern was observed with 2% sodium-alginate beads with a survival rate of 91.6% on MS-free and 58.3% on MS with 0.1 mg/L ABA, respectively. Meanwhile, adding mannitol to the culture media recorded the lowest values of survival percentage either with 3 % or 2 % sodium alginate.

Fig. (2). Encupsulated nodal segments coverd by alginate (shape of beads).

Table (1). Effects of sodium alginate, abscisic acid, and mannitol on survival percentage of encapsulated Manfaloty nodal segments

Treatments	Sodium alginate 2%	Sodium alginate 3%
MS medium + 0.1mg/L (ABA)	58.3b	83.3ab
MS medium + 0.2mg/L (ABA)	50.0b	66.6bc
MS medium + 0.3mg/L (ABA)	41.6b	50.0cd
MS medium + 1 g/L mannitol	50b	50.0cd
MS medium + 2 g/L mannitol	25bc	25.0de
MS medium + 3 g/L mannitol	8.3c	16.6e
MS medium free	91.6a	100.0a
F. Test	**	**

Concerning the effect of sodium-alginate concentration, abscisic acid and mannitol on the regrowth percentage of Manfaloty pomegranate explants, the highest regrowth to survival percentage (100 %) was recorded by both 2% and 3% sodium-alginate beads (Table 2) that cultured on free MS media (control treatment), followed by those beads of 3% sodium-alginate that cultured on MS medium with 0.1 mg/L ABA that gave 80% regrowth percentage to survival. Moreover, beads planted in mannitol at 3 g/L concentration recorded the lowest value of regrowth with the concentration 2% sodium-alginate (0 %).

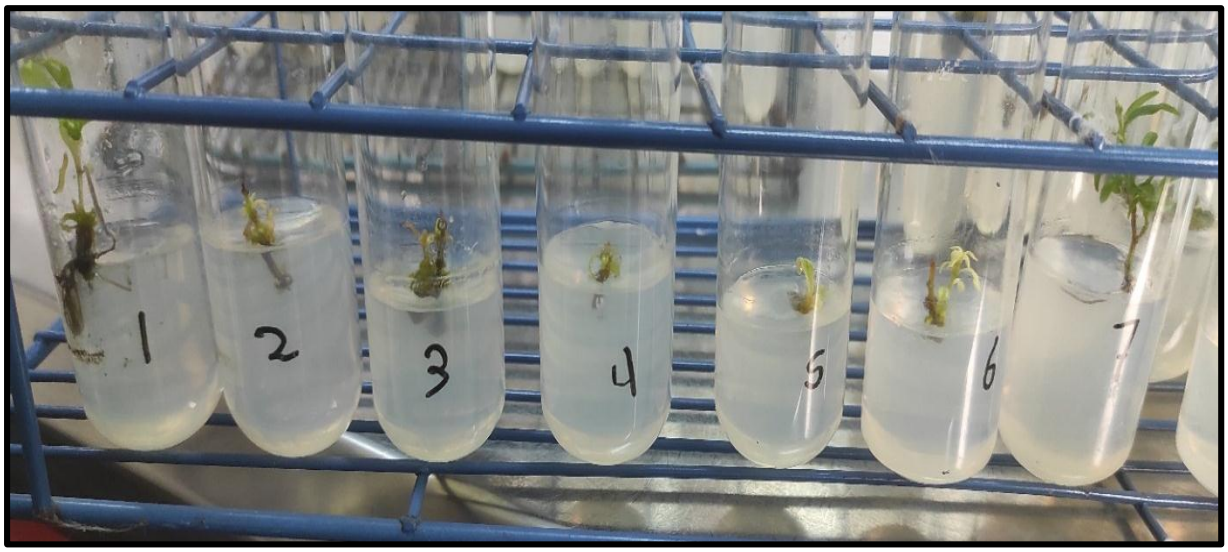

Fig. (3). performance of encupsulated nodal segments on different media after six weeks.

Table (2). Effects of sodium-alginate, abscisic acid, and mannitol on regrowth percentage to survival of Manfaloty encapsulated nodal segments

	Treatments	**Sodium alginate 2%**	**Sodium alginate 3%**
1	MS medium + 0.1mg/L (ABA)	71.4b	80.0b
2	MS medium + 0.2mg/L (ABA)	50.0c	62.5c
3	MS medium + 0.3mg/L (ABA)	40.0d	50.0d
4	MS medium + 1 g/L mannitol	50.0c	50.0d
5	MS medium + 2 g/L mannitol	33.3e	33.3e
6	MS medium + 3 g/L mannitol	0.00f	50.0d
7	MS medium free	100.0a	100a
	F. Test	**	**

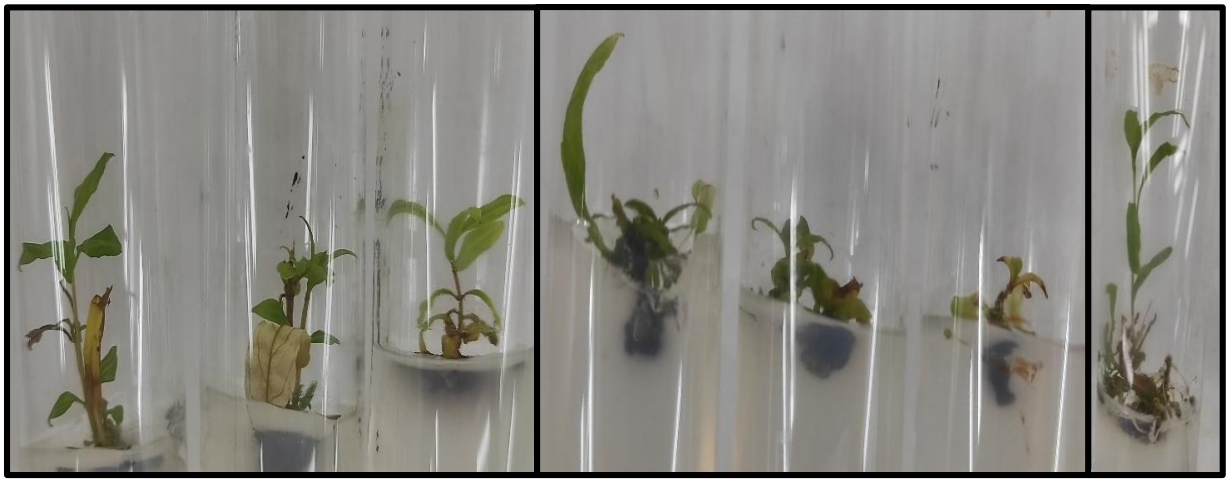

Fig. (4). Performance of encapsulated nodal segments on different media after twelve weeks

From the present results, it is clarified that survival and regrowth rates were affected by sodium-alginate, ABA, and mannitol concentrations. High concentrations of ABA and mannitol as inhibitory and osmotic substances tended to suppress the regrowth averages compared to the free medium. The reduction in regrowth and survival rates under moderate and high levels of the additive substances might be attributed to their inhibitory actions, which extend the preservation period of the stored pomegranate germplasm using in vitro procedures. These results are in accordance with **Pliego-Alfaro *et al*., (1996)**, who mentioned that high ABA concentrations (750-1750 µM) almost completely suppressed germination and shoot development in Mango. Moreover, mannitol hindered the development of nucellar embryos at concentrations between 7.5 and 12.5%, most likely because of osmotic effects. Furthermore, **Roberts (1991)** indicated that higher concentrations of mannitol inhibited the formation of globular embryos when applied with or without ABA.

Conversion of encapsulated Manfaloty nodal segments as affected by storage Durations

As shown in Table 3, the encapsulated nodal segments (propagules) were taken without storage (0 duration), cultured on MS proliferation medium, and incubated at 25°C ±2°C for 16 hrs., photoperiod a day. The regrowth and survival percentages were 100% before storage. While storage duration increased at 7°C, the regrowth and survival percentages gradually declined; the percentages of survival and regrowth; the percentages of survival and regrowth to survival of encapsulated nodal segments were 40.0 and 45.0%, respectively, after 12 weeks. These results are in harmony with those found by **Abd Alhady (2017)**, who stated that encapsulated shoot tips of white sapote (*Casimiroa edulis* L.) able to store at a low temperature (4°C) for for 12 weeks with a survival and regrowth of 46.67 and 40.00%, respectively. This decline of encapsulated nodal segments growth rate stored at low temperatures might be due to respiration inhibition of plant tissues caused by alginate cover (**Naik and Chand 2006**).

Finally, our results could be a promising method to preserve pomegranate germplasm for short to medium periods.

Table (3). Storage durations at 7° C, survival and regrowth percentages of Manfaloty encapsulated nodal segments.

Duration (Weeks)	Survival (%)	Regrowth to survival (%)
0	100 a	100 a
2	87.5 b	85.7 b
4	75.0 c	83.3 b
6	62.5 d	80.0 c
8	50.0 e	75.0 d
10	50.0 e	75.0 d
12	40.0 f	45.0 e

In general, the potential of the encapsulated technique becomes clear; especially, for its extensibility and possibility of moving small-sized micropropagated plant material between laboratories and countries while reducing phytosanitary and quarantine issues (**Piccioni and Standardi 1995; Naik and Chand 2006**). Encapsulation techniques might be a valuable method for plant material conservation, especially for long or medium -term preservation (**Benelli 2016**).

Conclusion

Encapsulation and *in vitro* preservation are useful techniques for storing "Manfaloty" pomegranate cultivar tissues for up to 12 weeks at 7°C with 3% sodium-alginate as nodal segment beads. Moreover, adding plant growth regulators such as abscisic acid and an osmotically active reagent such as mannitol to the culture medium promoted and prolonged the preservation period of pomegranate explants. Therefore, our results are considered preliminary studies for further experiments to extend the conservation period using the encapsulation technique.

Funding Statement This research was funded by Science, Technology & Innovation Funding Authority (STDF), grant number 33483: Strategies and facing pomegranate exportation challenges in Egypt by implementing some novel techniques through breeding programs and improvement fruit quality.

Conflict of interest E.A.H. Ahmed, T. K. El-Bolok, S. El-Habashy, S.A. Ahmed and Nahla.A. Awad declare that they have no competing interests.

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