



Communication

# Encapsulation of Shoot Tips and Nodal Segments for *In Vitro* Storage of “Kober 5BB” Grapevine Rootstock

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**Abstract:** *In vitro* preservation of the “Kober 5BB” rootstock (*Vitis berlandieri* × *Vitis riparia*) was assessed with the encapsulation technique and slow growth storage. Shoot tips and nodal segments excised from *in vitro* cultures were encapsulated in calcium-alginate beads. A 30 min ion exchange time proved optimal for forming proper beads. The encapsulated and naked explants were stored at 4 °C in the dark or light. After 9 months of cold storage, the highest regrowth, 83.3%, was recorded for the encapsulated shoot tips maintained in darkness. The development of the encapsulated nodal segments was 55.6% under the same storage conditions. The encapsulated explants had a better regrowth capacity after storage than the naked explants.

**Keywords:** alginate bead; grapevine rootstock; *in vitro* culture; slow growth storage

## 1. Introduction

The encapsulation technique for creating synthetic seeds is an important application for *in vitro* culture. Shoot tips, axillary buds or nodal segments may be used to develop the synthetic seeds. Synthetic seeds have been defined as artificially encapsulated somatic embryos or non-embryogenic *in vitro*-derived propagules and are used for sowing under *in vitro* or *ex vitro* conditions [1–3]. Synthetic seed technology combines the advantages of clonal propagation with those of seed propagation (i.e., storability, easy to handle and transport, protection against diseases and pests). The most recent application foresees the use of synthetic seeds in medium and long-term storage. The encapsulation technique is used today in advanced procedures of cryopreservation such as encapsulation-dehydration and encapsulation-vitrification methods [4], obtaining very promising results for the long-term preservation of plant germplasm [5–7]. The encapsulation of shoot tips, axillary buds or nodal segments has been reported for various species [8–13], and slow growth storage has been applied as a method for medium-term conservation [14–16]. Slow growth storage involves limiting development by reducing temperature and/or light intensity, adding osmotic compounds such as mannitol or sucrose in the culture medium, and use of growth retardants [17]. Among these, the most commonly used are the reduction of temperature and light intensity. These two parameters have physiological consequences that result in a significant reduction in cell metabolism and, consequently, shoot growth. With *in vitro* slow growth storage, it is possible to extend the intervals between subcultures, thus reducing the cost of stock plant maintenance as well as the risk of contamination during subculturing [15,18].

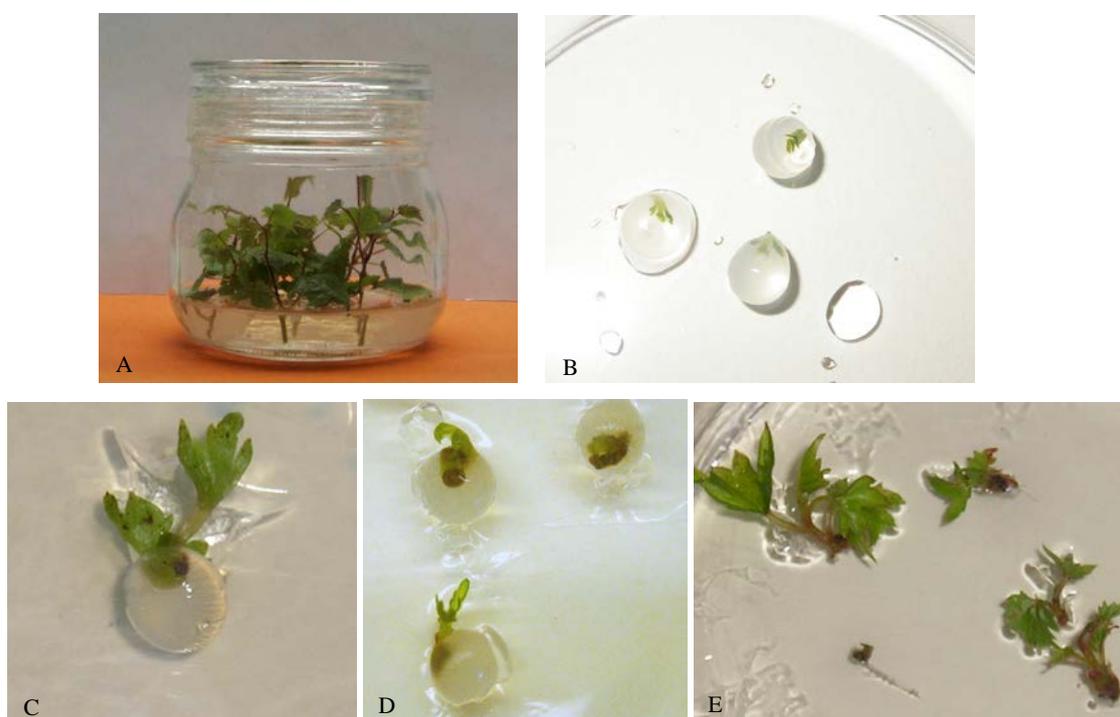
There are a large number of grapevine cultivars and rootstocks, many of which are unique to small, remote environments, and are therefore important for preserving biodiversity and reducing genetic erosion. The aim of this study was to optimize an encapsulation and storage protocol for shoot tips and nodal segments of “Kober 5BB” grapevine rootstock (*Vitis berlandieri* × *V. riparia*) as an alternative to traditional conservation methods. The experiments evaluated the effect of storage on the

regrowth ability of encapsulated shoot tips and nodal segments following different storage conditions. This report describes the initial results of an investigation aimed at the development of a protocol for encapsulation of “Kober 5BB” explants.

## 2. Materials and Methods

### 2.1. Culture Media and Conditions

In vitro stock cultures of grapevine rootstock “Kober 5BB” were proliferated on MS semisolid medium [19] with 30 g/L sucrose at pH 5.7 (Figure 1A). The proliferation medium contained 1.5 mg/L benzyladenine (BA) and 3 g/L Gelrite®. The salts, vitamins and gelling agent used for the culture media were purchased from Sigma-Aldrich (St. Louis, MI, USA). The medium was autoclaved for 20 min at 121 °C. The cultures were maintained at  $23 \pm 1$  °C in a growth chamber under a 16 h photoperiod with light intensity  $60 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (standard culture conditions).



**Figure 1.** (A) In vitro stock culture of “Kober 5BB” rootstock; (B) Encapsulated explants in 3% sodium alginate, 100 mM  $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$  with ion exchange time of 30 min. Regrowth of explants under standard conditions (21 days) after 3 months of storage at 4 °C in darkness; (C) encapsulated shoot tip; (D) encapsulated nodal segments and (E) naked shoot tips.

### 2.2. Encapsulation Procedure and Storage

Shoot tips (2–3 mm) and nodal segments (5–6 mm) were excised from in vitro stock cultures of “Kober 5BB”. For encapsulation, the explants were plunged into a solution of 3% (*w/v*) sodium alginate (medium viscosity, Carlo Erba, Cornaredo, Italy) and MS liquid medium. Drops of alginate solution with shoot tip or nodal segment were sucked into a micropipette with sterile plastic tips and dropped into MS liquid medium supplemented with 100 mM calcium chloride ( $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ ) for complexation. All operations were performed under sterile conditions. In order to achieve polymerization and prepare beads of an ideal shape and size with a uniform texture (Figure 1B), in the first experiment, different time periods (20, 30 or 40 min) for  $\text{Na}^+/\text{Ca}^{2+}$  ion exchange in the calcium chloride solution were tested. After each incubation period in the complexing agent, the encapsulated explants were

retrieved and rinsed three times in sterile distilled water in order to remove traces of calcium chloride. Regrowth of the explants was then assessed as described below.

The following experiment was carried out on both encapsulated, with 30 min of ion exchange time, and naked explants, which were placed in 90 cm diameter Petri dishes containing semisolid, hormone-free MS medium and held at 4 °C in a growth chamber in darkness or in light (30  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , 8 h photoperiod) for 3, 6 or 9 months of storage.

Regrowth of the explants (encapsulated and naked) was evaluated for bead incubation time and for each cold storage period after transferring them onto proliferation medium for 21 days, under standard conditions. Regrowth ability of encapsulated explants was determined as the time required for the shoot to appear and break through the gel [20]. Ten explants of each type were placed in a Petri dish, and dishes were replicated three times for each incubation time or treatment combination by storage period.

### 2.3. Data Analysis

The average regrowth time was calculated as follows:  $\Sigma (N_x T_x) / \text{number of developed shoots}$ ; where  $N_x$  is the number of developed shoots within consecutive intervals of time,  $T_x$  is the number of days between the beginning of the test and the end of the specific time interval.

Data on regrowth were recorded and presented as means with standard error of the mean (SEM). Significant differences among means were analyzed following analysis of variance using Duncan's Multiple Range Test at  $p < 0.05$ . Statistical analysis of percentages was carried out by a non-parametric Chi square test ( $p \leq 0.05$ ) for pairwise comparisons. All statistical tests were performed with Systat 13 (Systat Software, Inc., San Jose, CA, USA).

## 3. Results

### 3.1. Shoot Regrowth from Beads

Based on regrowth, optimal ion exchange between 3% sodium alginate and 100 mM calcium chloride was observed at 30 min (Table 1). This proved to be the most effective time interval for producing reasonably smooth beads around the explants. With this time, more than 96% of "Kober 5BB" beads containing shoot tips and 85% containing nodal segments were regrown in the shortest average regrowth time, 15 and 25 days, respectively. Low percentages of regrowth ability were observed when the complexation period was shorter or longer than 30 min. In addition, at 20 min of complexation time, the beads were too soft and difficult to handle, while they showed a well-defined shape but regrowth was slower at 40 min. Following encapsulation, shoot tips developed more quickly (15 vs. 25 days) and at a higher percentage (96% vs. 85%) than nodal segments.

**Table 1.** Effect of ion exchange time on regrowth ability and average regrowth time of encapsulated explants of "Kober 5BB" rootstock.

Ion Exchange Time (Min)	Shoot Tips (%)	Average Regrowth Time (Days)	Nodal Segments (%)	Average Regrowth Time (Days)
20	53.3 $\pm$ 2.6 b	18	46.7 $\pm$ 2.8 b	25
30	96.7 $\pm$ 0.3 a	15	85.3 $\pm$ 0.6 a	25
40	33.7 $\pm$ 2.0 b	19	31.2 $\pm$ 2.1 b	26

Means  $\pm$  standard error mean (SEM). Within each column, different letters indicate significantly different percentages using Duncan's Multiple Range Test,  $p < 0.05$ .

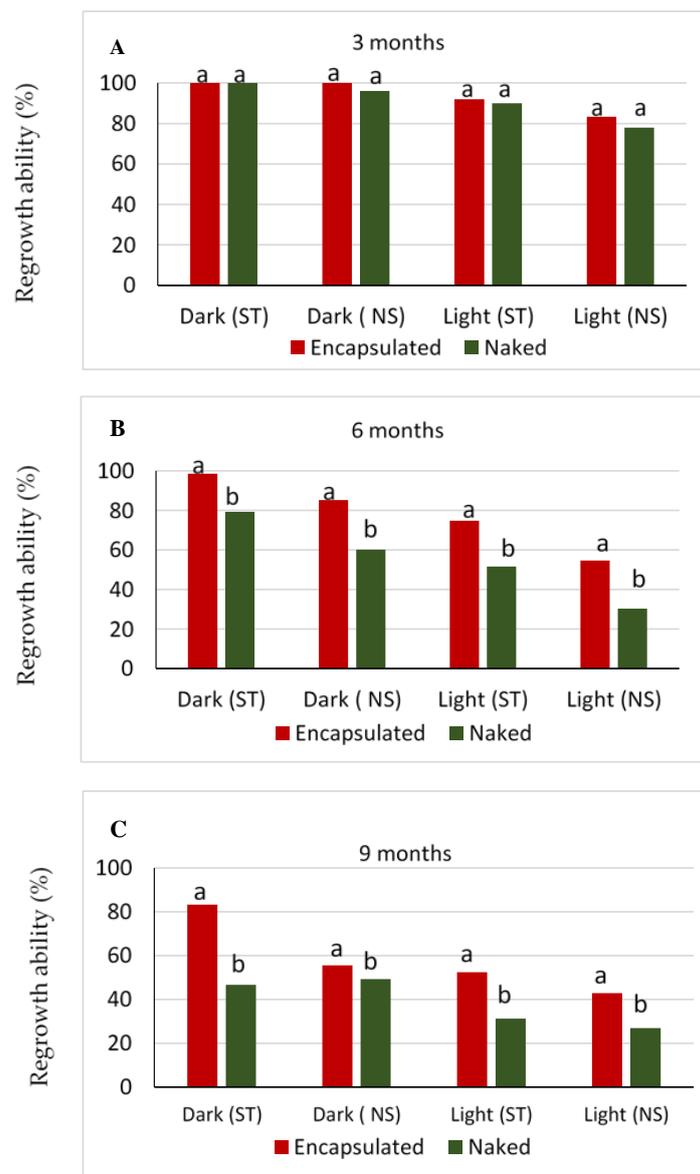
### 3.2. Storage in Slow-Growth Conditions

Irrespective of tissue type and storage condition, the explants exhibited some level of regrowth through 9 months of cold storage (Figure 2). No significant differences were observed in the regrowth ability of encapsulated versus naked shoot tip or nodal explants at 3 months, but encapsulated shoot

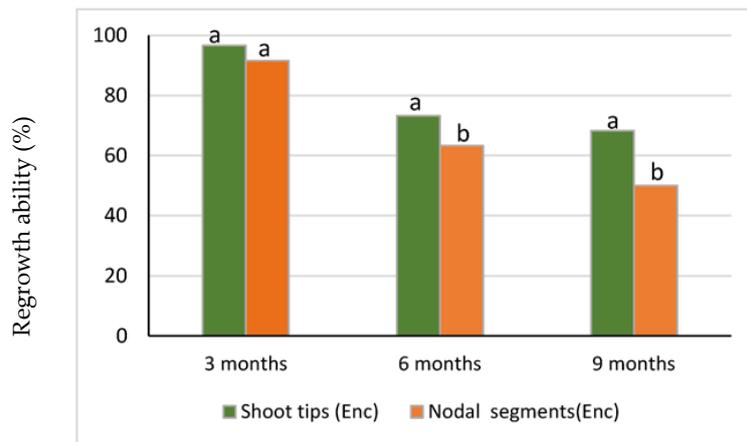
tip and nodal explants exhibited significantly higher regrowth percentages than naked explants in both light and dark storage at 6 and 9 months (Figure 2B,C).

Regrowth percentages of all explants in both growing conditions decreased over storage time (Figures 2 and 3), and the decrease was most evident between 3 and 6 months of storage. At 6 and 9 months, shoot tip explants showed higher regrowth ability than nodal explants (Figure 3). Furthermore, the highest regrowth ability at 6 and 9 months was recorded for the encapsulated shoot tips kept in the dark (Figure 2).

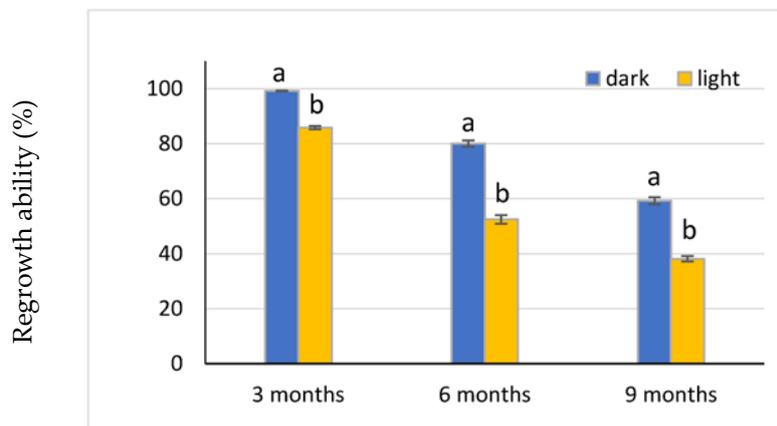
Overall, darkness had a positive influence on the regrowth of the explants at each storage period (Figure 4). The preservation conditions assessed proved to be more suitable for the encapsulated shoot tips than for the encapsulated nodal segments. No morphological changes were observed for the encapsulated explants that developed into shoots.



**Figure 2.** Regrowth ability (%) of encapsulated and naked shoot tips (ST) or nodal segments (NS) of “Kober 5BB” rootstock stored at 4 °C in darkness and light conditions for each storage period. (A) three months; (B) six months; (C) nine months. Different letters indicate percentages of encapsulated versus naked means within tissue type and light versus dark conditions that significantly differed ( $\chi^2$  test,  $p \leq 0.05$ ) at each storage period.



**Figure 3.** Regrowth ability of encapsulated shoot tips versus nodal segments after 3, 6, and 9 months of 4 °C storage. Different letters indicate percentages of shoot tip versus nodal segments that significantly differed ( $\chi^2$  test,  $p \leq 0.05$ ) at each storage period.



**Figure 4.** Effect of dark versus light on explant regrowth after 3, 6, and 9 months of 4 °C storage. Different letters indicate percentages for dark versus light that significantly differed ( $\chi^2$  test,  $p \leq 0.05$ ) at each storage period.

#### 4. Discussion

Encapsulation of explants is affected by sodium alginate and calcium chloride concentrations, and by ion exchange time, which together can give beads their optimum characteristics [11]. Sodium alginate and calcium salt have proven to be the best combination for encapsulation since they are low cost, non-damaging, easy to use and give high propagule-to-plant conversion [21]. The hardness of beads mainly depends on the number of sodium ions exchanged with calcium ions, and complexing time should be optimized in order to form uniformly spherical and firm beads. The exchange time generally has ranged between 20–40 min [22]. For “Kober 5BB” beads, the best results were obtained with the 30 min hardening process; this ion exchange time showed the best regrowth for shoot tips (96.7%) and nodal segments (85.3%). Most studies on fruit, ornamental and vegetable species suggest that a 30 min exchange time is best for obtaining a uniform spherical shape and a good level of sprouting from the beads [9,23–29]. The shortest average regrowth time of encapsulated “Kober 5BB” shoot tips was 15 d when the encapsulated explants were hardened for 30 min, while it was 25–26 days for nodal segments, for the ion exchange time tested. Regrowth time has varied with species; for example, when applying the same protocol for encapsulation used in this

study, regrowth in *Photinia fraserii* occurred in 10 days [30], while for *Nerium oleander* and *Kalanchoe* spp., it took 18 and 19 days, respectively [8].

The percentage of encapsulated and naked explants that formed shoots depended on the duration and conditions of storage. Under slow growth in vitro culture, the encapsulated explants of “Kober 5BB” were well suited for the storage conditions. Their better regrowth compared to naked explants could be attributed to their inclusion in the gel matrix which acts as an “artificial endosperm” for the explants. The present results confirmed the effectiveness of the protective coating. This agreed with previous literature which reported that the sodium alginate matrix provided protection and supplied nutrients to explants resulting in a higher regrowth percentage of encapsulated nodal segments than naked nodal segments [31].

The encapsulation technology could represent a useful technique for plant material conservation when used for medium or long-term preservation. In cryopreservation, this technique has been used for different species [32–34]. Moreover, various studies have described encapsulation for short and medium conservation (from 1 month to 1 year) without losing explant viability [35–38] and for increasing the interval between subcultures by reducing growth.

## 5. Conclusions

Implementation of the encapsulation technique may be used for clonal propagation and for effective storage at low temperatures. This study has shown that in vitro explant encapsulation could be used for storing “Kober 5BB” rootstock tissues up to 9 months at 4 °C without subcultures. The results indicated that the method could have great potential for preserving desirable elite genotypes of grapevine for medium periods, and were a useful prerequisite for further studies extending the storage period in this species.

In some plant species, poor regrowth of encapsulated propagules into plants has been a limitation for commercial application of the encapsulation technique, so further evaluation of the technique is needed. In addition, plant nurseries may be interested in achieving the development and growth of plants from synthetic seeds in soil without the aseptic conditions required for in vitro culture.

**Conflicts of Interest:** The author declares no conflict of interest.

## References

1. Murashige, T. Plant cell and organ cultures as horticultural practices. *Acta Hortic.* **1977**, *78*, 17–30.
2. Aitkens-Christie, J.; Kozai, T.; Takayama, S. Automation in plant tissue culture: General introduction and overview. In *Automation and Environmental Control in Plant Tissue Culture*; Aitken-Christie, J., Kozai, T., Smith, M.A.L., Eds.; Kluwer Academic Publication: Dordrecht, The Netherlands, 1995; pp. 1–18.
3. Standardi, A.; Piccioni, E. Recent perspectives on the synthetic seed technology using non-embryogenic vitro-derived explants. *Int. J. Plant Sci.* **1998**, *159*, 968–978.
4. Dereuddre, J.; Blandin, S.; Hassen, N. Resistance of alginate-coated somatic embryos of carrot (*Daucus carota* L.) to desiccation and freezing in liquid nitrogen: 1. Effects of preculture. *CryoLetters* **1991**, *12*, 125–134.
5. Sakai, A.; Engelmann, F. Vitrification, encapsulation-vitrification and droplet-vitrification: A review. *CryoLetters* **2007**, *28*, 151–172. [[PubMed](#)]
6. Fabbri, A.; Ganino, T.; Lambardi, M.; Nisi, R. Crioconservazione di gemme di portinnesto ‘Kober’ 5BB (*Vitis berlandieri* × *Vitis riparia*): Aspetti anatomici. *Ital. Hortus* **2007**, *14*, 82–86.
7. Benelli, C.; de Carlo, A.; Engelmann, F. Recent advances in the cryopreservation of shoot-derived germplasm of economically important fruit trees of *Actinidia*, *Diospyros*, *Malus*, *Olea*, *Prunus*, *Pyrus* and *Vitis*. *Biotechnol. Adv.* **2013**, *31*, 175–185. [[PubMed](#)]
8. Lambardi, M.; Benelli, C.; Ozudogru, E.A.; Ozden-Tokatli, Y. Synthetic seed technology in ornamental plants. *Floricult. Ornam. Plant Biotechnol. Adv. Top. Issues* **2006**, *2*, 347–354.
9. Piccioni, E.; Standardi, A. Encapsulation of micropropagated buds of six woody species. *Plant Cell Tissue Org. Cult.* **1995**, *42*, 221–226. [[CrossRef](#)]

10. Micheli, M.; Hafiz, I.A.; Standardi, A. Encapsulation of in vitro-derived explants of olive (*Olea europaea* L. cv. Moraiolo) II. Effects of storage on capsule and derived shoots performance. *Sci. Hortic.* **2007**, *113*, 286–292. [[CrossRef](#)]
11. Rai, M.K.; Asthana, P.; Singh, S.K.; Jaiswal, V.S.; Jaiswal, U. The encapsulation technology in fruit plants—A review. *Biotechnol. Adv.* **2009**, *27*, 671–679. [[CrossRef](#)] [[PubMed](#)]
12. Reddy, C.M.; Murthy, K.S.R.; Pullaiah, T. Synthetic seeds: A review in agriculture and forestry. *Afr. J. Biotechnol.* **2012**, *11*, 14254–14275.
13. Kulus, D.; Zalewska, M. In vitro plant recovery from alginate-encapsulated *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam shoot tips. *Propag. Ornam. Plants* **2014**, *14*, 3–12.
14. Nower, A.A.; Ali, E.A.; Rizkalla, A. Synthetic seeds of pear (*Pyrus communis* L.) rootstock storage in vitro. *Aust. J. Basic Appl. Sci.* **2007**, *1*, 262–270.
15. Benelli, C.; Ozudogru, E.A.; Lambardi, M.; Dradi, G. In vitro conservation ornamental plants by slow growth storage. *Acta Hortic.* **2012**, *961*, 89–93. [[CrossRef](#)]
16. Cordeiro, S.Z.; Simas, N.K.; Henriques, A.B.; Sato, A. In vitro conservation of *Mandevilla moricandiana* (Apocynaceae): Short-term storage and encapsulation–dehydration of nodal segments. *In Vitro Cell Dev. Biol. Plant* **2014**, *50*, 326–336. [[CrossRef](#)]
17. Grout, B.W.W. In vitro conservation of germplasm. In *Plant Tissue Culture: Application and Limitations*; Bhojwani, S.S., Ed.; Elsevier: Amsterdam, The Netherlands, 1990; pp. 394–411.
18. Ikhlaiq, M.M.; Hafiz, I.A.; Micheli, M.; Ahmad, T.; Abbasi, N.A.; Standardi, A. In vitro storage of synthetic seeds: Effect of different storage conditions and intervals on their conversion ability. *Afr. J. Biotechnol.* **2010**, *9*, 5712–5721.
19. Murashige, T.; Skoog, F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plantarum.* **1962**, *15*, 473–497. [[CrossRef](#)]
20. Standardi, A.; Micheli, M. Encapsulation of in vitro-derived explants: An innovative tool for nurseries. In *Protocols for Micropropagation of Selected Economically-Important Horticultural Plants*; Lambardi, M., Ozudogru, E.A., Jain, S.M., Eds.; Humana Press: New York, NY, USA, 2013; pp. 397–418.
21. Sharma, S.; Shahzad, A.; Teixeira da Silva, J.A. Synseed technology—A complete synthesis. *Biotechnol. Adv.* **2013**, *31*, 186–207. [[CrossRef](#)] [[PubMed](#)]
22. Standardi, A. Encapsulation: Promising technology for nurseries and plant tissue laboratories. *AgroLife Sci. J.* **2012**, *1*, 48–54.
23. Sicurani, M.; Piccioni, E.; Standardi, A. Micropropagation and preparation of synthetic seed in M.26 apple rootstock I: Attempts towards saving labor in the production of adventitious shoot tips suitable for encapsulation. *Plant Cell Tissue Org. Cult.* **2001**, *66*, 207–216.
24. Lisek, A.; Orlikowska, T. In vitro storage of strawberry and raspberry in calcium-alginate beads at 4 °C. *Plant Cell Tissue Org. Cult.* **2004**, *78*, 167–172. [[CrossRef](#)]
25. Naik, S.K.; Chand, P.K. Nutrient-alginate encapsulation of in vitro nodal segments of pomegranate (*Punica granatum* L.) for germplasm distribution and exchange. *Sci. Hortic.* **2006**, *108*, 247–252. [[CrossRef](#)]
26. Germanà, M.A.; Micheli, M.; Pulcini, L.; Standardi, A. Perspective of the encapsulation technology in the nursery activity of *Citrus*. *Caryologia* **2007**, *60*, 192–195.
27. Daud, N.; Taha, R.M.; Hasbullah, N.A. Artificial seed production from encapsulated micro shoots of *Saintpaulia ionantha* Wendl. (African Violet). *J. Appl. Sci.* **2008**, *8*, 4662–4667.
28. Sundararaj, S.G.; Agrawal, A.; Tyagi, R.K. Encapsulation for in vitro short-term storage and exchange of ginger (*Zingiber officinale* Rosc.) germplasm. *Sci. Hortic.* **2010**, *125*, 761–766.
29. Nor Asmah, H.; Nor Hasnida, H.; Nashatul Zaimah, N.A.; Noraliza, A.; Nadiyah Salmi, N. Synthetic seed technology for encapsulation and regrowth of in vitro-derived *Acacia* hybrid shoot and axillary buds. *Afr. J. Biotechnol.* **2011**, *10*, 7820–7824.
30. Ozden-Tokatli, Y.; de Carlo, A.; Gumusel, F.; Pignattelli, S.; Lambardi, M. Development of encapsulation techniques for the production and conservation of synthetic seeds in ornamental plants. *Propag. Ornam. Plants* **2008**, *8*, 17–22.
31. Singh, S.K.; Rai, M.K.; Asthana, P.; Sahoo, L. Alginate-encapsulation of nodal segments for propagation, short-term conservation and germplasm exchange and distribution of *Eclipta alba* (L.). *Acta Physiol. Plant* **2010**, *32*, 607–610. [[CrossRef](#)]

32. Gonzalez-Arno, M.T.; Engelmann, F. Cryopreservation of plant germplasm using the encapsulation-dehydration technique: Review and case study on sugarcane. *CryoLetters* **2006**, *27*, 155–168. [[PubMed](#)]
33. Engelmann, F.; Arno, M.T.G.; Wu, Y.; Escobar, R. Development of encapsulation dehydration. In *Plant Cryopreservation: A Practical Guide*; Reed, B., Ed.; Springer: New York, NY, USA, 2008; pp. 59–75.
34. Padrò, M.D.A.; Frattarelli, A.; Sgueglia, A.; Condello, E.; Damiano, C.; Caboni, E. Cryopreservation of white mulberry (*Morus alba* L.) by encapsulation-dehydration and vitrification. *Plant Cell Tissue Org. Cult.* **2012**, *108*, 167–172.
35. Hung, C.D.; Trueman, S.J. Encapsulation technology for short-term preservation and germplasm distribution of the African mahogany *Khaya senegalensis*. *Plant Cell Tissue Org. Cult.* **2011**, *107*, 397–405. [[CrossRef](#)]
36. Hung, C.D.; Trueman, S.J. Alginate encapsulation of shoot tips and nodal segments for short-term storage and distribution of the eucalypt *Corymbia torelliana* × *C. citriodora*. *Acta Physiol. Plant* **2012**, *34*, 117–128.
37. Sakhanokho, H.F.; Pounders, C.T.; Blythe, E.K. Alginate encapsulation of Begonia microshoots for short-term storage and distribution. *Sci. World J.* **2013**. [[CrossRef](#)]
38. Srivastava, V.; Khan, S.A.; Banerjee, S. An evaluation of genetic fidelity of encapsulated microshoots of the medicinal plant: *Cineraria maritima* following six months of storage. *Plant Cell Tissue Org. Cult.* **2009**, *99*, 193–198. [[CrossRef](#)]



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