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Short-Term Conservation of *Juglans regia* L. via Synthetic Seed Technology

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Abstract: *Juglans regia* L. is a crucial species as a forest tree and for its nutritional and medicinal values. It is also included in the list of endangered species in Albania and thus, there is a need to find methodologies to ensure its rapid regeneration and ex situ conservation. This research, investigated the regeneration of plantlets from synthetic seeds containing shoot tips of four native walnut varieties: 'Përmet', 'Korçë', 'Peshkopi', and 'Tropojë'. First, in vitro-derived shoot tips from walnut seedlings are encapsulated using sodium alginate. After that, the regeneration potential of the encapsulated shoot tips and the influence of incubation conditions are evaluated. The synthetic seeds were incubated at either 25 °C or 8 °C, with and without dehydration treatment, in 0.5 M sucrose solution for 3 h. The synthetic seeds in both temperature regimes (25 °C and 8 °C) develop plantlets and provid conservation potential without the need for subcultures for 4 and 3.5 months, respectively. Furthermore, all walnut varieties incubated in these conditions achiev a high regeneration rates.

Keywords: native walnut varieties; *in vitro* culture; encapsulated shoot tips; conservation period

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1. Introduction

The Persian walnut (*J. regia* L.) represents a crucial woody plant species for its nuts' nutritional, medicinal, and commercial values and, in some areas, its timber. In addition, it has numerous valuable qualities, including the high content of essential components beneficial to human health [1,2], its use in the furniture sector [3], the use of extract from its leaves and bark as a dye [4,5], and the importance as a forest tree for improving and preventing land erosion [6].

In Albania, the walnut tree is considered a native species distributed primarily in the north-east (Peshkopi, Tropojë) and south-east (Korcë, Përmet) with varieties that are considered to be quite adaptable to different environmental conditions [7]. However, despite this, walnut trees have consistently been subject to deliberate tree cutting and arson, which have reduced their population. For this reason, in Albania, this species is included in the list of endangered plants (EN) [7,8]. Furthermore, Paź-Dyderska et al. [9] reported that walnut trees tend to be invasive in northern Europe, whereas there has been a reduction of their population in southern Europe. The authors suggested that this situation, which was caused by climatic changes, is expected to become more pronounced soon. Thus, developing a strategic plan to prevent the loss of specific walnut varieties is highly recommended.

Usually, walnut trees are propagated by seed, but high yields are significantly slowed due to their dormant embryos [10]. In vitro regeneration methods using zygotic embryos enable overcoming the barriers in hybridization [11,12], obtaining higher and faster multiplication rates of plants of an elite genotype [10]. According to Lambardi et al. [13] and Rios Leal et al. [14], in vitro propagation is a method that creates opportunities for obtaining

homogeneous plant material with high genetic stability if explants, such as apical buds or shoot tips, are used as initial plant material. The socioeconomic significance of walnut trees associated with their wide range of uses, requires the development of procedures to ensure the rapid regeneration of selected lines. Applying in vitro techniques for the mass production of plants can ensure the production of a homogeneous material and allow biodiversity conservation [15–17]. The encapsulation technique for creating synthetic seeds is essential for *in vitro* culture. Artificial or synthetic seeds, also known by other names, such as "synseeds," were first described by Murashige [18]. They are defined as artificially coated somatic embryos; other plant parts such as zygotic embryos, shoot buds, cell aggregates, axillary buds; or any other micropropagules that can be sown like seeds and grown into plants *in vitro* or *ex vitro* [19,20]. Growing these artificial seeds involves synseed germination, leading to plantlet formation, shoot growth (synseed conversion), and new cell proliferation (synseed regrowth) [21]. Artificial seeds should also be able to retain their conversion and regrowth abilities for an extended period [22–24] without decreased vitality during the storage time [25–28].

Synthetic seed technology, as an alternative to natural seeds, has potential advantages such as efficient mass production, the rapid delivery of plantlets, easy handling and transportation, increased efficiency of in vitro propagation in terms of space, time, and labor, and cost-effective and efficient short-mid-term storage (minimal growth) or long-term storage (cryopreservation) [29–36]. In recent years, encapsulation technology has expanded, and the successful production of synthetic seeds has been reported in several plant species, with numerous advantages for their propagation and preservation [37–43].

For the encapsulation of explants, many gelling agents have been tested. However, sodium alginate is the most effective and widely applied due to its low cost, non-toxic nature, and gelling qualities [24,39]. Encapsulation within the alginate matrix offers protection to the explants from physical and environmental injury. The parameters of encapsulation, such as the alginate percentage used and time of bead hardening, should be optimized for selected species [23,44–46]. This study aims to evaluate the encapsulation technique in four native walnut varieties of Albania and to assess suitable in vitro conditions for synthetic seed regrowth and the possible duration for short-term storage.

2. Materials and Methods

2.1. Plant Material Collection and Explant Sterilization

Mature seeds of the Përmet, Korçë, Peshkopi, and Tropojë varieties were collected in their natural habitats (Përmet: 40.2362° N, 20.3517° E; Korçë: 39°56′53.29″ N, 20°5′44.12″ E; Peshkopi: 41°41′25.04″ N, 20°25′20.95″ E; Tropojë: 42°17′40″ N, 20°17′55″ E) The seeds were double-sterilized for 10 min before and after removing the seed coats with $HgCl_2$ 0.01% and then rinsed three times with distilled sterile water. Zygotic embryos were excised in aseptic conditions under laminar flow and used as primary explants for establishing and stabilizing walnut in vitro cultures.

2.2. In Vitro Culture for Walnut Zygotic Embryo Development

Zygotic embryos from the four varieties underwent in vitro culture for shoot and root organogenesis induction. For this purpose, three basal media were tested, specifically MS [47], WPM [48], and DKW [49]. Sucrose at 3% was used as a carbon source, and 0.6% agar was used as a gelling agent. No plant growth regulators (PGRs) were added at this stage. The zygotic embryo cultures were incubated at 25 °C ± 2 °C in a 16-h light 24-h photoperiod. Then, 21 days after the explants' inoculation, biometric parameters such as shoot and leaf number and shoot length were measured to evaluate embryonic regeneration potential and in vitro plantlet development.

2.3. Encapsulation Procedure

In vitro shoot tips derived from seedlings of the walnut varieties were immersed in a solution composed of calcium-free MS with sodium alginate at 3% (Sigma Aldrich, St.

Louis, MO, USA; medium viscosity) (Figure 1a). Then, the explants and a small quantity of sodium alginate solution were taken with a Pasteur pipette (Figure 1b) and dropped into 100 mM of $CaCl_2 \cdot 2H_2O$ solution (Figure 1c). The shoot tips were left in these conditions for about 30 min to achieve polymerization of sodium alginate in the presence of Ca^{2+} cations. After that, synthetic walnut seeds were produced (Figure 1d).

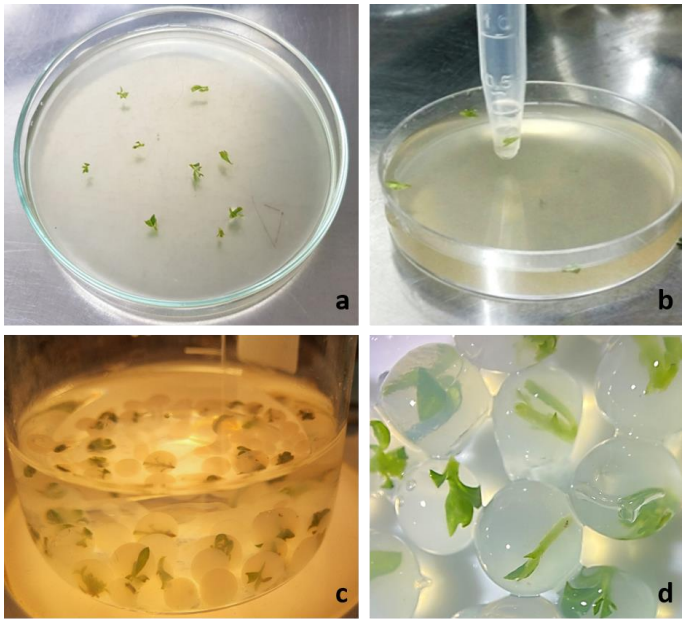


Figure 1. (**a**) In vitro-derived shoot tips in sodium alginate solution; (**b**) pipetting of an explant and a small quantity of sodium alginate solution; (**c**) dropping to $CaCl_2 \cdot 2H_2O$ solution; (**d**) synthetic seed formation after complete polymerization.

2.4. Effect of In Vitro Conditions on Encapsulated Explants

The explants of walnut varieties coated with artificial beads (synthetic seeds) were exposed to two incubation temperatures (25 °C or 8 °C) and to dehydration treatment with sucrose solution at 0.5 M for three hours. The synthetic seeds from the walnut varieties were divided into four groups: (I) incubation at 25 °C, (II) incubation at 25 °C after sucrose treatment, (III) incubation at 8 °C, and (IV) incubation at 8 °C after sucrose treatment. In all conditions, the synthetic seeds were maintained in culture vessels in MS medium regrowth, with 1 mg L^{-1} 6-benzyl amino purine (BAP), 0.1 mg L^{-1} 1-naphthaleneacetic acid (NAA), agar at 0.6% and sucrose at 3%. The culture vessels during the regrowth stage were maintained at a 16-h light/8-h dark photoperiod. In addition, experiments were conducted to assess the capability of encapsulated explants to break the gel coat, leading to normal growth with shoot development and to evaluate the possibility of preserving the synthetic seeds for a short time.

Data on germination (breaking of the beads from the explants) and regrowth (explants showing regrowth and conversion to shoots) percentages were recorded.

Periodic monitoring of the synthetic seed germination and regrowth was conducted immediately after planting them in a growth medium for three months, without subculturing, for groups I and II. Conversely, after three months of storage at 8 °C, groups III and IV were transferred to standard growth conditions at 25 °C for four weeks prior to monitoring.

2.5. Elaboration Data and Statistical Analysis

Thirty explants were used for each treatment. The tests were repeated three times for the micropropagation stage and for synthetic seed production and storage. Experimental data were elaborated by using Student's *t*-test ($p \leq 0.05$) and analysis of variance (ANOVA) with JMP 7.0 statistical software.

3. Results

3.1. In Vitro Germination of Walnut Zygotic Embryos

One week later, zygotic embryos (ZE) of all varieties showed strong organogenic responses with seedling induction (Figure 2a), regardless of the basal medium used. In addition, the growth of entire plantlets was observed after a few weeks (Figure 2b). The findings showed considerable disparities in the germination and proliferation rates of zygotic embryos in the initial stage of micropropagation. The variety of basal media used significantly impacted these differences (Figure 3).

The 'Tropojë' variety, followed by the 'Përmet' one, showed the highest values for all of the biometric parameters measured, regardless of the basal medium used. Concerning the medium applied, for the 'Tropojë' and 'Përmet' varieties, the DKW allowed having the highest number of shoots (2.75 and 2.25, respectively), number of leaves (6.33 and 4.85, respectively), and length of shoots (1.33 and 1.07 cm, respectively). However, no statistical differences in the biometric parameters were observed for the 'Përmet' variety when using the DKW or MS media. This last performance was also observed for the 'Korçë' and 'Peshkopi' varieties without significant differences between the MS and DKW media. The WPM medium gave low data for each parameters in the assessed varieties, except 'Tropojë' variety, for which, values above averages were recorded (Figure 3).

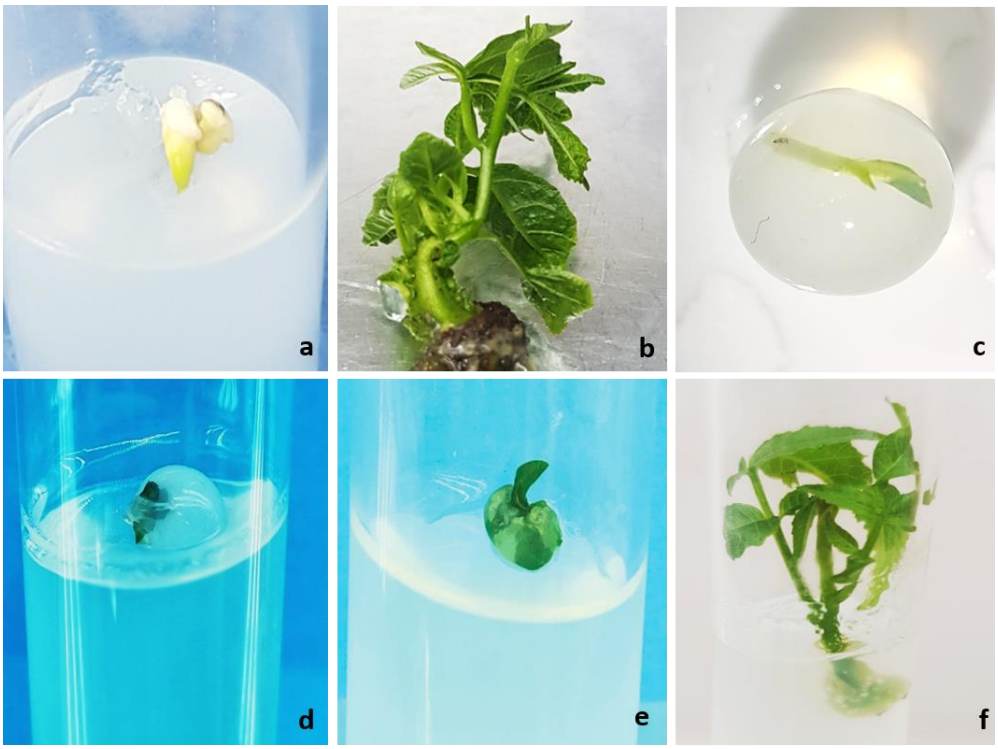
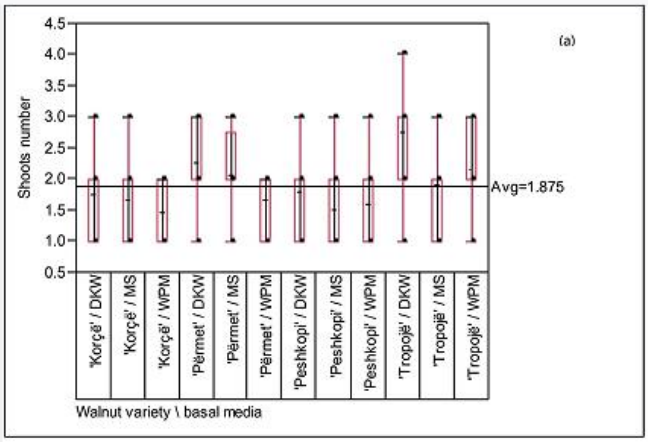
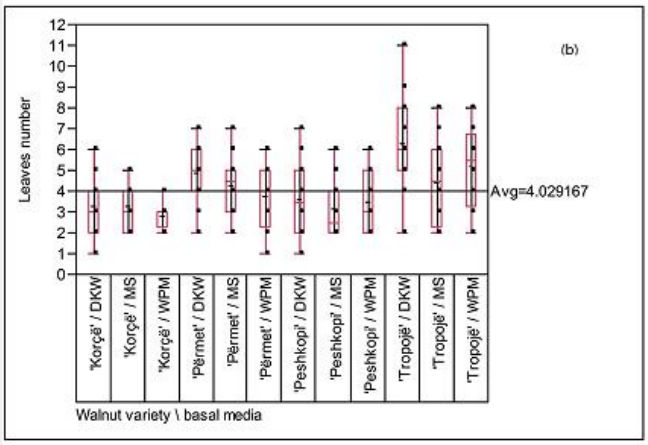


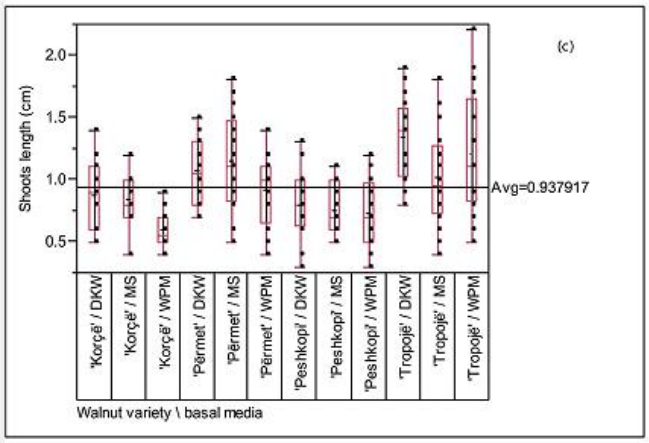
Figure 2. (**a**) Seedling induction of walnut zygotic embryo; (**b**) in vitro seedling development; (**c**) encapsulated shoot tip of walnut seedling; (**d**,**e**) plantlet regeneration from synthetic seeds; (**f**) proliferation of walnut plantlets from synthetic seeds via subcultures.



Walnut variety / basal media	Shoots number (Mean ± Std. dev.)
'Tropojë' / DKW	2.75 ± 0.85[a]
'Përmet' / DKW	2.25 ± 0.72[b]
'Korçë' / DKW	1.75 ± 0.72[cde]
'Peshkopi' / DKW	1.82 ± 0.76[cde]
'Tropojë' / MS	1.90 ± 0.72[bcd]
'Përmet' / MS	2.05 ± 0.68[bc]
'Korçë' / MS	1.65 ± 0.67[de]
'Peshkopi' / MS	1.51 ± 0.61[ef]
'Tropojë' / WPM	2.15 ± 0.67[bc]
'Përmet' / WPM	1.65 ± 0.48[def]
'Korçë' / WPM	1.45 ± 0.51[f]
'Peshkopi' / WPM	1.60 ± 0.59[ef]



Walnut variety / basal media	Leaves number (Mean - Std. dev.)
'Tropojë' / DKW	6.33 - 2.17[a]
'Përmet' / DKW	4.85 - 1.35[b]
'Korçë' / DKW	3.30 - 1.38[d]
'Peshkopi' / DKW	3.65 - 1.57[cd]
'Tropojë' / MS	4.41 - 1.87[bc]
'Përmet' / MS	4.31 - 1.45[bc]
'Korçë' / MS	3.25 - 1.07[d]
'Peshkopi' / MS	3.15 - 1.35[d]
'Tropojë' / WPM	5.20 - 1.98[b]
'Përmet' / WPM	3.75 - 1.58[cd]
'Korçë' / WPM	2.82 - 0.52[d]
'Peshkopi' / WPM	3.45 - 1.35[cd]



Walnut variety / basal media	Shoots length (cm) (Mean ± Std. dev.)
'Tropojë' / DKW	1.33 ± 0.31[a]
'Përmet' / DKW	1.07 ± 0.26[b]
'Korçë' / DKW	0.87 ± 0.28[de]
'Peshkopi' / DKW	0.79 ± 0.26[ef]
'Tropojë' / MS	1.01 ± 0.38[bc]
'Përmet' / MS	1.15 ± 0.39[b]
'Korçë' / MS	0.83 ± 0.18[de]
'Peshkopi' / MS	0.75 ± 0.21[ef]
'Tropojë' / WPM	1.21 ± 0.48[ab]
'Përmet' / WPM	0.92 ± 0.28[cde]
'Korçë' / WPM	0.59 ± 0.13[g]
'Peshkopi' / WPM	0.72 ± 0.26[fg]

Levels not connected by the same letter are significantly different (p = 0.05)

Figure 3. Variability chart for (**a**) shoot number, (**b**) leaf number, and (**c**) shoot length depending on the basal media used and walnut variety.

3.2. Effect of Different Temperatures and Sucrose Treatment on the Regrowth Potential of Synthetic Walnut Seeds

The percentage of sodium alginate (3%) used in the solution and the Na^+/Ca^{2+} ion exchange time (30 min) resulted in optimal conditions to have suitable calcium alginate beads for explant regrowth (Figure 2c–f).

The germination and regrowth rates of the encapsulated explants for each treatment were evaluated and compared (Figure 4).

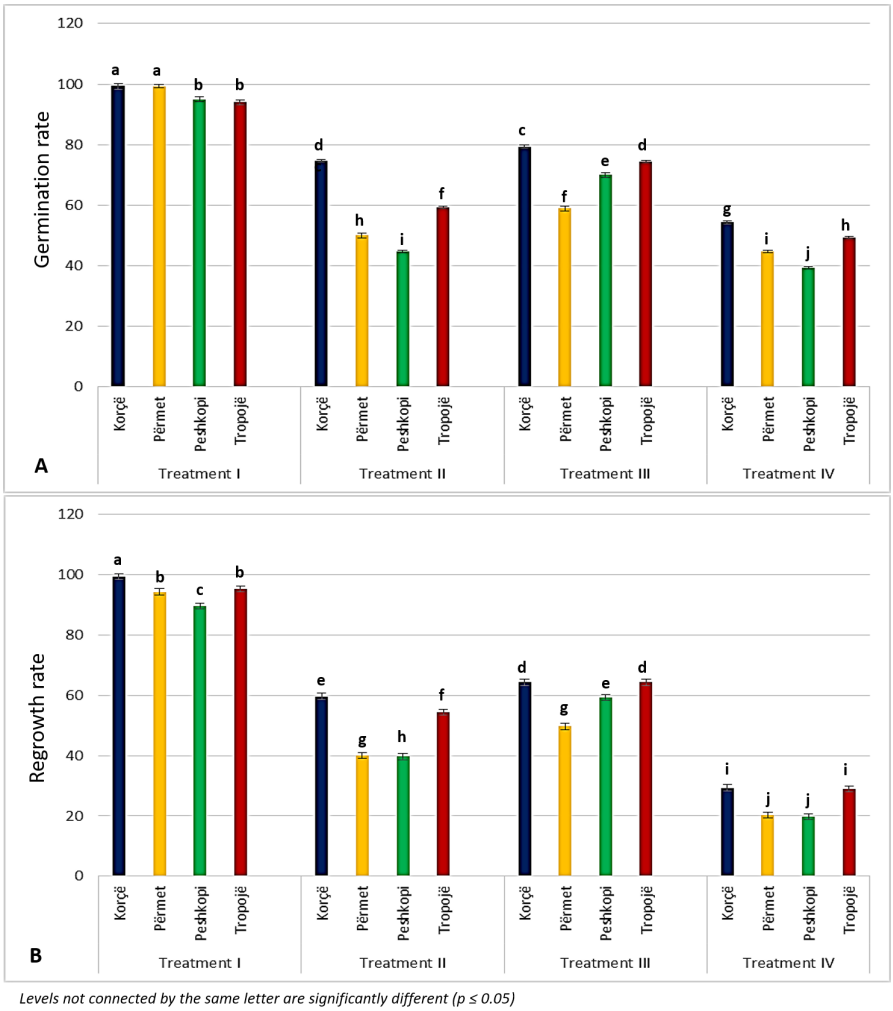


Figure 4. (**A**) Germination and (**B**) Regrowth rates of walnut varieties for different treatments: (I) 25 °C; (II) 25 °C and dehydration treatment; (III) 8 °C; (IV) 8 °C and dehydration treatment.

In this study, the synthetic seeds of the four varieties responded positively regarding germination and regrowth rates after Treatment I. 'Korçë' and 'Përmet' showed a 100% rate of germination, whereas 'Peshkopi' and 'Tropojë' showed 95% and 94% germination, respectively. Under the same conditions, the highest of the synthetic seeds' regrowth rates was recorded in 'Korçë', followed by 'Përmet' and 'Peshkopi' with a similar trend, and by 'Tropojë', whose rate was significantly lower. The standard temperature for the optimal walnut micropropagation process was 25 °C, and it helped the plantlets to recover from the synthetic seeds. The results of Treatment III, which involved storage at a temperature of 8°C, revealed a germination rate ranging from 60% to 80% and a regrowth rate ranging from 65% to 50% of the synthetic seeds after three months of preservation.

In Treatments II, III, and IV, the monitored parameters (germination and regrowth) were significantly reduced, and they were lower in Treatments II and IV than in Treatment III. These results suggest that dehydration treatment may be a stress factor that significantly reduces the explants' development from synthetic seeds in in vitro culture. However, the sucrose treatment permits low moisture content in the explant. This condition is essential when applying the encapsulation technique for mid-term conservation and cryopreservation.

Overall, the 'Korçë' and 'Tropojë' varieties were best adapted to all of the treatments. Despite the differences exhibited by the varieties within the same treatment, the lowest germination and regeneration values were observed for all varieties in Treatment IV, in

which 8 °C and dehydration treatment were combined. However, achieving 50–60% germination of explants after the conservation period (3 months) was considered adequate.

The application of specific growth conditions during minimal-growth storage, both physical and chemical, is aimed at the short-term or medium-term conservation of encapsulated explants [15]. The time between subcultures depends on the physicochemical alterations to which the encapsulated explants are exposed. Applying specific parameters affects the time of explant adaptation, the beginning of growth, and the speed of plant regeneration, consequently affecting the time necessary to move to the subsequent subculture [15,20]. Some physicochemical conditions (temperature, light/darkness, and osmotic compounds) that tend to slow down metabolic processes constitute stressful conditions for explants and sometimes decrease germination or regenerative potential [16,17,40–43]. However, at the same time, these conditions are helpful for the explants' preservation under minimal-growth storage, where a reduction in growth is required.

Explant regrowth time is when encapsulated explants react by increasing their size or rupturing the alginate beads until shoot formation [40,41]. In this study, regrowth time was monitored periodically from the first day in incubation at 25 °C and immediately after 8 °C incubation up to a maximum of four weeks. Figure 5 shows the average values for regrowth time for each walnut variety in all applied treatments. If an explant showed no signs of viability over four weeks, it was considered to not have survived. Depending on the treatment applied, the regrowth started from four days after inoculation up to twenty days after. New plantlet recovery was observed in all walnut varieties (Figure 2e,f).

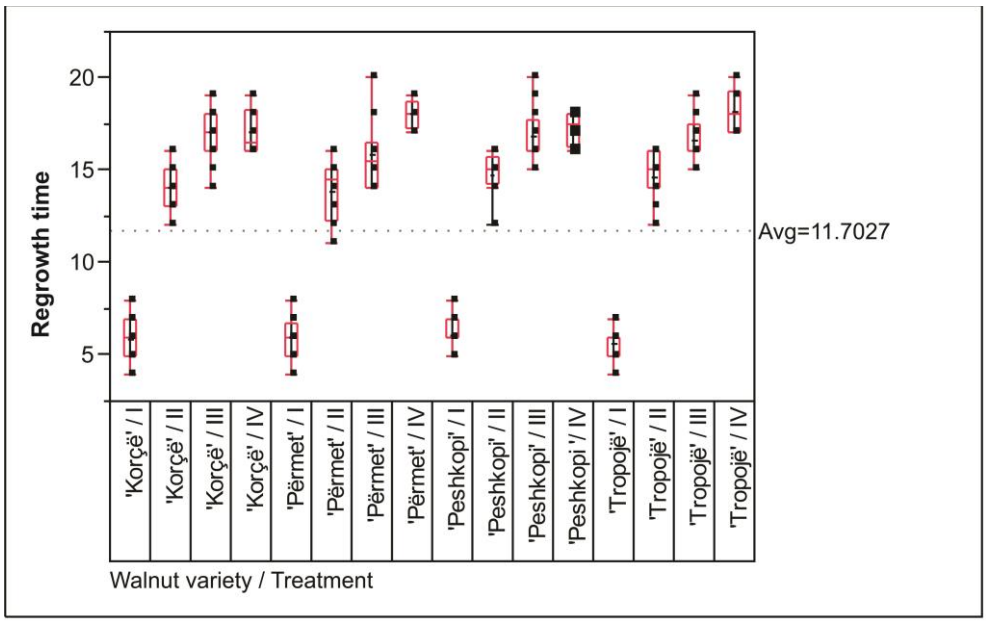


Figure 5. Regrowth time in synthetic seeds of walnut varieties.

Among the applied treatments, the shortest regrowth time was observed for Treatment I, with an average value of approximately 5.5–6 days in the varieties assessed (Figure 5 and Table 1). For Treatment II, the average proliferation time was about 14 days, and for Treatments III and IV, it was about 17 days. There was no difference in the regrowth time of the encapsulated explants in Treatments III and IV, which may be related to the conditions created by the temperature reduction. As lowering the temperature is a method that slows down plant metabolism, it is a condition that requires time to overcome or adapt when the explants are transferred to standard growth conditions (25 °C). This adaptation time is reflected in the longer regrowth times in Treatments III and IV versus Treatment II, where the explants were subjected to only dehydration treatment while maintaining the standard temperature (25 °C).

Table 1. Summary of the treatments, regrowth times, and conservation periods.

Group/Incubation Conditions	Variety	Regrowth Time (Mean ± Std. Dev.)	Period of the First Subculture
I Incubation at 25 °C	Korçë Përmet Peshkopi Tropojë	5.85 ± 1.18 [a] 5.95 ± 0.92 [a] 6.11 ± 0.81 [a] 5.58 ± 1.72 [a]	3 months (no signs of necrosis)
II Incubation at 25 °C after synthetic seed dehydration procedure	Korçë Përmet Peshkopi Tropojë	14.00 ± 1.28 [b] 13.87 ± 1.72 [bc] 14.75 ± 1.28 [b] 14.63 ± 1.93 [b]	4 weeks (conspicuous signs of necrosis)
III Incubation at 8 °C	Korçë Përmet Peshkopi Tropojë	17.08 ± 1.52 [ef] 16.00 ± 2.07 [cd] 16.83 ± 1.58 [de] 16.61 ± 0.82 [de]	3 + 1 = 4 months (slight signs of necrosis)
IV Incubation at 8 °C after synthetic seed dehydration procedure	Korçë Përmet Peshkopi Tropojë	17.00 ± 1.26 [ef] 18.00 ± 0.81 [ef] 17.25 ± 0.95 [ef] 18.16 ± 1.33 [f]	3 + 0.5 = 3.5 months (severe signs of necrosis)

Note: Levels not connected by the same letter, are significantly different between ($p \leq 0.05$)

Regarding the conservation period, the obtained results were promising and created the possibility of application of some of the assessed treatments for the short- or medium-term preservation of encapsulated explants. As noted in Table 1, Treatment I ensured the maintenance of synthetic seeds for three months without subcultures or signs of shoot necrosis for all walnut varieties. The synthetic seeds undergoing Treatment II developed over 14 days, but after four weeks, the shoots showed conspicuous signs of necrosis that advised subsequent subcultures to avoid material loss.

The first subculture was performed for Treatments III and IV after 4 and 3.5 months, respectively. Relatively high germination and regrowth rates of the explants were observed in Treatment III, but the combination of low temperature and dehydration in Treatment IV resulted in severe necrosis in the shoots in this last treatment. Thus, Treatment III is considered to be an effective short-term preservation procedure.

For Treatments II and IV, the surviving plants initially showed regenerative ability, but the subcultures later failed to produce healthy shoots and provide proliferation. Conversely, shoots recovered from Treatments I and III successfully produced new shoots that could be subcultured in further micropropagation steps.

The shoot quality from synthetic seeds was most suitable and appropriate at a storage temperature of 25 °C, but even during storage at 8 °C, the synthetic seeds remained viable and developed well after storage.

4. Discussion

The present study provides useful information regarding the possibility of walnut tree in vitro propagation and conservation using synthetic seed technology. The obtained results strengthen the findings of some of the reports so far on the in vitro propagation of walnut trees, and it also provides new information about conservation through synthetic seeds of this recalcitrant plant species. Zygotic embryos were used as primary explants and they have proven to be a good starting point for obtaining in vitro seedlings of the walnut varieties tested, taking into consideration that walnut is a recalcitrant plant species and thereby presents a low in vitro regeneration rate [50]. Studies have reported the efficiency of zygotic embryos for the in vitro propagation of many fruit-tree species [51–54] and also for the avoidance of post-zygotic incompatibility in some cases [55]. Due to their autotrophic nature, zygotic embryos from mature seeds show high organogenic responses in a PGR-free nutrient medium [56]. Particularly in walnut, a species characterized by a low percentage of seed germination and long propagation cycle, due to its recalcitrance, the application

of regeneration from in vitro cultured embryos [53,54] can allow walnut trees to obtain a higher and faster plant multiplication rate.

The development potential of zygotic embryos is determined at several levels, with the embryos' genotype being an essential factor. Kaur et al. [53] investigated embryo germination in five walnut cultivars (*J. regia* L.) via in vitro culture, reporting significant differences in percentage embryo germination among the cultivars. This result agrees with our study.

The variation on regeneration rates quickly demonstrates this concept in embryogenic responses, which are often observed among genotypes within the same species [51]. Hence, the genotypes significantly impact growth parameters in specific cultural conditions. Several authors have reported the effects of different varieties on plant proliferation or in vitro regeneration rates under the same cultivation conditions [53,54,57–59]. These differences result from interaction between external physicochemical factors and internal genetic and hormonal factors. The functional genomic component affects plant growth, proliferation, and hormonal components [12,60]. This can explain the different responses shown in zygotic embryos growth among the walnut varieties evaluated in our study. Nevertheless, despite these differences, in vitro cultures can allow the acquisition of zygotic embryos from selected lines with superior characteristics.

This research highlights that the success of walnut in vitro culture is influenced by the specific responses of different varieties and the effectiveness of the media used. Different culture media have been applied for the micropropagation of *J. regia* L. [12,49]. Driver and Kuniyuki [49] reported that DKW basal solution was effective for the in vitro regeneration of walnut seedlings. Yari et al. [61] tested three basal media, MS, WPM, and DKW, in the micropropagation of two walnut varieties. They found that DKW basal medium was the most effective by a significant margin compared to the other two media. Our findings were similar: zygotic embryos' best development and growth occurred in the DKW medium. On the other hand, other studies have identified the effective use of MS basal solution for the in vitro establishment and regeneration of walnut seedlings [54,62,63].

Woody plant medium (WPM) has been reported as effective for woody species [64–66], but it was less effective for the walnut varieties in this study. Long et al. [67] considered WPM efficiency for the *J. nigra* micropropagation protocol in combination with specific hormonal ratios. DKW medium is a relatively high-salt medium resembling MS in its nitrogen content but also containing high concentrations of several other ions. At the same time, WPM is a low-salt medium compared to DKW and MS media. For this reason, it is considered a poor medium for walnut tissue culture [68].

Another important finding provided by this research is that the application of the encapsulation technique on walnut varieties explants had promising results. As mentioned previously, recalcitrant species present difficulties for in vitro regeneration. Thus, optimizing protocols for short- and mid-term conservation is considered to be a challenge [69]. Indeed, all varieties developed plantlets from synthetic seeds, and, to our knowledge, this technology was employed for the first time in this species. Encapsulation technology is widely used in forming synthetic seeds for various purposes, especially for propagation and plant germplasm conservation [70–74]. Sodium alginate is a very effective gelling agent for forming the beads that surround the explant. This is due to the properties of sodium alginate in terms of viscosity and changing physical conditions in the presence of divalent Ca^{2+} or Mg^{2+} ions. The viscosity of the alginate, the number of bivalent ions, and the time of exposure to these ions play essential roles in gelling [24,75–77]. In our study, shoot tip beads prepared with 3% sodium alginate and 30 min of polymerization had uniform and isodiametric sizes and there were no negative impacts on plantlet development or decreased regrowth rate, as some investigations have reported [15,16]. The alginate concentration and polymerization time significantly impact the rigidity and hardness of synthetic seeds, which in turn can influence other factors, such as germination and storage ability [25,78–80].

In addition, in our research, two groups of synthetic walnut seeds were dehydrated with sucrose solution at 0.5 M for three hours. These synthetic seeds showed lower germination and regrowth percentages than those without dehydration treatment. These findings show that the short-term conservation of walnut using synthetic seed technology is possible, but the procedure should not include a dehydration stage. Some reports have indicated that rapid dehydration can increase the survival and germination of recalcitrant seeds compared to slow dehydration [81,82].

Different compounds can be used for synthetic seed desiccation treatment [80]. In a study on *Stevia rebaudiana* [83] and sugarcane [84], KNO_3 was applied before synthetic seed germination. Usually, treatment with sucrose permits low moisture content of the explant through dewatering due to low water potential in the external environment. It increases tolerance to dehydration and maintains tissue viability. This condition benefits applications requiring limited storage space and short- to medium-term preservation. For example, applying carbohydrates as osmotic agents during slow-growth storage (medium-term conservation) reduces cell division. It limits the development of shoots [15,16], prolonging the time between subcultures and reducing the number of subcultures. For plant germplasm conservation, achieving an optimum balance between extending conservation time and maintaining explants' vitality is essential. In this study, synthetic seeds treated with sucrose showed low germination and regrowth. Indeed, only this treatment combined with lower temperatures allowed preservation until 3.5 months for the walnut explants with severe signs of necrosis.

To date, the application of encapsulation technology to preserve various explants, with minimal growth, has been reported as adequate by several authors to provide short- or medium-term conservation by modifying chemicals (nutrient medium modification) or physical factors (such as temperature) [15,16,40,41,85–87]. In terms of temperature, the incubation of synthetic walnut seeds at 8 °C allowed a suitable regrowth time and a conservation period of 4 months, with slight signs of shoot necrosis, whereas incubation at 25 °C showed a better shoot quality, but a shorter conservation period.

5. Conclusions

The walnut tree is considered one of the most recalcitrant species, thereby presenting difficulties regarding its regeneration rate. Therefore, for its in vitro propagation and conservation it is necessary to define the optimal culture conditions and medium that are optimal for its establishment and proliferation.

In this study, walnut zygotic embryos proved to be a good choice for tissue culture. Theywere also found to have better potential to develop seedlings in the DKW medium than in the other media tested. Moreover, the production and storage of encapsulated shoot tips from in vitro seedlings was successfully applied for the in vitro regeneration and conservation of four walnut varieties. An important finding provided by this research is that the encapsulated explants showed high germination and regrowth rates, indicating potential propagation and short-term conservation applications. Specific conditions affected the shoots' shelf life and regenerative capacity. Synthetic seeds at 25 °C and 8 °C allowed maintaining the walnut explants for 3.5 and 4 months respectively without subcultures. These findings are promising, given the satisfactory regrowth in all tested walnut varieties after short-term storage, and the way for the application of encapsulation and storage in other *Juglans* spp. Moreover, encapsulation technology can be used in cryopreservation procedures for the long-term conservation of this species.

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References

1. Aryapak, S.; Ziarati, P. Nutritive Value of Persian Walnut (*Juglans regia* L.) Orchards. *Am. Eurasian J. Agric. Environ. Sci.* **2014**, *14*, 1228–1235.
2. Xiaoying, M.; Yufei, H.; Guogang, C. Amino Acid Composition, Molecular Weight Distribution and Gel Electrophoresis of Walnut (*Juglans regia* L.) Proteins and Protein Fractionations. *Int. J. Mol. Sci.* **2014**, *15*, 2003–2014.
3. Vassiliou, V.G.; Voulgaridis, E.V. Wood properties and utilization potentials of walnut wood (*Juglans regia* L.) Grown in Greece. *Acta Hortic.* **2005**, *705*, 535–542. [CrossRef]
4. Bukhari, M.N.; Shabbir, M.; Rather, L.J.; Shahid, M.; Singh, U.; Khan, M.A.; Mohammad, F. Dyeing studies and fastness properties of brown naphtoquinone colorant extracted from *Juglans regia* L on natural protein fiber using different metal salt mordants. *Text. Cloth. Sustain.* **2017**, *3*, 3. [CrossRef]
5. Tutak, M.; Benli, H. Colour and fastness of fabrics dyed with walnut (*Juglans regia* L.) base natural dyes. *Asian J. Chem.* **2011**, *23*, 566–568.
6. Vahdati, K.; Sarikhani Khorami, S.; Arab, M.M. Walnut: A potential multipurpose nut crop for reclaiming deteriorated lands and environment. *Acta Hortic.* **2018**, *1190*, 95–100. [CrossRef]
7. Vangjeli, J. *Atlas of the Albanian Flora*; Academy of Sciences of Albania: Tirana, Albania, 2016; p. 58. (In Albanian)
8. Vangjeli, J.; Ruci, B.; Mullaj, A. *The Red Book: Threatened and Rare Plant Species of Albania*; Academy of Sciences of Albania: Tirana, Albania, 1995. (In Albanian)
9. Paź-Dyderska, S.; Jagodziński, A.M.; Dyderski, M.K. Possible changes in spatial distribution of walnut (*Juglans regia* L.) in Europe under warming climate. *Reg. Environ. Change* **2021**, *21*, 18. [CrossRef]
10. Payghamzadeh, K.; Sayyed, K.K. In vitro propagation of walnut—A review. *Afr. J. Biotechnol.* **2011**, *10*, 290–311.
11. Hormaza, J.I. Early selection in cherry combining RAPDs with embryo culture. *Sci. Hortic.* **1999**, *79*, 121–126. [CrossRef]
12. Bridgen, M.P. A review of plant embryo culture. *HortScience* **1994**, *29*, 1243–1246. [CrossRef]
13. Lambardi, M.; Ozodogru, E.A.; Jain, S.M. *Protocols for Micropropagation of Selected Economically-Important Horticultural Plant*; Springer: New York, NY, USA; Heidelberg, Germany; Dordrecht, The Netherlands; London, UK, 2013; p. 490. [CrossRef]
14. Ríos Leal, D.; Sánchez-Olate, M.; Avilés, M.; Materan, M.E.; Uribe, M.; Hasbún, R.; Rodríguez, R. Micropropagation of *Juglans regia* L. In *Protocols for Micropropagation of Woody Trees and Fruits*; Jain, S.M., Häggman, H., Eds.; Springer: Dordrecht, The Netherlands, 2007; pp. 381–390.
15. Benelli, C.; Tarraf, W.; Izgu, T.; De Carlo, A. In vitro Conservation through Slow Growth Storage Technique of Fruit Species: An Overview of the Last 10 Years. *Plants* **2022**, *11*, 3188. [CrossRef] [PubMed]
16. Lambardi, M.; Ozudogru, E.A. Advances in the safe storage of micropropagated woody plants at low temperature. *Acta Hortic.* **2013**, *988*, 29–42. [CrossRef]
17. Lambardi, M.; Shaarawi, S. Importance of in vitro culture for developing cryopreservation strategies of woody plants. *Acta Hortic.* **2017**, *1187*, 177–188. [CrossRef]
18. Murashige, T. Plant cell and organ cultures as horticultural practices. *Acta Hortic.* **1977**, *78*, 17–30. [CrossRef]
19. 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.
20. Micheli, M.; Standardi, A.; Fernandes da Silva, D. Encapsulation and synthetic seeds of olive (*Olea europaea* L.): Experiences and overview. In *Synthetic Seeds*; Springer: Cham, Switzerland, 2019; pp. 347–361.
21. Lambardi, M.; Benelli, C.; Ozudogru, E.A.; Ozden-Tokatli, Y. Synthetic seed technology in ornamental plants. In *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues*; Teixeira da Silva, J.A., Ed.; Global Science Books: London, UK, 2006; pp. 347–354.
22. Ara, H.; Jaiswal, U.; Jaiswal, V. Synthetic seed: Prospects and limitation. *Curr. Sci.* **2000**, *78*, 1438–1444.
23. Daud, M.; Taha, M.Z.; Hasbullah, A.Z. Artificial seed production from encapsulated micro shoots of *Sainpaulia ionantha* Wendl. (African Violet). *J. Appl. Sci.* **2008**, *8*, 4662–4667. [CrossRef]
24. Saiprasad, G.V.S. Artificial seeds and their applications. *Resonance* **2001**, *6*, 39–46. [CrossRef]
25. Ahmad, N.; Anis, M. Direct plant regeneration from encapsulated nodal segments of *Vitex negundo*. *Biol. Plant.* **2010**, *54*, 748–752. [CrossRef]
26. 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]
27. Ravi, D.; Anand, P. Production and applications of artificial seeds: A review. *Int. Res. Biol. Sci.* **2012**, *1*, 74–78.
28. Mohanty, P.; Das, M.C.; Kumaria, S.; Tandon, P. High-efficiency cryopreservation of the medicinal orchid *Dendrobium nobile* Lindl. *Plant Cell Tissue Organ Cult.* **2012**, *109*, 297–305. [CrossRef]
29. Standardi, A.; Micheli, M. Encapsulation of in vitro-derived explants: An innovative tool for nurseries. *Methods Mol. Biol.* **2013**, *11013*, 397–418. [PubMed]
30. Vdovitchenko, Y.M.; Kuzovkina, I.N. Artificial Seeds as a Way to Produce Ecologically Clean Herbal Remedies and to Preserve Endangered Plant Species, *Mosc. Univ. Biolog. Sci. Bull.* **2011**, *66*, 48–50. [CrossRef]

31. Rai, M.K.; Jaiswal, V.S.; Jaiswal, U. Encapsulation of shoot tips of guava (*Psidium guajava* L.) for short-term storage and germplasm exchange. *Sci. Hortic.* **2008**, *118*, 33–38. [CrossRef]
32. Nyende, A.B.; Schittenhelm, S.; Mix-Wagner, G.; Greef, J.M. Production, storability, and regeneration of shoot tips of potato (*Solanum tuberosum* L.) encapsulated in calcium alginate hollow beads. *Vitr. Cell. Dev. Biol. Plant* **2003**, *39*, 540–544. [CrossRef]
33. Gantait, S.; Kundu, S.; Ali, N.; Sahu, N.C. Synthetic seed production of medicinal plants: A review on influence of explants, encapsulation agent and matrix. *Acta Physiol. Plant.* **2015**, *37*, 1847. [CrossRef]
34. Bekheet, S.A. A synthetic seed method through encapsulation of *in vitro* proliferated bulblets of garlic (*Allium sativum* L.). *Arab. J. Biotech.* **2006**, *9*, 415–426.
35. Roy, B.; Mandal, A.B. Development of synthetic seeds involving androgenic and pro-embryos in elite indica rice. *Indian J. Biotechnol.* **2008**, *7*, 515–519.
36. Ray, A.; Bhattacharya, S. Storage and plant regeneration from encapsulated shoot tips of *Rauvolfia serpentinea*–an effective way of conservation and mass propagation. *S. Afr. J. Bot.* **2008**, *74*, 776–779. [CrossRef]
37. Singh, A.K.; Chand, S. Plant regeneration from alginate encapsulated somatic embryos of *Dalbergia sissoo* Roxb. *Indian J. Biotechnol.* **2010**, *9*, 319–324.
38. Andlib, A.; Verma, R.N.; Batra, A. Synthetic seeds an alternative source for quick regeneration of a zero calorie herb–*Stevia rebaudiana* Bertoni. *J. Pharm. Res.* **2011**, *4*, 2007–2009.
39. Cheruvathur, M.K.; Kumar, G.K.; Thomas, T.D. Somatic embryogenesis and synthetic seed production in *Rhinacanthus nasutus* (L.) Kurz. *Plant Cell Tissue Organ Cult.* **2013**, *113*, 63–71. [CrossRef]
40. 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]
41. 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. Hort.* **2007**, *113*, 286–292. [CrossRef]
42. Shatnawi, M.A.; Engelmann, F.; Frattarelli, A.; Damiano, C. Cryopreservation of apices in vitro plantlets of almond (*Prunus dulcis* Mill.). *Cryoletters* **1999**, *20*, 13–20.
43. Plessis, P.; Leddet, C.; Dereuddre, J. Resistance to dehydration and to freezing in liquid nitrogen of alginate coated shoot tips of grapevine (*Vitis vinifera* L. cv. Chardonnay). *C. R. Acad. Sci.* **1991**, *313*, 373–380.
44. Gantait, S.; Kundu, S. Artificial Seed Technology for Storage and Exchange of Plant Genetic Resources. In *Advanced Technologies for Crop Improvement and Agricultural Productivity*; Agrobios (International): Jodhpur, India, 2017; Chapter 7; pp. 135–159.
45. Kumari, P.; Kumar, V.; Chandra, S. Synthetic Seeds: A Boon for Conservation and Exchange of Germplasm. *Biomed. Res.* **2014**, *1*, 1–11.
46. Benelli, C.; Micheli, M.; De Carlo, A. An improved encapsulation protocol for regrowth and conservation of four ornamental species. *Acta Soc. Bot. Pol.* **2017**, *86*, 1–12. [CrossRef]
47. Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **1962**, *15*, 473–497. [CrossRef]
48. Lloyd, G.; McCown, B. Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by the use of shoot tip culture. *Proc. Plant Prop. Soc.* **1981**, *30*, 421–427.
49. Driver, J.A.; Kuniyuki, A.H. In vitro propagation of Paradox walnut *Juglans hindsii* × *Juglans regia* rootstock. *HortScience* **1984**, *19*, 507–509. [CrossRef]
50. McGranahan, G.H.; Driver, J.A.; Tulecke, W. Issue Culture of Juglans. In *Cell and Tissue Culture in Forestry*; Bonga, J.M., Durzan, D.J., Eds.; Forestry Sciences; Springer: Dordrecht, The Netherlands, 1987; Volume 3, pp. 261–271.
51. Elhiti, M.; Stasolla, C. The use of zygotic embryos as explants for in vitro propagation: An overview. In *Plant Embryo Culture*; Thorpe, T., Yeung, E., Eds.; Methods in Molecular Biology; Humana Press: Totowa, NJ, USA, 2011; Volume 710, pp. 229–255. [CrossRef]
52. Leslie, C.; McGranahan, G. Micropropagation of Persian walnut (*Juglans regia* L.). In *Biotechnology in Agriculture and Forestry, High-Tech and Micropropagation I*; Bajaj, Y.P.S., Ed.; Springer: Berlin/Heidelberg, Germany; New York, NY, USA, 1992; Volume 18, pp. 136–150.
53. Kaur, R.; Sharma, N.; Kumar, K.; Sharma, D.R.; Sharma, S.D. In vitro germination of walnut (*Juglans regia* L.) embryos. *Sci. Hortic.* **2006**, *109*, 385–388. [CrossRef]
54. Toosi, S.; Dilmagani, K. Proliferation of *Juglans regia* L. by in vitro embryo culture. *J. Cell Biol. Genet.* **2010**, *1*, 12–19.
55. Ramming, D.W. The use of embryo culture in fruit breeding. *HortScience* **1990**, *25*, 393–398. [CrossRef]
56. Raghavan, V.; Srivastava, P.S. Embryo culture. In *Experimental Embryology of Vascular Plants*; Johri, B.M., Ed.; Springer: Berlin, Germany, 1982; pp. 195–230.
57. Sanchez-Zamora, M.A.; Diego Frutos Tomas, J.C.T.; Garcia-Lopez, R. Embryo germination and proliferation in vitro of *Juglans regia* L. *Sci. Hortic.* **2006**, *108*, 317–321. [CrossRef]
58. Kepenek, K.; Kolağasi, Z. Micropropagation of walnut (*Juglans regia* L.). *Acta Phys. Pol.* **2016**, *130*, 150–156. [CrossRef]
59. Scaltsoyiannes, A.; Tsoulpha, P.; Panetsos, K.P.; Moulalis, D. Effect of genotype on micropropagation of walnut trees (*Juglans regia* L.). *Silvae Genet.* **1997**, *46*, 326–332.
60. Tantikanjana, T.; Young, W.H.J.; Letham, D.S.; Griffith, M.; Hussain, M.; Ljung, K. Control of axillary bud proliferation and shoot architecture in Arabidopsis through supershoot gene. *Genes Dev.* **2001**, *15*, 1587–1588. [CrossRef]

61. Yari, M.G.; Gholami, M.; Khazaei, I. Impact of media and different cytokinins concentrations on *in vitro* shoot multiplication of Persian walnut (*Juglans regia* L.). *Int. J. Farming Allied Sci.* **2014**, *3*, 203–209.
62. Cossio, F.; Minolta, G. Prove preliminari di coltura in vitro di embrioni isolati di noce (*Juglans regia* L.) e confronto tra differenti combinazioni di sali minerali. *Riv. Fruttic. Ortofloric.* **1983**, *67*, 287–298.
63. Gruselle, R.; Boxus, P. Walnut micropropagation. *Acta Hortic.* **1990**, *284*, 45–52. [CrossRef]
64. Yao, Y.X.; Sun, Y.W.; Li, G.G.; Li, G.H. Regeneration of plants from in vitro culture of petioles in *Prunus domestica* Lindl (European Plum). *Biotechnol. Biotechnol. Equip.* **2011**, *25*, 2458–2463. [CrossRef]
65. Liu, X.; Pijut, P.M. Agrobacterium-mediated transformation of mature *Prunus serotina* (black cherry) and regeneration of transgenic shoots. *Plant Cell Tissue Organ Cult.* **2010**, *101*, 49–57. [CrossRef]
66. Blando, F.; Chiriac, L.; Gerardi, C.; Lucchesini, M.; Rampino, P. Sweet Cherry (*Prunus avium* L.) 'Giorgia', Adventitious Regeneration from Leaves of Microplants. *Eur. J. Hort. Sci.* **2007**, *72*, 138–143.
67. Long, L.M.; Preece, J.E.; Van Sambeek, J.W. Adventitious regeneration of *Juglans nigra* L. (eastern black walnut). *Plant Cell Rep.* **1995**, *14*, 799–803. [CrossRef]
68. Saadat, Y.A.; Hennerty, M.J. Factors affecting the shoot multiplication of Persian walnut (*Juglans regia* L.). *Sci. Hortic.* **2002**, *95*, 251–260. [CrossRef]
69. Berjak, P.; Pammenter, N.W. Implications of the lack of desiccation tolerance in recalcitrant seeds. *Front. Plant Sci.* **2013**, *4*, 478. [CrossRef]
70. Wang, Q.; Tanne, E.; Arav, A.; Gafny, R. Cryopreservation of in vitro-grown shoot tips of grapevine by encapsulation-dehydration. *Plant Cell Tissue Organ Cult.* **2000**, *63*, 41–46. [CrossRef]
71. Gonzales-Arnao, M.T.; Englmann, F.; Huet, C.; Urra, C. Cryopreservation of encapsulation apices of sugarcane: Effect of freezing temperatures and histology. *Cryoletters* **1993**, *14*, 303–308.
72. Faisal, M.; Ahmad, N.; Anis, M. In vitro Plant Regeneration from Alginate–Encapsulated Microcuttings of *Rauvolfia tetraphylla* L. *Am.-Eurasian J. Agric. Environ. Sci.* **2006**, *1*, 01–06.
73. Ganapathi, T.R.; Suprasanna, P.; Bapat, V.A.; Rao, P.S. Propagation of Banana through encapsulated shoot tips. *Plant Cell Rep.* **1992**, *11*, 571–575. [CrossRef] [PubMed]
74. Piccioni, E.; Standardi, A. Encapsulation of micropropagated buds of 6 woody species. *Plant Cell Tissue Organ Cult.* **1995**, *42*, 221–226. [CrossRef]
75. Kikowska, M.; Thiem, B. Alginate-encapsulated shoot tips and nodal segments in micropropagation of medicinal plants. A review. *Herba Pol.* **2011**, *57*, 45–57.
76. Bapat, V.A.; Rao, P.S. In vivo growth of encapsulated axillary buds of mulberry (*Morus indica* L.). *Plant Cell Tissue Org. Cult.* **1990**, *20*, 69–70. [CrossRef]
77. Sakamoto, Y.; Mashiko, T.; Suzuki, A.; Kawata, H.; Iwasaki, A. Development of encapsulation technology for synthetic seeds. *Acta Hort.* **1992**, *319*, 71–76. [CrossRef]
78. Mondal, P.K.; Bhattacharya, A.; Sood, A.; Ahuja, P.S. Propagation of tea (*Camella sinensis* L. O. Kuntze) by shoot proliferation of alginate-encapsulated axillary bud stored at 4 °C. *Curr. Sci.* **2002**, *83*, 941–944.
79. Sarmah, D.K.; Borthakur, M.; Borua, P.K. Artificial seed production from encapsulated PLBs regenerated from leaf base of *Vanda coerulea* Grifft. ex. Lindl.—An endangered orchid. *Curr. Sci.* **2010**, *98*, 686–690.
80. Rihan, H.Z.; Kareem, F.; El-Mahrouk, M.E.; Fuller, M.P. Artificial Seeds (Principle, Aspects and Applications). *Agronomy* **2017**, *7*, 71. [CrossRef]
81. Pritchard, H.W. Water potential and embryonic axis viability in recalcitrant seeds of *Quercus rubra*. *Ann. Bot. Lond.* **1991**, *67*, 43–49. [CrossRef]
82. Farrant, J.M.; Berjak, P.; Pammenter, N.W. The effect of drying rate on viability retention of recalcitrant propagules of *Avicennia marina*. *S. Afr. J. Bot.* **1985**, *51*, 432–438. [CrossRef]
83. Ali, A.; Gull, I.; Majid, A.; Saleem, A.; Naz, S.; Naveed, N.H. In vitro conservation and production of vigorous and desiccate tolerant synthetic seeds in *Stevia rebaudiana*. *J. Med. Plants Res.* **2012**, *6*, 1327–1333.
84. Ali, A.; Iqbal, M.; Majid, A.; Naveed, N.; Rehman, A.; Afghan, S. In vitro conservation and production of vigorous and desiccate tolerant synthetic seed formation in sugarcane (*Saccharum officinarum* L.). In Proceedings of the Conference: Annual Convention PSST, Rawalpindi, Pakistan, 9–10 September 2013; Volume 47.
85. Nassar, A.H. Slow Growth Storage of Encapsulated Germplasm of *Coffea arabica* L. *Int. J. Agric. Biol.* **2003**, *5*, 517–520.
86. Soneji, J.R.; Roa, P.S.; Mhatre, M. Germination of Synthetic seeds of pineapple (*Ananas comosus* L. Merr.). *Plant Cell Rep.* **2002**, *20*, 891–894. [CrossRef]
87. Pattnaik, S.; Chand, P.K. Morphogenetic response of the alginate–encapsulated axillary buds from *in vitro* shoot cultures of six mulberries. *Plant Cell Tissus Org. Cult.* **2000**, *60*, 177–185. [CrossRef]