

In Vitro Conservation Tactics for a Sweet Genotype of *Amygdalus scoparia*, an Endangered Medicinally Important Tree Species by Propagation Using Synthetic Seeds

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Abstract

Bud stems arisen from *in vitro* cultures of *A. scoparia* were encapsulated in calcium alginate pieces for short term stockpile and germplasm interchange. The maximum frequency (88.96%) of conversion of encapsulated nodal segments into plantlets and the highest node number (7.20) was performed on a murashige and Skoog's medium (MS medium) containing 2.2 μ M banzyl adenine (BA). The highest length of re-growing shoots was achieved when MS medium was supplied with 2.2 μ M BA and 0.5 μ M NAA. However, the number of shoots produced was higher (5.30 and 5.10) on MS medium supplemented with 2.2 μ M BA and 0.5 μ M NAA and MS medium with 2.2 μ M BA, respectively than on the hormone-free media. Treatment with 19.6 μ M IBA resulted in the highest conversion of encapsulated nodal segments into plantlets. The frequency of conversion (89.6% - 88.6%) was retained at 25°C for up to 2 weeks without significant change. The highest frequencies (61.1%) of plantlet formation from encapsulated nodal segments were obtained by transferring synthetic seeds onto peat mass and perlite (2:1) (v/v) mixture substrate. When transplanted into the peat mass and perlite (2:1) (v/v) mixture, these plantlets showed greater plantlet high, leaf number, shoot number and root number per plantlet than those of the other substrates. The synthetic seed technology offered a promising way for short term storage without refrigerating, germplasm conservation exchange for improvement and an alternative clonal propagation method for this endangered genotype of *A. scoparia*.

Keywords

Amygdalus scoparia, Micropropagation, Plantlet Conversion, Short Term Storage, Synthetic Seeds

1. Introduction

Amygdalus scoparia is an important medicinal and commercial species from *Rosaceae* that tolerates heat and drought. The seed of *A. scoparia* includes a special compound called amygdalin ($C_{20}H_{27}NO_{11}$) which is traditionally used to treat cardiovascular and respiratory diseases, rheumatism, and headaches; for wound healing; and also as an appetizer. A significant antihyperglycemic effect of *A. scoparia* also supported its traditional application in the treatment of diabetes. *A. scoparia* normally produces bitter seeds (bitter genotype); however, only one rootstock produces sweet seed (sweet genotype) that is found in Chah-Gorbeh mountain located in Naein, a city of Isfahan province in the central part of Iran. Considering the great importance of this sweet genotype rootstock, its non-sexual reproduction through micro-propagation to preserve the existing genetic source is considered [1] [2] [3]. The encapsulation technology which is useful in germplasm conservation of endangered plants by using appropriate storage technique presents a promising tool for exchange of plant material between laboratories for short- and medium-term storage of valuable plant material [4] [5]. Alginate encapsulation is very useful technique due to its cost effectiveness in comparison to cryopreservation in a wide range of woody plant species [6] [7]. In crop plants such as almonds that are difficult or even impossible to propagate through cuttings, the planting efficiency could be considerably improved by the use of synthetic seeds instead of cuttings [4]. Encapsulation of *in vitro* derived explants has been reported for olive [8], pomegranate [4], white cedar [9], eucalyptus [10] and many other fruit crops [11]. However, to date, there is no report on the development of a synthetic seed system for *A. scoparia*. Due to the importance of *A. scoparia* with sweet seed, which is a rare and endangered variety, synthetic seed technology using encapsulation of *in vitro* derived nodal segments of *A. scoparia* could become a potentially cost effective mass clonally propagation system. Therefore, in this paper, the first successful attempt of the utilizing of synthetic seed technology for this rare and endangered medicinal plant using nodal segments from *in vitro* culture for encapsulation is reported. The morphological response of the encapsulated nodal segments was also evaluated in various planting media. Efforts were also made to test the ability of the encapsulated nodal segments to retain viability following different durations of storage, so that this technology could be useful for transfer of viable and elite germplasm of *A. scoparia* to nurseries and laboratories.

2. Material and Methods

2.1. Explants Source

Seeds of *A. scoparia* (sweet genotype) collected from the natural habitat located in Chah-Gorbeh Mountain in Nain, were surface sterilized according to San *et al.* [12] by immersion into 3.75% (v/v) sodium hypochlorite solution containing two to three drops of Tween 20 for 25 min followed by rinsing three times for 5 min in sterile distilled water. The embryos were carefully excised from the coty-

ledons under sterile conditions and were cultured on MS medium [13], containing 2.2 μM BA as shown in **Figure 1(a)**. The plant material for experiments was obtained from *in vitro* proliferated *Amygdalus scoparia* (**Figure 1(a)**). Nodal segments (NS) approximately 0.5 cm long were aseptically excised from *in vitro* cultures of *A. scoparia* for use as encapsulated micropropagules in the following experiments.

2.2. Experiment 1: Shoot Regrowth from Capsules

This experiment aimed to optimize culture medium for regrowth of shoots from encapsulated nodal segments of *A. scoparia*. Calcium - alginate beads were produced according to Hung and Trueman [10], using 3% (w/v) low-viscosity sodium alginate (purchased from Sigma chemical Co) as gelling matrix and 100 mM CaCl_2 (purchased from Merck chemical Co) as the complexing agent. Nodal explants were cut in 0.5 cm in size and mixed in sterile sodium alginate solution containing full-strength liquid MS medium with 2.2 μM BA, as applied for

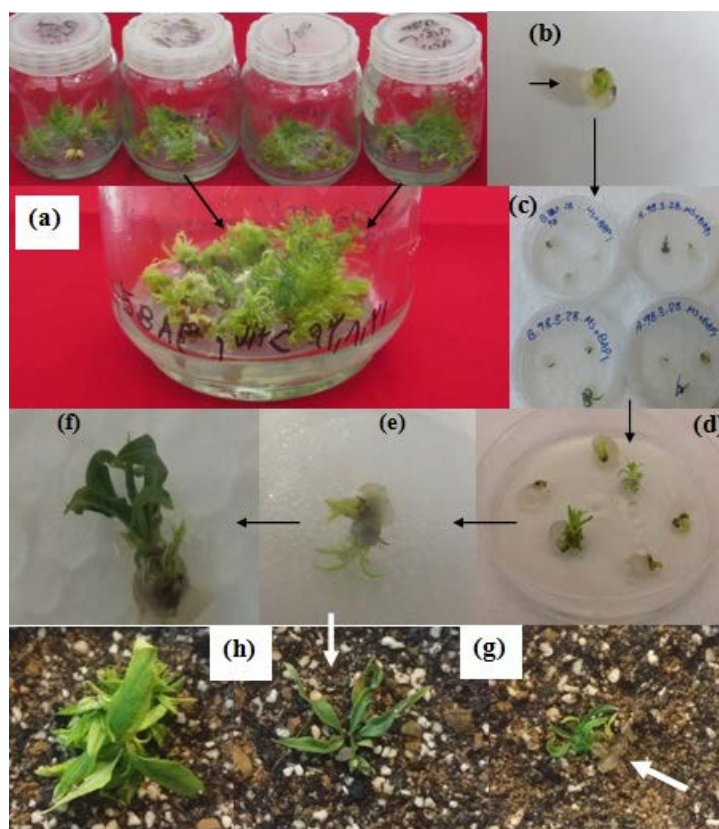


Figure 1. Development of encapsulated explants of *in vitro* raised nodal segments of *A. scoparia* under aseptic and non-aseptic conditions. (a) *In vitro* micropropagation of zygotic embryos isolated from mature seeds of *A. scoparia*. (b) A Capsule formed by encapsulation of a nodal segment. (c and d) Shoot regrowing from capsules after 6 weeks on different sowing media. (e) Multiplication of shoot in a synthetic seed. (f) Roots induced in a synthetic seed. (g) Plantlet formed directly from synthetic seeds after conversion and placement for 4 weeks on different non-sterile substrates. (h) 2 and 3-week-old potted plantlet.

proliferation of non-encapsulated embryo cultures of *A. scoparia*. For encapsulation experiment, the explants were picked up and placed individually into a sterile solution of CaCl_2 for 30 min. The encapsulated nodal segments (**Figure 1(b)**) were retrieved by decanting off the CaCl_2 solution, washing thrice with sterilized distilled water, and culturing in 60-mm diameter, 20-mm deep Petri dishes containing 10 mL of one of four sowing media for shoot regrowth: 1) 1/2 MS (M1); 2) MS (M2); 3) MS + 2.2 μM BA and 4) MS + 2.2 μM BA + 0.5 μM naphthalene acetic acid (NAA) (M4) as shown in **Figure 1(c)**. All media contained 3% sucrose with the pH adjusted to 5.7, solidified with 0.8% (w/v) agar (purchased from Merck chemical Co) and autoclaved at 104 kPa, 121°C for 15 min. The cultures were maintained for 6 weeks, at 25°C under a 16-h photoperiod at a photosynthetic photon flux density of 4000 lx to determine the percentage of capsules with shoot regrowth, the maximum shoot length from each re-growing capsule, and the numbers of nodes and shoots emerged from each re-growing capsule.

2.3. Experiment 2: Direct Conversion under Aseptic Conditions

This experiment was aimed to find the best concentration of IBA for conversion of synthetic seeds into plantlet when transferring synthetic seeds to sowing medium. The concentrations of IBA were selected based on the research conducted by Hung and Trueman [14]. Excised nodal segments of *A. scoparia* were treated in darkness for 24 h on agar-gelled 1/2 MS medium containing 0, 4.9, 19.6 and 74.4 μM IBA (purchased from Sigma chemical Co) and 3% sucrose. The treated explants were suspended in sodium alginate solution containing hormone free liquid MS medium and then placed individually into a sterile solution of CaCl_2 for 30 min for encapsulation. After encapsulation, synthetic seeds got transferred into 56-mm diameter, 20-mm deep Petri dishes moistened with 2 mL of sterile distilled water, tightly sealed with plastic film to prevent desiccation and kept in the dark at room temperature (23°C - 26°C) for 1 week to allow the formation of root primordia (**Figure 1(f)**). The synthetic seeds were then transferred to glass jars, each containing 50 mL of sterile agar-gelled hormone-free 1/2 MS medium, and incubated for 4 weeks at 25°C under a 16-h photoperiod at 4000 lx to determine the percentage of synthetic seeds that converted into plantlets and the number of nodes produced per plantlet.

2.4. Experiment 3: Short-Term Storage of Synthetic Seeds

The third experiment was conducted to investigate the potential of synthetic seeds to convert into plantlets after short-term storage at cold and room temperature. Nodal segments were treated with 0.04 mM IBA, prior to encapsulation, for induction of root primordia. The synthetic seeds containing pre-treated explants were then stored in moistened plastic Petri dishes (as described in experiment 2) for 0, 1, 2, 3, 4 and 8 weeks in darkness at 4 and 25°C. after each storage period, the synthetic seeds were removed from Petri dishes and cultured on agar-solidified 1/2 MS medium and maintained for 4 weeks in the light (16-h

photoperiod at 4000 lx) at 25°C to determine the percentage of synthetic seeds that converted into plantlets.

2.5. Experiment 4: Direct Conversion under Non-Aseptic Conditions

This experiment evaluated the suitability of different substrates for the formation and the growth of plantlets from synthetic seeds. Synthetic seeds obtained by treatment of nodal segments with 0.04 mM IBA, prior to encapsulation, for induction of root primordia (as described in experiment 3), were maintained under light (16-h photoperiod at 4000 lx) at 25°C in moistened Petri dishes for 4 weeks. The synthetic seeds were then placed in one of three non-sterile substrates: 1) peat mass and sand (2:1) (v/v) mixture (PS1), 2) peat mass and perlite (2:1) (v/v) mixture (PS2) and peat mass and loam soil (2:1) (v/v) mixture (PS3) kept for 4 weeks under 16-h photoperiod at 4000 lx to determine the percentage of synthetic seeds that converted into plantlets, plantlet height and the numbers of new leaves and adventitious roots per plantlet.

In all experiments, each treatment consisted of 10 replicates arranged in a completely randomized design and each experiment was repeated twice. The results are expressed as a mean of four independent experiments. The data were analyzed statistically using one-way analysis of variance (ANOVA) and the significant differences between means were assessed by LSD test at $P < 0.05$.

3. Results and Discussion

3.1. Experiment 1: Shoot Regrowth from Capsules

The most favorable attribute of the encapsulated nodal segments is their talent to preserve viability in terms of regrowth abilities after encapsulation [15]. In this study, shoot regrowth from encapsulated nodal segments was performed within 6 weeks on all sowing media (**Figure 1(d)** and **Figure 1(e)**). The lowest regrowth percentages (30.13% and 49.49%) were recorded with hormone-free half-strength and full-strength MS media, respectively (**Figure 2**). However, MS medium supplemented with 2.2 μM BA (M3) provided the highest mean (88.96%) for shoot regrowth from encapsulated nodal segments (**Figure 2**). The length of re-growing shoots on MS medium supplemented with 2.2 μM BA and 0.5 μM NAA (M4) was increased over other media (**Table 1**). The use of MS medium supplemented with 2.2 μM BA resulted in production of the highest node (7.20) from encapsulated nodal segments (**Table 1**). Higher regrowth frequencies on BA supplemented MS medium accorded with results from encapsulated explants of *Morus* sp. [16], *Punica granatum* [4] and *Salix tetrasperma* Roxb., [17] from which shoot regeneration were reported to be at higher frequencies on MS sowing media containing BA but contrasted with the finding of Kumar *et al.* [18] and Hung and Trueman [14], from which shoot regrowth were reported to be higher frequencies on hormone-free MS medium. However, the number of shoots produced was higher (5.30 and 5.10) on MS medium supplemented with

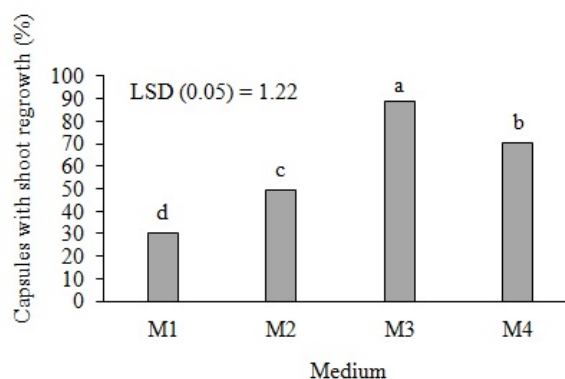


Figure 2. Percentages of encapsulated nodal segments of *A. scoparia* that developed shoots after 6 weeks of culture on different sowing media (M1: half-strength MS medium, M2: full-strength MS medium, M3: full-strength MS medium with 2.2 μ M BA and M4: full-strength MS medium with 2.2 μ M BA and 0.5 μ M NAA. Means with different letters are significantly different at $P \leq 0.05$ (ANOVA and LSD test, $n = 10$ replicates).

Table 1. Length, node number and shoot number of shoot clusters from *A. scoparia* encapsulated explants after 6 weeks' culture on one of four sowing media.

Medium	Mean shoot length (cm)	Nod number	Shoot number
M1	7.17 ^d	1.30 ^d	0.60 ^b
M2	10.68 ^c	2.10 ^c	1.10 ^b
M3	12.19 ^b	7.20 ^a	5.10 ^a
M4	14.74 ^a	6.00 ^b	5.30 ^a
LSD (0.05)	0.51	0.65	0.65

Means with different letters are significantly different at $P \leq 0.05$ (ANOVA and LSD test, $n = 10$ replicates). M1: half-strength MS medium, M2: full-strength MS medium, M3: full-strength MS medium with 2.2 μ M BA and M4: full-strength MS medium with 2.2 μ M BA and 0.5 μ M NAA.

2.2 μ M BA and 0.5 μ M NAA (M4) and MS medium with 2.2 μ M BA (M3), respectively than on the hormone-free media (M1 and M2) (**Table 1**). Our results are also in agreement with Shaheen and Shahzad [9], where they reported that the BA and NAA supplemented medium resulted in higher frequency of conversion from encapsulated nodal segments of white cedar (*Tecomella undulata*).

3.2. Experiment 2: Direct Conversion under Aseptic Conditions

Conversion frequencies from nodal segments at the studied IBA doses were ranged from 2.8% to 75.70% (**Table 2**). The highest conversion of encapsulated nodal segments into plantlets was recorded with 19.6 μ M IBA (**Table 2**). Therefore, the optimal IBA concentration for conversion of *A. scoparia* synthetic seeds was considered to be 19.6 μ M. The optimal IBA concentration (19.6 μ M) resulted in the highest mean numbers of nodes (5) from nodal segments (**Table 2**). Because the number of nodes on the plantlet indicates the frequency of *in vitro* proliferation [14], it was considered as an important factor in synthetic seed production of *A. scoparia*. Our results are in corroboration with the findings of

Shaheen and Shahzad [9], who reported that treatment with IBA enhanced root induction and helped in the development of roots which further supported our results. However, Swamy *et al.* [19] reported that although the addition of auxins was beneficial for root induction, half strength MS medium was enough to get sufficient rooting. Similar to our results Hung and Trueman [10] reported that the use of 19.6 μM IBA provided the highest conversion from encapsulated nodal segments of eucalypt plants. However, Chand and Singh [20] reported that IBA at 4.9 μM was the most suitable for conversion in *Dalbergia sissoo*.

3.3. Experiment 3: Short-Term Storage of Synthetic Seeds

Conversion of synthetic seeds of *A. scoparia* into plantlet significantly decreased with enhancing duration of storage at 4°C (**Figure 3(a)**). After one week, the frequency of conversion from encapsulated nodal segments dropped from 90.5%

Table 2. Percentages of synthetic seeds of *A. scoparia* that converted into plantlets on 1/2 MS medium containing different concentrations of IBA.

IBA concentration (mM)	Conversion (%)	Nod number
0.0	2.80 ^d	2.00 ^c
4.9	61.90 ^b	4.10 ^b
19.6	75.70 ^a	5.00 ^a
74.4	57.70 ^c	1.70 ^c
LSD (0.05)	1.16	0.66

Means with different letters are significantly different at $P \leq 0.05$ (ANOVA and LSD test, $n = 10$ replicates).

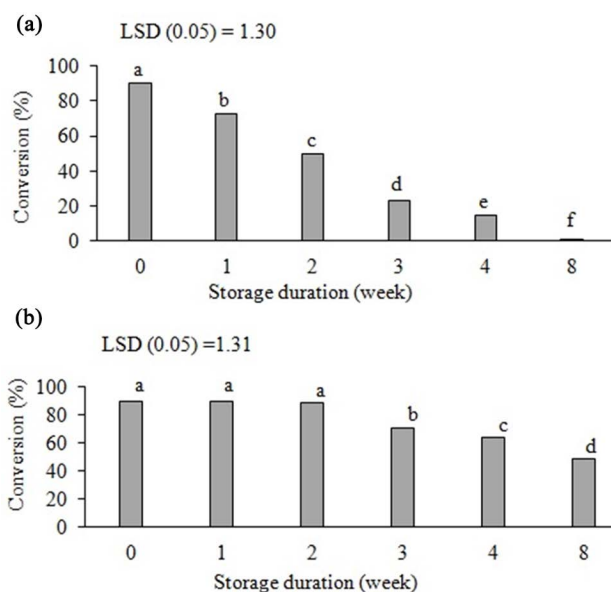


Figure 3. Percentages of synthetic seeds of *A. scoparia* that converted into plantlets on 1/2 MS medium after nodal segments were pre-treated with 0.4 μM IBA and the synthetic seeds then stored in darkness for different periods at 4°C (a) and 25°C (b). Means with different letters are significantly different at $P \leq 0.05$ (ANOVA and LSD test, $n = 10$ replicates).

to 72.6%, while these frequencies decreased to 14.3% after 4 weeks. Conversion capacity was almost totally lost (0.4%) after 8 weeks at 4°C. However, the frequency of conversion (89.6% - 88.6%) was maintained at 25°C for up to 2 weeks without significant change (**Figure 3(b)**). The decrease in conversion capacity was observed from week 4 onwards at 25°C. Although it decreased further after 8 weeks at 25°C, 48% of encapsulated nodal segments could still be converted into plantlets at this temperature (**Figure 3(b)**). The more positive impact of storage at 25°C compared to refrigeration at 4°C in our experiment were also in line with the results obtained in another study [10], further supporting these results. However, contrary to our results, cold temperature storage has been reported to be more effective than room temperature for encapsulated explants of *Camellia sinensis* [21], *Coelogyne breviscapa* [22] and *Olea europaea* [23]. The effect of storage temperature on conversion capacity appears to be highly dependent on plant type. The current study also confirmed that the encapsulation method of *A. scoparia* makes handling and the possibility to store the propagules for a reasonable period easier.

The storage at room temperature could be suggested for *A. scoparia* synthetic seeds without refrigerated containers and around 30 days period of storage without any loss of regrowth potential. These findings accorded with results from encapsulated explants of olive [24], from which conversion capacity was at higher frequencies after storage. After 8 weeks of storage at 4°C synthetic seeds lost their viability completely, while after 8 weeks at 25°C, 48% of the synthetic seeds were still alive. Oxygen deficiency in the calcium alginate bead may result in decreasing plant recovery from stored encapsulated explants [25]. Loss of synthetic seeds viability up to 50% after 8 weeks of room temperature storage (25°C) might be attributed to the cracks and dehydration of the beads [19]. In accordance to our findings, Naik and Chand [4] reported that the conversion frequency of encapsulated nodal segments of *Punica granatum* L. declined significantly following storage at low temperature which further supported these results in terms of *A. scoparia*.

3.4. Experiment 4: Direct Conversion under Non-Aseptic Conditions

Peat mass and perlite (2:1) (v/v) mixture (PS2) showed the most suitable substrate for direct conversion (**Figure 4**). Conversion frequency in this substrate was 61.1% from nodal segments (**Figure 4**). Peat mass and loam soil (2:1) (v/v) mixture (PS3) was the poorest substrate for direct transfer from synthetic seeds, with conversion frequency of 16.1% (**Figure 4**). Plantlet high, leaf number, shoot number and root number per plantlet were higher in PS2 substrate and, to a lesser extent, in PS1 substrate than in PS3 substrate (**Table 3**). Mortality of plantlet was also high in PS2 substrate, whereas plantlets in PS2 substrate were vigorous (**Figure 1(h)**) and acclimatized successfully in the greenhouse conditions. This study provided an efficient conversion for direct transfer of encapsulated

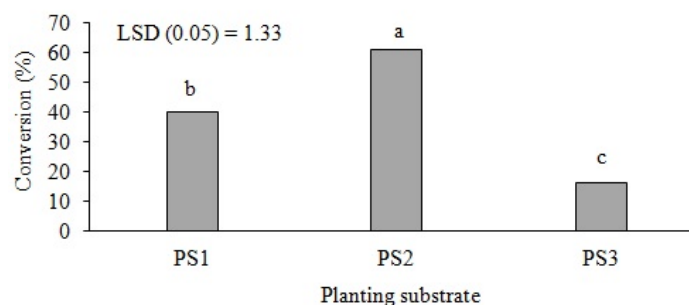


Figure 4. Percentages of converted synthetic seeds of *A. scoparia* that developed into plantlets on different non-aseptic substrates: peat mass and sand (2:1) (v/v) mixture (PS1), peat mass and perlite (2:1) (v/v) mixture (PS2) and peat mass and loam soil (2:1) (v/v) mixture (PS3). Means with different letters are significantly different at $P \leq 0.05$ (ANOVA and LSD test, $n = 10$ replicates).

Table 3. Height and numbers of shoots, leaves and roots of *Amygdalus scoparia* plantlets developed from converted synthetic seeds after transfer to one of three non-aseptic substrates for 4 weeks.

Medium	Plantlet height (mm)	Shoot number	Leaf number	Root number
PS1	13.20 ^b	2.20 ^a	3.00 ^b	1.70 ^b
PS2	17.20 ^a	2.50 ^a	4.30 ^a	2.50 ^a
PS3	9.60 ^c	1.40 ^b	1.80 ^c	1.40 ^b
LSD _(0.05)	0.78	0.52	0.65	0.59

PS1: peat mass and sand (2:1) (v/v) mixture, PS2: peat mass and perlite (2:1) (v/v) mixture and PS3: peat mass and loam soil (2:1) (v/v) mixture. Means with different letters are significantly different at $P \leq 0.05$ (ANOVA and LSD test, $n = 10$ replicates).

nodal segments (synthetic seeds) to non-sterile potting media. Similarly, Hung and Trueman [10] reported an *in vivo* conversion of synthetic seeds in non-sterile potting media. Practical use of *in vivo* conversion is hampered due to the high cost of sterilizing potting media [14].

But pre-converting the synthetic seeds of *A. scoparia* in moistened Petri dishes prior to transferring to non-sterile potting media, according to the method described by Hung and Trueman [14], circumvented the problem. High success of plantlet development in non-sterile soils could be attributed to reduced nutrient content of the synthetic seed matrix during pre-conversion and pre-acclimation during the pre-conversion period [14]. Our results proved that peat mass and perlite (2:1) (v/v) mixture (PS2) was more helpful in successful conversion of encapsulated nodal segments than the other substrates. Naik and Chand [4] found that vermin-compost was the most suitable potting substrate compared to garden soil which was consistent with our results regarding the weakness of loam soil for direct transfer from synthetic seeds. Our findings are also in accordance with Zamanidis *et al.* [25] and Shaheen and Sahzad [9] who reported that efficient growth depend on composition and quality of the potting substrate.

4. Conclusion

The results of the present study clearly indicated that the low temperature storage is a less suitable technique for *A. scoparia* than room temperature storage condition. The storage of encapsulated nodal segments of *A. scoparia* under room temperature makes it cost-effective (without using refrigerating machinery) and facilitates transport of this sterile and desirable elite genotype of *A. scoparia* to extension centers and laboratories of outlying places. Therefore, this offers the possibility of using synthetic seed technology for germplasm conservation of this genotype. Conversion of encapsulated nodal segments into plantlets in peat mass and perlite (2:1) (v/v) mixture showed that this method could be useful in developing a cost-effective propagation system for *A. scoparia*.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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