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Research article

Synthetic Seed Production and Germplasm Conservation of *Enicostema axillare* (Lam.) raynal ssp. *littoralis* (Blume) raynal Using Nodal and Root Explants

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Abstract

Keywords

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storage;
germination;
hardening

In the present study, synthetic seed production of the medicinally important plant *Enicostema axillare* through nodal and root explants were attempted. *Enicostema axillare* plantlets were germinated through the production of synthetic seeds using *in vitro* nodal and root explants. Synthetic seed prepared from 3% Na^+ and 100 mm calcium chloride was the optimum concentration for uniform and compact synseed formation. Alginate beads containing *in vitro* derived nodal explants were successfully entrapped with 3% sodium alginate prepared in half-MS, quarter-MS, full-strength MS, and double-distilled water and 100 mM of $CaCl_2$ solution. Synthetic seeds prepared from full strength MS medium exhibited the highest number of multiple shoots on benzyl amino purine (BAP) 2 mgl^{-1} and kinetin (KIN) 0.5 mgl^{-1} in combination with 2 mgl^{-1} GA_3. The capsulated nodal explants germinated and produced multiple shoots of 15.0±1.87 at a high rate (87%) in 2 mgl^{-1} BAP and KIN 0.5 mgl^{-1} in combinations with 2 mgl^{-1} GA_3 with a shoot length of 6.24±1.03 cm along with a root number of 28.4±5.50. Synthetic seeds stored at 18ºC were found to be favorable temperature for the preservation of synthetic seeds, and it increased seed germination frequency. The synseed nodal explants prepared using ¼ strength MS medium and stored at 18ºC were preserved for up to 80 days and more without losing germination ability (80%). The fully developed plantlets were hardened on vermiculite (100%) with a survival rate of 71%. This protocol could be used for the conservation of germplasm for long-term storage of *E. axillare*.

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1. Introduction

Enicostema axillare is an important medicinal plant belonging to the family Gentianaceae. The plant has been used to treat gastrointestinal problems, and diabetes, and its extracts possess purgatives and anti-venom properties [1, 2]. It has also used in the treatment of leucoderma [3], veterinary diseases [4], diabetes [5], inflammation [6], cancer [7], and diabetes mellitus [8]. In addition, the swertiamarin content in *E. littorale* (7.7%) was observed to be high compared to *Swertia chirata* (0.9%), which enhances the commercial value of this plant in the pharmaceutical industry [9]. Techniques like plant tissue culture, ionizing radiation, and application of magnetic fields were applied to plants that were difficult to reproduce by means of seeds. Recently, the use of magnetic fields and radiation such as gamma rays in breaking seed dormancy has piqued interest in the cultivation of plants such as *Lathyrus chrysanthus* [10]. In addition, synthetic seeds (synseeds) have been defined as artificially encapsulated plant parts such as somatic embryos, shoot buds or other tissues that can retain their totipotency to regenerate themselves even after storage for a longer period [11]. Shoot tips and nodal segments are often used for synthetic seed production for various medicinal plant species as they maintain a high level of genomic stability without any somaclonal differences [12].

Germplasm conservation is the most successful method used to conserve the genetic traits of endangered and commercially valuable species. Germplasm is a live information source for all the genes present in the respective plant. It can be conserved for long periods and regenerated whenever it is required in the future. Though *E. axillare* has high seed production, the percentage of germination was found to be too poor in the wild conditions [13]. Also, the demand for this type of plant has increased, especially in the pharmaceutical sector, where there is a drive for the isolation of swertiamarin and traditional medicines. Due to this reason, excess removal of these plants from the wild population has made them threatened. Micropropagation is a promising strategy that can be used to ensure continuous supply of plant material, and the conservation of sporadic and threatened medicinal herbs. The effect of matrix, plant growth regulator, and temperature on the conversion ability of synthetic seeds was determined using *in vitro* nodal of *E. axillare* in the present study. There are some reports on *in vitro* propagation of *E. axillare* from nodal and leaf explants [13]. However, the synthetic seed production and germplasm conservation of *E. axillare* have not been reported so far to the best of our knowledge. Therefore, the present study was well established for plantlet regeneration from the synthetic seeds and germplasm conservation of *E. axillare* produced from *in vitro* nodal and root explants. The protocol will fulfill the need for *E. axillare* plants and will sustain the plant's existence through germplasm conservation.

2. Materials and Methods

2.1 Explant source

Ex-situ derived nodal and root explants (2-3 cm) cut from 6th week-old grown nodes and roots were used as explants for synthetic seed production of explants, which were maintained using a method as termed described in former work of our team [13].

2.2 Encapsulation of explants and culture conditions

To entrap the synthetic seed, sodium alginate (from Sigma chemicals) was autoclaved at 121ºC for 20 min. Encapsulation was achieved through entrapping both nodal and root explants into the sodium alginate solution formulated in either distilled water or different concentrations of MS

medium (full-strength, half strength, and quarter strength) [14], devoid of plant hormones. Then the solutions were dropped into the different concentrations of calcium chloride solution (25, 50, 75, 100, and 200 mM). The droplets of 3% of sodium alginate enclosing the nodal explants were kept for 15-20 min in a $CaCl_2$ solution and entrapped nodal explants were washed more than three times with sterilized, double-deionized water (2-3 times) to eliminate drops of calcium chloride. For synthetic seed production, solutions of sodium alginate were formulated in the range of 3 (w/v), however, $CaCl_2$ solution was equipped in various concentrations (25, 50, 75, 100, and 200 mM) either in sterile deionized water [14] or in MS medium of various strengths (full, half, and quarter) devoid of plant growth hormones. Synthetic seeds were achieved by mixing nodal and root explant separately into sodium alginate solution and dipping them into calcium chloride solution. Entrapped nodal explants were recovered and washed several times with sterilized double deionized water.

For *in vitro* plant regeneration, stored and non-stored nodal explants were inoculated on MS medium, MS medium with the addition of only BAP, and MS medium in combination with KIN and GA_3, namely SY1 [MS medium with BAP (2 mgl^{-1}) and KIN (0.5 mgl^{-1})], SY2 [MS medium with BAP (2 mgl^{-1}) and KIN (0.5 mgl^{-1}) in combination with GA_3 (2 mgl^{-1})], and K5 [MS medium with BAP (2 mgl^{-1})]. For storage, nodal explants were transferred to sterile conical flasks and stored for 10, 15, 20, 30, 40, 50, and 60 days at 25ºC, 18ºC, and 4ºC. After each storage period, nodal explants were inoculated on MS medium containing BAP individually and in blend with the combination of KIN and GA_3 for conversion into plantlets. The pH was kept at 5.7 for the medium prior to autoclaving for 15 min at 121ºC and 1.1 kg cm^{-2} pressure. Cultures were kept at 25ºC with a 16/8 h (in light/dark) photoperiod at a photon flux of 50-70 µmol m^{-2} s^{-1} from white fluorescent tubes (Phillips, India).

2.3 Storage of synthetic seeds

Entrapped nodal explants were stored in various temperatures (18ºC, 24ºC and 4ºC) for different durations (0, 10, 15, 20, 30, 40, 50, and 60 days). After the storage period, nodal explants were directly inoculated with the various concentrations of growth regulators (BAP alone and in combination with KIN and GA_3) and placed under optimum conditions for the germination and regeneration of synthetic seed.

2.4 Germination frequency

The synthetic seeds were stored for various time intervals from 10-60 days at different temperatures (4ºC, 18ºC, and 24ºC). The frequency of seed germination was calculated using this formula given;

$$\text{Germination frequency} = \frac{\text{Total number of seed germinated}}{\text{Total number of seed stored}} \text{ x } 100$$

2.5 Acclimatization

Fully developed plantlets developed from synthetic seeds (nodal explants) were transferred to paper cups containing vermiculite alone moistened with sterile double distilled water. Plantlets were trapped in plastic bags to retain the level of moisture and kept at the temperature of 24-25ºC, with a photon flux density of 50 µmol m^{-2} s^{-1} being provided by fluorescent tubes.

2.6 Experimental design and data analysis

Plantlet regeneration from synthetic seeds was determined as the percentage of entrapped nodal explants showing both shoot and root out of the total number of nodal explants inoculated. For the above investigation, 5 replicates were used for all treatments, and all experiments were continual for 3 days. The mean standard error and one-way ANOVA were calculated using SPSS (version 17.0) software. The mean separations were carried out using Duncan's multiple range tests and significance was calculated at $P < 0.05$.

3. Results and Discussion

3.1. Encapsulation of nodal and root explant

Calcium alginate (50-200 mM) beads changed qualitatively with different concentrations of sodium chloride (1-4%). The condition of 3% sodium alginate and 100 mM calcium chloride was found to be the most suitable concentration for the formation of stable, transparent, and uniform beads (Table 1, Figure 1A). The synthetic seed droplets formed with 2% sodium alginate were delicate and irregular in form, while at higher concentrations of sodium alginate (4%), the beads were uniform but were tough enough to cause an extensive delay in shoot and root development. Therefore, 3% sodium alginate produced more uniformly compact synthetic seeds with a translucent complexion (Figure 1A and Table 1). Hence, it was selected for the encapsulation of the nodal and root explants in further experiments (Table 2, Figures 1B and 1C, respectively). Similar results were also observed in *Solanum tuberosum* [15], *Punica granatum* L. [16], and *Phyllanthus amarus* [17]. Entrapment of nodal explants and development of uniform beads were determined by the concentration of sodium alginate and calcium chloride, and gel complexion and bead hardiness depended on ion exchange between Na^+ and Ca^+ ions [18]. In the present study, synthetic seeds were prepared from various concentrations of MS medium, namely full, half, and quarter strength in 3% of sodium alginate solution. In the case of *Gentiana pneumonanthe* L. apical and axillary shoot buds, 3% sodium alginate in water (2.0 µM IAA) and MS medium, and 100 mM calcium chloride were used for synthetic seed formation. The endosperm is necessary for seedlings until their photosynthetic system develops and they can prepare their own food. To compensate for the role of endosperm in synthetic seeds, the addition of nutrition and carbon is essential. A source of carbon is necessary for the survival of the synseed during storage for a longer period. The addition of sucrose enhances the germination percentage of synthetic seed and also the conversion capacity [19, 20]. Nodal explants showed a great response towards germination and storage for more than 80 days. The germination of root explants on MS medium with BAP (2 mgl^{-1}) and KIN (0.5 mgl^{-1}) in combination with GA_3 (2 mgl^{-1}) from nodal explant exhibited hyperhydration (Figure 1D). However, root explants were found to be less responsive compared to nodal explants (Table 2). The duration of complex formation of synthetic seeds was an important factor for the production of synseeds (Table 1). Timing of 20-30 min was necessary for the formation of compact, transparent beads for synthetic seed production. Similar results were observed in most of medicinal plants such as the *Acacia* hybrid [21].

Table 1. Preparation of synthetic seed using various concentrations of sodium alginate and calcium chloride

Na-Alginate (%) + $CaCl_2$ (in mM)	Type of encapsulation (nature of synthetic seed)	Color of encapsulation	Encapsulation quality
1+50	No	---	---
1+100	No	---	---
1+150	Irregular	Translucent	+
1+200	Irregular	Translucent	+
2+50	Irregular	Translucent	+
2+100	Delicate and soft	Translucent	++
2+150	Delicate and soft	Translucent	++
2+200	Uniform and soft	Translucent	+++
3+50	Irregular	Translucent	++
3+100	Uniform and compact	Translucent	++++
3+150	Uniform and hard	Translucent	+++
3+200	Uniform and hard	White	+++
4+50	Irregular	Transparent	++
4+100	Uniform and compact	White	+++
4+150	Uniform and hard	White	+++
4+200	Uniform and hard	White	+++

'+' indicates positivity in the texture, complexion and encapsulation of explants; '-' indicates negativity in the formation of synthetic seeds

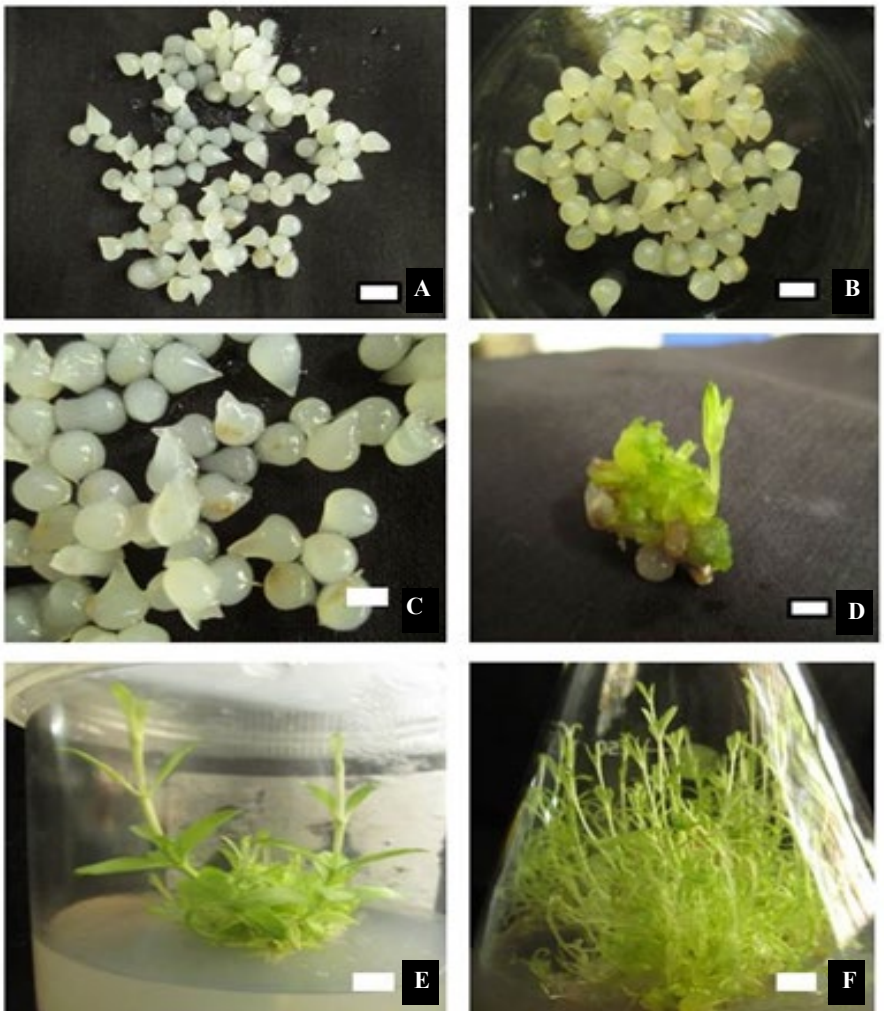

Figure 1. Prepararion and germination of synthetic seeds of *Enicostema axillare* prepared from full-strength MS medium: A- Synthetic seeds prepared from 3% of Na-Alginate (%) + in 100mM of $CaCl_2$; B and C- Synthetic seed with nodal and root explants, respectively; D- Germination of root explants on MS medium with BAP (2mgl-1) and KIN (0.5 mgl^{-1}) in combination with GA_3 (2 mgl^{-1}) from nodal explant exhibiting hyperhydration; E and F- Regenerated synthetic seeds from MS medium BAP (2 mgl^{-1} and KIN (0.5 mgl^{-1}) in combination with GA_3 (2mgl^{-1}) from nodal explants (Bar-1cm)

3.2. Regeneration of plantlets from nodal explants

The results of synthetic seeds prepared from various strengths of MS medium on germination and multiple shoot induction were tabulated in Table 2. Synthetic seeds prepared at full strength MS medium had the highest number of multiple shoots from nodal explants germinated on all the media tested (Figures 1E and 1F). Synthetic seeds from nodal explants exhibited shoot and root development after 7 days of culture on MS medium. Among the various strengths of MS medium tested for the development of nodal explants into plantlets, the best response for both shoot and root occurrence (Table 1, Figure 2E) from nodal explants was recorded in synthetic seeds prepared from full strength MS medium on SY1 medium (Table 2, Figure 2D). The germination and production of multiple shoots from synthetic seeds were found to be highest on MS medium containing BAP (2 mgl^{-1}) and KIN (0.5 mgl^{-1}) in combination with GA_3 (2 mgl^{-}1, SY2) (15.0±1.87 shoots per explant with 6.24±1.03 cm of shoot length along with root number of 28.4±5.50 (Table 2, Figures 1E and 1F), followed by SY1 medium which produced 7.2 shoots (MS medium with BAP (2 mgl^{-1}) and KIN (0.5 mgl^{-1}) (Figure 2D, Table 2). BAP at 2 mgl^{-1} was inferior to other media tested (Figures 2B and 2C) for germination of synthetic seeds. However, GA_3 was found to be inferior in root induction in *Physalis peruviana* L., whereas in the case of *Enicostema axillare*, GA_3 promotes both root and shoot induction (Table 2, Figure 1G). Also, the quality of the shoot was found to be superior in GA_3 containing media when compared to other media tested [21]. Synthetic seeds germinated by means of *in vitro* nodal stem segments give rise to axillary buds. Root explant regenerated shoots exhibit hyperhydricity (vitrification) and have a glossy appearance in all the media tested (Figure 1D). Hence, root explants were not suitable for synthetic seed production, and results were not tabulated. Other concentrations of MS medium, such as half and quarter-strength prepared synthetic seeds were interior to the full strength MS medium entrapped seeds (Table 2). Furthermore, no results were observed in the synthetic seeds germinated on MS medium till 15 days (Figure 2F). Moreover, the synthetic seed derived plants from MS medium were not healthy and fail to elongate further (Figures 2G and 2H). The formation of shoots and roots was observed in all the media tested, which adds an advantage to the present study (Figure 2E). Similar results were observed in *Physalis peruviana* L. where both shoots and roots were produced from synthetic seeds in shooting medium [22]. Fully developed plantlets were hardened in 100% vermiculite-containing paper cups (Figures 2I and 2J) with a 71% survival rate for more than 60 days, and the plantlets were gradually moved to greenhouse conditions.

Table 2. Germination of synthetic seeds of *E. axillare* using various strengths of MS medium on different culture media

Medium	Concentration	Number of shoots	Shoot length	Number of roots	Root length in cm
MS MEDIUM	SY1	15.0±1.87[a]	6.24±1.03[a]	28.4±5.50[a]	2.3±0.29[a]
	SY2	7.2±0.83[b]	1.88±0.72[b]	7.2±1.09[b]	1.8±0.23[b]
	K5	5.2±0.83[c]	0.54±0.15[c]	3.4±0.89[c]	1.0±0.13[c]
	Control	0.0±0.0[d]	0.0±0.0[d]	0.00[d]	0.00[d]
HALF MS	SY1	7.8±0.83[a]	2.4±0.52[a]	15.4±1.14[a]	1.72±0.31[a]
	SY2	3.6±1.14[b]	1.44±0.45[b]	4.0±0.70[b]	0.84±0.54[b]
	K5	3.8±1.30[b]	0.40±0.07[c]	0.4±2.20[c]	1.02±0.13[b]
	Control	0.00[c]	0.00[d]	0.00[d]	0.00[c]
QUATER MS	SY1	3.40±0.54[a]	1.92±0.45[a]	13.4±1.34[a]	1.76±0.59[a]
	SY2	2.80±0.44[a]	0.50±0.22[b]	6.60±2.07[b]	1.56±0.49[a]
	K5	1.80±0.83[b]	0.28±0.44[b]	3.20±0.44[c]	0.48±0.44[b]
	Control	0.00[c]	0.00[c]	0.00[d]	0.00[b]
WATER	SY1	2.2±0.44[a]	1.3±0.13[a]	0.00.0±0.0	0.0±0.0
	SY2	2.2±0.44[a]	0.46±0.05[b]	0.0±0.0	0.0±0.0
	K5	1.6±0.54[b]	0.26±0.054[c]	0.0±0.0	0.0±0.0
	Control	0.00±00[c]	0.00±00[d]	0.0±0.0	0.0±0.0

Note: In a column, means followed by a common letter are not significantly different at the 5% level by DMRT. SY1- MS medium with BAP (2 mgl^{-1}) and KIN (0.5 mgl^{-1}); SY2- MS medium with BAP (2 mgl^{-1}) and KIN (0.5 mgl^{-1}) in combination with GA_3 (2 mgl^{-1}); K5- MS medium with BAP (2 mgl^{-1}).

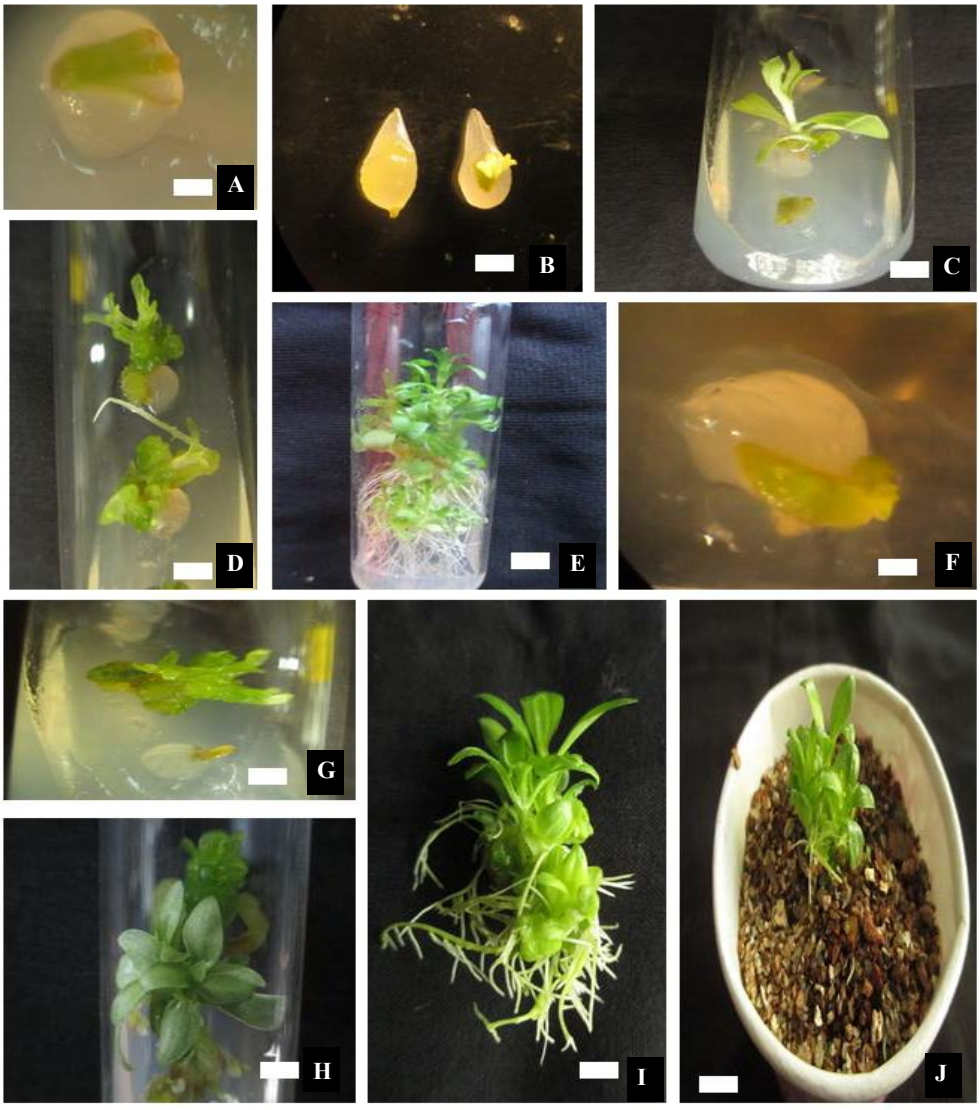

Figure 2. Multiple shoot and root induction from synthetic seeds of *Enicostema axillare* A- Nodal explant using 3% of Na-Alginate (%) + in 100mM of $CaCl_2$ in full strength MS medium; Bar-0.5cm; B and C- Germinated nodal explants on MS medium with BAP (2 mgl^{-1}); Bar-1cm; D- Synthetic seeds germinated on MS medium with BAP (2 mgl^{-1}) and KIN (2 mgl^{-1}); Bar-1cm; E- shoot and root induction on MS medium with BAP (2 mgl^{-1}) and KIN (0.5 mgl^{-1}); Bar-1cm; F and G- Germinated synseeds on MS medium devoid of growth regulators (control); F- Bar-1cm and Bar-0.5cm; H- Shoots developed from root explants; Bar-1cm; I and J- Developed plantlets from synthetic seeds and plantlets hardened on vermiculite, respectively; Bar-1cm.

3.3. Synthetic seed storage and germination

Synthetic seeds prepared from various strengths of MS medium were stored for up to 80 days. Quarter-strength MS medium prepared synthetic seeds was found to be the best for storage without losing viability for at least 80 days. Experiments were conducted to determine the frequency of synthetic seed germination under storage at different temperatures (25°C, 18°C and 4°C) for 10, 15, 20, 30, 40, 50, and 60 days (Table 3). The SY1 medium was used to determine the frequency of germination from synthetic seeds. Among the various temperatures tested, 18°C was found to be favorable for the storage of synthetic seeds of *E. axillare* in which about 80% of synthetic seeds were found to be viable for more than 100 days (Figure 2A and Table 3). Both 18°C and 25°C were found to be best for the storage of synthetic seeds. However, a low temperature (4°C) was not preferred for the storage. Temperature is one of the important factors to be considered in the storage of synthetic seeds. The influence of synthetic seed storage at low temperatures significantly reduces the viability of synthetic seeds and their potential for conversion to normal plants [23]. The storage of synthetic seeds of *E. axillare* with maintenance of temperature at 18°C is quite easy and affordable (Table 3). This may be due to the tropical nature of the plant, *E. axillare*. This allows a reduction in the cost of germplasm conservation of this medicinally important plant. Synthetic seed technology has been increasing applied to rare and endangered medicinal plants which are difficult to propagate from seeds and by vegetative means. Further, synthetic seed techniques were employed in germplasm conservation by long-term storage of threatened and extinct plant species [24]. In the present study, we were able to observe that the percentage of germination was influenced by the storage period.

Table 3. Germination frequency of *E. axillare* synthetic seeds stored on various temperatures prepared using 100 mM calcium chloride solution in various concentrations of MS medium

Medium	Storage Temperature (°C)	Frequency of germination (%)						
		days						
		10	**15**	**20**	**30**	**40**	**50**	**60**
Half MS	4	70	50	20	5	-	-	-
	18	90	70	55	45	40	35	30
	25	95	80	60	45	33	20	10
Quarter MS	4	80	55	24	15	-	-	-
	18	100	90	85	88	85	82	80
	25	100	90	80	74	50	20	-
Full strength	4	80	54	33	27	12	-	-
	18	96	87	45	30	22	10	-
	25	90	50	43	23	12	5	-
Sterile distilled water	4	50	42	20	5	-	-	-
	18	53	34	21	13	-	-	-
	25	45	34	23	10	-	-	-

*Frequency of germination is calculated by total number of seeds germinated/total number of synthetic seeds inoculated ×100

4. Conclusions

In the present study, a protocol was developed for the synthetic seed production of *E. axillare* and its germplasm conservation. From the present results, synthetic seed production and storage were developed to overcome the scarcity of this highly medicinal plant which is used in the fields of medicine and industry, allowing the isolation of its active compounds. The formation of both shoots and roots on the germinating medium offers additional benefits and reduces time consumption. The optimal temperature for storing synthetic seeds of *E. axillare* helps in the germplasm storage in tropical plant species. The protocol offers a cost-effective method to prepare synthetic seeds and to store them. It makes possible the conservation of this germplasm for future use.

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