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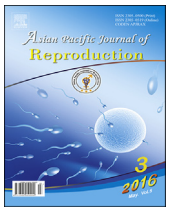

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Somatic embryogenesis and *in vitro* flowering in *Hybanthus enneaspermus* (L.) F. Muell. – A rare multipotent herb

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ABSTRACT

Objective: Present study reports the various factors affecting somatic embryogenesis and *in vitro* flowering in *Hybanthus enneaspermus* (L.) F. Muell.

Methods: The effect of the salts strength of Murashige and Skoog's (MS) medium, concentration of sucrose and plant growth regulators were analyzed for the induction of direct somatic embryogenesis using nodal segments as explants.

Results: High frequency of somatic embryogenesis was reported on full strength MS medium (with 3% sucrose) and additives supplemented with 0.5 mg/L 6-benzylaminopurine (BAP) and 0.25 mg/L indole-3-acetic acid (IAA). Maximum somatic embryos (207.0 ± 4.2) were germinated on 1/2 strength MS medium augmented with 0.5 mg/L BAP. Microscopic studies revealed the typical developmental patterns in somatic embryogenesis from globular to heart-shaped and followed by bipolar torpedo-shaped somatic embryos from nodal explants. The plantlets raised from the somatic embryos resulted in flowering on full strength MS medium augmented with 1.0 mg/L each of BAP and Kinetin (Kin) + 0.5 mg/L IAA at 50 μmol/(m^2·s) SFPD for 13 h/d photoperiod. About 92% plantlets were successfully acclimatized in the greenhouse. Field transferred plants exhibit normal flowering and fruit setting.

Conclusions: The study could be exploited for large scale propagation of true to type plants as conservation strategies of this rare and endemic medicinal plant.

1. Introduction

Hybanthus enneaspermus (*H. enneaspermus*) (L.) F. Muell. (family Violaceae) is an ancient Indian medicinal plant traditionally valued for its aphrodisiac and stimulant activity [1], popularly known as Rathanapurush (Sanskrit). This plant has been disappeared from the Western Ghats [2] and considered as rare and endemic species of Deccan Peninsular India [3,4]. Recently this plant has attracted much attention due to its multipotent bioactivities as antirheumatic, anti-infertility [5], antioxidant [6] and antidiabetic [7]. Coumarins, alkaloids, flavonoids, saponins, tannins, glycosides and triterpenoids are major bioactive compounds of *H. enneaspermus* [8].

Over harvesting for medicinal use, sporadic distribution, poor seed viability and germination are the major threats to this plant [9]. The conventional breeding methods are unable to improve the sustainability of *H. enneaspermus*. Plant tissue culture techniques represent a useful tool for mass propagation as well as an attractive alternative to conventional breeding [10]. *In vitro* culture of plants is increasingly used in conservation of biodiversity, especially for rare and endemic species, and considered as an important component of plant genetic resource management [11].

Somatic embryogenesis represents an important *in vitro* tool for large scale propagation of elite genotypes and it is one of the important prerequisites for genetic interventions. Somatic embryogenesis and plantlet regeneration have been achieved in *Leucojum vernum* [12], *Acacia senegal* [13], *Curcuma longa* [14], and Sugarcane [15] for successful propagation. Somatic embryogenesis has added advantages over organogenesis because it leads to the formation of bipolar structures possessing both root and shoot meristems [16]. Somatic embryo resembles the genotype of parent cells, assumed to be originating from single cell and will result in the formation of number of embryos per cell mass volume [17].

Somatic embryogenesis involves a process of development of embryogenic mass derived from the somatic explant *in vitro* and

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their subsequent development to regenerate plants directly. This potential mechanism aids to understand a change from vegetative to reproductive phase [18]. *In vitro* flowering can be obtained repeatedly in the shoots raised from somatic embryos. According to Simpson *et al.* the competent bud meristems are responsive to environmental or autonomous signals that eventually lead to flower formation [19]. Somatic embryogenesis and *in vitro* flowering has been achieved in Bamboo species [20], *Chamomilla recutita* [21] and *Boerhaavia diffusa* [22].

So far, there are no reports on direct somatic embryogenesis and *in vitro* flowering in *H. enneaspermus*. Earlier reports are available on indirect somatic embryogenesis through callus regeneration from leaf and stem explants [23–25]. The absence of direct somatic embryogenesis protocol is probably the reason that there is no report of stable genetic transformation and disease free stock production protocols in this plant. The present study introduced a rare species *H. enneaspermus* to direct somatic embryogenesis and flowering *in vitro* which can be used as strategy of conservation of this rare plant species.

2. Materials and methods

2.1. Plant material and surface sterilization

H. enneaspermus was selected from Coromandel Coast (Kanchipuram, Villupuram, Puducherry, Cuddalore, Nagapattinam and Karaikal districts) of India for the present study. Young emerging slender stems were used as the source of explants from a 2 months old mature plant. The nodal segments (approximately 3.0 cm in length) were harvested from the 2 months old field grown plant. The explants were sterilized with the systemic fungicide (0.1% Bavistin; BASF India Ltd., India) and then with 0.1% $HgCl_2$ (w/v) for 4–5 min. The sterilized explants were washed with autoclaved double distilled water for 5–6 times to remove the adhered traces of $HgCl_2$.

2.2. Medium and culture conditions

The sterilized explants were cultured on Murashige and Skoog (MS) medium [26] containing 3% sucrose, additives (50 mg/L ascorbic acid, 25 mg/L each of citric acid, L-arginine and adenine sulfate) [13] and different concentrations of indole-3-acetic acid (IAA), α-naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP) for the induction of somatic embryogenesis. The medium was solidified with 0.8% (w/v) agar, and the pH was brought to 5.8 with 0.1 mol/L NaOH or HCl and autoclaved at 121 °C for 15 min. All the chemicals used in the present study were procured from Himedia, Mumbai, India. The cultures were maintained in growth/culture room at (25 ± 2) °C under a photoperiod of 12 h/d with a light intensity of 30–40 μmol/(m^2·s). Spectral flux photon density (SFPD) was maintained using cool white fluorescent lamps (Philips Kolkata, India).

2.3. Microscopic studies of somatic embryogenesis

In order to understand the development of somatic embryogenesis, microscopic analysis of *in vitro* regenerated tissues was performed. The tissue samples were fixed in Formalin, acetic acid, ethyl alcohol, FAA (1:1:3) and the thin sections (12 μm) were stained with 1.0% (w/v) safranin and observed under the microscope (Labomed iVu 3100, USA) for histological studies.

2.4. Effects of growth hormones, strength of MS salts and sucrose on induction of somatic embryogenesis

Juvenile green nodal explants were cultured on MS medium incorporated either alone or in combination of BAP (0–2.0 mg/L) and IAA/NAA (0–1.0 mg/L). To evaluate the effect of strength of MS salts on the induction of somatic embryogenesis, explants were cultured on optimized concentration of growth hormones (0.5 mg/L BAP and 0.25 mg/L IAA + additives) supplemented in full, 1/2 and 1/4 strength of MS salts and 3.0%, 1.50% and 0.75% sucrose. The explants were inoculated horizontally on growth medium to study the induction of somatic embryos.

2.5. Germination of somatic embryos and shoot elongation

In order to germinate the somatic embryos, the meristemoid portion with mother explants were excised and cultured on different strength (full, 1/2 and 1/4) of MS medium supplemented with different concentrations of BAP ranging from 0.25, 0.50, 0.75 and 1.0 mg/L. The embryogenic callus was transferred to fresh medium for the maturation of embryos *in vitro*. Initially cultures were maintained in dark for a week. After induction of embryogenic potential by the explants on MS medium, the proembryo masses have been transferred to auxin free medium for elongation of adventitious shoots.

2.6. In vitro flowering

The fully elongated shoots (3–5 cm in length) of 30 d old shoot clumps from proliferated cultures were used for *in vitro* flowering. These were cultured on MS medium supplemented with different concentration of BAP and Kin along with IAA. To evaluate the optimum *in vitro* environment of light and temperature, these cultures were maintained at (25 ± 2) °C to (28 ± 2) °C under a photoperiod of 12–15 h/d with the light intensity of 50–70 μmol/(m^2·s) SFPD.

2.7. Hardening and field transfer

Fully developed plantlets were carefully separated from culture vessels and the traces of medium removed. The rooted plantlets were transferred to the paper cups containing soilrite® (a combination of perlite with peat moss and exfoliated vermiculite procured from KelPerlite, Bangalore, India), moistened with 1/4 MS salts and kept in the greenhouse. After 5 weeks the plantlets were shifted to nursery bags containing garden soil, soilrite®, manure and vermi compost (1:1:1:1) and finally transplanted to the natural fields.

2.8. Experimental design, data collection and statistical analysis

The experiments conducted with 20 explants for each treatment and each experiment was repeated thrice. The frequency of embryogenesis was calculated as the percentage of cultures showing somatic embryos. The results were expressed as mean ± SD of triplicates. The data were statistically analyzed using SPSS ver. 16 (SPSS Inc., Chicago, USA) and the

significance of differences among mean values was carried out using Duncan's multiple range test (DMRT) at $P < 0.05$.

3. Results

3.1. Induction of somatic embryogenesis

The nodal shoot segments were inoculated on the MS medium with plant growth regulators to induce somatic embryos from the surface of the explants. The resulted bipolar structures were closely resembles the zygotic embryo morphologically (Figure 1A). The maximum development of meristemoids (92%) was observed when the explants were cultured on full strength MS medium supplemented with 3.0% sucrose, 0.50 mg/L BAP and 0.25 mg/L IAA for 4 weeks (Table 1). The frequency and intensity of somatic embryogenesis were considerably high when the explants cultured on combination of BAP and IAA. BAP alone with the 1/2 and 1/4 strength MS medium fortified 1.50% and 0.75% sucrose did not support the formation of somatic embryoids (Table 2). The transition of somatic embryos from globular, heart-shaped and bipolar (torpedo) embryo were the critical steps in somatic embryogenesis (Figure 1B–D).

Table 1
Effect of BAP and IAA in induction of somatic embryogenesis.

Growth regulators (mg/L)		Frequency of somatic embryogenesis (%)
IAA	BAP	
0.00	0.25	0.0 ± 0.0[a]
0.00	0.50	2.7 ± 0.0[a]
0.00	0.75	6.9 ± 0.1[a]
0.00	1.00	2.3 ± 0.0[a]
0.25	0.25	67.1 ± 1.0[h]
0.25	0.50	92.4 ± 1.8[j]
0.25	0.75	80.0 ± 0.7[i]
0.25	1.00	61.3 ± 0.3[g]
0.50	0.25	42.6 ± 1.5[c]
0.50	0.50	55.5 ± 1.2[f]
0.50	0.75	49.0 ± 1.0[e]
0.50	1.00	46.3 ± 0.9[d]
0.75	0.25	33.2 ± 0.4[b]
0.75	0.50	37.9 ± 1.4[b]
0.75	0.75	35.1 ± 0.6[b]
0.75	1.00	30.4 ± 1.3[b]

Medium: Full strength MS salts + 3% sucrose + additives. Mean values in each column followed by different letters are significantly different according to DMRT at $P < 0.05$.

3.2. Microscopic analysis of somatic embryos

Development of globular somatic embryos was observed after 10 d of inoculation. The globular embryos proceed further in the formation of heart, torpedo and cotyledonary stage in another 30 d (Figure 2A–C). Asynchronous development of somatic embryos with different stages in the development of embryos was observed on the same explant. Initiation of new meristemoids was observed throughout the culture period (meristemoid, primordium, bud and shoots) as developmental stages. Microscopic studies of the intact embryogenic cell masses observed similar in appearance, size but different in shapes. It has been reported that the somatic embryos were originated from single epidermal or subepidermal cells (Figure 3A–H).

3.3. Germination of somatic embryos and elongation of shoots

Low concentration of cytokinin (BAP) with 1/2 strength MS medium was used for germination of somatic embryos into

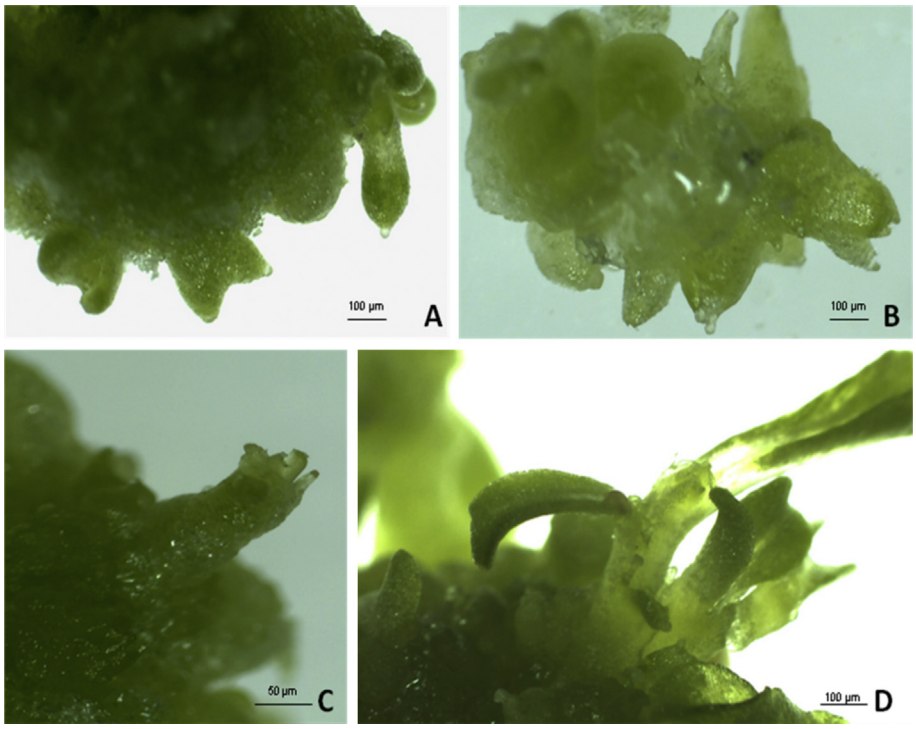

Figure 1. Different stages in the development of somatic embryos.
(1A and 1B) Globular and bipolar embryos from the surface of explants. (1C and 1D) Advanced stages in somatic embryogenesis.

Table 2
Effect of strength of MS salts and concentration of sucrose on induction of somatic embryogenesis.

Strength of MS salts	Concentration of sucrose (g/L)	Frequency of somatic embryogenesis (%)
Full strength	3.00	92.0 ± 2.3[d]
Full strength	1.50	62.9 ± 0.7[c]
Full strength	0.75	44.1 ± 1.3[b]
1/2 strength	3.00	20.5 ± 0.9[a]
1/2 strength	1.50	16.7 ± 0.2[a]
1/2 strength	0.75	9.2 ± 1.9[a]
1/4 strength	3.00	12.6 ± 2.8[a]
1/4 strength	1.50	10.8 ± 1.0[a]
1/4 strength	0.75	7.3 ± 0.4[a]

Medium: MS salts at various strength + sucrose at various concentration + 0.5 mg/L BAP and 0.25 mg/L IAA. Mean value in each column followed by different letters are significantly different according to DMRT at $P < 0.05$.

plantlets. Somatic embryos were converted into well developed plantlets (shoots and roots) within 4 weeks when the meristemoids transferred onto 1/2 strength MS medium containing 1.50% sucrose and additives supplemented with BAP 0.50 mg/L (Figure 4A and C). Full and 1/4 strength MS medium were reported comparatively less effective in plantlet regeneration from the somatic embryos. The regular subculture of these cultures on fresh medium with the same medium combinations was able to yielded 207 plantlets per culture vessel in this study (Figure 4B and Table 3).

3.4. In vitro flowering in H. enneaspermus

In vitro induction of flower buds was achieved by the subculture of elongated shoots into different culture environment and growth regulators. Among the plant growth regulators examined, full strength MS medium fortified with additives and 1.0 mg/L each of BAP and Kin + 0.5 mg/L IAA was reported suitable for flower bud induction from the shoots (Table 4). The present study reveals that the lengthy shoots (4–5 cm long) only responded for photoperiod for *in vitro* flowering.

Among the different types of medium considered for flower bud induction, full strength MS medium was responded positively. There was no flower bud observed on 1/2 and 1/4 strength MS medium with the same hormone concentrations even after 8 weeks of incubation on various SFPD. The maximum number of flower buds (2.8) formed at 50 µmol/(m^2·s) SFPD for 13 h/d photoperiod (Figure 4D).

3.5. Hardening and field transfer

The well rooted plantlets were separated from culture vessels and transferred to the soilrite® containing cups, moistened with aqueous 1/4 MS salts solution and maintained in the greenhouse for hardening and then to the nursery poly-bags containing garden soil, soilrite®, manure and vermi compost in 1:1:1:1 ratio after 5 weeks (Figure 4E). The plantlets were hardened in the greenhouse for about 2 months. Normal flowering and fruiting has been observed while hardening in the greenhouse (Figure 4F). About 92% of the plants were survived in the greenhouse and the hardened plants were finally shifted to the natural field.

4. Discussion

Somatic embryos were induced by the series of morphological and biochemical changes in the somatic tissues under specific culture conditions. Direct somatic embryogenesis from the explants is subjected to induce embryogenic competence by culture conditions and plant growth regulators in the medium. The maximum development of meristemoids was observed when explants cultured on BAP and IAA in this study and the bipolar structures formed were morphologically similar to the zygotic embryos. The combined effect of BAP and IAA in direct somatic embryogenesis was also reported in *Nicotiana* species [27] and *Solanum lycopersicum* [28]. About 60% meristemoids were observed with 0.50 mg/L BAP and 0.25 mg/L NAA. These findings are in contrast with the results of Ramaswamy *et al.* [29] in *Solanum surattense* and Kintzios and Michaelakis [21] in *Chamomilla recutita*, where NAA with BAP played significant role in formation of somatic embryos.

BAP alone in the 1/2 and 1/4 strength MS medium with 1.50% and 0.75% sucrose did not support the formation of somatic embryoids in present study. Auxins, especially 2,4-D (2,4-dichlorophenoxy acetic acid) alone or in combination with other auxins play a key role in the induction of somatic embryogenesis. Even combination of auxin and cytokinin can produce sufficient number of somatic embryos [30]. Altered response to different combination of growth hormones may be due to differences in genetic make-up among different plant species [31]. Juvenile explants on full strength MS medium supplemented with 0.50 mg/L BAP and 0.25 mg/L IAA induced the expression of cellular totipotency in *H. enneaspermus* plant cells.

Normal development of somatic embryos required a fine temporal and spatial regulation of cell division, elongation and differentiation [32]. The growth medium and culture conditions promoted the plant cell to undergo a series of complex

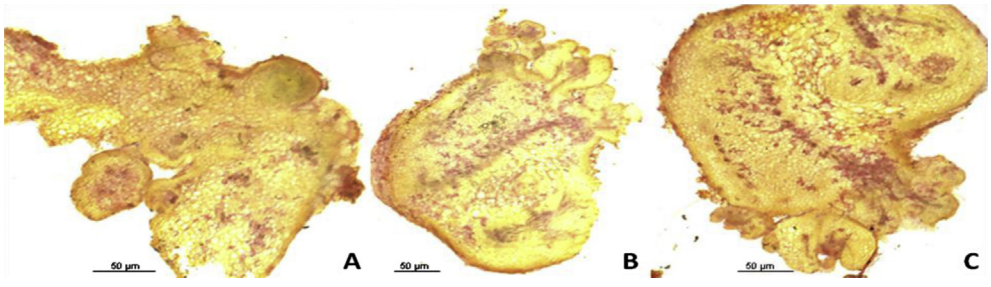

Figure 2. Microscopic study of the somatic embryogenesis from the explants.
(2A and 2B) Globular and torpedo-shaped embryos. (2C) Germination of embryos and formation of shoot primordia.

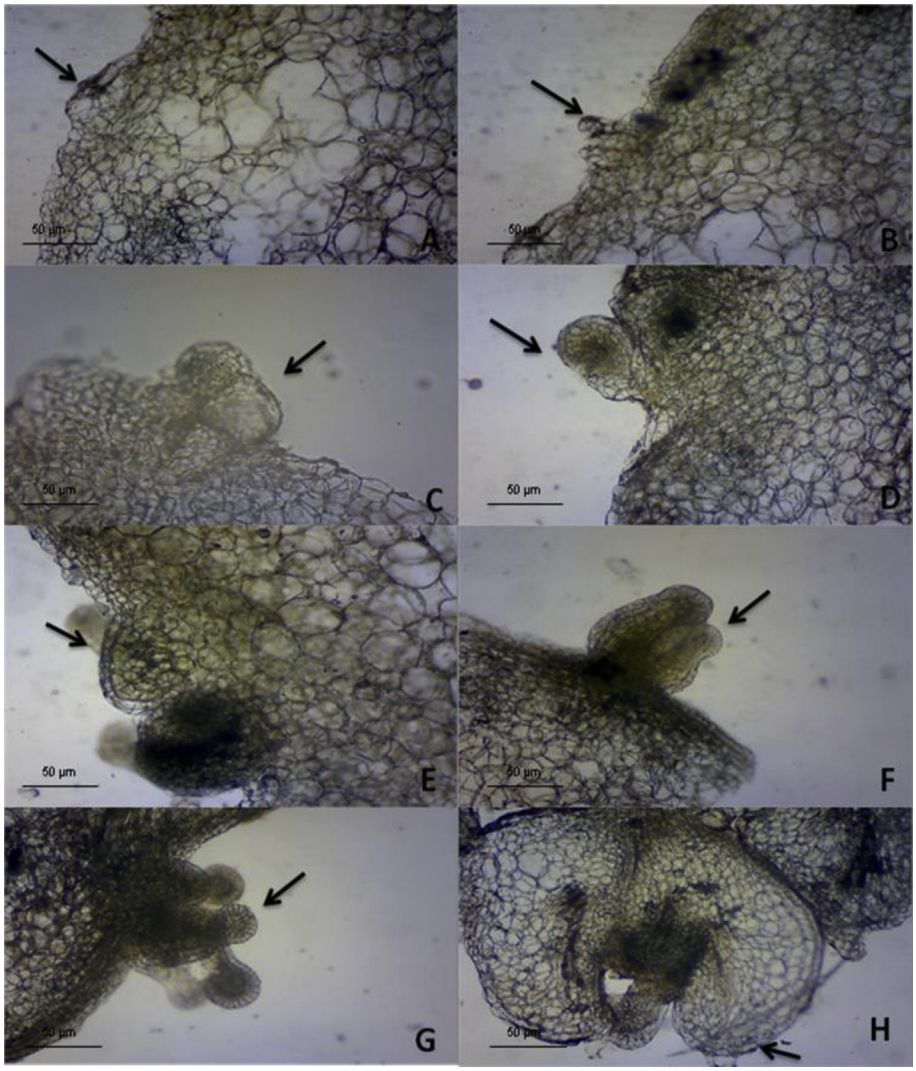

Figure 3. Developmental stages in the formation of somatic embryos.
(3A and 3B) Induction of meristemoid on the surface of the explant. (3C and 3D) Development of meristemoid into a tiny globular somatic embryo. (3E) Differentiation into embryoid. (3F) Explant surface showing masses of somatic embryos. (3G and 3H) Differentiation into a shoot primordium.

metabolic and morphological coordinated steps to produce a complete and normal sporophyte [30,33].

The direct somatic embryogenesis was further confirmed through microscopic observations. It was reported from the embryogenic cell masses that the somatic embryos were originated from single epidermal or subepidermal cells. Maturation of somatic embryos is an essential phase between embryo development and germination. The accumulated storage proteins in embryos assist to develop into normal plants [34,35]. The full strength MS salts medium played a significant role in maturation of somatic embryos due to the synthesis of certain metabolites and storage proteins.

Low concentration of BAP in 1/2 strength MS medium was reported suitable for germination of somatic embryos. Since rooting was also achieved on the same medium combination from bipolar somatic embryos, further rooting experimentation was exempted. The results revealed that an optimum level of BAP concentration was needed for germination of somatic embryos and multiplication of shoots *in vitro*. Use of full and 1/4 strength MS medium resulted in less number of shoots with compact mass of roots caused difficulty in separation of the plantlets.

The role of plant growth regulators, carbon, nitrogen source and minerals in development of floral organs has been discussed widely in number of plants [36–38]. *In vitro* flower buds formation was achieved by the subculture of elongated shoots into full strength MS medium at 50 µmol/(m^2·s) SFPD for 13 h/d photoperiod. *In vitro* flowering through somatic embryogenesis has been achieved in *Brassica nigra* [18] and *Dendrocalamus hamiltonii* [39].

The selection of explants from the mother plant determined the ability of the tissue to regenerate flower buds directly via somatic embryogenesis. The regeneration of floral organs probably achieved through transport of the flowering stimuli and due to the consecutive subculturing. *In vitro* flowering of the plantlets regenerated via somatic embryogenesis from nodal explants of *H. enneaspermus* was demonstrated for the first time. It could provide better understanding of nature of various factors influence the *in vitro* flowering in this plant. The plantlets were hardened in the greenhouse and transferred to the natural field conditions after 6 weeks. Plantlets regenerated through direct somatic embryogenesis and direct shoots induction have been reported to be genetically uniform [40–42] so that the genetic stability of *in vitro* propagated plants could be true to type.

Figure 4. Different stages of somatic embryogenesis and hardening of the plantlets.
(4A) Germination of somatic embryos. (4B) Multiplication of shoots. (4C) Rooted shoots. (4D) *In vitro* flower bud formation. (4E) Hardening of the plantlets in the greenhouse. (4F) Flowering and fruiting in the *in vitro* raised plantlets.

Table 3
Effect of BAP on germination of somatic embryos.

BAP + Kin conc. (mg/L)	Number of flower buds
0.00	0.0 ± 0.0[a]
0.25	0.0 ± 0.0[a]
0.50	1.8 ± 0.7[b]
0.75	2.4 ± 0.5[c]
1.00	2.8 ± 0.0[d]
2.00	1.6 ± 0.2[b]

Medium: 1/2 strength MS salts + 1.5% sucrose + additives. Mean values in column followed by different letters are significantly different according to DMRT at $P < 0.05$.

Table 4
Effect of cytokinins in flower bud induction on MS medium containing 0.5 mg/L IAA.

BAP conc. (mg/L)	No. of shoots per culture vessel
0.00	0 ± 0.0[a]
0.25	129 ± 0.6[c]
0.50	207 ± 4.2[e]
0.75	164 ± 2.7[d]
1.00	105 ± 2.0[b]

Medium: MS full strength + additives. Mean value in column followed by different letters are significantly different according to DMRT at $P < 0.05$.

In conclusion, the development of somatic embryos from sporophytic cells of *H. enneaspermus* has offered a great potential for the production of plantlets and its biotechnological manipulation, this could be exploited for the mass propagation of *H. enneaspermus*. The somatic embryos were germinated efficiently and *in vitro* flowering was observed. Direct somatic embryogenesis and *in vitro* flowering in *H. enneaspermus* were reported without intervention of callus phase. *In vitro* flowering offers better understanding of culture environments required by the plant for transition from vegetative to reproductive phase. The study could be used for mass scale production of this multipotent plant species.

Conflict of interest statement

The authors disclose that they have no conflict of interest in this publication.

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