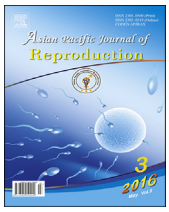
Contents lists available at ScienceDirect

Asian Pacific Journal of Reproduction

journal homepage: www.apjr.net



Original research http://dx.doi.org/10.1016/j.apjr.2016.04.005

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

Mahipal S. Shekhawat*, M. Manokari

Biotechnology Laboratory, Department of Plant Science, M.G.G.A.C., Mahe, Pondicherry, 673311, India

ARTICLE INFO

Article history:
Received 11 Sep 2015
Received in revised form 19 Dec 2015
Accepted 6 Jan 2016
Available online 19 Apr 2016

Keywords:
Hybanthus enneaspermus
Somatic embryogenesis
In vitro flowering
Conservation

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

*Corresponding author: Mahipal S. Shekhawat, Biotechnology Laboratory, Department of Plant Science, M.G.G.A.C., Mahe, Pondicherry, 673311, India.
Tel: +91 8943157187
E-mail: smahipal3@gmail.com
Peer review under responsibility of Hainan Medical College.

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