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Full Length Research Paper

High frequency induction of somatic embryos and plantlet regeneration from nodal explants of *Hygrophila spinosa* T. Anders

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An efficient protocol is described for the rapid *in vitro* plant regeneration of a medicinally important plant, *Hygrophila spinosa* through direct somatic embryogenesis from nodal explants excised from 4 week old aseptic seedlings. Somatic embryos differentiated directly from nodal explants on Murashige and Skoog (MS) medium supplemented with 1.0 µM 6- benzyladenine (BA) after 1 week of culture. The highest number (110.40 ± 3.55) of somatic embryos were recorded on MS medium supplemented with 1.0 µM BA and 0.5 µM NAA, growth regulator free half strength MS medium was found suitable for maturation and conversion of somatic embryos into complete plantlets. The well developed *in vitro* regenerated plantlets were successfully established in pots containing garden soil and grown in a greenhouse with 85% survival rate. The regenerants present an appearance similar to the seedlings obtained from normal seeds. The described method can be successfully employed for large scale multiplication and long term *in vitro* conservation of *H. spinosa.*

Key words: Acanthaceae, *Hygrophila spinosa*, somatic embryogenesis, medicinal plant, conservation, micropropagation.

INTRODUCTION

Hygrophila spinosa T. Anders (Acanthaceae), commonly known as Talimakhana, is a robust, erect, annual herb indigenous to India. The roots, leaves and seeds have been used in Indian system of medicine as diuretic and are employed to cure jaundice, dropsy, rheumatism and diseases of urino-genital tract. The fruits are used for curing menorrhagea (Anonymous, 2002). The conventional propagation of the plant is beset with problems of poor seed viability and low germination. Use of seed for medicinal purposes further curbs propagation via seed.

Plant tissue culture is a well known biotechnological tool for the rapid propagation of medicinal plants for the purpose of commercialization (Kitto, 1997), conservation (Anis et al., 2007) and cryopreservation (Decruse et al., 1999). Somatic embryogenesis and organogenesis have been the common pathways for the clonal propagation of superior medicinal plant species (Gary and Brent, 1986).

Somatic embryogenesis is an important application of plant biotechnology and offers an alternative and efficient system for plant regeneration. The technique has contributed a great deal of information for the genetic, morphological and physiological changes which take place during zygotic embryo formation *in vivo*. Somatic embryos are also important for management and conservation of *in vitro* produced materials for which they give an appreciably higher rate of multiplication compared to any other clonal propagation system (Malabadi and Nataraja, 2004). Moreover somatic embryos have been recognised as an attractive tool for synthetic seed production and mass propagation of elite genotypes (Redenbaugh et al., 1991, Verleysen et al., 2005). Somatic embryogenesis holds great promise not only for en masse propagation, but also for the improvement of the species through genetic engineering.

In this paper, we describe the first protocol for rapid *in vitro* plant regeneration of a medicinally important plant, *H. spinosa* through direct somatic embryogenesis from nodal explants followed by successful outdoor establishment of regenerated plants.

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Table 1. Effect of plant growth regulators on somatic embryogenesis from nodal explants of *H. spinosa*.

Growth regulators (µM)		Response (%)	Mean number of somatic embryos per explant
2,4-D	BA		
0.0		-	-
1.0		-	-
5.0		-	-
10.0		-	-
	0.0	-	-
	1.0	100	46.80 ± 1.280^{a}
	5.0	100	36.80 ± 1.067^{b}
	10.0	90	31.40 ± 1.208^{c}

Data taken after 6 wk. Values represent the mean ± SE from 10 replicates.
Mean followed by the same letter are not significantly different (P = 0.05) using DMRT.

MATERIALS AND METHODS

Establishment of aseptic seedlings

The seeds procured from A. K. Tibbiya college, AMU, Aligarh were washed thoroughly under running tap water for 30 min, treated with 5% (v/v) Teepol solution (a liquid detergent) for 15 min followed by washing in tap water. Because of high mucilaginous nature, the seeds were rinsed through cheese cloth and transferred to the laminar air flow hood. The seeds were dipped in 70% alcohol for 1 min and surface sterilized for 5 min in 0.1% (w/v) mercuric chloride solution and finally rinsed 5 times with sterile distilled water. The sterilized seeds were inoculated into MS basal medium supplemented with and without 0.5 µM GA_3 for germination. The nodal segments excised from 4 week old aseptic seedlings were used as explants.

Culture media and culture conditions

MS medium containing 3% (w/v) sucrose and 0.8% (w/v) agar was used in all the experiments. The pH of the medium was adjusted to 5.8 using 1N NaOH or 1N HCl prior to autoclaving at 121°C at a pressure of 1.06 kg/cm^2 for 15 min. All the cultures were incubated at 25 ± 2°C under 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 50 µM m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes with 60 - 65% relative humidity.

Induction of embryogenic tissues and multiplication

The nodal segments obtained from aseptic seedlings were cultured vertically on sterile MS basal medium (Murashige and Skoog, 1962) and on a medium supplemented with N^6- benzyladenine (BA) and 2,4- dichlorophenoxyacetic acid (2,4-D) at different concentrations (1.0, 5.0 and 10.0 µM) singly. Each culture tube was inoculated with 1 explant. For multiplication and maturation, the cluster of embryogenic tissues together with parental explant was transferred to MS medium supplemented with BA (1.0, 5.0 and 10.0 µM) in combination with either NAA or IBA (0.1, 0.2 and 0.5 µM).

Germination of somatic embryos

After 4 week of maturation, somatic embryos were transferred to the full, half and one- fourth strength MS medium devoid of plant growth regulators for germination. Subculturing was carried out at regular intervals.

Transplantation

Morphologically well developed embryogenic plantlets were washed with tap water and transferred to plastic pots containing sterile soil-rite and kept under diffuse light (16/8 h photoperiod) conditions. Potted plantlets were covered with transparent polythene bags to ensure high humidity. The bags were opened after 2 week in order to acclimatize plants to field conditions. After 4 week the plantlets were transferred to garden soil and maintained in glasshouse under normal day length conditions.

Statistical analysis

All the experiments were repeated thrice with 10 replicates for each treatment. The effect of different treatments was quantified and the data was analyzed using one way analysis of variance (ANOVA) and means were compared using the Duncan's multiple range test at 5% significance level.

RESULTS AND DISCUSSION

Induction of embryogenic tissues was achieved when nodal explants excised from 4 week old aseptic seedlings were cultured on MS medium supplemented with BA singly at different concentrations (Table 1). The explants grown in the presence of 2,4-D produced whitish calli which did not show any sign of differentiation (Table 1). No morphogenetic response was observed when the explants were cultured on MS medium devoid of plant growth regulators.

Initially, the explant swelled and acquired a loose whitish cluster of cells on nodal region without callus intervention. The formations of multiple nodules (small globular structures) were observed on MS medium supplemented with BA (1.0, 5.0 and 10.0 µM) after 1 week of

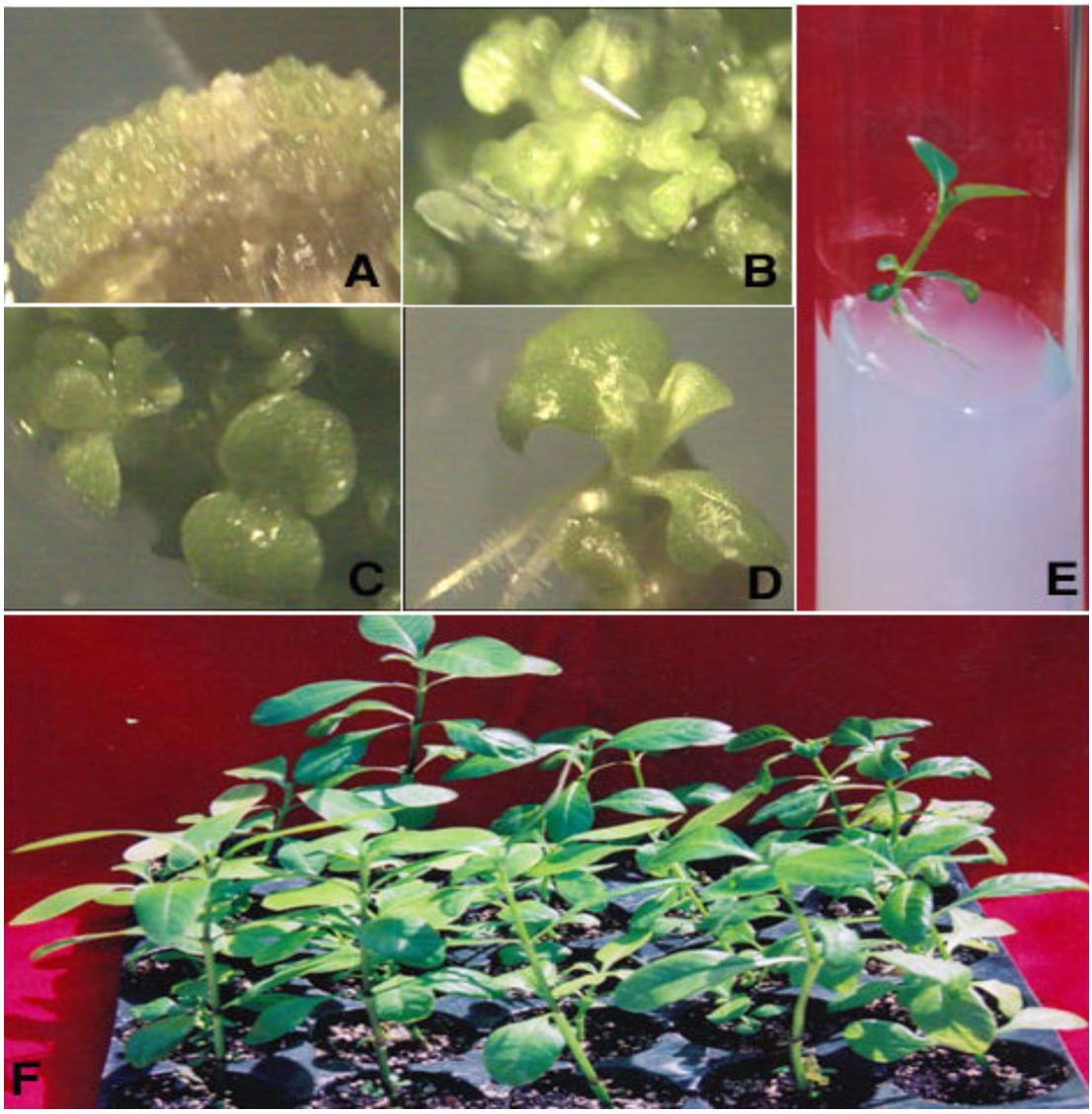

Figure 1A – F. (A) Globular somatic embryos induced directly on the surface of nodal segment cultured on MS + BA (1 µM). (B) Various developmental stages of somatic embryos and their multiplication on MS + BA (1µM) + NAA (0.5 µM). (C) Mature cotyledonary stage of somatic embryos on MS + BA (1 µM) + NAA (0.5 µM). (D) Germination of somatic embryo on half strength MS medium devoid of growth regulators. (E) Well developed plantlet from somatic embryo on half strength MS medium. (F) Acclimatized plants (2 month old).

incubation (Figure 1A). The development of embryogenic tissues was asynchronous, as evidenced by the presence of somatic embryos in the characteristic globular, heart shaped or early cotyledonary phase in the same explant (Figure 1B). The asynchrony of embryogenic cultures is a frequent process (Ibaraki et al., 2000) and has been reported in coffee (Etienne-Barry et al., 1999). The cluster of embryogenic tissues with parental explant were transferred to the MS medium supplemented with BA (1.0, 5.0 and 10.0 µM) in combination with either NAA or IBA (0.1, 0.2 and 0.5 µM) which allowed a faster differentiation and maturation of somatic embryos. Of the different PGRs used, BA (1 µM) in the presence of NAA or IBA at 0.5 µM concentration was found to be most effective for better differentiation of somatic embryos. The maximum number of somatic embryos (110.40 ± 3.55) were obtained on MS medium containing a combination of 1 µM BA and 0.5 µM NAA after 4 week of incubation and showed all developmental stages (globular, heart, torpedo and cotyledonary) similar to that of zygotic embryogenesis (Table 2). Development of the embryos was asynchronous and reasonably good % (60%) of embryos showed development to the cotyledonary stage on same medium (Figure 1C). On subculturing the large number of somatic embryos began to germinate from the loose cluster of embryogenic tissues after 15 days. However,

Table 2. Number of somatic embryos multiplied and germinated after 6 wk in cultures of nodal explants of *Hygrophila spinosa* on MS medium with different growth regulators.

Growth regulators (µM)			Mean number of somatic embryos	No. of somatic embryos germinated
BA	NAA	IBA		
1.0	0.1		58.80 ± 1.77^{ef}	35.40 ± 2.24^{e}
5.0	0.1		59.20 ± 2.55^{ef}	35.00 ± 2.07^{e}
10.0	0.1		50.20 ± 1.77^{gh}	25.20 ± 1.77^{f}
1.0	0.2		72.20 ± 2.22^{d}	38.00 ± 2.81^{e}
5.0	0.2		60.20 ± 1.77^{ef}	35.60 ± 2.06^{e}
10.0	0.2		58.40 ± 2.65^{ef}	28.60 ± 1.20^{f}
1.0	0.5		110.40 ± 3.55^{a}	85.40 ± 1.91^{a}
5.0	0.5		96.60 ± 2.65^{b}	74.40 ± 1.96^{b}
10.0	0.5		79.20 ± 3.12^{c}	75.40 ± 1.63^{d}
1.0		0.1	53.40 ± 1.43^{fg}	23.80 ± 1.52^{f}
5.0		0.1	37.00 ± 1.81^{i}	24.80 ± 1.71^{f}
10.0		0.1	28.80 ± 1.42^{j}	15.00 ± 1.58^{g}
1.0		0.2	64.00 ± 1.94^{e}	27.40 ± 1.50^{f}
5.0		0.2	50.00 ± 2.81^{gh}	24.40 ± 1.28^{f}
10.0		0.2	44.40 ± 1.50^{h}	14.80 ± 1.49^{g}
1.0		0.5	75.80 ± 1.74^{cd}	58.00 ± 2.00^{c}
5.0		0.5	57.20 ± 1.93^{ef}	55.00 ± 2.02^{c}
10.0		0.5	57.00 ± 2.09^{ef}	54.20 ± 1.24^{c}

Data taken after 6 wk. Values represent the mean ± SE from 10 replicates.
Mean followed by the same letter are not significantly different (P=0.05) using DMRT.

on prolong incubation (30 days) of primary somatic embryos on the multiplication and maturation medium, secondary somatic embryogenesis was induced.

Germination of somatic embryos occurred with cotyledons becoming green, the elongation of a radicle and a noticeable increase in the size of the somatic embryos when these were transferred into plant growth regulator free half strength MS medium (Figure 1D). The cotyledonary and bipolar stages were clearly observed within 4 week and in another 4 week, most plantlets had reached 2-3 cm in height and developed 2 to 3 pair of leaves (Figure 1E).

No additional growth regulators or transfer to a different media was required to achieve conversion, indicating that *H. spinosa* has a high potential for propagation through direct somatic embryogenesis. Thus, synergistic effect of reduced concentration of BA with NAA not only promoted the growth of shoot and root, but also helped in secondary embryo formation on the explants. After 8 week, 85% of the plantlets survived under greenhouse conditions and presented an appearance similar to the seedlings obtained from normal seeds (Figure 1F). The acclimatization to *ex-vitro* conditions was successful. In general, auxins particularly 2,4-D are the best studied inducers for obtaining embryogenic cells and the efficiency of this plant growth regulator in the induction of somatic embryos and subsequent transfer to the basal medium for successive development and conversion to plantlet have been reported in *Cerus canadensis* (Trigiano et al., 1988), *Eleutherococcus sessiflorus* (Choi et al., 2002) and *Holostemma ada- kodien* (Martin, 2003) but 2,4-D has proved inhibitory and was not able to mediate the transition from somatic cells to embryogenic cells in *H. spinosa*. In the present study, 2,4-D induced whitish dry calli and showed no sign of differentiation. Similar results where 2,4-D has proved insignificant in inducing somatic embryogenesis is reported in number of plant species such as *Lactuca sativa* (Zhou et al., 1992), *Oncidium* (Chen et al., 1999), *Phalaenopsis* (Kuo et al., 2005) and *Rauvolfia micrantha* (Sudha and Seeni, 2006) regardless of explants types.

It was apparent that single cytokinin, BA, has proved to be effective for inducing direct somatic embryogenesis. In accordance to our findings, BA has been shown to provide sufficient stimulus for inducing somatic embryogenesis on a variety of explants in a number of plant species like *Solanum carolinens* (Reynold 1986), *Lycopersicon esculentum* (Newmann et al., 1996), *Arachis hypogea* (Venkatachalam et al., 1999) and *Carthamus tinctorius* (Mandal and Gupta, 2003). In *A. hypogea* (Venkatachalam et al., 1999), best response was achieved on MS medium containing 22.19 µM BA along with 2.68 µM NAA where frequency of embryogenesis varied between 10.7 - 80.2% in the cotyledon explants while in

the present study, the synergistic effect of BA (1 µM) and NAA (0.5 µM) was found to be most effective and has produced about 110.40 somatic embryos directly from nodal explants which implies that low concentration of BA in combination with auxin could produce more somatic embryos and similar observations have been demonstrated in plant like *Coffea arabica* (Neuensehwander and Baumann, 1992), *Echinochloa colona* (Samantaray et al., 1997) and *Cyminum cuminii* (Hussein and Batra, 1998).

In conclusion, we report a procedure for *in vitro* plantlets regeneration via direct somatic embryogenesis in *H. spinosa*. No noticeable morphological abnormalities in tissue culture plants were observed even after year of growth under field conditions. The protocol developed is simple, cost effective and can be used for raising genetically uniform plants which is important for the sustainable supply of plant materials to the pharmaceutical industries and for conservation of elite germplasm.

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