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In vitro Regeneration and Mass Propagation of *Hybanthus enneaspermus* (L.) F. Muell. from the stem explants through callus culture

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A rapid and efficient method of mass propagation has been developed from the stem explants of *Hybanthus enneaspermus*. The stem explants were cultured on MS medium supplemented with different concentrations of IAA, IBA and NAA. Green compact callus was obtained in combination with low concentration of BAP with the auxins. The highest frequency of 98.8% callus culture was obtained on MS medium supplemented with 15 µM NAA in combination with 5 µM BAP. Shoots were regenerated from the callus by administering cytokinins BAP or KIN alone or combination of both. Large numbers of shoots were produced from all the concentrations of both the cytokinins. The highest frequency of 100% shoot regeneration was observed on MS medium supplemented with all the concentrations of BAP and KIN. The number of shoots produced on the basal medium supplemented ranged between 62 and 74 with BAP and 40 and 50 with KIN 30 days after inoculation. When these two cytokinins were combined, shoot regeneration was accelerated and the number of shoots became 78 to 90 per callus. Excised shoots were transferred to rooting medium containing different concentrations of NAA and IBA for root induction. The root initiation was found to be slow and 5-8 roots were initiated on a medium fortified with 12 µM NAA 30 days after transfer. The rooted plantlets were transplanted to the paper cup for hardening and the well established plants were transferred to the field for acclimatization.

Key words: Mass propagation, callus, shoot regeneration, rooting, hardening, acclimatization.

Abbreviations IAA: Indole-3-acetic acid; IBA: Indole butyric acid; NAA: Naphthalene acetic acid; BAP- 6-benzylaminopurine; KIN - kinetin (6-furfurylaminopurine).

Introduction

Hybanthus enneaspermus (L.) F. Muell. (*Ionidium suffruticosum* Ging.), a member of Violaceae, is a small suffrutescent perennial herb distributed in the tropical and subtropical regions of the world. It grows 15-30 cm in height with

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many diffuse or ascending branches and is pubescent in nature. The plant is popularly known as Rathanapurush or Pursharathna (Sanskrit, Hindi) and Orithazhthamarai (Tamil). This herb is considered to be extremely beneficial to men, hence it is called Rathanapurush. Traditionally the plant is used as an aphrodisiac, demulcent, tonic, diuretic, in urinary infections, diarrhea, leucorrhoea, dysuria and sterility (Yoganarasimhan, 2000).

The plant kingdom shows many species of plants containing substances of medicinal value which have yet to be identified. India is a land of immense biodiversity and also one of the 12 mega biodiversity countries in the world (Dubey *et al.* 2004). One of the main purpose of ongoing research is to increase their production and reduce the price. Therefore, for mass clonal production of medicinal plants and to increase the yields of bioactive products, tissue culture technique is being put to use. Biotechnological tools are important for multiplication and genetic enhancement of the medicinal plants by adopting techniques such as *in vitro* regeneration and genetic transformations. It can also be harnessed for production of secondary metabolites using plants as bioreactors (Tripathi and Tripathi, 2003).

Among the different techniques of biotechnology, plant tissue culture is the one being applied in crop improvement programme (Prakash *et al.*, 1994). The technique of plant tissue culture, therefore, holds great promise to plant breeders pharmaceutical industries and others, besides helping in conservation of our precious natural wealth. Plant tissue culture is a suitable method for obtaining a large quantity of genetically homogenous and healthy plant material, which can be used for planting (Pierik, 1987). It overcomes the barriers in conventional vegetative propagation and fulfils the demand for large-scale cultivation in a short period by rapid mass multiplication. A large number of productive plants can be multiplied routinely through tissue culture (Rao *et al*. 1996). *In vitro* regeneration in *Hybanthus enneaspermus* is meager and few studies were conducted to obtain *in vitro* plantlets (Prakash *et al.,* 1999; Natarajan *et al.* 1999). Prakash *et al.* (1999) obtained light-yellow friable callus on MS medium containing NAA and BA within 4 weeks of incubation. After one year of subculture they established light-green compact callus from which shoots were regenerated on medium supplemented with 8.8 µM BA and 2.6 µM NAA. They obtained the callus induction frequency of 66.6% and 8.9 shoots in 4 weeks. The aim of this study was to enhance the competence of shoot regeneration from the stem of *Hybanthus enneaspermus* for crop improvement.

Materials and methods

Sterilization of Glasswares, Media and Explants

All the glassware were washed thoroughly with chromic acid (potassium dichromate and sulphuric acid, 2:1 w/v), rinsed in tap water and then with distilled water. Sterilization of glassware, forceps and scalpels for micropropagation was done in an autoclave at 121°C for 20 minutes at 1.06 kg cm^{-2}. MS basal medium (Musarhige and Skoog, 1962) was used along with different concentrations of Plant Growth Regulators for callus induction (IAA and IBA), shoot multiplication (BAP and KIN) and rooting (IBA and NAA). The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or 1 N HCl prior to autoclaving (121°C at 1.06 kg cm^{-2} for 20 min).

The explants were collected from the field grown plants of *Hybanthus enneaspermus* in and around the college campus. The internodal regions of stem were rinsed with running tap water with few drops of liquid soap (Teepol). The explants were then washed with distilled water for 3 or 4 times and further sterilization was carried out in the Laminar Air Flow chamber under aseptic condition prior to inoculation. The explants were sterilized with 70% alcohol for 30-45 sec and 0.1% (w/v) $HgCl_2$ for 5 min. in the chamber. The explants were then washed 4-5 times with sterile distilled water to remove the traces of mercuric chloride.

Inoculation Procedure

For callus induction, the leaves were punched with forceps and placed on MS medium supplemented with different concentrations of indole-3-acetic acid (IAA: 5-25 µM), indole butyric acid (IBA: 5-25 µM) and naphthalene acetic acid (IBA : 5-25 µM). To obtain green compact callus, the medium is supplemented with 5 µM BAP with the auxins. For shoot regeneration, benzylaminopurine (BAP: 5-25 µM) and kinetin (KIN: 5-25 µM) were supplemented to the basal medium alone and combination of both. *In vitro* raised shoots of 4 cm and above were excised from the culture tube and subcultured on MS medium supplemented with various concentrations of indole butyric acid (IBA: 3-15 µM) or naphthalene acetic acid (NAA: 3-15 µM).

Culture maintenance and conditions

All cultures were maintained at 25±2°C in a culture room under cool white fluorescent lamps (Phillips, India) at intensity of 50 µmol m^{-2} s^{-1} with 16 hrs photoperiod.

Hardening and Acclimatization

Plantlets with well-developed roots were dislodged from the culture medium and roots were washed gently under running tap water to remove the adhering medium. Plantlets were transferred to plastic cups (10 cm diameter) containing autoclaved garden soil, farmyard manure and sand (2:1:1). Each plantlet was irrigated with distilled water every 2 days for 2 weeks followed by tap water for one week. The potted plantlets were initially maintained under culture room conditions for 3 weeks and later transferred to normal laboratory conditions for 2 weeks. The potted plantlets were initially covered with porous polyethylene sheets to maintain high humidity and were maintained inside the culture room. The relative humidity was reduced gradually. After 30 days the plantlets were transplanted to the field under shade for 3 weeks and then transplanted to the soil for further growth and development.

Experimental Design, Data Collection and Statistical Analysis

The design of all the experiments was a complete randomized block and each experiment consisted of five explants per flask or five test tubes and three replicates. The parameters recorded were frequency (number of cultures responding in terms of callus induction, multiple shoot proliferation and root development), number of shoots per callus, shoot length, number of roots per shoot, root length and survival rate (%). All of the experiments were repeated three times. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means were compared using Duncan's Multiple Range Test (DMRT) at a 5% level of significance (Gomez and Gomez, 1976).

Results and discussions

Callus Induction

The stem explants of *Hybanthus enneaspermus* were treated with different concentrations IAA, IBA and NAA ranging from 5 µM to 25 µM. All the three auxins tested induced callus from the stem explants. However, the

calli were mostly white and friable. When these auxins were combined with low concentration of BAP (5 µM) green, compact, reproducible calli were produced from all the concentrations and combinations of the three auxins (Fig.1a,b; Table 1). Of the three auxins, 15 µM NAA in combination with 5 µM BAP possessed the highest frequency of 98.8% callus induction followed by IAA and IBA. The order of effectiveness of plant growth regulators for callus induction was NAA>IAA>IBA. Similar effects were obtained by several authors (Wagner and Gailing, 1996; Rehman *et al*., 2003; Velayutham *et al.*, 2006). Higher amount of callus was obtained in 45 days of culture. The combination of auxins with cytokinins was effective for prospective callus. A similar result was shown by Rehman *et al.* (2003) and Velayutham *et al.* (2006). The subcultured calli enhanced shoot regeneration potential after two weeks. Callus induction and shoot regeneration system are known to be very useful for the study of biosynthesis of natural products and the factors that influence them, giving some possibilities of controlled production. In several plant species, this approach has been applied (Smollny *et al*. 1992; Schimdt *et al*. 2000).

Table 1. Effect of different concentrations of auxins on callus induction from the stem of *Hybanthus enneaspermus* (L.) F. Muell.

Concentrations of Growth Regulators				Callus Induction
IAA	IBA	NAA	BAP	Frequency (%)
5 µM	---	---	5 µM	92.2 ± 0.96^{jk}
10 µM	---	---	5 µM	93.5 ± 0.85^{ij}
15 µM	---	---	5 µM	96.8 ± 0.24^{g}
20 µM	---	---	5 µM	94.8 ± 1.20^{h}
25 µM	---	---	5 µM	86.7 ± 1.44^{lm}
---	5 µM	---	5 µM	82.6 ± 1.96^{mn}
---	10 µM	---	5 µM	89.4 ± 1.76^{l}
---	15 µM	---	5 µM	94.0 ± 0.92^{hi}
---	20 µM	---	5 µM	80.0 ± 1.56^{op}
---	25 µM	---	5 µM	80.2 ± 2.20^{no}
---	---	5 µM	5 µM	97.4 ± 0.96^{de}
---	---	10 µM	5 µM	98.5 ± 0.00^{ab}
---	---	15 µM	5 µM	98.8 ± 0.00^{bc}
---	---	20 µM	5 µM	97.6 ± 0.00^{cd}
---	---	25 µM	5 µM	96.8 ± 2.16^{ef}

Values are Mean of 5 replicates recorded after 30 days of culture.
Values in the last three columns are Mean ± SE of Mean followed by the letters within the column indicating the level of significance at $P<0.05$ by Duncan's Multiple Range Test (same letter within the column of the respective growth regulator indicates the absence of difference; different letters indicate the significant difference; and combination of letters indicate no significant difference)

Shoot Regeneration and Multiplication

The well developed, green compact calli were transferred to regeneration medium containing BAP and KIN ranging from 5-25 µM. The callus was very responsive, and shoot regeneration initiated within a week after subculture. Shoot regeneration potentiality was 100% in all the concentrations of both the cytokinins. The number of shoots and height of shoot were also more or less similar in all the cultures with a little variation. (Fig.1c-e; Table 2). Of the two cytokinins BAP produced 62-70 shoots per callus with the maximum at 10 µM and mean shoot length of 4.31 cm. When the calli were culture on basal medium supplemented with 10 µM BAP in combination with 2-10 µM KIN, the number of shoots per callus was numerous. The number of shoots vary from 78-90 with the maximum at 10 µM BAP + 6 µM KIN. The beneficial (4–5 cm long) shoot length was observed in all the concentration and combinations of shoot regeneration medium after 4 weeks of culture (Fig.2a).

Table 2. Effect of different concentrations of cytokinins on shoot regeneration from the stem derived callus of *Hybanthus enneaspermus* (L.) F. Muell.

Concentrations of Cytokinins		Shoot Regeneration Frequency	Number of Shoots	Length of Shoots (cm)
BAP	KIN			
5 µM	---	100	62 ± 2.56^{ij}	4.12 ± 1.76^{gh}
10 µM	---	100	74 ± 3.02^{f}	4.31 ± 1.26^{f}
15 µM	---	100	70 ± 2.16^{fg}	4.20 ± 0.98^{fg}
20 µM	---	100	64 ± 3.76^{hi}	3.92 ± 1.72^{ij}
25 µM	---	100	66 ± 2.56^{h}	3.70 ± 1.56^{kl}
---	5 µM	100	42 ± 2.72^{mn}	3.61 ± 1.76^{lm}
---	10 µM	100	40 ± 3.52^{no}	3.42 ± 1.56^{n}
---	15 µM	100	50 ± 2.76^{k}	4.20 ± 0.96^{fg}
---	20 µM	100	45 ± 2.24^{lm}	3.94 ± 2.20^{i}
---	25 µM	100	48 ± 1.96^{kl}	3.83 ± 1.86^{jk}
10 µM	2 µM	100	78 ± 1.52^{de}	5.24 ± 0.76^{e}
10 µM	4 µM	100	82 ± 1.96^{bc}	5.56 ± 0.92^{cd}
10 µM	6 µM	100	90 ± 1.56^{a}	5.68 ± 0.46^{a}
10 µM	8 µM	100	85 ± 2.20^{ab}	5.60 ± 0.82^{bc}
10 µM	10 µM	100	78 ± 1.56^{de}	5.62 ± 0.56^{ab}

Values are Mean of 5 replicates recorded after 45 days of culture.

Values in the last two columns are Mean ± SE of Mean followed by the letters within the column indicating the level of significance at $P<0.05$ by Duncan's Multiple Range Test (same letter within the column of the respective growth regulator indicates the absence of difference; different letters indicate the significant difference; and combination of letters indicate no significant difference)

Though several growth regulators are available for shoot multiplication, BAP and KIN are widely used. Of the two cytokinins tested, BAP was more effective in shoot induction and proliferation than KIN. Similar to this, in several studies BAP was more effective in inducing and sprouting of a large number of shoots (Sahoo and Chand, 1998; Kadota and Niimi, 2003; Velayutham and Ranjithakumari, 2003; Martinussen *et al.* 2004; Vasudevan *et al*. 2004; Velayutham *et al.* 2006; Padmapriya *et al.* 2011). Several workers showed that the synergistic combination of two cytokinins was more effective for shoot differentiation (Velayutham *et al.* 2006; Selvaraj *et al.* 2006; Baskaran *et al.*,2008).

Rooting

Excised shoots were transferred to rooting medium containing different concentrations of IBA and NAA (3-15 µM) for root induction (Fig. 2b,c; Table 3). Root initiation was found to be slow and the numbers of roots produced were also low on both the auxins. Though roots were initiated on all the concentrations of both the auxins, number and growth were slow. Maximum number of 5.6 roots per shoot was observed on 12 µM IBA and 8.6 roots per shoot on 12 µM NAA. Mean root length of 2.82 cm was observed at 9 µM IBA and 3.42 cm at 12 µM NAA. These results showed that of the two auxins, NAA was better in root induction and growth when compared to IBA.

Table 3. Effect of different concentrations of auxins on root induction from the isolated shoots of *Hybanthus enneaspermus* (L.) F. Muell. (after 30 days)

Concentrations of Auxins		Root Induction	Number of	Root
IBA	NAA	Frequency (%)	Roots	Length (cm)
3 µM	---	95 ± 2.56 ^{b}	2.5 ± 1.96 ^{j}	1.20 ± 2.56 ^{j}
6 µM	---	100 ± 0.00 ^{a}	4.3 ± 0.95 ^{hi}	2.10 ± 1.76 ^{g}
9 µM	---	100 ± 0.00 ^{a}	5.0 ± 1.20 ^{ef}	2.82 ± 1.96 ^{cd}
12 µM	---	90 ± 1.96 ^{c}	5.6 ± 2.66 ^{cd}	1.96 ± 1.93 ^{gh}
15 µM	---	85 ± 2.56 ^{d}	4.8 ± 2.98 ^{gh}	1.90 ± 2.48 ^{hi}
---	3 µM	85 ± 3.56 ^{d}	5.2 ± 2.91 ^{de}	2.80 ± 2.76 ^{de}
---	6 µM	95 ± 1.92 ^{b}	4.9 ± 2.36 ^{fg}	2.46 ± 2.52 ^{f}
---	9 µM	100 ± 0.00 ^{a}	6.8 ± 1.20 ^{c}	2.90 ± 1.96 ^{c}
---	12 µM	90 ± 2.96 ^{c}	8.6 ± 2.52 ^{a}	3.42 ± 2.52 ^{a}
---	15 µM	80 ± 2.52 ^{e}	7.8 ± 2.88 ^{b}	3.40 ± 2.76 ^{ab}

Values are Mean of 5 replicates recorded after 30 days of culture.
Values in the last three columns are Mean ± SE of Mean followed by the letters within the column indicating the level of significance at P<0.05 by Duncan's Multiple Range Test (same letter within the column of the respective growth regulator indicates the absence of difference; different letters indicate the significant difference; and combination of letters indicate no significant difference)

In most of the studies IAA, IBA and NAA were used for root induction. High frequency of rooting was achieved by IBA in *Aristolochia indica* (Manjula *et al.* 1997), *Gymnema sylvestris* (Komalavalli and Rao, 2000), *Avicennia marina* (Al-Bahrany and Al-Khayri, 2003) and *Eclipta alba* (Baskaran and Jayabalan, 2005), *Melothria maderaspatana* (Baskaran *et al.*, 2008); and *Solanum nigrum* (Padmapriya, *et al.* 2011). Higher frequency of roots was observed in *Cichorium intybus* at 5 µM NAA (Velayutham and Ranjithakumari, 2003; Velayutham *et al.* 2006), *Rubus chamoemorus* (Martinussen *et al*., 2004), *Viburnum odoratissimum* (Schoene and Yeager, 2005), *Plumbago zeylanica* at 3 µM NAA (Velayutham *et al.* 2005) and *Solanum nigrum* (Jabeen *et al.* 2005).

Hardening and acclimatization

The well rooted plantlets were transplanted to the paper cup (Fig.2d) containing a mixture of autoclaved red soil, farm yard manure and sand in the ratio of 2:1:1. The survival rate of these plants was 70%. The established plants were transferred to the field for acclimatization.

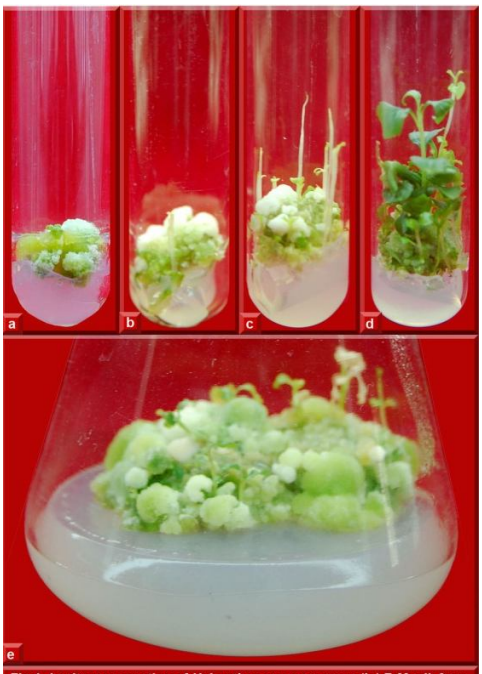

Fig.1. *In vitro* propagation of *Hybanthus enneaspermus* (L.) F. Muell. from stem explant. a. callus culture on MS basal medium supplemented with auxin 30 days after inoculation; **b.** shoot initiation from the callus 10 days after subculture; **c.** shoot proliferation and elongation after 20 days; **d.** proliferation and elongation of leafy shoots after 30 days; **e.** mass propagation of shoots from the stem derived callus .

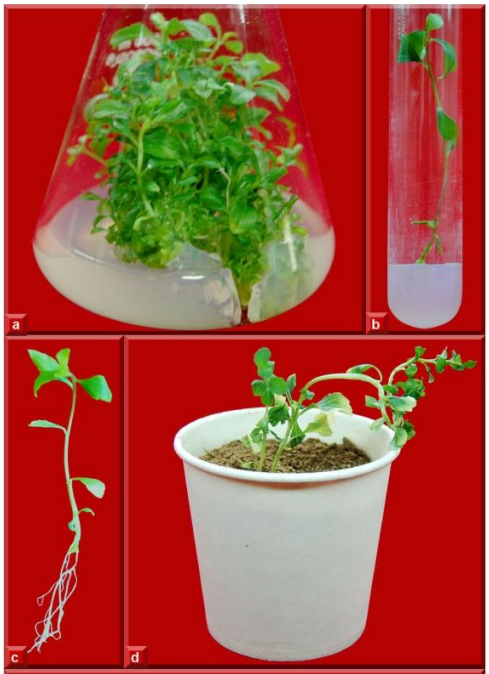

Fig.2. Mass propagation of *Hybanthus enneaspermus* (L.) F. Muell. from stem stem derived callus. a. mass proliferation and elongation of shoots 30 days after inoculation; **b.** initiation of roots from the isolated shoot; **c.** isolated shoot showing roots; **d.** hardening of rooted plant.

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