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RESEARCH ARTICLE

IN VITRO PROPAGATION AND CONSERVATION OF USEFUL ETHNOMEDICINAL PLANT OF *HYBANTHUS ENNEASPERMUS* (LINN.) F. MUELL. BELONGING TO THE VIOLACEAE FAMILY

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ABSTRACT

An efficient and reproducible protocol has been developed for in vitro propagation and conservation of Hybanthus enneaspermus using stem explants. Stem explant showed high frequency of callus induction potentiality on MS (Murashige and Skoog 1962) medium supplemented with 2.0mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) plus 0.8mg/l 6-Benzylamino purine (BAP) followed by 2.0mg/l α- naphthaleneacetic acid (NAA) plus 0.8 mg/l Kinetin (Kn). The highest frequency of shoot regeneration (88%) and number of shoots per explant (28.2) were obtained on medium supplemented with 2.5mg/l BAP plus0.6mg/l NAA. Rooting was best achieved on half-strength MS medium augmented with 1.5mg/l Indole-3-butyric acid (IBA) and optimum number of 19.7 roots per explants with average 9.3cm root length. The plantlets regenerated in vitro with well developed shoot and roots were successfully established in pots containing garden soil and grown in a greenhouse with 90% survival rate. The regenerated plants did not show any immediate detectable phenotypic variation. The described method can be successfully employed for large-scale multiplication and long term in vitro conservation of H. enneaspermus.

Key words: Ethnomedicine, *Hybanthus enneaspermus,* Stem explants, Violaceae *In-Vitro* Propagation, Conservation.

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INTRODUCTION

Hybanthus enneaspermus (Linn.) F. Muell. belonging to the Violaceae family, is a rare, perennial herb found in some of the warmer parts of the Deccan peninsula in India. This plant popularly called as 'Amburuha in Ayurvedic, Orilaithamarai in Siddha/Tamil and Ratna-purush in Folk. This ethnobotanical herb is known to have unique medicinal properties. The preparations made from the leaves and tender stalks of the plant are used in herbal medicine for its aphrodisiac, demulcent and tonic properties. The root is diuretic and administrated as an infusion in gonorrhea and urinary infections (The Wealth of India 1959). The plant gave a dipeptide alkaloid, aurantiamide acetate and a triterpene, iso-arborinol, and beta-sitosterol (Khare 2007), Anti arthritic potential (Tripathy et al 2009). The fruits and leaves are used as antidotes for scorpion stings and cobra bites by tribes (Sudarsanam and Sivaprasad 1995). However, the natural regeneration potential of this herb is very poor due to low seed viability. Because the seeds and developing capsules are often found on the ground, loss due to rodents and inundation is considerable.

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Increasing human and livestock population shave already affected the status of wild plants, particularly those used in herbal medicine. In view of its ethnomedicinal importance, there is a need to conserve the wild stock of *H. enneaspermus*. Plant tissue culture is a useful tool for the conservation and rapid propagation of a valuable ethnobotanical plant (Sanyal et al. 1998). However, there are a few reports on callus induction and plantlet regeneration using stem as explants. Thus, an efficient protocol for *in vitro* dedifferentiation and plant regeneration from callus culture is needed which could be useful in mass propagation and several *in vitro* manipulation studies. The preset communication describes a protocol for the establishment of rapidly proliferating callus cultures and efficient large-scale regeneration from stem explants of *H. enneaspermus* with successful acclimatization and field transfer.

MATERIALS AND METHODS

Source of explants and Surface Sterilization: *H. enneaspermus* were collected from ABS medicinal garden, Karippatti, Salem Districts and the plants were established and maintained in the greenhouse of Salem Sowdeswari College, Salem, South India for *in vitro* development.

Non meristematic explants like stem explants were excised with sterile blade and collected in a beaker. The excised stem explants were thoroughly washed with running tap water for 5 to 10 minutes. Thereafter, the explants washed with detergent (Teepol 2% v/v) solution for 3 minutes followed by fungicide (Bavistine 1% w/v) for 2 minutes and then soaked in 70% (v/v) ethanol for 30 seconds after the each treatment the explants were rinsed three times with distilled water. Further, sterilization treatments were done under a laminar-flow chamber. The explants were finally disinfected with 0.1% (w/v) $HgCl_2$ for 2 minutes and this these explants were then thoroughly washed 3-4 times with sterilized double distilled water to remove the traces of mercuric chloride, now the explants is ready for inoculation on required medium.

Inoculation of explants

The stem explants were inoculated on Murashige and Skoog (MS) basal medium supplemented with different concentration and combination of plant growth regulators (PGRs). By means of a long stainless steel forceps, one explant per tube was placed. It was a routine process to flame the mouth of the test tube after uncapping and before recapping the tubes to reduce contamination. To facilitate planting, two forceps were used alternatively to allow adequate time to cool, furthermore, to prevent burning the fingers and explants. The cultures were maintained at 25 ± 2˚C under 16/8 h light/dark conditions of $80\mu Ems^{-2}s^{-1}$ irradiance provided by fluorescent lamps (TL 40W/54 cool-day light) for callus induction and plantlets regeneration.

Callus induction

The stem explants were cultured on Murashige and Skoog (1962) basal medium containing 3% (w/v) sucrose, 0.8% (w/v) agar and various concentrations of 2,4-D and NAA alone or in combination with BAP and Kn for optimal callus induction. Data were recorded at 10, 20 and 30 days after inoculation of explants on the regeneration medium. The calli were graded according to their colour in a symbol of B - Brown; G - Green; W - White; LG - Light green; GY -Geenish yellow; GW - Greenish white; GB - Greenish brown; LB - Light brown; WB - White brown. The nature of callus was measured by the callus compactness and graded into three categories: compact (C), less compact (LC) and friable (F). Abundance of callus was measured by a transparent measuring ruler and graded according to their length scale: large (L) = 20 mm and above, medium (M) = 10 to 20 mm and small (S) = 10 mm below. The calli were subcultured and maintained in *in vitro* conditions. The effects of these quantitative characters with duration of the time for regeneration were estimated in percentage.

Shoot bud regeneration and multiplication

Dedifferentiated calli were transferred to regeneration medium containing 3% (w/v) sucrose, 0.8% (w/v) gel and different concentrations of BAP (1.0 – 3.0mg/l) and Kn (1.0 – 3.0mg/l) in combination with various concentrations of NAA which were used for shoot buds differentiation. After 4 weeks, the clumps of shoots were subcultured on MS medium with suitable growth regulators for multiplication and maturation of the shoots. The percentage of shoots and plantlets were estimated on the basis of the production of the plantlets from calli.

Proliferated multiple shoots were divided into small clusters of 2 - 3 shoots. They were subcultured on same medium for shoot elongation. After two weeks, shoots longer than 3.0 cm were counted and transferred to rooting medium.

Root induction and transplantation

The longer shoots (3 cm length) were excised and transferred to MS basal medium containing 3% (w/v) sucrose, 0.8% (w/v) agar and different concentrations of Indole-3-butyric acid (IBA at 0.1 – 2.0 mg/l) alone or in combination with BA (0.1 – 2.0 mg/l) / Kn (0.1 – 2.0 mg/l) for root induction. Rooting was observed from two to three weeks. Plantlets with well developed roots were removed from the culture tubes and after washing their roots in running tap water, they were grown in the mixture of red garden soil, river sand and saw dust in the ratio of 1:1:1 in paper cups for a month and subsequently transferred to pots. Potted plants were covered with transparent polythene membrane to high humidity and watered every three days with half strength MS salts solution for two weeks. Plantlets were left for a week in the paper cups at the controlled temperature (25 ± 2˚C) with 60% relative humidity. The survival percentage was observed in all the explants. After the initiation of new roots, they were kept in the green house and grown for maturity. Samples were photographed at different stages of growth period.

Statistical analysis

The cultures were examined periodically and the morphological changes were recorded on the basis of visual observation. Whenever possible the effects of different treatments were quantified on the basis of percentage of cultures showing the response per culture. The experimental design was Completely Randomized Design (CRD) and factorial with auxin and cytokinin as independent variables. Each treatment consisted of at least 7 explants and all the experiments were repeated five times. The data pertaining to frequencies of callus induction, shoot bud differentiation and multiplication, number of shoots, root induction and number of roots/culture were subjected to standard deviation. Mean separation was conducted by using Duncan's new Multiple Range Test (DMRT) and means were compared with $P < 0.05$ at level of significance.

RESULTS

The establishment of indirect *in vitro* regeneration protocol for *H. enneaspermus* was developed in an optimal medium for induction of morphogenetic responses. The morphogenetic response through the callus phase was observed from stem explants using different levels of auxins and cytokinins combination on MS medium. The highest percentage of culture response and optimal number of shoots from stem explants of indirect organogenesis is given in Figure 1.

Callus induction

The stem explants produced morphologically different callus on MS medium containing auxins and cytokinins. The plant growth regulators (PGR) and its concentrations screened were totally effective for inducing callus formation. The basal medium devoid of any plant growth regulators failed to induce callus in any of the cultured explants. Based on the preliminary experiments with different stages, maximum callus initiation

was noticed at the end of the week. Assessment of callusing behaviour was made in respect of colour, nature and size of callus at 10 and 20, days of inoculation (Fig. – 1).

Effect of auxin

Different concentrations of 2,4-D and NAA at the range of 0.5 – 2.5 respectively were used on MS medium. The frequency of callus induction, types of callus and size of callus were influenced by the explants and callus initiation was seen within 10 days of inoculation. Maximum callus initiation was observed at the cut ends of the stem explant on MS medium supplemented with 2,4-D at 2.0mg/l which was greenish brown and friable. On increasing concentrations of 2,4-D from 0.5 to 2.0mg/l, a gradual increase in size of callus was noticed and on further increase of concentration there was gradual decrease in the callus size. At high concentration of 2,4-D (above 3.0mg/l) there was nil callus response. Among the different concentrations of NAA tested, stem explant produced maximum, greenish brown and light compact callus at 2.0mg/l NAA within 10 days of incubation. An initial stage inoculums became swollen and showed curling. Sprouting of callus was first initiated from the cut ends of the explant. At low concentration, compact and green or light green callus was seen whereas at high concentration less compact and greenish brown colour callus was seen.

Effect of auxin with cytokinin

When the MS basal medium supplemented with auxin alone the explant produced callus, but the growth of callus was less and frequency of callusing was low than in combination. Therefore, a low concentration of cytokinin with auxin was added to increase the frequency of callusing. Of the 2,4-D with BA evaluated for their effect of callus initiation, after 20 days of inoculation the calli became light brown to brown and less compact in nature. The highest callus induction efficiency occurred when cultured on MS medium supplemented with higher concentration of 2,4-D (2.0mg/l) and low concentration of BAP (0.8mg/l). The stem explants cultured on MS medium containing 2,4-D (2.0mg/l) with Kn (0.6mg/l) callused.. The best callus formation also occurred on medium enriched with 2.0mg/l NAA and 0.8mg/l BAP and the callus was greenish yellow and compact. When the BAP concentration was increased the nature of callus became compact and low concentration of BA (0.2mg/l) with NAA produced compact with light green calli (Table - 1).

Regeneration

The concentration and combination of PGRs are the key factors for influencing the regeneration of plant. The different morphological callus growth and regeneration by BAP and Kn alone or in combination with NAA resulted in different modes of action from selected PGRs. A much reduced number of shoots or no signs of shoots were developed from stem derived calli cultured on MS basal medium. When the stem derived calli were subcultured on organogenic medium containing BA and Kn, they showed morphogenic differentiation. Among various growth regulators and different concentrations tested, the higher shoot regeneration frequency (80%) and highest number of shoots (21.8 ± 2.91) were recorded at 2.5mg/l BAP concentration. The callus when subcultured onto the MS medium containing different concentrations of Kn alone produced the highest regeneration frequency (77.1%) and number of 20.9 ± 2.78 shoots per callus clump, at a concentration of 2.0mg/l Kn (Table - 2). Increasing concentration of both PGR produced more shoots at optimum level of concentration and further increase in concentration declined shoot number gradually. The addition of NAA with optimal concentration of BAP or Kn significantly increased the frequency of shoot formation. Maximum frequency of regeneration (88.6%) with 28.2 shoots per callus clump was obtained at 2.5mg/l BAP + 0.6mg/l NAA (Fig. – 1d). The Kn (2.0mg/l) + NAA (0.6mg/l) showed maximum regeneration frequency (82.9%) with 24.2 shoots per callus clump. Increasing concentration of NAA with BA or Kn possessed organogenic potential with low frequency of regeneration.

Rooting and hardening of in vitro raised shoots

Individual shoots at least 2 – 3 cm long were separated and cultured on half strength MS medium supplemented with IBA (0.1 – 2.0mg/l) for root induction. The shoots that were below 1.0 cm in length did not produce any roots when cultured on rooting medium. The shoot produced roots at all concentrations of IBA but the highest efficiency of 88.6% rooting was observed in 1.5mg/l IBA. The development of an average number of 19.7 roots with 9.3 cm root length from a single shoot was noticed in IBA fortified medium at the end of the 20 – 25 days of culture. The IBA was comparatively better than with combination of IBA + Kn and IBA + BAP in terms of both percentage of the cultures that responded as well as number of roots and root length per shoot (Table - 3). The rooted shoots were successfully transplanted to paper cups containing garden soil, river sand and saw dust in the ratio of 1:1:1 and the humidity was maintained at approximately 80 – 90% by covering with polythene wrap. The plants were watered twice for every four days. After two months plants were transferred to large pots and after acclimatization, the 100 days old plants were transferred to the field.

DISCUSSION

Recent advances in plant tissue culture technology have opened up many new avenues for conducting basic genetic research on higher plants at the cellular level and have provided potentially powerful new tools in the hands of plant breeders for generating, selecting and propagation of novel and economically important plant varieties. The use of *in vitro* technology in the mass propagation of this valuable medicinal plant has been gaining momentum in the recent years (Sianipar et al., 2017 and Rajani Verma et al., 2017). A plant regeneration system via indirect shoot organogenesis has been established in this study for *Hybanthus enneaspermus*. Conditions encouraging callus proliferation and recovery of large number of adventitious shoots were identified. Application of *in vitro* techniques have been routinely practiced for the multiplication of many medicinal plants to meet the demand of pharmaceutical firms and to protect the natural populations of rare and endangered plant species (Rathod et al. 2008; Meratan et al. 2009; Shilpa et al. 2010; Majumder and Rahman 2016 and Ramakrishnan et al. 2017).

Callus induction

The essential findings of Skoog and Miller (1957) that organogenesis in tissue cultures is governed by the balance of auxin and cytokinin in the medium cannot be demonstrated universally due to the explant sensitivity or the original content

of endogenous growth regulators. Methods have been developed for reproducible plant regeneration system from cultured explants of *H. enneaspermus*. Results obtained from this experiment revealed that the explants vary in their different hormone requirements for callus induction, shoot bud regeneration, multiple shoot formation and root initiation. The explants were cultured on MS basal medium fortified with different concentrations of auxins (2,4-D, NAA) or in combination with BAP, Kn. Our results indicated that the addition of cytokinin effected with an increase in the frequency of callus induction. The callus induction frequency was different with different auxins. So, the present study clearly indicates that the auxin type greatly influenced the callus induction frequency. The callusing frequency increased with an increase in the concentration of NAA upto 2.5mg/l supplemented with BA 0.8mg/l / Kn 0.6mg/l in the medium. This is consistent with reports on the tissue culture study (Rathod et al. 2008; Arellano et al. 2009 and Sharma and Vashistha 2015). Similar results were also obtained on MS medium supplemented with 2,4-D and NAA alone or in combination for callus induction in the past by Meratan et al. (2009) and Roy and Bharadvaja (2017). The auxin alone or in combination with cytokinin supporting callus growth has been reported by Biswas et al. (2011) and Pandey et al. (2013). Moreover the combination of auxin in high concentration and cytokinin in low concentration was more effective for callus formation. Similar results were obtained in *Nicotiana tabacum* (Murnilawati et al. 2006) and *Plectranthus barbatus* (Thangavel et al. 2014). In our experiment it is seen that NAA 2.0mg/l and BA 0.8mg/l had optimum frequency of callus formation which was observed within 2 to 3 weeks of culture. Morphology, texture and colour of the callus were depending on the nature of hormones used. Auxin (2,4-D/NAA) individually resulted in greenish white or greenish yellow and less compact or friable calli whereas in combination with cytokinin (BA/Kn) embryonic light green calli were light brown and whitish brown in colour with nodular, less compact calluses. These results corroborate with the previous findings of Chen et al. (2005) and (Gopi and Vatsala 2006).

Shoot bud differentiation and multiple shoot regeneration

In the present study, we have obtained increased shoot bud regeneration efficiency from leaf explants derived callus by culturing them on MS basal medium containing different concentrations of BA/Kn (1.0 – 3.0mg/l) with low levels (0.2 – 1.0mg/l) of NAA. In the absence of cytokinin, however there was no induction of shoot bud regeneration. Majumder and Rahman (2016) reported that shoot regeneration was indirect and developed through calli. Auxins and cytokinins are the two types of phytohormones most often needed in culture and a relatively high ratio of 4:1 in cytokinin and auxin favored shoot formation. This is in agreement, with the present study whereas BA (2.5mg/l) with NAA (0.8mg/l) were as effective for shoot formation. Similar results were also reported by Murashige and Skoog (1962) who observed that a relatively low auxin; cytokinin ratio induced shoot formation in tobacco callus. Frequency of shoot formation from stem explant varied between 40.0 – 88.6 %, and the higher frequency was observed on medium containing 2.5mg/l BAP + 0.8mg/l NAA. This is in contrast with that of Meratan et al. (2009) who achieved an optimum frequency (78.6%) of shoot formation on medium containing NAA + TDZ and lowest frequency (6.56%) of shoot formation on 2,4-D + Kn fortified medium. Abubachker and Murugesan (1999) have reported the concentration of BAP (1.5mg/l) and NAA (0.5mg/l) to be most congenial for shoot regeneration in young stem explants followed by Roy and Bharadvaja (2017) who have reported that BAP (1.0mg/l) to be effective for shoot bud regeneration in nodal explants of *Plumbago zeylanica*. But in the present investigation BAP 2.5mg/l + NAA 0.8mg/l was effective for regeneration in stem explants derived callus which produced optimum of 28.21 ± 3.10 shoots per callus clump in this combination. Higher concentration of BAP (above 3.0mg/l) reduced the percentage of response, number of shoots and shoot length. Significant effects of subsequent shoot regeneration from calli on MS medium fortified with BAP 2.5mg/l alone was more effective than Kn because less shoot regeneration was observed on MS based medium supplemented with 2.0mg/l Kn alone. In contrast, Shen et al. (2007) have shown that 2iP was more effective than Kn. No morphogenetic response was observed from callus clumps cultured on MS basal medium which was devoid of PGRs. Callus tissue transferred to different shoot bud induction media became nodular and were found to be highly competent for shoot bud initiation. Similar results were obtained by Faisal et al. (2007) in *Tylophora indica,* and Majumder and Rahman (2016) in *Stevia rebaudiana.* Increasing the concentration of BA from 1.0 to 2.0mg/l or 2.5mg/l resulted in an increase in the rate of shoot regeneration ability and further increased concentration gradually decreased the shoot numbers. Similar results were earlier reported on *Tylophora indica* (Faisal and Anis 2005) Addition of NAA showed the synergistic effect with BAP and enhanced the induction of shoot bud from callus and increased the shoot morphogenetic response as compared to the other combinations of NAA + Kn. A similar observation is also reported in *Tylophora indica* (Faisal and Anis 2005) and *Stevia rebaudiana* (Majumder and Rahman 2016).

Rooting of *in vitro* shoots

Rooting of shoots is the most critical step in the production of complete plantlets and their subsequent survival. Growth regulators and nutrient contents of the medium play a vital role in the rooting process. The in vitro regenerated shoots were isolated and cultured for root induction on full and half strength MS basal medium. Among these, half strength MS medium was found sufficient for the induction of roots. The successful application of half strength MS medium for root inducing experiment have also been documented in various *in vitro* protocols viz *Tylophora indica* (Faisal et al., 2007) *Sarcostemma brevistigma* (Thomas and Shankar 2009), *Plectranthus barbatus* (Thangavel et al 2011 and Thangavel et al 2014) and *Stevia rebaudiana* (Majumder et al 2016). Half strength MS medium supplemented with different auxins were also used to enhance root formation. IBA were used individually and in combination with KN/BAP for rooting. In the present study IBA was found to be more potent for the highest percentage of rooting when compared to combinations. Thus, these results are analogous to the present observation whereas half strength MS medium containing IBA (1.5mg/l) produced highest (19.7) roots with root length 9.3 cm. IBA was found to be the best for root induction as reported by Jeyachandran et al (2005), Reshi et al (2017) and Rajani Verma et al (2017).

Acclimatization and hardening

Hardening refers to the process of acclimating plants from indoor temperatures to the outdoors.

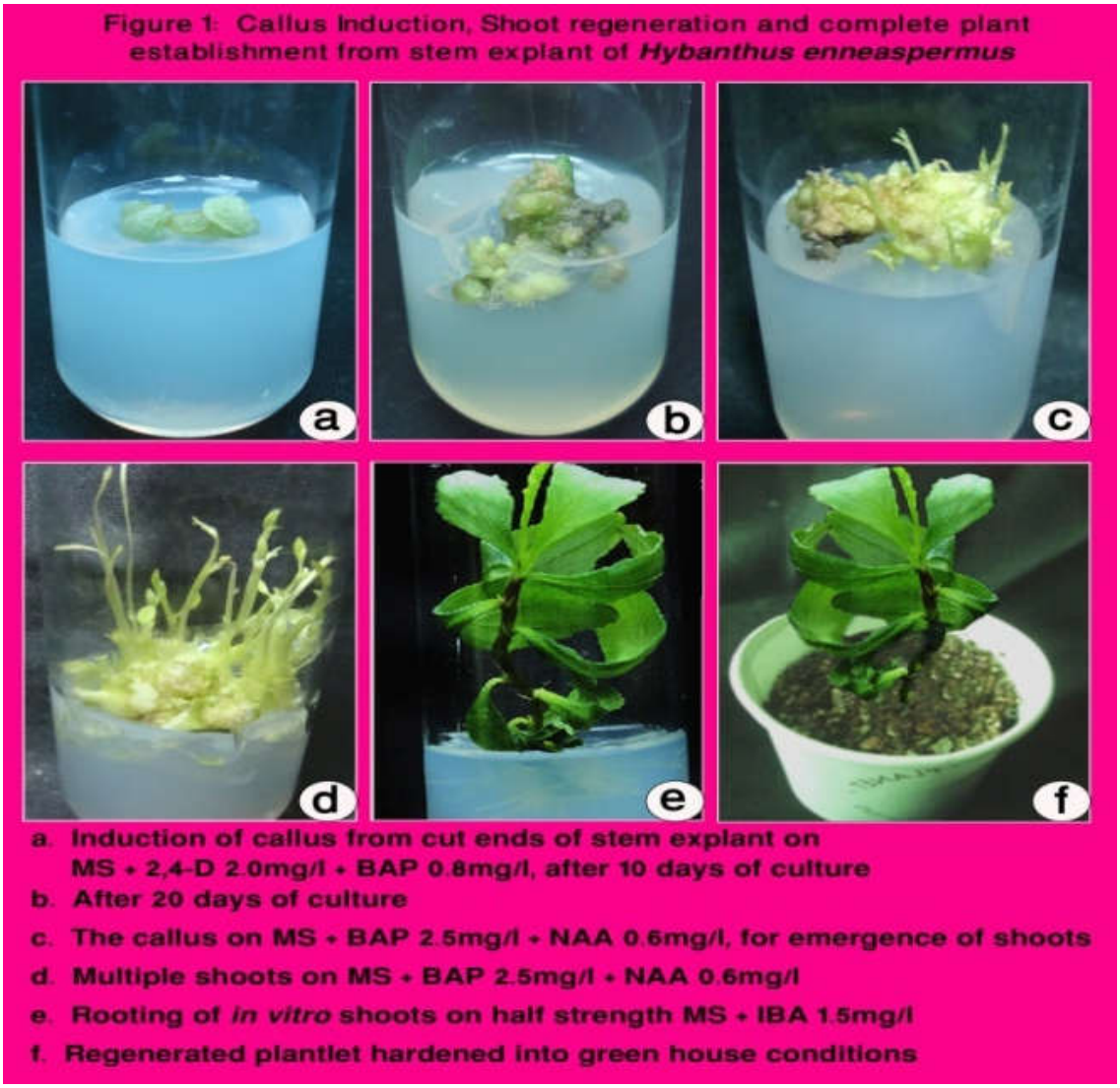

Table 1. Effect of different concentrations of auxins (2,4-D/NAA) alone or in combination with BAP/Kn on callus induction, colour and compactness from stem explants on MS medium, observation after 20 days of second subculture

Plant Growth Regulators (mg/l)				Types of Callus		
2,4-D	NAA	BAP	Kn	X	Y	Z
0.5	-	-	-	GW	S	LC
1.0	-	-	-	GW	S	LC
1.5	-	-	-	GB	M	F
2.0	-	-	-	GB	L	F
2.5	-	-	-	LB	M	F
-	0.5	-	-	G	S	C
-	1.0	-	-	G	M	C
-	1.5	-	-	GY	M	C
-	2.0	-	-	GB	M	LC
-	2.5	-	-	GB	L	LC
2.0	-	0.2	-	LB	S	LC
2.0	-	0.4	-	LB	M	LC
2.0	-	0.6	-	LB	M	LC
2.0	-	0.8	-	LB	L	LC
2.0	-	1.0	-	GY	L	LC
2.0	-	-	0.2	B	M	F
2.0	-	-	0.4	GB	L	F
2.0	-	-	0.6	LB	L	LC
2.0	-	-	0.8	LG	L	LC
2.0	-	-	1.0	LG	M	LC
-	0.5	0.2	-	LG	S	C
-	1.0	0.4	-	LG	M	C
-	1.5	0.6	-	GY	L	C
-	2.0	0.8	-	GY	L	C
-	2.5	1.0	-	G	L	C

X = Colour of the callus: B Brown; G Green; W White; LG Light green; GY Greenish yellow; GW Greenish white; GB Greenish brown; LB Light brown; WB White brown. Y = Size of the callus: S Small (below 10 mm); M Medium (10 - 20 mm); L Large (above 20 mm). Z = Nature of the callus: F Friable; LC Less Compact; C Compact.

Table 2. Effect of BAP and KN alone or incombination with auxin (NAA) on multiple shoots regeneration from stem explants derived callus of Hybanthus enneaspermus

Plant Growth Regulators (mg/l)			Frequency of cultures responded	Mean No. of shoots per callus
BAP	KN	NAA		
1.0	-	-	40.0e	6.58 ± 1.92cd
1.5	-	-	51.4d	8.32 ± 1.89c
2.0	-	-	71.4b	18.79 ± 2.84ab
2.5	-	-	80.0a	21.84 ± 2.91a
3.0	-	-	62.9c	16.79 ± 2.86b
-	1.0	-	40.0cd	6.63 ± 1.80de
-	1.5	-	54.3bc	13.79 ± 2.53bc
-	2.0	-	77.1a	20.95 ± 2.78a
-	2.5	-	62.9b	16.53 ± 2.20b
-	3.0	-	45.7c	8.95 ± 1.51d
2.5	-	0.2	51.4cd	12.05 ± 2.50d
2.5	-	0.4	71.4b	23.16 ± 3.08b
2.5	-	0.6	88.6a	28.21 ± 3.10a
2.5	-	0.8	60.0c	18.37 ± 2.56c
2.5	-	1.0	45.7d	9.37 ± 2.03de
-	2.0	0.2	45.7cd	8.95 ± 1.43d
-	2.0	0.4	68.6b	19.47 ± 2.91b
-	2.0	0.6	82.9a	24.26 ± 3.14a
-	2.0	0.8	54.3c	16.16 ± 1.54bc
-	2.0	1.0	40.0d	7.37 ± 1.54de

Mean values within column followed by different letters are significantly different from each other at P < 0.05 level comparison by DMRT.

Table 3. In vitro rooting of regenerated shoots of Hybanthus enneaspermus on half strength MS medium fortified with of IBA alone or in combination with cytokinins

Plant Growth Regulators (mg/l)			Frequency of culture responded	Mean No. of roots per shoot	Mean root length (cm)
IBA	Kn	BAP			
0.1	-	-	40.0ef	7.53 ± 1.26de	3.11 ± 0.81de
0.5	-	-	60.0c	12.42 ± 1.71bc	5.47 ± 1.17c
1.0	-	-	74.3b	13.95 ± 2.07b	7.16 ± 1.07b
1.5	-	-	88.6a	19.79 ± 2.44a	9.37 ± 1.21a
2.0	-	-	57.1cd	11.84 ± 1.98c	6.47 ± 1.84bc
1.5	0.1	-	60.0c	10.26 ± 1.91bc	6.89 ± 1.29bc
1.5	0.5	-	80.0a	16.84 ± 2.32a	8.68 ± 1.34a
1.5	1.0	-	71.4ab	12.63 ± 1.98b	7.11 ± 1.33b
1.5	1.5	-	54.3cd	8.84 ± 1.50c	5.16 ± 1.34d
1.5	2.0	-	42.9e	4.79 ± 1.36d	3.21 ± 1.58e
1.5	-	0.1	54.3bc	8.74 ± 1.85bc	5.21 ± 1.32bc
1.5	-	0.5	71.4a	12.58 ± 2.01a	7.26 ± 1.45a
1.5	-	1.0	54.3b	10.21 ± 1.65b	5.47 ± 1.17b
1.5	-	1.5	45.7c	5.16 ± 1.80d	4.16 ± 1.57c
1.5	-	2.0	37.1cd	3.79 ± 1.58de	2.05 ± 1.27d

Mean values within column followed by different letters are significantly different from each other at P < 0.05 level comparison by DMRT.

The hardening of *in vitro* raised plantlets is essential for better survival and successful establishment. Direct transfer of tissue culture raised plant to field is not possible due to high rate of cosseted environment with a very high humidity, varied light and temperature condition and being protected from the attack of microbial and other agents. The period of transition during the process of hardening after transfer from the *in vitro* to *ex vitro* environment is considered to be the most important step in tissue culture (Faisal et al., 2007 and Majumder et al., 2016). Tissue cultured plants lose their water rapidly, when moved to the external condition. *In vitro* plant leaf was very typical and incapable of complete stomatal closing under conditions of low relative humidity. For acclimatization plantlets were removed from rooting medium after two weeks at incubation and transferred to paper cups containing autoclaved soil tightly covered with perforated polythene bags to maintain (25 ± 2˚C) humidity and were kept under culture room conditions for a week. The present study is consistent with the reports of Sharma, and Vashistha (2015). During hardening, the plantlets were irrigated with half strength MS medium and tap water when required. Similar results were also reported by Rajani Verma et al. (2017).

Conclusion

In conclusion, the present investigation reports an efficient and reproducible regeneration protocol via indirect organogenesis of *Hybanthus enneaspermus*. The method is flexible, allowing incorporation of different concentration of 2,4-D, NAA, BAP, Kn and IBA effective in both callus induction, multiple shoot buds proliferation and rooting. MS medium supplemented with 2.0mg/l 2,4-D + 0.8mg/l BAP was the best for callus induction, 2.5mg/l BAP + 0.6mg/l NAA was best for highest rate of shoot proliferation and Half strength MS medium fortified with 1.5mg/l IBA was found to be the best treatment for root formation. There are many institutes and universities where the research work is going on but that work should be explored at higher level so that other researchers gain experience from that and help in conserving this medicinal plants otherwise this species also become rare. These *in-vitro* studies provide an efficient method for conservation and propagation of this over exploited valuable medicinal plant species. Biotechnological approaches are required in future also to promote its medicinal use.

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