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# *In vitro* multiplication, micromorphological studies and *ex vitro* rooting of *Hybanthus enneaspermus* (L.) F. Muell. – a rare medicinal plant

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**Abstract** – *Hybanthus enneaspermus* is a rare medicinal plant. We defined a protocol for micropropagation, *ex vitro* rooting of cloned shoots and their acclimatization. Surface-sterilized nodal segments were cultured on Murashige and Skoog (MS) medium with different concentrations of 6-benzylaminopurine (BAP) and kinetin (Kin). Medium supplemented with 1.5 mg $L^{-1}$ BAP was found optimum for shoot induction from the explants and 6.4±0.69 shoots were regenerated from each node with 97% response. Shoots were further proliferated maximally (228±10.3 shoots per culture bottle with 7.5±0.43 cm length) on MS medium augmented with 1.0 mg $L^{-1}$ each of BAP and Kin within 4–5 weeks. The shoots were rooted *in vitro* on half strength MS medium containing 2.0 mg $L^{-1}$ indole-3 butyric acid (IBA). The cloned shoots were pulse-treated with 300 mg $L^{-1}$ of IBA and cultured on soilrite® in a greenhouse. About 96% of the IBA-pulsed shoots rooted *ex vitro* in soilrite®, each shoot producing 12.5±0.54 roots with 5.1±0.62 cm length. The *ex vitro* rooted plantlets showed a better rate of survival (92%) in a field study than *in vitro* rooted plantlets (86%). A comparative foliar micromorphological study of *H. enneaspermus* was conducted to understand the micromorphological changes during plant developmental processes from *in vitro* to *in vivo* conditions in terms of variations in stomata, vein structures and spacing, and trichomes. This is the first report on *ex vitro* rooting in *H. enneaspermus* and the protocol can be exploited for conservation and large-scale propagation of this rare and medicinally important plant.

**Key words:** conservation, *ex vitro* rooting, *Hybanthus enneaspermus*, micromorphology, micropropagation, rare medicinal plant

## Introduction

*Hybanthus enneaspermus* (L.) F. Muell. (Formerly *Ionidium suffruticosum* Ging.), belongs to the family Violaceae. It is a rare multipotent herb, endemic to the Deccan Peninsula in India with various invigorating properties (Prakash et al. 1999, Sudeesh 2012). It is a small suffrutescent perennial herb found in India, Sri Lanka, Tropical Asia, Africa and Australia (Anand and Gokulakrishnan 2012). The plant grows up to 15–30 cm in height with many diffused branches (Kirtikar and Basu 1991). *H. enneaspermus* is traditionally known as Padmavati, Lakshmisheshta, Padmacharini or Purusharathna in India and considered a valuable healing herb in the Indian systems of medicine (Satheeshkumar 2011). It is well documented in the folklore medicine of India for its aphrodisiac and stimulant activity (Awobajo et al. 2009). *H. enneaspermus* has therapeutic applications. Moreover, the whole plant is used to treat diarrhea, painful dysentery, and strangury, burning sensations, urinary infections, leucorrhoea, dysuria and sterility (Tripathy et al. 2009). The plant is also valued for its antimicrobial (Retnam and Britto 2007), antiplasmodial, antiarthritic (Subramoniam et al. 2013), antimalarial, antirheumatic, emmenagogic, sedative, antispasmodic, antiasthmatic, anti-infertility (Nathiya and Selvi 2013), antibacterial, anticonvulsant, antidiabetic, antifungal (Arumugam et al. 2011), anti-allergic and analgesic, antinociceptive, antioxidant and aphrodisiac properties (Kumar et al. 2013). This plant contains various important phytochemicals like dipeptide alkaloids, aurantiamide acetate, isoarborinol and β- sitosterol, flavonoids, steroids, triterpenes, phenols, tannin, glycosides etc. (Krishnamoorthy et al. 2014).

There is no commercial cultivation of *H. enneaspermus* and the plants are collected from wild sources. It is facing genetic threat due to sporadic distribution, poor germina-

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tion of seeds, anthropogenic activities, overgrazing and over exploitation by herbal drug manufacturers (Arunkumar and Jayaraj 2011, Verma and Singh 2011). It has been disappearing from the large area of the Western Ghats of India due to widespread cultivation of rubber in the natural habitat of this plant (Joseph et al. 2000). *H. enneaspermus* is conventionally propagated through seeds. The seeds show poor viability and germination in the wild (Arunkumar and Jayaraj 2011). Conventional propagation methods are unable to meet the demand of the pharmaceutical industries and drug research. Therefore, it is necessary to develop a non-conventional method for propagation to fulfill the demands of the drug market (Rathore et al. 2008). *In vitro* propagation methods offer a powerful tool for conservation of germplasm and mass-multiplication of threatened plant species (Murch et al. 2000). They can support the *in situ* and *ex situ* conservation of this rare genotype. Since natural propagation is unable to support the demand, *in vitro* methods could be viable options. Some *in vitro* work on *H. enneaspermus* is available in the literature (Arunkumar and Jayaraj 2011, Velayutham et al. 2012, Premkumar et al. 2013, Sudharson et al. 2014). The present work is more effective in terms of number of multiple shoots regenerated per explant.

Survival of plantlets in field conditions is the major constraint in the micropropagation of *H. enneaspermus*. An *ex vitro* rooting method could help in better acclimatization which increases the chances of field adaptation of plantlets in the natural environment. Improved rooting and acclimatization can be achieved simultaneously with *ex vitro* rooting of *in vitro* propagated shoots (Baskaran and Van Staden 2013).This was found to reduce time, labor, energy involved and the cost factor of micropropagated plantlets (Patel et al. 2014). Therefore, the aim of the present study is to establish *in vitro* methodologies for mass production of this rare plant species using *ex vitro* rooting and to evaluate the optimum conditions for *in vitro* development of plantlets. This is the first report on *ex vitro* rooting of *in vitro* regenerated shoots in *H. enneaspermus*.

The widespread application of *in vitro* regeneration technologies is restricted by the difficulties during transfer of plantlets to the field conditions (Pospíšilová et al. 1999). This is due to the sudden change in the culture environment to relatively harsh environments. The ultimate success of micropropagation depends on successful hardening and field transfer of plantlets. The plants micropropagated in a culture vessel are partially heterotrophic; they acquire some developmental changes to make them fully autotrophic after being transferred to the field. The present study also aimed to investigate the foliar epidermal micromorphological changes during transfer of plantlets from an *in vitro* to a field environment.

## Materials and methods

### Plant material and surface sterilization

*Hybanthus enneaspermus* was selected from the Coromandel Coast (Kanchipuram, Villupuram, Puducherry, Cuddalore, Nagapattinam and Karaikal districts) of India for the present study. Slender young emerging stems were used as the source of explants. The nodal segments (approximately 3.0 cm in length) were harvested from two months old field-grown plant using sterilized surgical scissors. These explants were sterilized with a systemic fungicide (0.1% Bavistin; BASF India Ltd., India) and then under laminar air flow bench with 0.1% $HgCl_2$ (w/v) for 4–5 min. The sterilized explants were washed with autoclaved double distilled water 5–6 times to remove the adhered traces of $HgCl_2$.

### Medium and culture conditions

Murashige and Skoog medium (Murashige and Skoog 1962) augmented with 3% sucrose as carbon source and 50 mg $L^{-1}$ of ascorbic acid and 25 mg $L^{-1}$ each of arginine, adenine sulphate and citric acid were incorporated in the culture medium as additives to initiate the cultures. Culture medium was solidified by 0.8% Agar (Hi-Media, India) to support the proper position of the plant material in the medium. The pH of the medium along with plant growth regulators was adjusted to 5.8±0.02 prior to autoclaving. The cultures were maintained at 25±2 °C under a 12 h photoperiod light regime with a light intensity of 40–50 µmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux density (PPFD) implemented by cool white fluorescent lamps (Philips, India).

### Culture initiation and multiple shoot induction

To establish cultures *in vitro*, stout, green nodal explants were inoculated on MS medium containing different concentrations of cytokinins (6-benzylaminopurine, BAP; kinetin, Kin) (Hi-Media, India) ranging from 0.5–3.0 mg $L^{-1}$ to induce bud break.

Cultures showing bud break were further multiplied by subsequent transfer of *in vitro* regenerated axillary shoot clumps (5–7 shoots) with mother explants by subculturing onto fresh MS medium. The medium was supplemented with additives, BAP and Kin (0.1 to 2.0 mg $L^{-1}$) alone or combinations of optimized concentrations. Subculturing was performed at 4 weekly intervals.

### *In vitro* rooting

Shoots can be harvested after 3–4 subcultures for rooting experiments. For root induction under *in vitro* conditions, multiplied shoots longer than 4–5cm were separated individually from shoot clumps and transferred to different strengths of MS media (full MS, ½ MS and ¼$^{th}$ MS) with various concentrations of auxins (indole-3 acetic acid, IAA; indole-3 butyric acid, IBA; α-naphthalene acetic acid, NAA and naphthoxy acetic acid, NOA) (Hi-Media, India) (1.0 to 4.0 mg $L^{-1}$). The cultures were initially incubated under diffused light conditions (20–25 µmol $m^{-2}$ $s^{-1}$) for 2–3 days for *in vitro* root induction and thereafter transferred to an *in vitro* culture environment, and maintained at a light intensity of 40–45 µmol $m^{-2}$ $s^{-1}$ PPFD with a 12 h photoperiod per day.

### *Ex vitro* rooting of *in vitro* regenerated shoots

Experiments were carried out for *ex vitro* root induction from *in vitro*-produced shoots. Basal end (4–6 mm) of *in vi-*

*tro*-raised shoots were treated with different concentrations of auxins (IAA, IBA, NAA and NOA) (50–400 mg $L^{-1}$) for 5 min and transferred to eco-friendly paper cups containing sterile soilrite® (a combination of perlite with peat moss and exfoliated vermiculite procured from KelPerlite, Bangalore, India) and moistened with one fourth strength of MS basal salts. The cups were kept in the greenhouse for maintenance at 25±2 °C with 80–90% relative humidity (RH). After 4–5 weeks, the rooted plantlets were carefully taken out from the paper cups and transplanted to the nursery polybags in a greenhouse.

### Acclimatization and field transfer of regenerated plantlets

*In vitro* rooted plantlets were taken out cautiously from the culture tubes and rinsed with distilled water to remove adhered nutrients and agar. They were transferred to autoclaved soilrite® in bottles, moistened with 1/4$^{th}$ strength of MS basal salts and maintained in the greenhouse. The *ex vitro* rooted plantlets were acclimatized in paper cups which were covered with transparent polythene cups to provide enough space for gas exchange. The *in vitro* rooted plantlets were acclimatized by gradual loosening and then completely removing the transparent cup of the bottles. These plantlets were subsequently transferred to nursery polybags containing soilrite®, garden soil and organic manure (1:1:1) in the greenhouse for further acclimatization process. Plantlets were transferred to the field after 5 weeks of acclimatization.

### Foliar micromorphological studies of *in vitro* and field transferred plantlets

Experiments were conducted to study foliar micromorphological developments of veins (vein density and venation pattern), stomata types and density, and trichomes in leaves of plants grown *in vitro* after 4$^{th}$ subculture in multiplication phase and in those transferred to the field after 6$^{th}$ week. Plants were randomly selected from both the environments. The entire foliar apparatus (leaves) (10 from each stage of plantlets) third to seventh leaves from the base were excised manually for all the experiments. To observe the changes in structure and functioning of developing stomata, epidermal peels were separated manually by the traditional method (Johansen 1940) from the leaves. The leaves were fixed primarily in formalin–acetic acid–ethyl alcohol, FAA (1:1:3) and cleared in 70% ethanol (v/v) until the chlorophyll was removed (12–24 h), bleached with 5% (w/v) NaOH for 24–48 h, and rinsed three times in distilled water (Sass 1940). The leaves were then stained with 1% safranine (Loba chemie, India) aqueous solution for 4–8 min and rinsed carefully in water to remove excess stain and then mounted in distilled water and examined under microscope (Labomed iVu 3100, USA).

### Experimental design, data collection and statistical analysis

The experiments were performed with 20 replicates per treatment and repeated thrice. Data were subjected to analysis of variance and the significance of differences was calculated by Duncan's multiple range test using SPSS software (version 16.0). Observations were noted at 4 weeks interval.

## Results

### Establishment of cultures and multiplication of cultures *in vitro*

The fresh and light green colored nodal segments responded better than old and dark colored explants. Cultures were initially placed in diffused light (20–25 µmol $m^{-2}$ $s^{-1}$) to induce bud breaking, and further transferred to a culture room with a higher light intensity (40–50 µmol $m^{-2}$ $s^{-1}$) for proper establishment of culture. The MS medium supplemented with 1.5 mg $L^{-1}$ BAP was observed suitable for bud breaking and 97% of the explants responded with 6.4±0.69 shoots from each nodal explants (Fig. 1A, Tab. 1). Maximum

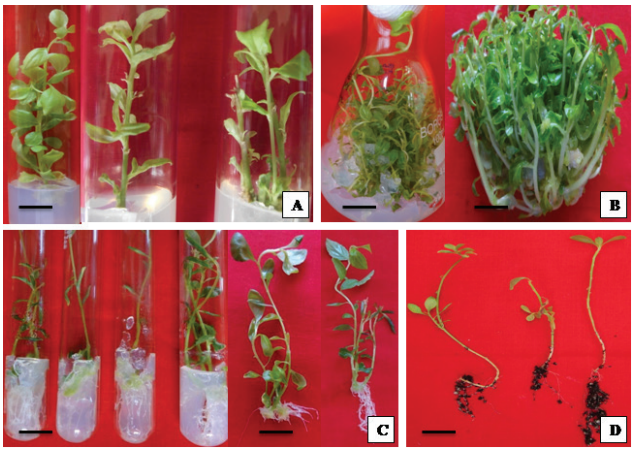


**Fig. 1.** Micropropagation of *Hybanthus enneaspermus*: induction of shoots from the nodal segments with 6-benzylaminopurine (A); multiplication of shoots on MS medium (B); *in vitro* rooting of the excised shoots on MS medium with indole-3 butyric acid (C); *ex vitro* rooted plantlets (D), scale bars = 2 cm.

**Tab. 1.** Effect of different concentrations of cytokinins, 6-benzylaminopurine (BAP) and kinetin (Kin) on bud breaking from nodal explants of *Hybanthus enneaspermus*. Significant variation between the concentrations was studied using Duncan's multiple range test at 0.5% level, SD – standard deviation.

| Cytokinins (mg $L^{-1}$) | Response (%) | Number of shoots (mean ± SD) | Shoot length (cm) (mean ± SD) |
|---|---|---|---|
| 0.00 | 0 | $0.0\pm0.00^{a}$ | $0.00\pm0.00^{a}$ |
| BAP | | | |
| 0.50 | 32 | $2.3\pm1.06^{abc}$ | $3.12\pm0.31^{bcd}$ |
| 1.00 | 56 | $4.2\pm0.28^{bcd}$ | $3.40\pm0.18^{cd}$ |
| 1.50 | 97 | $6.4\pm0.69^{d}$ | $5.60\pm0.49^{e}$ |
| 2.00 | 76 | $4.9\pm1.02^{cd}$ | $4.16\pm0.38^{de}$ |
| 2.50 | 50 | $4.1\pm0.57^{bcd}$ | $3.28\pm0.33^{cd}$ |
| 3.00 | 37 | $2.3\pm1.09^{abc}$ | $2.06\pm0.12^{bc}$ |
| Kin | | | |
| 0.50 | 26 | $2.1\pm0.54^{abc}$ | $1.32\pm0.43^{ab}$ |
| 1.00 | 41 | $2.9\pm0.76^{bc}$ | $2.07\pm0.64^{bc}$ |
| 1.50 | 68 | $3.0\pm0.43^{ab}$ | $2.78\pm0.21^{bcd}$ |
| 2.00 | 59 | $3.6\pm0.47^{bc}$ | $3.08\pm0.30^{bcd}$ |
| 2.50 | 50 | $2.0\pm0.53^{ab}$ | $3.12\pm0.36^{bcd}$ |
| 3.00 | 40 | $2.1\pm0.49^{ab}$ | $2.17\pm0.17^{bc}$ |

response of the explants was observed on MS medium augmented with BAP rather than with Kin in present study.

The maximum number of shoots (228±10.3 shoots with 7.5±0.43 cm length) was regenerated on a subculture of the *in vitro* regenerated shoot clumps on MS medium fortified with 1.0 mg $L^{-1}$ each of BAP and Kin within 4–5 weeks (Tab. 2). The shoot number and shoot length was increased by repetitive subculturing up to 3–4 passages onto fresh medium (Fig. 1B). Six fresh shoots from a single explant transferred to fresh medium yielded a maximum of 228 shoots (228/6=38) within 4–5 weeks. The rate of shoot multiplication increased more than 35 fold in 4–5 weeks. Cream colored callus was observed from the basal part of the cultures if the medium was augmented with auxins (IAA and IBA) along with cytokinins.

### *In vitro* rooting of the shoots

Among the different strengths of MS medium and auxins experimented for *in vitro* root induction, ½ strength MS medium supplemented with 2.0 mg $L^{-1}$ IBA was observed best for *in vitro* rooting (Fig. 1C). Maximum number of roots (25.7±3.90) with highest length (6.21±0.78 cm) were recorded with this medium combination (Tab. 3). Poor response with smaller root number and root length was reported on media fortified with IAA, NAA and NOA.

**Tab. 2.** Effect of different concentrations and combinations of cytokinins 6-benzylaminopurine (BAP), kinetin (Kin), and combination of BAP + Kin on shoot multiplication of *Hybanthus enneaspermus*. Significant variation between the concentrations was studied using Duncan's multiple range test at 0.5% level, SD – standard deviation.

| Cytokinins (mg $L^{-1}$) | Number of shoots (mean±SD) | Shoot length (cm) (mean±SD) |
|---|---|---|
| 0.00 | $0.0\pm0.00^{a}$ | $0.00\pm0.00^{a}$ |
| BAP | | |
| 0.10 | $14\pm0.10^{ab}$ | $4.0\pm0.54^{b}$ |
| 0.50 | $25\pm0.93^{abc}$ | $6.4\pm0.32^{hi}$ |
| 1.00 | $42\pm1.51^{d}$ | $6.6\pm0.23^{i}$ |
| 1.50 | $40\pm7.37^{d}$ | $5.3\pm0.17^{ef}$ |
| 2.00 | $34\pm6.24^{cd}$ | $6.4\pm0.62^{hi}$ |
| Kin | | |
| 0.10 | $19\pm8.91^{bc}$ | $5.0\pm0.44^{de}$ |
| 0.50 | $22\pm6.74^{bc}$ | $4.3\pm0.33^{bc}$ |
| 1.00 | $36\pm5.13^{cd}$ | $4.6\pm0.63^{cd}$ |
| 1.50 | $29\pm7.11^{abc}$ | $5.1\pm1.70^{def}$ |
| 2.00 | $20\pm8.83^{bc}$ | $4.9\pm0.54^{de}$ |
| BAP + Kin | | |
| 0.10 | $98\pm8.76^{e}$ | $5.6\pm1.30^{fg}$ |
| 0.50 | $172\pm9.27^{g}$ | $5.9\pm1.05^{gh}$ |
| 1.00 | $228\pm10.3^{h}$ | $7.5\pm0.43^{j}$ |
| 1.50 | $121\pm10.1^{f}$ | $6.1\pm0.79^{ghi}$ |
| 2.00 | $93\pm9.22^{e}$ | $5.1\pm0.43^{def}$ |

**Tab. 3.** Effect of auxins indole-3 acetic acid (IAA), indole-3 butyric acid (IBA), α-naphthalene acid acid (NAA), and naphthoxy acetic acid (NOA) on *in vitro* root induction, number and length of roots on half strength MS medium. Significant variation between the concentrations was studied using Duncan's multiple range test at 0.5% level, SD – standard deviation.

| Auxins (mg $L^{-1}$) | Response (%) | Number of roots (mean±SD) | Length of root (cm) (mean±SD) |
|---|---|---|---|
| 0.00 | 0 | $0.00\pm0.00^{a}$ | $0.0\pm0.00^{a}$ |
| IAA | | | |
| 1.0 | 26 | $12.9\pm0.16^{d}$ | $2.98\pm0.83^{h}$ |
| 2.0 | 40 | $16.6\pm10.75^{h}$ | $3.00\pm0.38^{h}$ |
| 3.0 | 34 | $16.0\pm1.56^{gh}$ | $2.35\pm0.33^{f}$ |
| 4.0 | 31 | $14.8\pm0.40^{f}$ | $2.05\pm0.20^{de}$ |
| IBA | | | |
| 1.0 | 63 | $18.5\pm3.43^{i}$ | $2.15\pm0.26^{e}$ |
| 2.0 | 98 | $25.7\pm3.90^{k}$ | $6.21\pm0.78^{k}$ |
| 3.0 | 71 | $21.0\pm2.37^{j}$ | $4.18\pm0.39^{j}$ |
| 4.0 | 59 | $13.7\pm1.70^{e}$ | $3.24\pm0.49^{i}$ |
| NAA | | | |
| 1.0 | 29 | $10.5\pm0.80^{b}$ | $1.78\pm0.13^{c}$ |
| 2.0 | 33 | $13.6\pm0.75^{de}$ | $2.90\pm0.23^{h}$ |
| 3.0 | 49 | $15.9\pm0.23^{gh}$ | $1.90\pm0.91^{cd}$ |
| 4.0 | 32 | $11.5\pm0.10^{c}$ | $1.95\pm0.26^{cd}$ |
| NOA | | | |
| 1.0 | 30 | $11.9\pm1.43^{c}$ | $2.71\pm0.83^{g}$ |
| 2.0 | 42 | $13.6\pm1.90^{de}$ | $3.32\pm0.38^{i}$ |
| 3.0 | 54 | $15.3\pm2.37^{fg}$ | $2.93\pm0.33^{h}$ |
| 4.0 | 50 | $10.5\pm1.20^{b}$ | $1.43\pm0.20^{b}$ |

### *Ex vitro* rooting of *in vitro* produced shoots

The basal end of *in vitro*-produced micro-shoots was treated (5 min) with root-inducing growth regulators and transferred to a greenhouse environment. The lower part (4–6 mm) of *in vitro*-regenerated shoots evaluated with 300 mg $L^{-1}$ IBA exhibited about 96% rooting (Tab. 4). A maximum of 12.5±0.54 roots per shoot with 5.10±0.62 cm length was observed within 4 weeks (Fig. 1D). Poorer rooting than with IBA was recorded with all the concentrations of IAA, NAA, and NOA in this study.

### Acclimatization and field transfer of regenerated plantlets

*In vitro* and *ex vitro* rooted plants were acclimatized efficiently in a greenhouse (Figs. 2A–2C). A profusely branched root system was observed in *ex vitro* rooted plantlets during transfer to the field. It resembled the conventional root system obtained under natural conditions. The hardened plantlets were successfully transferred to the field with 92% survival rate (Fig. 2D) but the survival rate of *in vitro* rooted plantlets was only 86%.

### Micromorphological studies of micropropagated plantlets

The plants developed under *in vitro* conditions possessed normal leaves with hairs and denticulate margins. The mid-

**Fig. 2.** Hardening of *Hybanthus enneaspermus* plantlets in the greenhouse (A-C), and *in vitro* raised plantlets under field conditions (D).

**Tab. 4.** Effect of auxins indole-3 acetic acid (IAA), indole-3 butyric acid (IBA), α-naphthalene acid acid (NAA), and naphthoxy acetic acid (NOA) on *ex vitro* root induction from *in vitro* raised shoots. Significant variation between the concentrations was studied using Duncan's multiple range test at 0.5% level, SD – standard deviation.

| Auxins (mg $L^{-1}$) | Response (%) | Number of roots (mean±SD) | Length of root (cm) (mean± SD) |
|---|---|---|---|
| 0.00 | 2 | $0.43\pm0.13^{a}$ | $1.21\pm0.32^{b}$ |
| IAA | | | |
| 50 | 30 | $0.45\pm0.30^{a}$ | $1.19\pm0.54^{b}$ |
| 100 | 45 | $0.62\pm0.11^{e}$ | $2.56\pm0.36^{h}$ |
| 200 | 51 | $1.93\pm0.15^{f}$ | $4.01\pm0.44^{m}$ |
| 300 | 50 | $3.22\pm0.32^{f}$ | $3.92\pm0.19^{l}$ |
| 400 | 43 | $2.07\pm0.28^{cd}$ | $2.05\pm0.30^{g}$ |
| IBA | | | |
| 50 | 35 | $0.96\pm0.21^{c}$ | $3.15\pm0.73^{k}$ |
| 100 | 42 | $2.78\pm0.59^{h}$ | $3.84\pm0.61^{l}$ |
| 200 | 56 | $4.89\pm0.41^{i}$ | $4.10\pm0.49^{n}$ |
| 300 | 96 | $12.5\pm0.54^{l}$ | $5.10\pm0.62^{p}$ |
| 400 | 84 | $5.83\pm0.49^{j}$ | $4.25\pm0.53^{o}$ |
| NAA | | | |
| 50 | 23 | $0.39\pm0.33^{a}$ | $1.03\pm0.69^{a}$ |
| 100 | 35 | $0.73\pm0.21^{b}$ | $1.41\pm0.74^{d}$ |
| 200 | 47 | $2.73\pm0.19^{h}$ | $2.05\pm0.21^{g}$ |
| 300 | 44 | $6.21\pm0.48^{l}$ | $3.91\pm0.28^{l}$ |
| 400 | 39 | $6.10\pm0.30^{k}$ | $3.01\pm0.35^{j}$ |
| NOA | | | |
| 50 | 33 | $0.11\pm0.45^{c}$ | $1.33\pm0.30^{c}$ |
| 100 | 47 | $0.29\pm0.29^{d}$ | $1.49\pm0.49^{e}$ |
| 200 | 56 | $2.39\pm0.16^{g}$ | $1.90\pm0.24^{f}$ |
| 300 | 41 | $3.26\pm0.72^{h}$ | $2.94\pm0.92^{j}$ |
| 400 | 38 | $2.30\pm0.64^{d}$ | $2.70\pm0.62^{i}$ |

rib was fairly prominent projecting equally on both the sides, but bluntly conical on the adaxial side and hemispherical on the abaxial side. Veins and vein-islets were fewer in *in vitro* than in field transferred plantlets (Figs. 3A and 3B). The vein density and distinct vein-islets were increased during the hardening period, and became distinct, rhomboidal and rectangular in shape after field transfer of the plantlets.

The stomata were more frequent in the inter-coastal areas than in the coastal areas, facing all directions with irregular distribution. The stomatal frequency was greater in the *in vitro* environment than in the field transferred plants with anisocytic stomata predominating, but these were non-functional as they were always in open condition (Figs. 3C and 3D). Anisocytic (cruciferous), paracytic (rubiaceous), diacytic (caryophyllaceous), anomocytic (rununculaceous), anisotricytic, isotricytic, tetracytic, staurocytic, desmocytic and pericytic stomata were observed in the *in vitro* produced leaves. Anomocytic and pericytic stomata were occasionally observed in these leaves. The field-transferred plants possessed the aforementioned stomatal types except for the anomocytic and pericytic. Anisocytic and paracytic stomata were prominent but diacytic and desmocytic stomata were rare in this plant. Trichomes were simple, unicellular or uniseriate emerging from the epidermis. Under *in vitro* conditions, the trichomes were unicellular, less frequent and underdeveloped but these were fully developed in field transferred plants after 6 weeks (Fig. 3E). The uniseriate and unicellular hairs were frequent but the bicellular and tricellular hairs were occasionally observed after plantlets were transferred to the field (Fig. 3F). Adaxial surface possessed numerous shaggy trichomes, and the trichome density was found maximum in field transferred plantlets compared to

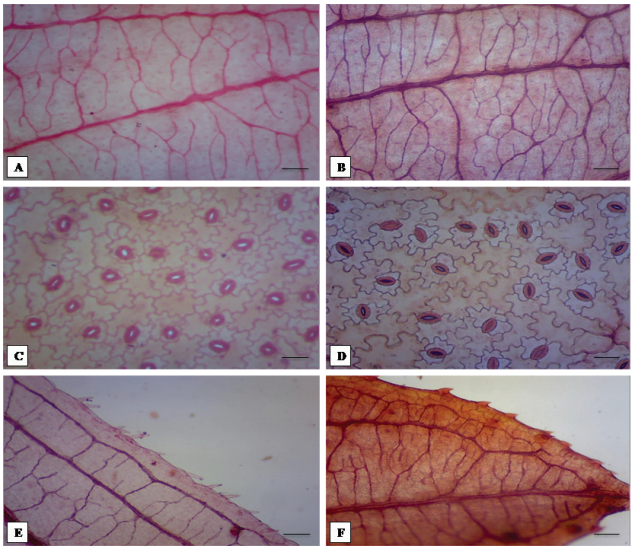

**Fig. 3.** Micromorphological studies of *Hybanthus enneaspermus*: venation pattern in leaves of *in vitro* shoots (A); and field plant (B); stomatal pattern in leaves of *in vitro* shoots (C), and field plant (D); and trichomes in leaves of *in vitro* shoots (E), and field plant (F). Tissues were stained with 1% safranine aqueous solution. Scale bars = 100 μm.

the *in vitro* grown leaves. Mucilaginous cells were also observed in field transferred plants but these were totally absent in the *in vitro* grown plantlets.

## Discussion

The success of tissue culture experiments basically depends on the selection of starting material. The mature explants responded later than the fresh and light green colored nodal segments under diffused light conditions in the present experiment. BAP induced more shoots on MS medium than Kin. Similar results were reported by many researchers recently in a number of plant species (Panwar et al. 2012, Premkumar et al. 2013, Rathore et al. 2013a, Sudharson et al. 2014). Shoot multiplication was achieved by repetitive transfer of mother explants with regenerated shoots onto fresh medium and by subculturing of freshly regenerated shoots isolated from the mother explants. This approach of shoot multiplication has been used in several plant species (Rai et al. 2010, Patel et al. 2014, Shekhawat and Manokari 2016). The higher rate of shoot multiplication during repeated transfer may be due to inhibition of apical dominance which stimulates the basal dormant meristematic cells to produce young shoots (Phulwaria et al. 2013). A maximum of 228 shoots were induced per culture vessel within 4–5 weeks in this study. Premkumar et al. (2013) induced the most (52.3) shoots, when the regenerated shoots were subcultured on MS medium containing IAA along with Kin and BAP. Contrary to this report, callus formation was observed when the medium was supplemented with IAA and IBA along with BAP and Kin in the present study. Sudharson et al. (2014) reported maximum of 11.8 shoots, when the cultures of *H. enneaspermus* were inoculated on MS medium supplemented with 2.0 mg $L^{-1}$ BAP. Maximal 90 shoots were reported in this plant by Velayutham et al. (2012) from the callus cultures on MS medium augmented with BAP and Kin. This supports our findings where the most shoots were regenerated on BAP and Kin, but our results were far better than the earlier reports in multiple shoot formation.

The shoots were rooted maximally on half strength MS medium augmented with IBA. The half strength MS salts and sucrose in medium was appropriate for *in vitro* rooting and supports many authors' findings in different plant species (Rai et al. 2010, Premkumar et al. 2013, Patel et al. 2014). We report more roots (25.7±3.90) per shoot in this study than were found in earlier works on *H. enneaspermus*. Maximum 2.8 roots per shoot was reported by Prakash et al. (1999), 5–8 roots by Velayutham et al. (2012) and 21.3 roots by Premkumar et al. (2013) in this plant species. The superiority of IBA over other auxins for root induction has been recognized by several researchers in a number of plants (Barreto and Nookaraju 2007, Rai et al. 2010, Rathore et al. 2013b). Plants rooted under *ex vitro* environment were better suited to natural conditions and reported easy to harden (Yan et al. 2010). It has been reported that *ex vitro* rooted plants are better suited to tolerate environmental stresses (Pospíšilová et al. 1999, Tiwari et al. 2002, Shekhawat et al. 2015a). About 96% shoots were rooted with IBA with maximum 12.5 roots per shoot in this report. IBA is more effective than NAA and NOA in *ex vitro* root induction in many plant species, and applied economically worldwide (Debergh et al. 1992, Yan et al. 2010, Ranaweeraa et al. 2013, Shekhawat et al. 2015b). This is the first report on *ex vitro* rooting of *in vitro* regenerated shoots in *H. enneaspermus* with maximum rate of survival under natural conditions. The rooted plantlets were hardened in greenhouse with development of profusely branched root system in *ex vitro* rooted plantlets. About 92% *ex vitro* rooted and 86% *in vitro* rooted plantlets survived in the field conditions. *Ex vitro* rooting reduced the time, energy of production of plantlets and mortality during hardening and field transfer. Normal flowering and fruiting was observed in the field transferred plantlets.

These micromorphological studies of micropropagated plantlets were performed to understand the developmental changes in the leaves of plantlets, when they were transferred to field conditions. The stomata were present on both surfaces of the leaf but the frequency was less on the adaxial surface (Narayanaswamy et al. 2006, Retnam and Britto 2007) therefore, the abaxial surface was further considered for the study. The specific artificial conditions *in vitro* are responsible for the structural changes occurring in micropropagated plantlets. The lesser stomatal density under field conditions may help to check the rate of transpiration and prevent water loss (Singh et al. 2003). Transitional types of stomata between anisocytic and paracytic are also present in *H. enneaspermus* (Inamdar 1969). Anisotricytic and isotricytic stomata could be the transitional form between anisocytic and paracytic types of stomata. Unicellular, less frequent and underdeveloped trichomes were observed under *in vitro* conditions but fully developed trichomes were reported in field-transferred plants. The mucilaginous cells were not observed with the *in vitro* leaves but found in field-transferred plants. Our findings are supported by the results of various researchers (Chandra et al. 2010, Rathore et al. 2013b, Lodha et al. 2015). Understanding the changes in foliar micromorphology of *in vitro* grown and hardened plantlets could be useful in improvement of *in vitro* clonal propagation protocols and for large scale production of plants.

## Conclusion

An efficient *in vitro* propagation protocol has been developed using various plant growth regulators for successful conservation of this rare plant species. Excellent rate of shoot multiplication was achieved *in vitro*. *Ex vitro* rooting has been successfully demonstrated in *H. enneaspermus,* which could save time, labor and energy in production of plantlets. The hardened plantlets were successfully transferred to the field with a 92% survival rate. The results of foliar micromorphological studies could help in understanding the response of plantlets under field conditions. The data could contribute significantly to meeting the market demand for this multipotent healing herb and conservation of this valuable genotype through biotechnological interventions.

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