Physiol Mol Biol Plants (July–September 2015) 21(3):395–406
DOI 10.1007/s12298-015-0302-6

RESEARCH ARTICLE

L-Dopa production and antioxidant activity in *Hybanthus enneaspermus* (L.) F. Muell regeneration

Ganeshan Sivanandhan [1,2] · Venkatachalam Vasudevan [1] · Natesan Selvaraj [3] · Yong Pyo Lim [2] · Andy Ganapathi [1]

Received: 9 February 2015 / Revised: 26 April 2015 / Accepted: 2 June 2015 / Published online: 21 June 2015

Abstract *Hybanthus enneaspermus* is an ethanobotanical plant extensively used in Indian traditional medicine. Quick and efficient in vitro mass propagation of this plant species was established for commercial utilization from leaf and node explants using various concentrations and combinations of plant growth regulators and polyamines. The maximum number of multiple shoots per leaf explant (40 shoots) was achieved on MS medium supplemented with 20 mg/l spermidine in combination with 4 mg/l BA+1.5 mg/l IAA after 8 weeks of culture. The elongated shoots were rooted (16 roots/shoot) on MS medium with the best concentration of IBA (1.5 mg/l) and in combination with 20 mg/l putrescine after 5 weeks of culture. The plants were successfully acclimatized (98 %) in the sand: soil: vermiculite mixture (1:1:1 *v/v/v*) in the greenhouse. An increased antioxidant activity was recorded in vitro regenerated shoots when compared to in vitro-induced roots. L-Dopa content was recorded higher in leaves (8.31 mg/g DW) followed by stem (6.22 mg/g DW) and root (3.22 mg/g DW) of leaf-derived plants than the field-grown parent plant after 5 weeks. By adopting this protocol, the regenerated-plants could be used for drug production and pharmacology work with as an alternative to field-grown plants.

Keywords Antioxidant · *Hybanthus enneaspermus* · HPLC · L-Dopa · Polyamines · *Violaceae*

Abbreviations

2-iP	6-(α,α-Dimethylallylamino)-purine
2,4-D	2,4-Dichlorophenoxyacetic acid
BA	6-Benzyladenine
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
HPLC	High Performance Liquid Chromatography
MS	Murashige and Skoog medium
PAs	Polyamines
TDZ	Thidiazuron
PAR	Photosynthetic Active Radiation
RH	Relative Humidity
Kn	Kinetin
Zea	Zeatin
DPPH	2 2-diphenylpicrylhydrazyl

Introduction

Hybanthus enneaspermus (L.) F. Muell, (*Violaceae*), is a small, diffuse, perennial herb or under shrub found in the tropical and subtropical regions of the world (Sahoo et al. 2006). This plant has been used for centuries in traditional medicine for the treatment of gonorrhoea, urinary infections, antitussive, strangury, vomiting, wandering of the mind, urethral discharges, blood disorders, asthma, epilepsy, cough, as aphrodisiac, demulcent, anticonvulsant and for its tonic

✉ Ganeshan Sivanandhan
gsivabdu@gmail.com

✉ Andy Ganapathi
aganapathi2003@rediffmail.com

[1] Department of Biotechnology and Genetic Engineering, School of Biotechnology, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India

[2] Molecular Genetics and Genomics Laboratory, Department of Horticulture, College of Agriculture and Life Sciences, Chungnam National University, Daejeon 305 764, South Korea

[3] Department of Botany, Periyar E. V. R College (Autonomous), Tiruchirappalli 620 023, Tamil Nadu, India

properties (Hemalatha et al. 2003; Boominathan et al. 2004; Weniger et al. 2004; Sahoo et al. 2006). In addition to that, the drugs obtained from this plant are used for antiplasmodial (Weniger et al. 2004), anti-inflammatory (Boominathan et al. 2004), anti-arthritic (Tripathy et al. 2009), hepatoprotective, curative, and anti-diabetic (Patel et al. 2011). The plant contains alkaloids, flavonoids, triterpenes, phenols, flavones, anthraquinones, L-dopa and Diosgenin (Anbu and Subramanian 2011; Patel et al. 2013). Among them, L-Dopa has majorly used as a drug for the treatment of Parkinson's disease. L-Dopa is commercially available in many trade forms such as Stalevo®, Sinemet®, Parcopa® and Atamet® (Ali and Haq 2006). Annual production of L-Dopa is 250 t and the total pharmaceutical market value is $101 billion/year (Koyanagi et al. 2005). Due to the presence of high valued medicinal properties as well as high market demand, the plant has been uprooted and utilized by many pharmaceutical industries for commercial drug preparation (Shantha et al. 2001). 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 huge. Increase in human and livestock population have already affected the status of field-grown plants, mostly those utilized in Ayurvedic and Siddha (Prakash et al. 1999). Owing to its importance, there is essential to conserve and propagate the *H. enneaspermus*. Under these circumstances, it is, therefore, dynamic to open steps for large-scale multiplication of this plant for sustainable production of secondary metabolites and its conservation. To overcome these conditions, biotechnological approaches can be applied to mitigate secondary metabolite production through cell/organ cultures to sustain the demands (Sivanandhan et al. 2012a, b).

Internationally, plant cell, tissue and organ culture has persisted as an indispensable plant biotechnology platform for the production and conservation of plants (Amoo et al. 2012). Production of a large number of plants from selected superior chemotypes using a single explant by in vitro culture has been suggested as an attractive means of propagation to maintain product uniformity (Sivanandhan et al. 2011). Moreover, by implementing plant cell, tissue and organ culture approaches, secondary metabolites production in association with pharmacological important medicinal plants can be greatly increased and developed. Recently, in our earlier studies, we have significantly enhanced secondary metabolites production in various in vitro techniques (Sivanandhan et al. 2011). In many cases, differentiated cultures tend to accumulate secondary metabolites in quantities higher than dedifferentiated cultures (Matkowski 2008; Amoo et al. 2012).

The only two reports available till now are restricted to in vitro regeneration of *H. enneaspermus* (Prakash et al. 1999; Premkumar et al. 2013) but failed to reveal any application due to low frequency of in vitro response. However, there is a need to optimize the protocol for increased production and to overcome innate tissue culture-related problems. Among the various factors, type and concentration of cytokinin and auxins, interaction of cytokinins with auxins or vice versa, type and concentrations of polyamines (PAs), explant type and selection of superior chemotype are very crucial for successful mass propagation and secondary metabolites production. None of the developed in vitro regeneration protocols, to the best of our knowledge, has evaluated the roles of different types and concentrations of polyamines in addition to cytokinin and auxin for mass propagation in *H. enneaspermus*. Exogenous PAs treatment in plant regeneration prompted growth and differentiation, and led to shoot induction and proliferation. Hence, the current experiment was designed to develop an effective regeneration system for rapid propagation by applying PAs in growth medium (Martin-Tanguy 2001). L-Dopa production and antioxidant activities were performed in in vitro plants as well as parent plants for the first time in *H. enneaspermus.*

Materials and methods

Plant material

Wild population of *H. enneaspermus* plants were collected from Tiruchirappalli, Tamil Nadu, India and taxonomically identified (Shantha et al. 2001). The collections were designated as 'HE-TRY01'. Mature seeds of HE-TRY01 were collected and they were sown in the Experimental garden, Dept. of Biotechnology and Genetic Engineering, Bharathidasan University, Tiruchirappalli to raise plants for explant source.

Surface sterilization of the explants

The leaf and nodal explants were cut from 2-month-old field-grown plants and they were washed thoroughly in running water to remove the dust and other particulate matters adhering on the surface. Then, they were washed with distilled water and drenched in Teepol® (2.5 %; *v/v*) for 5 min. After that the explants were washed thoroughly 3–4 times with sterile distilled water. The cleaned explants were moved to the inoculation chamber and surface sterilized with 0.1 % $HgCl_2$ (*w/v*) for 1 min followed by rinse in EtOH for 1 min. Finally the explants were washed thoroughly in sterilized distilled water to ensure that the last traces of $HgCl_2$ and EtOH were removed.

Culture media and culture conditions

The nutrient medium consisting of MS mineral salts (Murashige and Skoog 1962), and vitamins with 3 % (*w/v*) sucrose (SRL, India) and 0.2 % (*w/v*) phytagel (Sigma, USA) was used in all the experiments. The pH was adjusted to 5.8 by

adding 1 N NaOH/1 N HCl prior to autoclaving at 1.06 kg cm^{-2} (121 °C) pressure for 20 min. The MS medium was distributed in 10 and 30 ml aliquots in test tube and 150 ml Erlenmeyer flask (Borosil, India), respectively. All cultures were incubated in a culture room at 25±2 °C under a 16/8 h photoperiod with 50 μmol m^{-2} s^{-1} photosynthetic active radiation (PAR) provided with cool-white fluorescent tubes (4 tubes, 40 W, Philips, India) and 60±5 % of relative humidity (RH).

Optimization of cytokinin type and concentration on multiple shoot production

To standardize the optimum concentration and type of cytokinins, node (10 mm) and leaf segments (15 mm) were placed onto MS medium supplemented with various concentrations of BA (0–5 mg/l); Kin, Zea, 2iP and TDZ (0.5–2 mg/l). The MS medium without cytokinin was used as a control. The cultures were retained as reported earlier for 8 weeks and subcultured once at the end of fourth week. After 8 weeks of culture, the frequency of responding explants, mean number of shoots per responsive explant, and mean shoot length were assessed.

Optimization of cytokinin interaction with auxins on multiple shoot production

To validate the contact of cytokinin with auxins, both types of explants were placed on MS medium containing BA (4.0 and 3 mg/l for leaf and nodal explants respectively) with different concentrations (0.5–2.0 mg/l) of IAA, NAA and IBA for higher recovery of multiple shoot production. Optimal concentration of BA was selected based on the preceding experiment of this investigation. The MS medium without auxin was used as a control. The cultures were maintained and subcultured as described earlier. After 8 weeks of culture, the frequency of responding explants, mean number of shoots per responsive explant, and mean shoot length were assessed.

Effects of type and concentrations of auxins on root induction

After 8 weeks of culture, shoots longer than 5 cm regenerated from leaf and node explants were selected and transferred to MS medium containing IBA, IAA, and NAA (0.5–2.0 mg/l) individually for 5 weeks to optimize root induction. The MS medium without auxin was used as a control. The cultures were maintained as described earlier. After 5 weeks of culture, the frequency of responding shoots, mean number of roots per responsive shoot, and mean root length were assessed.

Impact of polyamines on shoot multiplication and rooting

PAs such as spermidine, spermine and putrescine at the concentration of 5, 10, 15, 20 and 25 mg/l were added individually to the optimal concentration of BA (4 and 3 mg/l) and NAA (1.5 mg/l) to further enhance the shoot proliferation from both the explants. The control was served as the explant cultured in the MS medium without PAs. The cultures were incubated and subcultured as described above. After 8 weeks of culture, the shoot number, their length, and frequency of cultured explants producing shoots were recorded and tabulated. After 8 weeks, shoots longer than 5–6 cm were selected and transferred to MS medium supplemented with different concentrations of PAs (spermidine, spermine and putrescine-5, 10, 15, 20 and 25 mg/l) in a combination with IBA (1.5 mg/l) for root induction. Appropriate control was maintained for each experiment. After 5 weeks of culture, the root number, length and response of cultured explants producing roots were recorded and tabulated.

Acclimatization

After 5 weeks, the rooted plants were washed thoroughly in running tap water to remove the phytagel and transferred to paper cups containing a mixture of autoclaved sand, soil, and vermiculite (1:1:1 *v*/*v*/*v*). Potted plants were grown in a growth chamber at 75 % relative humidity (RH) for 4 weeks, and then moved to greenhouse for 4 weeks before transfer to the field. Initially, plants were covered with polyethylene bags to maintain high humidity (75 %) and supplied with water daily. The polyethylene bags were gradually removed when the plants had shown signs of acclimatization and subsequently transferred to the garden.

Comparative DPPH radical scavenging activity in in vitro and field-grown plant parts

Antioxidant assay of the various plant samples was performed as described by Baskaran et al. (2014).

HPLC analysis and L-Dopa estimation

The dried powder of in vitro and in vivo plant parts (shoot and root) were extracted with analytical grade methanol. Known weights of the samples (1 g DW) were soaked in the analytical grade methanol and the samples were sonicated for 30 min. After 30 min sonication, the samples were kept at 48 °C under reduced pressure in dark. The solvent was swapped every day for seven consequent days to assure ample extraction before drying under reduced pressure. After 7 days, the samples were subjected to centrifugation at 8000 rpm for 15 min (at room temperature). The supernatant was filter-sterilized through 0.22 μm syringe filter prior to HPLC analysis. L-Dopa

estimation was determined using a Shimadzu High Throughput HPLC system (Shimadzu Analytical Pvt. Ltd., India) equipped with a UV detector and Agilent® column C18 (5 μm; 250×4.6 mm). Twenty microliters of a syringe-filtered (0.22 μm) samples were injected into the column and eluted isocratically with HPLC-grade methanol and water (65:35 *v*/*v*) (Himedia, Mumbai, India) at a flow rate of 1.0 ml/min. L-Dopa were detected with a UV detector at a wavelength of 250 nm. Standard sample of L-Dopa (RT 2.6 min) were obtained from Sigma-Aldrich (USA).

Statistical analysis

A completely randomized design was used for the present study. The tests were repeated thrice with three replicates. Data were presented as the mean ± standard error (SE). The mean separations were carried out using Duncan's multiple range test and significance was determined at the 5 % level (SPSS 17.5).

Results and discussion

Optimization of cytokinin type and concentration on multiple shoot production

In order to estimate the ability of different explants (leaf and nodal segments) to induce multiple shoots, different cytokinins at different concentrations were tested. Leaf and nodal explants reared on control medium (MS basal medium) did not stimulate meristematic cells formation and axillary bud initiation and eventually attained necrosis, whereas, the presence of plant growth regulators supported axillary bud initiation and substantial improvement in culture was accomplished. The cytokinin type and its concentration had impressive impacts on the mean number of shoots produced per cultured explant type and shoot length. The morphogenic response of both explants is summarized in Table 1. The leaf explants cultured on MS medium augmented with cytokinins caused enlargement of leaf and swelling over the explants after 3 days of culture, whereas, bulging of the nodal explants was noticed after 2 days. In these conditions, the both explants have a capability to concurrently regenerate the multiple shoots from them. After 1 week of culture, multiple shoot buds were initiated at the axillary regions and meristematic region of node and leaf explants respectively. These multiple shoot buds with their explants were subcultured recurrently on the same concentration of fresh medium for effectual multiplication and proliferation. Among the various cytokinins analysed, MS medium supplemented with 4 and 3 mg/l BA was most effective for multiple shoot production with a mean number of 28 shoots/leaf explant and 18 shoots/nodal explants respectively after 8 weeks of culture followed by Zea at 1.5 mg/l for leaf (23 shoots/explant) and node (14 shoots/explant) on same culture period (Table 1). Nevertheless, multiple shoot bud induction was encouraged in all the concentrations of BA and Zea tested, the highest number of shoots and maximum percent of shoot regeneration from both the explants were documented on MS medium augmented with BA only when compared to Zea. Kaminek (1992) stated that changes in the morphogenic responses of cytokinins like BA can be clarified by their several translocation rates to meristematic regions and metabolic process, in which the cytokinins may be degraded and conjugated with physiologically inert compounds. Similarly, BA has extensively influenced the multiple shoot regeneration efficiency in *Chrysanthemum morifolium* (Song et al. 2011), *Withania somnifera* (Sivanandhan et al. 2011; Sivanandhan et al. 2013) and *Naravelia zeylanica* (Benson et al. 2014). Multiple shoot production rates stated in the present study are apparently very high 3.5-and 2.25-fold from leaf and nodal explants respectively when compared to those reported by previous investigations; Prakash et al. (1999) obtained maximum 11 shoots from seed-derived light-green compact callus of *H. enneaspermus* on MS medium containing 1.86 mM KH_2PO_4, 500 mg/l casein hydrolysate, 8.8 μM BA and 2.6 μM NAA. Premkumar et al. (2013) reported the production of 6 shoots and 8 shoots per young leaf explant in MS medium containing 4.44 μM BAP and 4.64 μM Kn respectively after 4 weeks of culture. However, in our study, we significantly obtained increased number of multiple shoots from both the explants. Overall, number of multiple shoots as well as percent of shoot regeneration response for both the explants was increased by increasing the cytokinins concentrations up to optimal level. Multiple shoot bud regeneration was gradually decreased when the cytokinin concentration was increased beyond the optimal level. Exogenously applied cytokinins alter the development of axillary meristems, promote proliferation of the meristematic cells in the axillary buds and increase the number of bud primordia from the pre-existing meristems (Carmen et al. 2001). Sivanandhan et al. (2013) reported that the nutrient components in the medium and exogenously added cytokinins stimulated and favoured the development of growth of multiple axillary buds and two to three fold enhancement in shoot production can be fabulously attained within a shorter period under optimized culture conditions. In the present investigation, increased multiple shoot production was noticed at all cytokinin concentrations tested with the exclusion of treatments with 2, iP for both types of explants. Moreover, TDZ had impeded effects on both shoot multiplication and its elongation. When TDZ was added to the culture medium, bulky hard calli were appeared at the base of explants, and correspondingly with increasing the concentration of TDZ, the detrimental effects on shoot multiplication was perceived and the cultures could not be sustained (data not presented). In the present study, we

Table 1 Effects of types and concentrations of cytokinins on multiples shoot production in *H. enneaspermus* after 8 weeks of culture

Cytokinins concentration (mg/l)	Multiple shoot regeneration frequency (%)		No. of multiple shoots/explant		Length of the shoot (cm)	
	Leaf	Node	Leaf	Node	Leaf	Node
Control	0.0g	0.0f	0.0l	0.0k	0.0j	0.0g
BA						
1	59.6d	51.4c	12.15±0.70h	10.21±0.72de	4.52±0.34cd	4.35±0.24ab
2	68.3c	62.5b	17.21±0.63e	15.27±0.71b	4.82±0.35bc	4.61±0.16a
3	74.5b	78.7a	22.28±0.74c	18.22±0.52a	5.26±0.26ab	4.81±0.18a
4	82.7a	65.9b	28.23±0.76a	14.28±0.63b	5.44±0.18a	4.59±0.18a
5	78.6b	52.3c	24.26±0.54b	11.28±0.34cd	5.13±0.24ab	4.39±0.18ab
Zea						
0.5	56.5d	45.3d	7.29±0.23j	4.26±0.25i	4.05±0.16def	3.82±0.16cd
1.0	65.3c	57.8c	14.23±0.31fg	10.29±0.25d	4.28±0.17cde	4.02±0.19bc
1.5	78.7b	68.6b	23.21±0.44bc	14.27±0.32b	4.85±0.20abc	4.76±0.06a
2.0	62.4c	52.0c	17.28±0.29e	12.29±0.27c	4.49±0.23cd	4.38±0.24ab
TDZ						
0.5	61.7c	56.5c	8.28±0.34j	6.21±0.29g	3.33±0.25ghi	3.13±0.26ef
1.0	68.5c	63.9b	14.23±0.35fg	11.28±0.22cd	3.54±0.12fg	3.21±0.21ef
1.5	57.6d	52.7c	19.29±0.30d	15.23±0.23b	3.81±0.04efg	3.44±0.18de
2.0	50.4d	47.3d	12.24±0.17h	10.29±0.28d	3.71±0.17efg	3.34±0.10de
Kin						
0.5	46.4e	44d	12.28±0.26h	8.28±0.14e	3.37±0.13ghi	3.06±0.06ef
1.0	64.7c	50c	17.27±0.30e	12.27±0.16c	3.51±0.12fgh	3.23±0.13ef
1.5	55.4d	47d	15.29±0.11f	7.29±0.1f	3.34±0.10ghi	3.15±0.09ef
2.0	52.3d	41d	10.24±0.09i	4.27±0.23i	3.20±0.08ghi	3.09±0.13ef
2iP						
0.5	38.5f	34e	4.28±0.90k	2.28±0.05j	2.82±0.07i	2.70±0.09f
1.0	45.8e	42d	8.27±0.05j	4.27±0.13i	2.80±0.10i	2.86±0.15ef
1.5	54.6d	49d	13.29±0.25gh	9.25±0.10de	3.07±0.14hi	2.93±0.19ef
2.0	42.4e	39e	7.25±020j	5.26±0.03gh	2.91±0.15hi	2.86±0.26ef

Control: MS basal medium. Values represent the mean ± standard error of three experiments. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5 % level

reached a rapid and higher number of multiple shoots from leaf and nodal explants. From the present investigation, analysis of variance shown that means shoot number as well as percentage of response were significantly affected by the type and concentration of cytokinins tested; BA can induce in vitro development of meristems from both explants of *H. enneaspermus*.

Optimization of cytokinin interaction with auxins on multiple shoot production

The morphogenic responses of leaf and nodal explants assessed to different auxins (IBA, IAA and NAA) at various concentrations (0.5–2.0 mg/l) with optimal concentration of BA (4 and 3 mg/l for leaf and nodal explants respectively) showed an obvious result on multiple shoot production. Of three different auxins tested, NAA was found to be more efficient in combination with BA than other auxins with regard to induction and subsequent proliferation of multiple shoots from both explants (Table 2). In most cases, inclusion of NAA with BA in the medium significantly improved shoot number when compared to other auxins, suggesting a synergistic/additive effect of NAA with BA on shoot induction and proliferation in *W. somnifera* (Sivanandhan et al. 2011). Auxins have known to reveal synergistic, antagonistic and additive interactions with cytokinins at multiple levels (depending on the plant species and tissue type) in regulating physiological response (Coenen and Lomax 1997). Nordström et al. (2004) described that while the active cytokinin level in plant can be regulated by auxin and vice versa and in addition to that, they explicated that auxin is a hasty and potent regulator of cytokinin biosynthesis compared to the reverse (where cytokinin regulated auxin synthesis). NAA is the only auxin that does not require active uptake to easily

Table 2 Effects of types and concentrations of auxins along with optimal concentration of cytokinin on multiples shoot production in *H. enneaspermus* after 8 weeks of culture

Auxins concentration (mg/l)	Multiple shoot regeneration frequency (%)		No. of multiple shoots/explant		Length of the shoot (cm)	
	Leaf	Node	Leaf	Node	Leaf	Node
Control	82.7a	78.7b	28.24±0.49d	18.25±0.33f	5.41±0.22a	4.85±0.03b
IBA						
0.5	79.4b	75.3b	26±0.31ef	21±0.83e	5.30±0.17a	5.10±0.28ab
1.0	83.3a	79.4b	30.2±1.11c	25.4±0.50c	5.40±0.11a	5.20±0.11ab
1.5	77.7b	72.2b	24.4±0.50fg	18.6±0.50f	5.36±0.12a	5.14±0.12ab
2.0	73.8b	68.1c	20.8±0.58h	15.4±0.50g	5.20±0.08a	5.26±0.10ab
NAA						
0.5	80.1a	75.3b	26.4±0.50e	21.4±0.97e	5.44±0.10a	5.22±0.11ab
1.0	84.4a	79.7b	30.2±0.66c	25.4±0.50c	5.50±0.05a	5.38±0.08a
1.5	88.6a	83.5a	34.2±0.58a	29.4±0.50a	5.66±0.16a	5.40±0.15a
2.0	82.7a	76.6b	28.4±0.50cd	23.6±0.50d	5.52±0.17a	5.30±0.16ab
IAA						
0.5	76.5b	72.7b	24.2±0.48g	19.2±0.37f	5.36±0.15a	5.14±0.12ab
1.0	80.8a	76.8b	28.4±0.67cd	23.2±0.37d	5.44±0.08a	5.22±0.19ab
1.5	85.2a	80.5a	32±0.31b	27.2±0.37b	5.50±0.15a	5.32±0.16ab
2.0	79.1b	73.4b	26.2±0.48e	21.4±0.50e	5.28±0.5a	5.20±0.15ab

Control: MS medium supplemented with 4 mg/l BA or 3 mg/l BA for leaf and nodal explants respectively. Values represent the mean ± standard error of three experiments. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5 % level

pass through the plasma membrane into plant cells (Nordström et al. 2004). According to these statements, in the present study, a maximum frequency (88 %) of multiple shoot induction was obtained on MS medium supplemented with NAA (1.5 mg/l) and BA (4 mg/l) which produced 34 shoots/leaf explant with an average shoot length of 5.6 cm after 8 weeks of culture. Similarly, the highest overall number of shoots (29 shoots/ nodal explant) and maximum response (83 %) were recorded for nodal explants in the MS medium containing NAA (1.5 mg/l) supplemented with BA (3 mg/l) after 8 weeks of culture (Table 2). The auxins role in combination with cytokinin on explants regeneration ability may be responded differentially due to the attribution of endogenous hormones level in the explants (Sivanandhan et al. 2011). Consequently, other auxins tested in this experiment did not favour multiple shoot production when compared to NAA levels. Increasing or decreasing the level of auxins in the culture medium led to negative effect on multiple shoot proliferation. Despite these observations, a higher range of auxins concentrations (beyond the optimal level) in the medium has adversely affected the regeneration potential of both explants. It might be due to the increased level of endogenous or exogenous auxins concentrations in the medium. This ultimately barred the meristematic cell divisions, resulting in the decreased reactions as opined by Wernicke and Milkovitz (1987). The same trend was exactly documented by Sivanandhan et al. (2011) in multiple shoot culture of *W. somnifera*. The hormonal treatment optimised in the current experiment provided a 1.1- and 1.6-fold increment in the number of regenerated shoots from leaf and nodal explants, respectively when compared to the highest number perceived in the present experiment in treatments with cytokinin alone. Other literatures have correspondingly reported the synergistic/additive effect of auxins with cytokinins on shoot proliferation in many medicinal plants, for instance *W. somnifera* and *Aloe arborescens* (Sivanandhan et al. 2011; Amoo et al. 2012; Sivanandhan et al. 2013).

Effects of type and concentrations of auxins on root induction

Successful hardening and acclimatization process depends on efficient rooting of regenerated shoots and existence of the plants under field situation (Sivanandhan et al. 2011). In the present study, root induction was observed within 10–15 days in shoots regenerated from leaf explants whereas those regenerated from nodal explants took 15–20 days to initiate roots from the shoots on MS medium containing auxins. The MS basal medium served as a control exhibited no response in rooting (Table 3). Addition of any of these tested auxins was essential for root induction in elongated shoots of *H. enneasperms*. All the treatments supported root induction, but the rate of response differed significantly between the tested auxins. Shoots derived from both explants showed differential

Table 3 Effects of auxins on rooting of in vitro elongated-shoos derived from leaf and nodal explants of *H. enneaspermus* after 5 weeks of culture

Auxins concentration (mg/l)	Rooting frequency (%)		No. of roots/shoot		Length of the root (cm)	
	Leaf	Node	Leaf	Node	Leaf	Node
Control	0.0c	0.0d	0.0i	0.0h	0.0e	0.0f
IBA						
0.5	78.4b	70.4a	6.2±0.37f	2.4±0.24f	5.30±0.07b	4.64±0.05bc
1.0	82.1a	74.5a	9.2±0.37cd	5.2±0.48cd	5.60±0.08ab	4.80±0.18ab
1.5	86.3a	78.8a	12.2±0.37a	8.8±0.37a	5.82±0.03a	5.10±0.13a
2.0	83.7a	75.3a	10.6±0.24b	6.2±0.48bc	5.70±0.07a	5.08±0.08a
IAA						
0.5	74.4b	64.5b	4.2±0.37g	2.6±0.24ef	4.54±0.08cd	4.16±0.30d
1.0	79.8b	70.7a	8.4±0.24de	4.8±0.37d	4.52±0.09cd	4.18±0.12d
1.5	83.6a	74.8a	10.2±0.37bc	6.6±0.40b	4.72±0.19c	4.26±0.06cd
2.0	80.7a	71.1a	9.6±0.40bc	5.4±0.24cd	4.60±0.11cd	4.30±0.20cd
NAA						
0.5	67.2	59.6c	1.4±0.24h	1.2±0.20g	4.24±0.14d	3.50±0.11e
1.0	73.3b	64.8b	4.8±0.37g	2.6±0.24ef	4.32±0.22d	3.64±0.09e
1.5	77.6b	68.2b	7.8±0.37e	4.8±0.37d	4.48±0.11cd	3.74±0.10e
2.0	75.5b	66.7b	5.2±0.20g	3.6±0.24e	4.30±0.08d	3.60±0.07e

Control: MS basal medium. Values represent the mean ± standard error of three experiments. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5 % level

parameters upon different type and concentrations of auxin treatment. The maximum rooting percentage (86 %) and highest number (12 roots/shoot) of roots with an average root length (5.8 cm/shoot) were achieved on MS medium supplemented with IBA (1.5 mg/l) after 5 weeks of culture (Table 3). Similarly, for nodal explants, the greatest response of rooting was 8 roots per shoot, obtained on medium supplemented with 1.5 mg/l IBA. Shoots from nodal explants on this treatment had the average root length of 5.1 cm and the highest percentage (78 %) of rooting shoots (Table 3). In the present study, other auxins such as IAA and NAA did not show significant role on rooting from the regenerated elongated shoots when compared to IBA. Minocha (1987) pointed out that IBA has been widely used as root-inducing hormone both under in vitro or in vivo conditions. Siva et al. (2009, 2012) reported NAA and IBA influenced high frequency of rooting in *Oldenlandia umbellate*. The provoked role of IBA on root induction has also been reported in many medicinal plants like *H. enneaspermus* (Prakash et al. 1999);*W. somnifera* (Sivanandhan et al. 2011); *Aloe arborescens* (Amoo et al. 2012). Prakash et al. (1999) recorded a mean number of 2.8 roots/shoot on MS medium supplemented with IBA 4.8 μM. Inclusion of higher levels of IBA beyond or below the optimal level reduced the percentage of shoots producing roots, number of roots per shoot and root length. The shoots regenerated from both explants transferred on NAA containing medium produced callus interspersed with very thin roots (data not shown) as observed by Nitnaware et al. (2011) in *Phyllanthus amarus*. Such shoots failed to survive in soil during hardening. Callus formed at the shoot base can interfere with the connection between the shoot and root, resulting in poor root formation and on transfer, such plantlets do not survive under field conditions (Nitnaware et al. 2011).

Impact of polyamines on shoot multiplication and rooting

In order to explore the regenration ability with higher multiple shoots, various levels of PAs have examined with continuous of the preceding study in this experiment. A varied pattern was noticed with different concentrations of different polyamines as summarized in Table 4. MS medium supplemented with different concentrations of spermidine (5–25 mg/l) stimulated meristematic cells from lead explants after 5 days of culture, and 20 mg/l spermidine in combination with 4 mg/l BA + 1.5 mg/l NAA was found to be an optimum for maximum (40 shoots/leaf explant) shoot induction with 94 % responses after 8 weeks of incubation and at the end of the culture, each shoot grew to a length of 6.7 cm (Table 4; Fig. 1a). Similarly, maximum shoot induction response (89 %) and multiple shoot production (34 shoots/nodal explant) were recorded on MS medium supplemented with 20 mg/l spermidine with optimal concentrations of PGRs (3 mg/l BA + 1.5 mg/l NAA) after 8 weeks of culture for nodal explants (Table 4). In the current investigation, shoot elongation was thoroughly observed while the shoot induction and proliferation were noted in both explants on medium containing PGRs or PAs. Irrespective of the explants tested, further increase in spermidine

Table 4 Effects of types and concentrations of polyamines on multiples shoot production in *H. enneaspermus* after 8 weeks of culture

PAs concentration (mg/l)	Multiple shoot regeneration frequency (%)		No. of multiple shoots/explant		Length of the shoot (cm)	
	Leaf	Node	Leaf	Node	Leaf	Node
Control	88.4b	83.3a	34.80±0.37d	29.40±0.50d	5.60±0.08b	5.44±09b
Spermidine						
5	81.3b	79.9b	28.80±0.37f	24.60±0.40f	6.56±0.08a	6.30±0.10a
10	85.7b	84.2a	32.00±0.70e	26.60±0.40e	6.60±0.21a	6.46±0.21a
15	90.5a	85.5a	37.20±0.37b	31.40±0.50bc	6.60±0.07a	6.40±0.10a
20	94.4a	89.6a	40.40±0.50a	34.20±0.48a	6.72±0.06a	6.54±0.09a
25	89.8b	83.7a	35.80±0.37cd	30.80±0.37c	6.52±0.11a	6.42±0.11a
Spermine						
5	76.4c	71.1b	26.20±0.48g	23.60±0.40f	5.36±0.17b	5.26±0.06bc
10	80.6b	75.6b	29.40±0.50f	26.20±0.37e	5.50±0.10b	5.34±0.09bc
15	85.2b	81.5a	33.20±0.48e	29.20±0.48d	5.52±0.08b	5.30±0.18bc
20	90.1a	85.3a	36.60±0.40bc	32.20±0.37b	5.62±0.13b	5.40±0.11bc
25	84.7b	79.0b	32.20±0.37e	28.60±0.40d	5.54±0.13b	5.30±0.07bc
Putrescine						
5	70.0c	66.5c	16.20±0.37j	10.80±0.37j	5.30±0.09b	5.02±0.13d
10	74.6c	69.4c	19.60±0.24i	14.40±0.24i	5.42±0.11b	5.14±0.10bc
15	78.8c	74.9b	24.60±0.24h	18.60±0.40h	5.40±0.17b	5.12±0.17bc
20	83.4b	77.5b	28.20±0.37f	22.20±0.48g	5.52±0.21b	5.28±0.09bc
25	86.5b	80.8a	32.20±0.37e	26.40±0.24e	5.52±0.14b	5.36±0.12bc

Control: MS medium supplemented with 4 mg/l BA + 1.5 mg/l NAA or 3 mg/l BA + 1.5 mg/l NAA for leaf and nodal explants respectively. Values represent the mean ± standard error of three experiments. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5 % level

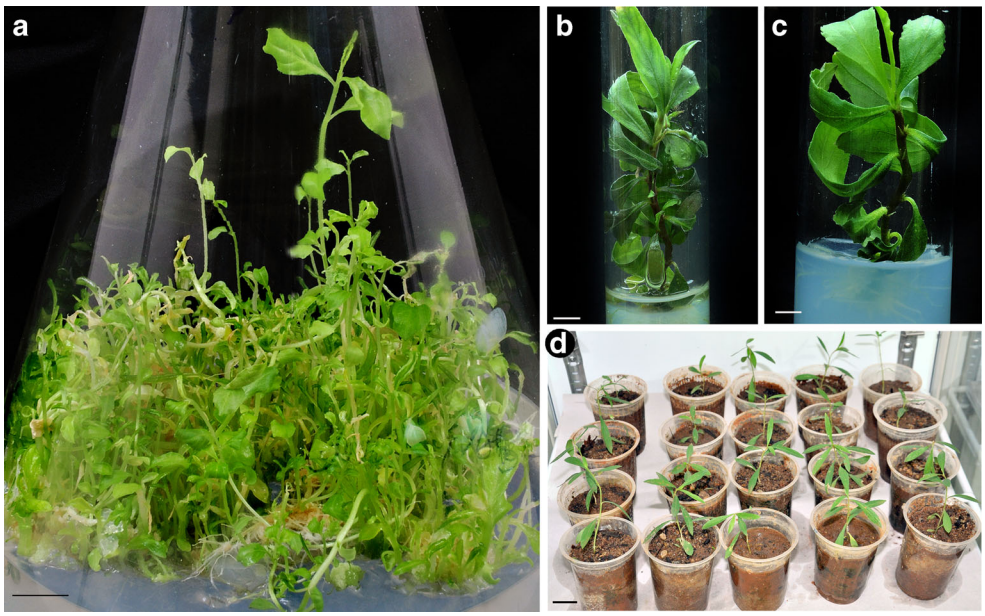

Fig. 1 A combined effect of plant growth regulators and polyamines on multiple shoot regeneration in *H. enneaspermus*. **a** Mass multiplication of shoots from leaf explants cultured on MS medium containing spermidine (20 mg/l) and BA (4 mg/) + NAA (1.5 mg/l) after 8 weeks of culture. **b** Elongation of shoot derived from leaf explant on MS medium supplemented with spermidine (20 mg/l) and BA (4 mg/) + NAA (1.5 mg/l). **c** Rooting of elongated shoot derived from leaf explant on MS medium augmented with 20 mg/l putrescine and 1.5 mg/l IBA after 5 weeks of culture. **d** Hardened plants in growth chamber. All *bar* = 1 cm

Table 5 Effects of polyamines on rooting of in vitro elongated-shoos derived from leaf and nodal explants of *H. enneaspermus* after 5 weeks of culture

PAs concentration (mg/l)	Rooting frequency (%)		No. of roots/shoot		Length of the root (cm)	
	Leaf	Node	Leaf	Node	Leaf	Node
Control	86.4b	78.6c	12.60±0.24c	8.80±0.20e	5.56±0.10b	5.16±0.19b
Spermidine						
5	0.0c	0.0d	0.0f	0.0g	0.0c	0.0c
10	0.0c	0.0d	0.0f	0.0g	0.0c	0.0c
15	0.0c	0.0d	0.0f	0.0g	0.0c	0.0c
20	0.0c	0.0d	0.0f	0.0g	0.0c	0.0c
25	0.0c	0.0d	0.0f	0.0g	0.0c	0.0c
Spermine						
5	0.0c	0.0d	0.0f	0.0g	0.0c	0.0c
10	0.0c	0.0d	0.0f	0.0g	0.0c	0.0c
15	0.0c	0.0d	0.0f	0.0g	0.0c	0.0c
20	0.0c	0.0d	0.0f	0.0g	0.0c	0.0c
25	0.0c	0.0d	0.0f	0.0g	0.0c	0.0c
Putrescine						
5	84.2b	81.0b	9.20±0.20e	7.60±0.24f	6.20±0.23a	5.74±0.06a
10	86.7b	84.3b	11.60±0.40d	9.20±0.37e	6.28±0.09a	5.68±0.15a
15	88.5b	86.8b	13.80±0.37b	10.80±0.37c	6.32±0.08a	5.82±0.16a
20	96.6a	92.2a	16.40±0.24a	12.00±0.44a	6.40±0.31a	5.90±0.16a
25	93.8a	88.6b	14.20±0.37b	11.40±0.24ab	6.32±0.10a	5.80±0.04a

Control: MS medium supplemented with 1.5 mg/l IBA. Values represent the mean ± standard error of three experiments. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5 % level

concentration beyond or below the optimal level, did not indicate any increment in multiple shoot production, rather a lessening trend in all the parameters estimated was apparent. The diminution in the shoot regeneration potential appeared to be owing to the negative effect of higher or lower concentration of spermidine. This spermidine response on the both the explants was followed by spermine and putrescine. The number of multiple shoots produced in spermidine treatment was significantly greater (3 fold) when compared to those in BA or BA and NAA combination (Table 4). PAs play a very important role in plant physiology and development such as cooperated with phytohormones, represent as plant growth regulator or hormonal secondary messengers, and used as a source of carbon and nitrogen to growing cells (Couee et al. 2004). Altamura et al. (1991) suggested that besides the key roles of cytokinin and auxin on shoot induction, very important function in multiple shoot induction and differentiation process can be accelerated by polyamines in the cultured medium. Furthermore, they suggested that polyamines have a broad spectrum of action with some resemblances both with auxin and cytokinin and in co-operation with plant phytohormones modulated morphogenic process. Sivanandhan et al. (2011, 2013) suggested that regeneration and differentiation could be drastically improved by application of polyamines in *W. somnifera* shoot culture. The shoots which having about 6–6.5 cm were removed from the both explants and cultured on MS medium containing PAs for root initiation along with optimal concentration of IBA (1.5 mg/l). There were no root induction in the elongated shoots grew in spermidine and spermine supplemeted medium. The root induction was exhibited in the putrescine at 20 mg/l and reduction was noticed in lower or higher concentration of putrescine (Table 5; Fig. 1c). At 20 mg/l putrescine, the shoot formed 16 roots/shoot with 6.4 cm lenght when compared to IBA in the similar culture time. The result has corroborated with Couee et al. (2004) and they postulated that putrescine involved in a vital role of root development and root architecture. Thus, we may postulate that a particular type and concentration of exogenously added polyamines in the culture medium would enable the explants to respond and produce multiple shoots and roots.

Acclimatization

The well-rooted plants were hardened for 4 weeks in the growth chamber and subsequently in the greenhouse for 4 weeks, of which nearly 98 % plantlets survived when transferred to field conditions (Fig. 1d). The acclimatized plants were healthy and exhibited normal growth. The field-transformed plants were periodically watered and after

Table 6 Comparative DPPH radical scavenging activity in in vitro and field-grown plants of *H. enneaspermus*

Nature of plant parts	DPPH radical scavenging activity (%)		
	31.25 μg/ml	62.5 μg/ml	125 μg/ml
Ascorbic acid	96.4	96.5	96.3
Field-grown plant			
Shoot	92.5	93.7	93.9
Root	34.6	53.6	78.4
In vitro-grown plant			
Shoot	75.8	84.4	88.6
Root	18.4	22.5	34.6

Values represent the mean ± standard error of three experiments. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5 % level

2 weeks, the plants were randomly selected and treated for further antioxidant study and HPLC analysis.

Comparative DPPH radical scavenging activity in in vitro and field-grown plant parts

Free-radical scavenging activity was performed to estimate the antioxidant activity of in vitro-raised and field-grown plant parts (Table 6). The field-grown shoots and roots of *H. enneaspermus* showed higher radical scavenging activity when compared to in vitro-grown plant parts. Similarly, in vitro-raised shoot exhibited higher DPPH radical scavenging activity when compared to in vitro-induced roots (Table 6). There was an improvement in free radical scavenging activities with an improvement in the concentration of plant extracts obtained from the aerial parts and the roots. This observation possibly specifies the occurrence of more (potent) antioxidant compounds in the shoots and followed by roots of field-grown and in vitro-grown plant. The radical scavenging activity of extracts can be attributed to the presence of its major phenolic compounds (Guimaraes et al. 2010). The antioxidant activity of phenolic compounds is related to the hydroxyl groups linked to the aromatic ring, which are capable of donating hydrogen atoms with electrons and stabilizing free radicals (Yanishlieva et al. 2006).

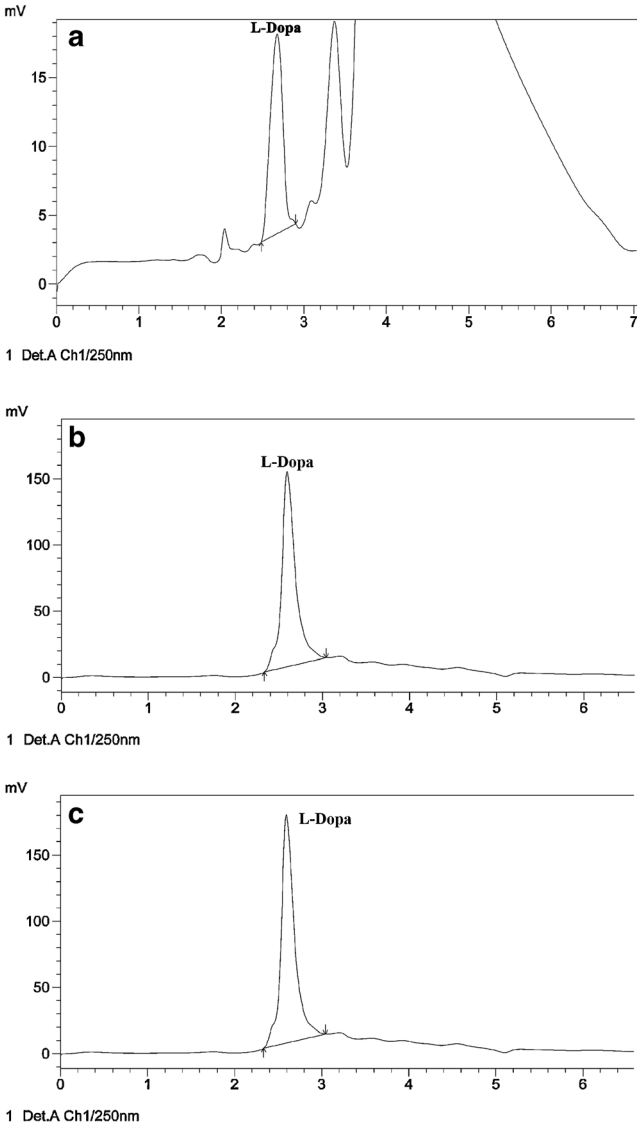

Fig. 2 HPLC analysis of L Dopa estimation in methanolic extract of *H. enneaspermus*. **a** L Dopa standard. **b** Leaves from parent plants. **c** Leaves from in vitro plants

HPLC analysis and L-Dopa estimation

The quantity of L-Dopa present in the methanolic extracts of in vitro and field-grown plant parts (leaves, stems, and roots) were analyzed by HPLC. The L-Dopa content in the leaves (8.31 mg/g DW), stems (6.22 mg/g DW), and roots (3.22 mg/

Table 7 The quantity of L-Dopa extracted from in vitro plants regenerated from leaf and nodal explants of *H. enneaspermus* after 5 weeks of culture

Types	Leaf	Stem	Root
In vivo parent plants	6.78±0.24c	4.33±0.21c	2.11±0.24b
In vitro plants regenerated from leaf explant	8.31±0.27a	6.22±0.27a	3.22±0.22a
In vitro plants regenerated from node explant	7.43±0.28b	5.02±0.26b	2.86±0.20b

Values represent the mean ± standard error of three experiments. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5 % level

g DW) of in vitro plants derived from leaf explant exhibited a marked increase over the respective parts (6.78 mg/g DW, 4.33 mg/g DW and 2.11 mg/g DW respectively) of field-grown parent plants (Table 7; Fig. 2). L-Dopa content was 1.22, 1.43 and 1.52-fold more in the leaves, stems and roots of in vitro plants cultured from leaf than field-grown plant's leaves, respectively. Similarly, in vitro plants derived from node explant recorded significantly higher levels of L-Dopa content than that of control (Table 7). Production of secondary metabolites varied depending upon the culture conditions as well as selection of explant for establishment of in vitro plants as previously described by Sharada et al. (2007) in *W. somnifera* multiple shoot culture. The authors documented that the withanolides production were recorded higher in leaf explant as well as the content was noticed lower in shoot tip explant in *W. somnifera* regeneration. Same trend was obtained by Sivanandhan et al. (2011) in *W. somnifera* regeneration. In the present study, we used three different types of explants to induce and establish the cultures. The shoots derived from the leaf explants produced L-Dopa with the maximum level followed by stems and roots of in vitro derived plants. The regeneration method could be used for secondary metabolite production as well as for conservation of elite germplasm of this important medicinal plant species.

Conclusions

The present investigation demonstrates that leaf explants of *H. enneaspermus* are a good starting material for in vitro large-scale propagation. The observed results exhibited that the efficacy of PA, spermidine with PGRs was superior when compared to other cytokinins and its combination with auxins in improving multiple shoot production in in vitro of *H. enneaspermus*. Exogenously applied BA and IAA interacted with spermidine in a synergistic/additive manner on multiple shoot proliferation and production, thus intensifying the number of multiple shoots 4–5-fold. Best rooting was achieved with a combination of IBA and putrescine. In vitro-raised shoots recorded higher levels of antioxidant activity. The results of the present study are important since the in vitro-micropropagated plants of *H. enneaspermus* may be an attractive alternative way to the wild-growing plants.

Acknowledgments Prof. A. Ganapathi is thankful to University Grants Commissions (UGC), Government of India, for the award of UGC-BSR (Basic Scientific Research Fellowship). Dr. N. Selvaraj is grateful to UGC for the award of UGC-Emeritus Fellowship. V. Vasudevan is thankful to Bharathidasan University for his University Research Fellowship to carry out his Doctoral Research.

References

Ali S, Haq I (2006) Innovative effect of illite on improved microbiological conversion of L-Tyrosine to 3, 4 dihydroxy phenyl L-alanine (L-DOPA) by *Aspergillus oryzae* ME2 under acidic reaction conditions. Curr Microbiol 53:351–357

Altamura MM, Torrigiani P, Capitani F, Scaramagli S, Bagni N (1991) De novo root formation in tobacco thin layers affected by inhibition of polyamine biosynthesis. J Exp Bot 42:1575–1582

Amoo SO, Aremu AO, Van Staden J (2012) In vitro plant regeneration, secondary metabolite production and antioxidant activity of micropropagated *Aloe arborescens* Mill. Plant Cell Tissue Organ Cult 111:345–358

Anbu K, Subramanian P (2011) Serial extraction of the crude extract, separation of the lyophilized extracts: Identification of major peaks and their elution in Hybanthus enneaspermus and pedalium murex. In: Subramanian P (ed) An anthology of articles on aquatic research. Nitheeshprabha Pathippagam., Karaikudi, pp 88–94

Baskaran P, Moyo M, Van Staden J (2014) In vitro plant regeneration, phenolic compound production and pharmacological activities of *Coleonema pulchellum*. S Afr J Bot 90:74–79

Benson A, Joe MM, Karthikeyan B, Sa T, Rajasekaran C (2014) Role of *Achromobacter xylosoxidans* AUM54 in micropropagation of endangered medicinal plant -*Naravelia zeylanica* (L.) DC. J Plant Growth Regul 33:202–213

Boominathan R, Parimaladevi B, Mandal SC, Ghoshal SK (2004) Anti-inflammatory evaluation of Ionidium suffruticosam Ging. in rats. J Ethnopharmacol 91:367–370

Carmen SJM, Ballester A, Vieitez AM (2001) Effect of thidiazuron on multiple shoot induction and plant regeneration from cotyledonary nodes of chestnut. J Horticult Sci Biotechnol 76:588–595

Coenen C, Lomax TL (1997) Auxin-cytokinin interactions in higher plants: old problems and new tools. Trends Plant Sci 2:351–356

Couee I, Hummel I, Sulmon C, Gouesbet G, Amrani AE (2004) Involvement of polyamines in root development. Plant Cell Tissue Org Cult 76:1–10

Guimaraes R, Sousa MJ, Ferreira ICFR (2010) Contribution of essential oils and phenolics to the antioxidant properties of aromatic plants. Ind Crops Prod 32:152–156

Hemalatha S, Wahi AK, Singh PN, Chansouria JPN (2003) Anticonvulsant and free radical scavenging activity of Hybanthus enneaspermus: a preliminary screening. Indian J Tradit Knowl 2:383–388

Kaminek M (1992) Progress in cytokinin research. Trends Biotechnol 10: 159–164

Koyanagi T, Katayama T, Suzuki H, Nakazawa H, Yokozeki K, Kumagai H (2005) Effective production of 3, 4-dihydroxyphenyl l-alanine (L-dopa) with Erwinia herbicola cells carrying a mutant transcriptional regulator TyrR. J Biotechnol 115:303–306

Martin-Tanguy J (2001) Metabolism and function of polyamines in plants: recent development (new approaches). Plant Growth Regul 34:135–148

Matkowski A (2008) Plant in vitro culture for the production of antioxidants—a review. Biotechnol Adv 26:548–560

Minocha SC (1987) Plant growth regulators and morphogenesis in cell and tissue culture of forest trees. In: Bonga JM, Durjan DJ (eds) Cell and tissue culture in forestry, vol 1. Martinus Nijhoff Publ, Dordrecht, pp 50–66

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Nitnaware KM, Naik DG, Nikam TD (2011) Thidiazuron-induced shoot organogenesis and production of hepatoprotective lignan phyllanthin and hypophyllanthin in *Phyllanthus amarus*. Plant Cell Tissue Organ Cult 104:101–110

Nordström A, Tarkowski P, Tarkowska D, Norbaek R, Astot C, Dolezal K, Sandberg G (2004) Auxin regulation of cytokinin biosynthesis in *Arabidopsis thaliana*: a factor of potential importance for auxin-

cytokinin regulated development. Proc Natl Acad Sci U S A 101: 8039–8044

Patel DK, Kumar R, Prasad SK, Sairam K, Hemalatha S (2011) Antidiabetic and in vitro antioxidant potential of *Hybanthus enneaspermus* (Linn) F. Muell in streptozotocin-induced diabetic rats. Asian Pac J Trop Biomed 1:316–322

Patel DK, Kumar R, Sairam K, Hemalatha S (2013) *Hybanthus enneaspermus* (L.) F. Muell: a concise report on its phytopharmacological aspects. Chin J Nat Med 11:199–206

Prakash E, Sha Valli Khan PS, SairamReddy P, Rao KP (1999) Regeneration of plants from seed derived callus of *Hybanthus enneaspermus* L. Muell., a rare ethnobotanical herb. Plant Cell Rep 18:873–878

Premkumar G, Arumugam N, Muthuramkumar S, Varatharaju G, Rajarathinam K (2013) Improved micropropagation in *Hybanthus enneaspermus* L. Muell. Am J Plant Sci 4:1169–1172

Sahoo S, Kar DM, Mohapatra S, Rout SP, Dash SK (2006) Antibacterial activity of *Hybanthus enneaspermus* against selected UTI pathogens. Indian J Pharm Sci 68:653–655

Shantha TR, Pashupathy S, Shetty JK, Vijayalakshmi B, Kandavel P, Bikshapathy T (2001) Pharmacognostical studies on orilaitamarai-*Hybanthus enneaspermus* (L) f. Muell-(violaceae). Anc Sci Life 21: 38–50

Sharada M, Ahuja A, Suri KA, Vij SP, Khajuria RK, Verma V, Kumar A (2007) Withanolide production by in vitro cultures of Withania somnifera and its association with differentiation. Biol Plant 51:161–164

Sivanandhan G, Arun M, Mayavan S, Rajesh M, Mariashibu TS, Manickavasagam M, Selvaraj N, Ganapathi A (2012a) Chitosan enhances withanolides production in adventitious root cultures of *Withania somnifera* (L.) Dunal. Ind Crop Prod 37:124–129

Sivanandhan G, Arun M, Mayavan S, Rajesh M, Jeyaraj M, Kapil Dev G, Manickavasagam M, Selvaraj N, Ganapathi A (2012b) Optimization of elicitation conditions with methyl jasmonate and salicylic acid to improve the productivity of withanolides in the adventitious root culture of *Withania somnifera* (L.) Dunal. Appl Biochem Biotechnol 168:681–696

Sivanandhan G, Mariashibu TS, Arun M, Rajesh M, Kasthurirengan S, Selvaraj N, Ganapathi A (2011) The effect of polyamines on the efficiency of multiplication and rooting of *Withania somnifera* (L.) Dunal and content of some withanolides in obtained plants. Acta Physiol Plant 33:2279–2288

Sivanandhan G, Rajesh M, Arun M, Jeyaraj M, Kapil Dev G, Arjunan A, Manickavasagam M, Muthuselvam M, Selvaraj N, Ganapathi A (2013) Effect of culture conditions, cytokinins, methyl jasmonate and salicylic acid on the biomass accumulation and production of withanolides in multiple shoot culture of *Withania somnifera* (L.) Dunal using liquid culture. Acta Physiol Plant 35:715–728

Siva R, Rajasekaran C, Mudgal G (2009) Induction of somatic embryogenesis and organogenesis in *Oldenlandia umbellata* L. a dye-yielding medicinal plant. Plant Cell Tissue Organ Cult 98:205–211

Siva R, Mayes S, Behera SK, Rajasekaran C (2012) Anthraquinones dye production using root cultures of *Oldenlandia umbellata* L. Ind Crop Prod 37:415–419

Song JY, Mattson NS, Jeong BR (2011) Efficiency of shoot regeneration from leaf, stem, petiole and petal explants of six cultivars of *Chrysanthemum morifolium*. Plant Cell Tissue Organ Cult 107:295–304

Tripathy S, Sahoo SP, Pradhan D, Sahoo S, Satapathy DK (2009) Evaluation of anti-arthritic potential of *Hybanthus enneaspermus*. Afr J Pharm Pharmacol 3:611–614

Weniger B, Lagnika L, Vonthron-Sénécheau C, Adjobimey T, Gbenou J, Moudachirou M, Brun R, Anton R, Sanni A (2004) Evaluation of ethnobotanically selected Benin medicinal plants for their in vitro antiplasmodial activity. J Ethnopharmacol 90:279–284

Wernicke W, Milkovitz L (1987) Effect of auxin on mitotic cell cycle in cultured leaf segments at different stages of development in wheat. Plant Physiol 69:16–22

Yanishlieva NV, Marinova E, Pokorny J (2006) Natural antioxidants from herbs and spices. Eur J Lipid Sci Technol 108:776–793