academicJournals

Vol. 13(11), pp. 1308-1313, 12 March, 2014
DOI: 10.5897/AJB2013.12969
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

In vitro microrhizome production in *Decalepis hamiltonii*

K. Thangavel[1]*, P. Ravichandran[2], M. G. Ebbie[2] and V. Manimekalai[3]

[1]School of Biological Sciences, Madurai Kamaraj University, Madurai - 625 021. India.
[2]Plant Biotechnology Laboratory, Sri Paramakalyani Centre for Environmental Sciences Manonmaniam Sundaranar University, Alwarkurichi - 627 412, India.
[3]Department of Botany, Sri Parasakthi College for Women (Autonomous), Courtallam - 627 802, India.

Received 28 June, 2013; Accepted 24 January, 2014

Microrhizomes were produced from leaf disc derived callus of a threatened endemic medicinal plant - *Decalepis hamiltonii.* Murashige and Skoog (MS) medium supplemented with 2 μM 6-benzyleaminopurine (BAP) and 6 μM 1-napthaleneacetic (NAA) acid was found to be optimum for rapid callus induction and establishment from leaf disc explants. Further differentiation of callus into microrhizome was conquered in MS medium supplemented with 4 μM indole-3-butyric acid (IBA) and 8 μM NAA. A maximum of 20 microrhizomes in a cluster was produced within 90 days. Yeast extract (0.05%) and polyvinylpyrrolidone (0.05%) further enhanced the microrhizome formation when supplemented along with plant growth regulators (PGRs).

Key words: *Decalepis hamiltonii*, microrhizome, herbal medicine, secondary metabolites.

INTRODUCTION

In recent years, there has been a gradual revival of interest in the use of medicinal plants in developed as well as developing countries since herbal medicines have been reported to be safe without side effects (Bahadur et al., 2007). Consequently, several medicinal plants becoming rare, endangered and threatened (RET) categories. *Decalepis hamiltonii* Wight and Arn. (Asclepiedaceae) is a glabrous extensively climbing shrub growing in moist deciduous forests, scrub jungles of Deccan peninsula and the Western Ghats of India. This is an endemic and endangered medicinal plant of southern peninsula. It prefers to grow along rocky slopes, big rock boulders and rocky crevices and small mounds at an altitude from 300 to 1200 m (Ravikumar and Ved, 2000). Rhizome extract of this plant is reported to have antioxidant, antibacterial, hepatoprotective, antiinflammatory, insecticidal, antidiabetic, anti-atherosclerotic, immunomodulatory and antitumour potentials due to the presence of bioactive principles such as decalepin, salicylaldehyde, vanillin anisaldehyde, 2-hydroxy 4-methoxy benzaldehyde and 5,7,4-trihydroxy flavanone 4'-o-β-D-glucoside (Srivastava and Shivanandappa, 2006, 2010; Harish and Shivanandappa, 2011; Ashalatha et al., 2010; Naveen and Khanum, 2010; Sumalatha et al., 2010; Thangavel et al., 2011). In natural habitat, this species is under severe threat due to poor seed setting and ruthless harvesting by traditional medical practitioners (Ravikumar and Ved, 2000). Among

*Corresponding author. E-mail: thangavel@dbtiplsmku.org; thangamspkces@gmail.com. Tel: 91-9952385778.

Abbreviations: MS, Murashige and Skoog medium; **NAA,** 1-napthaleneacetic; **BAP,** 6-benzyleaminopurine; **IBA,** indole-3-butyric acid.

different forms of plant cell and tissue culture systems, specific organ cultures such as root, rhizome and tubers have been reported to hold tremendous potential for *in vitro* production of uniform, high quality plant based medicines (Tripathi and Tripathi, 2003; Subathra et al., 2006; Majumdar et al., 2010; Poojadevi et al., 2013.

Micro-rhizomes have been induced in *Curcuma aromatica*, *Curcuma longa* and the efficiency of microrhizome formation was found to be determined by the concentration of cytokinins, sucrose and coconut milk (Naik and Naik, 2006). Production of microrhizomes *in vitro* has many advantages compared to plantlet production. Besides, the use for extraction of secondary compounds, microrhizomes are feasible to facilitate germ plasm exchange across national borders. Hence, a rapid reproducible protocol for microrhizome production from leaf disc derived callus of *D. hamiltonii* have been devised in this present study.

MATERIALS AND METHODS

Procurement of plant material

Young saplings of *D. hamiltonii* was collected from Sirumalai hills, Dindigul district, Tamil Nadu, potted and maintained in the department (SPKCES, M. S. University) green house for routine culture work. Identity of the plant was authenticated by Dr. K. Ravikumar, Assistant Director, Repository for Medicinal Plant Resources, FRLHT, Bangalore. Voucher specimen was deposited in departmental herbarium (F.No:144: SPKCESH). Chemicals and plant growth regulators (PGRs) used in this present study were procured from Himedia Laboratories, Mumbai, India.

Preparation of culture medium

Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) was used throughout the study. The basal medium was modified by adding plant growth regulators (PGRs) such as 1-naphthaleneacetic acid, indole-3-butyric acid and 6-benzylaminopurine (2 to 10 µM), 1 to 3% (w/v) sucrose, media adjuvants such as coconut milk [5 to 15% (v/v)], yeast extract [0.05% (w/v)] and polyvinylpyrrolidone [0.05% (w/v)]. pH of the medium was adjusted to 5.6 to 5.8. After adding 0.8 to 1.2% (w/v) agar, medium was melted in a microwave oven and dispensed in culture vials, then sterilized in an autoclave at 15 Psi and 121°C, for 15 min.

Surface sterilization of explants

Fresh leaves of *D. hamiltonii* were collected, thoroughly washed in running tap water and 0.5% (v/v) sodium hypochlorite (NaOCl) with a few drops of "Teepol" for 5 min. This was followed by a thorough wash in running tap water. Then leaves were transferred to sterile laminar air flow cabinet and surface sterilized in 0.05% (w/v) mercuric chloride ($HgCl_2$) solution for 5 min and rinsed thoroughly in sterile distilled water.

Callus establishment and microrhizome initiation

Surface sterilized leaves were trimmed into optimum size (0.5 to 1.0 cm). Two discs were aseptically placed per culture vial with sterile MS medium. Culture vials were incubated under complete darkness at 25 ± 2°C. Proliferated callus was sub-cultured in 10 to 15 days interval for further establishment. After establishment, 0.5 g of callus was transferred to liquid MS medium (50 ml) supplemented with indole-3-butyric acid (IBA) and 1-napthaleneacetic (NAA: 2 to 10 µM), coconut milk [5 to 15% (v/v)] and yeast extract [0.05% (w/v)]. Suspension cultures were incubated at 24°C in orbital shaker at 100 rpm. Callus was sub-cultured in 15 days interval by filtering the callus mass with several layers of sterile cheese cloth. Partially differentiated microrhizomes were sub-cultured into solid medium for further development. 10 replicates were maintained for each media combination, fresh weight of callus was measured 20 days after sub culture. Callus induction efficiency of PGRs in different concentration and combination were evaluated through one way ANOVA. All culture sets were maintained with 10 replicates. Mean values were calculated (Table 1) and interpreted with control. Significance was taken at the level of $P< 0.01$.

RESULTS

Among different PGR combinations used, MS medium supplemented with NAA (6 µM) along with BAP (2 µM) exhibited callus proliferation at the maximum level (2.9 ± 0.64 g) after 20 days from sub-culture (Table 1), (Figure 1c). Leaf explants often leached phenolic substances that inhibited the callus growth considerably, but this was suppressed by the addition of 0.05% polyvinylpirrolidone (PVP).

Among various levels of sucrose and agar tested, 2 and 1.2% respectively were found to be ideal for rapid callus induction. After establishment, callus was transferred into microrhizome differentiation (liquid) medium, sub-cultured at 15 days interval. In liquid medium, differentiation and development of microrhizomes were observed in a slow rate (Figure 1d and 1e). Hence, the partially developed microrhizome clusters were transferred to solid medium where they grow faster and produced higher number of microrhizomes (Table 2) within 20 days (Figure 1f). Microrhizome differentiation potential was evoked by NAA in combination with IBA, YE and CM (Table 2). Culture medium supplemented with 8 µM NAA + 4 µM IBA + 0.05%YE and 10% CM exhibited maximum efficiency (average: 16 numbers) on microrhizome development and was considered very ideal for producing large number of microrhizomes. During initial stages, microrhizomes were white in color and after 15 days, they turned into pale brown color (Figure 1f).

DISCUSSION

Plant tissue culture techniques have been useful for conserving germplasm of rare and endangered species and considered as an alternative to conventional field gene banks to safeguard against pests and environmental stresses (Dodds, 1991). Tissue culture of medicinal plants holds tremendous potential for *in vitro* production and exploration of herbal therapeutics. In this

Table 1. Callus proliferation from leaf disc explants.

Medium composition					Callus induction efficiency	Initial weight of callus (g)	Weight (g) after 20 days[a] (10 replicates)	ANOVA[b]	
								P - value	F - value
MS					-	0.5	0.5	-	-
NAA (µM)	**2,4- D (µM)**	**S (%)**	**A (%)**	**PVP (%)**					
2.0	-	2.5	0.8	0.05	+	0.5	0.68±0.18	0.1042^{NS}	2.927
4.0	-	2.5	0.8	0.05	+	0.5	0.72±0.16	0.0019**	13.198
6.0	-	2.5	1.0	0.05	+++	0.5	0.72±0.21	0.0019**	13.198
8.0	-	2.0	1.0	0.05	+		0.76±0.25	1.97E-06**	47.287
10.0	-	2.0	1.2	0.05	+	0.5	0.78±0.19	3.85E-06**	42.682
-	2.0	2.0	1.2	0.05	+	0.5	0.80±0.21	5.38E-07**	57.241
-	4.0	2.0	1.2	0.05	+	0.5	0.80±0.24	5.38E-07**	57.241
-	6.0	2.0	1.2	0.05	+ +	0.5	1.60±0.50	1.11E-20**	2443.606
-	8.0	2.0	1.2	0.05	+ +	0.5	1.70±0.53	1.37E-20**	2386.983
-	10.0	2.0	1.2	0.05	+ +	0.5	1.70±0.62	1.37E-20**	2386.893
NAA (µM)	**BAP (µM)**	**CM (%)**	**S (%)**	**A (%)**	**PVP (%)**				
2.0	2.0	10	2.0	1.2	0.05	+ +	0.5	1.78±0.59	3.34E-23**	4674.064
4.0	2.0	10	2.0	1.2	0.05	+ +	0.5	1.8±0.61	1.37E-20**	2387.344
6.0	2.0	10	2.0	1.2	0.05	+ + + +	0.5	2.9±0.64	8.98E-26**	9039.249
2,4-D (µM)	**BAP (µM)**	**CM (%)**	**S (%)**	**A (%)**	**PVP (%)**				
2.0	2.0	10	2.0	1.2	0.05	+ + +	0.5	2.0±0.58	2.9E-21**	2839.667
4.0	2.0	10	2.0	1.2	0.05	+ + +	0.5	2.1±0.62	1.49E-22**	3956.045
6.0	2.0	10	2.0	1.2	0.05	+ +	0.5	1.6±0.56	1.27E-19**	1860.319
6.0	2.0	10	2.0	1.2	0.05	+ +	0.5	1.55±0.58	7.07E-17**	912.910
6.0	2.0	10	2.0	1.2	0.05	+ +	0.5	1.52±0.72	1.79E-19**	1789.952

+ Delayed response with very minimum callus growth, + + delayed response with normal callus growth, + + + quick response with normal callus growth, + + + + quick response with maximum callus growth; NS, Not Significant; ** significant at P< 0.01; b - all values were compared with MS basal medium, a - all values are mean weight (20 days after sub-culture) of 10 replicates ± SD; CM, coconut milk; S, sucrose; A, agar; PVP, polyvinylpyrrolidone

this present study, a rapid, reproducible protocol has been devised for the development of microrhizome from leaf disc derived callus of *D. hamiltonii*. Plant growth regulators were amended at different concentrations and combinations. However, the specific combination of the medium resulted in different extent of callus induction and differentiation of microrhizome. With the stimulus of endogenous or by addition of exogenous

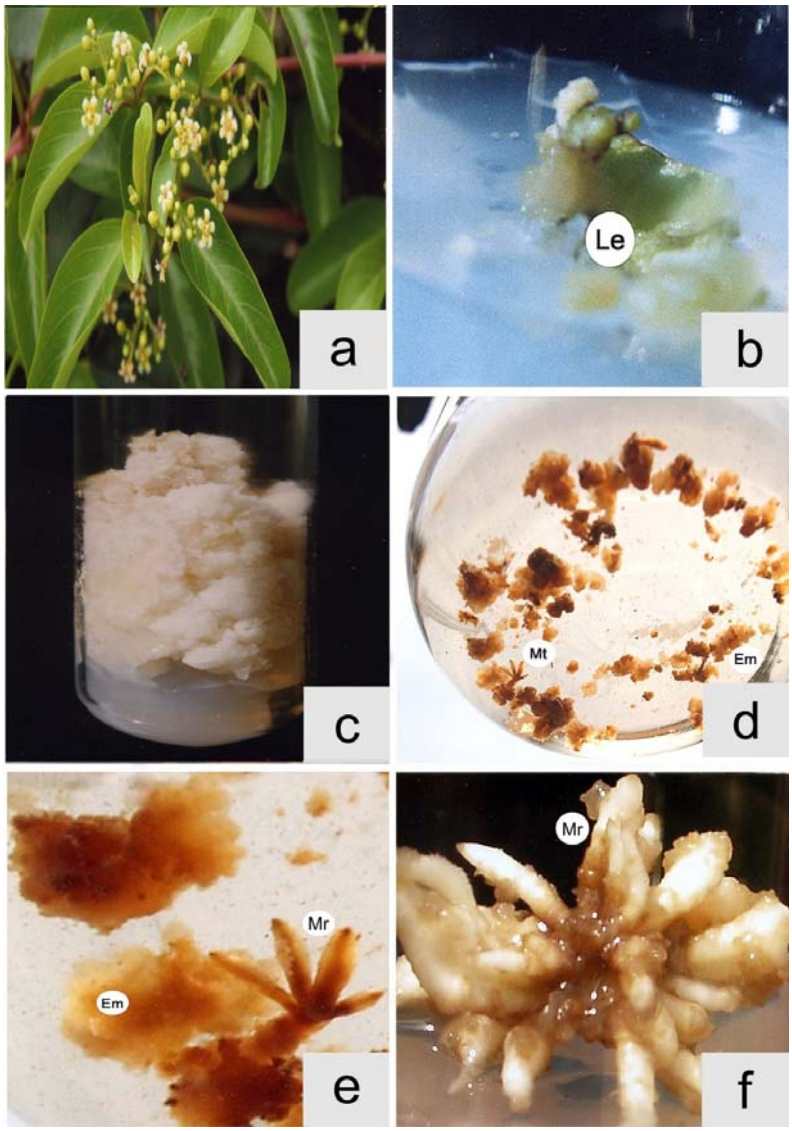

Figure 1. *In vitro* microrhizome development from leaf derived callus. **a.** *D. hamiltonii* - A twig with flower; **b.** leaf disc (Le) cultured on MS medium supplemented with 6 µM NAA + 2 µM BAP + 10% CM + 0.05% PVP. Callus proliferation after 10 days of inoculation; **c.** further establishment of friable callus (30 days after inoculation) - a stage suitable for liquid culture; **d.** friable callus after transfer into MS liquid medium supplemented with 8 µM NAA + 4 µM IBA + 10% CM + 0.05% PVP (developing microrhizome - Mr); **e.** further development of microrhizomes after 90 days; **f.** close up view of microrhizome cluster after transfer into solid medium.

growth regulators to the nutrient medium, cell division, and tissue differentiation could be induced. Callus obtained in this present study was friable in nature (Figure 1c).

Effect of PGRs on callus induction and establishment

Supplementation of auxins at suitable level is a generic requirement for any explant to induce callus. Callus was proliferated initially from the cut surfaces of leaf discs cultured in MS medium supplemented with NAA (Figure 1b). Within 15 days, well proliferated callus covered the entire explant.

Basal medium as well as BAP supplemented medium did not induce callus. In case of medium supplemented with BAP along with NAA callus, the proliferation was rapid. MS medium consist of 6 µM NAA, 2 µM BAP and 0.05% PVP promoted quick induction and rapid proliferation of callus compared to other combinations

Table 2. Microrhizome development from leaf disc derived callus.

Medium composition						Number of microrhizomes*
MS						-
NAA (µM)	**IBA (µM)**	**S (%)**	**YE (%)**	**CM (%)**	**A (%)**	
2.0	-	1.5	-	10	-	-
4.0	-	1.5	-	10	-	-
6.0	-	1.5	-	10	-	2 ± 0.13
8.0	-	1.5	-	10	-	5 ± 0.14
6.0	-	1.5	0.05	10	-	6 ± 0.17
8.0		1.5	0.05	10	1.2	7 ± 0.21
10.0	-	1.5	0.05	10	1.2	9 ± 0.24
8.0	2.0	1.5	0.05	10	1.2	12 ± 0.23
8.0	4.0	1.5	0.05	10	1.2	16 ± 0.26
8.0	6.0	1.5	0.05	10	1.2	11 ± 0.28

*All values are average of 10 replicates ± SD (after 90 days); YE, yeast extract; CM, coconut milk; S, sucrose; A, agar.

(Figure 1c). NAA could promote callus induction in several plants where 2,4-D or 2,4,5-T fails (Pawar et al., 2002). Callus induction and organogenesis was successful in several medicinal plants like *Petunia inflata*, *Withania somnifera*, *Commiphora wightii* and *Iphigenia indica* (Beek and Camper, 1991) from leaf disc explants with the supplementation of NAA in various concentrations.

In this present study, NAA in combination with BAP enhanced callus multiplication at the maximum level (Table 1) and this observation corroborates the previous reports (Giridhar et al., 2004). In *D. hamiltonii*, *in vitro* hairy root production was reported with low biomass (Giridhar et al., 2004; Sudha and Seeni, 2001). However, high efficiency of micro-rhizome production is achieved in this present study and it could be a better tool for large scale production of high value secondary metabolites available in the rhizome of this medicinally important plant.

Effect of modified sucrose and agar concentrations

Establishment of callus was successful only when the sucrose and agar concentrations were altered. In general, this plant stores high quantity of starch in all vegetative organs. Reducing the normal concentration of sucrose from 3 to 2% induced regenerative callus and the callus survived a longer time. Reduced level of sucrose in culture medium has been reported in medicinal plants like *Ficus lyrata* and *Solanum melongena* (Mukherjee et al., 1991).

Increasing the concentration of agar (1.0 to 1.2%) enhanced the survival and curtailed hyperhydricity based suppression during callus establishment. This could be attributed to the natural conditions where this plant is exposed to xeric habitats. Thus, creating a near natural environment facilitated growth of callus and differentiation of microrhizome significantly. Such a similar observation was made earlier in *Solanum surattense* by Pawar et al. (2002).

Effect of PGRs on microrhizome differentiation

Specialized organ cultures such as root, rhizome and tubers will provide an efficient means of biomass production due to fast growth and stable metabolite production. Callus mass was established and transformed into microrhizome in suspension and later on solid medium. During the course of such differentiation, profound influence of exogenous plant growth regulators was observed. As the bioactive principles of this plant are produced in rhizome, the *in vitro* rhizome production would be of much commercial interest and value. Microrhizome was differentiated at the maximum level in MS medium supplemented with IBA (4 µM), NAA (8 µM), yeast extract (0.05%) and coconut milk (10%).

This combination induced only four to seven microrhizomes in 90 days in suspension culture under agitated condition (Figure 1d). Rate of differentiation, further growth and development of microrhizome was successful only after transfer into solid medium containing similar hormone and adjuvant combinations (Figure 1f). Each cluster had a maximum of 20 microrhizomes and an average of 16 on solid medium. Callus mediated microrhizome was also produced from *Hemidesmus indicus* (Sreekumar et al., 1998), *Bunium persicum* (Grewal, 1996) and *Solanum tuberosum* (Piao et al., 2003) with various hormone supplementations. Microrhizomes have also been developed *in vitro* from various rhizomatous medicinal plants such as *Zingiber*

officinale (Sharma and Singh, 1995), *Curcuma aromatic* (Nayak, 2000) and *Curcuma longa* (Sunitibala et al., 2001). In all these reports, supplementation of PGRs such as NAA and IBA (2 to 15 µM), adjuvants like coconut milk, maleic hydrazide, activated charcoal and yeast extract were found to be crucial for microrhizome development. Photoperiod was reported to play a key role in induction of storage organs such as rhizome and tuber *in vitro* (Jean and Cappadocia, 1991). In the present study, cultures incubated in 16 h photoperiod produced more number of microrhizomes. Under 16 h light regime, large number of tubers have been produced in *Dioscorea abysinica* and *Dioscorea alata*, whereas continuous dark conditions hindered the tuberization process. Compared to other forms of cell aggregates, microtubers, microrhizomes and adventitious tuberous roots could serve as better alternative for large scale extraction of secondary metabolites (Jean and Cappadocia, 1991; Xie et al., 2000). Accordingly, the protocol devised in this present study will find an immense application in pharmaceutical industries for extraction and formulation of herbal products.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. K. Ravikumar, Assistant Director, Repository for Medicinal Plant Resources, FRLHT, Bangalore for identification of plant specimen. We are also grateful to the Coordinator and Chairperson, DBT-MKU-IPLS and UGC-NRCBS programmes, School of Biological Sciences, Madurai Kamaraj University for providing necessary facilities.

REFERENCES

Ashalatha K, Venkateswarlu Y, Moushumi Priya A, Lalitha P, Krishnaveni P, Jayachandran S (2010). Anti inflammatory potential of *Decalepis hamiltonii* (Wight and Arn.) as evidenced by down regulation of pro inflammatory cytokines—TNF-α and IL-2. J. Ethnopharmacol. 130:167-170.

Bahadur B, Janardhan Reddy KH, Rao MLN (2007). Medicinal Plants - An Overview; Advances in Medicinal Plants. Janardhan Reddy K, Bahadur B, Bhadraiah B, Rao MLN, Eds., Hyderabad, University Press (India), 14-36.

Beek NJ, Camper ND (1991). Shoot regeneration from *Petunia* leaf discs as a function of explant size, configuration and benzyladenine exposure. Plant Cell Tiss. Organ Cult. 26:101-106.

Dodds JH (1991). *In vitro* Methods of Conservation of Plant Genetic Resources. Chapman and Hall, London.1991.

Giridhar P, Kumar V, Ravishankar GA (2004). Somatic Embryogenesis, organogenesis and regeneration from leaf callus culture of *Decalepis hamiltonii* Wight and Arn.- an endangered shrub. *In Vitro* Cell Dev. Biol. Plant. 40:567-571.

Grewal S (1996). Micro-tubers from somatic embryos of *Bunium persicum*. Indian J. Exp. Biol. 34:813-815.

Harish R, Shivanandappa T (2011). Hepatoprotective potential of *Decalepis hamoltonii* against carbon tetrachloride induced hepatic damage in rats. J Pharm Bioallied Sci. 2(4):341-345.

Jean M, Cappadocia M (1991). *In vitro* tuberization in *Dioscorea alata* L. 'Brazo fuerte and 'Florida' and *D. abyssinica* Hoch. Plant Cell Tiss. Organ Cult. 26:147-152.

Majumdar S, Garai S, Jha S (2010). Genetic transformation of *Bacopa monnieri* by wild type strains of *A. rhizogenes* stimulates production of Bacopa saponins in transformed calli and plants. Plant Cell Rep. 30:941-954.

Mukherjee SK, Rathinasabapathi B, Gupta N (1991). Low sugar and osmotic requirement of shoot regeneration from leaf pieces of *Solanum melogena* L. Plant Cell Tiss. Organ Cult. 25:13 -16.

Murashige T, Skoog T (1962). A revised medium for rapid growth requirements of tabacco tissue cultures. Physiol. Plantarum. 15, 473 - 497.

Naik S, Naik PK (2006). Factors affecting *In vitro* microrhizome formation and growth in *Curcuma longa* L. and improved field performance of micropropagated plants. Sci. Asia. 32:31-37.

Naveen S, Khanum F (2010). Antidiabetic, antiatherosclerotic and hepatoprotective properties of *Decalepis hamiltonii* in streptozotocin-induced diabetic rats. J. Food Biochem. 34:1231-1248.

Nayak S (2000). *In vitro* multiplication and microrhizome induction in *Curcuma aromatica* Salisb. Plant Cell Tiss. Organ Cult. 32: 41-47.

Pawar, PK, Pawar CS, Narkhede BA, Teli NP, Bhalsing SR, Maheswari VL (2002). A technique for rapid propagation of *Solanum surattense* Burm. Indian J. Biotechnol. 1:201-204.

Piao XC, Chakrabarthy D, Hahn EJ, Paek KY (2003). A simple method for mass production of potato microtubers using a bioreactor system. Curr. Sci. 84:1129-1132.

Poojadevi S, Harish P, Neeta S (2013). Hairy root cultures: A suitable biological system for studying secondary metabolic pathways in plants. Eng. Life Sci. 13(1):62-75.

Ravikumar K, Ved DK (2000). 100 Red Listed Medicinal Plants of Conservation Concern in Southern India, Foundation for Revitalization of Local Health Traditions, Bangalore, India.

Sharma TR, Singh B (1995). *In vitro* microrhizome production in *Zingiber officinale* Rosc. Plant Cell Rep. 15:274-277.

Sreekumar S, Seeni S, Pushpangadan P (1998). Production of 2 - hydroxy 4- methoxy benzaldehyde using root cultures of *Hemidesmus indicus*. Biotechnol. Lett. 20:631-635.

Srivastava A, Shivanandappa T (2006). Hepatoprotective effect of aqueous extract of the roots of *Decalepis hamiltonii* against ethanol-induced oxidative stress in rats, Hepatol. Res. 35:267-275.

Srivastava A, Shivanandappa T (2010). Neuroprotective effect of *Decalepis hamiltonii* roots against ethanol-induced oxidative stress. Food Chem. 119:626-629.

Subathra DC, Murugesh S, Srinivasan M (2006). Gymnemic acid production in cell suspension cultures of *Gymnema sylvestre.* J. Appl. Sci. 6:2263-2268.

Sudha CG, Seeni S (2001). Establishment and analysis of fast - growing normal rot culture of *Decalepis arayalpathra*, a rare endemic medicinal plant. Curr. Sci. 81:371-374.

Sumalatha G, Vidya Sagar J, Ragini V, Suresh K (2010). Extraction and evaluation of roots of *Decalepis hamiltonii* for antidiabetic activity. J. Pharm. Phytochem. Res. 2(3):20-25.

Sunitibala H, Damayanti M, Sharma G (2001). *In vitro* propagation and rhizome formation in *Curcuma longa* L. Cytobios. 105:71-89.

Thangavel K, Ebbie MG, Ravichandaran P (2011). Antibacterial potential of *Decalepis hamiltonii* Wight & Arn. callus extract. Pharm. Tech. 1(1):14-18.

Tripathi L, Tripathi JN (2003). Role of Biotechnology in Medicinal Plants. Trop J. Pharm Res. 2(2):43-253.

Xie D, Wang L, Ye H, Guofeng LI (2000). Isolation and production of artemisinin and stigmasterol in hairy root cultures of *Artemisia annua.* Plant Cell Tiss. Organ Cult. 63:161-166.