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IN-VITRO ORGANOGENESIS OF *OPHIORRHIZA MONGOOSE LINN* FROM ROOT AND INTERNODE EXPLANTS

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ABSTRACT

Camptothecin (CPT) analogues and derivatives serve as a novel class of effective anticancer agents that exert their action against DNA topoisomerase-I. This paper presents procedures for the rapid, high frequency regeneration of a camptothecin producing plant. *Internode* explants cultured on Murashige and Skoog (MS) medium *Ophiorrhiza mongoose Linn* via shoot organogenesis. The concentrations of plant growth regulators and explant types exhibited discrete roles in the efficacy of shoot induction. N6- Benzyladenine (BA) was the most effective cytokinin for the induction of shoots. MS medium with 8.83 µM BA and 2.46 µM indole-3-butyric acid (IBA) yielded the highest number of shoots from leaf and internode explants (76.3 and 90.2 shoots respectively). In the case of leaf explants, explants from the proximal end produced a higher number of shoots than those from the mid and distal end. Leaf supplemented with a α-naphthaleneacetic acid a NAA and BA developed shoots, calli and roots. Excision and culture of internode and proximal leaf explants from the established cultures on MS basal medium significantly enhanced the number of shoots and yielded a mean of 18.3 and 13.7 shoots respectively within 40 days. Shoots cultured on half-strength MS basal medium with 10.74 µM NAA and 2.32 µM Kinetine (Kn) produced a mean of 48.2 roots per shoot. Direct transfer of rootless healthy shoots showed a 50% survival rate, whilst it was 100 percent in the case of in vitro rooted shoots.

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KEYWORDS

Camptothecin, Ophiorrhiza mongoose Linn, Murashige and Skoog medium, Benzyl Adenine, Indole Butyric acid(IBA), Kinetin (Kn)

INTRODUCTION

Camptothecin (CPT) is a monoterpene indole alkaloid isolated and characterized from Camptotheca acuminate Family: Nyssaceae[1], a native of North China. Members of the Icacinaceae, Olacaceae, Rubiaceae, and Apocynaceae families are also reported to produce camptothecin. CPT analogues and derivatives are a novel class of effective anticancer agents that exert their action against DNA topoisomerase (topo) I[2]. The worldwide market size of camptothecin derivatives (e.g. topotecan and irinotecan) reached 1.5 billion US dollars in 2002. Due to the cytotoxicity of camptothecin itself, the CPT derivatives, irinotecan and topotecan, are used throughout the world for the treatment of various cancers, and over a dozen or more CPT analogues are currently at various stages of clinical development[3,4]. However, they are synthesized from natural camptothecin which is extracted from plants. The use of CPTs as inhibitors of replication, transcription, and packing of double stranded DNA-containing adenoviruses, papovaviruses, and the single-stranded DNA-containing autonomous parvoviruses has been studied[5]. It appears that CPTs could be powerful antiviral drugs for several DNA viruses, which are causative agents for a large number of diseases. Since 1994, CPT has been in use clinically in Japan for the treatment of lung, ovarian and uterine[6]. The therapeutic values of CPT derivatives are highlighted against colon cancer[7], uterine cervical and ovarian cancer[8], and falciparum malaria[9]. The genus Ophiorrhiza belongs to the family Rubiaceae, which comprises 150 species. The roots of Ophiorrhiza species, O.prostrata and O.pumila have been reported as the sources of CPT and 10-methoxycamptothecin[11,12]. The Ophiorrhiza spp. is also used to provide remedies for ulcers, helminthiasis, snake poison, poisonous wounds, gastropathy, leprosy, and hydrophobia[13]. The rate of plant propagation is critical to meet the pharmaceutical demand for camptothecin. A slow propagation rate in *Ophiorrhiza mongoose Linn,* because of low seed viability and germination rate as well as a small number of propagules (stem cuttings), has restricted the natural dissemination of the plant. Thus, the large-scale demand necessitates rapid multiplication of the plant within a short timeframe without a negative impact on the natural resources. In vitro morphogenesis without a callus phase is regarded as the most faithful strategy to obtain plants with high speed as well as genetic fidelity. Direct shoot induction as an easy way for the rapid plant propagation has been reported in several medicinal plants viz[14,15].

This study establishes rapid propagation protocols using leaf and internode explants in order to meet the demand, which could curtail the impact on the natural population and prevent the plant from becoming endangered.

MATERIALS AND METHODS

Healthy young shoots of *Ophiorrhiza mongoose Linn,* were collected from potted plants and used as the starting material. Young leaves and internode segments were washed separately under running tap water followed by 5% (v/v) solution of neutral liquid detergent for 5 min followed by surface sterilization using 0.1% (w/v) mercuric

chloride. Leaf segments were sterilized for 7-9 min, while internode segments were treated for 10-12 min. Decanting of the mercuric chloride was followed by repeated washes (3 times 5 min each) in sterile water. The sterilized tissues were cut into appropriate sizes (leaf explants of 10 mm2 and internode of 7-15 mm) using sterile forceps and knife. For the induction of direct shoot formation, the explants were cultured on MS medium[16] supplemented with different levels of plant growth regulators (Table 1). Subcultures were carried out on MS medium with various Subcultures were carried out on MS medium with various concentrations of plant growth regulators (PGRs) as specified in the text. For indirect organogenesis and in vitro rooting MS media supplemented with different PGRs were used as shown respectively in Table 2 and Table 3. Test tubes (25 x 200 mm), 100 ml conical flasks, and jam bottles (53 x 125 mm; 2 mm thick) were used as culture vessels. Test tubes and conical flasks were closed by cotton plugs, while jam bottles were capped with polypropylene autoclavable lids (14 mm height x 48 mm inner diameter autoclavable lids (14 mm height x 48 mm inner diameter with 2 mm thick). The media were adjusted to pH 5.8, and were solidified with 0.8% (w/v) agar. They were sterilized at a pressure of 1.06 kg cm^{-2} at 121ºC for 20 min in an autoclave. All cultures were incubated at 2 5 ± 2ºC with 16 hrs light (at an irradiance of 25 µmol m-2 s^{-1})/8 h dark cycle under white fluorescent tubes.

Plantlets (derived the rough in vitro rooting) as well as well-grown shoots without roots (ex vitro rooting) from the shoot multiplication medium were transferred to small pots containing soil and sand (1:1), covered with moistened polyethylene bags and kept at room temperature (33 ± 2ºC) for acclimation, and subsequently transferred to field conditions.

The experiments were performed in a completely randomized design. Twenty replicates were used for each treatment, and all the treatments were repeated twice for confirmation. The mean values of different treatments were analyzed using Duncan's multiple range tests.

Table 1

Direct organogenesis from internode and proximal leaf explants of Ophiorrhiza ngoose Linn, on MS media with various plant growth regulators

Plant Growth regulators (µM)				Percentage of explants forming shoots		Number of shoots per explant	
BA	**Kn**	**NAA**	**IBA**	**Leaf**	**Internode**	**Leaf**	**Internode**
0	0	0	0	80	90	2.9	4.1
13				100	100	31.1	35.2
17.5				100	100	24.3	29.4
22.2				100	100	12.4	16.8
	4.65			100	100	19.2	25.6
	7.11			100	100	18.2	20.1
	9.1			100	100	7.1	13.2
8.83	0.45			100	100	25.3	30.2
8.83	2.51			100	100	21.3	24.3
8.83	4.53			100	100	15.7	22

8.83	0.58		100	100	28.5	36.2
8.83	2.71		100	100	23.8	27.2
8.83	5.31		100	100	19.2	25.3
8.83		0.48	100	100	45.3	63.2
8.83		2.46	100	100	76.3	90.2
8.83		4.33	100	100	38.7	53.33

Table 2

Organogenesis from callus on MS medium with different plant growth regulators.

Data represents the mean of 20 replicates. Mean values followed by the same letters within columns are not significantly different at the 5% level. Culture duration was 40 days.

Plant Growth regulators (µM)				Number of Shoots per culture
BA	Kn	NAA	IBA	
2.22				5.5
4.44				8.4
6.55				15.5
8.83				8.2
13	0.45			11.3
8.83	2.51			7.8
8.83		0.58		12.3
8.83		2.71		9.9
8.83			0.48	14.1
8.83			2.46	21
8.83			4.33	12.6

Table 3

Efficacy of half strength MS medium with different plant growth regulators in the induction of roots on in vitro-derived shoots

Plant Growth regulators (µM)				Percentage of explants forming shoots	Number of shoots per explant Internode Leaf
BA	Kn	NAA	IBA		
0	0	0	0	95	3.4
0.54				95	5.9
2.69				100	19.1
5.37				100	35.1
	0.49			100	4.3
	2.46			95	12.1
	4.90			100	10.6
		0.57		95	5.0
		2.85		100	13.4
		5.71		100	11.6
			4.65	95	8.8
10.74			2.32	100	48.2
10.74			4.65	100	26.3

In vitro propagation of *O. mongoose* through Organogenesis.

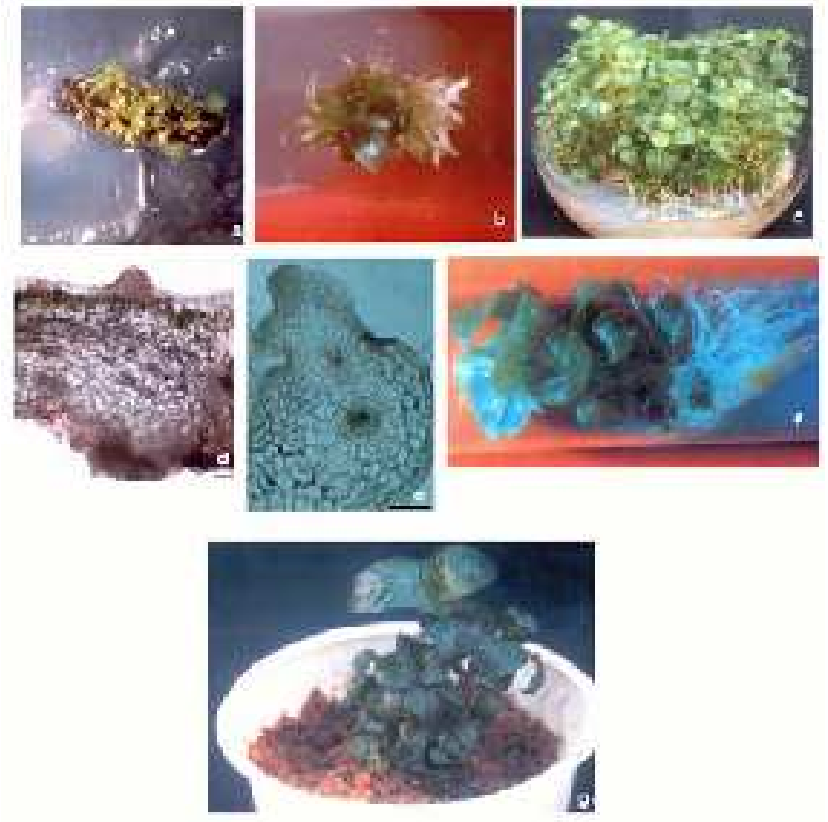

Figure 1

(a) Shoots from internode explants on MS medium with 8.83 µM BA and 2.46 µM IBA.
(b) Shoots from leaf explants on MS medium with 8.83 µM BA and 2.46 µM IBA.
(c) Shoot multiplication on the above medium during subculture.
(d) Shoot origin from sub-epidermal cells of leaf explants.
(e) Shoot origin from sub-epidermal cells of internode explants.
(f) Rooted shoot on half-strength MS medium with 10.74 mM NAA and 2.32 mM IBA.
(g) Established plant (after 45 days).

RESULTS AND DISCUSSION

Direct organogenesis

Leaf and internode explants cultured on MS basal medium produced directly a mean of 2.9 and 4.1 shoots respectively. Addition of PGRs induced shoot formation and the number of shoots depended on the types and concentrations of PGRs, BA in particular (Table 1) as well as the explant types. MS medium containing BA alone was superior to that containing Kn alone for the induction of shoots (Table 1). On medium with 8.83 µM BA, internode explants developed a mean of 51.5 shoots, while the proximal leaf explants produced a mean of 38.0 shoots within 40 days. Upon medium with 8.83 µM BA, leaf explants initiated shoots within 14 days, while the internode explants induced shoots within 12 days. Lower concentrations of BA induced callus with a reduction in number of shoots. Higher levels of BA increased the amount of callus. The calli later developed shoot buds.

Increasing BA concentrations above 8.83 µM exhibited a negative effect on the height of shoots.

Synergy between BA and auxins exhibited a positive effect in the induction of shoots. MS medium with 8.83 µM BA and 2.51 µM IBA developed the highest number of shoots; 76.3 and 90.2 shoots from leaf and internode explants respectively (Table 1; Figure 1a and Figure 1b). As on medium with other PGRs, the shoots on leaf explants were developed adaxially, however, with a few shoots on the abaxial side. Of the different regions in the leaf, explants from proximal region were superior in the induction of shoots. Increasing IBA concentration favored callus induction especially from the cut ends of explants, which later regenerated into shoots. The shoots were longer on media containing BA and auxins than those in media containing BA alone. The regenerated shoots developed roots following further culture. The combination of BA and NAA was inferior for shoot formation (Table 1) but facilitated the formation of a higher amount of callus.

Direct organogenesis is regarded as the most reliable method for clonal propagation because it upholds genetic uniformity among the progenies. As in the present study, direct formation of shoots without an exogenous trigger has been reported in different explants/species, e.g. from internodes of Bacopa monniera[17], and leaf explants of Drosera binata[18]. Reportes are also available for spontaneous formation of shoots from hairy roots of Ophiorrhiza pumila[12]. The development of shoots on basal media may be due to stimulation by endogenous hormones or some signals related to wounding, which play a vital role during the induction of regeneration, or the ratio of ions present in the medium. The difference in the number of shoots formed in leaf and internode explants may be a result of differences in the regeneration potential of different explants, which is attributed by the physiological state, age and cellular differentiation among the constituent cells[19]. Enhancement in the induction of shoots by the synergy of BA and auxins observed in the present study has been documented in Stevia rebaudiana[20], Aloe vera[21] and Murraya koeningii[14]. Though Kn was inferior for shoot formation in O. mongoose, efficacy of Kn or other cytokinins either alone or in combination with BA/auxins indirect shoot formation has been demonstrated in Asparagus maritimus, Robinia pseudoacacia and Bixa orellana. High shoot regeneration potential of proximal end explants as to leaf tip explants as in the present study has been emphasized in Beta vulgaris[22], Anthurium andraeanum cultivars Tinora Red and Senator[23], and Euphorbia nivulia[15]. A report also suggest that high potential of the proximal end to the distal may be due to the difference in the maturity between proximal and distal end of the leaf, and which is supported by the fact that leaves reach maturity first at distal (tip) and subsequently in a basipetal progression[24]. High frequency shoot induction at the proximal region may also be due to the higher level of IAA and abscisic acid[25].

Indirect Organogenesis

Callus initiated on MS medium with NAA alone or in combination with BA or Kn upon subculture developed adventitious shoots. MS medium with 8.83 M BA and 2.46 µM IBA was the best with a mean of 20.1 shoots within 40 days (Table 2). Increasing the concentration of BA prevented elongation of shoots. Higher concentrations of BA favored callus proliferation and subsequent culture of the callus favored better shoot morphogenesis. Callus mediated shoot morphogenesis has been well accomplished in several medicinal plants: Tylophora indica[26, 27], Saussurea obvallata[28] and Euphorbia nivulia[29] and Cassia angustifolia[30]. High frequency callus

mediated shoot regeneration can be utilised for the induction of somaclonal variation for the improvement of this valuable medicinal plant.

Shoot Multiplication

The internode and leaf explants derived from established in vitro cultures on MS basal medium produced high numbers of shoots with means of 18.3 and 13.7 respectively. A high yield of shoots was achieved by culturing stem and leaf (proximal) segments as well as shoot clumps excised from the primary cultures on medium optimal for direct shoot multiplication (MS medium with 8.83 µM BA and 2.46 µM IBA). The number of shoots developed was difficult to count and was further increased in subsequent cultures (Figure 1c). The shoots cultured on MS medium without PGRs developed long shoots with roots. The roots turned to reddish brown from white through golden yellow. The colour of the roots may be due to the accumulation of secondary metabolites (CPT and its derivatives).

Ontogeny of Shoots

The shoots were initiated from sub-epidermal cells especially from the region above the vascular bundles of the explants (Figure 1d and Figure 1e). Both palisade and spongy cells developed shoots. The shoots were shown to contain an extension of the vascular bundles from the source tissue. In the case of leaf explants, the shoots originated from subepidermal cells above the vascular traces. Shoots originating from subepidermal cells have been reported in tobacco stem explants[31]. The ability of different tissues to form shoots directly in other species has been demonstrated including leaf epidermis[32], vascular cambium[33], cortex[11] and cortical, subcortical[34] or epidermis or cortex of root .

Rooting

In vitro as well as ex vitro root induction was preceded by transfer of healthy shoots with more than 3.0 cm height grown in the shoot induction or shoot multiplication medium to rooting media or soil. Shoots transferred to half strength MS medium free of PGRs induced more roots than those on full-strength MS medium. Auxins at lower concentrations, supplemented to half-strength MS medium induced a large number of roots (Table 3). Of the different auxins, NAA at 5.37 µM was superior, which induced a mean of 35.1 roots per shoot within 30 days. Auxins also facilitated callus formation in a small number of shoots. The callus developed roots later. Shoots cultured on halfstrength MS medium with 4.65 µM Kn alone also induced roots (Table 3). Addition of Kn to auxin containing medium augmented root induction. MS medium with high concentration of NAA and Kn favoured better in vitro rooting than with auxins alone. Half-strength MS medium containing 10.74 µM NAA and 2.32 µM Kn produced a mean of 48.2 roots per shoot (Figure 1f). Increased rooting by the addition of cytokinins has been emphasized in Drosera cunefolia[18]. The roots developed on all media became reddish brown through golden yellow from white. The high numbers of roots produced under optimal plant growth regulator regimes opens the possibility of producing camptothecin as the root is the main source for the chemicals.

Transfer of rootless shoots directly into pots containing sand and soil (ex vitro rooting) resulted in a survival rate of 50% shoots. The shoots resumed growth after 15 days of transplantation. Ex vitro rooting may reduce the micropropagation cost and also the time from laboratory to field. Rooting extra vitrum has been reported in Rotula aquatica[36], Eupatorium triplinerve[37] and Prunus spp.[38]. In the present study, ex vitro shoots exhibited only 50% survival. A report also noticed a high survival rate of plantlets through ex vitro propagation[39]. This study showed ex vitro rooting gave a lower percentage of survival

than in vitro rooting though ex vitro rooting looks promising considering the reduction in cost by avoiding the in-vitro rooting and use of auxins and the reduction in labour and time of plantlet establishment from laboratory to land. Plantlets derived after in vitro rooting showed 100% survival in field conditions. The plantlets transferred revived growth after 9 days and grew well (Figure 1g). The field established plants were identical in morphology and growth pattern to that of source plant.

CONCLUSION

The protocol described in this study enables production of more than 75 plants within two months using single internode explant. This may realize the demand of pharmaceutical industries and minimize the impact of over exploitation of the plants. Besides the propagation of elite cultivars and conservation of this rare medicinal plant, a highly efficient regeneration protocol opens a way for improvement of the plant through genetic transformation strategies.

REFERENCES

1. M.E.Wall, M.C.Wani, C.E.Cook, K.H.Palmer,G.A.Sim. Plant anti-tumor agents: The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from Camptotheca acuminata. Journal of the American Chemical Society, vol.88, no.16:3888- 3890 (1966).
2. Redinbo,R. Matthew, Styewart, Lance, Kuhn, Peter, Champoux, James J. and Hol, Wim G.J. Crystal structures of human topoisomerase I in covalent and monocovalent complexes with DNA. Science, vol.279, no. 5356:1504-1513, (1998).
3. Lorence, Argelia and Nessler, Craig L. Camptothecin, Over four decades of surprising findings. Phytochemistry, vol. 65, no. 20, p. 2735-2749, (2004)
4. Lorence, Argelia, Medina-Bolivar, F. and Nessler, Craig L. Camptothecin and 10-hydroxycamptothecin from Camptotheca acuminata hairy roots. Plant Cell Reports, vol. 22, no. 6, p. 437-441, (2004).
5. Pantazis, Panayotis, Han, Zhiyong, Chatterjee, Devasis and Wyche James. Water-insoluble camptothecin analogues as potential antiviral drugs. Journal of Biomedical Science, vol. 6, no. 1, p. 1-7, (1999).
6. Japan Pharmaceutical Information Center. Drugs in Japan, Ethical Drugs. Yakugyo Jiho Co, Tokyo, p. 169-270, (1995)
7. Giovanella, B.C, Stehlin, J.S, Wall, M.E, Wani, M.C, Nicholas, A.W, Liu, L.F, Silber, R. and Potmesil, M. DNA topoisomerase I-targeted chemotherapy of human colon cancer in xenografts. Science, vol. 246, no. 4933, p. 1046-1048, (1989).
8. S.Takeuchi, K.Dobashi, S.S.Fujimoto, K.Tanaka. A late phase II study of CPT-11 in gynecologic cancers. Japanese Journal of Cancer and Chemotherapy, vol. 18, no.10:1681-1689, (1991).
9. Bodley, L.Annette, Cumming, N.Jared and Shapiro. Effects of camptothecin, a topoisomerase inhibitor, on Plasmodium falciparum. Biochemical Pharmacology, vol. 55, no.5: 709-711, (1998).

10. K.Saito, H.Sudo, M.Yamazaki, M.Kosekinakamura, M.Kitajima, H.Takayama, N.Aimi . Feasible production of camptothecin by hairy root cultures of Ophiorrhiza pumila. *Plant Cell Reports, March 2001, vol. 20, no.3:267-271.*
11. M.Arai, T.Saito, Y.Kaneko and H.Matsushima. Cellular origin and ultrastructural changes of regenerating shoots from tobacco (Nicotiana tabacum) internodes cultured in vitro. Physiologia Plantarum, vol.99, no. 4: 523-528, (1997).
12. I.Watase, H.Sudo, M.Yamaki, K.Saito .Regeneration of transformed Ophiorrhiza pumila plants producing camptothecin. Plant Biotechnology, vol.21, no.5:337-342, (2004).
13. Kirtikar, K.R. and Basu, B.D. *Indian Medicinal Plants Vol. II. 2nd ed. M/S Bishen Singh Mahendrapal Singh, New Delhi, India, 1975. p. 1268-1269.*
14. Rout, Gyana Ranjan. Direct plant regeneration of curry leaf tree (Murraya koenigii Koenig.), an aromatic plant. In-Vitro Cellular and Development Biology - Plant, vol. 41, no.2:133-136, (2005).
15. Martin, K.P, Sunandakumari, C, Chithra, M. and Madhusoodanan, P.V. Influence of auxins in direct in vitro morphogenesis of Euphorbia nivulia, a lectinacious medicinal plant. In Vitro Cellular and Development Biology - Plant, vol. 41, no. 3, p. 314-319, (2005).
16. Murashige, T. and Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, vol. 15, no. 43, p. 473-497, (1962).
17. V.Tiwari, B.D.Singh, K.N.Tiwari .Shoot regeneration and somatic embryogenesis from different explants of Brahmi [Bacopa monniera (L.) Wettst.]. Plant Cell Reports, vol.17, no.6-7:538-543, (1998).
18. Kawiak, Anna, Krolicka, Aleksandra and Lojkowska, Ewa. Direct regeneration of Drosera from leaf explants and shoot tips. Plant Cell, Tissue and Organ Culture, vol. 75, no. 2, p. 175-178, (2003).
19. Murashige, T. Plant propagation through tissue cultures. Annual Review of Plant Physiology, vol. 25, p.135-166, (1974).
20. Sivaram, Latha, Mukundan, Usha . In vitro culture studies on Stevia rebaudiana. In Vitro Cellular and Development Biology - Plant,vol. 39, no.5:520-523, (2003).
21. Liao, Zhihua; Chen, Min; Tan, Feng; Sun, Xiaofen and Tang, Kexuan. Micropropagation of endangered Chinese aloe. Plant Cell, Tissue and Organ Culture, vol. 76, no. 1, p. 83-86, (2004)
22. Zhang, Chun-Lai,Chen, Dong-Fang; Elliot, C.Malcolm , Slater. Thidiazuron-induced organogenesis and somatic embryogenesis in sugar beet (Beta vulgaris L). In Vitro Cellular and Development Biology - Plant, vol. 37, no.2:305-310, (2001).
23. Martin, K.P.; Joseph, D.; Madassery, J. and Philip, V.J. Direct shoot regeneration from lamina explants of two commercial cut flower cultivars of Anthurium andraeanum Hort. In Vitro Cellular and Development Biology - Plant, vol. 39, no. 5, p. 500-504, (2003).
24. M.Welander. Plant regeneration from leaf and stem segments of shoots raised in vitro from mature apple trees. Journal of Plant Physiology, vol.132, no.6:738- 744, (1988).
25. Rajasekharan, K, Hein, M.B, Davis, G.C, Carnes, M.G. and Vasil, I.K. Endogenous growth regulators in leaves and tissue cultures of Pennisetum purpureum Schum. Journal of Plant Physiology, vol. 130, no. 1, p.13-25, (1987).
26. Dennis,T. Thomas, Boban, Philip , Thidiazuron induced high-frequency shoot organogenesis from leaf derived callus of a medicinal climber, Tylophora indica (Furm. f.) Merrill. In Vitro Cellular

and Development Biology - Plant, vol. 41, no.2:124-128., (2005).
27. Faisal, M. and Anis, M. An efficient in vitro method for mass propagation of Tylophora indica. Biologia Plantarum, vol. 49, no. 2, p. 257-260, (2005).
28. Dhar, Uppeandra and Joshi, Mitali. Efficient plant regeneration protocol through callus for Saussurea obvallata (DC.) Edgew. (Asteraceae): effect of explant type, age and plant growth regulators. Plant Cell Reports, vol. 24, no. 4, p. 195-200, (2005).
29. C.Sunandakumari, Zhang, K.P Martin,A.Slater, P.V.Madhusoodanan . Effect of auxins on indirect in vitro morphogenesis and expression of gusA transgene in a lectinacious medicinal plant, Euphorbia nivulia Buch.-Ham. In Vitro Cellular and Development Biology - Plant, vol. 41, no.5:695-699, (2005).
30. V.Agrawal, P.R.Sardar,. In vitro propagation of Cassia angustifolia through leaflet and cotyledonderived calli. Biologia Plantarum, vol.50, no.1:118-122, (2006).
31. J.Creemers-Molenaar, J.C.Hakkert, M.J.Vanstaveren, L.J.Gilissen. Histology of the morphogenetic response in thin cell layer explants from vegetative tobacco plants. Annals of Botany, vol.73, no.5:547-555, (1994).
32. Lo, K.H, Giles, K.L. and Sawhney, V.K. Histological changes associated with acquisition of competence for shoot regeneration in leaf discs of Saintpaulia ionantha X confusa hybrid (African violet) cultured in vitro. Plant Cell Reports, vol. 16, no. 6, p. 421-425, (1997).
33. C.L.Wenzel, D.C.Brown . Histological events leads to somatic embryo formation in cultured petioles of alfalfa. In Vitro Cellular and Development Biology - Plant, vol.27:190-196, (1991).
34. C.T.Twyford, S.H.mantell . Production of somatic embryos and plantlets from root cells of the greater yam. Plant Cell, Tissue and Organ Culture, vol.46, no.1: 17-26, (1996).
35. Knoll, K.A, Short, K.C, Curtis, I.S Power, J.B. and Davey, M.R. Shoot regeneration from cultured root explants of spinach (Spinacia oleracea L.): A system for Agrobacterium transformation. Plant Cell Reports, vol. 17, no. 2, p. 96-101, (1997).
36. Martin, K.P. Rapid in vitro multiplication and ex vitro rooting of Rotula aquatica Lour, a rare rhoeophytic woody medicinal plant. Plant Cell Reports, vol. 21, no. 5, p. 415-420, (2003).
37. Martin, K.P. Rapid axillary bud proliferation and ex vitro rooting of Eupatorium triplinerve. Biologia Plantarum, vol. 47, no. 4, p. 589-591, (2003).
38. Pruski, Kris; Astatkie, Tess and Nowak, Jerzy. Tissue culture propagation of Mongolian cherry (Prunus fruticosa) and Nanking cherry (Prunus tomentosa). Plant Cell, Tissue and Organ Culture, vol. 82, no.2, p. 207-211, (2005).
39. Liu, Z. and Li, Z. Micropropagation of Camptotheca acuminata decaisne from axillary buds, shoot tips and seed embryos in a tissue culture system. In Vitro Cellular and Development Biology - Plant, vol. 37, no. 1, p. 84-88, (2001).
40. De pavia neto,Vespasiano Borges; Da Mota, Tiago Ribeiro and Otoni, Wagner Campos. Direct organogenesis from hypocotyl-derived explants of annatto (Bixa orellana). Plant Cell, Tissue and Organ Culture, vol.75, no.2:159-167, (2003)
41. Jose, B. and Satheeshkumar, K. In vitro mass multiplication of Ophiorrhiza mungo Linn. Indian Journal of Experimental Biology, vol. 42, no. 6, p. 639-642, (2004).
42. G.Roja. Comparative studies on the camptothecin content from Nothapodytes foetida and Ophiorrhiza species. Natural

Product Research, vol. 20, no.1:85-88, (2006).
43. N. Shrivastava, M. Rajani . Multiple shoot regeneration and tissue culture studies on Bacopa monnieri (L.) Pennell. Plant Cell Reports, vol.18, no.11:919-923, (1999).
44. Q.Y.Shu, G.S Liu,QI, C.C.Chu, J.Liu . An effective method for axillary bud culture and RAPD analysis of cloned plants in tetraploid black locust. Plant Cell Reports, vol. 22, no.3:175- 180, (2003).
45. Stajner, Natasa,Bohanec,Borut,Jakse, Marijana . In vitro propagation of Asparagus maritimus – A rare Mediterranean salt-resistant species. Plant Cell, Tissue and Organ Culture, vol.70, no.3: 269-274, (2002).
46. Sudo, Hiroshi, Yamakawa, Takashi; Yamazaki, Mami. Bioreactor production of camptothecin by hairy root cultures of Ophiorrhiza pumila. Biotechnology Letters, vol.24, no.5:359-363, (2002).
47. S.Tafur, J.D.Nelson, D.C.Delong, G.H.Svoboda. Antiviral components of Ophiorrhiza mungos isolation of camptothecin and 10-methoxycamptothecin. Lloydia, vol. 39, no. 4: 261-262, (1976).