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AN EFFICIENT PROTOCOL FOR *IN VITRO* SHOOT REGENERATION FROM DIFFERENT EXPLANTS OF *MURRAYA KOENIGII* (L.) SPRENG

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Murraya koenigii (L.) Spreng, commonly known locally as "curry patta" or "mitha neem" in India, is a valuable medicinal plant known for its biochemical and aromatic properties. This paper presents a protocol for the rapid and high frequency shoot regeneration from nodal and inter-node explants from matured plant and epicotyl, cotyledons, cotyledonary node (embryonic axis), juvenile leaf, hypocotyl and root segment of *in vitro* derived seedling via axillary and adventitious shoot formation of *M. koenigii*. Adventitious regeneration, which is a pre-requisite in most genetic transformation studies using Agrobacterium and ballistics, needs to be developed as a protocol for micropropagation of *M. koenigii*. Cytokinins benzylaminopurine (BAP), Kinetin (Kin), adenine sulphate (ADS) and indole-3-acetic acid (IAA) were used for multiple shoot induction. Addition of an auxin along with cytokinin improved the shoot production capacity. Shoot buds could be initiated from all the explants tested, with epicotyl explants producing the highest average number of shoots/explant. N6-benzyle adenine (BA), kinetin, adenine sulphate (ADS) and indole-3-acetic acid (IAA) in combination were the most effective PGRs for shoot induction.

Keywords: Adenine sulphate; *in vitro* shoots; *Murraya koenigii*, Regeneration; Rutaceae.

Introduction

Murraya koenigii, commonly known as curry leaf plant belonging to family Rutaceae. Of the 14th global species belonging to the genus *Murraya* (Rutaceae), only two are available in India, viz. *Murraya koenigii* Spreng and *Murraya paniculata* Linn. Jack (syn. with *M. exotica* Linn.). Almost every part of this plant has a strong characteristic odor. Its leaves are slightly pungent, bitter, and acidulous in taste. Fresh and dried leaves are used extensively as a flavoring agent in many Indian culinary practices. The aromatic components of this tree are widely utilized in the medicinal field. The presence of several monomeric, binary carbazole alkaloids[1,2] and simple furo- and pyranocoumarin[3] in various plant parts which are bioactive enabled new vistas in several scientific investigations. These alkaloids are proved to be antimicrobial[4], oxidative[5], diabetic[6] and trichomonal[7]. Some

of them showed anti carcinogenic properties in a cultured human leukemia cell line[8,9]. The presence of numerous valuable medicinal properties and other usage established its potential demand for export[10]. Recent phyto-chemical studies have revealed the presence of antioxidant protein in leaves[11] of *M. koenigii*.

The population of *M. koenigii* in nature is very spars because of its poor rate of fruit set and short viability period of seed with poor rate of germination. Biotechnological approaches for plant regeneration have potential ways for utilization and conservation of plant genetic resources. Therefore, an efficient and rapid *in vitro* regeneration technology for *M. koenigii* is prerequisite for its mass propagation, conservation and genetic improvement through transformation techniques. A few studies were reported on *in vitro* propagation of *M. koenigii* using different explants[12-17]. The present study was aimed to develop an efficient plantlet regeneration protocol through axillary, adventitious shoot using different types of explants such as ten years old matured nodal, inter-nodal segments and epicotyl, cotyledons, cotyledonary node (embryonic axis), juvenile leaf, hypocotyl and root segment of *in vitro* derived seedling.

Materials and methods

(i) Plant material and explant preparation:

Nodal and inter nodal explants were collected from ten year old plant growing in the botanic garden of SPC Government College Ajmer, Rajasthan, India. The explants were first washed twice with Laboline (liquid Soap) rinsed thoroughly with distilled water, treated with 0.1% (w/v) mercuric chloride for 5-6 min to surface sterilize then washed 5 times for 3 min each with autoclaved distilled water. The surface sterilized explants were cut into 2.0 - 2.5 cm pieces. The explants were inoculated on 0.8% agar gelled as well as liquid MS basal medium[18] supplemented with a various concentrations and combinations of 6-benzylaminopurine (BAP), kinetin, indole 3-acetic acid (IAA) and adenine sulfate (ADS).

(ii) Axillary and adventitious shoot multiplication:

Four week old (1-2cm) *in vitro* axillary shoots (regenerated from nodal explants) and adventitious shoots (regenerated from inter - nodal explants) were excised from explants and were cultured on 0.8% agar gelled MS basal medium supplemented with BAP (0.0 – 17.7µM) kinetin (0.0 - 13.93 µM) and adenine sulfate (0.0 - 244.34µM) to optimize the concentration and combination of PGR for enhanced shoot multiplication.

The fruits of *Murraya koenigii* were collected from surrounding areas of Ajmer, Rajasthan, India, the seeds were carefully taken out by removing the pulp of fruits with the help of scalpel and were washed in running tap water for 15 min. Seeds were first washed with liquid detergent (Teepol; Qualigen, India) and then soaked with 0.1% solution of Bavistin fungicide (BASF, India) and rinsed with distilled water. The seeds were surface sterilized with an aqueous solution of 0.1% (*w/v*) $HgCl_2$ (Hi Media, India) for 5-6 minutes followed by four to five washes with sterile distilled water to remove all the traces of mercuric chloride. Disinfected seeds were germinated in 200 ml screw-capped glass jars containing 40 ml seed germinating 0.8% agar gelled half strength Murashige and Skoog (MS) basal medium devoid of plant growth regulators.

Different explants: epicotyl, cotyledons, cotyledonary node (embryonic axis), juvenile leaf, hypocotyl and root

segment were excised from 60 days old *in vitro* derived seedlings.

The explants were inoculated on 0.8% agar gelled MS basal medium supplemented with BAP (0.00-22.21µM), Kinetin (0.00- 13.96 µM), IAA (0.00-4.57µM) and adenine sulfate (0.0 - 244.34µM)

(iii) Shoot Multiplication:

Four week old (1-2cm) shoots were excised from explants and were cultured on 0.8% agar gelled MS basal medium supplemented with BAP, (0.0 to 17.38 µM), Kinetin (0.0 to 13.96 µM), adenine sulfate (0.0 - 244.34µM) and IAA (0.0 to 4.57µM) alone or in combination to optimize the concentration and combination of PGR for enhanced shoot multiplication.

The cultures were incubated in a culture room at 25 ± 2 °C, 55–60% relative humidity (RH), under 12 h per day (hd) photoperiod with a light intensity of 35–40 µmol m−2 s−1 spectral flux photon (SFP) of photo-synthetically active (460–700 nm) radiations (provided by cool white florescent tubes Philips, Mumbai, India).

(iv) Root regeneration and acclimatization:

The healthy and elongated shoots were cultured on 0.8% agar-gelled MS salts supplemented with Indole-3-butyric acid (IBA) and IAA with full strength of MS medium was also evaluated for in vitro root regeneration. The rooting response was recorded after 4 weeks. The in vitro rooted plantlets were taken out of the culture vessels and washed thoroughly with sterile water to remove adhered nutrient agar to avoid microbial contaminations. The healthy and elongated shoots were pulse-treated with different concentrations of IBA (0.0–24.65µM) and IAA (0.0–5.95 µM) for 3–5 min and transplanted into plastic bags containing a mixture of peat moss, perlite and vermiculite in the ratio of 1: 1: 1 for *ex vitro* rooting and subsequent acclimatization under green house conditions. After 8 weeks the acclimatized plantlets were transplanted into large polythene bags or earthen pots containing garden soil and farmyard manure and transferred under natural conditions.

(v) Statistical analysis:

The experiments were carried out in a completely randomized design with 10 replicates per treatment and each experiment was repeated three times. Mean values were subjected to analysis of variance (ANOVA) and statistical significances between means were assessed using new Duncan's multiple range test (DMRT) at $P < 0.05$[19].

Results

(i) Effect of cytokinins and auxin on shoot regeneration from matured axillary bud and inter node explants:

Different kind of cytokinins and auxin were tested for their morphogenetic potential in the regeneration from nodal explants, inter-nodal explants, epicotyl, cotyledons, cotyledonary node (embryonic axis), juvenile leaf, hypocotyl and root segment. Experiments were conducted to achieve maximum number of shoots from different explants on MS medium supplemented with various combinations and concentrations of BAP, Kinetin, ADS and IAA (data of all the experimental not shown, only optimal concentrations of PGR are shown in Table 1). The combination of BAP (11.09 µM), Kinetin (11.61 µM) and ADS (81.44 µM) was found more effective for regeneration of 8.8 ± 0.02 shoots in 97 ± 2.28 percent axillary bud explants as compare to internode explant in which 6.9 ± 0.06 shoots were regenerated on MS basal medium with 13.01 µM BAP, 8.87 µM kinetin and 136.52 µM ADS (Table 1). The shoots regenerated from nodal (axillary bud) explants (Fig.1A) attained a length of 42.6 ± 0.05 mm whereas the shoots regenerated from inter-node

Table 1. Effect of different concentrations of plant growth regulators on shoot regeneration from different explants of *Murraya koenigii* after 8 weeks of cultures.

S. No.	Explants	M.S Medium + PGRs (µM)				Shoot regenerated from different types of explants		
		BAP	Kinetin	ADS	IAA	Explant response (%) for shoot initiation (Mean ± S.D.)	Average no. of shoots per explant (Mean ± S.D)	Average shoot Length (mm) (Mean ± S.D)
1.	Axillary bud	11.09	11.61	81.44	-	97.00 ± 2.28 ef	8.8 ± 0.02ef	42.6 ± 0.05 a
2.	Inter-node	13.01	8.87	136.52	-	82.02 ± 0.08cc	6.9 ± 0.06b	15.2 ± 0.06gh
3.	Epicotyl	12.54	9.21	-	2.87	95.20 ± 0.07bd	8.9 ± 0.09fd	30.0 ± 0.05bd
4.	Leaf	12.75	8.98	152.74	-	72.80 ± 0.08kh	6.3 ± 0.07g	16.4 ± 0.09ac
5.	Cotyledons	12.95	8.98	159.32		95.00 ± 0.58g	8.6 ± 0.04ef	19.3 ± 0.09ij
6.	Cotyledonary node	12.54	9.21	-	2.87	89.40 ± 0.37h	6.7 ± 0.09aa	28.4 ± 0.07cd
7.	Hypocotyl	12.95	8.98	159.32	-	76.20 ± 0.06gh	7.4 ± 0.01a	15.3 ± 0.05ed
8.	Root segment	13.05	9.15	148.45	-	94.50 ± 0.05abc	8.8 ± 0.07bc	19. 9± 0.05cc

$P < 0.05$; Each value represents the mean ± Standard deviation (SD) of ten replicates per treatment in three repeated experiments, PGRs plant growth regulators, BAP 6- benzylaminopurine, ADS Adenine sulphate, IAA indole-3-acetic acid MS medium- Murashige and Skoog medium

Table 2. Effect of different concentrations of plant growth regulators on shoot multiplication from different explants of *Murraya koenigii*

S. No	Explants	M.S Medium + PGRs (µM)				Shoot multiplication from different types of explants
		BAP (µM)	Kinetin (µM)	ADS (µM)	IAA (µM)	Multiplication Rate (Mean ± S.D.)
1.	Axillary bud	11.09	11.69	81.44	-	3.4 ± 0.03fd
2.	Inter-node	9.35	4.56	186.07	-	4.8 ± 0.02 bb
3.	Epicotyl	8.52	4.62	-	0.58	4.1 ± 0.06d
4.	Leaf	9.05	4.56	186.94	-	2.8 ± 0.07 ac
5.	Cotyledons	8.59	4.62	183.62	-	4.6 ± 0.03b
6.	Cotyledonary node	8.52	4.62	-	0.58	3.9 ± 0.08ad
7.	Hypocotyl	8.59	4.62	183.62	-	2.8 ± 0.04k
8.	Root segment	8.85	4.66	190.18	-	4.2 ± 0.06 ab

$P < 0.05$; Each value represents the mean ± Standard deviation (SD) of ten replicates per treatment in three repeated experiments; PGRs plant growth regulators, BAP 6- benzylaminopurine, ADS Adenine sulphate, IAA indole-3-acetic acid MS medium- Murashige and Skoog medium

explants (Fig.1B) attained a length of 15.2 ± 0.06 mm in 8 weeks. The in *vitro* raised shoots were multiplied by subsequent sub-culturing on the same medium in the interval of 4 weeks (Fig.1C).

(ii) Effect of cytokinins and auxin on shoot regeneration from different parts of in vitro raised seedling:

Different parts epicotyl, cotyledonary node (embryonic axis), intact cotyledons, juvenile

Fig. 1. *In vitro* regeneration of shoots from **A** - matured nodal (axillary bud) explant, **B**- inter-nodal explant, **C**- Multiplication of axillary and adventitious shoots, Regeneration of shoots from different part of *in vitro* seedling, **D**- Epicotyl, **E**- Cotyledonary Node (embryonic axis), **F**- Cotyledons, **G**- Leaf, **H**- Hypocotyl, **I**- Root, **J**- Shoot multiplication, **K**- *ex- vitro* rooted plants of *M. koenigii*, L- hardened *in vitro* raised plants of *M. koenigii.*

Table 3. Effect of MS medium and different concentrations of rooting hormones on root induction from *in vitro* raised shoots of *Murraya koenigii*

S. No.	Different types of explants	MS Medium + PGRs (µM)		% Rooting	Length of roots (mm) (Mean ± S.D)
		IBA	IAA		
1.	Axillary	12.30	5.13	95.6 ± 1.14e	24.0 ± 1.30gh
2.	Inter-node	19.68		91.4 ± 0.02 g	23.1 ± 0.83ab
3.	Epicotyl	9.98	-	93.4 ± 2.30 b	25.5 ± 1.41fg
4.	Leaf	14.86	-	76.2 ± 0.15ef	20.0 ± 0.83cb
5.	Cotyledons	17.26	-	84.8 ± 0.13cg	23.5 ± 1.58ef
6.	Cotyledonary node	9.98	-	88.0 ± 1.00df	25.0 ± 1.40 c
7.	Hypocotyl	17.26	-	64.4 ± 0.11aa	21.3 ± 0.44df
8.	Root	19.72	-	95.8 ± 0.79ad	22.7 ± 1.06k

$P < 0.01$; Each value represents the mean ± Standard deviation (SD) of ten replicates per treatment in three repeated experiments; PGRs plant growth regulators, IBA indole-3-butyric acid, IAA indole-3-acetic acid, MS medium- Murashige and Skoog medium

leaf, hypocotyl, and root segments of four weeks old *in vitro* raised seedlings were used as explants for regeneration of shoots (Fig. D - I). The highest 95.2 ± 0.07 percent epicotyl explants were responded for regeneration of 8.9 ± 0.09 shoots per explants on MS basal medium supplemented with 12.54 µM BAP, 9.21 µM kinetin and 2.87 µM IAA (Fig.1 D). Different concentrations of ADS with BAP and kinetin were required for shoot regeneration from all the seedling explants except Cotyledonary node (Table.1).

(iii) Effect of cytokinins and auxin on shoot multiplication from different parts of in vitro raised seedling-

Regenerated *in vitro* shoots were excised from different explant and sub- cultured to the MS medium augmented with combinations of BAP, Kinetin, ADS and IAA in different concentrations (data of all the experimental not shown, only optimal concentrations of PGR are shown in Table-2). Compact shoot clumps were formed in a period of 3-4 week (Fig. J). The highest multiplication rate (4.6 ± 0.03) was observed on MS medium supplemented with BAP 8.59 µM, Kinetin 4.62 µM and ADS 183.62 µM compact clumps of shoots were formed from cotyledons (Table-2).

(iv) Effect of different concentrations of IBA and IAA on root induction from shoots regenerated from different explants:

The shoots regenerated from different explants were kept separately and were inoculated on MS basal medium supplemented with different concentrations of IBA and IAA individually or in combination (Fig. K). IBA 12.30 µM in combination with IAA 5.13 µM was found suitable for induction of roots in 95.6 ± 1.14 percent shoots of axillary bud explants (data of the entire experimental not shown, only optimal concentrations of PGR are shown in Table 3). The root lengths in all the shoots were regenerated from different explants ranging between 20.0 ± 0.83mm to 25.5 ± 1.41 mm (Table- 3).

(v) Establishment of plantlets:

In vitro plantlets were hardened in small earthen pots containing a mixture of Soil Rite (peat moss: perlite: vermiculite in the ratio of 1: 1: 1 at 70-80% relative humidity and temperature gradient of 28-36°C under green house conditions for 21 days. These plants were then transferred to field conditions (Fig. L).

Discussion

A plant regeneration system via shoot proliferation from axillary bud explant, inter node explant, epicotyl, cotyledons, cotyledonary node (embryonic axis), leaf, hypocotyl and root segments of M*urraya koenigii* was successfully revealed through this investigation.

However, the present study suggested that MS medium responded better than WPM media. The type of explant is an important factor for organogenesis in tissue culture[20]. The regeneration studies have been reported in a number of plant species using stem cuttings and intact seedling[21,16] and also reported in other species of c*itrus* and *Aegle marmelos*[22,23]. Which are close relatives of *Murraya koenigii*? In our study Cotyledons and Leaf explants were used which do not have any apparent pre-existing meristems. The adventitious bud formation efficiency of cultured explants showed varied response and seems to be dependent more precisely on type of explant, culture medium, concentrations and combination of PGRs. Cytokinin either alone or in combination has significant effects on shoot induction and their subsequent multiplication[24-27]. Adenine sulphate is known to be precursor of adenine during the DNA replication in cell, which supposed to

be indirectly helps in the rejuvenation of plant vigor, therefore, the explants and the shoots in the adenine sulphate supplemented in MS medium exhibited rejuvenation after each sub culture[16,28]. Similar observation was noted in present investigation in which the highest shoot proliferation was recorded on BAP, Kinetin and Adenine sulphate added MS basal medium. In general, lower concentration of adenine sulphate or without ADS the cultures could not maintain their vigor for longer time under *in vitro* conditions. Similar observation was recorded in our study that ADS at the concentration 159.32 µM favored the induction of maximum number of shoots from axillary explants and in higher concentration 186.07 µM showed higher rate of shoot multiplication of shoots from inter-node explants of *Murraya koenigii.* Type of auxin and their optimized concentration in the medium was found to be the critical factor in the regeneration of healthy roots. Superiority of IBA over other auxins in root formation has also been reported in other plant species such as *Cunila galoide, Clitoria ternatea* and *Cassia siamea*[29-31]. The IBA has been reported to have a stimulatory effect on root induction in many tree species including *Alnus glutinosa* and *Morusindica*[32,33]. In present study, the highest rooting percentage (95.8 ± 0.79) was achieved in the shoots originate from root segment on MS medium supplemented with 19.72 µM IBA. In general, *in vitro* raised plantlet grow in microbe free and control conditions, therefore the hardening these plants in pre requisite for their field transfer. In our study rooted plantlets were hardened prior to their field transfer. Six weeks old hardened plantlets with tap roots were transferred to poly bags containing a mixture of perlite, vermiculite and peat moss in equal ratio (1: 1: 1).

Conclusion-

The protocol described in this study enables production of over one hundred plants from single explant by re-culturing on fresh medium to produce micro-shoots for rooting. The protocol reported in present research may meet the raw material demands of pharmaceutical industries for isolation of target compounds from *M. koenigii*. Furthermore, the investigation will potentially address the issues of large scale micropropagation and genetic transformation of *M. koenigii* and its close relatives.

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References

1. Chakrabarty M, Nath A, Khasnobis S, Chakrabarty M, Konda Y, Harigaya Y and Komiyama K 1997, Carbazole alkaloids from *Murraya koenigii*. *Phytochemistry* **46**(4) 751-755.
2. Nutan MTH, Hasnat A and Rashid MA 1998, Antibacterial and cytotoxic activities of *Murraya koenigii. Fitoterapia,* **69**(2) 173-175.
3. Gupta GL and Nigam SS 1970, Chemical examination of the leaves of *Murraya koenigii*. *Planta Medica,* **19** 83.
4. Rahman MM and Gray AI 2005, A benzoisofuranone derivative and carbazole alkaloids from *Murraya koenigii* and their antimicrobial

activity. *Phytochemistry,* **66** 1601-1606.

5. Tachibana Y, Kikuzaki H, Lajis NH and Nakatani N 2003, Antioxidative activity of carbazoles from *Murraya koenigii* leaves. *J Agric Food Chem,* **49** 5589–5594.
6. Kesari AN, Kesari S, Singh SK, Gupta RK and Watal G 2007, Studies on the glycemic and lipidemic effect of *Murraya koenigii* in experimental animals. *J Ethnopharmacol,* **112** 305–311.
7. Adebajo AC, Ayoola OF, Iwalewa EO, Akindahunsi AA, Omisore NO, Adewunmi CO and Adenowo TK 2006, Anti-trichomonal, biochemical and toxicological activities of methanolic extract and some carbazole alkaloids isolated from the leaves of *Murraya koenigii* growing in Nigeria. *Phytomedicine,* **13** 246–254.
8. Roy MK, Thalang VN, Trakoontivakorn G and Nakahara K 2004, Mechanism of mahanine-induced apoptosis in human leukemia cells (HL-60). *Biochem Pharmacol,* **67** 41–51.
9. Ito C, Itoigawa M, Nakao K, Murata T, Tsuboi M, Kaneda N and Furukawa H 2006, Induction of apoptosis by carbazole alkaloids isolated from *Murraya koenigii*. *Phytomedicine*, **13** 359–365.
10. Rao LJM, Ramalakshmi K, Borse BB and Raghavan B 2007, Antioxidant and radical-cavenging carbazole alkaloids from the oleoresin of curry leaf (*Murraya koenigii* Spreng.). *Food Chem,* **100** 742–747.
11. Ningappa MB, Dinesha R and Srinivas L 2008, Antioxidant and free radical scavenging activities of polyphenol-enriched curry leaf (*Murraya koenigii* L.) extracts. *Food Chem,* **106** 720–728.
12. Bhuyan AK, Pattanaik S and Chand PK 1997, Micropropagation of curry leaf tree [*Murraya koenigii* (L.) Spreng.] by axillary proliferation using intact seedlings. *Plant Cell Rep,* **16** 779–782.
13. Mathew D and Prasad MC 2007, Multiple shoot and plant regeneration from immature shoot and plant regeneration of *in vitro* origin in curry leaf (*Murraya koenigii* Spreng) *Indian J. Plant Physiology,* **12**(1) 18-22.
14. Khatik N and Joshi R 2014, Efficient plantlet regeneration system via enhanced adventitious shoot proliferation in *Murraya koenigii* (L.) Spreng. *J. Phytol. Res.* **27** (1 & 2) 33-40.
15. Khatik N and Joshi R 2016, *In vitro* adventitious shoot regeneration from cotyledon and hypocotyl explants of *Murraya koenigii* (l) spreng. *J. Phytol. Res.* **29** (1 & 2) 7-16.
16. Rout GR 2005, Direct plant regeneration of curry leaf tree (*Murraya koenigii* koenig.) an aromatic plant. *In Vitro Cell Dev Biol-Plant*, **41** 133–136.
17. Nirmal Babu K, Anu A, Remashree AB and Praveen K 2000, Micropropagation of curry leaf tree. *Plant Cell Tissue Organ Cult,* **61** 199–203.
18. Murashige T, Skoog F 1962, A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant,* **15** 473–497.
19. Duncan DB 1955, Multiple range and multiple F tests. *Biometrics* **11** 1-42.
20. Ntui VO, Thirukkumaran G, Iioka S and Mii M 2009, Efficient plant regeneration via organogenesis in

"Egusi" melon (*Colocynthis citrullus* L). *Sci Hort*, **119** 397– 402.
21. Anonymous 1988, The Wealth of India: A dictionary of Indian raw materials and industrial products, CSIR Publication, New Delhi, India, **6** S446–448.
22. Rattanpal HS, Kaur G and Gupta M 2011, *In vitro* plant regeneration in rough lemon (*Citrus jambhiri* Lush) by direct organogenesis. *African Journal of Biotechnology*, **10**(63) 13724-13728.
23. Behera PR, Thirunavoukkarasu M and Chand PK 2013, Adventitious plantlet regeneration from different explants of *Aegle marmelos* (L.) Corr. *Journal of Medicinal Plant Research*, **7(**37) 2761-2768.
24. Gaj MD 2004, Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. *Plant Growth Regul*, **43** 27–47.
25. Dam A, Paul S and Bandyopadhyay TK 2010, Direct somatic embryogenesis and plant regeneration from leaf explants of *Limonium sinensis* (Girard) Kuntze. *Sci Hortic*, **126** 253– 260.
26. Nanda RM and Rout GR 2003, *In vitro* somatic embryogenesis and plant regeneration in *Acacia Arabica*. *Plant Cell Tissue Organ Cult*, **73** 131–135.
27. Carra A, De Pasquale F, Ricci A and Carimi F 2006, Diphenylurea derivatives induce somatic embryogenesis in *Citrus*. *Plant Cell Tiss Organ Cult*, **87** 41–48.
28. Tejavathi DH, Padma S, Gayathramma K and Pushpavathi B 2010, *In vitro* studies in *Sauropus androgynous* (L.) merr. *Acta Hortic*, **865** 371-375.
29. Fracro F, Echeverrigaray S 2001, Micropropagation of *Cunila galoides*, a popular medicinal plant of South Brazil. *Plant Cell Tiss Org Cult*, **64** 1-4.
30. Shahzad A, Faisal M and Anis M 2007, Micropropagation through excised root culture of *Clitoria ternatea* and comparison between *in vitro* regenerated plants and seedlings. *Ann Appl Biol*, **150** 341-349.
31. Parveen S, Shahzad A and Saema S 2010, *In vitro* plant regeneration system for *Cassia siamea* Lam., a leguminous tree of economic importance. *Agrofor Syst*, Doi: 10.1007/s10457-010-9301-3.
32. Perinet P and Lalonde K 1983, *In vitro* propagation and nodulation of the actinorhizal host plant, *Alnus glutinosa* (L.) Gaertn. *Plant Sci Lett*, **29** 9–17.
33. Chand PK, Sahoo Y, Pattnaik SK and Patnaik SN 1995, *In vitro* meristem culture – an efficient ex situ conservation strategy for elite mulberry germplasm, In: Mohanty RC (ed) Environment: change and management, Kamla Raj Enterprises, New Delhi, India, pp 127–133.