Int J Pharm Bio Sci 2012 Oct; 3(4): (B) 659 - 668

Research Article **Plant Bio technology**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

Micropropagation of *Clerodendrum indicum* (L.)Kuntze: An Unexplored Medicinal Plant

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ABSTRACT

A simple micropropagation protocol has been achieved using nodal region and apical leaf of *Clerodendrum indicum*,(L) kuntze; an important medicinal plant of Verbenaceae. The explants were cultured in MS medium supplemented with NAA, BA, 2,4-D,Kn, IBA in various combination and concentrations (0.1- 10.0 µM). Response to callogenesis were diverse, ranging from white vigorous compact callus (1.0µM NAA), compact green calli in NAA:BA(0.5:0.1µM) & (1.0:1.0 µM), white compact calli (1.0:3.0µM) NAA: Kn. Only 2,4D expectedly generated profuse watery, vigorous, friable calli (1.0µM).The nodal region was differentiated into shoot directly in combinations with NAA and BA(0.1:1.0µM, 1.0:3.0µM, 0.5:1.0µM, 0.1:0.5µM). Multiple shooting were generated in NAA: BA combination (0.5:0.3µM). Moderate rooting were initiated at high IBA level in (10 µM) in full strength MS Medium. Simultaneous emergence of root & shoot were noticed from callus in triple combination of NAA, BA and IBA,in triple combination(5.0:1.0:1.0µM). The well rooted plant were transferred in the sterile soilrite covered with polybag in the culture room for acclimatization for 15 days. Such plants were transferred into the pot with 90% survivality.This is the maiden report of micropropagation of *Clerodendrum indicum*(L.)Kuntze.

KEY WORDS: ***Clerodendrum indicum*. Growth regulators, Nodal region, Apical leaf, callogenesis, *in vitro* shooting , Rooting, plantlets, Acclimatization.**

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INTRODUCTION

Clerodendrum indicum (L.) Kuntze, a woody herbaceous, perennial plant of Verbenaceae, is a native of India, commonly known as Bamonhati(Bengali) . This plant originated in eastern India and found dispersed throughout south eastern Asia, India, Nepal, Bhutan, Sri Lanka, and Southern China[1]. In India, it is found mainly in West Bengal and Assam. The plant is Shrub about 3m long, leaves are stalked, 6-7 cm long with broad base, lanceolate having acute apex with sub-entire margin. Inflorescence raceme, white flowers develop at the axillary or terminal branches from March to June . Fruits are dark bluish green in colour.

The aqueous extract of the plant has traditional use for treatment of cough, serofulous infection, buboes problem, skin disease. In addition to this, it has been employed as vermifuge and febrifuge[2]. Rheumatism, asthma and other inflammatory disease can also be treated with leaf and root extract(aqueous) of the plant[3]. The juice of the leaf has wider application in hepatic eruption and pemhigus[4].The methanolic extract of the plant has been shown to inhibit lipid peroxidation in bovine brain [5].

Few important phytochemicals like few phenolics, flavonoids alkaloids, steroids were reported from the different parts of the plant. The phenolic compound known as Cleroindicin (A-F) were isolated from aerial part of the plant[6,7]. Two characteristic flavonoids namely, hispidulin and pectolinarigenin were reported from the stem and the root of the plant [4.] A novel hydroquinone diterpenoid, clerodendrone was isolated from the plant *Clerodendrum indicum* [8].Alkaloids and few steroids were reported to occur in leaf and flower [9]. The plant extract was significant antibacterial activity against twelve pathogenic bacterial strains [4.]

The seeds of the plant are highly abortive & low seed-setting rate pose vital problem for its survival in wild conditions. Conventionally, the propagation is done only by stem cutting, but the regeneration of the plants is too poor due to high mortality rate in field. The paper addresses this problem by proposing an easy micropropagation protocol for rapid multiplication of the plant using *in vitro* techniques. This protocol assumes importance not only from conservation aspect of this medicinal plant but also serves the purpose of explorative search for novel drugs till unknown to pharmaceutical science.

MATERIALS AND METHODS

a) Plant material

The plantlet of *Clerodendrun indicum* (L) Kuntze. and its mature seeds were collected from the medicinal garden of Hatgobindopur College, Burdwan, India. Collected plants were maintained in the pot. We used nodal region, apical leaf, axillary leaf and seeds of the plant for selection of perfect explants.

b) Selection & sterilization of explant

All the explants were washed thoroughly in tap water for 10-15 min. Then it was immersed into 1% bavistin solution stirred vigorously for 10 min in 30-35°C to provide better surface contact. These were rewashed in running tap water to remove the traces of Bavistin. After that, the explants were treated with freshly prepare 0.1% (w/v) $HgCl_2$ (Merck, India) solution under laminar air flow for 1-7 minutes (vary according to the explant types- For nodal Region, apical leaf, axillary leaf -1-3 min, seeds 5-7 min). Finally the explants were washed in sterilized distilled water for 4-5 times to complete the surface sterilization process.

c) Treatment and germination of seeds

The surface sterilized seeds were treated with distilled water and GA (100-500µM) for 1-4 hours. Then it were transferred to sterilized

petridishes embedded with deionized water soaked blotting paper and kept at 25±2°C and RH 65 ± 5% under continuous white fluorescent light (450-640uW.cm^{-2}) emitted by 40W Phillips tubes . The germination rates were very low because germinated seeds were found be to infected with endogenous fungal mats. So, the cotyledonary leaves were not used as explants and instead nodal region and apical leaf were used.

d) Culture Medium and Culture Condition

Explants (Nodal Region, apical leaf axillary leaf) were transferred into sterilized MS Medium with different PGRs in various concentration .The explants length were 1-2cm long and put into the semisolid MS medium (pH-5.8) containing 3% sugar and 1% agar with different combinations of PGRs. Approximately, 10ml medium with PGRs are dispensed into the culture tube 2.5cm×15cm test tube (Borosil) plugged with non – absorbent cotton and autoclaved at 1.06kg cm $^{-2}$ at 121°C for 15 mins.

Cultures were maintained in the culture room at 25 ±2°c temperature and RH 65±5% under continuous white fluorescent light (450-640uW.cm^{-2}) emitted by 40WPhilips tubes. The explants were transferred into MS medium with different concentrations of NAA, BA, 2, 4-D, Kn, IBA etc. from 0.1-10.0µM within the culture tube. Subculturing was routinely done at interval of 30-45 days for maintenance of the calli. Subsequently calli were differentiated into plantlets within 30 days.

RESULT

a) Callogenesis

Apical leaf explants when placed in MS medium supplemented with NAA, BA 2, 4 D & Kn either in combination or separately, began to form different types of calli. Few of these combinations produced microshoots (through both direct and indirect embryogenesis). Poor responses were generally found in different combinations and concentrations of PGRs both in callogenesis and regeneration when axillary leaf used as explant. Only NAA (1.0µM) produced white, friable calli with moderate growth. NAA with two different combination of BA produced green, compact, embryogenic calli. The two combinations of NAA:BA generated calli were different in terms of rate of callogenesis. NAA:

e) Scanning Electron Microscopy Study

For scanning electron microscopic study, Calli were fixed in 2% gluteraldehyde in potassium phosphate buffer (pH 6.8) dehydrated with graded alcohol and amyl acetate series, critical point drying with CO_2, and sputter coated with gold. Scanning electron photographs were obtained using Hitachi S-530, 15 KV SEM.

(f) Acclimatization and field transfer

For acclimatization, the in vitro raised plantlets were taken out of the test tubes, washed thoroughly with tap water and dipped in 1% bavistin for 5 mins and rinsed again with sterile distilled water. Such plantlets were then transferred to pre-sterilized soilrite kept in the beaker and watered with sterilized distilled water thrice a week up to 15 days in the culture room condition. The beaker was covered with polybag to check excessive water loss After 15 days, the plastic cover were removed and the plant were kept in the room temperature for 1 week for acclimatization. Then the healthy plants were transplanted to the pot containing pre-autoclaved garden soil and kept in field condition for one month for complete adaption.

(g) Statistical analysis

Results are expressed as mean ± SE. The mean value calculated on the basis of the five replicates in each experiment, each experiment was repeated thrice. Data were statistically analyzed using ANOVA with SAS software (SAS Institute 1999). The data were not significantly different at p≤0.05 level.

BA(0.5:0.1µm)produced calli within 30±5 days whereas NAA:BA(1.0:1.0) produced calli within 21±2 days.(Fig.1)

Figure .(1)
Embryogenic calli produced in NAA:BA (0.5:0.1µm)

Only 2,4-D (1.0µM) expectedly produced watery, friable rapidly growing calli in high concentration. Kn were shown poor response in callogenesis, but Kn used in combination with NAA and 2, 4-D separately. NAA: Kn (1.0:3.0µM) were produced white, compact, vigorous calli (Fig.2). 2, 4-D: Kn (0.5: 3.0µM) were produced white friable calli but the growth was vigorous . All the calli developed within 28 -30 days. The responded combination of PGRs and nature of calli were shown in Table 1.

TABLE: 1
Responded Concentration of different PGRs for callogenesis and its type.

NAA	BA	2,4D	KN	Type of calli	Growth Response	Culture grow in days
1.0	-	-	-	White, loose,	Moderate	30±2
0.5	0.1	-	-	Green, compact, embryogenic	Moderate	30±2
1.0	1.0	-	-	Green, compact, embryogenic	Vigorous	21±5
1.0	-	-	3.0	White ,compact,	Vigorous	21±2
-	-	1.0	-	Watery, friable ,	Vigorous	21±2
-	-	-	5.0	Whitish, friable	Vigorous	21±5
-	-	0.5	3.0	White friable,	Vigorous	21±5

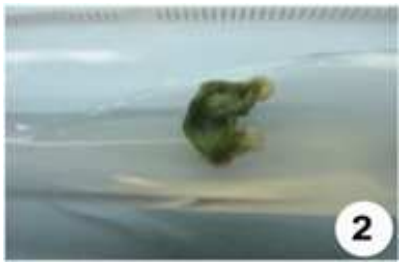

Figure.(2)
White calli in NAA:Kn(1.0:3.0µm)

b) Development of shoots

Among several explants, only nodal segments produced shoots directly or via callus formation. The different combinations of NAA: BA stimulated shooting directly from the explant. The combination of NAA: BA (0.1:1.0µM) produced shooting (No. of shoots-2) .The double shoots (with average shoot length 2.1±0.3cm) were showing polar regeneration at the cut ends of the nodes. (Fig.3 & 6).Multiple shooting generated in combination of NAA: BA (0.5:0.3µm) within 30±5 days of culture. The number of shoots were seven, and shoots regenerated from one end of the nodal segment (Fig4 & 5). The average shoot length was 3.3±0.15cm. It was transferred into the rooting medium by cutting each shoot separately. Single shoots developed in various combination of NAA:BA (1.0:3.0µM,average shoot length 3.3± 0.1^{d} cm), (0.5:1.0µM, average shoot length 3.1 ± 0.09^{cd} cm), (0.1:0.5µM, average shoot length 2.9 ± 0.06^{bc} cm) & NAA:KN (0.1:10.0µM, average shoot length 2.8 ± 0.1^{b}cm) . The combinations of PGRs, no. of shoots and average shoot length were shown in Table 2.

A single triple combination of NAA:BA: IBA (5.0:1.0:1.0) produced shooting and rooting simultaneously via callogenesis. The root and shoots were developed from the calli around one and half month of culture. The average shoot length was 3.45 ± 0.1cm and root length 4.32± 0.16 cm developed by indirect embryogenesis (Fig7 & 8).

Figure .(3) & Figure.(6)
Polar regeneration of shoot by direct embryogenesis in NA:BA(0.1:1.0µm)

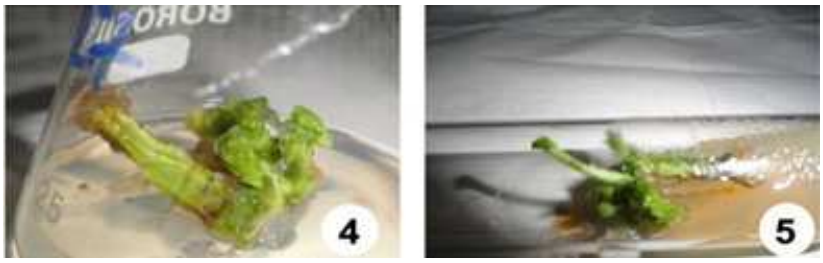

Figure (4)&Figure (5)
Multiple shoot regeneration by direct embryogenesis NAA:BA(0.5:0.3µm)

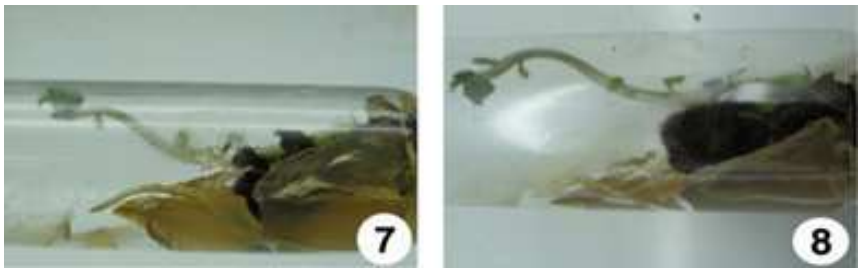

Figure (7) & Figure (8)
Triple combination of NAA:BA:IBA (5.0:1.0:1.0µm) generated shooting and rooting simultaneously.

TABLE: 2
Responded Concentration of PGRs for Regeneration of shoots(by direct embryogenesis)

NAA(µM)	BA (µM)	2,4D(µM)	Kn(µM)	NO OF SHOOT	AVARAGE SHOOT LENGTH WITH STANDARD DEVIATION(cm)
0.1	1.0	-	-	2	2.1 ± 0.3^{a}
1.0	3.0	-	-	1	3.3 ± 0.1^{d}
0.5	1.0	-	-	1	3.1 ± 0.09^{cd}
0.1	0.5	-	-	1	2.9 ± 0.06^{bc}
0.1	-	-	10.0	1	2.8 ± 0.1^{b}
0.5	0.3			7	3.3 ± 0.15^{a}

(c) Development of Roots
All the shoots (developed by direct and indirect embryogenesis) were transferred into MS medium supplemented with IBA (1.0µM) and normal roots emerged within 25±2 days from the base of the shoots(Fig.9).

Figure (9)
Rooting of excised shoot in the IBA(1.0µm) +MS medium

c) Scanning electron micrographs of callus
In scanning electron microscopic study, the differentiated embryogenic callus of NAA:BA (1.0:1.0µM) reveals profuse development of vascular structure. The vasculature were represented mainly by xylem strands. Tiny, sessile glands were found scattered throughout the surface of the callus tissue mass. Stomata were also present beside the xylem strand. (Fig.11 & 12).

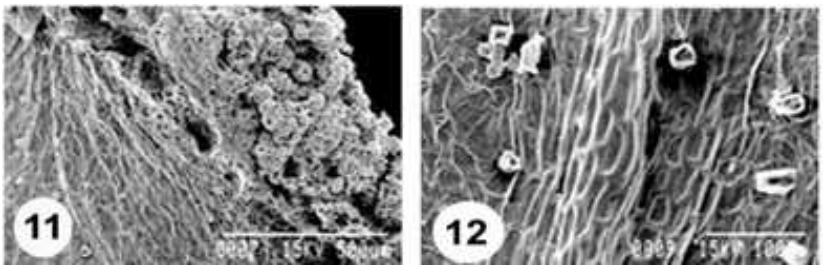

Figure (11)
SEM Photograph of embryogenic calligrown in NAA:BA(0.5:0.1µm) showing profuse vascular structure(×100)

Figure (12)
Vascular structure of embryogenic calli with stomata and glands(×300)

(d) Acclimatization and field transfer
The *in vitro* raised plantlets were taken out of the test tubes and were subjected to routine hardening process. The plant washed thoroughly with tap water and dipped into 1% bavistin for half an hour and rewashed in sterilized distilled water, transferred to sterile soilrite kept in beaker, covered with polybag. Then the plants were watered with sterile distilled water thrice a week up to 15 days for complete acclimatization (Fig.10). After that, the plants were transplanted to the pot containing pre-autoclaved garden soil and maintained in constant humidity (70%) in the culture room condition for next 7 days. Finally, the plant were transferred to the pot and allowed to grow normally just like a field plant. The percentage of survival in the field was about 90% after 3 months.

Figure (10)
Acclimatization of the plantlets in culture room

DISCUSSION

Vegetative propagation of the plant *Clerodendrum indicum*(L.)Kuntze. has been found extreamly difficult because of three reasons: (1)fruits are mostly abortive in nature (2)germination rates of the seeds are remarkably poor due to presence of endogenous pathogen which infect germinated seedling. (3) non- availability of elite germplasm. Clonal multiplication through explants is advantageous over conventional propagation methods as it overcomes these hindrances and a large number of plants can be produced within a limited time. Among the several explants (apical leaf, axillary leaf, cotyledonary leaf and nodal segment), only apical leaf and nodal segment responded properly.

Apical leaf explants generally produced various types of calli in *C indicum* . Cytokinin could not induced proper callogenesis at any concentration (0.1-10.0µM). BA failed to induced any callus production (0.1-10.0 µM) while kinetin produced slow and poor callogenesis (0.1-10.0 µM) excepting at 5.0 µM concentration where rapid, friable calli developed regularly. In *Vitax negundo* ,BA and Kn singly not responded to callogenesis at any concentration was reported [10].

Auxins, mainly 2,4-D are known for rapid cell division and callus induction up to optimum concentration. In our experiment, 2,4-D expectably produced, first growing watery, friable calli in higher concentration (1.0µM) but beyond this concentration calli did not form. 2,4-D induced white friable calli with Kn and best response in this combination was 2,4-D:Kn at 0.5:3.0µM concentration.

On the other hand, green, embryogenic callus induction was noticed in various combination of NAA : BA. As expectedly this two combinations generated embryogenic calli but the growth rate was comparatively slow than other auxin or cytokinin treated sets. On the other hand, NAA: BA induced (0.5:0.1µM) green compact callus with moderate growth within 30 days while NAA: BA (1.0:1.0 µM) supported rapid growth of callus within 21 days. In *Vitax negundo*, a plant of Verbenaceae family, generated calli in low concentration of NAA and high concentration of BA[10].

In SEM study, embryogenic calli showed that profuse vascular structure and stomata which indicate the differentiation of the calli into the shootlets. Few glands were also found on the surface of the calli. NAA individually at 1.0µM concentration induced white, loose, friable callus of moderate growth , but in combination NAA: Kn (1.0: 3.0 µM) combination induced white compact ,vigorous callogenesis. Poor response were generally found in different PGRs ranging from 0.1-10.0µM (combination or singly) both in callogenesis and regeneration where the explants was axillary leaf.

Among the several explants, only nodal explants induced shooting in various combination of NAA: BA and NAA: KN in different concentration, but NAA: BA (0.1:1.0µm) and (0.5:0.3µm) showed best

response compared to others. The NAA: BA (0.1:1.0µm) combination induced emergence of double shoots from both sides of nodal explant. Multiple shoot regeneration was noticed in NAA: BA combination (0.5:0.3µm) and no of shoot was seven (7) per explants developed from one side of the nodal explants. Good responses on shoot morphogenesis using supplemented cytokinin and auxin were also reported. Among the several auxin–cytokinin combination, NAA: BA found to produce good response compared to NAA:2i-P in *in vitro* clonal propagation of *Vitex negundo* (L.)[10]. In *Clerodendrum incisum*(L.) a low concentration of BA was found to be optimum for highest number of shoot multiplication [11]. Number of shoots were more in BA containing medium as compared to Kn . Superiority of BA over KN was also demonstrated by several workers[12]. BA proved superior to other cytokinin for multiple shoot induction in *Clerodendrum colebrookianum* [13].At high concentration of BA or Kn, the rate of shoot proliferation declines. Similar findings have been reported in *Psoralea corylifolia* [14], *Cleistanthus callinus* [15] ,*Decalepis arayalpathra* [16] .Shooting were also produced by indirect embryogenesis in triple combination of NAA:BA:IBA(5.0:1.0:1.0µm) where root and shoot initiation were noticed to occur simultaneously. The root length was more than the shoot at the juvenile stage of the plant. This is the first report of indirect regeneration of *Clerodendrun indicum* (L.)Kuntze *in vitro*. Other species of this family does not show such indirect regeneration.

The regenerated shoots of *C.indicum* grew normally to produce nodes and internodes bearing opposite decussately arranged leaves just like wild plant. Rooting was started from the base and each node of the micro-shoots. Root initiation was stimulated by IBA at 1.0µM concentration in full MS medium did not respond at half strength MS condition. Contrarily, *Clerodendrum incisum* produce roots in combination of IBA with 1/2MS medium [11].Several authors reported that IBA to be an effective auxin in the induction of roots in different ornamental , medicinal and fruit plant like Chrysanthemum ,Carnation , Neem , Apple . After root initiation, the *in vitro* raised healthy plantlets were established in the field via routine hardening process and survivality of plant in the field was appreciably high (90%) after three months.

CONCLUSION

The present findings could be used for conservation purpose and to set up large-scale propagation of this medicinally important plant species for further phytochemical explorations.

ABBREVIATIONS

PGRs-plant growth regulators; NAA – naphthalene acetic acid; BA – N6 –benzyladenine ;2,4-D – 2,4-dichlorophenoxyacetic acid ; Kn – kinetin; IBA – indole-3-butyric acid; MS –Murashige & Skoog's medium; GA-Gibberalic acid, µM-micromolar

REFERENCES

1) Manandhar N.P.and Manandhar,2002,The useful plant of Nepal,In plants and people of Neal Timber press London,U.K.isbn-13 PP160.

2) Raheman,A.U,S.Begum,S.Saied,M.I.Choudhury and F.Akhter,1997,A steroidal glycosides from *Clerodendrun inerme,*Phytochemistry,45:1721-1722.

3) Shrivastava.N, patel T; Clerodendrum and heathcare: an overview medicinal and aromatic plant science and biotechnology ©2007 global science books
4) Rahmen M.Z.A, ATMZ AZAM and M.A. Gafur; In vitro antibacterial principal of extract and two flavonoids from C.indicum Linn.Pak.J.of Biol sci,3.2002
5) Kumer K.C.S. and K.Muller 1999, Medicinal plant from Nepal II Evaluation as inhibitor of lipid peroxidationin Biological membrane,J.Ethnopharmacol64:135-139.
6) Tian J, Qin-Shi Zhao, Hong-Jie Zhang, Zhong-Wen Lin, and Han-Dong Sun, New Cleroindicins from *Clerodendrum indicum* ,Journal of Natural Products., *60* (8), pp 766–769(1997).
7) Chen J,Jun Tian, Feng E wu, Norio Kawabe, Masahiko Tokuda First Total Synthesis of Cleroindicin B, ( ) Cleroindicin C and E,Chinese Chemical Letters Vol. 12, No. 9, pp 771 – 774, 2001.
8) Ravindranath, N.; C. ramesh, .; K. H.Kishore, .; U.S.N Murty.; B.Das,Clerodendrone, a novel hydroquinone diterpenoid from clerodendrum indicum journal of chemical reserach volume 2003, number 7, july 2003 , pp. 440-441(2).
9) Venkata S. S. N.Kantamreddi, . Nagendra Lakshmi and V. V. V. Satyanarayana Kasapu, preliminary phytochemical analysis of some important Indian plant species international journal of pharma and bio sciences vol.1/issue-4/oct-dec.2010.
10) Islam M.R., R. Khan, S. N. Hossain,G. Ahmed, L.Hakin; In vitro clonal propagation of Vitax negundo (L.)-an important medicinal plant,plant tissue culture & Biotech.19(1)113-117,2009 (June).
11) Goyel S. A. Shahzad, M.Anis and S.Khan;Multiple shoot regeneration in C.incisum; an ornamental woody shrub Pak.J.Bot;42(2):873-878,2010.
12) Rech, E.L. and M.J.P. Pires. 1986. Tissue culture propagation of Mentha species by use of axillary buds. Plant Cell Rep., 5: 17-18.
13) Mao AA, Wetten A, Fay M, Caligari PDS (1995) In vitro propagation of Clerodendrum colebrookianum Walp, a potential natural anti- hypertension medicinal plant. Plant Cell Rep 14:493–496.
14) Saxena, C., G.R. Rout and P. Das. 1998. Micropropagation of Psoralea corylifolia J. Medicinal and Aromatic Plant Sci., 20: 15-18.
15) Quraishi, A., V. Koche and S.K. Mishra. 1996. *In vitro* micropropagation from nodal segment of *Clustanthus collinus*, *Plant Cell Tissue and Organ Culture*, 45: 87-93.
16) Gangaprasad, A., S. William Decruse, S. Seeni and G.M. Nair. 2005. Micropropagation and ecorestoration of *Decalepis arayalpathra* (Joseph & Chandia) Venler – An endemic and endangered ethnomedicinal plant of western Ghats, *Indian Journal of Biotechnology*, 4: 265-270.