

Direct and indirect method of plant regeneration from root explants of *Caesalpinia bonduc* (L.) Roxb.— A threatened medicinal plant of Western Ghats

S R Santosh Kumar, V Krishna*, Venkatesh, K Pradeepa, K Girish Kumar & A U Gnanesh
P.G. Department of Studies and Research in Biotechnology and Bioinformatics, Kuvempu University,
Shankaraghatta 577 451, India

Received 21 May 2011; revised 7 September 2012

An *in vitro* regeneration protocol has been standardized via direct and indirect methods from excised root explants of *C. bonduc*, a threatened woody legume used for the treatment of contagious diseases, inflammation, leprosy, antiperiodic, febrifuge, anthelmintic, urinary disorders, leucorrhoea, piles and to heal wounds. MS medium supplemented with 17.75 μmol BAP and 2.46 μmol IBA, induced a mean of 3.40 ± 1.07 shoots directly from the surface of excised root explant. Subsequently, the shoots rooted readily on MS half strength medium with out growth regulators. In indirect organogenesis, callogenic frequency was optimized (96.66%) at the concentration of 9.04 μmol 2, 4-D and 0.88 μmol BAP. An average, 15.30 ± 5.25 shoots were differentiated from the root callus at the concentration of 17.57 μmol BAP and 2.85 μmol IAA. Shoots regenerated through callus were rooted well on MS half strength medium with growth regulators at 2.95 μmol IBA. Rooted plantlets were transferred to the pots containing sterilized soil and were successfully hardened at greenhouse condition for three weeks then exposed to the natural environment. Survival rate was more (95%) in plantlets derived through direct organogenesis than (60%) the plantlets regenerated through root calli.

Keywords: *Caesalpinia bonduc*, Plant regeneration, Root explants

Caesalpinia bonduc (L.) Roxb. (Caesalpinaceae) is a dioecious scrambling woody liane with a spiny stem and bipinnate leaves up to 1 m long. The plant is threatened and very sparsely distributed in the deciduous forests of the Western Ghats of India¹. It also reported to be critically endangered in Malaysia^{2,3}.

In Ayurvedic system of medicine the plant is popularly known as fevernut and has being used for treating tuberculosis, cancer, eye sores, haemorrhages, leprosy, inflammations, asthma, toothache and fever^{4,5}. The seeds have been screened for antimicrobial activity^{6,7}, anti-hypoglycaemic, anti-hypolipidemic^{8,9}, antitumour, antioxidant¹⁰, antipyretic and analgesic activities¹¹, anti-filarial activity¹², anxiolytic activity¹³, immunomodulatory activities¹⁴. *C. bonduc* seeds are the major component of famous Ayurvedic drug- 'Ayush', used against malaria¹⁵. The plant is propagated through round marble-sized seeds born in borne in a two seeded

Pods. The glossy and hardy seeds are frost-tolerant and it requires more than 5 years of stratification for germination. Due to longer duration of dormancy and thick seed coat, viability is very poor and it does not germinate unless the scarifying action of microbes, weathering, insects, or rodents which eventually break the glossy coat and allows water to enter the seeds. Further, unscientific overexploitation of the plant parts like seeds and bark for medicinal purposes and destruction of habitat by anthropogenic activities drag this species towards the threshold of threatening condition and will become extinct if proper steps are not taken for its conservation and listed under endangered medicinal plant category¹⁶.

Since, the plant is having immense medicinal value previous investigators were attempted to derive *in vitro* protocol using epicotyl¹⁷ and stem explants¹⁸. As compared to chlorophyllus areal explant culture, reports are scanty on exploration of *in vitro* regenerative potentialities of the root explants^{19,20}. Therefore the objective of the present study is to investigate the regenerative potentialities of root explants of *C. bonduc* and to develop a reliable system for mass propagation with or without an intervening callus phase.

*Correspondent author
Telephone: (+91) 08282-256235
Fax: (+91) 08282- 256255
E-mail: krishnabiotech2003@gmail.com

Materials and Methods

Mature seeds of *Caesalpinia bonduc* were collected from Bhadra Wild Life Sanctuary (1 km from the Kuvempu University) of the Western Ghats, Karnataka, India. The plant material and seeds was authenticated by Prof. Y. L. Krishnamurthy, Department of Applied Botany, Kuvempu University, Shankaraghatta (Voucher specimen number: KUAB 301). The seeds were washed under running tap water treated with few drops of Tween 20 for 15 min followed by 3 times distilled water then 70% ethanol for 5 min. The dormancy of the seeds was over come by acid scarification using conc. H_2SO_4 for 60 min followed by washing with double distilled water for 4-5 times. The seeds were soaked in distilled water for 72 h and disinfested with 0.1% HgCl_2 for 8 min followed by 3 to 4 times wash with sterilized distilled water in aseptic condition. Seeds were subjected to germination on MS medium²¹ supplemented with 3.48 μmol kinetin (Kn). Root segments of 1.0-1.5 cm in length were aseptically harvested from the 45 days old culture and used as explants for the exploration of callogenic and caulogenic potentialities.

For direct organogenesis, aseptically excised root segments were placed horizontally on to MS medium fortified 5.42 μmol Adenine sulfate, 300 mg^{-1} polyvinyl pyrrolidone (PVP) and a range (4.43 to 22.19 μmol) of 6-Benzylaminopurine (BAP) and 2.46 to 9.84 μmol of 3-Indolyl butyric acid (IBA). For indirect organogenesis, the callogenic media consisted MS basal salts supplemented with 10.85 μmol Adenine sulfate, 500 mgL^{-1} PVP and the growth regulators 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and BAP are at the range of 3.39–13.57 μmol and 0.44 to 2.21 μmol respectively. For shoot bud differentiation the media fortified with a range of BAP (8.87–26.63 μmol) and IAA (2.85–11.41 μmol). The shoot buds organized either directly from the explants or through the callus phase, grew above 5–6 cm with well developed biparipinnately compound leaves were aseptically excised and transferred to MS half strength media consists of 2.95 μmol IBA and 500 mgL^{-1} PVP. The growth regulators were added to the medium before autoclaving. The pH of the medium was adjusted to 5.6 to 5.8, autoclaved at 121 °C at 15 psi (1.06 kg/cm^2) pressure for 20 min. The cultures were incubated at 12 h photoperiod with a light intensity of 2000 Lux at 22 ± 2 °C with 65–70% RH. The data were statistically evaluated by using ezANOVA

software (0.98 versions). The regenerated plantlets were primary hardened for two weeks in culture condition and then transferred to the greenhouse for secondary hardening.

Results and Discussion

Till date there are many reports available on the culture of achlorophyllous root explants of woody species and derive reliable protocol for mass multiplication of plantlets^{22,23}. It has been suggested that tissue culture of medicinal plants from root explants has been regarded as a good explant source not only for mass multiplication of plantlets but also for the production of secondary metabolites from the calli^{24,25}. In this study, regenerants were successfully produced from the excised root explants of *C. bonduc* both from direct and indirect organogenesis methods.

Direct organogenesis

The root explants harvested from the *in vitro* grown seedling are considered as an excellent material for culture initiation as compared to mature plant explant and the degree of contamination is very less. Further, culture of stem explants of *C. bonduc* observed the major problem of endogenous microflora contamination²⁶. For culture initiation of *Melia azedaracta*²⁷, also harvested the root explants from the seedlings was used. Although, this species had an immense medicinal value, rigorous investigations has not been carried out because of recalcitrant nature and browning of the explants during the initial stage of culture. Due to phenolic exudation, most of the woody species explants does not induces morphogenic responses in culture condition and it is very poor among woody legumes^{28,29}. In absence of growth adjuvant, adenine sulfate and antioxidant, PVP browning of the explant noticed even with the presence of optimal concentration of growth regulators. Within a week of culture phenolic exudation noticed from the explants. The phenolic oxidation was controlled by the supplementation of 5.42 μmol adenine sulfate and 300 mgL^{-1} PVP.

The quality and quantity of growth regulator would have an immense effect on *in vitro* organogenesis and significant results obtained due to the interaction of optimal concentrations of exogenously supplemented growth regulators with the endogenous growth regulators of cultured explants. In many woody legumes, the supplementation of BAP alone in different concentrations or in combination with auxins induced shoot bud organogenesis³⁰ and its caulogenic



Fig. 1—Plant regeneration from root explants from *C. bonduc*. (A) shoots bud initiation from the cultured root explants, (B) multiple shoot buds organization on root explants, (C) growth of shoot with serrate margined leaves, (D) further elongation of shoot with peripinnately compound leaves, (E) rooting from the organized shoot, (F) hardened and soil acclimatized plantlets.

frequency varies depending upon the type of explants cultured. The reason for effectiveness of the BAP may lie in its ability to stimulate the plant tissues to metabolize the natural endogenous hormones or could induce the production of natural hormone system for the induction of shoot organogenesis³¹.

The root explants excised from the seedlings of *C. bonduc* when cultured on MS culture medium supplemented with BAP alone (4.43–22.19 μmol) or in combination with IBA (2.46–9.84 μmol) induced adventitious shoot bud. After 15 days of culture photosynthetic loci appeared on the surface of root explants and these were transformed in to shoot buds (Fig. 1A and 1B). Subotic *et al.*³⁰ investigated the effects of six cytokinins [kinetin (Kn), N6-benzylaminopurine (BAP), 6-g, g-dimethylallylaminopurine (2IP), N-(2-chloro-4-pyridyl)-N0-phenylurea (CPPU), 1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea (TDZ) and 6-[4-hydroxy-3-methyl-but-2-enylaminopurine (ZEA)] on the induction and development of adventitious buds from the root explants of *Centaurium erythraea* and found that the urea-type cytokinin such as CPPU and TDZ were more effective than adenine-type cytokinin for adventitious buds formation with normal morphology. But in the present study, BAP the Adenine-type cytokinin would provoke better shoot organogenesis on interacting with the auxin IBA. The shoot bud organogenesis was optimized at the concentration of 17.75 μmol BAP and 2.46 μmol IBA with a mean of 3.40 ± 1.07 shoots per explant (Table 1), off which, only one or two shoots grew up well bearing biparipinnately compound leaves (Fig. 1C and D). The shoot buds turns to brown and dried on the explant. This may be due to well differentiated vascular bundles of the selectable shoots. It is also observed that as the concentration of BAP decreases with the increased concentrations of IBA, the caulogenic potentialities of the root explant was decreased. The effect of BAP on bud differentiation from root explants has been reported in other woody plant species such as *Citrus mitis*³², *Populus tremula*³³, *Piper colubrinum*²². The regenerated shoots rooted readily on MS basal medium without plant growth regulators (Fig. 1E). The plantlets were acclimatized to greenhouse for hardening in polythene bags containing sterilized soil (Fig. 1F).

Indirect organogenesis

The root segments of *C. bonduc* inoculated onto MS medium augmented with 2, 4-D, BAP showed the

sign of organogenic response and pale yellow, callus initiation was noticed from surface of the explants after 15 days of inoculation, at the concentration of 3.39 to 13.57 μmol of 2, 4-D alone (Fig. 2A). The frequency of callogenesis increases with the augmentation of BAP at the range of 0.44 to 2.21 μmol . In the culture of epicotyls explants of *C. bonduc*, Meena *et al.*¹⁷ also noticed that presence of auxin alone showed poor callusing from the explants and interaction ^c auxins with cytokinines favored callogenesis. But the percentage of callus induction was increased considerably to 96% in the interaction of 5.70 μmol of IAA with 17.75 μmol BAP. In the present study the caulogenic frequency of the root explants of *C. bonduc* was highest (96.66%) at the concentration of 9.04 μmol 2, 4-D and 0.88 μmol BAP (Table 2). When increased concentrations of

Table 1—Synergetic effect of BAP and IBA on shoot bud differentiation from the Root explants of *C. bonduc*

Growth regulators (μmol)		Number of shoot buds/ root explant (mean $\pm$ SD)
BAP	IBA	
04.43	0.00	1.20 $\pm$ 0.79
04.43	2.46	0.90 $\pm$ 0.74
04.43	4.92	0.00 $\pm$ 0.00
04.43	7.38	0.00 $\pm$ 0.00
04.43	9.84	0.00 $\pm$ 0.00
08.87	0.00	1.60 $\pm$ 0.97
08.87	2.46	2.20 $\pm$ 0.63
08.87	4.92	1.10 $\pm$ 0.74
08.87	7.38	0.00 $\pm$ 0.00
08.87	9.84	0.00 $\pm$ 0.00
13.31	0.00	2.70 $\pm$ 0.95
13.31	2.46	3.90 $\pm$ 1.20
13.31	4.92	2.50 $\pm$ 0.85
13.31	7.38	0.40 $\pm$ 0.52
13.31	9.84	0.30 $\pm$ 0.67
17.75	0.00	3.10 $\pm$ 0.99
17.75	2.46	3.40$\pm$1.07
17.75	4.92	2.70 $\pm$ 0.95
17.75	7.38	1.30 $\pm$ 0.95
17.75	9.84	0.50 $\pm$ 0.71
22.19	0.00	3.50 $\pm$ 1.27
22.19	2.46	3.20 $\pm$ 0.79
22.19	4.92	3.00 $\pm$ 1.15
22.19	7.38	1.70 $\pm$ 0.82
22.19	9.84	2.30 $\pm$ 0.82
F Value		26.4

The value of each combination consisted of mean $\pm$ SD of 10 replicates.

The F-value is significantly different when $P < 0.05$.



Fig. 2—Plant regeneration from root calli explants from *C. bonduc*. (A) callus formation on root explants, (B) initiation of greenish nodal like shoots bud organization, (C) sprouting of multiple Shoots differentiation, (D) growth of shoot with serrate margined leaves, (E) rooting on half strength MS medium with 0.6% IBA, (F) soil acclimatization of plantlets derived from root calli.

BAP hindered callogenic efficiency of the explant. This variation in callogenic response from different explants of the same species is due to the presence of endogenous growth regulators at varied concentrations.

Prior to callus initiation, the root explants bulges into moniliform knot like structures. After 25 days of culture whitish fleshy callus protruded from these knots (Fig. 2A). Sub culturing of the primary callus on to the same media resulted in the formation of whitish hard nodular mass (Fig. 2B) and no sign of caulogenesis even after five subculture onto the same media. On the contrary, interaction of higher concentrations of BAP (8.87–26.63 μmol) with lower concentrations of IAA (2.85–11.41 μmol) shoots bud differentiation from the root callus (Table 3). On shoot bud differentiation media, the whitish callus turns to pale brown initially, later organization of pale greenish protuberances were noticed all over the surface of the root callus and in

four week old culture, these protuberances were transformed into shoot buds. Sub-culturing of these differentiating calli on to the same media resulted in the growth of buds in to multiple shoots with photosynthetic simple linear lanceolate leaves (Fig. 2C). At optimal concentration (17.75 μmol BAP and 2.85 μmol IAA) a mean of 15.30 ± 5.25 shoots counted per callus (Table 3). However, only two to three shoots were capable to grew up well with biparipinnately compound leaves (Fig. 2D). In the callus culture of woody legumes *Entada purseatha*³¹, *Acacia senegal*³⁴ also noticed the development of simple leaves in the early stage of shoot bud differentiation and the growth of minimal number of efficient shoots with biparipinnately compound leaves at later stages of development.

Table 2—Effect of 2, 4-D and BAP on the frequency of callus formation from the root explants of *C. bonduc*

Growth regulators (μmol)		Frequency of callus formation (%)
2,4-D	BAP	
03.39	0.00	10.00
03.39	0.44	46.66
03.39	0.88	23.33
03.39	1.33	26.66
03.39	1.77	00.00
03.39	2.21	00.00
04.52	0.00	26.66
04.52	0.44	36.66
04.52	0.88	33.33
04.52	1.33	73.33
04.52	1.77	26.66
04.52	2.21	16.66
09.04	0.00	56.66
09.04	0.44	76.66
09.04	0.88	96.66
09.04	1.33	66.66
09.04	1.77	23.33
09.04	2.21	16.66
13.57	0.00	43.33
13.57	0.44	66.66
13.57	0.88	36.66
13.57	1.33	00.00
13.57	1.77	00.00
13.57	2.21	00.00

The value of each combination consisted of percentage of callus induction from the mature stem of 3×10 replicates.

Table 3—Synergetic effect of BAP and IAA on shoot bud differentiation from the Root callus of *C. bonduc*

Growth regulators (μmol)		Number of shoot buds/callus (mean $\pm$ SD)
BAP	IAA	
08.87	00.00	07.40 $\pm$ 1.43
08.87	02.85	04.40 $\pm$ 2.88
08.87	05.70	01.20 $\pm$ 1.23
08.87	08.56	00.50 $\pm$ 0.71
08.87	11.41	00.10 $\pm$ 0.32
13.31	00.00	09.00 $\pm$ 2.05
13.31	02.85	07.80 $\pm$ 2.35
13.31	05.70	05.60 $\pm$ 3.13
13.31	08.56	01.60 $\pm$ 0.70
13.31	11.41	00.40 $\pm$ 0.70
17.75	00.00	10.90 $\pm$ 2.13
17.75	02.85	15.30$\pm$5.25
17.75	05.70	09.70 $\pm$ 1.89
17.75	08.56	06.70 $\pm$ 3.06
17.75	11.41	03.60 $\pm$ 2.07
22.19	00.00	11.40 $\pm$ 2.67
22.19	02.85	09.10 $\pm$ 1.66
22.19	05.70	08.40 $\pm$ 1.65
22.19	08.56	06.60 $\pm$ 3.63
22.19	11.41	04.90 $\pm$ 2.13
26.63	00.00	13.50 $\pm$ 1.65
26.63	02.85	12.30 $\pm$ 1.57
26.63	05.70	10.90 $\pm$ 2.23
26.63	08.56	09.30 $\pm$ 2.67
26.63	11.41	07.10 $\pm$ 1.66
F Value		35.9

The value of each combination consisted of mean $\pm$ SD of 10 replicates.

The F-value is significantly different when $P < 0.05$.

Rooting of regenerated shoots

One of the major tasks in the regeneration of woody legumes is the rooting of microshoots. Generally on high ionic strength and liquid media withering of leaflets and exudation of phenolics are common. Half strength media augmented with antioxidants and the growth regulator IBA or IAA is suitable for induction of roots from the *in vitro* differentiated shoots of woody legumes^{35,36}. When the shoots attain the length of 5 to 6 cm with two to four biparipinnately compound leaves were transferred to rooting medium augmented with 2.95 μmol IBA as analogous to the report of Meena *et al.*¹⁷. Root developed from shoots with 100% survival rates (Fig. 2E). The regenerants were successfully hardened and acclimatized on soil (Fig. 2F). The well rooted regenerated plantlets were transferred to plastic cups containing sterilized garden soil acclimatized in greenhouse condition. As compared to the plantlets regenerated through root calli (60%), the survival of plantlets was more (95%) in the plantlets derived through direct organogenesis.

Conclusion

This study developed an efficient and reproducible regeneration protocol via direct and indirect organogenesis from the root explants. The results presented here are expected to be helpful for *ex-situ* conservation and for the production of secondary metabolites from the root calli.

Acknowledgement

The authors are grateful to the Registrar, Kuvempu University for financial support and Prof. Y. L. Ramachandra, Chairman, Department of Biotechnology for laboratory assistance and facilities.

References

- 1 Ved D K, Prathima C L, Morton N & Dharsan Shnakar, Conservation of India's medicinal plant diversity through a novel approach of establishing a network of in situ gene banks, in *Forest genetic resources—Status, threats and conservation strategies*, edited by R. Uma Shaankar, K.N. Ganeshaiah and Kamaljit S. Bawa (Oxford and IBH Publishing Co. New Delhi, India) 2001, 183.
- 2 Hou D, Larsen K & Larsen S S, *Caesalpinaceae. Flora Malesiana*, Series 1, 12 (1996) 409.
- 3 Tan H T W, Tan K X, Ali bin Ibrahim P T, Chew K S, Chua H, Duistermaat S K, Ganesan M W K, Goh A T, Gwee R, Kiew S M L, Lee P, Leong J, Lim A F S L, Lok A H B, Loo S K Y, Lum T, Morgany Saifuddin Suran S, Sim Haji Samsuri bin Haji Ahmad Y C, Wee K F, Yap C K, Yeo & Yong J W H, Checklists of threatened species—Seed Plants, in *The Singapore red data book*, 2nd edition, edited by GWH Davison, PKL Ng and HC Ho (The Nature Society Singapore, Singapore) 2008, 213.
- 4 Said H M, *Hamdard pharmacopoeia of Eastern Medicine*. (Times Press, Karachi) 1970, 367.
- 5 Gopalan G, *Medicinal plants of India*. (Indian Council of Medical Research, New Delhi) 1976, 64.
- 6 Simi, Khaliq-uz-Zaman S M & Ahmad V U, Antimicrobial activity of seeds extract and bondenolide from *Caesalpinia bonduc*, *Phytother Res*, 15 (2000) 437.
- 7 Arif T, Mandal T K, Kumar N, Bhosale J D, Hole A, Sharma G L, Padhi M M, Lavekar G S & Dabur R, *In vitro* and *in vivo* antimicrobial activities of seeds of *Caesalpinia bonduc* (Lin.) Roxb, *J Ethnopharmacol*, 123 (2009) 177.
- 8 Sharma S R, Dwivedi S K & Swarup D, Hypoglycemic, antihyperglycemic and hypolipidemic activities of *Caesalpinia bonduc* seeds in rats, *J Ethnopharmacol*, 58 (1997) 39.
- 9 Kannur D M, Hukkeri V I & Akki K S, Antidiabetic activity of *Caesalpinia bonduc* seed extracts in rats, *Fitoterapia*, 77 (2006) 46.
- 10 Gupta M, Mazumder U K, Kumar R S, Sivakumar T & Vamsi M L, Antitumor activity and antioxidant status of *Caesalpinia bonduc* against Ehrlich Ascites Carcinoma in Swiss albino mice, *J Pharmacological Sci*, 94 (2004) 177.
- 11 Archana P, Tandan S K, Chandra S & Lal J, Antipyretic and analgesic activities of *Caesalpinia bonduc* seed kernel extract, *Phytother Res*, 19 (2005) 376.
- 12 Gaur R L, Sahoo M K, Dixit S, Fatma N, Rastogi S, Kulshreshtha D K, Chatterjee R K & Murthy P K, Antifilarial activity of *Caesalpinia bonduc* against experimental filarial infections, *Indian J Med Res*, 12 (2008) 865.
- 13 Venkat Rao N, Shalam M D, Shantakumar S M, Altaf Ali, Shivaraj Gouda T & Jeevan Mane Babu, Anxiolytic activity of seed extract of *Caesalpinia Bonduc* (Roxb) in Laboratory, *Internet J Pharmacol*, 5 (2008).
- 14 Shruti Shukla, Archana M, Jinu J, Pradeep M, Suresh Prasad Vyas & Savita Shukla, Immunomodulatory activities of the ethanolic extract of *Caesalpinia bonduc* seeds, *J Ethnopharmacol*, 125 (2009) 252.
- 15 Vaidyaratnam P S, *Indian medicinal plants- A compendium of 500 species* (Orient Longman, Madras) 1, 1994, 33.
- 16 Hutton I, *Rare plant surveys- Report to NSW Scientific Committee* (Lord Howe Island, Sydney) 2001.
- 17 Meena K C, John B & Thuruthiyil D T, Callus induction and shoot regeneration from epicotyls explants of ethnomedicinally important *Caesalpinia bonduc* (L.) Roxb, *Iranian J Biotechnol*, 8 (2010).
- 18 Kannan P, Premkumar A & Ignacimuthu S, Organogenesis from stem explants of *Caesalpinia bonduc*, *J Trop Med Plants*, 7 (2006) 95.
- 19 Vinocur B, Carmi T, Altman A & Ziv M, Enhanced bud regeneration in aspen (*Populus tremula* L.) root cultured in liquid media, *Plant Cell Rep*, 19 (2000) 1146.
- 20 Shankar Murthy K, Krishna V & Rahiman R, Plant regeneration from root culture of *Embelia ribes* Burm- a threatened medicinal plant, in *Recent trends in plant sciences*, edited by T. Pullaiah (Regency Publications, New Delhi) 2005, 48.
- 21 Murashige T & Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiol Pl*, 15 (1962) 473.

- 22 Kelkar S M & Krishnamurthy K V, Adventitious shoot regeneration from root, internode, petiole and leaf explants of *Piper colubrinum* L., *Plant Cell Rep*, 17 (1998) 721.
- 23 Yadav R, Arora P, Kumar D, Katyal D, Dilbaghi N & Chaudhury A, High frequency direct plant regeneration from leaf, internode, and root segments of Eastern Cottonwood (*Populus deltoides*), *Pl Biotech Rep*, 3 (2009) 175.
- 24 Kusakari K, Yokoyama M & Inomata S, Enhanced production of saikosaponins by root culture of *Bupleurum falcatum* L. using two step control of sugar concentration, *Pl Cell Rep*, 19 (2000) 1115.
- 25 Krolicka A, Kartanowicz R, Wosinski S A, Szpitter A, Kaminski M & Lojkowska E, Induction of secondary metabolite production in transformed callus of *Ammi majus* L. grown after electromagnetic treatment of the culture medium, *Enz Microbial Technol*, 39 (2006) 1386.
- 26 Santosh Kumar S R, Krishna V, Venkatesh, Pradeepa K, Gnanesh U A & Girish kumar K, Indirect organogenesis from stem derived callus of *Caesalpinia bonduc* (L.) Roxb—a medicinal plant of Western ghats, *Int J Curr Res*, 4 (2012) 22.
- 27 Silvavi Ila, Anag Onzalez, Hebe Rey & Luism Rogins, Plant regeneration, origin, and development of shoot buds from root segments of *Melia azedarach* L. (Meliaceae) seedlings, *In Vitro Cell Dev Biol - Pl*, 41 (2005) 746.
- 28 Furze M J & Cresswell C F, Micropropagation of *Eucalyptus grandis* and *nitens* using tissue culture techniques, *S Afr For J*, 135 (1985) 20.
- 29 Bonga J M, Clonal propagation of mature trees - problems and possible solutions, in *Cell and tissue culture in forestry* edited by J.M. Bonga (Martinus Nijhoff) 1, 1987, 249.
- 30 Subotic Angelina, Jevremovic Slapana & Grubisic Dragoljub, Influence of cytokinins on *in vitro* morphogenesis in root cultures of *Centaurea erythraea*—valuable medicinal plant, *Sci Hort*, 120 (2009) 386.
- 31 Vidya S M, Krishna V, Manjunatha B K & Shankar murthy K, Micropropagation of *Entada pursaetha* DC- an endangered medicinal plant of Western Ghats, Indian, *J. Biotech*, 4 (2005) 561.
- 32 Sim G E, Goh C J & Loh C S, Micropropagation of *Citrus mitis* Blanco-multiple bud formation from shoot and root explants in the presence of 6-benzylaminopurine, *Plant Sci*, 59 (1989) 203.
- 33 Vinocur B, Tzfira T, Ziv M, Vainstein A & Altman A, Bud regeneration and growth from transgenic and non-transgenic aspen (*Populus tremula*) root explant, in *Biology of root formation and development*, edited by A Altman & Y Waisel (Plenum Press, New York) 1997, 217.
- 34 Khalafalla M M & Daffalla H M, *In vitro* Micropropagation and micrografting of gum arabic tree [*Acacia senegal* (L.) Wild], *Int J Sustain Crop Prod*, 3 (2008) 19.
- 35 Reza M A, Islam R, Mamun A N K & Joarder O I, *In vitro* organogenesis of a tree legume- *Albizia lebbeck* L., *Plant Tissue Cult*, 6 (1996) 67.
- 36 Bari M A, Ferdaus K M K B & Hossain M J, Callus induction and plantlet regeneration from *in vivo* nodal and internodal segments and shoot tip of *Dalbergia sissoo* Roxb, *J bio-sci*, 16 (2008) 41.