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Micropropagation of *Cochlospermum religiosum* (L.) Alston

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ABSTRACT

An *in vitro* micropropagation system has been developed for *Cochlospermum religiosum* (L.) Alston using node segments, obtained from 15-day-old aseptically grown seedlings, cultured on MS medium containing 6-benzyladenine (BA) and kinetin (Kn) either alone, or in combination. The regeneration medium with combined BA (4.44 µM) and Kn (4.65 µM) showed highest mean number of shoots (3.01 ± 0.32), increased shoot length (1.21 ± 0.06), and 80% regeneration response within 21 days. To initiate subculture, when separated and transferred to fresh medium with different levels of BA (4.44, 6.66 and 8.87 µM) and Kn (4.65, 6.97 and 9.29 µM), the medium containing BA (8.87 µM) and Kn (4.65 µM) resulted in further elongation of regenerated shoots. This phytohormone combination caused an increase in shoot length (2.23 ± 0.05) within 42 days from the beginning of culture. The regenerated shoots showed optimal rooting response (80%) on medium containing half strength MS salts, 0.8% agar and 4.90 µM indole-3-butyric acid (IBA). The plantlets established *in vitro* were transferred to pots containing sterilized soil and vermiculite (1:1) mixture. On transfer to the greenhouse, 65-70% plants survived. The present investigation for the first time describes an efficient micropropagation protocol for *C. religiosum.*

Keywords: cytokinins, cotyledon node, gum-kateera, micropropagation
Abbreviations: BA, 6-benzylaminopurine; **IBA**, indole-3-butyric acid; **Kn**, kinetin; **MS**, Murashige and Skoog

INTRODUCTION

Cochlospermum religiosum (L.) Alston (family Cochlospermaceae), commonly known as yellow silk cotton tree, is native to India, Bangladesh and Myanmar. This tree is of high economic importance, yields a valuable 'gum-kateera', a product that finds use in calico printing and paper making (Jain and Babbar 2002). In India, the gum is used for leather dressing. Several million pounds of this gum is annually exported from India for use abroad in the cigar and ice cream industries (Ojha *et al*. 2008). Particularly the gum is considered stomachic and sedative; it is used in Unani medicine to treat gonorrohoea, syphilis and asthma. The gum is sweet and cooling in effect. It is also used as remedy for eye troubles and trachoma. In addition the gum is applied cosmetically to soften the skin and to cure skin diseases (Jagtap *et al*. 2005). The dried leaves, flowers and latex of this tree are used to cure asthama and mouth ulcer (Khyade *et al.* 2010). The powdered bark of this tree is taken orally with water to treat jaundice (Dinesh and Aruna 2010).

There is a considerable demand for this plant in India. The wounding of the tree results in gum secretion; however, a severe decline in populations of natural habitat is observed due to destructive tapping methods for gum collection and poor vegetative means of propagation. Over exploitation, severe habitat destruction, restricted distribution, hard seed coat in conjunction with poor seed germination (8%; www.forest.ap.nic.in), and short time seed viability (approximately 2 months), are some of the major constraints on sustainable production of *C. religiosum*. In view of this, *in vitro* propagation can be used as an effective alternate for multiplication and conservation of this valuable tree species. To the best of our knowledge there are no reports available on *in vitro* propagation of *C. religiosum*. We describe for the first time an efficient protocol for micropropagation of *C. religiosum* using seedling node explants.

MATERIALS AND METHODS

The fruits of *C. religiosum* were collected from single tree during May to July from plants grow in a medicinal arboretum maintained by the Forest Department, Warangal, India. The fruits were dried; the fruit wall (5-8 cm long) was manually broken and seeds were collected. The seeds were placed in boiling water (15 min) to break dormancy, and were soaked in cold water for 24-48 h. These seeds were mechanically scarified, where the seed coat was rubbed using sand paper, and were soaked in detergent Tween-20 (0.1% v/v) (Merck, India) for 10 min followed by washing (5-6 times) with distilled water. Seeds were surface sterilized with $HgCl_2$ (0.1% w/v) (SD Fine Chemicals, India) for 4-6 min followed by 4-5 rinses (each of 10-15 min) with sterile distilled water. This was followed by cracking gently the seed coat and imbibing the seeds in double distilled water for 10 h. After rinsing for 5-6 times with sterile water, the seeds were aseptically blotted on Whatman paper and transferred to screw capped bottles (10x8.5 cm) containing 50 ml of Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose (Hi-media, India). After 4 week, the node segments (1.0-1.5 cm) were excised from seedlings (5 cm) and implanted vertically on MS medium (with 2% w/v sucrose) containing 6-benzyladenine (BA, 2.22, 3.11, 4.44, 6.66 and 8.87 µM) (Hi-media) or kinetin (Kn, 2.32, 3.25, 4.65, 6.97 and 9.29 µM) (Hi-media) that were tested either alone or in combinations. The nodal explants were cultured for 3 week on these media using 28°C day, 24°C night, under white fluorescent light (40-60 µmol $m^{-2}s^{-1}$; IS 2416 L 7434877, two Philips, India) (65 µE/m^2/s) with a 16-h photoperiod. The proliferated shoots (1.2 cm) for further growth and propagation were excised and sub cultured on to fresh MS medium supplemented with BA (4.44, 6.66 and 8.87 µM) in combination with Kn (4.65, 6.97 and 9.29 µM). On these media, the proliferated shoots were cultured for a period of 3 more weeks. MS medium without cytokinins, BA or Kn served as control.

For rooting, individual microshoots (2.0-2.2 cm) with 2-3 leaves were isolated and transferred to tubes containing MS medium of full, ½, or ¼ of its strength supplemented with different levels of IBA (2.46, 4.90, 7.36 and 9.80 µM) (Hi-media).

Received: 23 July, 2009. Accepted: 23 September, 2010.

Original Research Paper

MS medium without IBA served as rooting control. *In vitro* rooted shoots (3-week-old) were removed from culture tubes and washed thoroughly with water to remove agar and medium constituents. Individual shoots were potted in plastic jars containing sterilized soil and vermiculite (1:1) mixture. Plantlets were covered with polyethylene sheets to minimize loss of moisture and transferred to a greenhouse (28°C day, 24°C night, 65% RH).

All media pH were adjusted to 5.7 before adding agar (0.8% w/v, Hi-media) and autoclaved at 121°C for 15 min. Glass tubes (Borosil, 150 × 25 mm) containing 20 ml of medium, closed with cotton plug and wrapped in cheese cloth were used. During establishment and shoot proliferation, cultures were incubated under cool fluorescent light (65 µE/m^2/sec) for a 16-h photoperiod at 25 ± 2°C. For node explants, data (shown in **Table 1**) pertaining to proliferation response (i.e. percentage of explants exhibiting shoot development and number/length of shoots) was recorded 3 week after culture on regeneration medium. Data related to sub culturing (shown in **Table 2**) was recorded on day 42 from the beginning of culture. Rooting data (shown in **Table 3**) was obtained at day 21 following transfer of regenerated shoots to root induction medium containing IBA. All data were subjected to analysis of variance followed by Duncan's multiple range test (DMRT) for mean comparison ($P \leq 0.05$). For *in vitro* shoot multiplication/elongation and rooting, 10 explants were used in each of the two replicates for each treatment and the experiment was repeated twice.

RESULTS

During our investigation, culture conditions were established for *in vitro* seed germination using the method of scarification and other mechanical means to break seed coat dormancy of *C. religiosum*. Seeds were germinated within 21 days on MS medium containing 3% sucrose in the absence of growth regulators, and this protocol supported a high per cent (49%) of seed germination (**Fig. 1A**).

Seedling node explants showed optimal response for regeneration in *C. religiosum*. These explants when inoculated on MS medium with cytokinins, BA or Kn either alone or in combination showed altered responses *in vitro* (**Table 1**). Significant differences were observed in terms of number and length of the shoots that were regenerated on cytokinin supplemented media. The combined effect of BA and Kn promoted enhanced node proliferation and shoot development with early axillary bud sprouting (6–7 d) whereas, BA (10–11 d) or Kn (12–13 d) delayed this response. All tested combinations of BA and Kn responded positively to shoot development and 45-80% explants showed the regeneration response. The medium containing BA (4.44 µM) in combination with Kn (4.65 µM) showed 80% regeneration response, and this combination was most effective in terms of number (3.01 ± 0.32) and elongation (1.21 ± 0.06) of shoots that was achieved in first 3 week of culture (**Fig. 1B**). Low concentrations of BA (2.22, 3.11 µM) or Kn (2.32, 3.25 µM) did not induce shoot regeneration response (**Table 1**). Higher levels of BA (4.44, 6.66, 8.87 µM) or Kn (4.65, 6.97, 9.29 µM) induced, on an average, 1.0-1.7 shoots per node explant (**Fig. 1C**). At these concentrations of BA or Kn, 40-65% regeneration response was observed (**Table 1**). A single shoot emerged when nodal segments were cultured at higher levels of BA (8.87 µM) or Kn (9.29 µM) alone. However, a significant reduction in shoot length was observed at these hormone concentrations (**Table 1**). In control experiments, no shoot development was noticed.

From the above experiments, the higher levels of BA and Kn were found to play positive role in proliferation of seedling node explant cultures of *C. religiosum*. So, for further growth and propagation of these shoots, combinations of higher levels of BA (4.44, 6.66 and 8.87 µM) and Kn (4.65, 6.97 and 9.29 µM) were tried. After 3 week of culture, the microshoots (1.2 cm) were excised and cultured individually on these media. The cytokinin BA (4.44 µM) in combination with Kn (4.65, 6.97, 9.29 µM) induced 55-65% of response with increase in mean shoot length in the range from 1.42 ± 0.03 to 2.03 ± 0.13. A low BA (4.44 µM) and high Kn (9.29 µM) elicited 65% response with increase in mean shoot length to 2.03 ± 0.13 (**Table 2**). A significant decrease was observed in the mean shoot length when BA (6.66 µM) and Kn (4.65, 6.97, 9.29 µM) were used (**Table 2**). A higher concentration of BA (8.87 µM) used in combination with Kn (4.65, 6.97, 9.29 µM) resulted in a 60-75% response with average shoot length from 1.21 ± 0.06 to 2.23 ± 0.05. Within these hormone combinations, a high BA (8.87 µM) in combination with low Kn level (4.65 µM) showed a 75% response with 2.23 ± 0.05 as the average shoot length (**Fig. 1D**). The remaining combinations of BA (8.87 µM) and Kn (6.97, 9.29 µM) did not show a significant increase in shoot length (**Table 2**). These responses for shoot elongation of microshoots in the presence of BA and Kn were analyzed after 3 weeks of culture.

For rooting, individual microshoots (2.0-2.2 cm) were isolated and placed on ¼-, ½-, or full-strength MS medium fortified with various levels of IBA (2.46, 4.90, 7.36 and 9.80 µM). Half-strength MS medium supplemented with 4.90 µM IBA produced healthy roots (3.00 ± 0.51 roots per microshoot) from cut ends of the microshoot (**Fig. 1E**). At this IBA concentration, a high rooting percentage (80%)

Fig. 1 ***In vitro*** **multiplication of** ***Cochlospermum religiosum*** **(L.) Alston.** (**A**) *In vitro* germination of seeds on MS medium with 3% sucrose. (**B**) Shoot regeneration on MS medium with BA (4.44 µM) plus Kn (4.65 µM). (**C**) Induction of multiple shoots from node culture at a higher BA level (4.44 µM). (**D**) Elongated shoots on MS medium with BA (8.87 µM) plus Kn (4.65 µM). (**E**) Shoot with well developed roots on half-strength MS medium supplemented with IBA (4.90 µM). (**F**) Plants in soil in a greenhouse.

Table 1 Effect of BA or Kn alone, or in combinations, on cotyledon node explants of *C. religiosum*.

Growth regulators (µM)		% Response	Mean no. of shoots (± S.E.)	Mean shoot length (cm ± S.E.)
BA	Kn			
2.22	-	NR	0.00 ± 0.00 a	0.00 ± 0.00 a
3.11	-	NR	0.00 ± 0.00 a	0.00 ± 0.00 a
4.44	-	65	1.72 ± 0.18 b	0.81 ± 0.09 b
6.66	-	50	1.40 ± 0.16 c	0.70 ± 0.05 c
8.87	-	40	1.00 ± 0.11 d	0.42 ± 0.07 d
-	2.32	NR	0.00 ± 0.00 a	0.00 ± 0.00 a
-	3.25	NR	0.00 ± 0.00 a	0.00 ± 0.00 a
-	4.65	60	1.40 ± 0.15 c	0.80 ± 0.08 b
-	6.97	50	1.22 ± 0.14 e	0.62 ± 0.07 c
-	9.29	40	1.02 ± 0.11 d	0.53 ± 0.05 d
2.22	2.32	45	1.83 ± 0.21 f	0.40 ± 0.06 d
3.11	3.25	70	2.00 ± 0.21 g	1.03 ± 0.07 e
4.44	4.65	80	3.01 ± 0.32 h	1.21 ± 0.06 e
6.66	6.97	70	2.00 ± 0.25 g	0.60 ± 0.06 c
8.87	9.29	60	1.44 ± 0.16 c	0.55 ± 0.06 d

NR - no response
Values are mean of 40 explants ± S.E.
In each column mean followed by same letter were not significantly different ($P \leq 0.05$) according to Duncan's multiple range test

Table 2 Elongation of microshoots derived from cotyledon node cultures of *C. religiosum* after subculture on MS medium with BA and Kn.

Growth regulators (µM)		% Response	Mean length of shoot (cm ± S.E.)
BA	Kn		
4.44	4.65	55	1.42 ± 0.03 a
4.44	6.97	60	1.62 ± 0.09 b
4.44	9.29	65	2.03 ± 0.13 c
6.66	4.65	60	1.22 ± 0.08 d
6.66	6.97	60	1.20 ± 0.06 d
6.66	9.29	65	1.30 ± 0.06 d
8.87	4.65	75	2.23 ± 0.05 c
8.87	6.97	65	1.31 ± 0.04 d
8.87	9.29	60	1.21 ± 0.06 d

Values are mean of 40 explants ± S.E.
In each column mean followed by same letter were not significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

Table 3 Effect of MS medium composition supplemented with different IBA levels on the rooting ability of *C. religiosum* microshoots.

MS medium (strength)	IBA (µM)	% Cultures with roots	Mean no. of roots per shoot (± S.E.)	Mean root length (cm ± S.E.)
Half MS	2.46	70	2.00 ± 0.23 a	1.80 ± 0.07 a
	4.90	80	3.00 ± 0.51 b	4.00 ± 0.13 b
	7.36	65	3.00 ± 0.43 b	2.00 ± 0.08 a
	9.80	60	2.00 ± 0.24 a	1.60 ± 0.05 c
Quarter MS	2.46	60	2.00 ± 0.35 a	2.00 ± 0.10 a
	4.90	75	2.00 ± 0.29 a	2.10 ± 0.05 a
	7.36	50	2.00 ± 0.25 a	2.00 ± 0.08 a
	9.80	40	1.00 ± 0.17 c	1.40 ± 0.10 c

Values are mean of 40 explants ± S.E.
In each column mean followed by same letter were not significantly different ($P \leq 0.05$) according to Duncan's multiple range test

was obtained within 3 week of culture and the roots attained an average length of 4.00 ± 0.13 (**Table 3**). No callus tissue was developed from the base of the cut shoot at this IBA level (**Fig. 1E**). The half-strength MS medium with 7.36 µM IBA also induced 3.00 ± 0.43 roots per microshoot however the number of such cultures was less (65%) and a decrease in the mean root length (2.00 ± 0.08) was observed (**Table 3**). At other IBA levels (2.46 and 9.80 µM), significant decline in the number of roots per microshoot, and also a decrease in the mean root length was observed (**Table 3**). The use of aforesaid levels of IBA in full-strength MS medium was ineffective to induce roots from the *in vitro* regenerated shoots. Moreover, these treatments produced profuse callus from cut ends of the microshoot. The roots that were developed on ¼ strength MS medium in the presence of different IBA (2.46, 4.90, 7.36, 9.80 µM) concentrations were brittle and easily damaged during transfer of shoots to soil. No roots were developed in our control experiments. The shoots with well developed roots were transferred to pots containing sterilized soil and vermiculite (1: 1) mixture and hardened under greenhouse conditions (**Fig. 1F**).

DISCUSSION

In vitro clonal multiplication is an important alternative to regenerate plants that are economically and medicinally important, and difficult to propagate *via* conventional means. Conclusively, a reproducible protocol for *in vitro* propagation of valuable tree species *C. religiosum* is developed as part of this study. Using seedling node explants, shoot regeneration was obtained for the production of true-to-type plants.

Several factors are responsible for seed dormancy; among which seed dormancy due to hard seed coat can be overcome using various mechanical and/or scarification methods. During our study, such methods were employed to break hard seed coat dormancy of *C. religiosum* that resulted in improved seed germination (49%). In addition, the shoot cultures were initiated using juvenile node explants derived from the seedling cultures. Such juvenile explants were frequently used to establish *in vitro* regeneration protocols for woody trees (Bindu and Vivekanandan 1998; Nand *et al.* 2004; Ramesh *et al*. 2005; Rajeswari and Paliwal 2006).

The regenerated shoots developed from node cultures were smaller in size when BA, or Kn, or BA+KN were used. These shoots did not elongate further, lost vigor, and were vitrified when allowed to remain on the same medium for more time period (> 4-5 weeks). Transfer of proliferated shoots on to fresh medium appears mandatory for shoot elongation in *C. religiosum*. A similar observation was reported earlier for other tree species (Lakshmi and Seeni 2003; Chaturvedi *et al*. 2004).

It is known that BA is superior cytokinin that can govern *in vitro* development in wide range of plant species (Pradhan *et al*. 1998; Sinha *et al*. 2000; Ramesh *et al*. 2005). Our findings are consistent with these reports. However, the combination of cytokinins, BA and Kn promoted the development of multiple shoots, thereby demonstrating the requirement of two-way interaction of cytokinins for multiple shoot induction in *C. religiosum*. The combination of two or more cytokinins for shoot induction from various explants has been reported for *Gymnocladus dioicus* L. (Geneve 2005), *Eclipta alba* (Baskaran and Jayabalan 2005), *Albizia odoratissim* (Rajeswari and Paliwal 2006), and *Momordica tuberosa* Roxb (Aileni *et al*. 2008).

In addition to physical environment, nutrient media, including their mineral constituents together with auxin supplement is crucial for root induction under *in vitro* conditions. The auxin, IBA is frequently used for *in vitro* rooting of woody trees (Ramesh *et al*. 2002; Ramesh *et al.* 2005; Kaul 2008). Our present studies on root induction showed that IBA at all levels induced root formation on ¼- and ½-strength MS medium. No rooting from the cut ends of the microshoots was noticed when IBA was added to full-strength MS medium. Our results are consistent with previous reports where the applicability of ½-strength MS medium with IBA was found to be superior for *in vitro* root inductios (Dhar and Joshi 2005; Roy *et al*. 2007).

To conclude, the present study reports an efficient, simple, and reproducible tissue culture system for micropropagation of a valuable gum-yielding and medicinal tree, *C. religiosum*. The methodology applied for plant regeneration has successfully complimented root induction protocol that is developed for the first time for this woody tree. This protocol will strengthen large scale plantation activities

towards conservation and restoration of the threatened and valuable *C. religiosum*.

ACKNOWLEDGEMENTS

YR is thankful to Andhra Pradesh Council for Science and Technology (APCOST) for providing partial financial assistance in the form of A. P. Y. S. Award. The authors are thankful to Prof. V. S. Raju, Plant Systematics Laboratory, Department of Botany, Kakatiya University, Warangal, India, for the help with identification of plant material.

REFERENCES

Aileni M, Kota SR, Venugopal Rao K, Pavan U, Sadanandam A (2009) Efficient *in vitro* regeneration and micropropagation of medicinal plant, *Momordica tuberosa* Roxb. *Journal of Herbs, Spices and Medicinal Plants* **15**, 141-148

Baskaran P, Jayabalan N (2005) An efficient micropropagation system for *Eclipta alba*: A valuable medicinal herb. *In Vitro Cellular and Developmental Biology – Plant* **41**, 532-539

Bindu RC, Vivekanandan M (1998) Hormonal activities of 5-aminolevulinic acid in callus induction and micropropagation. *Plant Growth Regulation* **26**, 15-18

Chaturvedi R, Razdan MK, Bhojwani SS (2004) *In vitro* clonal propagation of an adult tree of neem (*Azadirachta indica* A. Juss.) by forced axillary branching. *Plant Science* **166**, 501-506

Dhar U, Joshi M (2005) Efficient regeneration protocol through callus for *Saussurea obvallata* (DC.) Edgew. (Asteraceae): Effect of explant type, age and plant growth regulators. *Plant Cell Reports* **24**, 195-200

Dinesh KD, Aruna J (2010) Ethnobotanical studies on plant resources of Tahsil Multai, District Betul, Madhya Pradesh, India. *Ethnobotanical Leaflets* **14**, 694-705

Geneve RL (2005) Comparative adventitious shoot induction in Kentucky coffee tree root and petiole explants treated with thidiazuron and benzylaminopurine. *In Vitro Cellular and Developmental Biology – Plant* **41**, 489-493

Jagtap SD, Deokule SS, Bhosle SV (2005) Some unique ethnomedicinal uses of plants used by the Korku tribe of Amravati district of Maharashtra. India. *Journal of Ethnopharmacology* **107**, 463-469

Jain N, Babbar SB (2002) Gum katira – a cheap gelling agent for plant tissue culture media. *Plant Cell, Tissue and Organ Culture* **71**, 223-229

Kaul K (2008) Variation in rooting behavior of stem cuttings in relation to their origin in *Taxus wallichiana* Zucc. *New Forests* **36**, 217-224

Khyade MS, Awasarkar UD, Deshmukh RR, Petkar AS (2010) Ethnobotanical reports about few important diseases from Akole Tehasil of Ahmednagar District (MS) India. *Asian Journal of Experimental Biological Sciences* **1 (2)**, 393-403

Lakshmi GN, Seeni S (2003) *In vitro* multiplication of *Calophyllum apetalum* (*Clusiaceae*), an endemic medicinal tree of the Western Ghats. *Plant Cell, Tissue and Organ Culture* **75**, 169-174

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures *Physiologia Plantarum* **15**, 473-497

Nand N, Drew RA, Ashmore S (2004) Micropropagation of two Australian native fruit species, *Davidsonia pruriens* and *Davidsonia jerseyana* G. Harden & J.B. Williams. *Plant Cell, Tissue and Organ Culture* **77**, 193-201

Ojha AK, Maiti D, Chandra K, Subhas M, Roy DDSK, Ghosh K, Syed I (2008) Structural assignment of a heteropolysaccharide isolated from the gum of *Cochlospermum religiosum*. *Carbohydrate Research* **343**, 1222-1231

Pradhan C, Kar S, Pattnaik S, Chand PK (1998) Propagation of *Dalbergia sissoo* Roxb. through *in vitro* shoot proliferation from cotyledonary nodes. *Plant Cell Reports* **18**, 122-126

Rajeswari V, Paliwal K (2006) *In vitro* propagation of *Albizia odoratissima* L.F. (Benth.) from cotyledonary node and leaf nodal explants. *In Vitro Cellular and Developmental Biology – Plant* **42**, 399-404

Ramesh M, Pavan U, Prasad SS, Rao AV, Sadanandam A (2002) *In vitro* regeneration of plants from mature nodal segments of *Terminalia arjuna* Bedd. *Sericologia* **41**, 75-80

Ramesh M, Pavan U, Venugopal Rao K, Sadanandam A (2005) Micropropagation of *Terminalia bellirica* Roxb. – A sericulture and medicinal plant. *In Vitro Cellular and Developmental Biology – Plant* **41**, 320-323

Roy AT, Leggett G, Koutoulis A (2007) Development of a shoot multiplication system for hop (*Humulus lupulus* L.). *In Vitro Cellular and Developmental Biology – Plant* **37**, 79-83

Sinha RK, Majumdar K, Sinha S (2000) *In vitro* differentiation and plant regeneration of *Albizia chinensis* (Osb.) Merr. *In Vitro Cellular and Developmental Biology – Plant* **36**, 370-373