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Research Article

IN VITRO PROPAGATION AND CONSERVATION OF *ANTHOCEPHALUS CADAMBA* THROUGH APICAL BUD AND NODAL EXPLANTS– A VALUABLE MEDICINAL PLANT

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ABSTRACT
Anthocephalus cadamba (Rebox) Miq. (Rubiaceae) is widely distributed throughout the greater part of India, especially at low levels in wet places. The genus *Anthocephalus* is one of the important members of the family Rubiaceae and comprises of only three species. The frequency of bud break and no. of shoots developed per explant was highest in M S medium followed by B5 medium, White's medium. Consequently MS medium is used in all our experiments. For nodal explants, On the MS medium containing BAP 2mg/L and IAA 0.5 mg/L 92% of cultures showed bud break within 30 days and produce 5 no. of shoots per explant. The explants cultured on these media showed their first response by initial enlargement of the existing axillary buds followed by bud break. After initial proliferation of shoots in medium containing BAP 2mgL, 3mg/L with IAA 0.5 mg/L, shoots along with mother explant subcultured on medium containing BAP 2mg/L, 3mg/L for 90 days results in 3 fold multiplication of shoots from basal end of explants of *A.cadamba*. Each explant produces 3-4 shoot clumps. For root induction regenerated individual shoots of *A.cadamba* were excised cultured on White medium supplemented with IBA of different concentrations (0.1,0.5,1.0 ,2.0,3.0 mg/L). IBA at 0.5 mg/L in White medium was found as most effective for rooting of *Anthocephalus cadamba* and produced highest no. of roots. This protocol can be useful for conservation of endangered *Anthocephalus cadamba.*

Keywords: *Anthocephalus cadamba, Micropropagation, Apical Bud, Nodal Explants, in Vitro Rooting*

INTRODUCTION
Anthocephalus cadamba (Rebox) Miq. (Rubiaceae) is widely distributed throughout the greater part of India, especially at low levels in wet places. The genus *Anthocephalus* is one of the important members of the family Rubiaceae and comprises of only three species. Out of which only one species namely *A. Cadamba* is distributed in several part of India (Santapan and Henry, 1973). According to Hindu Mythology, In India *A. cadamba* has a mythological association with Lord Krishna. About 2000 years ago vast kadamba forests existed in the vrindaban region, some of which are still there today. Hindu mythological text describes its beauty, shade and medicinal values (Bose and Choudhary, 1991). *A. Cadamba* is used as medicine to make breast attractive & normal, enhance sperm count and increase milk production. It is aphrodisiac and used in mouth ulcer also. It contains cinchotannic acid which was used to control fever long before the knowledge of quinine to be effective against malaria (Bhatnagar *et al.*, 1948; Visharad, 1966). The timber is used for plywood, light construction, pulp and paper, boxes and crates, dug-out canoes, and furniture components. *Kadamba* yields a pulp of satisfactory brightness and performance as a hand sheet. The wood can be easily impregnated with synthetic resins to increase its density and compressive strength. The alkaloids cadamine and isocadamine are isolated from the leaves of *Kadamba*. Fruits also have medicinal properties. Seeds have 'Anti-poison' medical properties. Crush the seeds, dissolve them in water and drink it as medicine for "Anti-poison". Yellow dye can be extracted from the root bark. Due to its heavy leaf shedding nature, O.C content of the soil can be increased. As there is no distinct heart wood, tree can be easily sawed. Thus, the tree is described as gem of tree, wonder tree and miracle tree in Philippines (Lopez, 1966). Depletion of natural population of *A. cadamba* due to its poor seed germination, lack of seed viability and poor efficacy of rooting in conventional method of propagation are the serious concern for conservation of this precious tree (Bose and

Choudhary, 1991). Vegetative propagation of *A. cadamba* by conventional methods is not successful due to high sensitivity of nodal segments against mechanical injury and poor rooting. Besides, this tree at early stage of growth is susceptible to pests, especially nematodes (Gupta and Dalal, 1973) and disease known as 'sudden death of cadamba' (Gibson and Nylund, 1976). Depletion of natural population of *A. Cadamba* due to its poor seed germination, lack of seed viability and poor efficacy of rooting in conventional method of propagation are the serious concern for conservation of this precious tree (Bose and Choudhary, 1991). Due to the failure in propagation of *A. cadamba* by conventional methods by seed and rooted cuttings, distribution of this sacred tree becomes very limited demands micropropagation techniques for large-scale production of this tree for conservation of this tree. Therefore, restoration and conservation of this tree is very important as it has many medicional values. However, multiple shoot regeneration has been reported in its closely related species, the *A.* Indica (Haque *et al.*, 1991). Induction of somatic embryos from inter nodal segment reported, but there was no complete plant regeneration from zygotic embryos and field establishment (Apurva and Thakur, 2009). However, only few work was done so far to develop micropropagation protocol for *A. cadamba* through direct regeneration method using apical bud and nodal explants, reported in *A.cadamba* (Kavitha *et al.,* 2009), which is considered as the most reliable method of propagation as compared to somatic embryogenesis. Therefore, the aim of our present work was to develop a protocol for micropropagation of *A. cadamba* using apical bud and nodal explants of mature field grown tree to overcome its problem of propagation and conservation.

MATERIALS AND METHODS

Plant Material

Around 100 year old Candidate Plus tree of *A. cadamba* grown in chopasni shrinathji temple, Jodhpur, Rajasthan become the source of explants for the present study. Shoots form the mother plant, either with active or dormant phase of vegetative growth in different seasons were collected and used for various experiments for developing the protocol for explant preparation and regeneration of shoots from apical bud and nodal segments. Apical bud and nodal segments measuring about 1.0-1.5 cm were cut from the shoots and rinsed thoroughly with tap water for 5 min. The segments were subjected to surface sterilization using tween -20 for 10 minutes, then with distilled water 4-5 times. After, these were sterilized with bavistin for 5-10 minutes, then with distilled water 4-5 times. In laminar air flow bench these were surface sterilized with 70% ethanol for 30 seconds, then with 0.1% (wt/vol) mercuric chloride (BDH, India) for 3-6 min depending upon the maturation of explants. Thereafter, the segments were washed 5 - 7 times with autoclaved distilled water. The stipules which enclose the apical bud and axillary buds in the nodal segments were carefully removed under aseptic condition and blotted on sterile filter paper before inoculated on the medium.

Culture Medium and Conditions

Different mediums were tried like M.S. medium (Murashige and Skoog, 1962), Gamborg's B5 medium (Gamborg *et al.,* 1968), White's medium (White, 1963) for inoculation of explants but the best medium was MS medium. Murashige and Skoog's medium supplemented with sucrose (3%) and various growth regulators used for our current study. The pH of the medium was adjusted to 5.8 before gelling with 0.8% agar. Molten medium was dispensed into culture tubes or jam bottles depending upon the requirement. The culture tubes containing media were autoclaved at 104 kPa and 121°C for 20 min. The processed explants were inoculated vertically on the culture medium. All the cultures were grown under 16-h photoperiod maintained by florescent light at25±2°C .The number of explants cultured in each treatment varied from 40 – 50depending upon the experiments.

Effect of Antibiotics on Bacterial Contamination

In order to test the efficacy of certain selected antibiotics on bacterial contamination during *in vitro* culture of apical buds and nodal explants of *A. cadamba*, different commercially used antibiotics such as norfloxacin, trimethoprim and rifampicin were incorporated into the medium at various concentrations (0, 10, 50, 100 mg/L). These antibiotics were filter sterilized in order to ensure their efficacy. MS medium supplemented with BAP (2mg/L) and IAA (0.5mg/L) become the basic and common medium to all the

treatments. In each concentration of antibiotic, 20 explants were cultured. Cultures were incubated under diffuse natural light for 30 days and observation was done to analyse bacterial contamination.

Effect of Various Surface Sterilants on Contamination of Explants

Two commonly used surface disinfectants, namely NaOCl (1%) and $HgCl_2$ (0.1%) were tested for their effect in removal of microbial contamination. The processed apical buds and nodal segments of *A. cadamba* measuring about 1.5 cm length were surface sterilized with above disinfectants for different durations (2, 3, 5 and 7 min.) and were thoroughly rinsed with autoclaved distilled water for 4-5 times under aseptic condition. Bavistin was also used as an effective fungicide. The processed apical buds and nodal segments of *A. cadamba* measuring about 1.5 cm length were surface sterilized with the bavistin for different time intervals (5,6,7and 10 minutes) before sterilization with NaOCl and $HgCl_2$ and thoroughly washed with distilled water for 4-5 times. The basal end of the apical buds were slightly trimmed and implanted vertically on MS medium supplemented with BAP (2 mg/L) and IAA (0.5mg/L). In each treatment, a total of 60 explants were cultured with two replication, each with 30 explants. After 40 days of culture, data was recorded for % of contamination in each treatment.

Effect of Season of Explants Collection on Shoot Regeneration

Apical buds and nodal explants were inoculated on MS medium supplemented with BAP (2 mg/L) and IAA (0.5 mg/L) in four different seasons. These includes (January - April), (May - August), (September-December). After 25 days, observation on explant recovery, percentage of response, bud break and shoot regeneration were recorded.

Effect of Growth Regulators on Shoot Proliferation

For shoot proliferation, apical bud and nodal segments were collected from actively growing shoots during may - august and inoculated on MS medium supplemented with different concentrations of BAP (0.5, 1.0 ,2.0, 3.0, 4.0. 5.0 mg/L) and Kin (0.5, 1.0 ,2.0, 3.0, 4.0. 5.0 mg/) in combination with IAA(0.5 mg/L). For each experiment 24 replicates were taken and repeated twice. Explants cultured for 30 days were observed and Data on bud break and shoot regeneration was recorded and analyzed. Once the culture conditions for actively growing shoots from explants were established, the *In vitro* produced shoots were transferred on to same medium for shoot proliferation for obtaining adequate no. of shoots and grown for another 60 days, bringing the total culture period to 90 days. Data on bud break and shoot regeneration were recorded and analyzed.

Effect of Explanting Season on Bud Break and Shoot Regeneration

Apical buds and nodal explants were processed as described above and inoculated on MS medium fortified with BAP (2 mg/L) and IAA (0.5mg/L) in 3 different seasons. These includes (January – April), (May-August), (September - December). After 30 days, observation was recorded on explant recovery, percentage of response, bud break and shoot regeneration .The actively growing shoots were transferred on to the same medium for shoot proliferation for obtaining adequate number of shoots.

In Vitro Rooting and Complete Plant Regeneration

About 50 healthy micro shoots with 2 - 3 pairs of leaves regenerated on MS medium supplemented with BAP (2 mg/L) and IAA (0.5 mg/L) were taken out from the culture vessel and agar which is adhering on the stem of micro shoots were removed under running tap water. The basal end of the micro shoots were slightly trimmed in order to expose the fresh layer of tissues for facilitating the absorption of auxin and transferred to White's medium supplemented with IBA (0.5 mg/L). These shoots were retained on the same medium for 40 days and observation was recorded on root and shoot development.

RESULTS AND DISCUSSION

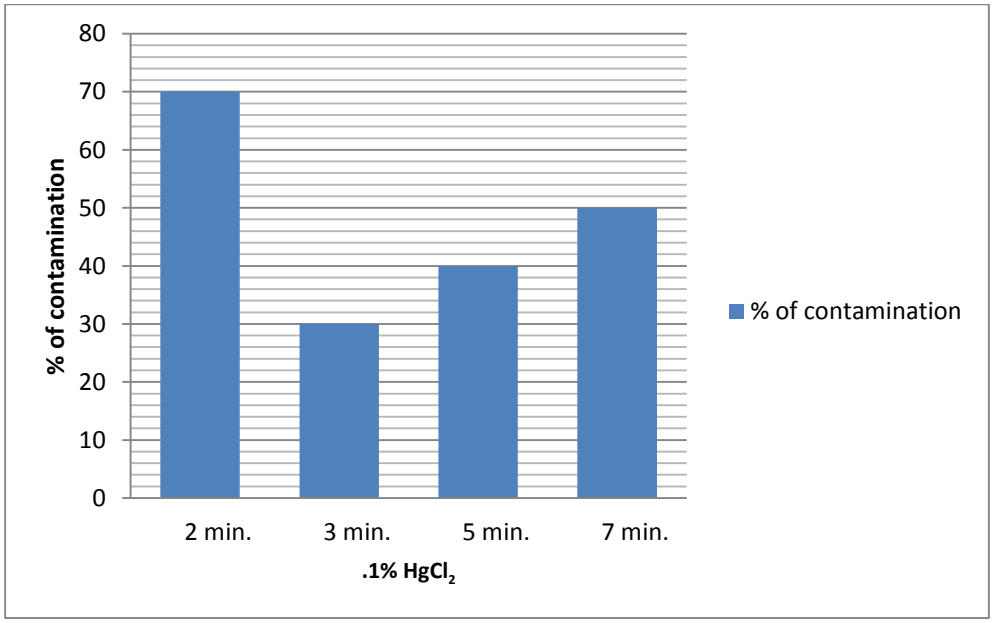
Effect of Different Sterilants on Disinfection of Apical and Nodal Segments

Effect of different sterilants on disinfection of apical bud and nodal explants of *A.cadamba* was summarized in (Table 1). Following the selection of explants, the next effort was to initiate maximum contamination free cultures, which is usually difficult and problematic, due to high rate of contamination, when the explants are collected from the field grown plants. When $HgCl_2$ was used alone, the rate of contamination was 70%. Since there was fungal contamination even after surface sterilizing with

$HgCl_2$solution, the fungicides Bavistin at various time durations was found to be effective, when used along with $HgCl_2$. However explants surface sterilized with NaOCl(1%) and $HgCl_2$(.1%) for different time intervals showed varying level of % of contamination. Explants treated with NaOCl and $HgCl_2$ for short duration recorded high microbial contamination and further increase in duration shows lessmicrobial contamination, further increase in duration results in browning of explant. However sterilization of apical bud and nodal explant with 0.1% $HgCl_2$ for 3 minutes was more effective than NaOCl (1%). Maximum contamination free cultures were obtained when the explants were disinfected with 0.1% $HgCl_2$ for 3 minutes along with bavistin. In general $HgCl_2$ was reported to be an effective disinfectants and widely used in micropropagation of several woody species such as coffee (Rajasekaran and Mohankuma, 1993), tea (Rajakumar and Ayyappan, 1992; Rajasekaran and Raman, 1993). $HgCl_2$ is better than NaOCl was reported in *A. Cadamba* (Kavitha *et al.,* 2012)**.**

Table 1: Effect of different sterilants on disinfection of apical and nodal segments of *Anthocephaluscadamba* on MS medium supplemented with BAP (2mg/L) and IAA (0.5 mg/L) for 30 days

Bavistin	0.1%HgCl2	% of contamination
5 min.	2 min.	70
6 min.	3 min.	30
7 min.	5 min.	40
10 min.	7 min.	50



Effect of Different Antibiotics on Bacterial Contamination

Effect of different antibiotics on bacterial contamination is summarized in (Table 2). All the explants cultured on antibiotic free medium show bacterial contamination. The explants cultured on medium with different concentrations of antibiotics showed different percentage of bacterial contamination. Of the three antibiotics tested norfloxacin, trimethoprim and rifampicin, trimethoprim (50 mg/l) shows mimimum percentage of bacterial contamination. At lower concentrations all antibiotics were ineffective. The shoots produced on medium containing trimethoprim has optimum growth with minimum contamination. Explants were cultured on MS medium supplemented with BAP (2mg/L) and IAA (0.5 mg/L) with different antibotics for 30 days. A similar results was reported when a range of antibiotics were tested for control bacterial contaminationin cultures of A. Cadamba (Kavitha *et al.*, 2012), also in

shoot tip cultures of several woody plants by (Young *et al.,* 1984), also in Yam plantlets by (Sherifah and Edith, 2012).

Table 2: Effect of different antibiotics on microbial contamination in cultured apical bud and nodal explants of *A. cadamba*. on MS medium supplemented with BAP (2mg/L) and IAA (0.5 mg/L) for 30 days

Antibiotics	Conc.(mg/L)	Percentage of contamination
Norfloxacin	10	70
	50	50
	100	40
Trimethoprim	10	75
	50	15
	100	30
Rifampicin	10	68
	50	25
	100	38

Effect of Different Seasons on Bud Break and Shoot Proliferation

Bud break frequency and shoot proliferation was influenced by age of lateral shoots and season in which cultures were initiated. Apical bud and Nodal segments collected from (January – April) shows 25% bud break response, these cultures produced 1 shoots per explant. Initiation of shoot culture during (May-August) showed maximum number of bud break response and these cultures produced 3-4 shoots per explant in apical and nodal explants. Initiation of cultures during (September- October) showed reduction in bud break with 2 shoots per explant (Table-3). A similar results were reported in *Wrightia tinctoria* by (Purohit and Kukda, 2004), in *A. cadamba* (Kavitha *et al*., 2012).

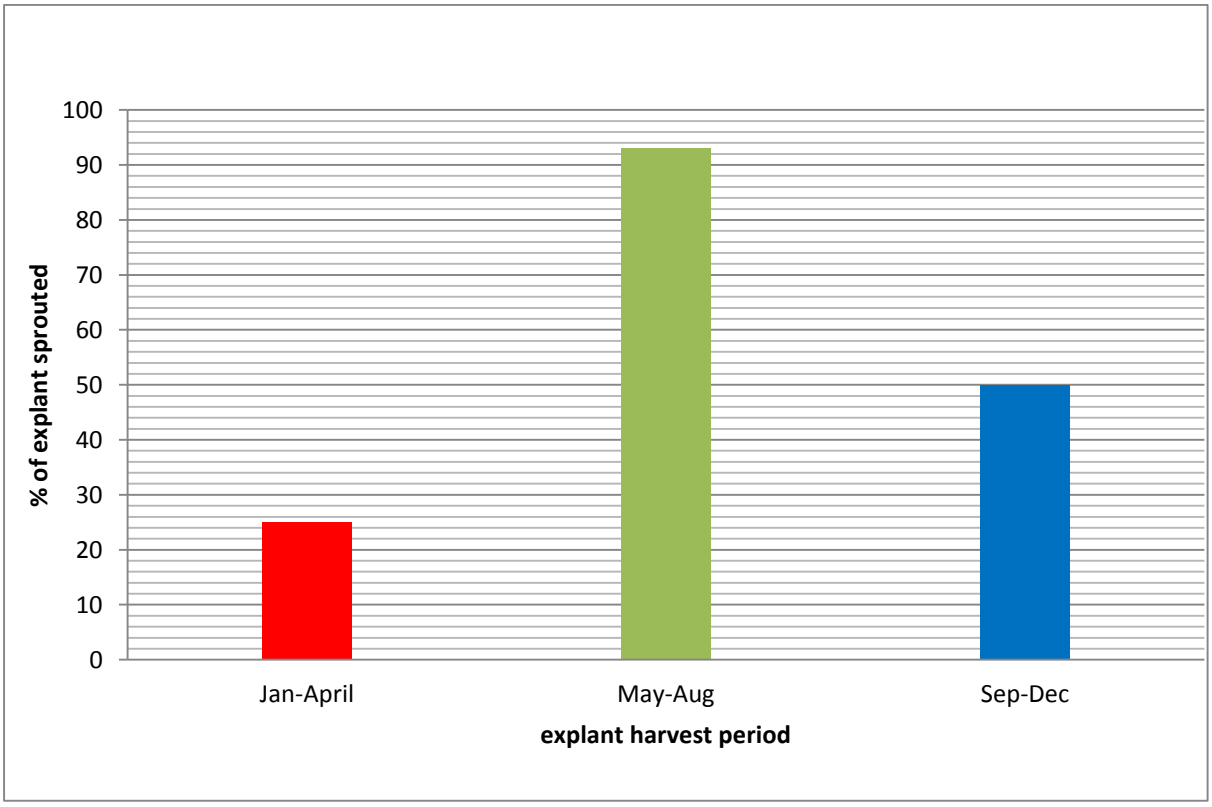


Table 3: Effect of season of explant collection on shoot regeneration of *Anthocephalus cadamba* on MS medium supplemented with BAP (2 mg/L) and IAA (0.5 mg/L). Duration of culture 25 days

Explant harvest period	Percentage of explant	mean no. of sproutedshoots ±SD	mean length of shoots±SD (cm)
Jan-April	25	1.3±0.6	1.2±0.2
May-August	93	3.9±0.4	3.5±0.8
Sep-Dec	50	2.2±0.8	2.3±0.6

Initiation of Shoot Cultures

The frequency of bud break and no. of shoots developed per explant was highest in M S medium followed by B5 medium, White's medium. Consequently MS medium is used in all our experiments. Apical bud and Nodal explants collected from actively growing shoots when cultured on MS medium supplemented with different conc. of BAP (0.5- 5.0 mg/L), Kin (0.5- 5.0 mg/L) in combination with IAA (0.5 mg/L) percentage of explant sprouted, mean no. of shoots and mean length of shoots were varied in different concentrations and also promoted multiple shoot formation.

For nodal explants, On the MS medium containing BAP 2mg/L and IAA 0.5 mg/L 92% of cultures showed bud break within 30 days and produce 5 no. of shoots per explant. The explants cultured on these media showed their first response by initial enlargement of the existing axillary buds followed by bud break (Table 4). Subsequently different conc. of Kin in combination with IAA was tested. BAP 2mg/L with IAA 0.5 mg/L showed maximum bud break 92% as well as maximum no. of shoots per explant. Shoot proliferation was better on BAP than Kin. However combination of auxin and cytokinine promoted elongation of shoots.

Table 4: Effect of various conc. of PGR's on shoot proliferation from stem nodal explants of *Anthocephalus cadamba*

MS + conc. of PGR'S		Percentage of explant	Mean no. of shoots (mg/L) sprouted ± SD	Mean length of shoots ±SD (cm)
BAP	**IAA**			
0.5	0.5	62.0	2.6±0.16	1.6±0.38
1.0	0.5	76.0	3.6±0.49	3.4±0.78
2.0	0.5	92.0	5.4±0.38	3.8±0.86
3.0	0.5	66.0	3.4±0.54	2.9±0.54
4.0	0.5	52.0	3.2±0.73	2.3±0.16
5.0	0.5	44.0	2.8±0.36	1.6±0.32
Kin	IAA			
0.5	0.5	52.0	1.4±0.42	1.3±0.26
1.0	0.5	50.0	1.8±0.19	1.6±0.68
2.0	0.5	66.0	2.4±0.15	2.9±0.36
3.0	0.5	58.0	1.8 ±0.34	1.6±0.28
4.0	0.5	52.0	1.3±0.24	0.8±0.10
5.0	0.5	42.0	1.2±0.18	0.6±0.08

Data from 20 replicates in two experiments (Mean±SD)

For apical bud, on MS medium with BAP 3 mg/L with IAA 0.5 mg/L 86% of cultures showed bud break within 30 days and produced 10 number of shoots per explants (Table 5). Number of shoots per explant increases with increase in conc. of BAP upto 3 mg/L. Kin is less effective than BAP as no. of shoots per explant is very less. Shoot formation from apical bud has been reported in many medicinal plants like *Phyllnthusamarus* (Bhattacharya and Bhattacharya, 2001), *Lippiajunelliana* (Juliani *et al.*, 1999). The BAP is more effective than Kin in direct shoot regeneration was reported earlier in many medicinal plants

like *Ocimum* spp (Pattnaik and Chand, 1996), *Wex negundo* (Sahoo and Chand, 1998b) and *Bacopa monniefa* (Tiwari *et al.*, 2001). The addition of auxin promotes shoot elongation was reported earlier in *Eucalyptus grandis* (Luis *et al.*, 1999). Direct multiple shoot regeneration from nodal explants has been reported in many medicinal plants (Sahoo and Chand, 1998b; Nobre *et al.*, 2000; Tiwari *et al.*, 2000). For the shoot regeneration, cytokinin is effective when used in combination with an auxin (Nike *et al.*, 1999). Beneficial effects of BAP or kinetin in combination with IAA on shoot induction have been observed in melon (Moreno *et al.*, 1985; Kathal *et al.*, 1986).

After initial proliferation of shoots in medium containing BAP 2mgL, 3mg/L with IAA 0.5 mg/L, shoots along with mother explant subcultured on medium containing BAP 2mg/L, 3mg/L for 90 days results in 3 fold multiplication of shoots from basal end of explants of *A. cadamba*. Each explant produce 3-4 shoot clumps.

Growth period 30 days

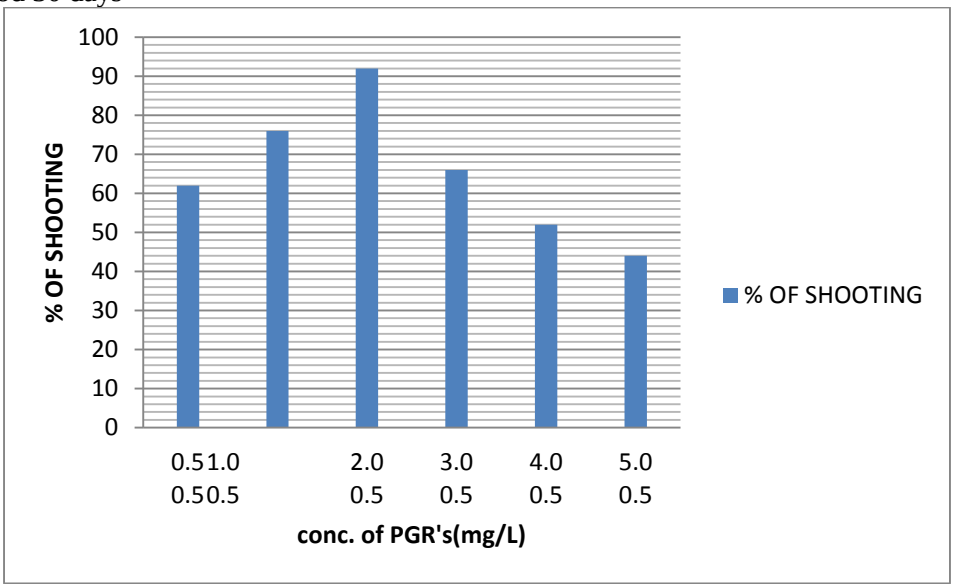
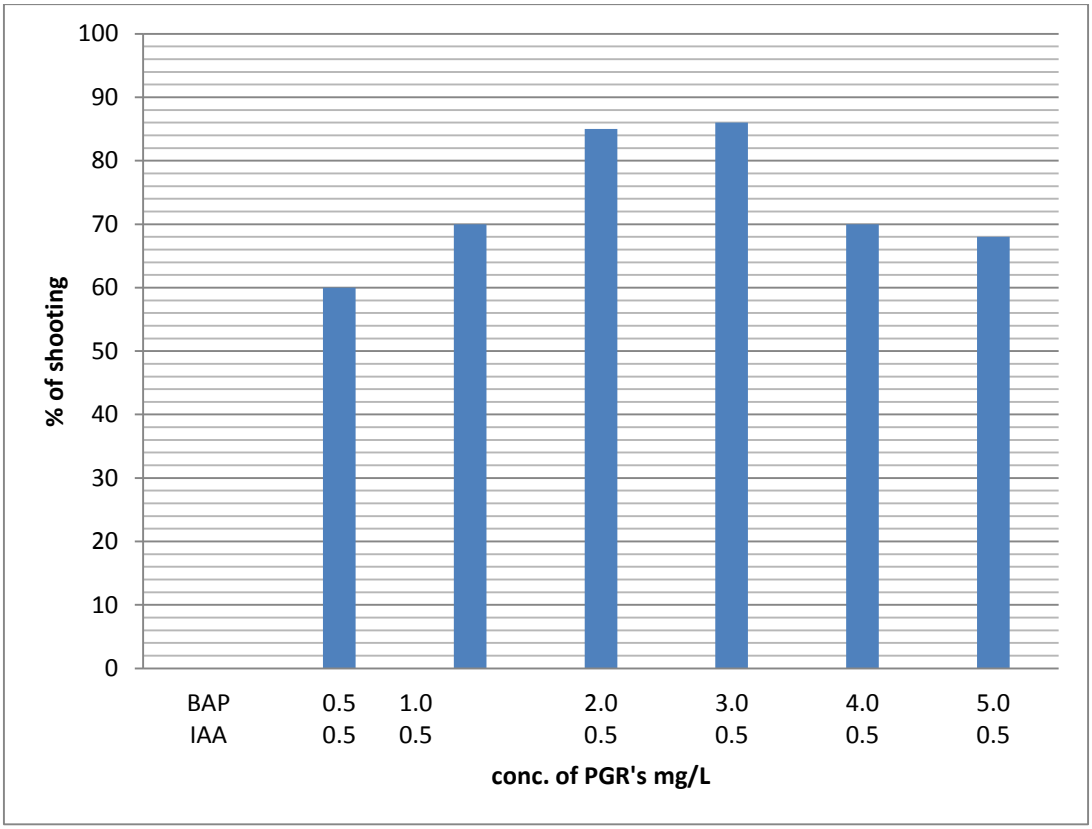


Table 5: Effect of various conc. of PGR's on shoot proliferation from apical bud explants of *Anthocephalus cadamba*

MS + conc. of PGR'S		Percentage of explant	Mean no. of shoots (mg/L) sprouted ±SD	Mean length of shoots ±SD (cm)
BAP	**IAA**			
0.5	0.5	60.0	3.2±0.41	2.6±0.68
1.0	0.5	70.0	6.4±0.28	3.8±0.28
2.0	0.5	82.0	8.8±0.78	3.4±0.46
3.0	0.5	86.0	10.2±0.18	4.6±0.16
4.0	0.5	68.0	8.4±0.33	2.9±0.74
5.0	0.5	58.0	4.4±0.18	2.8±0.26
Kin	IAA			
0.5	0.5	28.0	1.8±0.34	1.3±0.24
1.0	0.5	58.0	2.4±0.18	2.5±0.36
2.0	0.5	68.0	2.9±0.18	2.8±0.28
3.0	0.5	52.0	2.3±0.16	2.2±0.33
4.0	0.5	46.0	1.9±0.28	1.8±0.18
5.0	0.5	38.0	1.4±0.16	1.8±0.12

Data from 24 replicates in two experiments (Mean±SD)

Growth period 30 days



In vitro Rooting

For root induction regenerated individual shoots of *A.cadamba* were excised cultured on White medium supplemented with IBA of different concentrations (0.1,0.5,1.0 ,2.0,3.0 mg/L). IBA at 0.5 mg/L in White medium was found as most effective for rooting of *Anthocephalus cadamba* and produced highest no. of roots 3 with highest frequency 80% (Table 6). Shoots produced healthy roots directly from the basal end of shoots without producing any callus at the end of 40 days. Apurva and Thakur (2009) have reported that NAA (5.4 µM) induced roots directly from the internodal explants of *A. cadamba*. In our present studies, IBA (0.5 mg/l) also induced rooting within 40 days of culture. Therefore, a combination of NAA and IBA may be useful to promote rooting as these two auxins were reported to be having synergistic effect in enhancing rooting (Ganesh and Sreenath, 1997, 2008). The optimum conc. of IBA is 0.5 mg/l for inducing rooting within 40 days of culture. These shoots could be hardened in plastic pots containing soilrite mix. Upon further hardening, there was increase in plant height and no. of leaves.

Table 6: Effect of white medium supplemented with different concentrations of IBA on in vitro rooting of *Anthocephalus cadamba*

IBA (mg/L)	Percentage of rooting	Mean no. of roots ± SD	Mean length of roots ±SD(cm)
0.1	60	1.8±0.22	2.2±0.56
0.5	80	2.9±0.76	3.6±0.48
1.0	75	2.4±0.26	2.7±0.36
2.0	70	1.8±0.18	2.3±0.32
3.0	68	1.4±0.24	1.6±0.15

Data from 24 replicates in two experiments (Mean ±SD)

Growth period 40 days

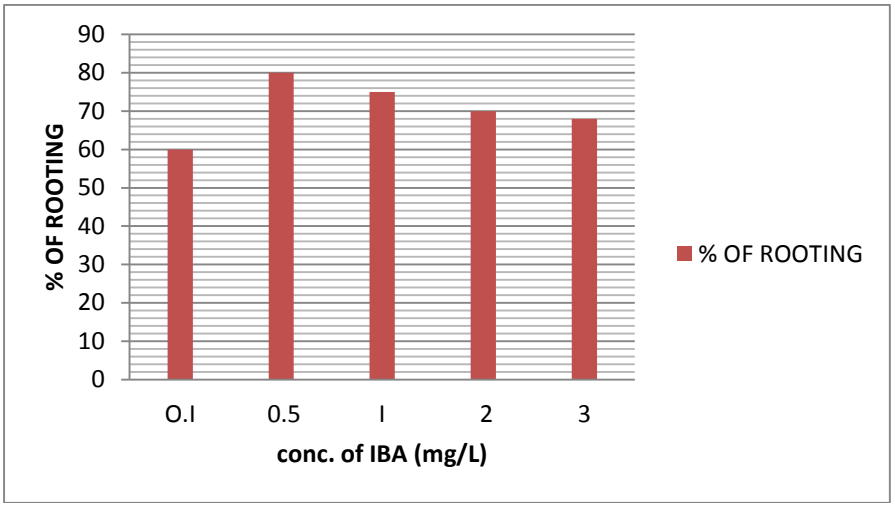


Figure A: In vitro shoot initiation, B: Shoot multiplication, C: E—In vitro rooting, D: Multiple shoot formation, F: Acclimatization of *in vitro* raised *Anthocephalous cadamba* from nodal and apical explants

Conclusion

Anthocephalus cadamba is an important medicinal and sacred plant of India. Due to its continuing exploitation of natural populations, natural hazards, cultural, political and economical issues, pose a great threat to plant genetic resources. Thus in vitro cell and tissue culture methodology is envisaged as a mean for germplasm conservation to ensure the survival of endangered plant species. Information on micropropagation of *A. cadamba* is very less. Protocols developed in the present experiment for direct regeneration from apical bud and nodal explants using cytokinin in combination with auxin followed by in vitro rooting is first report for rapid propagation and ex *situ* conservation of important sacred and medicinal tree *Anthocephalus cadamba*.

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