

In vitro Plant Regeneration from Apical Bud and Nodal Segments of *Anthocephalus Cadamba* – An important sacred and medicinal tree

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ABSTRACT : Multiple shoot induction and plant regeneration using apical bud and nodal explants of 100 year old tree of *Anthocephalus cadamba*, an important sacred and medicinal tree in India was achieved for the first time. Aseptic explants cultured in Murashige and Skoog (MS) medium augmented with different concentrations of BAP (0.1, 0.5, 1.0, 2.5, 5.0 and 10 mg/l), when maintained for 60 days, healthy shoots were induced in presence of BAP (1 mg/l). Lower concentrations of BAP (0.1 - 0.5 mg/l) induced only one shoot per explant. Increase in number of shoots per explant was observed in presence of higher concentrations of BAP (2.5, 5.0 and 10 mg/l). However, elongation of shoots was completely inhibited. Bud break and shoot regeneration was largely associated with seasonal factors. Apical buds cultured during June to August exhibited early bud break within two weeks of initial culture. In rest of the months, bud break and shoot regeneration was very slow irrespective of the various concentrations of BAP used in the medium. Explants sourced from three different maturity levels of shoots indicated that actively growing shoots from the mother plant with 1 - 2 nodal segments was more suitable for culture initiation than the explants collected from mature shoots at dormant stage. Regenerated shoots with 2 - 3 pairs of leaves when transferred to half strength MS medium fortified with IBA (1 mg/l), 60% of the shoots induced healthy roots, indicating the possibility of large scale micropropagation.

Keywords : *Anthocephalus cadamba*, Shoot proliferation, Micropropagation, Plant regeneration, Conservation

INTRODUCTION

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, *A. cadamba* is considered as an important sacred tree in India. It is reported that well established tree provides beauty and shade besides having wide range of medicinal (Brown and Chapple, 1976; Kapil et.al. 1995) and timber values (Soerianegara and Lemmens, 1993). The extract of leaves is aphrodisiac in nature and used for curing mouth ulcer. It also contains cinchotannic acid used for controlling fever (Bhatnagar et al. 1948; Visharad, 1985). 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). Unfortunately, seed propagation of *A. cadamba* also ends with limited success due to lack of seed viability resulting in poor germination (Bose and Chaudhary, 1991). Therefore, distribution of this sacred tree becomes very limited and demands for other alternative methods of propagation. Presently, many of the trees that are distributed in southern part of India become very old and several of the existing trees attained the age of more than 500 years. Therefore, restoration and conservation of this tree is very important in the context of its multiple values.

Induction of somatic embryos from inter nodal segment

of *A. cadamba* was reported, but there was no mention of complete plant regeneration from zygotic embryos and field establishment (Apurva and Thakur, 2009). Regeneration of multiple shoots from apical bud explants was reported only from the related species, namely *A. indica* (Haque et al. 1991). However, no attempt was made so far to develop micropropagation protocol for *A. cadamba* through direct regeneration method which is considered as the most reliable method of propagation as compared to somatic embryogenesis. Therefore, our present work is aimed at developing micropropagation protocol for restoration of *A. cadamba* using apical bud and nodal explants of mature field grown tree. In addition, the effect of explanting seasons and nature of explants in culture establishment and efficient shoot regeneration were also studied. The utility of this finding in large scale propagation and conservation of *A. cadamba* is discussed.

Materials and Methods

Plant material

Around 100 year old tree of *A. cadamba* grown in Sivasailam Hindu Temple, Alwarurichi, close to the Western Ghats in southern part of India become the source of explants for the present study. Shoots from the mother plant, either with active or dormant phase of vegetative growth in different seasons were collected and used for various experiments. Apical bud and nodal segments measuring about 1.5 cm were cut from the shoots and rinsed thoroughly with water for 10 - 15 min. The segments were subjected to surface sterilization using 0.1% (wt/vol) mercuric chloride (BDH, India) for 4 - 7 min depending upon the maturation of explants. Thereafter, the segments were washed 5 - 7 times with sterile distilled water. The stipules which enclose the apical bud and axillary buds in the nodal segments were carefully removed under aseptic condition. The basal end of the explants was trimmed using a sterile surgical blade under a mixture of ascorbic and citric acid solution (0.1% each) and blotted on sterile filter paper before implanting on the medium.

Culture medium and conditions

The culture medium used for the present work includes Murashige and Skoog's (1962) medium supplemented with sucrose (3%) and various growth regulators. The pH of the medium was adjusted to 5.8 before gelling with 0.8% agar (Hi-media, India). All the chemicals used in the present study were of analytical grade (British Drug House, Sigma, Merck, and Hi-media). Molten medium was dispensed into 200-ml screw-capped glass jars or 150-ml Erlenmeyer flasks (Borosil, India) or test tubes depending upon the requirements. The culture vials containing the media were autoclaved at 104 kPa and 121°C for 20 min. The processed explants were implanted vertically on the culture medium. All the cultures were maintained at 25±2°C and grown under 16 hr photoperiod irradiance provided by cool white fluorescent tubes (Philips, India). The number of explants cultured in each treatment varied from 30 - 40 depending upon the experiments.

Effect of BAP on shoot proliferation

To optimize shoot proliferation, apical bud and nodal segments from actively growing shoots were collected during June - August and inoculated on MS medium fortified with different concentrations of BAP (0, 0.1, 0.5, 1, 2.5, 5 and 10 mg/l). Explants cultured initially for 30 days were observed and uncontaminated explants with good sign of response were transferred on to fresh media of same composition and grown for another 60 days, bringing the total culture period to 90 days. Data on bud break and shoot regeneration was 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 (1 mg/l) in four different seasons. These includes intermediate (December - February), dry season (March - May), South west monsoon (June - August) and North east monsoon (September - November). After 30 days, ob-

servation on explant recovery, percentage of response, bud break and shoot regeneration was recorded. The recovered cultures with actively growing shoots were transferred on to the same medium to enhance shoots to proliferate for obtaining adequate number of shoots. The data on the response of explants in different seasons were analyzed after 60 days of total culture period.

Effect of maturity on shoot proliferation

Shoots with three different stages of maturity such as i) young actively growing shoots, ii) intermediate shoot showing slow growth, iii) mature shoots at complete stage were collected from the mother plants. Apical bud explants were processed from the above maturity levels of shoots and cultured on MS medium fortified with BAP (1 mg/l). The response of explants with regard to bud break and shoot regeneration was recorded and analyzed after 60 days of initial culture.

In vitro rooting and complete plant regeneration

About 60 healthy micro shoots with 2 - 3 pairs of leaves regenerated on MS medium fortified with BAP (1 mg/l) were taken out from the culture vessel and basal end of the micro shoots were slightly trimmed and transferred to half strength MS medium supplemented with

IBA (1 mg/l). These shoots were retained on the same medium for 30 days without subculture. Observation on shoot and root development was recorded.

Results and Discussion

Establishment of aseptic explants becomes extremely difficult in *A. cadamba* due to microbial contamination and browning of explants. However, considerable number of aseptic cultures could be established with the protocol described in this report. Observation of freshly inoculated explants revealed that fungal contaminants were mostly developed from the stipules of apical bud and nodal segments. However, removal of stipules along with gummy substances before implanting the explants on sterile medium reduced the percentage of fungal contamination. Browning of explants due to phenolic oxidation was commonly observed and this problem could be overcome by incubating the freshly inoculated explants under dark for a few days of initial culture. Endogenous bacterial contamination severely interfered with culture initiation.

Apical bud and nodal explants collected from actively growing shoots when cultured on MS medium fortified with different concentrations of BAP, percentage of cultures responding with bud break and shoot proliferation was varied in different concentrations (Table 1). Explants cultured at lower concentrations of BAP (0.1 and 0.5 mg/l) pro-

Table 1. Effect of BAP on bud break and shoot regeneration in cultured apical bud explants of *A. cadamba* for 90 days. Data represents the mean value of 20-25 explants depending upon the final recovery. Total number of explants cultured in each treatment was 50.

BAP (mg/l)	No. of explants recovered		No. of shoots/explant		Shoot length (cm)	
	Apical bud	Node	Apical bud	Node	Apical bud	Node
Control	03 (06)	05 (10)	1.00 ± 0.00	1.00 ± 0.00	1.78 ± 0.26	1.62 ± 0.24
0.1	05 (10)	04 (08)	2.30 ± 0.24	2.13 ± 0.14	2.24 ± 0.32	2.43 ± 0.32
0.5	08 (16)	07 (14)	2.40 ± 0.32	2.22 ± 0.21	3.24 ± 0.26	3.48 ± 0.32
1.0	19 (38)	12 (24)	3.18 ± 0.24	2.34 ± 0.32	4.62 ± 0.34	2.72 ± 0.26
2.5	14 (28)	16 (32)	5.78 ± 0.32	5.42 ± 0.34	3.24 ± 0.24	2.62 ± 0.24
5.0	08 (16)	12 (24)	2.76 ± 0.24	3.14 ± 0.24	2.34 ± 0.32	1.42 ± 0.22
10	06 (12)	10 (20)	***	***	***	***

*** Shoots were remained with single shoot with more callus proliferation at the basal end of the explants. Data within the parentheses are percentages.

duced only one shoot per explant. These shoots were lean and lanky with longer internodes (Fig. 1a). Increase in concentration of BAP (1 mg/l) enhanced the shoot induction and 2 - 3 axillary shoots were produced per explant. However, the main shoot was more elongated into healthy shoot, dominating the axillary shoots due to apical dominance (Fig. 1b). Higher concentrations of BAP (2.5 mg/l) induced axillary branching from apical bud and nodal explants during initial culture (Fig. 1c & d). Maintenance of these culture on the same concentrations for 90 days resulted in regeneration of high frequency multiple shoots from the basal end of the explants. Each explant produced 5 - 6 smaller shoot clumps with reduced leaf size and inter nodes (Fig. 1e). Presence of BAP at 5 and 10 mg/l induced bud break within two weeks of culture. The basal end of these explants produced compact yellowish to brown callus and limited the shoot development (Fig. 1f).

Varying response of explants was noticed in different seasons with regard to bud break and shoot regeneration (Table 2). Explants cultured during December - February (Intermediate season) shown 22% and 25% sprouting in

apical bud and nodal explants respectively and bud break was observed only after 3-4 weeks in case of apical bud and 2-3 weeks in nodal explants. These cultures produced 2 - 3 shoots per explant. Initiation of cultures during March - May (summer season) shown decrease in response with significant reduction in number of shoots per explant. Response of apical bud and nodal explants increased significantly during June - August (South West monsoon) with an average of 4 - 5 shoots per explant in apical bud and nodal explants. Decline in response was noticed during September - November (North East Monsoon) with significant reduction of shoots per explants.

Response of explant was largely associated with physiological conditions of the shoots from which explants were collected for initiation of cultures (Table 3). Explants collected from freshly emerging shoots with active vegetative growth showed maximum response (72%) within a week of culture and each explant produced an average of five shoots per explant with a longer shoot (5.2 cm). Use of apical bud explants from mature shoots without active

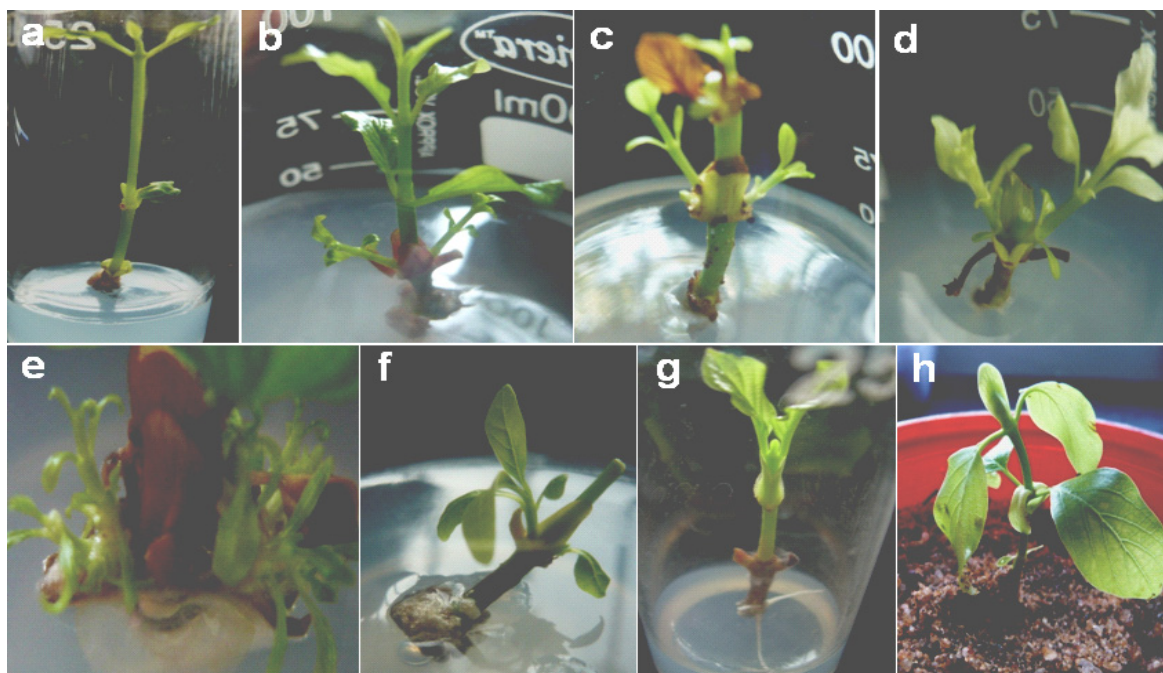


Fig. 1. a) Induction of lean and lanky shoots of *A. cadamba* at BAP (0.5 mg/l), b) healthy long shoot with axillary branching in presence of BAP (1 mg/l), c & d) Slow growth of axillary shoot proliferation in apical bud and nodal explants during initial culture, e) 90 days old culture showing multiples shoot clumps, f) Callus proliferation and slow sprouting of shoots in BAP (2.5 mg/l), g) Rooting of microshoots in presence of IBA (1 mg/l), h) Regenerated shoots under hardening.

vegetative growth and under dormant phase did not encourage the initiation of culture due to very poor response (12%). Many of the explants did not proliferate even after a month of initial culture. Explants collected from the shoots of intermediate nature shown 38% sprouting. The response of apical buds sourced from three different maturity levels of shoots is showed in Figure 2.

Our efforts in optimizing the protocol for induction of multiple shoots in *A. cadamba* revealed that BAP at 1 mg/l is an optimal level for regeneration of healthy shoots since higher levels of BAP induced weaker shoots. Shoots obtained in presence of higher levels of BAP though induced more number of shoots per explants, the shoots were weaker with shorter inter nodes with reduced leaf size. The basal end of the shoot clumps produced compact green callus. BAP is one of the widely used effective

cytokinins for induction bud break and shoot regeneration in a variety of tree species such as *Quercus suber* (Romano et al. 1992), *Coffea arabica* (Ganesh and Sreenath, 1997), *Camellia sinensis* (Rajasekaran and Mohankumar, 1990), *Theobroma cacao* (Mallika et al. 1996), *Eucalyptus* (Das and Mitra, 1990) and in several other woody species. In our study, although we have not evaluated other cytokinins, BAP induced healthy long shoots from apical bud and nodal explants of *A. cadamba*. Experiments for improving shoot regeneration using a combination of cytokinins with several other adjuvants are in progress.

In our experiment, despite the use of growth hormones, the response of explants was found largely dependent on several other factors. Apical bud and nodal explants inoculated in all the months revealed that the budbreak and shoot regeneration is closely associated with seasonal

Table 2. Effect of explanting season on bud break and shoot regeneration in cultured apical bud (A) and nodal (N) explants of *A. cadamba*. Explants were cultured on MS medium with BAP (1 mg/l) for 60 days. Data represents the mean value of 30 explants.

Seasons	Months	Response (%)		Time taken for bud break (weeks)		No. of Shoots/explant	
		A	N	A	N	A	N
Intermediate Season	Dec -Feb	22	25	3 - 4	2 - 3	2.42 ±0.16	2.82±0.34
Summer	Mar - May	14	17	2 - 4	3 - 4	1.63 ±0.16	2.26±0.24
South-West Monsoon	Jun - Aug	64	62	1 - 2	1 - 2	5.26 ±0.36	4.36±0.23
North - East Monsoon	Sep - Nov	52	47	1- 2	1 - 2	2.62 ± 0.23	2.16±0.32

Table 3. Effect of maturity of shoots on bud break and shoot regeneration in cultured apical bud explants of *A. cadamba*. Explants were cultured on MS medium with BAP (1 mg/l) for 60 days. Total number of explants cultured in each treatment was 50. Data represents the mean value of 35 explants.

Nature of shoots	Response (%)	Time taken for response (weeks)	No. of Shoots/explants	Length of shoot (cm)
Immature, soft, actively growing shoots	72	One week	5.26±0.32	5.26±0.36
Intermediate between the above types	38	3 - 4 weeks	2.62±0.36	3.64±0.36
Semi woody, mature without active growth	12	No response	**	**

factors which normally control the vegetative and reproductive phase of this tree. The south Western Ghats of India has four definite seasons (Nair, 1991). These seasons control the vegetative and reproductive phase of the plants. In *A. cadamba*, very active vegetative growth is observed only in two seasons namely South-West monsoon (June - August) and North-East monsoon (September - November). The rest of the two seasons, namely Intermediate season (December - February) and Dry season (March - May) is dominated by reproductive phase of this tree. The better response of explants with regard to bud break and shoot regeneration is largely dependent on vegetative growth phase of *A. cadamba* as evidenced by highest recovery of responding explants during South-West monsoon (June - August) with 64% and 62% in apical bud and nodal explants respectively, followed by North - East (September - November) monsoon season.

A similar results were reported in several other tree

species. It was reported that most of the callus and organ forming explants is controlled by genotypic and seasonal factors (Pence, 1989). Das and Mitra (1990) have reported that the bud break and shoot regeneration in *Eucalyptus* is dependent on the season as well as the physiological condition of the shoots from which explants were prepared for culture initiation. A similar results were also reported while axillary buds of *Rhododendron* was used for micro-propagation (Bojarezuk, 1996). Explants collected from mature shoots under complete dormant phase did not respond well even at the optimum concentration of BAP that we have identified. This is possibly due to dormancy of apical and axillary buds during reproductive phase of *A. cadamba*.

When explants were sourced from the shoots of three different maturity levels (Table 3), higher recovery of responding cultures was established only from actively growing shoots. These explants responded well within a week of culture and proliferated into multiple shoots. Apical bud

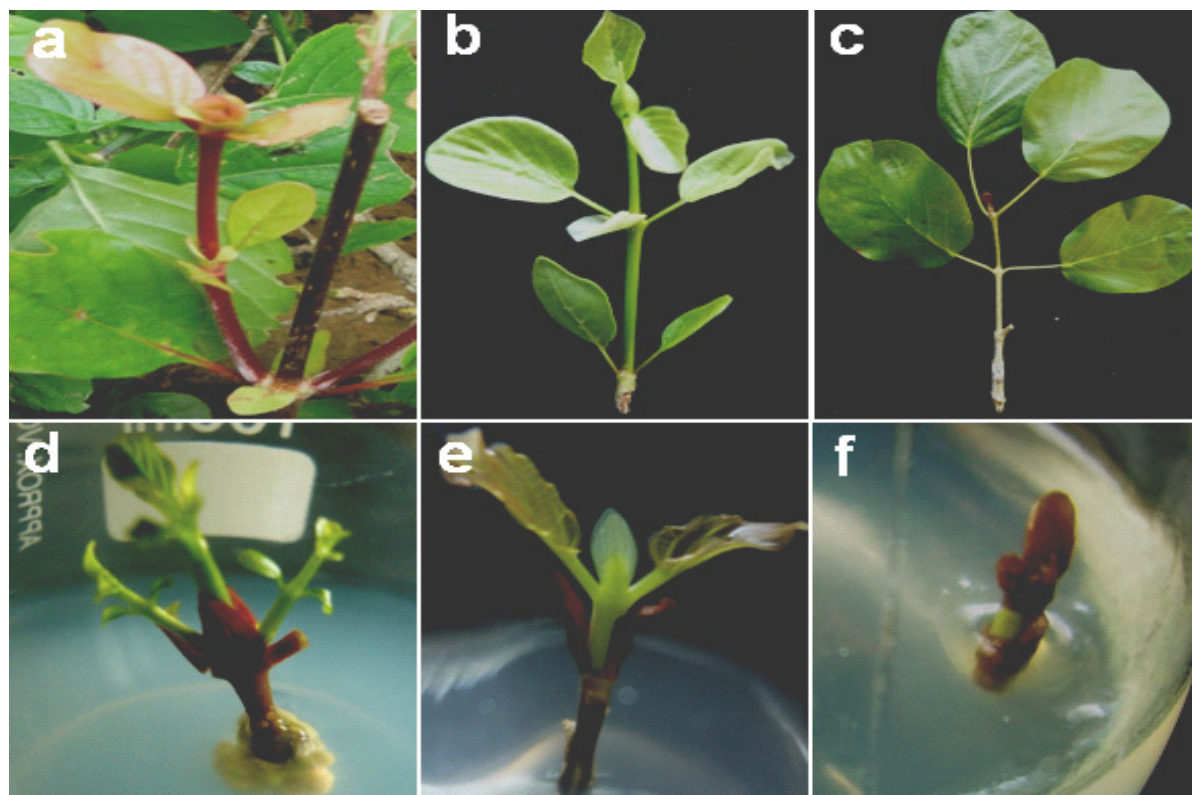


Fig. 2. Three different maturity levels of shoots used for culture initiation namely a) Immature actively growing shoots, b) intermediate type of shoots and c) semi hard and mature shoots. Response of explants when different maturity levels of shoots used for culture initiation (d, e and f). Note the better response in d and e when actively growing and intermediate types of shoots were used for culture establishment.

explants collected from the shoots that were attained either partial or complete maturity did not respond well as evidenced by very slow response and these explants could not be established well due to poor shoot regeneration. These findings become very useful in sampling of explants for improving culture initiation and shoot multiplication. A similar results were reported while arthotropic branches of three different maturity levels were tested for inducing bud break and shoot regeneration in coffee (Ganesh, 2000).

Regenerated shoots of *A. cadamba* could be successfully converted into plantlets by inducing roots on half strength MS medium fortified with IBA (1 mg/l). Shoots produced healthy roots directly from the basal end of the shoots without producing any callus and these shoots could be hardened in a plastic pot containing vermicompost (Fig. 1g and h). Recently, Apurva and Thakur (2009) have reported that NAA (2.5 and 5.4 μ M) induced roots directly from the internodal explants of *A. cadamba*. In our study, IBA (1 mg/l) also induced rooting within 30 days of culture. Therefore, a combination of NAA and IBA may be useful to enhance rooting as these two auxins were reported to be having synergistic effect in enhancing rooting (Zok, 1987; Ganesh and Sreenath, 1997, 2008).

In India, *A. Cadamba* is considered as an important species not only as a sacred tree but also for its multiple uses. Hence, this tree is highly regarded and planted as shade tree and grown along avenues, road sides and villages several years ago. Presently, several of the established trees had attained the age of more than 500 years in most of the temples in India, posing the threat of extinction due to lack of viable propagation methods. Besides, susceptibility of this tree to insect pest (*Arthroschista hilalaris*) and fungal disease (*Scytalidium lignicola*) during its early stage of establishment in the field is one of the major bottlenecks for new establishment (Gibson and Nylund, 1976). Published information on micropropagation of *A. cadamba* is very limited and so far only one report is on record with regard to somatic embryogenesis and root proliferation from internodal segment (Apurva and Thakur,

2009). However, no further effort is made either to refine the micropropagation technique through somatic embryogenesis or by direct regeneration, employing shoot tip and nodal explants. Therefore, this is the first report on micropropagation of *A. cadamba* using direct regeneration method. In our study, we have demonstrated the possibility of micropropagation of *A. cadamba* through induction of shoots followed by *in vitro* rooting. The experiments with regard to seasonal factors and nature of explants provided useful results for further refining the micropropagation protocol of *A. cadamba*. These results are expected to be useful for exploiting the *in vitro* technique not only for regeneration of large number of plants but also for conserving this important sacred and medicinal tree in India.

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