Publications
**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, Alwarkurichi, close to the Western Ghats in southern part of India 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. 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-
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Observation 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 form 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>
<thead>
<tr>
<th>BAP (mg/l)</th>
<th>No. of explants recovered</th>
<th>No. of shoots/explant</th>
<th>Shoot length (cm)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Apical bud</td>
<td>Node</td>
<td>Apical bud</td>
</tr>
<tr>
<td>Control</td>
<td>03 (06)</td>
<td>05 (10)</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>0.1</td>
<td>05 (10)</td>
<td>04 (08)</td>
<td>2.30 ± 0.24</td>
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<tr>
<td>0.5</td>
<td>08 (16)</td>
<td>07 (14)</td>
<td>2.40 ± 0.32</td>
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<tr>
<td>1.0</td>
<td>19 (38)</td>
<td>12 (24)</td>
<td>3.18 ± 0.24</td>
</tr>
<tr>
<td>2.5</td>
<td>14 (28)</td>
<td>16 (32)</td>
<td>5.78 ± 0.32</td>
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<tr>
<td>5.0</td>
<td>08 (16)</td>
<td>12 (24)</td>
<td>2.76 ± 0.24</td>
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<tr>
<td>10</td>
<td>06 (12)</td>
<td>10 (20)</td>
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*** 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.](image-url)
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 \textit{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 \textit{Quercus suber} (Romano
et al. 1992), \textit{Coffea arabica} (Ganesh and Sreenath, 1997),
\textit{Camellia sinensis} (Rajasekaran and Mohankumar, 1990),
\textit{Theobroma cacao} (Mallika et al. 1996), \textit{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 \textit{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

\begin{table}
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  \begin{tabular}{|c|c|c|c|c|c|c|c|c|c|}
    \hline
    Seasons & Months & \multicolumn{2}{|c|}{Response \(\%\)} & \multicolumn{2}{|c|}{Time taken for} & \multicolumn{2}{|c|}{No. of} \\
    & & \(\text{A}\) & \(\text{N}\) & \(\text{bud break \ (weeks)}\) & \(\text{Shoots/explant}\) & \(\text{A}\) & \(\text{N}\) \\
    \hline
    Intermediate & Dec - Feb & 22 & 25 & 3 - 4 & 2 - 3 & 2.42 ±0.16 & 2.82±0.34 \\
    \hline
    Summer & Mar - May & 14 & 17 & 2 - 4 & 3 - 4 & 1.63 ±0.16 & 2.26±0.24 \\
    \hline
    South-West & Jun - Aug & 64 & 62 & 1 - 2 & 1 - 2 & 5.26 ±0.36 & 4.36±0.23 \\
    Monsoon & Sep - Nov & 52 & 47 & 1 - 2 & 1 - 2 & 2.62 ± 0.23 & 2.16±0.32 \\
    \hline
  \end{tabular}
  \caption{Effect of explanting season on bud break and shoot regeneration in cultured apical bud (A) and nodal (N) explants of \textit{A. cadamba}. Explants were cultured on MS medium with BAP (1 mg/l) for 60 days. Data represents the mean value of 30 explants.}
\end{table}

\begin{table}
  \centering
  \begin{tabular}{|c|c|c|c|c|c|c|}
    \hline
    Nature of shoots & Response \(\%\) & Time taken & No. of & Length of \\
    & & & for response \(\text{(weeks)}\) & Shoots/explant & shoot \(\text{(cm)}\) \\
    \hline
    Immature, soft, actively growing shoots & 72 & One week & 5.26±0.32 & 5.26±0.36 \\
    \hline
    Intermediate between the above types & 38 & 3 - 4 weeks & 2.62±0.36 & 3.64±0.36 \\
    \hline
    Semi woody, mature without active growth & 12 & No response & * & * \\
    \hline
  \end{tabular}
  \caption{Effect of maturity of shoots on bud break and shoot regeneration in cultured apical bud explants of \textit{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.}
\end{table}
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 micropropagation (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

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**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 arthrotropic 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 µM) 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 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 (*Arthrochista hilalari*s) 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.

Acknowledgement

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Standardization of protocol for explant preparation and plant regeneration from apical bud and nodal explants of *Anthocephalus cadamba*

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Abstract

Experiment was carried out for disinfection of apical bud and nodal explants of mature tree of *Anthocephalus cadamba*. Two commonly used disinfectants such as NaOCl (1%) and HgCl₂ (0.1%) was tested for different duration ranging from 1 – 10 min and disinfection of apical bud and nodal explants with HgCl₂ for three min was more effective due to higher recovery of explants for initiation of aseptic cultures. Though NaOCl was effective with increase in duration of treatment, loss of explants due to browning followed by leaching of chlorophyll was observed in actively grown apical buds and nodal segments. The present experiment revealed that HgCl₂ was more potent for effective disinfection of apical bud and nodal explants. Experiments conducted with six different antibiotics such as streptomycin, erythromycin, norfloxacin, chloromphenicol, oxytetracycline and rifampicin for control of bacterial contamination revealed promising results. Incidence of bacterial contamination was varying depending upon the type of antibiotics used. All the six antibiotics were found to be effective at their higher concentration above 75 mg/l. Of the six antibiotics tested, streptomycin, erythromycin, chloromphenicol and oxytetracycline were found more effective than norfloxacin and rifampicin.

Shoots cultured on MS medium supplemented with different concentrations of antibiotics along with BAP (1 mg/l) had produced healthy shoots without phytotoxic effects and those shoots were converted into complete plantlets by treating the basal end of the microshoots under IBA (5000 ppm) followed by planting of shoots under the controlled condition.

Key words: Plant regeneration, microbial contamination, micropropagation, ex vitro rooting.

Introduction

*Anthocephalus cadamba* Miq (Rubiaceae) is an important fast growing evergreen trees in tropical and subtropical regions in many countries such as Australia, China, India, Indonesia, Philippines, Singapore, South Africa, Taiwan and Costa Rica. According to Hindu Mythology, this tree is considered as most popular sacred in India (Santapan and Henry, 1973). *A. cadamba* is reported to be a suitable tree for development of agroforestry since this tree sheds large quantities of leaf which are easily decomposed. Leaf litter of this tree contain high organic content and thus improve physical and chemical properties of soil.

Dried bark of this holy tree forms an important component in folk medicines for anaemia, uterine complaint besides its several other medicinal properties such as astringent, mucolytic, analgesic, anti-inflammatory, febrifuge and antiseptic (Patel and Kumar, 2008). Mature trees of *A. cadamba* is used in preparation of matchstick boxes, tea boxes, bobbins, veneer, playwood, crates and furniture (Chudnoff, 1984; Zabala and Manarapau, 1968). 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 Chaudhary, 1991).

Failure in propagation of *A. cadamba* by conventional methods by seed and rooted cuttings demands micropropagation techniques for large-scale production of this tree. Preliminary report on regeneration of plantlets by
in vitro culture through somatic embryogenesis was not promising due to lack of reproducibility (Apurva and Thakur, 2009). Further, there was no serious attempt to develop micropropagation protocol for large scale production of *A. cadamba* using apical bud and nodal explants. Regeneration of plantlets using apical bud and nodal explants is one of the safest methods for cloning large number of plants since this method offer true-to-type of plants without genetic variation. *A. cadamba* is a perennial tree and difficult to propagate by tissue culture since the explants sourced from mature tree often encountered with high incidence of microbial contamination. Efficient method of explant preparation is one of the important steps to overcome the problem of microbial contamination. In the present study, two commonly used surface disinfectants such as NaOCl and HgCl₂ were tested for their efficacy. In addition few commonly used broad spectrum antibiotics were tested to control the problem of bacterial contamination. Methods were also developed to produce plantlets by *ex vitro* rooting and hardening. The results on this study and its application in conservation of *A. cadamba* are discussed.

Materials and Methods

Source of plant material

About 500 year old well maintained huge tree of *A. cadamba* in Sivasailam Hindu Temple, Alavarkurichi, close to the Western Ghats in southern part of India in Tirunelveli District, Tamilnadu become the mother plant for collection of explants throughout the present investigation (Fig1a). Orthotropic shoots from 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.

Medium and culture conditions

The basic culture medium used for the present study includes Murashige and Skoog (1962) medium supplemented with sucrose (3%) and various growth regulators depending upon the experimental design. The pH of the medium was adjusted to 5.8 before gelling with 0.8% agar (Hi-media, India). All the chemicals, including growth regulators 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 or test tubes (Borosil, India) 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 with the light intensity of 40 μmol m⁻² s⁻¹ provided by cool white fluorescent tubes (Philips, India). The number of explants cultured in each treatment varied from 50 - 150 depending upon the experiments.

Efficacy of NaOCl and HgCl₂ on apical buds

Two commonly used surface disinfectants, namely NaOCl (1%) and HgCl₂ (0.1%) were tested for their efficacy in elimination of microbial contamination. The processed apical buds of *A. cadamba* measuring about 1 cm length were surface sterilized with above disinfectants for different durations (1, 2, 3, 5, 7 and 10 min) and were thoroughly rinsed with sterile distilled water for 5 - 6 times under aseptic condition. The basal end of the apical buds were slightly trimmed and implanted vertically on MS medium supplemented with BAP (1 mg/l). Explants cultured without disinfection formed the control. In each treatment, a total of 180 explants were cultured with three replications, each with 60 explants. After 60 days of culture, data on the loss of explants due to microbial contamination and browning, including recovery was recorded and analysed.

Efficacy of NaOCl and HgCl₂ on disinfection of nodal segments

A similar experiment as described above was carried out with nodal explants of *A. cadamba*. After disinfection with NaOCl and HgCl₂, both the ends of the nodal explants were trimmed under aseptic condition and cultured on the medium as described in the above experiment. Data on microbial contamination was collected after 60 days of culture as similar to the previous experiment and subjected to analysis for optimizing the duration of treatments.

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 of *A. cadamba*, six different commercially used antibiotics such as
streptocycline, erythromycin, norfloxacin, chloramphenicol, oxytetracycline and rifampicin were incorporated into the medium at various concentrations (0, 25, 50, 75, 100 mg/l). These antibiotics were filter sterilized before the use in order to ensure their efficacy. MS medium supplemented with BAP (1 mg/l) become the basic and common medium to all the treatments. In each concentration of antibiotic, 50 explants were cultured. Cultures were incubated under diffuse natural light for 60 days and observation on the incidence of bacterial contamination was carried out and analyzed.

Regeneration of micro shoots and ex vitro rooting

Considerable number of microshoots consisting of well developed shoot system with 2 - pairs of leaves was taken from the culture vessels and agar medium adhering on the stem of microshoots were gently removed under running tap water. The basal end of the shoots was trimmed in order to expose the fresh layer of tissues for facilitating the absorption of auxin. Basal end of the shoots were briefly dipped for 5 min in sterile IBA solution (3000 ppm) and implanted on sterile substrate containing soil, sand and vermicompost (6:2:1) and maintained for 45 days. The implanted microshoots were covered with polythene bags and frequently watered to maintain high humidity under natural shade. Shoots that were remained fresh with active shoot growth were removed after 60 days of observation was carried out to calculate the percentage of rooting.

Statistical analysis

Majority of the experiments were analyzed with three replications. However, the number of explants for the treatment in various experiments was variable due to differences in final recovery of explants. The effect of the different treatments on various parameters was quantified and the level of significance was determined by analysis of variance (ANOVA) using SPSS version 11.0 and level of differences between the treatments were assessed by Duncan’s New multiple range Test (DMRT) at P≤0.05.

Results and Discussion

Effect of NaOCl and HgCl₂ on disinfection of apical buds

Effect of different concentrations of NaOCl and HgCl₂ on disinfection of apical bud explants of A. cadamba was summarised in table 1 and 2 respectively. In control, all the explants were lost within a week of culture either due to fungal or bacterial contamination. However, explants disinfected with NaOCl (1%) and HgCl₂ (0.1%) for different durations showed varying levels of recovery depending upon the duration of treatment. Explants treated with NaOCl and HgCl₂ for short durations (1 – 2 min) recorded high incidence of microbial contamination and further increase in duration improved the recovery of explants significantly. Browning of apical bud explants was increased with increase in duration of treatment. In this experiment, disinfection of apical bud explants with NaOCl (1%) recorded the highest recovery with responding explants. However, disinfection of apical bud explants with HgCl₂ (0.1%) for 1 – 3 min was more effective than NaOCl (1%). Leaching of chlorophyll from young shoots and leaves was noticed when apical shoots were subjected to disinfection with NaOCl (Fig. 1b). Exposure of apical bud explants to HgCl₂ for longer duration (3 – 7 min) did not reveal any phytotoxic effect as evidenced by improved bud break and shoot regeneration.

Effect of NaOCl and HgCl₂ on disinfection of nodal explants

Results on the efficacy of NaOCl and HgCl₂ on disinfection of nodal explants of A. cadamba was summarized in table 3 and 4 respectively. The loss of explants due to fungal contamination was higher than bacterial contamination when nodal explants were disinfected with NaOCl (1%). On the other hand, fungal contamination was reduced significantly in all the treatments when nodal explants were treated with HgCl₂. In general, the overall recovery of explants was improved when nodal explants was disinfected with HgCl₂ than NaOCl. There was no difference between the treatments of nodal explants with NaOCl and HgCl₂ with regard to browning of explants. In general apical bud explants were found to respond well for bud break and shoot regeneration irrespective of the type of disinfectants used. The above experiment clearly revealed that apical buds were more suitable for the initiation of cultures due to higher recovery with better responses over nodal explants. In the present study, bud break and shoot regeneration could be obtained from node and apical bud explants (Fig1.c & d).
Table 1: Effect of different duration of surface sterilization with NaOCl (1%) on microbial contamination in cultured apical bud explants of A. cadamba. Explants were cultured on MS medium supplemented BAP (1 mg/l). Duration of culture 60 days.

<table>
<thead>
<tr>
<th>Treatments (min)</th>
<th>No. of explants with microbial contamination</th>
<th>Browning</th>
<th>Recovery</th>
<th>Response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria</td>
<td>Fungus</td>
<td>Bacteria/Fungus</td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>23.4(^a)</td>
<td>9.23(^a)</td>
<td>20.3(^a)</td>
<td>7.22(^a)</td>
</tr>
<tr>
<td>1.0</td>
<td>8.23(^b)</td>
<td>27.1(^b)</td>
<td>5.33(^b)</td>
<td>3.05(^b)</td>
</tr>
<tr>
<td>2.0</td>
<td>12.1(^c)</td>
<td>27.2(^c)</td>
<td>8.24(^c)</td>
<td>4.33(^c)</td>
</tr>
<tr>
<td>3.0</td>
<td>6.41(^d)</td>
<td>26.1(^d)</td>
<td>6.12(^d)</td>
<td>2.13(^d)</td>
</tr>
<tr>
<td>7.0</td>
<td>7.09(^e)</td>
<td>21.3(^e)</td>
<td>7.08(^e)</td>
<td>9.42(^e)</td>
</tr>
<tr>
<td>10</td>
<td>6.32(^f)</td>
<td>7.09(^f)</td>
<td>6.33(^f)</td>
<td>22.3(^f)</td>
</tr>
</tbody>
</table>

Response of explants was calculated out of recovered explants. Data represent the mean values of three replications, each consist of 60 explants. Mean values within each column followed by the same letter in superscript are not significantly different (P < 0.05: Duncan’s New Multiple Range Test).

Table 2: Effect of different duration of surface sterilization with HgCl\(_2\) (0.1%) on microbial contamination in cultured apical bud explants on MS medium supplemented BAP (1 mg/l). Duration of culture 60 days.

<table>
<thead>
<tr>
<th>Treatments (Min)</th>
<th>No. of explants with microbial contamination</th>
<th>Browning</th>
<th>Recovery</th>
<th>Response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria</td>
<td>Fungus</td>
<td>Bacteria/Fungus</td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>26.7(^a)</td>
<td>10.9(^a)</td>
<td>13.1(^a)</td>
<td>9.33(^a)</td>
</tr>
<tr>
<td>1.0</td>
<td>2.84(^b)</td>
<td>2.22(^b)</td>
<td>3.07(^b)</td>
<td>3.10(^b)</td>
</tr>
<tr>
<td>3.0</td>
<td>5.25(^c)</td>
<td>4.22(^c)</td>
<td>4.22(^c)</td>
<td>3.10(^c)</td>
</tr>
<tr>
<td>5.0</td>
<td>3.87(^d)</td>
<td>1.93(^d)</td>
<td>3.33(^d)</td>
<td>8.43(^d)</td>
</tr>
<tr>
<td>7.0</td>
<td>4.74(^e)</td>
<td>1.64(^e)</td>
<td>6.14(^e)</td>
<td>15.1(^e)</td>
</tr>
<tr>
<td>10</td>
<td>4.65(^f)</td>
<td>3.13(^f)</td>
<td>4.15(^f)</td>
<td>23.3(^f)</td>
</tr>
</tbody>
</table>

Response of explants was calculated out of recovered explants. Data represents the mean values of three replications, each consist of 60 explants. Mean values within each column followed by the same letter in superscript are not significantly different (P < 0.05: Duncan’s New Multiple Range Test).

Table 3: Effect of different duration of surface sterilization with NaOCl (1%) on microbial contamination in cultured nodal explants on MS medium supplemented BAP (1 mg/l). Duration of culture 60 days.

<table>
<thead>
<tr>
<th>Treatments (min)</th>
<th>No. of explants with microbial contamination</th>
<th>Browning</th>
<th>Recovery</th>
<th>Response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria</td>
<td>Fungus</td>
<td>Bacteria/Fungus</td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>22.1(^a)</td>
<td>10.2(^a)</td>
<td>10.2(^a)</td>
<td>8.22(^a)</td>
</tr>
<tr>
<td>1.0</td>
<td>9.22(^b)</td>
<td>6.32(^b)</td>
<td>6.32(^b)</td>
<td>4.03(^b)</td>
</tr>
<tr>
<td>2.0</td>
<td>12.1(^c)</td>
<td>8.22(^c)</td>
<td>8.22(^c)</td>
<td>5.32(^c)</td>
</tr>
<tr>
<td>3.0</td>
<td>6.13(^d)</td>
<td>7.13(^d)</td>
<td>7.13(^d)</td>
<td>3.13(^d)</td>
</tr>
<tr>
<td>7.0</td>
<td>8.03(^e)</td>
<td>8.02(^e)</td>
<td>8.02(^e)</td>
<td>10.4(^e)</td>
</tr>
<tr>
<td>10</td>
<td>9.32(^f)</td>
<td>7.32(^f)</td>
<td>7.32(^f)</td>
<td>25.3(^f)</td>
</tr>
</tbody>
</table>

Response of explants was calculated out of recovered explants. Data represents the mean values of three replications, each consist of 60 explants. Mean values within each column followed by the same letter in superscript are not significantly different (P < 0.05: Duncan’s New Multiple Range Test).
Table 4: Effect of different duration of surface sterilization with HgCl₂ (0.1%) on microbial contamination in cultured nodal explants on MS medium supplemented BAP (1 mg/l). Duration of culture 60 days.

<table>
<thead>
<tr>
<th>Treatments (Min)</th>
<th>No. of explants with microbial contamination</th>
<th>Browning</th>
<th>Recovery</th>
<th>Response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacteria/Fungus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Response of explants was calculated out of recovered explants.

Data represents the mean values of three replications, each consist of 50 explants. Mean values within each column followed by the same letter in superscript are not significantly different (P< 0.05: Duncan’s New Multiple Range Test).

Table 5: Effect of different antibiotics on microbial contamination in cultured apical bud explants of J. cadamba. Explants were cultured on MS medium supplemented BAP (1 mg/l) and with different concentrations of antibiotics. Duration of culture 60 days.

<table>
<thead>
<tr>
<th>Antibiotics mg/l</th>
<th>Contamination</th>
<th>Bacteria/Fungal</th>
<th>Browning</th>
<th>Recovery</th>
<th>Response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Str</td>
<td>Ery</td>
<td>Nor</td>
<td>Oxy</td>
<td>Rif</td>
<td></td>
</tr>
</tbody>
</table>

Response of explants was calculated out of recovered explants.

Data represents the mean values of three replications, each consist of 50 explants. Mean values within each column followed by the same letter in superscript are not significantly different (P< 0.05: Duncan’s New Multiple Range Test).

Str: Streptomycin; Ery: Erythromycin; Nor: Norfloxacin; Chl: Chloramphenicol; Oxy- Oxytetracycline; Rif: Rifampicin

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Figure 1: a) About 500 year old tree of *Anthocephalus cadamba* used as mother plant for collection of apical bud and nodal explants; b) Leaching of chlorophyll pigments from leaves and stem of young shoots when subject to disinfection with NaOCl; c & d) Regeneration of shoots from nodal and apical bud segments on MS medium supplemented with BAP (1 mg/l); e) Microshoot showing poor growth in presence of bacterial contamination; f) Regeneration of single shoots on MS medium containing BAP (1 mg/l) and erythromycin (75 mg/l); g) Ex vitro rooting of microshoots; h) Plantlets showing enlarged leaves with improved shoot growth after induction of rooting.

**Efficacy of antibiotics on bacterial contamination**

Effects of different antibiotics on bacterial contamination in cultured apical bud explants of *A. cadamba* is summarized (Table 5). All the explants cultured on antibiotic free medium (control) developed bacterial contamination (Fig 1.e). However, those apical buds cultured on medium with different concentrations of antibiotics showed varying percentage of bacterial contamination. Of the six antibiotics tested, erythromycin (75 mg/l) recorded maximum control over bacterial contamination. All the antibiotics were ineffective at their lower concentration. However, increase in concentration of all the antibiotics from 50 - 100 mg/l improved the overall recovery of explants by minimizing the bacterial contamination. In general, though most of the cultures were observed with bacterial contamination, their growth on the medium was minimized. It was found that shoots regenerated on medium containing erythromycin, norflaxacin, chloromphenical oxytetracycline and rifampicin at 75 mg/l grown well without any sign of
phytotoxicity even after 60 days of culture. These shoots produced healthy leaves and considerable number of such microshoots were regenerated (Fig. 1f).

**Ex vitro rooting and hardening**

Out of 35 well developed microshoots of *A. cadamba* tested for ex vitro rooting, about eight (22.8%) shoot were found to produce healthy roots at the end of 30 days (Fig. 1g). The rooted shoots showed improved growth as evidenced by the production of 3 - 4 pairs of fresh leaves during ex vitro establishment. Upon further hardening, these shoots produced 4 - 5 cm long healthy shoot system with many lateral roots (Fig. 1h). Whereas the unrooted shoots did not show any further growth and remained with older leaves.

The present study revealed optimum concentration of HgCl₂ for effective disinfection of apical bud and nodal explants using HgCl₂. Explants disinfected with HgCl₂ (0.1%) for 1 - 3 min recorded considerable number of explants for establishment of aseptic cultures. In general HgCl₂ was reported to be an effective disinfectants and widely used in micropropagation of several woody species such as coffee (Rajakumaran and Mohankumar, 1993), cashew (D’Silva and D’Souza, 1993), tea (Rajakumaran and Ayyappan, 1992; Rajasekaran and Raman, 1993), Cocoa (Malik et al., 1996), and rubber (Genevirante and Wijesekara, 1996). The present study also indicated that HgCl₂ is a more effective sterilant than NaOCl for establishing apical bud and nodal explants of *A. cadamba*.

Experiments conducted using different antibiotics revealed only partial inhibition of bacterial contamination in apical bud culture of *A. cadamba*. However, the incidence and rate of bacterial growth was varied depending upon the type and concentration of antibiotic used. Among the six antibiotics tested for their efficacy, all the antibiotics were found effective only at their higher concentrations ranging from 50 – 100 mg/l. A similar results was reported when a range of antibiotics were tested for control bacterial contamination in shoot tip cultures of several woody plants (Young et al., 1984). In this study, rifampicin was also found effective for the control of bacterial contamination. In support of our findings, rifampicin was effective in controlling bacterial contamination in tissue culture of several species such as Helianthus tuberosus (Phillips et al., 1981), Nicotiana plumaginifolia (Pollock et al., 1983), Cryptocoryne and Cinchona (Pierik, 1987).

Ex-vitro rooting is very popular in commercial micropropagation of important plant species. In the present study, when microshoots of *A. cadamba* was experimented for ex vitro rooting, high percentage (22.8%) of rooting was obtained. Though this method was well demonstrated in other plant species by several workers (Ma and Wang, 1977; Fordham et al., 1982; Anderson, 1978; McCown and Lloyd, 1983; Kyte and Briggs, 1979; Econonou and Read, 1981 & 1986; Wong, 1981 and Ettinger and Precece 1983), in *A. cadamba*, there was no information about ex vitro rooting. The advantage of ex vitro rooting is that both root induction as well as acclimatization can be carried out simultaneously. In order to reduce in vitro manipulations and also to reduce the cost of production, ex-vitro rooting is very often adopted for commercial production of several horticultural and forest tree species. In the present study, this method has proved effective for rooting of microshoots of *A. cadamba*. It was concluded that the present protocol can be used for conservation of *A. cadamba*.

**Acknowledgements**

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**References**


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