In vitro regeneration of an endangered tree - Elaeocarpus blascoi Weibel. (Rudraksha) from Southern Western Ghats, Tamil Nadu, India

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Abstract
Elaeocarpus blascoi Weibel. A relative species of Rudraksha, of Elaeocarpaceae is an endemic and endangered evergreen forest tree from Palni hills, Southern Western Ghats. In vitro regeneration protocol from nodal segments of E. blascoi for clonal propagation was developed by employing different tissue culture media. Woody plant medium (WPM) was found to be suitable for E. blascoi shoot multiplication and regenerations. Phenolic secretion makes the explants recalcitrant and was overcome by supplement of 0.3% charcoal / 0.5% polyvinlypyrrolidone (PVP). Nodal segments were cultured in WPM medium supplemented with cytokinins including Thidiazuron (TDZ), 6-Benzylaminopurine (BAP), and Kinetin (KIN). Among the three plant growth regulators, TDZ induced multiple shoots. Shoot elongation was achieved by addition of 10 mg/l silver nitrate in the shoot elongation medium, and an average of 6 ± 0.47 shoots are produced per explants. The regenerated micro shoots were subsequently rooted in half-strength WPM supplemented with 2 mg/l Indole -3 Butyric Acid (IBA). Thus the protocol developed is utilised for successful conservation of the IUCN red listed species E. blascoi.

Keywords: Elaeocarpus blascoi, In vitro culture, WPM, nodal segments, Regeneration.

1. Introduction
The richness of flora makes India one of the mega diversity countries in the world with four biodiversity hotspots and three mega centres of endemism. Nayar and Daniel [1] have reported that there are 4000 species of flowering plants recorded from the Western Ghats and 1,500 (38%) of these are endemic. The Western Ghats of India is recognized as one of the 34 global biodiversity hotspots of the world with over one-third of its angiosperms being endemic [2]. The United Nations Educational, Scientific and Cultural Organization (UNESCO) have recognised the Western Ghats as one of the Natural World Heritage sites due to its importance of rich endemism [3]. The Palni hills are a part of Western Ghats which are one of the internationally recognized hotspots of the planet and is also known as an environmental marker zones [4]. There is an alarming threat for extinction of species which often increases in the wild. The reasons may be due to natural habitat degradation, followed by anthropogenic pressure and reproductive limitation. The conservation of red listed species is a critical issue for any attempt to mange and utilise in a sustainable manner.

The family Elaeocarpaceae has 12 genera and 605 species in tropical and temperate southern regions [5]. The genus Elaeocarpus has 350 species in the temperate, subtropical and tropical zones throughout South- East Asia, Australia, Chile, New Zealand and the West Indies [6-10]. Khan et al., [11] reported that 360 species belong to world wide and 120 species in Asia. In India there are about 29 species of which ten are endemic. Eleven Elaeocarpus species have been reported from Tamil Nadu. There are eight Elaeocarpus species which are confined to the Western Ghats including four steno-endemics viz., E. blascoi Weibel, E. gaussenii Weibel, E. recurvatus Corner and E. venustus Bedd [12].

Elaeocarpus blascoi is a woody, evergreen tree, first discovered in Kodaikanal, Palni hills, Western Ghats, India [13-15] (Fig 1). E. blascoi is an endemic and also red listed as endangered species by IUCN [16]. Mathew, who could not collect any specimens, then presumed the plant to be extinct in the wild; however the single tree was rediscovered in Kodaikanal forest area during the year 2000 by local conservation trust team [17]. Besides, a couple of individuals were relocated around the Vattakanal forests of Kodaikanal, Southern Western Ghats [18]. In general Elaeocarpus fruits are endowed with a hard and highly ornamental stony endocarp and germination of the seeds is very low and erratic since the nuts are unable to imbibe water [19]. As on now there is no successful report on the regeneration of Elaeocarpus blascoi hence, an attempt was made to establish an efficient and reproducible method for clonal propagation of E. blascoi from mature tree explants.

2. Materials and Methods
Plant Collection- Plant specimens of Elaeocarpus blascoi were collected from Vattakanal forest (10°12.59.6” N and 77°29.06.9” E) at an altitude of 2031m, Kodaikanal, Palni hills India. The voucher specimens were processed, mounted and identified with the help of Flora of Palni Hills and were also authenticated by Dr. K. Ravikumar, Deputy Director, FRLHT, Bangalore. The labelled voucher specimens (No. 896) have been deposited at the Herbarium, SPKCES, MSU, Alwarkuruchi, Tirunelveli 627 412.
Culture Media Conditions - The nodal explants were collected from the healthy disease-free mature branches of the existing plants in the field and brought to the laboratory in sealed polythene covers. Expanded leaves from the shoot apex were first trimmed, and the shoot tip explants (3.0 – 3.5 cm) were washed thoroughly under running tap water for 30 minutes, then the explants were immersed for 5 – 8 min in 10% sodium hypochlorite (Merck, Mumbai) with two or three drops of Teepol. After three consequent washes in sterile distilled water, explants were treated with 70% ethanol for 30 seconds and thoroughly rinsed in sterile distilled water. The explants were then surface sterilized with 0.05% mercuric chloride (Hi Media, Mumbai) which was followed by two or three washes with autoclaved distilled water, followed by immersion in antimycotic solution (Hi Media, Mumbai) for 5 min. Explants were finally trimmed to smaller pieces (2 – 2.5 cm) and were transplanted on Woody Plant Medium (WPM) of Lloyd and McCown [20] to initiate multiplication. The basal medium of WPM consisted of the mineral salts and nutrients with 2% sucrose and 0.8% agar. The basal medium of the in vitro cultures experiments was variously supplemented with factorial combination of different plant growth regulators such as TDZ, BAP, Kin, IBA, IAA and NAA at different concentrations ranging from 0. 22 µM to 4.5 µM for shoot induction, 2.4 µM to 17 µM for rooting. All the supplements were added to the molten agar at around 50 °C and the pH of the medium was adjusted to 5.8 before autoclaving at 121 °C for 15 min (15 lb). The cultures were maintained at 24 ± 2° under 16 h photo-period with light intensity of 60 – 70 µ Em⁻² s⁻¹ from Crompton cool white fluorescent lamp.

3. Results and Discussion

Explant size, surface contamination, phenolic exudation and regeneration capacity

Fungal contaminants cause a major threat at every stage of plant cultures and it is difficult to eradicate 100% from the in vitro cultures. Shoot induction and multiplication in E. blascoi was very difficult initially due to heavy fungal and bacterial growth. This condition may be due to its natural habitat characterized by low temperature (8°C- 23 °C) and high humidity (85 – 99%) throughout the year which favours microbial growth. It has been reported that the leaves of 27 endemic endangered rare taxa of the Southern Western Ghats harbour Asterinaeaceous fungi. The black colonies of these fungi increase the photosynthetic efficiency of the plants, affecting the hormonal and phenolic compound level thus, the efficiency of the plants in total [21]. Elaeocarpus species are also found to be associated with Asterina fungus persisting even after sterilization with Mercuric Chloride. As many as 1172 fungal colonies were reported from two Elaeocarpus species [22] and 200 fungal colonies were associated in a single Elaeocarpus tree [23]. Hosagoudar [24] reported that four Elaeocarpus species were associated with exogenous Asterina which are E. munronii, E. serratus, E. tectorius and E. tuberculatus. The leaves of E. blascoi in the field were also found with exogenous fungi (Fig.2). However, the contamination on the explants of E. blascoi was reduced to 10% – 20% in the present study after Mercuric Chloride treatment followed by antimycotic solution treatment for 5 min which helped to achieve 80% – 90% sterile cultures. Besides fungal and bacterial contamination another major problem with in vitro cultures of E. blascoi was phenolic exudation from explants and browning of the culture which often resulted in the necrosis. This result corroborates with the findings of micropropagation studies in other woody plants [25, 26]. The exudation of phenols and browning of the medium was overcome by addition of 0.3% charcoal and 0.5% polyvinyl pyrrolidone (PVP) in the medium, decreasing the passage time by frequent subcultures and placing the cultures in dark condition for few days. The successful in vitro propagation method requires the correct size and physiological status of explants which play a crucial role during regeneration. Healthy and younger nodes with 2-2.5 cm long (Fig. 3) respond very well in woody plant medium. The explants response recorded maximum (80%) among 2 – 2.5 cm long shoot explants. Among the different size of explants tested 2 – 2.5 cm gave a maximum survival rate. While the smaller sized explants lost their regeneration potential which lead to tissue blackening and death after two weeks.

Induction of shoot growth and multiplication

The node was initially cultured on woody plant basal medium with different concentrations of plant growth regulators such as TDZ, BAP and KIN. In the absence of growth regulators shoot multiplication was not observed from the nodal explant rather only a single shoot grew from it. However, in the presence of growth regulators the mean number of shoots varied significantly with the type of cytokinin in the medium. Results showed that TDZ induced 2 - 4 shoots per node (Tab. Figs. 9-10) when compared to BAP and KIN. Among the different concentrations 0.22 µM TDZ induced 2 - 4 shoots followed BAP at a concentration of 0.44 µM (1.83±0.76) and KIN at 1.16 µM (1.53±0.51) per node after 5 weeks. In TDZ (0.22 µM) medium showed a maximum number of 7 shoots per node in three subcultures over a period of 150 days. High concentrations above 3 µM induced profuse basal callusing in all the three plant growth regulator combinations treated. When compared to BAP and KIN, TDZ was very active in inducing basal callus even at low concentration (Figs. 6-7). Basal callus was compact type which did not regenerate into shoots when transferred to medium lacking plant growth regulators. During subculture newly formed shoots were transferred to fresh medium of same composition. The shoots continued to proliferate through several subcultures with an average of four new shoots per transfer. Even though, multiple shoots were produced on TDZ medium the shoots failed to elongate therefore, they were transferred to medium containing gibberellic acid (5- 20 µM) and silver nitrate 10 mg/l for elongation. The formation of stunted shoots on TDZ medium the shoots failed to elongate and they decrease the hormonal imbalance in the entire leaves and they decrease the photosynthetic efficiency of the plants, affecting the hormonal and phenolic compound level thus, the efficiency of the plants in total [21]. Elaeocarpus species are also found to be associated with Asterina fungus persisting even after sterilization with Mercuric Chloride. As many as 1172 fungal colonies were reported from two Elaeocarpus species [22] and 200 fungal colonies were associated in a single Elaeocarpus tree [23]. Hosagoudar [24] reported that four Elaeocarpus species were associated with exogenous Asterina which are E. munronii, E. serratus, E. tectorius and E. tuberculatus. The leaves of E. blascoi in the field were also found with exogenous fungi (Fig.2). However, the contamination on the explants of E. blascoi was reduced to 10% – 20% in the present study after Mercuric Chloride treatment followed by antimycotic solution treatment for 5 min which helped to achieve 80% – 90% sterile cultures. Besides fungal and bacterial contamination another major problem with in vitro cultures of E. blascoi was phenolic exudation from explants and browning of the culture which often resulted in the necrosis. This result corroborates with the findings of micropropagation studies in other woody plants [25, 26]. The exudation of phenols and browning of the medium was overcome by addition of 0.3% charcoal and 0.5% polyvinyl pyrrolidone (PVP) in the medium, decreasing the passage time by frequent subcultures and placing the cultures in dark condition for few days. The successful in vitro propagation method requires the correct size and physiological status of explants which play a crucial role during regeneration. Healthy and younger nodes with 2-2.5 cm long (Fig. 3) respond very well in woody plant medium. The explants response recorded maximum (80%) among 2 – 2.5 cm long shoot explants. Among the different size of explants tested 2 – 2.5 cm gave a maximum survival rate. While the smaller sized explants lost their regeneration potential which lead to tissue blackening and death after two weeks.

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weeks either alone or along with GA3. Bais et al., have reported that AgNO3 at 40 µM influenced root induction in Decalepis hamiltonii shoots in vitro by accelerating endogenous polyamines [35].

Table 1. Effect of plant growth regulators on shoot induction and multiplication from nodal segments of E. blascoi

<table>
<thead>
<tr>
<th>PGR</th>
<th>Concentration (µM)</th>
<th>% of Shoot Induction (%)</th>
<th>Number of shoots per explants ( \pm SE )</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDZ</td>
<td>(0.22 µM)</td>
<td>80.00±1.0</td>
<td>3.86±0.61</td>
</tr>
<tr>
<td></td>
<td>(0.45 µM)</td>
<td>46.66±2.3</td>
<td>2.06±0.40</td>
</tr>
<tr>
<td></td>
<td>(1.13 µM)</td>
<td>30.00±2.0</td>
<td>1.37±0.15</td>
</tr>
<tr>
<td></td>
<td>(2.27 µM)</td>
<td>16.66±0.57</td>
<td>1.50±0.50</td>
</tr>
<tr>
<td></td>
<td>(4.50 µM)</td>
<td>13.33±1.52</td>
<td>1.26±0.28</td>
</tr>
<tr>
<td>BAP</td>
<td>(0.22 µM)</td>
<td>28.33±2.08</td>
<td>1.50±0.50</td>
</tr>
<tr>
<td></td>
<td>(0.44 µM)</td>
<td>38.33±3.70</td>
<td>1.83±0.76</td>
</tr>
<tr>
<td></td>
<td>(1.10 µM)</td>
<td>45.00±1.00</td>
<td>1.39±0.22</td>
</tr>
<tr>
<td></td>
<td>(2.21 µM)</td>
<td>23.33±3.05</td>
<td>1.57±0.37</td>
</tr>
<tr>
<td></td>
<td>(4.43 µM)</td>
<td>18.33±2.00</td>
<td>1.30±0.26</td>
</tr>
<tr>
<td>KIN</td>
<td>(0.24 µM)</td>
<td>10.00±1.73</td>
<td>1.16±0.15</td>
</tr>
<tr>
<td></td>
<td>(0.46 µM)</td>
<td>20.00±2.00</td>
<td>1.58±0.36</td>
</tr>
<tr>
<td></td>
<td>(1.16 µM)</td>
<td>28.33±2.51</td>
<td>1.53±0.51</td>
</tr>
<tr>
<td></td>
<td>(2.32 µM)</td>
<td>11.66±0.57</td>
<td>2.26±0.95</td>
</tr>
<tr>
<td></td>
<td>(4.64 µM)</td>
<td>6.00±1.15</td>
<td>1.30±0.30</td>
</tr>
</tbody>
</table>

The value represents the mean (± SE) of three independent experiments. Twenty explants were used for each experiment.

**Root induction**

The elongated shoots were transferred to half strength woody plant medium containing either one of different concentrations ranging from 2.4 µM to 17.12 µM of auxin type PGR’s such as IBA, NAA, IAA and to a basal medium, treated as control. IBA 9.84 µM was found to be the ideal concentration for promoting rooting in E. blascoi. Out of three auxin type PGR’s, IAA did not induce rooting at any concentration as it is well known that supplements of direct auxin in the culture medium do not necessarily induce roots [36]. However, IBA and NAA were effective in inducing roots. A maximum 80% of shoots were rooted on half strength WPM+ IBA 9.84 µM with 3 to 5 weeks whereas 55% of shoots were rooted medium containing half strength WPM+8.05 µM NAA. The basal medium failed to induce any roots. The collection of time of explants play a vital role in inducing in vitro multiplication and rooting of E. blascoi. The explants collected during April to May induced shoots and roots on auxin containing medium. However, explants collected during other months and seasons showed a poor shoot multiplication and rooting response. Singh et al., [37] and Choudhary et al., [38] have reported that IBA alone can induce highest percentage of rooting in woody trees - Shorea robusta and Prunus dulcis. The findings of the present study also show that IBA alone induces maximum roots in E. Blascoi (Fig.13).

For hardening rooted plantlets were transferred to potting mixture which has a combination of farmyard manure, garden soil and river sand in the ration of 2:1:1. In vitro derived plantlets were successfully acclimated and trained to withstand the outer environmental conditions and are ready to be reintroduced into natural habitats.
Fig 5-13: *In vitro* clonal propagation of *Elaeocarpus blascoi* from nodal explants. 5. Shoot initiation from nodal explant in WPM+TDZ (0.227 µM); 6. Shoot and basal callus formation from nodal explant on WPM+TDZ (0.45 µM); 7. Compact callus induced from nodal segment on TDZ containing medium (1.13 µM); 8. Shoot and root induction on WPM medium; 9-10 Multiple shoot induction on WPM+TDZ (0.227 µM); 11-12. Shoot elongation on medium containing GA3 + Ag NO3 (10mg/l); 13. Well grown shoot on root induction medium WPM+ IBA

4. Conclusion

The clonal propagation through nodal culture of *E. blascoi* was successful using WPM supplemented with cytokinin type PGR’s. This is the first report on *in vitro* culture conservation attempt which was made to establish shoot multiplication and whole plant regeneration of *E. blascoi*. These results show that it is possible to propagate this *E. blascoi* endangered tree species of Western Ghats through *in vitro* clonal propagation in order to overcome the threat of the extinction.

5. Acknowledgment

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6. References


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