Micro propagation of *Eugenia singampattiana Bedd.* - A critically endangered plant in the southern western Ghats of India

1.2 Erkings Michael Y, Dharmar K

1, 2 Research Department of Botany, Pasumpon Thiru Muthuramalinga Thevar Memorial College, Kamuthi, Tamilnadu, India.

Abstract

*Eugenia singampattiana* Beddome belongs to the family Myrtaceae. It is an evergreen medicinal tree found at the tail end of Southern Western Ghats of India. It is a critically endangered species enlisted by IUCN and exists only in with two fragmented population. It was successfully propagated through *in vitro* culture on MS medium. BAP are most suitable than Kn that represent the 3.66±0.21 shoot number and 3.75±007 shoot elongation but shoot regeneration percentage more on Kn in 1.5 mg/l concentration (53%). 2.0 mg/l of 2, 4-D alone and 2.5 mg/l 2,4iP alone and combination of 2,4iP (2.0 mg/l)+Kn (0.5 mg/l) and 2,4iP (2.5 mg/l)+Kn (0.5 mg/l) are intense callus formation. The *in vitro* callus generation, shoot regeneration and root formation are prerequisite for the mass multiplication which is liable part of strategies for ex situ conservation.

Keywords: *Eugenia singampattiana*, IUCN, Callus, Shoot regeneration

Introduction

*Eugenia singampattiana* Beddome is a critically endangered small evergreen medicinal tree belongs to the family Myrtaceae and found at the tail end of Southern Western Ghats of India. It is proven as antineoplastic, antitumorous, antioxidative, antimicrobial, antifungal, antinflammatory, antihyperlipidemic and antidiabetic agents [1]. This species is categorized as endangered or possibly extinct by Botanical Survey of India. In 2013, IUCN enlisted this species as Critically Endangered. It is geographically distributed only with two fragmented population [2] which is caused by decline in area of occupancy and loss of habitat. The species grows only on sandy clay loam at slopes in different altitudes [3]. There were no seedlings noticed in field observation because seed germination requires 65 to 85 days [4]. Hence, this species requires an efficient *in vitro* plant regeneration system. Thus the present study deals with *in vitro* propagation of *Eugenia singampattiana* Bedd.

Materials and Methods

The chosen plant was collected from Karaiyar in Southern Western Ghats of India. It was identified and checked with the Herbarium of Jawaharlal Nehru Tropical Botanical Garden and Research Institute (JNTBGRI) and voucher specimen (Collection No.76845) was deposited in JNTBGRI.

Media selection and Shoot proliferation

MS (Murashige and Skoog’s, 1962) [5] basal medium supplemented with vitamins, 3% sucrose, micronutrients and solidified with 0.8% agar were used for shoot multiplication, regeneration and shoot elongation with different dilution of growth hormone. The media were adjusted to the pH of 5.6 for shoot and 5.8 for root, autoclaved at 108 kpa on 121 °C for 15 minutes. Different concentrations of BAP and Kn (0.5-2.5 mg/l) were used individually for shoot proliferation. BAP and Kinetin with combination of NAA (0.25-0.5mg/l) were used for proliferation of shoots from nodal explants. Cultures were incubated under 10 hours photoperiod at 25+2 °C and 60+5% relative humidity. A minimum of ten tubes were taken for each experiment. It was repeated thrice. The shoots produced *in vitro* were sub cultured on basal medium with subsequent growth regulators.

Callus induction and rooting

Different combination of Auxins (2, 4-D and 2, 4iP) (1.5-2.5 mg/l) and Cytokinin (BAP and Kn) were used for callus induction from nodal explants. The regenerated shoots from the nodal explants derived callus and or *in vitro* shoots were excised and transferred to MS medium and 1.0% sucrose with Auxins (NAA) alone in different concentrations (0.25-2.5mg/l) and solidified with 0.8% agar for rooting.

Results and Discussion

Shoot proliferation

*E. singampattiana* was propagated through *in vitro* culture successfully on MS medium supplemented with growth regulator BAP, Kn alone and combination with NAA. After 3 weeks of *in vitro* culture initiation, shoots were appeared on culture media (Table 1, Plate 1). MS medium supplemented without growth regulator did not initiate shoot proliferation. MS medium supplemented with growth regulator BAP, Kn and combination with NAA showed enhanced shoot proliferation. Both BAP and Kn 1.5 mg/l concentration alone promote optimum shoot regeneration. BAP and KN alone promote shoot induction in endangered plant *Psoralea corylifolia* [6] and *Saussurea esthonica* [7]. BAP are most suitable than Kn that represent 3.66±0.21 shoot number and 3.75±007 shoot elongation but shoot regeneration percentage more on Kn in 1.5 mg/l concentration (53%) because medium with BAP acted as trigger for initiating shoot proliferation. BAP act as inducing bud breaking for promote shoot initiation [8]. When concentration was increased above 1.5 mg/l concentration, both BAP and Kn showing reduced number of shoot proliferation and length of shoot.
Multiple shoot bud was induced maximum on MS medium supplemented with 2.0 mg/l Kn combination with Auxin in *Vitex negundo* [9]. The synergistic effect of cytokinin and auxin has been demonstrated in *Cedrela montana* [10]. The present investigation also showed more shoot induction by auxin combination with cytokinin. The positive modification of BAP and Kn with NAA shoot induction efficiency more because of synergistic effect of cytokinin and auxin. BAP and Kn 1.5 mg/l concentration combination with NAA enhanced shoot proliferation and 2.0 mg/l concentration of BAP and Kn indicate finest shoot proliferation.

**Callus induction**

Highest diameter of callus was observed on MS Medium supplemented with 2.0 mg/l of 2,4-D alone and 2.5 mg/l 2,4iP alone and combination of 2, 4iP (2.0 mg/l)+Kn (0.5 mg/l) and 2,4iP (2.5 mg/l)+Kn (0.5 mg/l) (Table 2). Intense callus mostly occur in the form of brownish. It may be due to the phenolic exudation or microbial contamination. Brownish callus tested with microbial culture which proved no evidence of microbial contamination. It was conformed that brownish callus due to phenolic exudation. The presence of Cytokinin in the medium causes browning [11]. The greenish and friable nature of callus was able to regenerate shoots initiation because of greenish nature is a sign of shoot regeneration [12]. White and pale yellow callus turn brownish or greenish due to phenolic exudation or shoots initiation. The obtained callus was sub cultured on shoot proliferation medium and initiate shoot regeneration.

### Table 1: Effect of plant growth regulators on shoot regeneration in *E. singampattiana*

<table>
<thead>
<tr>
<th>Growth regulators (mg/l)</th>
<th>Shoot regeneration (%)</th>
<th>No. of shoots/explants</th>
<th>Shoot length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP KN NAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 - - -</td>
<td>27</td>
<td>2.22±0.14</td>
<td>2.96±0.08</td>
</tr>
<tr>
<td>1.0 - - -</td>
<td>30</td>
<td>2.44±0.17</td>
<td>2.93±0.44</td>
</tr>
<tr>
<td>1.5 - - -</td>
<td>50</td>
<td>3.66±0.21</td>
<td>3.75±0.007</td>
</tr>
<tr>
<td>2.0 - - -</td>
<td>40</td>
<td>2.75±0.17</td>
<td>3.30±0.08</td>
</tr>
<tr>
<td>2.5 - - -</td>
<td>37</td>
<td>2.54±0.52</td>
<td>2.88±0.07</td>
</tr>
<tr>
<td>- 0.5 - -</td>
<td>33</td>
<td>2.30±0.15</td>
<td>2.73±0.08</td>
</tr>
<tr>
<td>- 1.0 - -</td>
<td>37</td>
<td>2.45±0.15</td>
<td>2.92±0.07</td>
</tr>
<tr>
<td>- 1.5 - -</td>
<td>53</td>
<td>3.47±0.71</td>
<td>3.65±0.04</td>
</tr>
<tr>
<td>- 2.0 - -</td>
<td>43</td>
<td>2.93±0.18</td>
<td>3.31±0.08</td>
</tr>
<tr>
<td>- 2.5 - -</td>
<td>40</td>
<td>2.58±0.14</td>
<td>2.98±0.03</td>
</tr>
<tr>
<td>0.5 - 0.25 -</td>
<td>33</td>
<td>2.40±0.16</td>
<td>2.77±0.08</td>
</tr>
<tr>
<td>1.0 - 0.25 -</td>
<td>37</td>
<td>2.54±0.15</td>
<td>3.02±0.07</td>
</tr>
<tr>
<td>1.5 - 0.25 -</td>
<td>40</td>
<td>2.58±0.14</td>
<td>3.09±0.06</td>
</tr>
<tr>
<td>2.0 - 0.5 -</td>
<td>53</td>
<td>3.33±0.18</td>
<td>3.85±0.03</td>
</tr>
<tr>
<td>2.5 - 0.5 -</td>
<td>-</td>
<td>3.00±0.19</td>
<td>3.34±0.06</td>
</tr>
<tr>
<td>- 0.5 0.25 -</td>
<td>37</td>
<td>2.36±0.15</td>
<td>2.67±0.06</td>
</tr>
<tr>
<td>- 1.0 0.25 -</td>
<td>40</td>
<td>2.41±0.14</td>
<td>2.88±0.06</td>
</tr>
<tr>
<td>- 1.5 0.25 -</td>
<td>43</td>
<td>2.58±0.14</td>
<td>3.04±0.06</td>
</tr>
<tr>
<td>- 2.0 0.5 -</td>
<td>57</td>
<td>3.41±0.19</td>
<td>3.90±0.03</td>
</tr>
<tr>
<td>- 2.5 0.5 -</td>
<td>47</td>
<td>2.92±0.16</td>
<td>3.18±0.09</td>
</tr>
</tbody>
</table>

Mean ±SE, n= 10 replicates, 3 times repeatedly

### Table 2: Effect of different hormones on callus proliferation in *E. singampattiana*

<table>
<thead>
<tr>
<th>Growth regulators (mg/l)</th>
<th>Callus proliferation scoring</th>
<th>Colour of callus</th>
<th>Morphology of callus</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D 2,4iP BAP KN KIN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 - - -</td>
<td>+++</td>
<td>Whitish green</td>
<td>Friable</td>
</tr>
<tr>
<td>2.0 - - -</td>
<td>++++</td>
<td>Dark green</td>
<td>Non Friable</td>
</tr>
<tr>
<td>2.5 - - -</td>
<td>++</td>
<td>White green</td>
<td>Friable</td>
</tr>
<tr>
<td>- 1.5 - -</td>
<td>+++</td>
<td>Whitish green</td>
<td>Non Friable</td>
</tr>
<tr>
<td>- 2.0 - -</td>
<td>++++</td>
<td>Dark green</td>
<td>Non Friable</td>
</tr>
<tr>
<td>- 2.5 - -</td>
<td>+++</td>
<td>Brownish green</td>
<td>Friable</td>
</tr>
<tr>
<td>1.5 - 0.5 -</td>
<td>++</td>
<td>Brownish green</td>
<td>Non Friable</td>
</tr>
<tr>
<td>2.0 - 0.5 -</td>
<td>+++</td>
<td>Brownish green</td>
<td>Non Friable</td>
</tr>
<tr>
<td>2.5 - 0.5 -</td>
<td>++++</td>
<td>Whitish green</td>
<td>Non Friable</td>
</tr>
<tr>
<td>1.5 - 0.5 -</td>
<td>++</td>
<td>Pale yellow</td>
<td>Friable</td>
</tr>
<tr>
<td>2.0 - 0.5 -</td>
<td>+++</td>
<td>Green</td>
<td>Friable</td>
</tr>
<tr>
<td>2.5 - 0.5 -</td>
<td>++++</td>
<td>Brownish</td>
<td>Non Friable</td>
</tr>
<tr>
<td>- 1.5 0.5 -</td>
<td>++</td>
<td>White</td>
<td>Friable</td>
</tr>
<tr>
<td>- 2.0 0.5 -</td>
<td>+++</td>
<td>Whitish green</td>
<td>Non Friable</td>
</tr>
<tr>
<td>- 2.5 0.5 -</td>
<td>++++</td>
<td>Brownish green</td>
<td>Non Friable</td>
</tr>
<tr>
<td>- 1.5 - 0.5 -</td>
<td>++</td>
<td>White</td>
<td>Friable</td>
</tr>
<tr>
<td>- 2.0 - 0.5 -</td>
<td>+++</td>
<td>Brownish green</td>
<td>Non Friable</td>
</tr>
<tr>
<td>- 2.5 - 0.5 -</td>
<td>++++</td>
<td>Brownish green</td>
<td>Non Friable</td>
</tr>
</tbody>
</table>

+++,+++ - Intense, +++ - Moderate, ++ - Eager
Rooting of shoots
The MS medium without PGR was failed to induce rooting of regenerated shoots. Maximum of 67% shoots were rooted with an average of 2.70±0.21 roots per shoot and average length of 2.87±0.05 cm on MS medium supplemented with 2.0 mg/l NAA (Table 3). NAA was most suited for root induction on *Stemona tuberosa* [13] and *Ceropegia intermedia* [14].

### Table 3: Effect of Auxin (NAA) on root induction from shoot of *E. singampattiana*

<table>
<thead>
<tr>
<th>Growth regulators (mg/l)</th>
<th>% of rooting</th>
<th>No. of roots/shoots</th>
<th>Root length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>27</td>
<td>1.50±0.28</td>
<td>1.95±0.11</td>
</tr>
<tr>
<td>0.5</td>
<td>40</td>
<td>1.50±0.22</td>
<td>2.31±0.09</td>
</tr>
<tr>
<td>1.0</td>
<td>47</td>
<td>1.71±0.18</td>
<td>2.58±0.07</td>
</tr>
<tr>
<td>1.5</td>
<td>53</td>
<td>1.88±0.20</td>
<td>2.64±0.07</td>
</tr>
<tr>
<td>2.0</td>
<td>67</td>
<td>2.70±0.21</td>
<td>2.87±0.05</td>
</tr>
<tr>
<td>2.5</td>
<td>33</td>
<td>1.80±0.37</td>
<td>2.14±0.15</td>
</tr>
</tbody>
</table>

### Conclusion
*Eugenia singampattiana* was micropropagated through *in vitro* conservation approach. BAP and Kn alone promote shoot induction and the combination of BAP and NAA, Kn and NAA promote more shoot induction because of synergistic effect of cytokinin and auxin. MS Medium supplemented with 2,4-D and 2,4iP alone favoured for induction of callus. The regenerated shoots were excised on basal medium supplemented with NAA for root formation. The *in vitro* callus, shoot regeneration and root formation are prerequisite for the mass multiplication of plantlets through tissue culture. It should be a valuable part of strategies for ex situ conservation of endangered plant.

### Acknowledgment
The authors are thankful to University Grant Commission (UGC), Government of India, New Delhi for financial assistance under Major Research Project.

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**Plate 1. In vitro regeneration of *Eugenia singampattiana* Bedd.**

a) Mother plant; b) Flower; c) Explant inoculation; d) Shoot proliferation; e) Shoot elongation; f) Shoot multiplication; g) Whitish callus h) Brownish callus i) Brownish green callus; j) & k) shoot initiation from callus; l) Root regeneration from callus
References