Conservation of Curcuma caesia Roxb.- A critically endangered species via in vitro plant regeneration from organogenic callus

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ABSTRACT

A novel protocol for callus mediated shoot regeneration and somatic embryogenesis was established for Curcuma caesia Roxb., a critically endangered species of North east India. Vigorous callus growth was observed in MS medium containing higher concentration of 5.0 mg l\(^{-1}\), 4-D from pseudostem explants. More than 70% of the pseudostem explants of this species responded for callus induction within 22 days of culture. After 4 subcultures these callus showed embryogenic response in MS medium containing 2.0 mg l\(^{-1}\) BAP with 0.05 mg l\(^{-1}\) NAA. Shoots were successfully regenerated on MS medium with a concentrations of 0.25 mg l\(^{-1}\) Kn and 0.05 mg l\(^{-1}\) GA\(_3\), within 25 days of transfer. Single shoots transferred into MS basal medium free of plant growth regulator rooted well with (80%) survivality under field condition.

Key words: Curcuma caesia, callus induction, somatic embryogenesis, plant regeneration

INTRODUCTION

Curcuma caesia Roxb. of Zingiberaceae Family, is a perennial, erect rhizomatous herb with large leaves and bluish-black rhizome. It is commonly known as black turmeric or kali khati, native to North-East and Central India. Fresh rhizomes are aromatic with intense camphoraceous odour contains camphor, ar-turmerone, (Z)-ocimene, ar-curcumene, 1, 8-cineole, borneol, bornyl acetate and curcumene as the major constituents. The rhizomes of this plant have a high economic importance owing to its putative medicinal properties. Traditionally, the rhizomes of Curcuma caesia Roxb. is used in the treatment of leucoderma, asthma, tumours, piles, bronchitis, rheumatic pains etc (Kaguyung et al., 2010). This herb also gives protection against Alzheimer’s disease and inflammatory bowel diseases such as ulcerative colitis and Crohn’s disease (Behar et al., 2013). Due to high medicinal value, black turmeric has been exploited indiscriminately from their natural habitat which make it under the category of critically endangered species (Behar et al., 2014; Kumar et al., 1998). Thus, efforts should be made to conserve and work for the betterment of this plant species. Vegetative modes of propagation and absence of seed setting in C. caesia prevented the production of new and improved cultivars through conventional plant breeding.

An alternative method for creating improved genotypes through selection of somaclonal variation has been reported in many species by several workers (Nayak et al., 2003; Mohanty et al., 2008). Callus induction and plantlet regeneration from different parts of other species of Curcuma has been reported earlier by other workers (Mohanty et al., 2008). However, very least report is available for callus culture of Curcuma caesia. Therefore, attempt has been made in the present work for plantlet regeneration from callus culture of C. caesia and in vitro somatic embryogenesis.

MATERIALS AND METHODS

Healthy rhizomes of Curcuma caesia Roxb. were collected and grown in the experimental garden of Department of Botany, Gauhati University, Guwahati. Dormant rhizome axillary buds were excised from the clean rhizome, which were used as the source of explants. Freshly collected rhizomes were cleaned with running tap water and immature buds were excised with sharp blade and washed with detergent (Tween-20, 0.1% v/v) for 15 min and subsequently rinsed with clean water. Explants were then surface sterilized in disinfectant (0.1% HgCl\(_2\) to which two – three drops of Tween-20 were added) for 10 minutes. Under sterile conditions, HgCl\(_2\) solution was decanted and the explants were rinsed five-six times with sterile distilled water. Sterilized explants were trimmed aseptically with a sharp and pre sterilized surgical blade. Trimmed rhizome axillary bud (3.0-4.0 mm size) served as the primary explant and initially cultured on shoot induction medium to obtain contamination free cultures. Murashige and Skoog (1962) MS basal media supplemented with 3% sucrose and 2.2 gl\(^{-1}\) Gelrite modified with various concentrations of N\(_6\)-Benzyil-aminopurine (BAP 0.05 to 3.0 mg l\(^{-1}\)) in combination with Kinetin (Kn; 0.5-2.0 mg l\(^{-1}\)), Indole-3-acetic acid (IAA; 0.5-1.0 mg l\(^{-1}\)), \(\alpha\)-naphthaleneacetic acid (NAA; 0.5-1.0 mg l\(^{-1}\)) and 2, 4-D (0.5-4.0 mg l\(^{-1}\)) were used for establishment of the culture. Laboratory reagent-grade sucrose was replaced by locally available commercial sugar (market sugar) as carbon source for reducing the cost of the media.

The pH of the media was adjusted to 5.8 prior to the addition of the gelling agent. The media was autoclaved at 121°C with 15 lbs pressure for 15 minutes. Excised buds were inoculated into culture media under laminar air flow. Cultures were incubated at 25±2°C under 16 hours of photoperiod from cool white fluorescent tube giving 12.5\(\mu\)mol m\(^{-2}\)s\(^{-1}\) at culture level. Most

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of the cultures sprouted within 7-12 days of inoculation and the emerged shoots (1.0-2.0 cm) were sub cultured in the same media for further multiplication. A total of 20 explants were used for each of the treatments and the number of shoots per explant was recorded after four weeks of inoculation. Each set of experiment was repeated thrice.

**Callus induction and shoots regeneration**

**Callus induction:** Leaf sheath, pseudostem and root segment derived from in vitro grown 4 weeks old seedlings were inoculated in freshly prepared media for callus induction. MS basal media supplemented with various concentrations of N6-Benzyl-aminopurine (BAP 0.05 to 3.0 mg/l) in combination with Kinetin (Kn; 0.5-2.0 mg/l), Indole-3-acetic acid (IAA; 0.5-1.0 mg/l), á-naphthaleneacetic acid (NAA; 0.5-1.0 mg/l) and 2, 4-D (0.5-4.0 mg/l) sucrose and 2.2 gl-1 Gelrite were used as callus inducing media. Cultures of all treatments were divided into two groups. The first group incubated and maintained under 16-h photoperiod. The second group maintained in dark. All cultures incubated at 26 ± 1° C for 8 weeks. After callus development the cultures were transferred in to new media for somatic embryogenesis and shoot regeneration.

**Embryogenic test**

Test of callus cells for the presence of embryos was carried out by acetocarmine stain (Gupta and Durzan, 1987). Acetic acid solution (45%) was prepared by mixing 45 ml of glacial acetic acid with 55 ml of sterile distilled water heated this solution and stir with glass rod. Continue boiling until the dye dissolves and then cool to room temperature. Filter the solution and stored in refrigerator.

Calli extracted from cultures responded for somatic embryogenesis are macerated gently to break down to small pieces. Small amount of cells are taken on a clear slide and treated with 1-2 drops of acetocarmine reagent on cells and stir well with a needle. Warm the slide for 1-2 minutes gently over the flame then washed the sample by destaining solution on warm water for 2-3 times and then examined under microscope. The resulted stained crimson red cells are embryogenic while unstained cells are non embryogenic.

**RESULT AND DISCUSSION**

**Callus induction and shoots regeneration**

Shoot multiplication of *Curcuma caesia* was found to be best in MS medium supplemented with BAP (1.0 mg/l) and 2, 4-D (0.25 mg/l) within 8 weeks in (23.80±0.51 shoots/explant). Out of the three explants (pseudostem, leaf sheath and root segment) studied, pseudostem showed vigorous callus growth in MS medium containing higher concentration of 2, 4-D (5.0 mg/l) (Plate-d). More than 70% of the pseudostem explants of this species responded for callus induction within 22 days of culture. It was followed by the MS media supplemented with 2.0 mg/l 2,4-D and 4.0 mg/l BAP, 4.0 mg/l 2, 4-D within 25-30 days of culture(Plate-a; Plate-b). The Calli thus generated from this combination was found to be highly friable, transparent white in colour, loose in nature with vigorous growth. During subcultures, secondary proliferated calli were subsequently produced from initially induced calli on the same medium. Similar results also reported by Roopadarsini (2010) on *in vitro* callus induction with the supplementation of 3.0 mg/l 2, 4-D in other *Curcuma* species. Callus induction was observed under dark treatment only while in
Callus induction of *Curcuma caesia* was also observed from leaf sheath explant in half strength MS medium fortified with 5.0 mg l\(^{-1}\) 2, 4-D within 20 days of culture. Tuan et al. (2011) reported higher callus induction in MS medium supplemented with 1.0 mg l\(^{-1}\) 2, 4-D, 1.0 mg l\(^{-1}\) BA and 2% sucrose, from leaf-base explants of *Curcuma zedoaria* (Table 1, Plate 1).

Among the explants studied the root explants of *Curcuma caesia* showed poor response for callus formation. Callus induction from root explants was observed in the medium containing ½ strength MS basal medium with 2.0 mg l\(^{-1}\) NAA and 0.5 mg l\(^{-1}\) 2, 4-D within 30 days of culture. In contrast to the present findings Miachir et al.(2004) reported callus induction from root segments of *Curcuma zedoaria* on MS medium supplemented with 1.0 mg l\(^{-1}\) NAA in dark.

**Somatic embryogenesis and Plantlet Regeneration**

Highly proliferated callus tissue of *Curcuma caesia* initiated in MS medium were aseptically transferred to the organogenesis inducing medium fortified with various auxins and cytokinins singly or in combinations. Embryonic callus were proliferated from the explants of *Curcuma caesia* after 30 days of transfer on MS medium.

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<table>
<thead>
<tr>
<th>Explant</th>
<th>Media</th>
<th>Percent of explant for callus induction</th>
<th>Days required for callus induction</th>
<th>Callus Growth</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf sheath</td>
<td>MS</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>No response</td>
</tr>
<tr>
<td></td>
<td>+ 1.0 mg/l 2,4-D</td>
<td>22</td>
<td>25</td>
<td>+</td>
<td>Callus induced with poor growth</td>
</tr>
<tr>
<td></td>
<td>+ 3.0 mg/l 2,4-D</td>
<td>40</td>
<td>22</td>
<td>+ + +</td>
<td>Highly friable callus with profuse growth</td>
</tr>
<tr>
<td></td>
<td>+ 5.0 mg/l 2,4-D</td>
<td>56</td>
<td>20</td>
<td>+ + + +</td>
<td>White friable callus induced</td>
</tr>
<tr>
<td></td>
<td>+ 8.0 mg/l 2,4-D</td>
<td>30</td>
<td>22</td>
<td>++</td>
<td>Callus induced, compact in nature</td>
</tr>
<tr>
<td></td>
<td>+ 1.0 mg/l BAP+ 2,4-D</td>
<td>50</td>
<td>24</td>
<td>+ + + +</td>
<td>Callus induced friable in nature</td>
</tr>
<tr>
<td></td>
<td>+ 1.0 mg/l BAP+ 0.5 mg/l NAA</td>
<td>28</td>
<td>26</td>
<td>+</td>
<td>Compact callus induced with poor growth</td>
</tr>
<tr>
<td></td>
<td>+ 0.5 mg/l 2,4-D+ NAA 2.0 mg/l</td>
<td>20</td>
<td>27</td>
<td>+</td>
<td>Little callus induced, compact in nature</td>
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<tr>
<td>Root</td>
<td>MS+ 1.0 mg/l 2,4-D</td>
<td>0</td>
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<td>-</td>
<td>No response</td>
</tr>
<tr>
<td></td>
<td>+ 2.0 mg/l NAA</td>
<td>10</td>
<td>38</td>
<td>++</td>
<td>Callus induced with moderate growth</td>
</tr>
<tr>
<td></td>
<td>+ 1.0 mg/l 2,4-D+ 0.5 mg/l NAA</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>No response</td>
</tr>
<tr>
<td></td>
<td>+ 1.0 mg/l 2,4-D+ 1.0 mg/l NAA</td>
<td>20</td>
<td>33</td>
<td>+</td>
<td>Callus induced, hard in nature</td>
</tr>
<tr>
<td></td>
<td>+ 0.5 mg/l 2, 4-D+ 2.0 mg/l NAA</td>
<td>23</td>
<td>30</td>
<td>++</td>
<td>Callus induced, Compact in nature</td>
</tr>
<tr>
<td></td>
<td>+ 2.0 mg/l NAA</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>No response</td>
</tr>
<tr>
<td>Pseudo-stem</td>
<td>MS+ 0.05 mg/l BAP+ 1.0 mg/l 2, 4-D</td>
<td>32</td>
<td>28</td>
<td>+</td>
<td>Callus induced with moderate growth</td>
</tr>
<tr>
<td></td>
<td>+ 1.0 mg/l BAP+ 4.0 mg/l 2,4-D</td>
<td>47</td>
<td>25</td>
<td>++++</td>
<td>Friable callus induced with high growth</td>
</tr>
<tr>
<td></td>
<td>+ 0.5 mg/l BAP+ 1.5 mg/l 2,4-D</td>
<td>45</td>
<td>27</td>
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<td>Callus induced</td>
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<tr>
<td></td>
<td>+ 0.5 mg/l 2,4-D+ 2.0 mg/l NAA</td>
<td>12</td>
<td>35</td>
<td>+</td>
<td>Compact Callus induced</td>
</tr>
<tr>
<td></td>
<td>+ 1.0 mg/l 2,4-D</td>
<td>35</td>
<td>30</td>
<td>+++</td>
<td>Whithish Friable Callus induced, with moderate growth</td>
</tr>
<tr>
<td></td>
<td>+ 2.0 mg/l 2,4-D</td>
<td>50</td>
<td>30</td>
<td>++++</td>
<td>Friable Callus induced</td>
</tr>
<tr>
<td></td>
<td>+ 2.0 mg/l 2,4-D</td>
<td>70</td>
<td>22</td>
<td>++</td>
<td>Highly friable callus induced, transparent white in colour</td>
</tr>
<tr>
<td></td>
<td>+ 0.5 mg/l 2, 4-D+ 2.0 mg/l NAA</td>
<td>12</td>
<td>30</td>
<td>+</td>
<td>Hard Callus with slow growth</td>
</tr>
</tbody>
</table>

Conservation of *Curcuma caesia* Roxb.
supplemented with 2.0 mg/l BAP with 0.05 mg/l NAA. Malamug et al. (1991) was also able to regenerate multiple shoots from callus derived shoots of ginger using BAP and NAA in modified MS medium. The embryogenic calli were routinely maintained on the same induction medium. Numerous somatic embryos of varied shapes were developed on the surface of the embryogenic callus. In this medium globular embryo developed within 30 days of transfer. Greenish friable callus with preshoots were developed while transferring these embryos to freshly prepared media fortified with 0.25 mg/l Kn and 0.05 mg/l GA3 within 25 days of transfer (Table 2) (Plate e). There is strong evidence that the combination of BAP and NAA enhances shoot regeneration from organogenic calluses of Alstroemeria (Gonzales-Benito and Alderson, 1990) and Dianthus chinensis (Jethwani and Kothari, 1996). The effect of combining these two growth regulators was also described for micropropagation of Curcuma amada (Barthakur and Bordoloi, 1992) and other members of the Zingiberaceae family (Babu et al., 1992; Reghunath and Priyadarshan, 1993; Dogra et al., 1994; Hung, 1995; Ilig and Faria, 1995). Addition of GA3 in the culture media promoted germination of somatic embryos of Curcuma caesia as reported earlier in Curcuma species by Prakash et al. (2001). Mature somatic embryos germinated readily and developed into normal plantlets after 4 weeks on half strength MS basal medium. Well rooted plantlets were successfully acclimatized in soil at high rate of survival (80%).

CONCLUSION

It may be concluded from this study that the protocol developed will be useful for rapid in vitro propagation of Curcuma caesia through somatic embryogenesis. Mass multiplication within a short period of time may provide a viable approach through callus culture and somatic embryogenesis for germplasm conservation of this species.

REFERENCES


