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In vitro propagation of a medicinal plant - mango ginger (*Curcuma amada* Roxb.)

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Abstract

This paper describes efficient *in vitro* propagation protocol of *Curcuma amada* Roxb., an important aromatic zingiberaceous plant in the countries of Indian subcontinent, using rhizome explants. Rhizome tip explants showed better response than rhizome bud explants in terms of shoot proliferation and hence excised rhizome tip explants were inoculated aseptically on Murashige and Skoog's (MS) medium supplemented with different 6-Benzyl adenine (BA) or 6-furfurylamino purine (Kn) concentrations (0.0, 2.0, 4.0, 6.0, 8.0, 10.0 µM) alone or in combinations with α- Naphthalene acetic acid (NAA) and Indole-3-butyric acid (IBA) (1.0, 1.5, 2.0 µM). Optimum proliferation was obtained on MS medium having 8.0 µM BA + 1.0 µM NAA. Shoot proliferation was maximal (99.8%) with 10.6 ± 0.26 shoots per explant after 6 weeks of culture. The successfully proliferated shoots with an average height of 38 cm were transferred to rooting medium augmented with NAA and IBA at four different concentrations (2.0, 4.0, 6.0, 8.0 µM) and 4.0 µM IBA was found to be best for *in vitro* adventitious rooting. Rooted plantlets were then transferred to plastic cups containing autoclaved garden soil and compost (1:1), gradually acclimatized and finally transferred to the field condition with 80% survival rate.

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Introduction

Mango ginger (*Curcuma amada* Roxb.) is an important aromatic plant in the countries of Indian subcontinent having morphological resemblance with ginger but imparts a raw mango flavour. It is a perennial, rhizomatous, aromatic herb belonging to the family Zingiberaceae and believed to have originated in Indian subcontinent and found in most provinces except dry ones, but cultivated in southern India (Prain, 1981). It grows wild in all over Bangladesh, especially in marshy and shady places of Gaibanda, Rangpur, Bogra, Rajshahi, Jessore and Natore but is not cultivated anywhere commercially (Ghani, 1998). Mango ginger is used medicinally as a coolant, astringent and to promote digestion. In addition, due to having a typical exotic flavour of raw unripe mango, mango ginger is used as a basic ingredient in pickles, preserves, candies, sauces, curries, salads and so on (Verghese, 1990; Shankaracharya, 1982). Its rhizome has carminative properties as well as being useful as a stomachic (Hussain *et al.*, 1992) and rhizome pest has traditionally been used for healing of wounds, cuts and itching (Srivastava *et al.*, 2001). This plant is normally multiplied by rhizome buds in the wild whereas it is propagated using portions of underground rhizomes during cultivation. Several common problems including slow propagation rate, soil born diseases, deterioration of rhizomes caused by bacteria and fungi and insect attacks etc. have already been reported for this plant (Balachandran 1990; Prakash *et al.* 2004). Besides, the harvest of this plant for traditional medicine purposes on a mass scale from their natural habitats is leading to a depletion of natural plant resources. Due to the afore-mentioned problems, proper step should be taken to conserve as well protect the existing germplasm of this plant species and *in vitro* propagation may play a vital role in this regard. *In vitro* clonal propagation of *Curcuma* species through rhizome buds has been reported (Nadgauda *et al.*, 1978; Mukhri and Yamaguchi, 1986; Balachandran *et al.*, 1990; Sit and Tiwari, 1997; Salvi *et al.*, 2002). Two reports have been found on *in vitro* propagation of *C. amada* - one partial report has been developed by Prakash *et al.* (2004) who used rhizome piece explant for multiple shoot induction and cultured them on MS medium fortified with BA alone or in combination with NAA and another report has been developed by Barthakur and Bordoloi (1992). However, no efficient work has been reported in Bangladesh yet on *in vitro* propagation of *C. amada*. In this study, we report an efficient protocol for large scale propagation of *C. amada* through rhizome explants. The protocol reported here could be used for the conservation, large-scale propagation and for further research on the biochemical composition and medicinal importance of this valuable medicinal herb.

Materials and methods

Plant material

Healthy rhizome with active buds were collected from the rhizome of *C. amada* maintained in the nursery bed of experimental garden of Plant Tissue Culture Laboratory, department of Botany, Rajshahi University. They were cut into 1.5 to 2 cm length with active buds intact and categorized into two separate groups- rhizome tip and rhizome bud (Fig. 2 A & B). These rhizome explants were initially washed with Savlon (Chlorhexidine gluconate 0.3% w/v + cetrimide 3% w/v solution) for 10 minutes and rinsed several times under running tap water. These excised explants were transferred to laminar air flow cabinet and surface sterilized with 0.1% $HgCl_2$ for 10 minutes and then washed with sterile distilled water thoroughly 3 to 4 times with sterile distilled water and soaked with sterile blotting paper and used as explants for *in vitro* cultures before the inoculation in to sterilized nutrient agar medium (MS).

Culture medium and growth conditions

The sterilized blotted explants were implanted on to the Murashige and Skoog's (1962) agar-gelled medium fortified with various concentrations/combinations of growth regulators. For shoot induction, the medium was supplemented with 0.0, 2.0, 4.0 6.0 8.0 and 1.0 µM BA and Kn alone or in combination with 1.0, 1.5 and 2.0 µM NAA and IBA. For root induction, *in vitro* raised

shoots grown in multiplication medium were cultured on MS medium supplemented with either NAA or IBA in concentration of 2.0, 4.0, 6.0 and 8.0 µM. The pH of the medium was adjusted to 5.8 before autoclaving at 1.2 kg/cm² pressure and 121°C temperature for 15 - 20 minute. Molten medium of 20 ml was dispensed into the culture tube and plugged with nonabsorbent cotton wrapped in one layer of cheesecloth. All the cultures were incubated in the culture room at 25 ± 2°C under a 16h light and 8h dark cycle with the light intensity of 2000 – 3000 lux provided by cool-white fluorescent tubes (36W). Each treatment had 15 culture tubes and the experiment was repeated thrice. The cultures were maintained by regular subcultures at 2 weeks intervals on fresh medium with the same compositions.

Hardening and acclimatization

Plantlets having a well-developed root system were removed from the culture tubes and the roots were washed under running tape water to remove agar. Then the plantlets were transferred to sterile small plastic cups containing autoclaved garden soil and compost (1:1) and maintained inside growth chamber set at temperature 28°C and 70-80% relative humidity. After four weeks they were kept under laboratory conditions for additional two weeks before transferring them to the Botanical Evaluation Garden and maintained there for acclimatization.

Culture observation and presentation of results

Fifteen cultures were used per treatment and each experiment was repeated three times. The data pertaining to percentage of cultures showing proliferation, average number of shoots/culture, percentage of rooting, mean number of roots and average length of roots were statistically analyzed by the Duncan's multiple Range Test at the $P < 0.05$ level of significance using a statistical software-SPSS.

Results and discussion

Shoot multiplication was remarkably effected by the type of explants used and significant number of shoots was produced from rhizome tip explants irrespective of growth regulator concentration and combinations (Fig. 1). The similar trends were also reported in *C. longa (*Keshavachandaran and Khader, 1989; Balachandran *et al.*, 1990 and Das *et al.*, 2010) and *Zingiber officinale* (Hoque *et al.*, 1999 and Kambaska *et al.*, 2009). No reports have been found on the reason of differential response of rhizome tip and rhizome bud explants in shoot multiplication of zingiberaceous plant. The result of this investigation, therefore, suggests to further research on the reason behind the difference of shoot multiplication efficiency between rhizome tip and rhizome bud explants.

The effect of different concentrations of BA and Kn singly or in combination with NAA and IBA on shoot proliferation is presented in Table 1. All the treatments resulted in variable responses to induce multiple shoots from rhizome tip explants. Among the 22 different concentrations and combinations of plant growth regulators except control treatment where a little proliferation had been observed, 8.0 µM BA together with 1.0 µM NAA showed a better response than any other treatments in terms of percentage of explant showed proliferation (99.8 ± 1.87 %) as well as average number of shoots per culture (10.6 ± 0.26) (Fig. 2. D). In addition, bud break was noticed within 10-12 days of culture in this media formulation (Fig. 2. C). A comparatively lower response was recorded when explants cultured on cytokinin alone or in combination with IBA. In the present study, higher NAA concentrations than 1.0 µM reduced the shoot numbers, length of shoots as well as percentage of response. The results revealed that NAA in lower concentration in combination with BA can increase shoot multiplication in *C. amada*, which is in agreement with a report on propagation of *C. amada* by Prakash *et al.* (2004) who found 4.44 µM BA + 1.08 µM NAA as optimal combination for multiple shoot induction from rhizome explants of *C. amada*. In addition, these results are also supported by several reports on zingiberaceous plant propagation (Kambaska *et al.*, 2010; Chougule, 2008; Islam *et al.*, 2004; Hoque *et al.*, 1999; Hosoki

et al., 1977; Bharalee *et al*., 2005 and Noguchi *et al*., 1988). However, Stanly and Keng (2007) found IBA as low as 0.5 mg/l along with BA as suitable for the induction of multiple shoots from in *C. zedoaria* and *Z. zerumbet*. Balachandran *et al.* (1990) reported that *C. domestica* could produce 3.4 shoots per explant, *C. caesia* produced 2.8 shoots per explant and *C. aeruginosa* produced 2.7 shoots per explant using MS medium supplemented with 3 mg/l BA alone. Nayak (2000) reported MS medium supplemented with 5 mg/l BAP alone was most effective for shoot multiplication of *C. aromatica* producing an average of 3.3 shoots per explant.

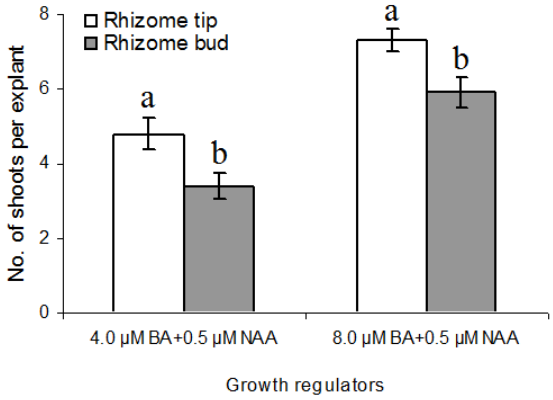

Fig. 1. Effect of explant type on *in vitro* shoot multiplication of *C. amada* Roxb. Mean number of shoots produced in the same growth regulator combination followed by different letter(s) are significantly different (t-test).

To induce rooting, elongated shoots were cultured on MS medium augmented with two types of auxins viz., NAA (2.0, 4.0, 6.0, 8.0 µM) and IBA (2.0, 4.0, 6.0, 8.0 µM) individually. A control group was also maintained. Among the two different auxins tested, percentage of response, number of roots and root length varied. Plantlets significantly developed lengthy roots and root induction was strengthened within 25 days of culture. IBA was found to be more adequate than NAA. Roots were visible within 5 - 10 days following transfer of elongated shoots to the rooting medium. After 2 weeks, the plantlets had developed the primary and secondary root system. The frequency of rhizogenesis was almost 98%. In agreement, IBA was reported as potential auxin for rooting in *C. aromatica* (Nayak, 2000), *C. longa* (Meenakshi *et al*., 2001; Ali *et al*., 2004; Rahman *et al.,* 2004; Nasirujjaman *et al*., 2005), *C. zedoria* (Chan and Thong, 2004; Bharalee *et al*., 2005), *Zingiber officinale* (Dogra *et al*., 1994; Pandey *et al*., 1997; Hoque *et al*., 1999; Sunitibala *et al*., 2001) and many other plant species. The average number of roots per shoot ranged from 10-11 and the maximum root length observed was 12.7 cm in 4.0 µM BA containing MS medium (Fig. 2. E). Increasing the concentration of IBA to over 4.0 µM gradually led to a decrease in the frequency of root regeneration, number of roots and root length. However, NAA formed slender roots. In contradiction to our report, NAA was reported as best for rooting in *C. longa* (Salvi *et al*., 2000 & 2001) and in *Z. officinale* (Inden *et al*., 2003). Shoots also produced roots when transferred to basal medium containing no growth regulators, but the rooting was higher with IBA (2.0 - 8.0 µM).

Fig. 2. *In vitro* propagation of *Curcuma amada* Roxb. using rhizome explant: Excised rhizome tip and rhizome bud, respectively (A & B); Shoot initiation from rhizome tip after 10 days of culture (C); Multiple shoots after 6 weeks of culture (D); *In vitro* rooting (E); Plantlets in plastic pots after hardening (F).

The successfully rooted plantlets were transferred to plastic cups containing sterile garden soil and compost (2:2) for hardening. Plantlets were initially maintained in the culture room (25 ± 2°C) conditions for 4 weeks and thereafter transferred to normal laboratory conditions for about 2 more weeks. Finally the plantlets were transferred to the

Botanical Evaluation Garden and maintained there. The initial growth rates of plant height were 14.9 to 25.3 cm during the first 4 weeks of acclimatization (Data not shown). However, in the following 2-3 weeks, substantial increases in plant height were observed (Fig. 2. F).

Table 1. Effect of plant growth regulators on shoot multiplication from rhizome tip explants of *C. amada* Roxb.

Plant growth regulators (µM)	% of explants showed proliferation (Mean ± SE)	Average No. of shoot/culture (Mean ± SE)
0.0	33.9 ± 1.17^{i}	1.09 ± 0.81^{l}
BA		
2.0	73.1 ± 1.29^{h}	3.8 ± 0.31^{jk}
4.0	81.5 ± 1.23^{g}	5.3 ± 0.29^{ghijk}
6.0	87.5 ± 1.17^{ef}	6.2 ± 0.34^{efghi}
8.0	98.2 ± 0.98^{ab}	7.6 ± 0.34^{bcdef}
10.0	93.6 ± 0.91^{bcd}	6.1 ± 0.33^{efghi}
Kn		
2.0	66.1 ± 1.28^{i}	3.4 ± 0.37^{k}
4.0	74.1 ± 1.18^{h}	3.9 ± 0.31^{jk}
6.0	81.5 ± 1.13^{g}	4.5 ± 0.26^{ijk}
8.0	86.2 ± 1.10^{fg}	4.7 ± 0.26^{hijk}
10.0	93.3 ± 1.08^{bcd}	5.7 ± 0.20^{fghij}
BA+NAA		
8.0+1.0	99.8 ± 1.87^{a}	10.6 ± 0.26^{a}
8.0+1.5	92.9 ± 1.12^{cd}	9.7 ± 0.46^{ab}
8.0+2.0	85.4 ± 1.80^{fg}	9.1 ± 0.24^{abcd}
BA+IBA		
8.0+1.0	96.8 ± 1.21^{abc}	9.2 ± 0.32^{abcd}
8.0+1.5	91.2 ± 1.03^{de}	8.7 ± 0.51^{abcd}
8.0+2.0	85.7 ± 1.37^{fg}	7.8 ± 0.51^{bcdef}
Kn+NAA		
10.0+1.0	86.3 ± 1.17^{fg}	8.7 ± 0.26^{abcd}
10.0+1.5	96.8 ± 1.16^{abc}	9.3 ± 0.55^{abc}
10.0+2.0	95.4 ± 1.09^{abcd}	8.6 ± 0.51^{abcd}
Kn+IBA		
10.0+1.0	84.1 ± 1.08^{fg}	7.1 ± 0.27^{cdefg}
10.0+1.5	92.5 ± 1.19^{cd}	8.3 ± 0.21^{abcde}
10.0+2.0	83.8 ± 1.27^{fg}	6.9 ± 0.23^{defgh}

Values represent means ± standard error of 15 explants per treatment in three repeated experiments. Means in a column with the same letter are not significantly different by Duncan's multiple Range Test at 5% significant level.

The survival rate was 80% upon hardening and acclimatization under the natural field condition. Initially, two to three healthy leaves were developed adjacent to the main shoot. The stem was very slender without branching and growth of minute hairs on the stem and on the leaf underside was observed. The number of leaves increased after 8-10 weeks of acclimatization. There was no detectable variation among the acclimatized plantlets with respect to morphological and growth characteristics. All micropropagated plants were free of external defects and morphologically similar to the mother plant.

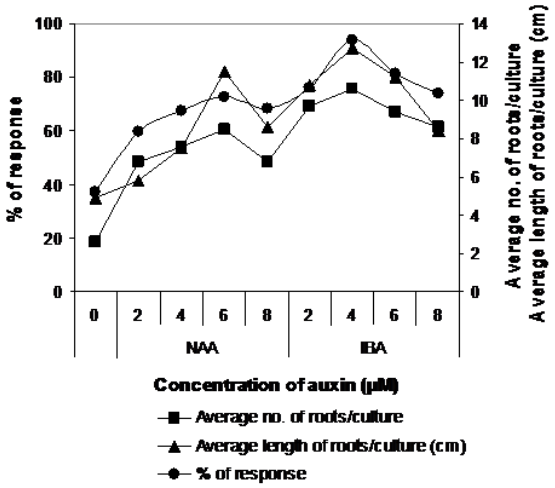

Fig. 3. Effect of auxins (NAA & IBA) on rooting of *in vitro*-grown shoots of *C. amada* Roxb.

In the present investigation the *in vitro* micro-plantlet multiplication system of *C. amada* has been optimized through rhizome tip culture. The described protocol is the first report for this species which can be used to produce a higher amount of propagules around the year. Production of *in vitro* plantlets would be a suitable methodology for direct regeneration of shoot lets as a source of disease free quality planting material that could be stored and transported easily and a step forward towards commercial scale of propagule production in mango ginger.

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