Research in Plant Biology, 5(6): 09-13, 2015 ISSN : 2231-5101
www.resplantbiol.com

Short Communication

Efficient *in vitro* regeneration of pathogen free mango ginger (*Curcuma amada* Roxb.) from rhizome bud explants

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***Curcuma amada* is an aromatic and medicinal plant belonging to Zingiberaceae. Like all other Zingiberous crops, the plant is propagated vegetatively by rhizome for which the pathogen is easily transmitted by the planting material and lack of seed set hinders breeding efforts by conventional techniques. *In vitro* regeneration techniques can be used to overcome this problem. Reports on production of disease free clones of *Curcuma amada* is lacking. So, the present study was designed. Young rhizome sprouts were cultured in Murashige and Skoog basal medium supplemented with BAP and Kinetin. BAP gave better results compared to kinetin. Combinations of cytokinins were more potential in regeneration rather than when they were used alone. Moderate rooting were observed in all the cultures. The regenerated plants showed a survival percentage of 92 % in poly cups and 98 % in field. The plantlets produced were found disease free.**

Key words: BAP, *Curcuma amada*, disease free, hardening, Kinetin, rhizome sprouts

Mango ginger (*C. amada* Roxb.) is a Zingiberous rhizomatous aromatic herb with a leafy tuft and 60-90 cm in height. It is cultivated in Gujarat and found wild in parts of West Bengal, U. P, Karnataka and Tamil Nadu. The common English name, mango ginger is due to its characteristic odour similar to raw mangoes (*Mangifera indica* L.). The essential oil contains a-pinene, a-and b-curcumene, camphor, cuminyl alcohol, myristic acid and turmerone. Car-3-ene and cis-ocimene contribute the characteristic mango odour of the rhizome. Rhizomes yield 1% essential oil containing d-a -pinene 18%, ocimene 47.2%, linalool 11.2%, linalyl acetate 9.1% and safrole 9.3% (Chopra *et al.*, 1980). The plant used as major ingredient in the pickles, candies, salads, sauces and chutneys (Mridula & Jayachandran, 2001). The rhizomes of this plant are useful in anorexia, dyspepsia, flatulence, colic, bruises, wounds, chronic ulcers, skin diseases, fever, constipations, cough, bronchitis, sprains, gout, inflammations (Warrier *et al.*, 1994) and for abdominal pain (Kumar & Bharati, 2014). The rhizomes are used externally in the form of paste as an application for bruises and skin diseases generally combined with other medicines. Tubers rubbed with the leaf-juice of *Caesalpinia bonduc* is given for worms (Nadkarni, 1982). Rhizome is also used in applications over contusions and sprains (Chopra *et al.*, 1980).

Mango ginger is propagated vegetatively by rhizome for which the pathogen is easily transmitted by the planting material. The planting materials of Zingiberaceae are susceptible to soft rot disease (Balachandran *et al*., 1990) and have a very low multiplication rate (Prakash *et al*., 2004). Hence, huge amounts of planting material are required every year for plantation purposes (Barthakur & Bordoloi, 1992). In addition, lack of seed set in this species hinders breeding efforts by conventional techniques (Sit *et al*., 2005; Bhattacharya and Sen, 2006; Prakash *et al*., 2004). So, *in vitro* regeneration techniques are important. *In vitro* regeneration of mango ginger (Barthakur & Bordoloi, 1992; Prakash *et al*., 2004) has been reported. To our knowledge there are no reports on disease free clones of *Curcuma amada.* In the present communication we report a rapid *in vitro* regeneration technique of disease free clones.

Materials and methods

Collection of plant materials and culture conditions

Matured rhizomes of *Curcuma amada* were collected from a medicinal plant grower of Jalpaiguri (West Bengal) and were washed under running tap water to remove the soil from the surface. They were treated with Diathane M 45 (0.1% in water) and were kept on trays filled with sand for germination. The rhizomes sprouted within one month and the sprouts were used as explants. The young sprouts were washed in running water to remove the soil of the germinating tray and dipped in 1% Extran for 10 minutes. They were washed several times with double distilled water. The explants were taken to laminar air flow cabinet and were dipped in mercuric chloride solution of 0.5% (w/v) for 10 minutes**.** The treated explants were washed several times with sterile double distilled water and dipped in 70% Ethyl alcohol for 1 minute. The traces of Ethyl alcohol were removed by washing them with sterile double distilled water for 5 times. The open ends of the explants were cut off and were inoculated on the culture media.

Murashige and Skoog (M.S.) basal medium (Murashige & Skoog, 1962) was used for the present study. Sucrose was used as the carbon source at the rate of 30 mg/l in all the experiments. The medium was supplemented with cytokines like benzyl amino purine (BAP) and kinetin at different concentrations and combinations. For solidifying the culture media, agar was used at a concentration of 8 mg/l. The pH was adjusted to 5.7 ± 0.1. The media was sterilized at 121ºC for 20 minutes at 1.08 Kg/cm^2 pressure. Filter sterilized vitamins and growth regulators were added to the media after autoclaving. The culture vessels after inoculation were incubated at 25 ± 2ºC and were given a photoperiod of 16 hours with a light intensity of 2000-2500 Lux provided by cool white fluorescent tubular lamps.

Hardening of plantlets

Healthy, *in vitro* regenerated plants with good number of roots were selected for hardening. The plantlets were taken out and washed carefully to remove all traces of agar sticking to the roots. They were then transplanted into plastic cups containing a mixture of garden soil and sand (1:1). They were kept covered with plastic bags with holes to provide 60-70% relative humidity. The hardened plants were transferred to the fields of Jalpaiguri for trial.

Detection of pathogen in regenerated plants

Two diagnostic tests were performed to detect the presence or absence of the pathogen.

1. Rhizome pieces of these clones were transferred to potato dextrose agar (PDA) and observed for 8 – 10 days for fungal growth on the medium.
2. Rhizomes harvested from the tissue culture-derived plants were stored in river sand, and the number of rotted and healthy

rhizomes was recorded after 6 months of storage.

Results and discussion

Establishment of culture

The explants responded within 21-25 days (Figure 1a & b). In the primary culture 30 and 45 days were required respectively for shoot and root initiation. Surface sterilization with 0.5% mercuric chloride for 10 minutes followed by 70% ethanol for 1 minute was the most preferred sterilization technique. The number of days required for shoot and root initiation increased with the increase in the concentration of mercuric chloride. In all the cases the number of days required for shoot and root initiation increased with the increase in the concentration and duration of mercuric chloride treatment. This may be due to cell death of explants during surface sterilization with higher concentrations of mercuric chloride. Similar results were obtained by Bhattacharya & Sen, 2013 in micropropagation of *Kaempferia galanga*.

Plant regeneration

In the cultures of *C. amada*, significant variations were observed in the results obtained by supplementing the media with different cytokinins (Table-1; Figure 1c & d). BAP gave better results compared to kinetin. In media supplemented with BAP, the maximum numbers of plantlets/explant (3.37 ± 0.18) at a concentration of 4 mg/l while the maximum height (4.43 ± 0.19 cm) was found with 3 mg/l. In the media supplemented with kinetin the maximum numbers of plantlets/explant (2.97 ± 0.14) were observed at 4 mg/l and the maximum height (2.77 cm) at 4 mg/l. Rooting were observed in all the cultures. Lower concentrations of cytokinins produced less number of shoots from the explants while it declined above a critical level. In some secondary cultures callusing of the explants was observed.

Table 1.Effect of cytokinin on regeneration of *Curcuma amada*

BAP mg/l	Kinetin mg/l	Shoot / explants	Height(Cm) / shoot
1		2.02 ± 0.17	2.42 ± 0.32
2		2.31 ± 0.11	3.23 ± 0.09
3		2.83 ± 0.15	4.43 ± 0.19
4		3.37 ± 0.18	3.77 ± 0.24
5		3.22 ± 0.10	3.46 ± 0.11
	1	1.93 ± 0.12	1.96 ± 0.12
	2	2.28 ± 0.31	2.32 ± 0.10
	3	2.66 ± 0.12	2.34 ± 0.18
	4	2.97 ± 0.14	2.77 ± 0.14
	5	2.45 ± 0.17	2.76 ± 0.14
1	1	2.62 ± 0.19	0.92 ± 0.21
1	2	2.87 ± 0.22	1.53 ± 0.26
1	3	3.48 ± 0.12	2.29 ± 0.31
2	1	3.11 ± 0.21	4.44 ± 0.19
2	2	4.41 ± 0.31	5.58 ± 0.22
2	3	4.68 ± 0.11	6.98 ± 0.10
3	1	4.89 ± 0.16	6.34 ± 0.34
3	2	5.74 ± 0.13	7.42 ± 0.15
3	3	6.06 ± 0.23	8.33 ± 0.25
4	1	5.32 ± 0.22	5.59 ± 0.28
4	2	5.61 ± 0.28	6.21 ± 0.13
4	3	5.37 ± 0.18	7.25 ± 0.19

Data ± standard deviation was taken 12 weeks following inoculation. All treatments had 5 replicates and were repeated thrice.

The regeneration of plantlets varied considerably with different combinations of BAP and kinetin. The maximum numbers of plantlets (6.06 ± 0.23) and maximum height (8.33 ± 0.25) were obtained in the media supplemented with 3 mg/l BAP + 3 mg/l kinetin. Regeneration of plantlets gradually increased with increase in hormone concentrations while it declined beyond 3 mg/l BAP + 3 mg/l kinetin. Among the cytokinins tried, BAP gave better results compared to kinetin. BAP was found to be very much effective in the generation of ginger (Sit *et al.,* 2005 and Bhattacharya & Sen, 2006) and *Kaempferia galanga* (Bhattacharya & Sen, 2013) tissue culture. Combinations of cytokinins were more potential in regeneration rather than when they were used alone. Moderate rooting were

observed in all the cultures which indicates that plant growth regulators does not have any influence on rooting. Lower concentrations of cytokinins produced less number of shoots from the explants while it declined above a critical level. This decline in the rate of shooting may be due to some inhibitory effect produced by higher concentrations of cytokinins. Similar results showing gradual increase and decline of the number of shoots after a certain level of cytokinins was observed in ginger tissue culture (Balachandran *et al.*, 1990 and Sit *et al.*, 2005). Regeneration of plantlets gradually increased with increase in hormone concentrations while it declined beyond a critical level. Combinations of cytokinins were more potential in regeneration rather than when they were used alone.

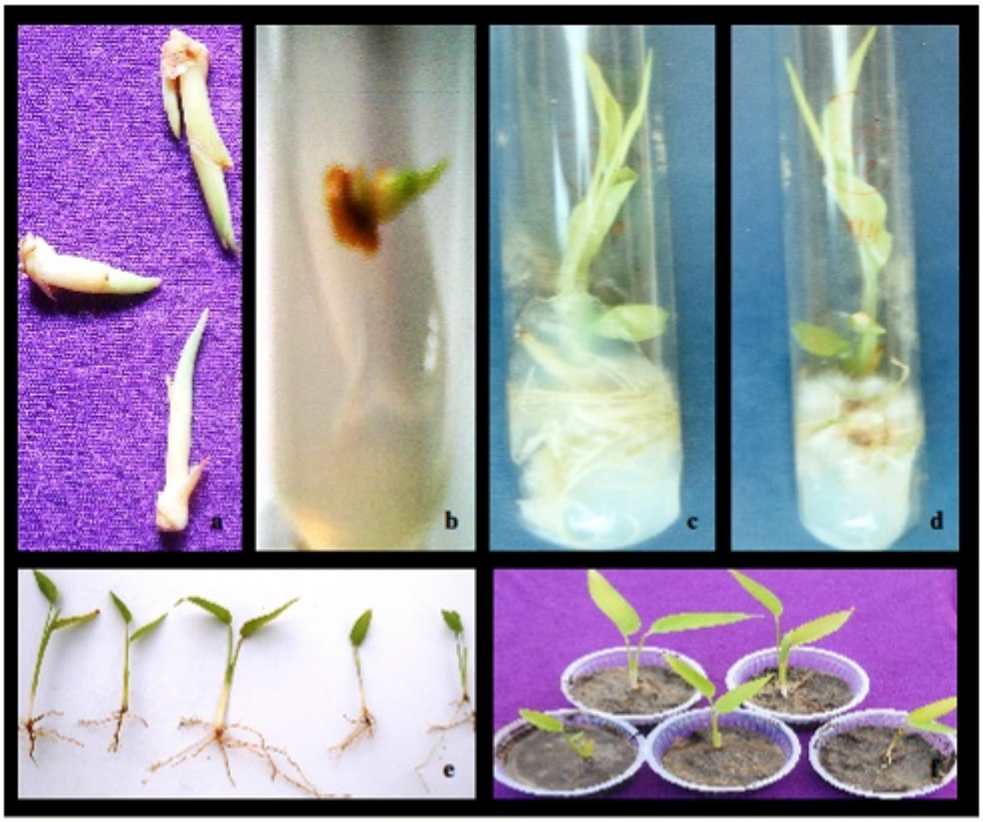

Figure: 2. a. Explants, b. *In vitro* sprouting of the explants, c. Regeneration in medium supplemented with Kinetin, d. Regeneration in medium supplemented with BAP, e. *In vitro* rooting and f. Hardening of plantlets.

Hardening of plantlets

The ultimate success of *in vitro* propagation lies in the successful establishment of plant in the soil. The regenerated plantlets of *Curcuma amada* after hardening were transferred to garden after one month (Figure 1e & f). The regenerated plants showed a survival percentage of 92 % in poly cups (green house) and 98 % in field (environment).

Detection of pathogen in regenerated plant

Rhizome pieces of micropropagated plants were transferred onto PDA to examine the infection, if any. Mycelia growths were not observed on medium after 8 – 10 days of incubation while 26 % of the pieces of

conventional rhizomes showed mycelia growth. Rhizomes obtained from the tissue culture-derived plants stored in sand for 3 months also did not show any rotting of rhizomes.

Conclusion

The high regeneration rate, shooting and rooting in the same media along with very high survival percentage indicates that this method could easily be adopted for *in vitro* regeneration of *Curcuma amada.* This protocol has the ability of detecting the presence of pathogen in the regenerated plantlets.

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