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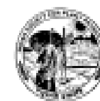

RAPID *IN VITRO* MULTIPLICATION OF DISEASE-FREE *ZINGIBER OFFICINALE* ROSC.

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SUMMARY

Ginger belonging to the family Zingiberaceae is a rhizomatous medicinal spice. Biotechnological improvement of ginger is important due to its lack of flowering and seed set. Direct *in vitro* regeneration of disease-free plantlets was achieved through tissue culture. Different media supplemented with different concentrations and combinations of cytokinins were studied. Murashige and Skoog media supplemented with 4 mg/l benzyl amino purine (BAP) provided the best regeneration compared to kinetin (kn) and zeatin (Zn) when they were used alone. Combination of 4 mg/l BAP and 3 mg/l Kn resulted in maximum number of shoots. Profuse rooting was observed in the same media. Hardenings of the healthy plantlets were done in mixture of garden soil and sand in the proportion of 1:1. Ninety four per cent of the plantlets survived hardening and all the plantlets got established in the field. Diagnostic tests- rhizome pieces were transferred to PDA to observe fungal growth on the medium, visual observations on the presence of ginger yellows symptoms and detection of the number of rotted rhizomes after storage on river sand were performed to detect the presence or absence of the pathogen in tissue culture-derived clones. Comparative studies of *in vitro* regenerated and conventional planting materials were performed in field. Superiority of the *in vitro* plantlets was established.

***Key words*: Clonal propagation, cytokinin, disease free, ginger, plant regeneration**

INTRODUCTION

Ginger (Z*ingiber officinale* Rosc. Cv. Gorubathan) of the family Zingiberaceae is a rhizomatous spice used all over the world in alternative systems of medicine. The whole part of the plant is aromatic, but it is the underground rhizome, fresh or preserved, that are the valuable commodities. It is rich in secondary metabolites such as oleoresin (Baghyalaxmi and Singh 1988). Heavy losses in yield of this variety of ginger have been reported due to bacterial wilt (caused by *Pseudomonas solanacearum*), soft rot (*Pythium aphanidermatum*) and nematodes (*Meloidogyne* spp.) (Hosoki and Sagawa 1977, de Lange *et al.* 1987). In India, rhizome rot and yellows caused by *Fusarium oxysporum* f. sp. *zingiberi* is a serious threat to this crop during storage and under field conditions. Breeding of disease free ginger is seriously handicapped by poor flowering and seed set. Most crop improvement programmes were confined to evaluation and selection of naturally occurring variations. *In vitro* culture techniques provide an alternative means of plant propagation and a tool for crop improvement (Vasil 1988). Rhizomes mainly transmit the disease so; the production of disease-free clones with a rapid multiplication rate is necessary for the successful cultivation of this crop.

To our knowledge there is no detailed report on the production of ginger clones of this variety free from *F. oxysporum* f. sp. *zingiberi* and the performance of

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tissue culture-derived plantlets under field conditions. Some workers have reported *in vitro* regeneration of ginger by using different plant parts (Hosoki and Sagawa 1977, Pillai and Kumar 1982, de Lange *et al.* 1987, Vasil 1988, Wang 1989, Nirmal Babu *et al.* 1992, Rout *et al.* 1998) but the effect of different combinations of cytokinins were never assessed. In the present communication we report the rapid *in vitro* multiplication of disease-free clones of ginger, the high-frequency establishment of tissue-culture plants in a simple potting mixture and their performance under field conditions and the control of the storage rot of rhizomes.

MATERIALS AND METHODS

Zingiber officinale Rosc. cv. Gorubathan was used for the present study. The rhizomes of the plant material were collected from the fields of Gorubathan (West Bengal). The rhizomes of *Z. officinale* Rosc. were washed under running tap water to remove the soil from the surface. They were treated with Diathane M 45 (1% in water) and were kept on trays filled with sand for germination. They sprouted within 2-4 weeks 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 min. They were washed several times with double distilled water. The explants were taken to laminar air flow cabinet and were dipped in autoclaved solution of 0.2 % (w/v) mercuric chloride for 10 min**.** The treated explants were washed several times with sterile double distilled water and dipped in 70% ethyl alcohol for 1 min. The traces of ethyl alcohol were removed by washing them 5 times with sterile double distilled water. The open ends of the explants were cut off and were inoculated on the culture media.

Murashige and Skoog (Murashige and Skoog 1962) basal media and Gamborg B5 (Gamborg *et al.* 1968) media was used for the present study. Cytokines like BAP, Kn and Zn at different concentrations and combinations were experimented. Sucrose was used as the carbon source at the rate of 30 mg/l in the experiments to access the effect of phytohormones. Sucrose (10-40 g/l) was added to the culture media to find out the ideal percentage of it in regeneration of ginger. For solidifying all the culture media, agar was used at a concentration of 8 mg/l. The pH of the media was adjusted to 5.7 ± 0.1. The media were sterilized at 121 °C for 20 min at 1.08 kg/cm^2 pressure. The filter sterilized vitamins and growth regulators were added to the media and they were poured to culture vessels. The culture vessels were inoculated and incubated at 25 ± 2°C providing a photoperiod of 16 h with a light intensity of 2000-2500 Lux provided by cool white fluorescent tubular lamps.

After the regeneration, healthy *in vitro* plantlets 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. The individual plantlets were separated from each other and were then transplanted into polycups 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. Subsequently they were transferred to the garden and planted in the field after one month.

Three diagnostic tests were performed to detect the presence or absence of the pathogen, *F. oxysporum* f. sp. *zingiberi* (causal agent of rhizome rot) in tissue culture-derived clones: (1) rhizome pieces of these clones were transferred to PDA and observed for 8 – 10 days for fungal growth on the medium; (2) visual observations on the presence of ginger yellows symptoms were recorded throughout the growing season; (3) rhizomes harvested from the tissue culture-derived plants were stored in river sand, and the number of rotted and healthy rhizomes were recorded after 6 months of storage. Micropropagated clones along with seed rhizomes containing a single bud from field-grown plants were planted. Data on disease infestation per cent; height of plants, number of shoots per plant and number of leaves per shoot; number of primary fingers and secondary fingers and yield (g/plant) of both tissue culture grown clones and non- tissue culture grown clones were recorded.

RESULTS AND DISCUSSION

The explants proliferated within 2-3 weeks. Twenty eight per cent of explant buds were discarded after 21 days of culture because of bacterial and fungal contamination. Surface sterilization with 0.2 % mercuric

chloride for 10 min followed by 70% ethanol for 1 min showed extremely low rate of contamination. Early sprouting was observed at lower concentration of mercuric chloride but the percentage of contamination was high (Hosoki and Sagawa 1977) also reported a high degree of contamination in *in vitro* cultures of ginger after sterilizing with 0.5% (v/v) sodium hypochlorite for 10 min. In the present study, once the contamination-free cultures of buds were established, these were easily maintained by subculturing on fresh medium.

Regeneration (Fig. 1) of plantlets varied with the medium (Table 1). Better regeneration was observed in the Murashige and Skoog (MS) media when compared to the Gamborg (B5) media using BAP at different concentrations. MS media supplemented with 4 mg/l BAP showed the maximum rate of shooting (8.33) and rooting (20.06) per explant. In the B5 media, shooting (4.67) and rooting (3.00) rate was maximum at 4 mg/l BAP. This indicates that there must be some requirements for the plant which is not available in B5 media.

Table 1. Effect of media on the regeneration of ginger. Data (mean ± se) was taken 12 weeks following inoculation. All treatments had 5 replicates and were repeated thrice. (Both the media contained 30g/l sucrose)

BAP (mg/l)	No. of shoots	No. of roots
	Murashige & Skoog medium	
1	2.73±0.25	5.87±0.25
2	4.66±0.05	8.40±0.33
3	6.26±0.57	13.20±1.18
4	8.33±0.41	20.06±1.22
5	6.50±0.34	18.21±1.17
	Gamborg B5 medium	
1	1.45±0.25	3.47±0.47
2	2.40±0.33	5.80±0.28
3	3.20±0.16	4.73±0.25
4	4.67±0.25	3.00±0.43
5	3.47±0.34	2.40±0.33

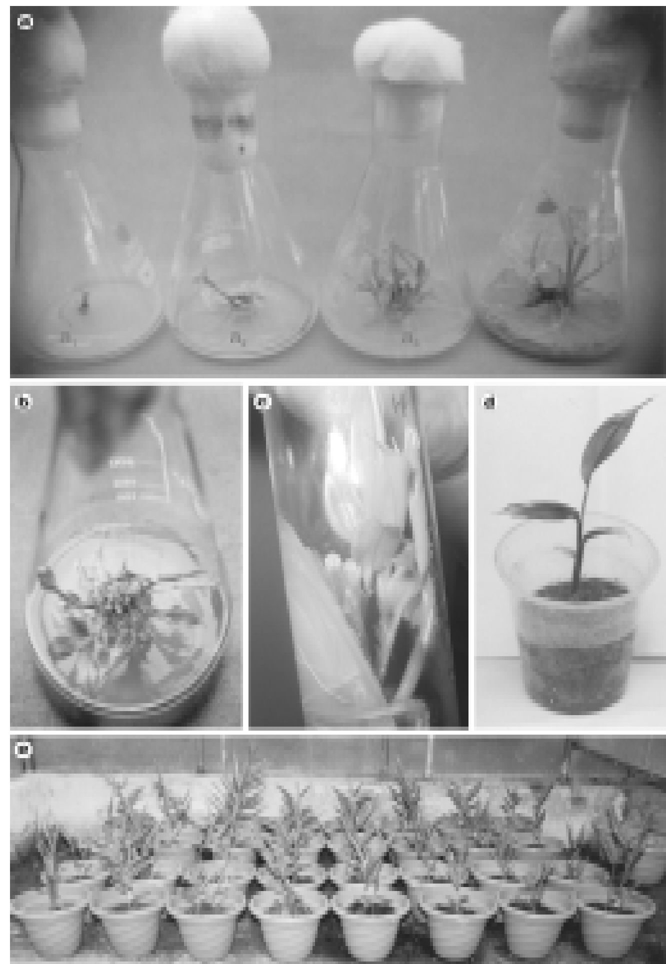

Fig. 1. a.Effect of regeneration by different % of sucrose, b. multiple shoot regeneration, c. rooting of plantlets, d. hardening of plantlet, e. hardened plantlets ready for field transfer

Regenerated plantlets in different concentrations of BAP were subcultured in MS media supplemented with different percentage of sucrose and BAP. It was observed that the quantity of sucrose in the media has a profound effect on the rate of regeneration as well as on the growth of the plantlets (Table 2). Maximum regeneration was observed in the media supplemented with 3% sucrose and 4 mg/l BAP (8.33) followed by 3 mg/l BAP (6.26). The regeneration and growth was extremely low in the media supplemented with 1% sucrose supplemented with 3 mg/l (1.53 shoot/explant and 1.73 cm height) and 4 mg/l (2.06 shoot/explant and 1.15 cm height) BAP, while in the media supplemented with 3% sucrose with 3 mg/l (6.26 shoot/explant and 4.61 cm height) and 4 mg/l (8.33 shoot/explant and 5.78 cm

Table 2. Effect of cytokinin on the regeneration of ginger. Data (mean ± se) was taken 12 weeks following inoculation. All treatments had 5 replicates and were repeated thrice. (M.S. media were used in all the cases)

Hormones (mg/l)	Regenerated plantlets	
	Shoot number	Shoot height (cm)
BAP		
1	2.73±0.25	2.51±0.26
2	4.66±0.50	3.55±0.27
3	6.26±0.57	4.61±0.13
4	8.33±0.41	5.78±0.17
5	6.50±0.34	6.90±0.54
Kinetin		
1	1.86±0.34	2.11±0.28
2	2.66±0.34	3.19±0.14
3	3.73±0.25	4.20±0.10
4	5.40±0.25	6.06±0.38
5	4.86±0.34	4.99±0.20
Zeatin		
1	1.93±0.34	1.15±0.15
2	2.46±0.25	2.13±0.19
3	3.80±0.49	2.97±0.21
4	5.33±0.10	3.87±0.07
5	7.20±0.65	4.67±0.06
BAP + Kinetin		
1 + 1	2.13±0.25	1.99±0.13
1 + 2	3.06±0.41	3.56±0.35
1 + 3	5.33±0.34	5.78±0.55
1 + 4	5.66±0.41	6.27±0.40
1 + 5	5.93±0.41	6.84±0.32
2 + 1	3.26±0.25	2.72±0.12
2 + 2	5.73±0.52	3.55±0.12
2 + 3	7.13±0.25	5.98±0.16
2 + 4	6.93±0.47	7.02±0.20
2 + 5	7.26±0.10	8.16±0.31
3 + 1	5.87±0.19	4.04±0.07
3 + 2	7.13±0.23	4.38±0.50
3 + 3	7.40±0.43	7.07±0.69
3 + 4	8.53±0.41	8.38±0.13
3 + 5	6.80±0.43	7.35±0.10
4 + 1	8.40±0.30	4.59±0.22
4 + 2	8.94±0.57	7.53±0.28
4 + 3	9.60±0.59	8.59±0.19
4 + 4	7.40±0.16	7.09±0.61

height) BAP was high. This indicates that the percentage of sucrose has substantial effect along with the concentration of plant hormones. The plantlets turned pale or white in the media supplemented with 1% and 2 % sucrose, this may be due to deficiency of elementary carbon.

Different cytokinins like BAP, Kn and Zn were added to the media to observe their effects on regeneration. It has been observed that the growth regulators elicit different responses. Among the cytokinins tried, BAP gave better results in terms of plantlets regenerated and plantlet height compared to Kn and Zn (Table 2). The maximum numbers of plantlets/explant (8.33) were observed in the media supplemented with BAP at a concentration of 4 mg/l while the maximum height (6.90 cm) was found with BAP at a concentration of 5 mg/l. In the media supplemented with Kn the maximum numbers of plantlets/explant (5.40) were observed at 4 mg/l and the maximum height (6.06 cm) at 4 mg/l. The highest numbers of plantlets/explant (7.20) regenerated with Zn were at 5 mg/l whereas the maximum height (4.67 cm) was at Zn 4 mg/l. Moderate to profuse rooting was observed in all the cultures. The high effectiveness of BAP was observed in turmeric tissue culture (Rahman *et al.* 2004). Lower concentrations of cytokinin 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.

The regeneration of plantlets varied considerably with different combinations of BAP and Kn (Table 3). The maximum number of plantlets/explant were obtained in the media supplemented with 4 mg/l BAP + 3 mg/l Kn (9.60) followed by 4 mg/l BAP +2 mg/l Kn (8.94), while the maximum plantlet height was obtained in the media supplemented with 4 mg/l BAP + 3 mg/l Kn (8.59) followed by 3 mg/l BAP + 4 mg/l Kn (8.38). Regeneration of plantlets gradually increased with increase in hormone concentrations while it declined above the combined concentrations of 7 mg/l. Plantlet regeneration was relatively less in the media supplemented with the combinations of low BAP and Kn. The plantlets rooted in the same medium and they were moderate to profuse in most of the combinations.

Similar results were observed in ginger tissue culture (Khatun *et al*. 2003). The rate of rooting was found to be proportional to the number of plantlets. It has been clearly observed that high BAP: Kn is more effective than high Kn: BAP and combinations of growth regulators work better than when they are used alone.

The ultimate success of *in vitro* propagation lies in the successful establishment of plant in the soil. *In vitro*-derived plantlets of ginger with a well-developed root and shoot system were successfully transferred to pots in potting mixture, The regenerated plantlets of ginger after hardening were transferred to the field. The regenerated plants showed a survival percentage of 94% and 100% in the polycups and field respectively.

The different morphological and yield parameters were studied in course of the experiment and wide variations were observed in the plants obtained from different sources (Fig. 2). This decrease in disease infestation may be due to *in vitro* and field selection of the tissue cultured plantlets. The yield per plant was 0.287 g for tissue culture derived plant and 0.142 g for plants propagated through conventional rhizomes, i.e. the increase of yield was 2-fold. The increases of yield of the tissue cultured plants were due to less susceptibility to pathogens and more number of shoots.

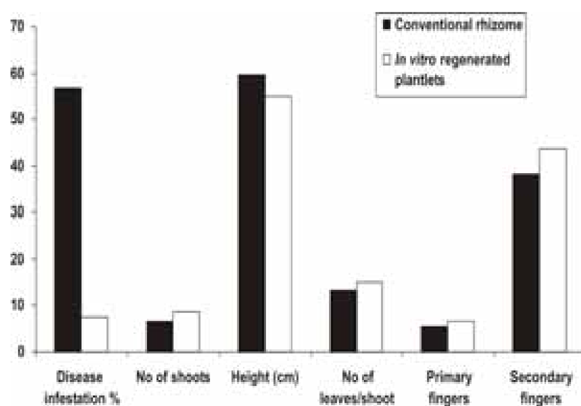

Fig. 2. Comparative data of plants from conventional and *in vitro* regenerated planting materials

In vitro-derived plants raised under field conditions did not show yellows symptoms until maturity. Rhizomes obtained from the tissue culture-derived plants stored in sand for 6 months also did not show any rotting of rhizomes due to *F. oxysporum* f. sp. *zingiberi* infection, whereas about 61% of the rhizomes of mother-derived plants rotted during storage. Rhizome pieces of micropropagated plants were transferred onto PDA to examine the infection, if any, of *F. oxysporum* f. sp. *zingiberi*. Mycelial growths were not observed on medium after 8 – 10 days of incubation while 79% of the pieces of conventional rhizomes showed mycelial growth.

This direct *in vitro* regeneration technique has the potential to produce huge number of disease-free somaclones from the desired cultivar within a very short time. The high regeneration rate and survival percentage indicates that this method could easily be adopted for *in vitro* regeneration of superior quality planting material.

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