A Novel Approach of Bacteria-free Rhizome Production of Ginger through Biotechnology

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Keywords: meristem culture, multiplication, rhizome production, *Zingiber officinale* Rosc.

Abstract

Ginger buds were sterilized and cultured on Murashige and Skoog (MS, 1962) medium at 29±1 °C air temperature in the dark condition for 1 month. The meristems of developed shoots were cut into 0.15±0.05, 0.35±0.05, 0.55±0.05, and 0.75±0.05 mm in diameter and cultured on MS medium supplemented with 15 % coconut water for rapid screening of bacterial infection. The bacteria-free shoots were multiplied on MS medium supplemented with 0, 2, 4, and 6 mg l^{-1} N^6-Banzylamine (BA) or 0, 2, 4, and 6 mg L^{-1} 6-(γ-dimethylallylamino) purine (2iP) at 25±1 °C air temperature for 5 weeks. The ginger plantlets were transferred to ex-vitro and produced the bacteria-free rhizome. The bacteria-free rhizomes were compared with those of rhizome produced through conventional method in the commercial scale. The lowest bacterial infection of meristem was observed in the shoots cut into 0.15±0.05 mm in diameter. The shoots multiplied on the medium supplemented with 4 mg L^{-1} BA were 1.9 times higher when compared with those multiplied on the medium supplemented without. The vigorous growth, high survival percentage and high yield were observed in the ginger plants produced through bacteria-free rhizome.

INTRODUCTION

Ginger (*Zingiber officinale* Rosc.), an herbaceous perennial usually grown as an annual, is produced mostly in Thailand, India, China, Taiwan, Australia, and Nigeria. It has been an important tropical horticultural plant valued all over the world as a spice crop and medicinal properties (Kacker et al., 1993). Ginger is vegetatively propagated through rhizomes. However, conventional multiplication produces only 10-15 lateral buds from the rhizome of a single plant after 8 months (Bhagyalakshmi and Singh, 1988). In addition, ginger rhizomes produced in conventional propagation were infected by various pathogens such as *Fusarium oxysporum* f., *Pseudomonas solanacearum*, *Pythium spp*. and nematodes. Dohroo (1989) reported that approximate 87% of conventional propagation was infected by *F. oxysporum*. It is imperative to produce bacteria-free clones using tissue culture techniques (Bhagyalakshmi and Singh 1988; Sharma et al., 1994; Thaveechai et al., 1997; Balachandran et al., 1990). Hosoki and Sagawa (1977) reported a maximum of 6 shoots per clump with a low survival percentage after transfer to ex vitro. The production of bacteria-free clones with a rapid multiplication rate and high survival percentage is necessary for the successful production of this crop. The objective of this investigation was to develop a high efficiency protocol for rapid multiplication. Efficiency of bacteria-free rhizome production was compared with those of conventional rhizome production.

MATERIALS AND METHODS

Plant Materials

Vigorous rhizomes of ginger (*Zingiber officinales* Rosc.) were obtained from the Kao Ko Agro-industry Co., Ltd. These bulk-rhizomes were kept to emerge the new clumps from bud at 28±2 °C air temperature for 2 months. Young germinated buds were trimmed by using blade, that was always soaked in 95 % ethyl alcohol for sterilization. Pre-surface sterilization was step wise applied to the germinated buds by washing them

Proc. XXVI IHC – Future for Medicinal and Aromatic Plants
Eds. L.E. Craker et al.
Acta Hort. 629, ISHS 2004
Publication supported by Can. Int. Dev. Agency (CIDA)

under running tap water for 10 min, soaking in 5 % Teepol® (Multi-purpose liquid detergent, Cherwood Chemical Limited, Thailand), and, finally, rinsing with distilled water and 70 % ethyl alcohol for 3 min. Surface sterilization of the buds was done by immersion in 1.3 % sodium hypochlorite with 2-3 drops of Tween 20® (Polyoxy ethylene-sorbitan monolaurate, Sigma, USA) and then in 0.3 % sodium hypochlorite with 2-3 drops of Tween 20® for 20 and 5 min, respectively, and rinsing three times aseptically with sterile distilled water. Outer leaves of germinated clumps were removed. Explants of 3±1 mm in diameter were placed in the culture medium, composed of 0.25 % Phytagel (Gellan gum, Sigma, USA) solidified Murashige and Skoog (MS, 1962) medium supplemented with 3% sucrose concentration and 2 mg l^{-1} N6-Banzylamine (BA). The pH of medium was adjusted to 5.8 before autoclaving at 1.05 kg cm^{-2} and 121 °C for 15 min. Four clumps were cultured on MS medium in a 250-ml glass vessel and sub-cultured on the same medium at five-week interval. All cultures were maintained at 25±1 °C air temperature, 60±5 % RH, 60±5 μmol m^{-2} s^{-1} photosynthetic photon flux (PPF) supplemented by cool-white fluorescent lamps (TLD 36W/84 3350 Im, Philips, Thailand) and 16 h d^{-1} photoperiod.

Meristem Culture

Vigorous shoots (shoot length: 1.0±0.5 cm) were incubated in an environmental control chamber at 29±1 °C air temperature and 40±5 % RH in the dark condition for 1 month. Incubated shoots were removed of outer leaves. Meristems on the apex of shoot were cut into pieces 0.15±0.05, 0.35±0.05, 0.55±0.05, and 0.75±0.05 mm in diameter under a microscope (Steni SV 6, ZEISS, Germany) and placed on the culture medium, composed of 0.25 % Phytagel® solidified MS medium supplemented with 3 % sucrose concentration and 15 % coconut water. The medium was adjusted to pH 5.8 before autoclaving at 1.05 kg cm^{-2}, and 121 °C for 15 min. They were cultured at 29±1 °C air temperature, and 40±5 % RH in the dark condition for 3 days and then transferred to culture at 29±1 °C air temperature, 40±5 μmol $m^{-2}s^{-1}$ PPF supplemented by cool-white fluorescent lamps (TLD 36W/84 3350 Im, Philips, Thailand) and 16 h d^{-1} photoperiod for 57 days. Percentages of bacterial contamination and survival were counted.

The experiment was Completely Randomized Design (CRD) with 5 replications. Data of bacterial contamination percentage and survival percentage were tested by analysis of variance (ANOVA) for 4 plantlets of each replication. DMRT (Duncan's Multiple Range Test) at $p \leq 0.05$ was used to determine significance of the meristem diameter.

Shoot Multiplication and Root Induction

In vitro-derived shoot clumps with 2-3 shoots were placed on Phytagel® solidified MS medium supplemented with 2 % sucrose concentration and 0, 2, 4, and 6 mg l^{-1} N^6-Banzylamine (BA) concentrations or 0, 2, 4, and 6 mg l^{-1} 6-(γ-dimethylallylamino) purine (2iP) concentrations. Media were autoclaved at 1.05 kg cm^{-2}, and 121 °C for 15 min. The pH of media was adjusted to 5.8. A Clump with 2-3 shoots was grown in a 350-ml glass vessel with plastic cap. All cultures were maintained at 25±1 °C air temperature, 60±5 % RH, 60±5 μmol $m^{-2}s^{-1}$ PPF supplemented by cool-white fluorescent lamps (TLD 36W/84 3350 Im, Philips, Thailand) and 16 h d^{-1} photoperiod. After culture for 5 weeks, the number of shoot and root, fresh weight and dry weight of shoot and root were collected.

The experimental design was a 2^4 factorial with 5 replications and 4 clumps per replication for the number of shoot and root, as well as dry weight and dry matter of shoot and root. Data were tested by analysis of variance (ANOVA). Least Significant Difference (LSD) at $p \leq 0.01$ was used to determine significance of the cytokinin concentration.

Ex-Vitro Rhizome Production

Shoots were cultured on the MS medium supplemented with 2 % sucrose concentration. A clump with 2-3 shoots was grown in a 350-ml glass vessel with plastic

cap. All cultures were maintained at 25±1 °C air temperature, 60±5 % RH, 60±5 μmol $m^{-2}s^{-1}$ PPF supplemented by cool-white fluorescent lamps (TLD 36W/84 3350 Im, Philips, Thailand) and 16 h d^{-1} photoperiod for 1 month. One hundreds plantlets were removed from the culture boxes, rinsed thoroughly of the substrate. In-vitro plantlets were planted into 1,000-ml plastic pots with fumigated soil, and placed immediately in the net house. Plantlets were grown at 22±13 °C air temperature, 50±20% RH, 12±1 h d^{-1}, and 450±100 kJ m^{-2} d^{-1} solar radiation. Plantlets were irrigated by dipping system and fertilized monthly with 5 g per clump 15N-15P-15K. After growing for 6 months, bacteria-free micro-rhizomes were collected. Bacteria-free micro-rhizomes and conventional rhizomes (weight: 20 g) were replanted into 1,000-ml plastic pots with fumigated soil, and placed in the net house at 22±13 °C air temperature, 50±20 % RH, 450±100 kJ m^{-2} d^{-1} solar radiation, and 12±1 h d^{-1} photoperiod for 8 months. The survival percentage, fresh weight, dry weight, dry matter and number of bud were collected.

The experiment was a CRD with 5 replications. Data of survival percentage, dry weight, dry matter and number of bud were tested by analysis of variance (ANOVA) for 4 plantlets of each replication. T test at $p \leq 0.05$ was used to test significant difference among the rhizome type.

RESULTS AND DISCUSSION

Meristem Culture

Meristem culture was especially applied in several agricultural crops, potatoes strawberry, tobacco, *Lolium*, raspberry, and grape (Pierik, 1987). In present experiment, ginger shoots were cut meristem at various diameters. Bacterial contamination and survival percentage of ginger decreased with decreasing the meristem diameter (Table 1). The lowest percentage of bacterial contamination and survival were found in the meristem cut into 0.15±0.05 mm in diameter. However, one percentage of contamination was observed in the meristem cut into 0.15±0.05 mm in diameter.

Shoot Multiplication and Root Induction

Young shoots were observed from the basal region within 5 weeks of culture. The number of shoot and dry weight increased with increasing culture period. The shoot multiplication and dry weight were strongly affected by the type of cytokinin (Table 2). Dry weight and number of shoot cultured on the medium supplemented with BA were higher than those cultured on the medium supplemented with 2iP. These results might be the effect of high concentration of 2iP. The positive effect on multiplication was reported in *Centaurea spachii* cultured on the medium supplemented with 0.2 mg l^{-1} 2iP (Cuenca and Amo-Marco, 2000). The medium supplemented with 4-mg l^{-1} BA was the most effective for multiple shoot formation producing an average of 8.1 shoots per clump. This multiplication rate of ginger was higher than that reported by Inden et al. (1988). The medium supplemented with 2-4 mg l^{-1} BA was optimum for shoot multiplication in other species of *Curcuma* and Ginger reported so far (Balachandran et al., 1990 and Sharma and Singh, 1995). The dry weight of shoot cultured on the medium supplemented with 4 mg l^{-1} BA was almost twice when compared with those cultured on the medium supplemented with 0 mg l^{-1}BA. Dry matters were similar for shoot cultured on the medium supplemented with 0, 2, 4, and 6-mg l^{-1} BA (Table 2).

Shoots cultured on the medium supplemented with 2iP produced the higher number of root when compared with those cultured on the medium supplemented with BA (Table2). It should be noted that 2iP not only effected on shoot multiplication but also root induction. Shoot cultured on the medium supplemented with 2iP demonstrated the shorter root than those cultured on the medium supplemented with BA. The highest number of root was found in the shoot cultured on the medium supplemented with 2-6 mg l^{-1} 2iP. While, the highest dry weight and dry matter of root were found in the shoot cultured on the medium supplemented with 4 mg l^{-1} 2iP.

Ex-vitro Rhizome Production

After bacteria-free rhizomes and conventional rhizomes were grown in the net house for 8 months. The conventional rhizomes demonstrated the symptom of bacterial infection. While, bacteria-free rhizome obtained from in-vitro culture did not show any rotting due to bacterial infection. The survival percentage of ginger grown from bacteria-free rhizome was 100 %, while the survival percentage of ginger grown from conventional rhizome was 63 % (Table 3). There are no significant difference in dry weight and dry matter of rhizome grown in the net house for 8 months. Number of bud on the rhizome was significantly higher in the ginger grown from bacteria-free rhizome than ginger grown from conventional rhizome. The high number of bud on the rhizome may be the accumulative effect of cytokinin in the multiplication stage. These high number of bud on rhizome should give a high number of shoot after growing, resulting the high quantity of rhizome production in the next generation.

CONCLUSION

The lowest bacterial infection of ginger was succeeded in the shoot cut the meristem into 0.15±0.05 mm in diameter. Bacteria-free ginger cultured on the medium supplemented with 4 mg l^{-1} BA demonstrated the highest multiplication rate. The survival percentage was approximately 1.6 times as high from ginger cultured from bacteria-free rhizome compared with those of ginger cultured from conventional rhizome. The present investigation indicates an efficient and practical protocol for in-vitro multiplication and ex vitro rhizome production to decrease the production cost in a commercial point of view.

ACKNOWLEDGEMENTS

We would like to thank National Center for Genetic Engineering and Bio-technology (BIOTEC), Thailand for research funding.

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Tables

Table 1. Effect of meristem diameter on bacterial contamination and survival percentages of gingers cultured in-vitro for 2 months.

Meristem diameter (mm)	Bacterial contamination (%)	Survival (%)
0.15±0.05	1 d	48 c
0.35±0.05	4 c	67 b
0.55±0.05	18 b	80 a
0.75±0.05	30 a	86 a

Means in a column on each day followed by same letters are not significantly different at 5 % level by Duncan's multiple range test.

Table 2. Effect of N^6-Banzylamine (BA) and 6-(γ-dimethylallylamino) purine (2iP) concentrations on fresh weight, dry weight, the number of shoot and root of gingers cultured in- vitro for 5 weeks.

Cytokinin		Shoot			Root		
Type	Concentration (mg l^{-1})	Number	Dry weight (mg)	Dry matter (%)	Number	Dry weight (mg)	Dry matter (%)
BA	0.0	4.2	45	7.7	7.1	50	8.8
	2.0	6.9	75	7.0	4.8	62	7.9
	4.0	8.1	90	6.7	3.2	66	7.7
	6.0	5.4	48	7.4	2.6	49	9.1
2iP	0.0	4.2	45	7.7	7.1	50	8.8
	2.0	3.9	52	7.6	12.6	52	8.8
	4.0	3.7	52	7.1	12.5	69	9.1
	6.0	3.6	54	6.7	13.9	47	6.4
ANOVA							
LSD $_{p\leq0.05}$		0.8	5	1.2	0.7	3	0.5
Type (T)		**	*	NS	**	NS	NS
Concentration (C)		**	*	NS	**	*	*
T × C		NS	NS	NS	*	NS	NS

*,** Significant at $P \leq 0.05$ and 0.01, respectively
^{NS} Nonsignificant

Table 3. Survival percentage, fresh weight, dry weight, dry matter and number of bud of rhizome grown in the net house for 8 months.

Rhizome source	Survival (%)	Dry weight (g)	Dry matter (%)	Number of buds
Bacteria-free rhizome	100 a	65 a	28 a	52 a
Conventional rhizome	63 b	62 a	27 a	41 b

Means in a column on each day followed by the same letters are not significantly different at 5 % level by T test.