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# Regeneration of Ginger Plant from Callus Culture Through Organogenesis and Effect of $CO_2$ Enrichment on the Differentiation of Regenerated Plant

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**Abstract:** A efficient and systematic protocol for complete plant regeneration via shoot apical meristem culture has been developed for *Zingiber officinale* L. Callus was initiated from shoot-tip of young plant on MS-media supplemented with a combination of Naphthalene acetic acid (0.1 mg $L^{-1}$) and Kinetin (1.0-2.0 mg $L^{-1}$) and Indole-3-acetic acid (0.1 mg $L^{-1}$) and 6-Benzylaminopurine (1.0-2.0 mg $L^{-1}$). Maximum shoot differentiation from callus occurred on MS medium supplemented with Indole-3-acetic acid (0.1 mg $L^{-1}$) and 6-Benzylaminopurine (1.0 mg $L^{-1}$). Complete plantlets were transferred into specially made plastic pot containing soilrite followed by their transfer to the field soil. Survival rate of the plantlets under *ex vitro* condition was 90%. Regenerated plants on MS medium supplemented with Naphthalene acetic acid (0.1 mg $L^{-1}$) and 6-Benzylaminopurine (0.1 mg $L^{-1}$) was exposed to elevated $CO_2$ concentration. A 400-4000 ppm increase in atmospheric $CO_2$ concentration led to an increase in adventitious bud and shoot primodium while the growth of adventitious bud and shoot primodium were reduced at 8000 ppm.

**Key words:** Callus culture, $CO_2$ enrichment, growth regulator, organogenesis, regeneration, *Zingiber officinale*

## INTRODUCTION

Ginger, *Zingiber officinale* Roscoe is a mono-cotyledonous herbaceous perennial belonging to the Zingiberdceae, a family of 47 genera and 1400 species (Purseglove and Zingiberaceae, 1975). Ginger rhizomes are used as spices, in herbal medicine and as raw material in the food beverage and pharmaceutical industries.

Plant tissue culture techniques have been useful in conservation of germplasm of vegetatively propagated crops and considered as an alternative to conventional field gene banks to safeguard against pests and environmental vagaries (Dodds, 1991). There are some early reports on *in vitro* culture of ginger (Balachandran *et al*., 1990; Rout and Das, 1997). It is important to note that, as is the case with ginger (Babu *et al*., 1992), the absence of seed made conventional breeding methods ineffective.

Various techniques have been developed to facilitate the acclimatization of the plantlets to the ambient environments. *In vitro* $CO_2$ enrichment is known as one of the ways of enhancing *ex vitro* survival rate, growth and quality of micropropagated plantlets of a variety of plant species (Kozai, 1991). In the vast majority of these studies, vegetative productivity has been significantly enhanced by increases in the air's $CO_2$ concentration (Raschi *et al*., 1997; Fernandez *et al*., 1998).

Traditionally, ginger was propagated by using rhizome. The rhizome cannot be stored for long time as it is susceptible to fungal diseases, which affect the quality of the tubers. To achieve high productivity, homogeneity and good quality tubers, pathogen-free planting material is crucial. Therefore, this experiment was carried out to investigate an efficient and systematic procedure for propagation with different combination of NAA, KN, IAA and BA of shoot-tip of *Zingiber officinale* through shoot apical meristem culture and whether $CO_2$ enrichment improve differentiation of regenerated plant productivity.

## MATERIALS AND METHODS

The study was conducted in Chonbuk National University, Korea during 2003. The shoot-tips of ginger (*Gingiber officinale* L. cv. Wanju) tubers were used in the

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Table 1: Detail of growth regulator used in this experiment

| Name of growth regulator | Medium | Media composition | Concentration (mg $L^{-1}$) |
|---|---|---|---|
| Naphthalene acetic acid (NAA) | MS I | NAA+KN | 0.1+1.0 |
| Kinetin (KN) | MS II | NAA+KN | 0.1+2.0 |
| Indole-3-acetic acid (IAA) | MS III | IAA+BA | 0.1+1.0 |
| 6-Benzylaminopurine (BA) | MS IV | IAA+BA | 0.1+2.0 |

experiment obtained from local market. The tubers were surface sterilized by immersing for 10s in 95% ethanol followed by 15 min immersion in diluted solution of 1.5% sodium hypochlorite (NaOCl) and washed 3 times with sterile distilled water. The shoot-tips were excised under stereomicroscope and placed on the medium. For shoot-tips culture of tuber, Murashige and Skoog (1962) medium supplemented with different plant growth regulators were used (Table 1). The media were autoclaved at 121°C for 15 min. The pH of the medium was adjusted to 5.8 with 1 N NaOH before autoclaving. Light conditions in the growth cabinet were fixed to 2,500 Lux and a photoperiod of 8 h of light/16 h of dark at 25±1°C, day and night. Survival organogenesis of shoot-tips was observed after 40 days of culture. Regenerated plants were removed and transferred to soilrite mixture. All the treatments were replicated thrice.

**Effect of $CO_2$ enrichment on regenerated plant:** Regenerated plantlets on media containing 0.1 mg $L^{-1}$ IAA and 0.1mg $L^{-1}$ BA were transferred to Magenta® GA-7 vessels at pH 5.8 on same media. Magenta® vessels were modified by adding a 1 cm, 0.2 μ $CO_2$ membrane to facilitate gas exchange. Each vessel contained two regenerated plantlets. The vessel was caped with transparent polypropylene film attached with a microporous polypropylene membrane and aluminum foil. Magenta® vessels containing plantlets were placed in growth chamber especially made for controlling internal $CO_2$ concentration, temperature and relative humidity. Tubes through the polypropylene walls of growth chamber supplied a mixture of $CO_2$ (liquid) and compressed air at a rate of 17 L $min^{-1}$. Mass debit controls made it possible to obtain different $CO_2$ concentrations (400-8000 ppm).

**Statistical analysis:** Data on number of adventitious bud and number of shoot primordium were recorded. Data analysis was performed by the SAS package (Allison, 1995) using analysis of variance (ANOVA) and means was separation in columns by Duncan's multiple range tests at 5% level.

## RESULTS

Culture incubation indicated that medium with concentration of Indole-3-acetic acid (0.1 mg $L^{-1}$) and 6-Benzylaminopurine (1.0 mg $L^{-1}$) showed a remarkable

Fig. 1: Regenerated plant of *Zingiber officinale* from callus culture through Organogenesis after 8 weeks

callus formation after 3 weeks. The calli obtained from this medium was faster growing, delicate and white creamy in colour (Table 1). While culturing in media amended with NAA and KN combination, data showed that calli were compact and green in colour with good frequency of callus formation at concentration of NAA and KN at 0.1 and 1.0 mg $L^{-1}$. The highest frequency of callus induction was 98% in media amended with IAA and BA combination while the lowest frequency of callus induction was 77.5% at the end of six weeks.

Embryogenic potentialities of the callus showed difference to some extend, which was supplemented by different growth regulators (Table 1). In media, all the concentration of NAA, KN, IAA and BA combination showed that somatic embryogenesis started form the shoot-tip callus after 3 weeks of subculturing. However, MS medium containing selected concentration of 0.1 and 1.0 mg $L^{-1}$ of each IAA and BA induced further somatic embryoids differentiation. The presence of both IAA and BA in this medium drastically improved the development of the embryos and allowed to obtain whole plantlets in 6 weeks (Fig. 1).

**Effect of $CO_2$ enrichment on the differentiation of *Zingiber officinale*:** It was observed that a 400-4000 ppm increase in atmospheric $CO_2$ concentrationled to increase in adventitious bud (Number of bud shoot) and shoot primodium (Number of primodium and shoot) growth of *Gingiber officinale* while the growth of adventitious bud and shoot primodium were reduced at 8000 ppm (Table 3 and 4). It was also observed that the vessel caped with transparent polypropylene film attached with a microporous polypropylene membrane had more adventitious bud and shoots primodium then the vessel caped with aluminum foil (Table 3 and 4).

Table 2: Effect of plant growth regulators on adventitious bud formation and shoot growth on MS medium from shoot tip of *Gingiber officinale*

| Medium[a] | Plant growth regulators (mg $L^{-1}$) | Adventitious bud (%+S.E) | Shoot growth (%) <3 mm | Shoot growth (%) >3 mm |
|---|---|---|---|---|
| MS I | NAA 0.1+KN 1.0 | 89.6+2.8b | 29.9 | 70.1 |
| MS II | NAA 0.1+KN2.0 | 77.5+8.4c | 29.4 | 70.6 |
| MS III | IAA 0.1+BA 1.0 | 98.1+6.6a | 11.7 | 88.3 |
| MS IV | IAA 0.1+BA2.0 | 82.7+9.9b | 26.5 | 73.5 |

a: Basal medium: MS+30 g $L^{-1}$ sucrose+3 g $L^{-1}$ agar; Means with the same letter(s) are not significantly different as determined by an analysis of variance with Duncan's multiple range test at 5% level

Table 3: Effect of $CO_2$ enrichment on adventitious bud in *Gingiber officinale* cultured on MS medium containing 0.1 mg $L^{-1}$ IAA and 0.1 mg $L^{-1}$ BA

| Adventitious bud | Cap type | $CO_2$ enrichment (ppm) Control | 400 | 2000 | 4000 | 8000 |
|---|---|---|---|---|---|---|
| No. of bud | A | 6.1f | 8.4d | 9.8cd | 13.4b | 12.7b |
| | B | 7.4e | 8.9d | 10.6c | 15.0a | 13.0b |
| No. of shoots | A | 3.0e | 3.4d | 3.7cd | 4.9a | 4.3ab |
| | B | 3.4d | 3.9c | 4.0c | 5.1a | 4.6ab |

A: Aluminum foil cap., B: Transparent polypropylene film attached with a microporous polypropylene membrane. Mean separation with columns by Duncan's multiple range test at 5% level

Table 4: Effect of $CO_2$ enrichment on shoot primordium in *Gingiber officinale* cultured on MS medium containing 0.1 mg $L^{-1}$ IAA and 0.1 mg $L^{-1}$ BA

| Shoot primordium | Cap type | $CO_2$ enrichment (ppm) Control | 400 | 2000 | 4000 | 8000 |
|---|---|---|---|---|---|---|
| No. of primordium | A | 18.2f | 22.4d | 24.8c | 30.0a | 27.0bc |
| | B | 21.0e | 24.0c | 27.5b | 31.2a | 28.9b |
| No. of shoots | A | 6.1f | 7.0d | 8.1bc | 9.3a | 8.4b |
| | B | 6.6e | 7.7c | 8.5b | 9.4a | 8.6b |

A: Aluminum foil cap., B: Transparent polypropylene film attached with a microporous polypropylene membrane. Mean separation with columns by Duncan's multiple range test at 5% level

## DISCUSSION

Application of different media such as MS, ½ MS, 1/3 MS and 1/4 MS with different concentrations of IAA, IBA and naphthalene acetic acid (NAA) did not produce satisfactory results (Jabbarzadeh and Khosh-Khui, 2005). In our experiment, the highest frequency of callus induction was obtained in MS-media amended with IAA and BA (Table 2). Based on the results obtained in this study, IAA (0.1 mg $L^{-1}$) in combination with BA (1.0 mg $L^{-1}$) was the most suitable treatment for *in vitro* multiplication of ginger. Similar kind of results was obtained by Balachandran *et al.* (1990). They observed good response towards multiple shoot regeneration of ginger using same hormonal supplements in MS medium. Similar combination of auxin with cytokinin for callus induction has been reported by Dode *et al.* (2003). Callus showed difference in embryogenic potentialities to some extend which depending on the growth regulators supplements (Table 1) in the media. The variation of response resulted due to the varying concentrations of growth regulators used in the medium and light condition as also reported earlier in *Zingiber officinale* (Rout and Das, 1997).

*In vitro* $CO_2$ enrichment is known as one of the ways of enhancing *ex vitro* survival rate, growth and quality of micro propagated plantlets of a variety of plant species (Kozai, 1991). This technique have reduced the length of the acclimatization period and improved the quality of strawberry, raspberry and asparagus plantlets (Desjardins *et al*., 1988). In this experiment, $CO_2$ enrichment also promotes the growth of plantlets during *in vitro* acclimatization up to 4000 ppm (Table 3 and 4). Similar kinds of results were obtained by Pospisilova *et al.* (1999) and Solarova and Pospisilova (1997). They observed that $CO_2$ enrichment also promotes the growth of plantlets during *in vitro* acclimatization. These responses are similar to those observed in other crop plants in terms of total productivity enhancement and differences in above and below ground growth stimulation. For root crops such as carrot and radish, for example, the biomass of the primary root storage organ is typically enhanced slightly more than the aboveground biomass (Idso and Kimball, 1991). In contrast, plants such as cotton and soybean, which do not possess root storage organs, generally experience a more equal above and belowground growth stimulation (Idso and Kimball, 1991).

The main objective of present study was to develop a protocol for multiplication of ginger from shoot tip, which is achieved by developing MS-media, amended with different combination of IAA and BA and it was also concluded that $CO_2$ enrichment up to 4000 ppm improve differentiation of regenerated plant productivity *in vitro* condition.

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