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In-vitro plant regeneration via somatic embryogenesis and multiple shoot production in an elite ginger cultivar

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Abstract. Direct production of somatic embryos from the meristematic tissue of elite ginger cultivar 'Garubathan' have been developed and more frequently plant regeneration with multiple shoots has been obtained through secondary embryogenesis. Different ex-plants were incubated in the Murashige – Skoog (MS) media with varied concentration of 2,4-D and BAP and sucrose for direct induction of embryogenic calli and promotion of multiple shoots. The rapid initiation and development of somatic embryos were noticed from the meristematic tissue ex-plants cultured in the MS medium with 2% sucrose, 10% coconut milk pulsed with 2,4-D 1mg/l and BAP 1mg/l. The frequency of secondary embryogenic cells production was also higher when cultured in the medium. Response of embryogenic calli regeneration and multiple shoot production varied depending on the strength of the MS medium and concentration of BAP and a consistent production of multiple shoots was achieved on ¾ strength MS medium with BAP 5mg/l. Scanning electron microscopy and histological examination of embryogenic calli revealed a clear organised globular and bipolar structures, which hold great promise for germplasm conservation and genetic manipulation of ginger.

Introduction

Ginger (*Zingiber officinale* Rosc.) is an important spice crop in India. It bears high export potential and has emerged as an important cash crop for Darjeeling Himalayan region. Ginger is propagated asexually through its rhizome parts. There are many varieties of ginger and they differ in their productivity. Agroclimatic conditions also determine its suitability for cultivation. The crop improvement programme is very slow owing to the totally sterile nature of the plant as well as rhizome rot disease complex which affects productivity adversely. Biotechnological approaches have potential for ginger production improvement. Plant regeneration in ginger has been successfully achieved in the past (Hung, 1995; Sharama and Singh, 1997; Malamug *et al*., 1991). Earning guidance from the past results, a protocol for *in vitro* plant regeneration through somatic embryogenesis will suit the requirement for efficient regeneration of plantlets and multiple shoot production per ex-plant.

There are several reportsof *in vitro* clonal multiplication and organogenesis of ginger plants either adventitiously or via somatic embryogenesis from different ex-plants like shoot tips and leaf pieces. For the regeneration protocol to be useful in the development of transgenic plants, it is desirable to develop a tissue culture system that results in repetitive production of embryogenic cells for transformation and several shoots per ex-plant. Genetic transformation has been used successfully in various crop plants for enhanced disease and pest resistance.

The present study aims specifically to develop efficient regeneration protocol in ginger through somatic embryogenesis and produce multiple shoots per ex-plants for future breeding through non-conventional methods.

Materials and Methods

The elite ginger cultivar 'gorubathan' of Darjeeling hills was used in this study and different ex-plants like leaf pieces, shoot tips and meristematic tissues were tried. The different ex-plants were thoroughly washed and sterilised (0.1% Mercuric chloride) and then placed on callus inducing media consisting of Murashige and Skoog (MS) as basal (Murashige and Skoog, 1962), supplemented with different concentration and combination of 2,4-D (0.5–2.0mg/l), benzyl aminopurine (BAP) (0.5-1.0mg/l), coconut milk (CM) 10% and 2.0% sucrose. The pH of the media throughout the study was adjusted to 5.8 (before phytagel application) and poured in the petriplates. All cultures were incubated at 24±2 °C with a 16-h light/8-h dark photoperiod. After few weeks, clumps of embryogenic calli were split into small pieces and placed on the same media for the production of secondary embryogenic cells. In order to regenerate plants from the cultured cells, the callus was incubated in ¾ strength of MS salts with varied concentration of BAP (0.5-5.0mg/l). Once the embryoids were differentiated, they were subcultured in the specified medium in conical flasks and the regeneration frequency was computed as percentage of embryoids showing at least one shoot.

For scanning electron micrography, embryogenic calli were fixed in 2% glutaraldehyde in 0.1M cacodylate buffer for overnight and post fixed overnight in 1% uranyl acetate. After washing and dehydration with graded alcohol series, tissues were dried with a Tousimis critical point dryer, using liquefied CO_2 as the transitional fluid. Samples were mounted on stubs, sputter-coated with gold and viewed using Phillips 500 scanning electron microscope. For histological studies, well developed embryogenic calli were fixed in formaldehyde-acetic acid-alcohol (FAA), dehydrated in alcohol series and embedded in sequential 5µm sections cut and stained with safranin-fast green.

Results and Discussion

When cultured on a MS medium containing various levels of 2,4-D (0.5-2.0mg/l) and BAP (0.5-1.0mg/l), most ginger ex-plants, with an exception of shoot tips induced both embryogenic and non- embryogenic calli. The frequency of callus formation was highest (83.3%) on culture of meristematic tissues pulsed with 2,4-D and BAP 1mg/l each (Table 1). In contrast, in the same media maximum callus formation from leaf ex-plant was 69.2% callus formation. Callus initiation was lowest when both growth regulators were included at reduced levels. The frequency of

Table 1: Effect of MS media with various concentrations of growth regulators on callus induction from different ex-plants of ginger.

Medium	Different explants of ginger initiated calli (%) *		
	Meristematic tissue	Shoot tips	Leaf pieces
MS salts + BAP 0.5mg/l + 2,4-D 0.5mg/l	25.0	7.69	15.38
MS salts + BAP 1.0mg/l + 2,4-D 0.5mg/l	35.71	12.50	30.76
MS salts + BAP 0.5mg/l + 2,4-D 1.0mg/l	68.0	21.41	38.46
MS salts + BAP 1.0mg/l + 2,4-D 1.0mg/l	83.33	50.0	69.23

* Average of three sets of experiments.

embryogenic calli formation was also higher in the ex-plant of meristematic tissue than leaf pieces. Of 83.3% callus from meristematic tissues, 75% was embryogenic, 17% non-embryogenic and 8% mixed. On the other hand, leaf ex-plants gave 40% embryogenic calli and the remaining was non-embryogenic and mixed types. The typical developmental stages are observed in somatic embryos, which appear distinctly greenish white with a yellowish background of the callus. In a previous report by Kackar *et al*. (1993), 70% embryogenic calli can be achieved from leaf pieces of leaf ex-plants of ginger cultivar 'Eruttupetta'.

Shoot tips of cultivar 'gorubathan' did not show potential of producing embryogenic callus in the test media. Although Malamug *et al*. (1991) suggested the use of MS major elements, Ringe-Nitsch minor elements and organics pulsed with 0.5mg and 1mg/l of 2,4-D and BAP respectively, were responsible for callus induction from shoot tip ex-plants and successful shoot regeneration.

In the subsequent experiments, the rate of regeneration and shoot elongation varied greatly on the four levels of BAP supplemented in ¾ strength of MS medium (Table 2). Multiple shoot buds were observed in the study, which appeared distinct from the somatic embryos from meristematic tissues. Maximum shoot regeneration was attained on MS medium fortified with BAP 5.0mg/l. The magnitude of shoot elongation was also high at high levels of BAP 5mg/l. It would seem that in order for shoots to regenerate from meristematic cell layers in ginger a higher concentration of BAP is required in the nutrient medium. The regenerated plants were well maintained in the MS media without any hormones and successfully established in soil. Ex-plants from leaf pieces and shoot tips have the lowest regeneration rate and multiple shoot formation. While there are many reports on production of ginger plants *in vitro*, most of these protocols are characterized by low frequency of shoot regeneration from embryogenic calli and direct plantlet formation from different ex-plants. Babu *et al*. (1992), Kackar *et al*. (1993) observed a successful shoot regeneration from calli of leaf ex-plants in other ginger cultivars.

Table 2: Effect of benzyl aminopurine (BAP) on the growth and multiple shoot formation in ginger via somatic embryogenesis.

Concentration of BAP	Maximum Number of Shoot/callus	Shoot length after one month of growth (cm)
¾ MS + 0.5 mg/l	2.30	3.0
¾ MS + 1.5 mg/l	3.40	3.0
¾ MS + 3.0 mg/l	5.75	5.0
¾ MS + 5.0 mg/l	8.15	7.5

Scanning electron microscopic observations provide an evidence of organised globular structures, formation of the scutellar notch and embryoids of direct origin from meristematic tissues. Histological examination of the well developed embryoids revealed a clear bipolar organisation of the shoot and root axis with a distinct scutellum, coleoptile and coleorhiza.

We have developed an expeditious protocol to induce somatic embryogenesis from meristematic tissues ex-plants of ginger cultivar 'gorubathan' from Darjeeling hills. It is evident from the results that the principal mode of plant regeneration in tissue culture of ginger is via somatic embryogenesis. The results also suggested that more regenerates can be obtained, because the meristematic zone located deeper in the tissue of ginger may also be induced to regenerate plants. This system has been employed in our laboratory to successfully regenerate ginger plants, which will promote rapid production of disease free planting materials and further breeding programme for the improvement of ginger.

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References

Babu, K.N., Samsudeen, K. and M.J. Rantambol. 1992. *In vitro* plant regeneration from leaf derived callus in ginger (*Zingiber officinale* Rosc.). Plant Cell Tissue Organ Culture 29: (2) 71-74.

Hung, J.H. 1995. *In vitro* propagation and preservation of ginger germplasm. Scientia Agricultura Sincta. 28: (2) 24-30.

Ikeba,L.R. and M.J. Tanabe. 1989. *In vitro* subculture application for ginger. Hort Science. 24: (1) 142-143.

Kackar, A, Bhat, S.R., Chandel, K.P.S. and S.K. Malik. 1993. Plant regeneration via somatic embryogenesis in ginger. Plant Cell Tissue Organ Culture 32: (3) 289-292.

Malamug, J.J.F., Iden, H. and T. Ashahira. 1991. Plantlet regeneration and propagation from ginger callus, Sci. Hortic (Amsterdam). 48: (1-2) 89-97.

Murashige, T, and F. Skoog. 1962 A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum.15:473-597.

Nadgauda, R.S., Kulkarni.D.D., Mascarenhas, A.F. and V. Jagannathan. 1980. Development of plantlets from cultured tissues of ginger (*Zingiber officinale* Roscoe). In: Proceedings of the 198 Plant Tissue Culture on Genetic Manipulation of Somatic Hybridization of Plant Cells symposium, BARC, Bombay, Pp. 358-365.

Sharma, T.R. and B.M. Sing. 1997. High frequency *in vitro* multiplication of disease-free *Zingiber officinale* Rosc. Plant Cell Reports.17: (1) 68-72.