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Direct multiple shoot induction from aerial stem of ginger (*Zingiber officinale* Rosc.)

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Abstract

An efficient and reproducible protocol for direct multiple shoot induction through *in vitro* culture of aerial stem (pseudostem) of ginger (*Zingiber officinale* Rosc.) is reported. Murashige and Skoog basal medium supplemented with BAP and NAA in different combinations (1:0.5, 1:1, 2:1, 3:1 mg l^{-1}) resulted in high frequency shoot / root organogenesis from the basal and middle segments of the aerial stem explants of *in vitro* grown ginger as compared to the other hormone combinations. Maximum of 3.1 shoots could be obtained. Genotypes differed in their *in vitro* response of multiple shoot induction. Anatomical studies revealed the origin of shoot primordial from axillary, apical and primary thickening meristem and of root primordial from primary thickening meristem. The regenerated plantlets were acclimatized and successfully established in the field conditions and harvested after 8 months.

Key words: Aerial stem, apical meristem, axillary meristem, direct regeneration, ginger, transformation, *Zingiber officinale* Rosc.

Introduction

Ginger (*Zingiber officinale* Rosc.) is a rhizomatous herbaceous perennial, the underground rhizome of which is an important spice. Ginger is commonly used all over world as a spice and in the indigenous systems of medicines. Ginger is believed to have originated in South East Asia (Bailey, 1949). *Zingiber* is included in the tribe Hedychieae of Zingiberaceae and in the series Zingibereae which contains only one genus, *Zingiber* (Mabberley, 1987).

Two main constraints that limit ginger production all over the world are rhizome rot caused by *Pythium aphanidermatum* and bacterial wilt caused by *Ralstonia solanacearum*. Infected rhizome bits are the primary source of inoculum, as rhizome is the planting unit in ginger. Many a times the rhizomes serve as carriers of dormant pathogenic fungi, bacteria and viruses. The use of disease free planting materials will help to check the spread of the disease.

Biotechnological tools can be used to produce disease free nucleus stock and for purification of germplasm. Use of plant parts other than the rhizomes for micropropagation has more relevance in this context. The use of pseudostem (aerial stem) explants will be an ideal option in this regard, if it can be regenerated directly in enough quantity. Direct regeneration will be relevant in genetic transformation too. Directly regenerated plantlets are known to have greater genetic and cytological fidelity as well (Maheswaran and Williams, 1984).

However, so far there is no report of direct plantlet regeneration from the aerial stem (pseudostem) of ginger, though micropropagation of ginger using vegetative buds and shoot tip have been reported by various workers (Hosoki and Sagawa, 1977; Nadgauda *et al.*, 1980; Pillai and Kumar, 1982; Sato *et al.*, 1987; Ilahi and Jabeen, 1987; Bhagyalakshmi and Singh, 1988; Inden *et al.*, 1988; Noguchi and Yamakawa, 1988; Sakamura and Suga, 1989; Saradha and Padmanabhan, 1989; Wang, 1989; Balachandran *et al.*, 1990; Oliver, 1996; Sharma and Singh, 1997). Direct regeneration of plantlets from immature inflorescence was also reported by Nirmal Babu *et al.* (1992). The present work is an attempt to induce plantlets from aerial stem of ginger under *in vitro* conditions, which will have more relevance in germplasm purification and genetic transformation of ginger.

Materials and methods

Plant material: Aerial stem cuttings taken from *in vitro* grown plantlets (3- 4 month old) of ginger cultivars, 'Jamaica' and 'Australia' were used as explants as according to earlier reports, explants from *ex vitro* grown plants failed to produce plantlets directly. Aerial stems, divided in to three equal parts *i.e.*, basal, middle and top portion of about 1- 1.5 cm. long, were cultured on MS medium without any pre treatment after removing the outer 2- 3 leaf sheaths.

Culture media and conditions: The basal medium used in the present investigation was that of Murashige and Skoog (1962) with 3% sucrose and 0.7% bacteriological grade agar. Medium was supplemented with different concentrations of BAP (6-benzylaminopurine) and NAA (1-naphthaleneacetic acid) prior to adjusting the pH (5.8). The growth hormone combinations tried were, BAP and NAA (1:0.5, 1:1, 2:1, 3:1 mg l^{-1}) besides BAP alone in two different concentrations, *i.e.* 1 and 2 mg l^{-1}. All cultures were raised in culture tubes (25 x 150 mm; Borosil) containing 15 ml medium and incubated at 25 ± 1° C with 16 hr. photoperiod and 3000 lux light intensity.

Histological studies: For anatomical studies, specimens were fixed in FAA (Formalin: Acetic acid: 70 % Alcohol- 5: 5:90) for 24 hr and were dehydrated through a graded series of alcohol and tertiary butyl alcohol, then embedded in paraffin wax. Serial sections of specimens, 10 μm in thickness, were cut using a rotary

microtome. The sections were dehydrated through a xylene–alcohol series, stained in Papanicolous solutions (Harries hematoxylin solution), mounted and examined under an Olympus B X 50 microscope and photographs were taken (Johansen, 1940).

Hardening and harvesting: Well rooted plantlets, after 2 months of sub culturing were removed from the culture flasks, washed in tap water to remove the adhering medium and transplanted in to a mixture of autoclaved soil, sand, coir dust and cow dung powder (2:1:1:1) in plastic cups, irrigated and kept covered with polythene bags to maintain humidity. After two weeks of hardening the plantlets were transferred to big pots containing soil, sand, coir dust and cow dung powder (2:1:1:1) under natural light condition. Data were obtained for the percentage survival of plants. Matured plants were harvested after 8 months.

Results and discussion

Multiple shoot induction – effect of explants and genotypes: Aerial stem explants produced root initials followed by shoot initials from second week of culturing without any callus phase. Out of the three different explant sources (basal, middle and top) 95% of cultures containing the basal part (axillary meristem) and 70% of cultures containing the middle part (apical meristem) of aerial stem gave good result. But the explants taken from the top portions of aerial stem did not produce any root or shoot. Among the two cultivars tried, 'Jamaica' gave good results *in vitro* direct organogenesis as compared to the 'Australia' variety. A maximum of 3.1 shoots could be observed in the variety 'Jamaica' (Table 1).

Effect of hormones: Direct organogenesis in the form of shoots and root was observed in cultures containing BAP and NAA in all combinations, leading to multiple shoots (Fig. 1). The details of hormone combinations and the culture responses are given in Table 1. Earlier workers reported that BAP and NAA combinations were best for shoot multiplication in ginger (Charlwood *et al.*, 1988; Sakamura and Suga, 1989). In case of cultures with BAP in two different concentrations (1 and 2 mg l^{-1}) the explants showed low rate of response as compared to the other hormone combinations tried. The plantlets obtained from these cultures were found very weak and rooting was also low. Shoot proliferation was not observed in these explants even after subsequent sub culturing.

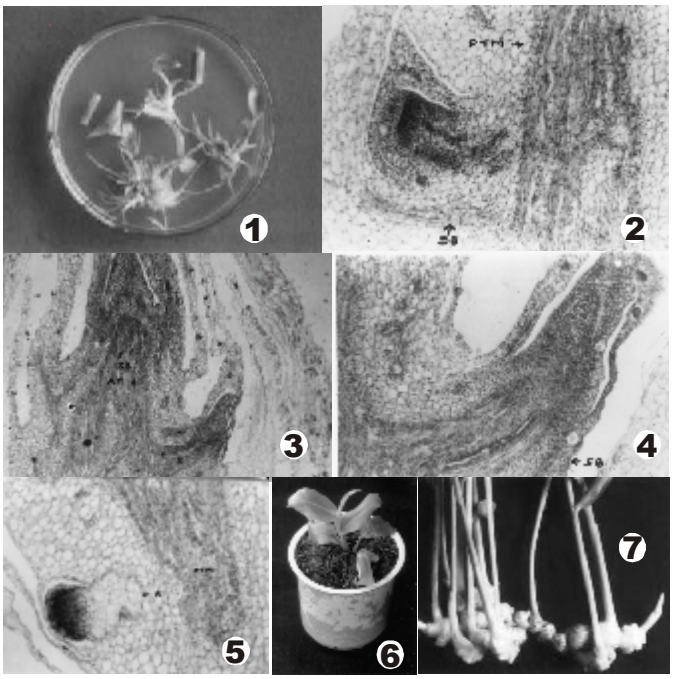

Fig. 1. Pseudostem explant showing multiple shoot formation on MS + BAP and NAA (2:1 mgl^{-1}). Fig. 2. Primary thickening meristem (PTM) showing shoot formation X 200. Fig. 3. Apical meristem (AM) showing shoot bud(SB) initiation X 200. Fig. 4. Activation of dormant axillary bud in to shoot X 200. Fig. 5. Root(R) primordial initiation from PTM X 200. Fig. 6. Hardened plantlet. Fig. 7. Matured harvested plant.

Histological analysis: In order to understand the ontogeny of the shoot / root initiations anatomical studies were done. The longitudinal sections of young aerial stem consist of two distinct regions, inner zone and outer zone. These two zones contain numerous meristamatic regions called primary thickening meristem (Fig. 2). Gifford and Bayer (1995) reported that, primary thickening meristem may be responsible for the stem thickening and production of shoots and roots in monocotyledons. Aerial stem of monocotyledons consists of nodes and inter nodes (Tomlinson, 1969). In immature aerial stem the nodes are constricted and each node carries an axillary bud and each axillary bud has its own accessory bud.

Anatomical studies revealed that, the basal portion containing axillary meristem produced maximum number of shoots and roots than the middle portion containing apical meristem (Table 2). In the present study no shoot / root bud development was observed in the top most aerial stem explant. Anatomical studies revealed lack of any meristamatic region in this portion.

The transverse and longitudinal sections of the multiple shoot induced aerial stem showed four types of differentiations: (a) the

Table 1. Effect of NAA and BAP concentrations on shoot and root formation from aerial stem explant of ginger

Hormones		Var. Jamaica		Var. Australia	
BAP	NAA	Number of shoots[a]	Number of roots[a]	Number of shoots[a]	Number of roots[a]
1.0	0	0.3 ± 0.12	0.9 ± 0.26	0.2 ± 0.11	0.6 ± 0.34
2.0	0	0.2 ± 0.11	0.8 ± 0.20	0.1 ± 0.89	0.9 ± 0.19
1.0	0.5	2.3 ± 0.24	17.2 ± 0.85	2.2 ± 0.27	4.6 ± 1.36
1.0	1.0	2.5 ± 0.12	17.0 ± 0.97	1.7 ± 0.97	12.9 ± 1.28
2.0	1.0	3.1 ± 0.29	13.5 ± 1.37	2.0 ± 0.19	12.4 ± 1.18
3.0	1.0	2.9 ± 0.32	16.6 ± 1.16	1.1 ± 0.18	9.1 ± 0.88

[a] (Values are mean ± standard error. Each treatment was replicated 10 times).

Table 2. Ontogeny of shoot / root initials from aerial stem of ginger (as per the LS and CS of the explants)

Growth hormones (mg l^{-1})		Explant (aerial stem cuttings)			
		Basal portion (axillary meristem)		Middle portion (apical meristem)	
BAP	NAA	Shoot[a] initiation	Root[a] initiation	Shoot[a] initiation	Root[a] initiation
1	0	0.6	2.6	_	0.4
2	0	_	3.2	0.2	_
1	0.5	2.0	6.4	0.8	2.6
1	1	3.4	6.8	1.4	3.0
2	1	3.2	5.4	1.2	4.2
3	1	3.8	6.8	1.4	4.8

[a]Mean values of shoot and root initiations (5 replications each)

dedifferentiation of primary thickening meristem in the outer region into shoots (Fig. 2), (b) from the apical meristem (Fig. 3) (c) the dormant axillary bud present in the nodal region of the aerial stem activates due to the hormonal action, leading to shoot formation (Fig.4), (d) the accessory bud present in the axillary bud also emerges out and produces shoot leading to multiple shoots. The root primordia get differentiated endogenously from the cells present in the endodermoid layer or primary thickening meristem of the explant (Fig. 5). Thus for the first time, direct *in vitro* organogenesis from aerial stem explant of ginger originating from apical and axillary meristem could be demonstrated. The comparatively high level of endogenous hormones in the *in vitro* raised plants as compared to the explants from *ex vitro* sources in the earlier study might be the possible reason for the successful organogenesis in the present experiment.

After two months these cultures were subcultured on to the same medium. Well developed plantlets (3- 5) were obtained within another 2 months of time from each explant. Eighty five percent of the plantlets could be hardened and got established in the field (Fig. 6). The hardened plants, after 8 months, yielded approximately 100 g fresh rhizome per plant (Fig. 7).

In the present investigation it has been possible to establish a reliable and reproducible protocol for *in vitro* multiple shoot induction from aerial stem of ginger and its successful hardening and harvesting. This regeneration system can be used effectively in germplasm conservation, purification (as some of the germplasm accessions (rhizomes) carry, dormantly viruses and other pathogens) and in transformation experiments too.

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