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Enhanced adventitious shoot regeneration from aerial stem explants of ginger using TDZ and its histological studies

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Abstract: A protocol for direct plantlet regeneration from aerial stem explants of ginger (*Zingiber officinale* Rosc.) has been developed. Adventitious shoots and roots were regenerated from in planta aerial stem explants of 2 ginger varieties viz. var. 'Jamaica' and var. 'Varada'. Maximum shoot and root regeneration were observed in the cultures containing TDZ and IBA (1:1; 1:0.1 mg L-1) in both varieties. Cultures containing TDZ alone showed poor shoot and root formation. Histological studies revealed that the shoot initials originated from the primary thickening meristem and apical meristem, while the root primordial originated from the primary thickening meristem present in the aerial stem. The hardening of in vitro plants was performed with different hardening media and successful acclimatization was obtained. The hardening medium containing soil: sand: coir dust: cow dung + 5 g *Trichoderma harzianum* / cup gave good result in all aspects like survival of hardened plants, plant height, number of leaves, and chlorophyll content.

Key words: Aerial stem, biological hardening, direct regeneration, ginger, primary thickening meristem, *Trichoderma harzianum*

Introduction

Ginger (*Zingiber officinale* Rosc.) is herbaceous, rhizomatous, perennial, and the rhizome of which forms one of the important spices and medicines used by people all over the world. Cultivation of ginger and conservation of genetic resources are threatened by various diseases and pests. None of the existing cultivars is resistant to these diseases and it causes heavy crop loss. Infected rhizome bits are the primary source of inoculum, as rhizome is the planting unit in ginger (Nirmal Babu et al., 1992). Use of disease-free planting materials will help to keep the spread of diseases under control. Biotechnological tools can be used to produce disease-free nucleus planting materials.

Being a monocotyledonous crop, ginger does not allow much explant diversity for micropropagation. Rhizome buds and shoot tips, which are often used as the explant sources, have been the responsive explants. However, rhizome explants often carry soil-borne pathogens and they are not available during the crop season. Therefore, in the present study, aerial stem explants were tried for plantlet regeneration, as a novel source.

Tissue culture propagated plants often require extensive hardening treatments to prevent high mortality after transfer to ex-vitro conditions. Biological and physiological stresses adversely affect the successful acclimatization of tissue cultured plants. In order to harden the micropropagated plantlets against such stresses *Trichoderma harzianum* can be used as a biological hardening agent, which helps in better establishment and survival, and faster growth in the hardening stage and field condition.

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Micropropagation of ginger using vegetative bud explants has been reported previously (Hosoki & Sagawa, 1977; Pillai & Kumar, 1982; Sakamura et al., 1986; Bhagyalakshmi & Singh, 1988; Inden et al., 1988; Balachandran et al., 1990; Choi & Kim, 1991; Choi, 1991; Dogra et al., 1994; Huang, 1995; Oliver, 1996; Pandey et al., 1997; Sharma & Singh, 1997; Palai et al., 1997; Tyagi et al., 1998; Devi et al., 1999; Jasrai et al., 2000; Adaniya & Shirai, 2001; Rout et al., 2001). Nirmal Babu et al. (1992) reported direct regeneration of plantlets from immature inflorescence of ginger. In vitro propagation of ginger using in vitro aerial stem (pseudostem) explants was reported by Ikeda and Tanabe (1989). Lincy et al. (2004) also reported multiple shoot induction from in vitro aerial stem of ginger.

Though biological hardening in ginger has not been reported, Miachir et al. (2004) reported mycorrhizal treatment (*Glomus etunicatum* Becger & Gerdemann) of in vitro raised *Curcuma zedoaria* (Berg.) Rosc. Biological hardening using AM fungus (*Glomus mosseae* (Nicol. & Gerd.) Gerdemann) and PGPRs (*Bacillus coagulans* and *Trichoderma harzianum*) was reported by Jayanthi et al. (2003) in *Ficus benjamina* (Miq.) Corner.

The present study is an attempt to develop successful and reproducible protocol for direct regeneration of plantlet from aerial stem explants and its successful hardening using *Trichoderma harzianum*. Direct regeneration protocol in ginger is relevant for genetic transformation, purification of germplasm, and in germplasm exchange.

Materials and methods

Abbrreviations

AM: Apical meristem, BAP: 6-Benzyl amino purine, IBA: Indole 3-butyric acid, NAA: α–Naphthalene acetic acid, PTM: Primary thickening meristem, R: Root, SB: Shoot bud, TDZ: N-Phenyl-N-1, 2, 3-thidiazol-5'–yl-urea.

Standardization of protocol for direct regeneration of plantlets from aerial stem

Two varieties of ginger, viz. var. 'Jamaica' and var. 'Varada', were used in this study. In planta aerial stem explants (taken from the middle portion containing the apical meristem) collected from disease-free plants were thoroughly washed in tap water and disinfected by washing with 5% detergent solution (Teepol) for 20 min. Then the explants were washed in sterile distilled water. The explants were cut into convenient sizes after removing 3-4 leaf sheaths. The cut pieces were surface sterilized with 0.1% $HgCl_2$ for 10 min and rinsed 4-5 times in sterilized double distilled water, then trimmed to 1.0-1.5 cm pieces. These explants were transferred onto the half strength MS medium (Murashige and Skoog, 1962) supplemented with TDZ (1.0 mg L^{-1}), TDZ:IBA (0.5:0.5; 1.0:0.5; 1.0:1.0 mg L^{-1}), TDZ:BAP (0.5:0.5 mg L^{-1}), TDZ:IBA:BAP (1.0:0.5:1.0 mg L^{-1}), TDZ:IBA:GA_3 (0.5:0.5:0.5 mg L^{-1}), and TDZ:BAP:GA_3 (0.5:0.5:0.5 mg L^{-1}).

All the cultures were maintained in an air conditioned room at 22 ± 2 °C and photoperiod regime of 16 h light and 8 h dark with a light intensity of 3000 lux, provided by Philips cool white fluorescent tubes.

The experiments were set to as a Completely Randomized Design. Days taken for regeneration of plantlets, percentage of explants regenerated, and the number of shoots and roots regenerated from each explant were recorded from 10 random culture tubes.

Shoot proliferation

In the case of the in planta aerial stem cultures, the explants having first shoot bud initials were transferred to liquid medium supplemented with different concentrations of TDZ + IBA (02:0.2,1.0:0.5 and 1.0:1.0 mg L^{-1}) and BAP + NAA (0.2: 0.2, 1.0:0.5 and 1.0:1.0 mg L^{-1}) and incubated on a shaker (Cetronat) to hasten the shoot bud proliferation. Then these cultures were again subcultured onto MS solid medium supplemented with BAP and NAA (1.0:1.0 mg L^{-1}).

Histological studies

Standard procedures (Johansen, 1940) were followed to carry out histological studies. Specimens at different developmental stages were fixed in FAA (5 mL formalin: 5 mL glacial acetic acid: 90 mL 70% alcohol) for 24 h, then washed under tap water for 5 h and dehydrated in an ethyl alcohol - tertiary butyl alcohol (TBA) series. The specimens were processed for gradual infiltration and embedded in melted paraffin wax (56-58 °C, Qualigens, Mumbai). The specimens were serially sectioned with 'Ernst Leitz Wetzlar GMBH' (Germany) microtome at 10 µm

thickness and stained with Papanicolous staining solution (Harries hematoxyline solution, Qualigens) for 20 min and washed in water. The slides were then dehydrated through TBA – Xylene series. Representative sections were photographed with an Olympus BX 50 microscope.

Acclimatization and establishment of regenerated plants

To standardize the best hardening medium, well rooted plantlets of 3-4 months old were transplanted onto different hardening media in disposable polythene cups (250 mL). The hardening media tried in the study were 1) coir dust, 2) coir dust + covering of plantlets with polythene cover (25 × 15 cm and 250 gauge), 3) coir dust + *Trichoderma harzianum* - 5 g/cup (CFU = 10^{14} g^{-1}), 4) soil: sand: coir dust: cow dung, and 5) soil: sand: coir dust: cow dung (sterilized): *Trichoderma harzianum* - 5 g/cup. Hardening of the tissue cultured plants was performed at room temperature and each treatment was replicated 10 times.

Before use, soil, sand, coir dust, and cow dung were sterilized by autoclaving at 120 °C for 1 h at 1.06 kg cm^{-2}.

The experiment was set as a randomized block design (RBD). Observation on survival of plants, plant height, number of leaves, and chlorophyll content in leaf tissues were taken from 5 randomly selected plants after 1 month. Standard procedures were used for the chlorophyll estimation study (Arnon, 1949; Witham et al., 1971).

Results

First shoot bud initiation from the explant was observed in nearly 15-18 days after culturing. Root regeneration was observed after 25-30 days of culturing in both varieties.

Percentage of shoot regeneration and the mean number of shoots and roots were observed in the 2 varieties with different hormone combinations are presented in Table 1. In all hormone combinations, shoot regeneration was observed but the percentage of shoot inducing cultures ranged between 70% and 100% in the var. 'Jamaica' and 60% and 100% in the var. 'Varada' (Table 1).

Table 1. In vitro shoot regeneration from in planta aerial stem on MS medium fortified with different growth hormones.

Growth regulators in basal medium (mg L^{-1})				Jamaica			Varada			Mean	
TDZ :	IBA :	BAP :	GA_3	% of shoot inducing culture	No. of shoots	No. of roots	% of shoot inducing culture	No. of shoots	No. of roots	No. of shoots	No. of roots
0.5 :	0.5 :	0.0 :	0.0	80	3.8	7.1	90	9.8	5.4	6.8	6.3
1.0 :	0.5 :	0.0 :	0.0	100	7.6	7.5	90	9.9	6.1	8.8	6.8
1.0 :	1.0 :	0.0 :	0.0	90	11.1	11.0	100	14.6	4.8	12.9	8.1
1.0 :	0.5 :	1.0 :	0.0	90	7.9	4	90	6.9	6.0	7.4	5.05
0.5 :	0.5 :	0.0 :	0.5	90	6.0	4.1	90	3.9	3.2	5.0	3.2
0.5 :	0.0 :	0.5 :	0.0	80	1.9	3.2	70	2.2	1.5	2.05	1.4
0.5 :	0.0 :	0.5 :	0.5	90	4.1	1.2	80	4.2	2.0	4.15	1.9
1.0 :	0.0 :	0.0 :	0.0	70	1.1	1.7	60	0.9	0.4	1.0	0.2
Mean					5.4	0.0		6.6	3.7		
CD (P = 0.05)						4.5					
Variety					0.99	0.69					
Hormone					1.99	1.38					
Variety × Hormone					2.81	1.95					

Among the 8 growth hormone combinations tried, TDZ + IBA (1:1 mg L^{-1}) gave the maximum number of shoots and roots in both varieties. In this medium, the var. 'Jamaica' produced 8-19 shoots (with a mean number of 11.1) and the var. 'Varada' produced 11-22 shoots (mean number 14.6) after 60 days of culture (Figure 1a and 1b). In the case of root regeneration, MS medium supplemented with TDZ + IBA (1:1 mg L^{-1}) was the most suitable medium in var. 'Jamaica'. In the var. 'Varada', the highest number of roots was observed in MS medium supplemented with TDZ + IBA (1:0.5 mg L^{-1}) (Table 1). The effects of variety, hormone, and variety × hormone interaction were significant for both shoot and root regeneration.

Cultures containing TDZ and IBA alone or in combination with other hormones showed high multiple shoot induction as compared to other combinations. However, explants inoculated onto medium supplemented with TDZ (1 mg L^{-1}) alone showed poor shoot and root regeneration (Table 1).

Nature of the growth regulators affects not only the number of shoots but also the morphology of plantlets. Shoots regenerated form cultures containing GA_3 showed long shoots (visual observations) as compared to other hormone combinations.

Shoot proliferation

Since the TDZ treatments (solid media) resulted in retarded and abnormal growth to hasten the shoot proliferation, the in planta explants with shoot and root initials were transferred onto liquid medium supplemented with different concentrations of TDZ + IBA and BAP + NAA. Shoot proliferation started after 7 days of culturing in the medium containing TDZ + IBA (1.0:0.5 and 1.0:1.0 mg L^{-1}). After 20 to 25 days, the shoots multiplied and formed a clump. The length of the shoot buds increased considerably but turned white in colour. The rest of the combinations had no effect on shoot proliferation.

For further shoot and root proliferation, the excised plantlets were transferred onto the MS solid medium with BAP and NAA (1:1 mg L^{-1}). These shoots turned green after 12 days of culturing. Well-developed plantlets could be obtained by 45 days of culturing in this medium. Profuse rooting was also observed (Figure 1c). No varietal variation was observed in this case.

Histology

The longitudinal sections of the in planta aerial stem revealed the presence of 2 distinct regions, inner zone and outer zone. These 2 zones contain numerous meristamatic regions called primary thickening meristem (PTM) (Figure 1d).

The first change, i.e. the onset of active cell division of PTM/apical meristem, observed after 5-10 days on regeneration medium. Continuous meristamatic activity in these cells resulted in the formation of adventitious shoot primordia that ruptured the explant surface.

The longitudinal and transverse sections of the multiple shoot induced aerial stem showed that the shoots regenerated from apical meristem and PTM of the explant (Figure 1e and 1f). The root primordia were regenerated from the cells present in the PTM (Figure 1g).

Acclimatization and establishment of regenerated plants

In the case of plant survival, all the treatments except treatment 2 (coir dust + polythene covering) gave good result (90%–100%). Plants grown in this treatment resulted in only 70% survival. Micropropagated plants hardened in the treatments containing *Trichoderma harzianum* showed 100% survival as compared to other treatments.

Among the 5 treatments, treatment 5 (soil: sand: coir dust: cow dung + 5 g *Trichoderma harzianum* / cup) gave good results in terms of plant height, the number of leaves, and total chlorophyll content in both varieties studied (Table 2). Treatment 4 (soil: sand: coir dust: cow dung) also showed good results. Poor results were observed in treatment 2 (sterilized coir dust + polythene covering) (Figure 1h). Through this biological hardening method, the tissue cultured plants could be hardened within 14 days and the plantlets could be transferred to the field condition.

Discussion

Shoots were regenerated directly from explants in numerous species; plants that are conventionally propagated adventitiously may be proliferated rapidly in vitro using not only the conventional organs as a source of explants, but also the other tissues not

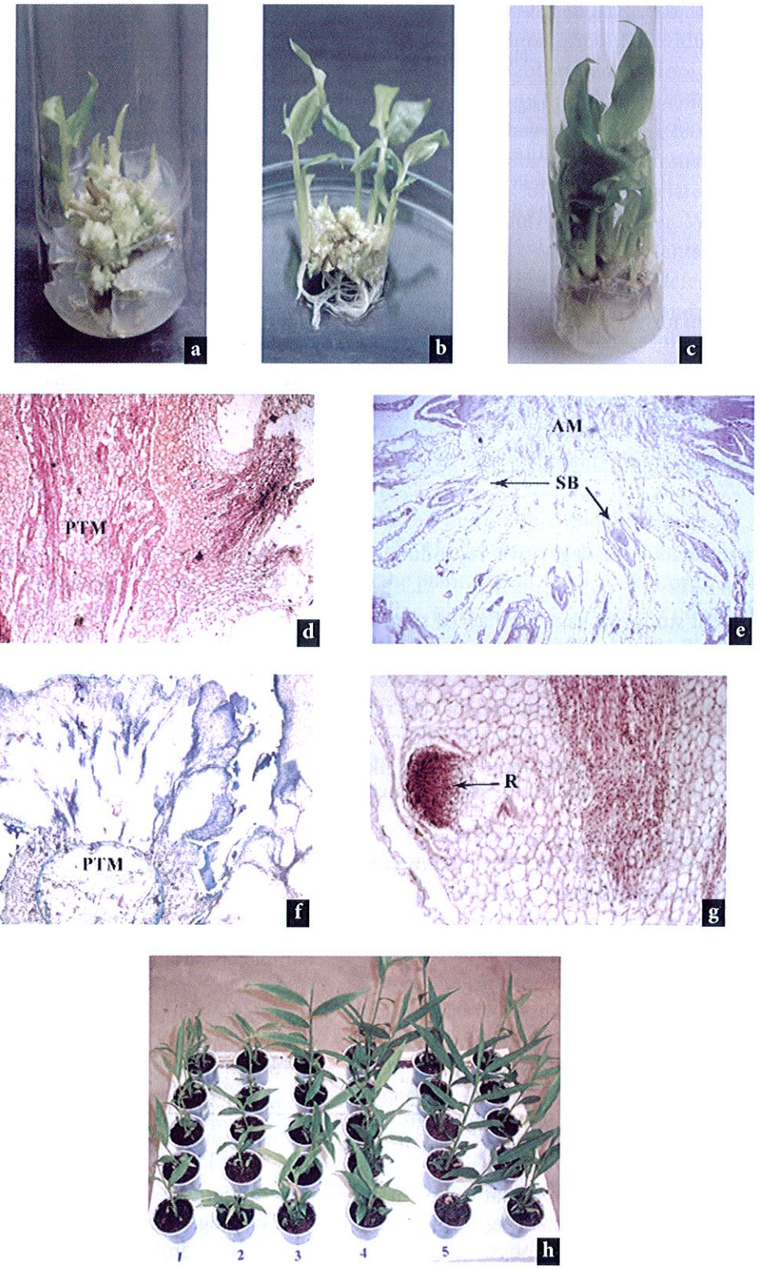

Figure 1. a. Multiple shoot induction from in planta explants of var. Jamaica; b. var. Varada (1 mg L-1 TDZ + 1 mg L-1 IBA); c. Well developed plantlets with profuse rooting (1 mg L-1 BAP + 1 mg L-1 NAA); d. Primary thickening meristem (PTM); e. Shoots regenerated from apical meristem; f. PTM of the explant; g. Root regeneration from PTM; h. Acclimatisation of plantlets.

Table 2. Mean plant height, no. of leaves, and chlorophyll content of the plantlets hardened in different medium.

Treatments*	Plant height (cm)			No. of leaves			Total chlorophyll (mg)		
	Jam.	Var.	Mean	Jam.	Var.	Mean	Jam.	Var.	Mean
1	10.8	9.30	10.1	8.00	8.40	8.2	0.78	0.95	0.87
2	6.40	8.00	7.2	7.40	9.20	8.3	0.77	0.81	0.79
3	15.0	11.5	13.3	11.2	9.00	10.1	0.97	1.00	0.99
4	15.7	12.9	14.3	12.0	10.0	11	1.40	1.16	1.28
5	22.6	19.0	20.8	13.2	15.2	14.2	1.73	1.67	1.70
Mean							1.13	1.12	
CD (P = 0.05)	14.1	12.1		10.4	10.4		NS		
Variety	0.86			NS			0.05		
Treatment	1.36			1.03			0.07		
Variety × Treatment	NS			1.46			6.75		
CV (%)	16.00			15.42					

normally associated with vegetative reproduction (Meins, 1986).

In the present study, we have used aerial stem of ginger, an unconventional explant. Genotypic differences were observed for shoot regeneration from this explant. Palai et al. (2000) reported that the rate of shoot bud regeneration varied with different genotypes of ginger using conventional explants like rhizome buds.

Planta aerial stem of ginger produced shoots only in the presence of TDZ. TDZ has been shown to exhibit stronger effect than other conventionally used cytokinins in a wide range of species (Iram & Anis, 2007; Li et al., 2002). Effective axillary shoot proliferation and adventitious shoot organogenesis have been reported to be caused by TDZ (Huetteman & Preece, 1993). TDZ may be involved in the synthesis or in accumulation of cytokinins in plant tissues (Capelle et al., 1983; Carvalho et al., 2000). This may be the reason for the high multiple shoot induction observed.

However, it was also observed that the plantlets grown in the solid medium containing TDZ showed stunted appearance. Even though the shoot multiplication was observed, the plant growth was retarded. The inhibition of shoot elongation may be due to the high cytokinin activity of TDZ. Cytokinins commonly stimulate shoot proliferation while inhibiting their elongation (Huetteman & Preece, 1993). TDZ is known to inhibit shoot proliferation especially in the solid cultures (Amutha et al., 2006).

The adverse effect of TDZ on shoot proliferation could be manipulated by changing the medium from solid to liquid. The subculture of the explant in the liquid medium supplemented with TDZ or BAP increased the organogenic potential and it helps to improve the regeneration efficiency in *Centaurium erythrace* Rafn and *Phaseolus vulgaris* L. (Piatczak et al., 2005; Veltcheva & Svetleva, 2005). According to Mohamed et al. (2006) the liquid culture changes the physiological nature of explants. The explants are completely submerged in the liquid medium, where they apparently uptake nutrients and growth hormones that favour the development of organogenesis. Similar results were observed in the present study.

Plantlets grown in the medium containing GA_3 showed long shoots compared to the other. Arous et al. (2001) observed that the role of GA_3 in differentiation and elongation of shoot buds is well acknowledged and it is fundamental for the good continuous growth of in vitro regenerated buds. Mohamed et al. (2006) reported that medium supplemented with GA_3 in combination with IBA and $AgNO_3$ was suitable for shoot elongation.

Root induction from in planta aerial stem explants of ginger was observed only in the presence of IBA. Bhagyalakshmi and Singh (1988) have reported that IBA was more effective than NAA for root regeneration in meristem culture of ginger. Successful shoot and root regeneration using TDZ and IBA in *Stachys sieboldii* Miq. and *Cassia angustifolia* Vahl. were also reported (Iram & Anis, 2007; Li et al., 2002).

The longitudinal sections of in vitro aerial stem revealed the presence of 2 distinct regions, inner zone and outer zone (Lincy et al., 2004). Gifford and Bayer (1995) reported that PTM may be responsible for the stem thickening and production of shoots and roots in monocotyledons. Tomlinson (1969) reported that aerial stem of monocotyledons consists of nodes and internodes. In the present study, it was observed that the aerial stem of ginger also involves nodes and internodes.

In vitro adventitious shoot development from aerial stem of ginger and ontogeny of shoot/root were reported by Lincy et al. (2004). Mc Kinless and Alderson (1991) reported the similar results in *Lapageria rosea* Ruiz & Pav., a rhizomatous plant. Anatomical study of in vitro shoot cultures of *L. rosea* revealed the presence of axillary buds in the leaf axis of the aerial stem and presence of vascular traces. Adventitious shoot formation from the leaf axillary buds of aerial stem of stressed field grown ginger plant is also reported (Sasikumar, 2005).

Development of adventitious shoot primordial continued under the influence of cytokinin activity and produced multiple shoots and resulted in a rosette appearance. A similar result was observed in *Lapageria rosea* (Mc Kinless & Alderson, 1991). Though aerial stem branching is not a general feature in ginger, stressed ginger plants are reported to produce adventitious shoots from the aerial stem possibly due to the change in the endogenous level of hormones caused by the stress (Sasikumar, 2005).

The hardening medium containing soil: sand: coir dust: cow dung + 5 g *Trichoderma harzianum* / cup gave good result in all aspects, such as the survival of hardened plants, plant height, number of leaves, and chlorophyll content. Earlier studies indicated that inoculation of in vitro raised plantlets with *Piriformospora indica* during acclimatization reduced the stress of acclimatization, providing faster growth, and better establishment (more than 90%) in *Nicotiana tobacum* L. and *Bacopa monniera* L. (Singh et al., 2000). Sahay and Varma (2000) observed that the plant – microbe interaction results in the synthesis and expression of defence related proteins and enzymes in a controlled manner. When a pathogen comes in contact with these plants, it is capable of boosting the production of defence mechanism related secondary metabolites and these chemicals provide protection, which results in higher plant survival. Treatments with endomycorrhizal (*Glomus etunicatum*) at the ex vitro transferring stage was beneficial for acclimatization, improving plant growth, and development of *Curcuma zedoaria* Rosc. (Miachir et al., 2004). Krishna et al. (2005) reported that the mycorrhizal inoculation at an early stage resulted in the accumulation of different biochemicals in the plant system, such as chlorophyll, carotenoids, proline, phenol, and enzymes like polyphenol oxidase and nitrate reductase. These plantlets showed enhanced survival and improved tolerance against stresses experienced during the weaning phase. The mycorrhizal plants also exhibited improved physiological and nutritional status and had higher relative water content and photosynthetic rate.

Through this protocol, large number of disease-free plants could be produced from aerial stem explants without passing through a callus phase and thus minimizing the probability of somaclonal variation. The biological hardening method using *Trichoderma harzianum* is viable and useful to tackle the survival and establishment problem associated with tissue cultured ginger plants.

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References

Adaniya S & Shirai D (2001). In vitro induction of tetraploid ginger (*Z. officinale* Rosc.) and pollen fertility and germinability. *Sci Hortic* 83: 277-287.

Amutha S, Muruganantham M & Ganapathi A (2006). Thidiazuron–induced high frequency axillary and adventitious shoot regeneration in *Vigna radiata* (L.) Wilczek. In vitro *Cell Dev Biol–Plant* 42: 26-30.

Arnon DI (1949). Copper enzymes in isolated chloroplasts. Polyphenol-oxidase in *Beta vulgaris*. *Plant Physiol* 24: 1-15.

Arous S, Boussaid M & Marrakchi M (2001). Plant regeneration from zygotic embryo hypocotyls of Tunisian chili (*Capsicum annum* L.). *J Appl Hort* 3: 17-22.

Balachandran SM, Bhat SR & Chandel KPS (1990). In vitro clonal multiplication of turmeric (*Curcuma* spp.) and ginger (*Zingiber officinale* Rosc.). *Plant Cell Rep* 8: 521-524.

Bhagyalakshmi N & Singh NS (1988). Meristem culture and micropropagation of a variety of ginger (*Zingiber officinale* Rosc.) with a high yield of oleoresin. *J Hort Sci* 63: 321-327.

Capelle SC, Mok DWS & Turner JE (1983). Effects of thidiazuron on cytokinin autonomy and the metabolism of N6-(D2-isopentenyl) [8-14C] adenosine in callus cultures of *Phaseolus lunatis* L. *Plant Physiol* 73: 796-802.

Choi SK (1991). Studies on the clonal multiplication of ginger through the in vitro cuttings. *Research Reports of the Rural Development Administration, Biotechnology* 33: 33-39.

Choi SK & Kim DC (1991). The study on the clonal multiplication of ginger through in vitro culture of shoot apex. *Research Reports of the Rural Development Administration, Biotechnology* 33: 40-45.

Cruz de Carvalho MH, Van Le V, Zuily-Fodil Y, Pham Thi AT & Tran Thanh Van K (2000). Efficient whole plant regeneration of common bean (*Phaseolus vulgaris* L.) using thin cell layer culture and silver nitrate. *Plant Sci* 159: 224-232.

Devi S, Taylor MB, Powaseu I & Thorpe P (1999). Micropropagation of ginger. *PRAP Report Pacific Regional Agricultural Programme* 7: 11-12.

Dogra SP, Korla BN & Sharma PP (1994). In vitro clonal propagation of ginger (*Zingiber officinale* Rosc.). *Hort J* 7: 45-50.

Gifford EM & Bayer DE (1995). Developmental anatomy *Cyperus esculentus* (Yellow nutsedge). *Int J Plant Sci* 156: 622- 629.

Hosoki T & Sagawa Y (1977). Clonal propagation of ginger (*Zingiber officinale* Rosc.) through tissue culture. *J Hort Sci* 12: 451-452.

Huang JH (1995). In vitro propagation and preservation of ginger germplasm resources. *Sci Agric Sinica* 28: 24-30.

Huetteman CA & Preece JE (1993). Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell Tiss Organ Cult* 33: 105 -119.

Ikeda LR & Tanabe MJ (1989). In vitro subculture applications for ginger. *Hort Sci* 24: 142-143.

Inden H, Asahira T & Hirano A (1988). Micropropagation of ginger. *Acta Hort* 230: 177-184.

Jayanthi S, Bagyaraj DJ & Sathyanarayana BN (2003). Enhanced growth and nutrition of micropropagated *Ficus benjamina* to *Glomus mossese* co-inoculated with *Trichoderma harzianum* and *Bacillus coagulans*. *World J of Microbiology and Biotechnology* 19: 69-72.

Iram S & Anis M (2007). In vitro shoot multiplication and plantlet regeneration from nodal explants of *Cassia angustifolia* (Vahl.): a medicinal plant. *Acta Physiologae Plantarum* 29: 233-238.

Jasrai YT, Patel KG & George MM (2000). Micropropagation of *Zingiber officinale* Rosc. and *Curcuma amada* Roxb. *Spices and aromatic plants: challenges and opportunities in the new century*, Calicut, Kerala, India, 20-23 September, 2000. p.p. 52-54.

Johansen D (1940). *Plant micro techniques*. Mc Graw Hill Publication, New York.

Krishna H, Singh SK, Sharma RR, Khawale RN, Grover M & Patel VB (2005). Biochemical changes in micropropagated grape (*Vitis vinifera* L.) plantlets due to arbuscular mycorrhizal fungi (AMF) inoculation during ex vitro acclimatization. *Sci Hortic* 106: 554-567.

Li W, Gao HH, Lu R, Guo GQ & Zheng GC (2002). Direct plantlet regeneration from the tuber of *Stachys sieboldii*. *Plant Cell Tiss Org Cult* 71: 259-262.

Lincy AK, Remashree AB & Sasikumar B (2004). Direct multiple shoot induction from aerial stems of ginger (*Z. officinale*). *J Appl Hort* 6: 99-101.

Mc Kinless J & Alderson PG (1991). An anatomical study of rhizome bud formation induced by Paelobutrazol and adventitious root formation in in vitro cultures of *Lapageria rosea* (Ruiz et Pav.). *Ann Bot* 67: 331-338.

Meins FJR (1986). Determination and morphogenetic competence in plant tissue culture. *In*: Yeoman MM. (ed.) *Plant Cell Culture Technology. Botanical monographs*. Vol. 23, Blackwell Scientific Publications, Oxford. p.p. 7-25.

Miachir JI. Romani VLM. Amaral AF, de C Mello MO, Crocomo OJ & Melo M (2004). Micropropagation and callogenesis of *Curcuma zedoaria* Rosc. *Sci Agric* 61: 427-432.

Mohamed SV, Sung JM & Wang CS (2006). Organogenesis of *Phaseolus angularis* L.: high efficiency of adventitious shoot regeneration from etiolated seedlings in the presence of N6-Benzyl aminopurine and Thidiazuron. *Plant Cell Tiss Org Cult* 86: 187-199.

Murashige T & Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Plant* 15: 473-497.

Nirmal Babu K, Samsudeen K & Ravindran PN (1992). Direct regeneration of plant lets from immature inflorescence of ginger (*Z. officinale* Rosc.) by tissue culture. *J Spices & Aromatic Crops* 1: 43-48.

Oliver JJ (1996). The initiation and multiplication of ginger (*Z. officinale* Rosc.) in tissue culture. *Inligtings bulletin, Instituut via. Tropiese en Subtropiese Gewasse* 291: 10-11.

Palai SK, Rout GR, Das P & Edison S (1997). Micropropagation of ginger (*Zingiber officinale* Rosc.) - interaction of growth regulators and culture conditions. In: Ramana KV, Sasikumar B, Babu KN & Eapen SJ (eds.). *Biotechnology of spices, medicinal & aromatic plants. Proc. Nat. Sem. Biotech. Spices and Aromatic Plants*, Calicut, India, 24-25 April, 1996. p.p. 20-24.

Palai SK, Rout GR, Samantaray S & Das P (2000). Biochemical changes during in vitro organogenesis in *Zingiber officinale* Rosc. *J Plant Biol* 27: 153-160.

Pandey YR, Sagwansupyakorn C, Sahavacharin O & Thaveechai N (1997). In vitro propagation of ginger (*Zingiber officinale* Roscoe). *Kasetsart Journal, Natural Sciences* 31: 81-86.

Piatczak E, Wielanek M & Wysokinska H (2005). Liquid culture system for shoot multiplication and secoiridoid production in micropropagated plants of *Centaurium erythraea* Rafn. *Plant Sci* 168: 431-437.

Pillai SK & Kumar KB (1982). Note on the clonal multiplication of ginger in vitro. *Ind J Agri Sci* 52: 397-399.

Rout GR, Palai SK, Samantaray S & Das P (2001). Effect of growth regulator and culture conditions on shoot multiplication and rhizome formation in ginger (*Zingiber officinale* Rosc.) in vitro. In vitro *Cell Dev Biol-Plant* 37: 814-819.

Sahay NS & Varma A (2000). A biological approach towards increasing the rates of survival of micropropagated plants. *Curr Sci* 78: 126-129.

Sakamura F, Ogihara K, Suga T, Taniguchi K & Tanaka R (1986). Volatile constituents of *Zingiber officinale* rhizome produced by in vitro shoot tip culture. *Phytochem* 25: 1333-1335.

Sasikumar B (2005). Stem fasciation in ginger. *Spice News* 16: 1.

Sharma TR & Singh BM (1997). High frequency in vitro multiplication of disease free clones of *Zingiber officinale* Rosc. *Plant cell Rep* 17: 68-72.

Singh A, Sharma J, Rexer KH & Varma A (2000). Plant productivity determinants beyond minerals, water and light: *Piriformospora indica*-A revolutionary plant growth promoting fungus. *Curr Sci* 79: 1550.

Tomlinson PB (1969). Zingiberaceae. *In*: Metcalfe CR. (ed), *Anatomy of the monocotyledons: Commelinales-Zingiberales*, Oxford, U.K., Clarendon Press. p.p. 353-354.

Tyagi RK, Bhat SR & Chandel KPS (1998). In vitro conservation strategies for spices crop germplasm - *Zingiber*, *Curcuma* and *Piper* species. In: Mathew NM, Kuruvilla JC, Licy J, Joseph T, Meenattoor JR & Thomas KK (eds.). *Developments* in plant*ation crops research. PLACROSYM XII*, Kottayam, India, 27-29 November 1996. p.p. 77-82.

Veltcheva MR & Svetleva DL (2005). In vitro regeneration of *Phaseolus vulgaris* L. via organogenesis from petiole explants. *J Central European Agric* 6: 53-58.

Witham FH, Blaydes DF & Devlin RM (1971). Experiments in Plant Physiology, Van Nostrand, New York. p. 245.