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Indirect and direct somatic embryogenesis from aerial stem explants of ginger (*Zingiber officinale* Rosc.)

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Protocols for direct and indirect somatic embryogenesis from aerial stem explants of ginger have been developed. Aerial stem explants of two ginger varieties were cultured on different concentrations of 2,4-D induced callus. An *in vitro* aerial stem produced hard, nodular and yellowish callus (Type I) and an *in planta* aerial stem gave rise to soft, sticky callus with pale white color (Type II). The proliferated Type II calli were subject to stress for 40–60 days without subculturing. The desiccated calli produced white friable calli which turned embryogenic and then produced somatic embryos in a medium containing 2 mg L^{-1} benzyl amino purine. The mature, club-shaped somatic embryos were germinated on a medium containing benzyl amino purine and α – naphthalene acetic acid in different concentrations. Type I callus of neither variety turned embryogenic but produced roots in all the cultures. Direct somatic embryogenesis was observed from the *in planta* aerial stem and leaf base explants with the use of thidiazuron alone or in combination with indole, 3-butyric acid. Histological studies revealed that the somatic embryos in ginger have a distinct single layered epidermis, scutellum, coleoptile, shoot apex and root apex.

Keywords: Aerial stem, *Zingiber*, histology, somatic embryogenesis

Abbreviations: BAP – Benzyl amino purine**, IBA** – Indole, 3-butyric acid, **NAA** – α – naphthalene acetic acid**, 2,4-D** – 2,4 Dichlorophenoxy acetic acid**, TDZ** – Thidiazuron

Introduction

Ginger is an important tropical, herbaceous, perennial crop, the rhizomes of which are valued all over the world either as a spice or herbal medicine. Ginger is normally propagated by its rhizome, with a low proliferation rate (10–15 buds from one unit per year) and it is easily infected by soil-borne pathogens like *Pythium aphanidermatum* and *P. vexans* (rhizome rot), *Ralstonia solanacearum* (bacterial wilt) and *Meloidogyne incognita* (nematode), resulting in heavy crop loss. Recombination breeding in ginger is handicapped by the absence of seed set. Though the crop is not amenable to conventional breeding, it is easily amenable to biotechnological approaches for crop improvement.

Genetic transformation is one of the most promising options for crop improvement in ginger, as many of the important traits have no effective resistance source available in the germplasm. A major prerequisite for genetic transformation is the availability of an *in vitro* regeneration system preferably via somatic embryogenesis, a process whereby a single somatic cell develops into a whole embryo and subsequently to a complete plantlet. Besides, somatic embryogenesis can be used directly in somatic hybridization, protoplast culture, synseed production and for inducing variability (KANTHARAJAH and GOLEGAONKAR 2004).

Many researchers have reported somatic embryogenesis in ginger using different explants. KACKAR et al. (1993) reported somatic embryogenesis from *in vitro* leaf explants. Induction of somatic embryos from ovary explants of ginger and its regeneration were described by NIRMAL BABU et al. (1996). GUO and ZHANG (2005) established somatic embryogenic cultures of four ginger cultivars from shoot tip explants. A protocol for plant regeneration via somatic embryogenesis from vegetative bud-derived callus cultures of ginger was reported by SUMA and KESHAVACHANDRAN (2005). Due to lack of seed set, the embryological studies in ginger are still in the initial stage. The histological studies of somatic embryos in the present study revealed the origin, evolution and structure of ginger embryos.

Barring few studies on the histology of indirect somatic embryogenesis in ginger (KACKAR et al. 1993, SUMA and KESHAVACHANDRAN 2005), there are no reports on direct somatic embryogenesis and its ontogeny in ginger. The present work reports both direct and indirect somatic embryogenesis in ginger including the histological and developmental changes during somatic embryogenesis.

Materials and methods

In vitro and *in planta* aerial stem explants taken from two varieties of ginger (*Zingiber officinale* Rosc.), var. Jamaica and var. Varada were used in this study. *In planta* explants collected from 3–7 months old disease-free plantswere thoroughly washed in tap water with 5% detergent solution (Teepol) for 20 minutes followed by 2–3 washes in sterile distilled water. The explants were cut into convenient sizes after removal of the leaf sheaths. The cut pieces were surface sterilized with 0.1% $HgCl_2$ for 10 minutes and rinsed 4–5 times with sterilized double distilled water and then trimmed to 1.0–1.5 cm size. These explants were transferred onto half strength MS medium (MURASHIGE and SKOOG 1962) supplemented with 2, 4-D (2, 4-dichlorophenoxy acetic acid) at different concentrations (0.5, 1.0, 2.0 mg L^{-1}). The *in vitro* aerial stem explants, collected from 3–4 months old plantlets cultured on a medium containing BAP and NAA in different concentrations, were cut into 1.0 –1.5 cm length and transferred to the same medium. Explants were maintained in an air conditioned room at 22±2 °C and photoperiod regime of 16 hr light and 8 hr dark with a light intensity of 3000 lux, provided by Philips cool white fluorescent tubes.

Callus cultures (about 2–2.5 g) derived from the aerial stem explants were subcultured onto MS medium containing 2,4-D + BAP in different combinations (0.2: 0.2, 0.5: 0.2, 1.0: 0.5, 2.0: 0.5, 1.0: 1.0 mg L^{-1}). These cultures were subjected to stress by keeping them without sub culturing for 40 – 60 days to induce embryogenic calli. The embryogenic calli were then transferred onto MS medium containing BAP in different concentrations (0.2, 0.5, 1.0, 2.0, 5.0 mg L^{-1}) for the induction of somatic embryos.

Mature somatic embryos were transferred onto MS medium with BAP + NAA (α – Naphthalene acetic acid) in different combinations (0.2: 0.2, 1.0: 0.5, 1.0: 1.0, 2.0: 0.2, 2.0: 1.0 mg L^{-1}).

Single explants were inoculated onto MS medium supplemented with TDZ and IBA (Thidiazuron and Indole, 3-butyric acid) at different concentrations (0.5: 0.5; 1.0: 0.5; 1.0:1.0 mg L^{-1}) and TDZ alone (0.2, 0.5, 1.0 mg L^{-1}).

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 hour, then washed under tap water for 5 hours 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 an Ernst Leitz Wetzlar GMBH (Germany) microtome at 10μm thickness and stained with Papanicolous staining solution (Harries hematoxyline solution, Qualigens) for 20 minutes and washed in water. The slides were then dehydrated through TBA – Xylene series. Representative sections were photographed with an Olympus BX 50 microscope.

Well-rooted plantlets of 3–4 months old were removed from the culture flasks, washed in tap water to remove the adhering medium and transplanted in disposable polythene cups (250 ml) containing sterile sand and coir dust and kept at room temperature for further growth.

The experiments were set according to completely randomized design. Observations recorded include days taken for morphogenic response from ten random tubes, percentage of culture responded, nature of callus – callus colour and texture, type of callus response (induction of somatic embryos, rhizogenesis and callus proliferation) besides number of shoots and roots regenerated from each somatic embryo culture. All experiments were performed in triplicate. Ten culture tubes constituted an experimental unit. Two factor ANOVA was performed to test the significance of treatment effect. Statistical procedures were performed using Mstat C.

Results

Induction of callus from aerial stem – effect of explants and genotypes

The *in vitro* aerial stem produced callus by the second week of culturing in both the varieties. However, the *in planta* aerial stem took 32 days in the var. Jamaica and 25 days in the var. Varada for callus induction. The *in vitro* aerial stem of the var. Jamaica produced profuse callusing in MS with 2,4-D (2 mg L^{-1}) and moderate callusing in 2,4-D (1 mg L^{-1}). The *in planta* aerial stem of this variety showed only moderate callusing with 2,4-D (2 mg L^{-1}). In the var. Varada, the *in vitro* aerial stem showed profuse callusing in 2,4-D (1 mg L^{-1} and 2 mg L^{-1}), while the *in planta* aerial stem produced moderate callusing in 2,4-D (2 mg L^{-1}) (Tab. 1).

Color and texture of callus – effect of explants

The quality and type of the callus produced from the different explants varied. Callus induced from the *in vitro* aerial stem was hard, nodular and yellowish in colour. The surface

Tab. 1. Effect of explants and genotypes on callus induction

Sl. No	Hormone mg L^{-1} (2,4-D)	Jamaica				Varada			
		In vitro aerial stem	% of callus induced cultures	*In planta* aerial stem	% of callus induced cultures	*In vitro* aerial stem	% of callus induced cultures	*In planta* aerial stem	% of callus induced cultures
1	0.5	+	30	–	–	+ +	40	–	–
2	1.0	+ +	70	+	20	+++	60	+	40
3	2.0	+ + +	90	+ +	60	+++	100	+ +	70

+ low callusing; + + moderate callusing; + + + profuse callusing (observations based on amount of callus in 10 cultures).

of the callus was covered with small hairy structures (hereafter referred to as Type I callus) while the *in planta* aerial stem produced soft, sticky callus with pale white or creamy colour of friable nature (hereafter referred to as Type II callus). No genotypic differences were observed.

Induction of somatic embryos

The Type II calli showed callus proliferation in all cultures containing 2,4-D and BAP in different concentrations, in both the varieties. These proliferated calli, when subjected to stress for 40 to 60 days without subculturing, became desiccated due to the depletion of water and nutrients. These desiccated calli started to produce good quality, white friable callus and on subculturing onto the medium containing 2 mg L^{-1} BAP produced somatic embryos. The other concentrations of BAP resulted in callus proliferation only. The percentage of somatic embryo induction showed variation with the different varieties. In the var. Jamaica, 100% of cultures produced somatic embryos by 30–35 days of culturing and in the var. Varada, only 50% of cultures resulted in somatic embryo induction after 45 days of culturing. The Type I callus as well as Type II callus that were not subjected to stress did not become embryogenic on repeated subculturing but produced roots in all the cultures, in both the varieties.

The embryogenic calli produced localized groups of cells, which differentiated into embryogenic forms on the peripheral region of the callus and latter differentiated into white globular embryoids (Fig. 1 a) after 30 days of culture. This process continued in the subsequent subculturing in the same medium leading to the production of oval shaped embryo (Fig. 1 b). After 45 days, these somatic embryos differentiated into a more mature, elongated club shaped or cylindrical stage, and cotyledon initiation, as well as signs of scutellum initiation, occurred at this stage (Fig. 1 c). After 60 days the coleoptile became green and the shoot completely differentiated with one or two leaf plumules. At this stage, the embryos started germinating and developed shoot. The pattern of embryo development was the same in both the varieties, though in case of the var. Jamaica the events occurred a little faster.

Secondary somatic embryogenesis was observed when the embryogenic calli with somatic embryos were transferred onto fresh medium with BAP (2.0 mg L^{-1}) or without any growth hormones (Fig. 1 d). The presence of BAP hastened the secondary somatic embryo induction.

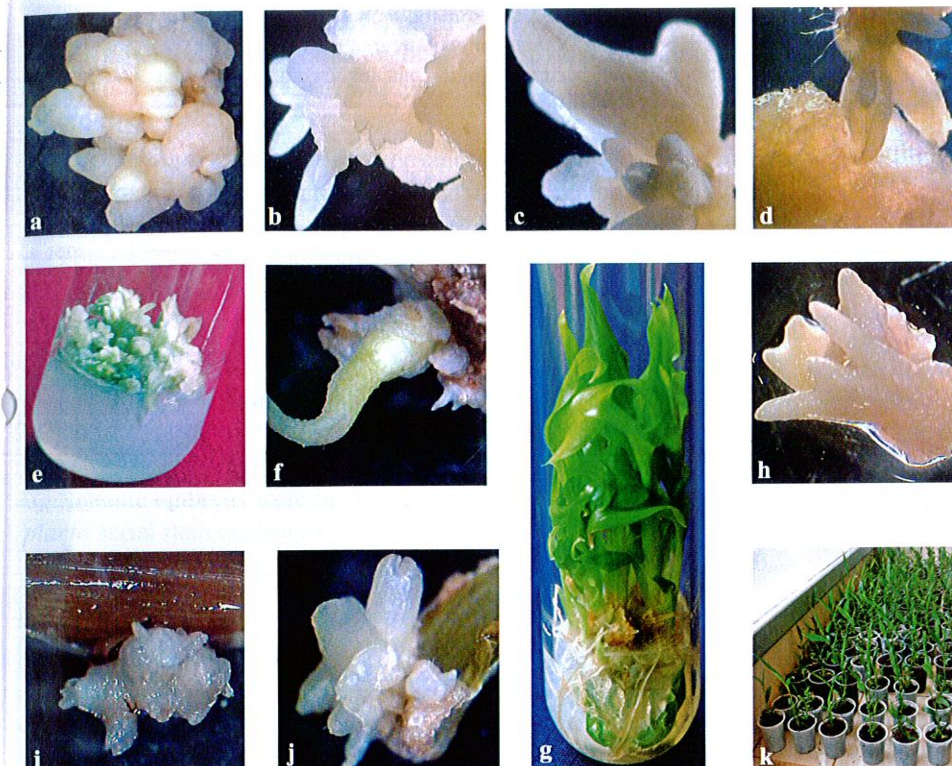

Fig. 1. Callus proliferation in ginger. a – white globular embryoids on the peripheral region of the callus cultured on medium supplemented with BAP 2 mg L^{-1}. b – globular and oval shaped embryos, c – club shaped embryo with cotyledon and scutellum initiation, d – secondary somatic embryogenesis, e – mature embryos showed green color after 12 days of culturing (1.0 BAP + 1.0 NAA mg L^{-1}), f – close-up view of somatic embryo germination, g – shoot and root proliferation from somatic embryos cultured on 2 BAP + 0.2 NAA mg L^{-1}, h – irregular and aberrant formation of somatic embryos, i – *in planta* aerial stem explant of var. Jamaica showing direct somatic embryo induction (0.5 TDZ + 1.0 IBA mg L^{-1}), j – leaf base explant of var. Varada showing direct somatic embryo induction (0.5 TDZ mg L^{-1}), k – acclimatization of plantlets).

Germination of somatic embryos

The germination of embryos was obtained by the second week of culturing in the regeneration medium containing BAP and NAA in different combinations. The mature embryos showed a green color after 12 days of culturing (Fig. 1 e, f). Then these embryos produced shoots and roots simultaneously (Fig. 1 g).

Germination of somatic embryos was observed in all the hormone combinations tried and the percentage of germination varied from 60 to 100% in both the varieties (Tab. 2).

Among the five hormone combinations tried, the optimum concentration for shoot regeneration was 2 mg L^{-1} BAP + 0.2 mg L^{-1} NAA in the var. Jamaica and 2 mg L^{-1} BAP + 1 mg L^{-1} NAA in the var. Varada. The effects of hormones on shoot regeneration were signif-

Tab. 2. *In vitro* shoot and root regeneration from somatic embryos on MS medium supplemented with different growth hormones. Values for each treatment are means of three replicas; ten culture tubes constituted one experimental unit. Data analyzed using two-way ANOVA. NS – non significant; CD – critical difference.

Growth regulators in basal medium (mg L^{-1})	Jamaica			Varada			Mean	
BAP : NAA	% of shoot inducing cultures	No. of shoots	No. of roots	% of shoot inducing cultures	No. of shoots	No. of roots	No. of shoot	No. of root
0.2 : 0.2	100	4.4	6.4	60	1.4	8.2	2.9	7.3
1.0 : 0.5	60	0.8	12.6	100	2.2	7.6	1.5	10.1
1.0 : 1.0	100	1.8	9.0	80	1.2	10.2	1.5	9.6
2.0 : 0.2	100	8.2	9.0	100	2.8	5.2	5.5	7.1
2.0 : 1.0	80	2.0	7.8	100	9.0	10.2	5.5	9.0
Mean		3.4	9.0		3.3	8.3		
CD (p <0.05)								
Variety		NS	NS					
Hormone		0.74	NS					
Variety X Hormone		1.04	NS					

icant. Maximum root regeneration was observed in BAP and NAA (1:0.5 mg L^{-1}) in the var. Jamaica and BAP and NAA (1:1 and 2:1 mg L^{-1}) in the var. Varada (Tab. 2).

Some well developed somatic embryos (20–40%) failed to germinate and formed irregular and aberrant structures (Fig. 1 h).

Induction of direct somatic embryos

In planta aerial stem explants of the var. Jamaica produced somatic embryos directly from 30% of explants in the cultures containing TDZ + IBA (0.5 + 1.0 mg L^{-1}) (Fig. 1) with 30 – 40 days of culturing. In the var. Varada, direct somatic embryos were induced from the *in planta* leaf base explants (20%) cultured on the medium containing TDZ (0.5 mg L^{-1}) after 45 days of culturing (Fig. 1 j). The other hormone combinations showed no morphogenic responses. However, no plantlet regeneration was observed in the direct regenerated somatic embryos in either of the varieties.

Histological studies – origin and development of somatic embryos

Anatomical sections prepared from the embryogenic cultures of ginger containing somatic embryos at different developmental stages revealed the ontogeny of somatic embryos. The histological sections showed groups of meristamatic cells formed at the peripheral region of embryogenic callus, which can be clearly identified from the normal non embryogenic calli. These meristamatic cells were small, compact and densely cytoplasmic with distinct nuclei. The embryos developed from single cells, which underwent continuous divisions and formed two, four, six and eight celled stages. After that, it formed a globular mass (proembryoids) that showed a distinct epidermis. Further differentiation of these structures led to the formation of globular, oval shaped embryos on the surface of the callus (Fig. 2 a). The embryo was attached to the embryogenic callus with a prominent multicellular stalk, the suspensor.

At the cylindrical stage of somatic embryo, the embryo primordium started to grow as a broad outgrowth and a notch arose at terminal region of the embryo (Fig. 2 a). The scutellum developed as a hump opposite the notch where the shoot apex is organized and the coleoptile developed as a circular primordium around the shoot apex. The shoot apex had a lateral position and the root apex developed opposite the shoot apex. Thus the somatic embryos had a scutellum, coleoptile, shoot apex and coleorhiza with an independent vascular system that is not connected to the maternal tissue (Figs. 2 b, c).

The primary and secondary somatic embryos at the peripheral region of the callus had a bunch-like appearance (Fig. 2 d). Secondary somatic embryos were developed due to the proliferation of cells in the coleorhizal part of mature primary somatic embryos (Fig. 2 d).

Somatic embryos were induced directly from the primary thickening meristem of *in planta* aerial stem explant and from the epidermal tissues of leaf base explant (Figs. 2 e, f) with distinct scutellum, coleoptile, shoot apex and root apex (Fig. 2 g).

Hardening and establishment of plantlets

Well developed plantlets were transferred to polythene cups containing sterile sand and coir dust and 85 – 90% of the plantlets could be hardened (Fig. 1 k). After 2 weeks of hardening the plantlets were transferred to field or experimental shed.

Discussion

Somatic embryos were induced indirectly from *in planta* aerial stem derived callus and directly from aerial stem as well as leaf base explants of ginger. The callus induced from the *in vitro* aerial stem explants of the two varieties was shown to be inappropriate for somatic embryo induction in the present study. The type of callus was also different with regards to different explant types. Source of explants and genotypes are considered to be important factors in the induction of callus and somatic embryo (GEORGE 1993). GUO and ZHANG (2005) reported variation over different genotypes on somatic embryogenesis in ginger as we observed in the present study. MA and GANG (2006) reported that the rate of callus formation in ginger varied dramatically depending on the tissues used.

In our study, the calli induced in medium containing 2,4-D were further proliferated in a medium with 2,4-D + BAP. The calli become embryogenic only in the presence of BAP. It is observed that complete removal of 2,4-D from the medium favoured somatic embryogenesis. Earlier workers in *Zingiber officinale* Rosc. and *Kaempferia galanga* L. observed the same results (GUO and ZHANG 2005, VINCENT et al. 1992).

A period of stress was found favorable to induce somatic embryogenesis from callus cultures of ginger in the present study. Stress-induced somatic embryogenesis has already been reported in carrot (HARADA et al. 1990). Different stresses (osmotic, heavy metal ion and dehydration stress) are implicated in somatic embryo induction in *Arabidopsis tha-*

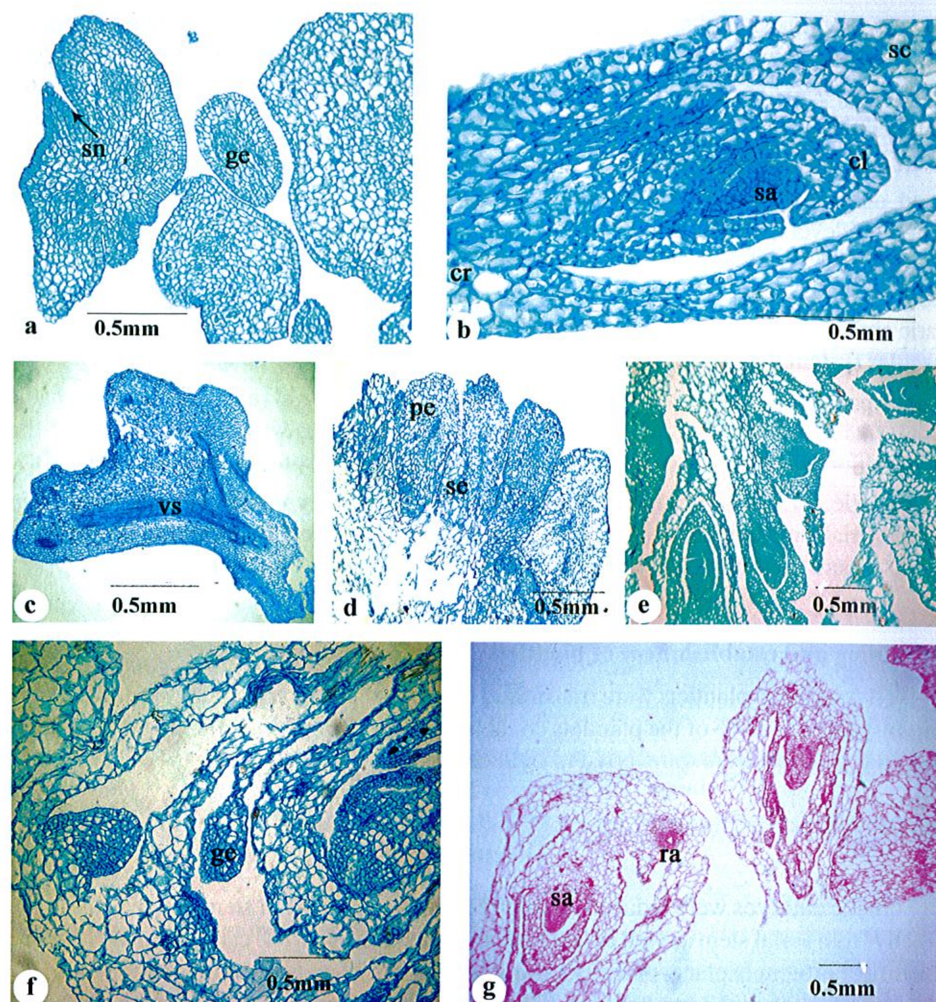

Fig. 2. Globular and oval shaped embryos on the surface of the callus (a). Arrow mark indicating the scutellar notch at the terminal region of the embryo. b – somatic embryo with scutellum, coleoptile, shoot apex and coleorhizae, c – somatic embryo with an independent vascular system, d – primary and secondary somatic embryos – secondary somatic embryos originating from the coleorhizal part of primary somatic embryo, e – direct somatic embryo induction from aerial stem explants, f – direct somatic embryo induction from leaf explant – globular stage, g – single somatic embryo with shoot apex and root apex. cl – coleoptile, cr – coleorhiza, ge – globular embryo, pe – primary embryo, ra – root apex, sa – shoot apex, sc – scutellum, se – secondary embryo, sn – scutellar notch, vs – vascular system. Bars denote 0.5 mm.

liana (IWAI et al. 2003). Probably, each stress treatment induces expression of some factors (genes) that control the start of somatic embryogenesis (IWAI et al. 2003).

Successful plantlet regeneration from somatic embryos was observed in the present study with BAP and NAA. GUO and ZHANG (2005) reported that BAP was effective for regeneration of somatic embryos also, besides induction of somatic embryos. KACKAR et al.

(1993) also reported that inclusion of BAP (0.5–1.5 mg L^{-1}) in the regeneration medium hastened the germination of somatic embryos in ginger. The somatic embryos directly induced were not regenerated in the present study. Long exposure of explants to TDZ inhibited the capacity of shoot regeneration and multiplication. GISBERT et al. (2006) reported that the development of shoots decreased when the concentration of TDZ increased. TDZ is known to inhibit shoot proliferation, especially in solid cultures (AMUTHA et al. 2006). The somatic embryos in ginger followed typical developmental stages described for other monocot systems: globular embryo stage, club shaped cotyledon initiation stage and the plumule initiation stage. SHAH (1982) reported that the monocotyledon embryo is cylindrical and shoe shaped with a slightly pointed distal end and broad blunt coleorhizal end. VASIL et al. (1984), GUIDERDONI and DEMARLY (1988), SMITH and KRIKORIAN (1991) also reported similar pattern of embryo development in other monocots like maize, sugar cane and day lily, respectively.

SMITH and KRIKORIAN (1991) reported that embryogenic callus of day lily yielded various 'neo morph-like' structures (developmentally abnormal or poorly developed pseudo-embryonic forms that did not yield plantlets and eventually died) comprising a root and a poorly developed or altogether lacking shoot tip meristem. Germination without plantlet recovery, i.e. the formation of root but not an epicotyl, may be due to malformation of the apical shoot meristem or else to the embryo being damaged during its isolation for transfer to germination medium (VALLADARES et al. 2006).

Histological studies of somatic embryogenesis in ginger and other monocotyledons were reported by earlier workers. KACKAR et al. (1993) observed the presence of nodular structures containing richly cytoplasmic cells delimited by a single layered epidermis. These authors also observed stalked somatic embryos with scutellum, coleoptile and coleorhiza in ginger. SWAMY (1982) observed that the quadrant and octant stages are invariable and necessary stages in the embryogenesis of both dicotyledons and monocotyledons. WARDLAW (1955) reported that in a monocotyledonous embryo, the shoot apex occupies a lateral position in the somewhat cylindrical embryo and the cotyledon is terminal. BECHTEL and POMERANZ (1978) and BATYGINA (1969) observed the presence of scutellum and coleoptile in the monocot embryo.

Secondary somatic embryos were originated from the coleorhizal part of the developed somatic embryos (primary somatic embryos). KUO et al. (2005) reported that secondary somatic embryogenesis occurred at the basal part of primary embryos and originated from outer cell layers, in *Phalaenopsis*, an orchid species. Direct somatic embryogenesis from epidermal tissues of leaf explants was also reported in *Phalaenopsis* (CHEN and CHANG 2006; KUO et al. 2005).

In the present study, we established a reliable and reproducible protocol for indirect somatic embryogenesis from aerial stem explants and direct somatic embryogenesis from aerial stem as well as leaf base explants of ginger. The results of the study revealed the origin and development of direct and indirect somatic embryogenesis. We were able to induce somatic embryos repeatedly from primary somatic embryos as well (secondary somatic embryogenesis). Such a system for cyclic somatic embryogenesis is useful in the production of large scale somatic embryo induction, which can be used for the genetic transformation of ginger.

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