MICROPROPAGATION

Direct somatic embryogenesis and plant regeneration from leaf sheath explants of mango ginger (*Curcuma amada* Roxb.)

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Abstract An efficient and simple procedure was systematically developed for inducing direct somatic embryogenesis and plantlet regeneration from leaf sheath explants of *Curcuma amada* Roxb. A two-step culture system was used to induce somatic embryogenesis. The optimized procedure resulted in direct somatic embryogenesis from 93.3% explants after 3-wk culture. Leaf sheath explants were incubated for 2 wk on medium containing 2.24 μM 2,4-dichlorophenoxyacetic acid and 1.11 μM 6-benzyladenine to initiate direct somatic embryogenesis. Thereafter, these explants were transferred to a medium containing 9.10 μM thidiazuron and 1.33 μM α-naphthaleneacetic acid. Elongated somatic embryos obtained from these cultures germinated readily, and the optimal frequency of plantlet development (86.7%) was achieved when embryos were cultured in darkness on 1/2 strength Murashige and Skoog medium containing 1.44 μM gibberellic acid. Histological and scanning electron microscopic studies showed that the initial cell divisions that led to embryo formation occurred in epidermal and subepidermal cells, followed by the development of globular and elongated structures that appeared to be somatic embryos. The presence of a clear protoderm in the globular structures and procambial strands in the elongated structures confirmed that these structures were true somatic embryos. Plantlets derived from somatic embryos were acclimatized successfully to *ex vitro* conditions at a survival rate of 87.43% and developed with normal phenotypes.

Keywords *Curcuma amada* · Mango ginger · Leaf sheath explant · Direct somatic embryogenesis · Protoderm · Thidiazuron

Introduction

Curcuma amada Roxb. is an aromatic spice crop of the family Zingiberaceae, a group of important tropical monocots which includes turmeric, ginger, and cardamom. It is commonly known as mango ginger and possesses a raw mango-like flavor blended with that of ginger (Banerjee *et al.* 2012). The aromatic smell raised from *C. amada* is mainly attributed to presence of car-3-ene and cis-ocimene compounds (Gholap and Bandyopadhyay 1984; Rao *et al.* 1989; Choudhury *et al.* 1996). Its rhizomes contain fibers, starch, and more than 68 volatile, aromatic essential oils which are used in food, beverages, cosmetics, and medicines (Srivastava *et al.* 2001; Mustafa *et al.* 2005; Policegoudra and Aradhya 2008).

C. amada possesses several medicinal properties, such as stomachic, carminative, aphrodisiac, antipyretic, and laxative properties, and is a potential source of compounds with cholesterol-lowering activities (Kirtikar and Basu 1984; Warrier *et al.* 1994; Srinivasan *et al.* 2008). The major bioactive compounds of *C. amada* include curcuminoids, curcumin, demethoxy curcumin, and bis-demethoxy curcumin. These compounds have been used for their antioxidant, anti-inflammatory, antidepresent, and platelet-aggregation inhibition activities (Policegoudra *et al.* 2011). It also contain a labdane-type diterpenoid (labda-8(17), 12-diene-15 and 16-dial), which exhibit activity against tuberculosis (Singh *et al.* 2010).

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C. amada is mainly propagated by division of rhizomes which are slow to multiply. Genetic improvement by conventional breeding is also difficult in *C. amada* because of a lack of sexual reproduction (Balachandran *et al.* 1990; Prakash *et al.* 2004). Furthermore, genetic engineering and molecular studies of its resistance to rhizome rot (caused by *Pythium* sp.) and bacterial wilt (caused by *Ralstonia solanacearum*) requires a standardized, simple morphogenic protocol (Prasath *et al.* 2011). Therefore, a reliable protocol for large scale propagation of this rhizomatous spice is necessary. Somatic embryogenesis represents a promising tool for mass propagation as well as for genetic transformation (Nhut *et al.* 2000; Manrique-Trujillo *et al.* 2013). Somatic embryos are preferred as a tissue for production of alginate-encapsulated synthetic seeds (Ganapathi *et al.* 2001; Remakanthan *et al.* 2013). Tissue culture studies in *C. amada* have shown only adventitious plantlet formation from rhizome and leaf sheath explants (Prakash *et al.* 2004; Das *et al.* 2010; Banerjee *et al.* 2012), and indirect somatic embryogenesis *via* callus induction (Soundar Raju *et al.* 2013). In this study, we report direct somatic embryogenesis and plant regeneration from *C. amada* leaf sheath explants, and a method that can be used for large scale production of elite *C. amada* in less time than with previous procedures.

Material and Methods

Plant material, preparation of explants, and culture condition *C. amada* plants, procured from Malappuram, Kerala, were established *in vitro* and used as a source of explants. The culture procedures and media were similar to those described by Soundar Raju *et al.* (2013). Leaf sheath explants were obtained from 3-mo-old plants. The innermost, tender leaf tips were avoided and only peripheral leaf sheath segments were considered. Leaf sheath segments (1.5 cm long) were inoculated on medium with the abaxial surface facing the medium.

The pH of media used for all experiments was adjusted to 5.7 ± 0.1 and autoclaved at 121°C and 104 kPa for 15 min. All experiments were performed with solid media gelled with 0.8% agar powder (Himedia®, Mumbai, India). Cultures were maintained at 25 ± 2°C, 16 h photoperiod (except as noted below) under 40 μmol m^{-2} s^{-1} light intensity provided by white fluorescent tubes and a relative humidity at 55–65%.

Induction of somatic embryogenesis All media used for somatic embryo induction included the Murashige and Skoog (MS) (Murashige and Skoog 1962) mineral formulation supplemented with B_5 vitamins (Gamborg *et al.* 1968) and 3% sucrose. Preliminary experiments to assess the potential of somatic embryo induction form leaf sheath explants were performed in somatic embryo induction medium 1 (SIM 1) supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D; 2.24, 4.49 μM) alone or with 1.11 μM 6-benzyl adenine (BA). Based on preliminary results obtained using SIM 1, the explants were transferred to somatic embryo induction media 2 (SIM 2) supplemented with BA (4.44, 8.88 μM), kinetin (Kn; 4.64, 9.29 μM), or thidiazuron (TDZ; 4.55, 9.10 μM) alone or in combination with α-naphthalene acetic acid (NAA; 1.33 μM) for 3 wk to determine the optimal levels of growth regulators for somatic embryogenesis.

Germination and hardening Somatic embryos cultured on SIM 2 medium for 3 wk were individually transferred to germination medium [1/2 strength MS medium supplemented with gibberellic acid (GA$_3$; 0.0, 0.72, 1.44, 2.16, 2.88 μM) and 2% sucrose] and cultured in either dark or light (16 h photoperiod) to induce germination. After 3 wk, tiny plantlets were transferred to growth medium, which consisted of 1/2 strength MS basal medium supplemented with 2% sucrose, and cultured in light with a 16 h photoperiod. Plantlets with 3–4 leaves and 4–5 roots were transferred to plastic cups containing an autoclaved mixture of sand, soil, and vermiculate (1:2:1). Cups were covered with perforated polythene bags to maintain high humidity. The bags were removed when the plants produced new leaves. Some of these primary-hardened plants were selected at random and were transferred to earthen pots containing sand, soil, and cattle manure (1:2:1) for secondary hardening under shade house condition. The survival rate of plantlets was calculated after 1 mo of primary hardening.

Histological and microscopic analysis Explants before and after differentiation of somatic embryos were selected for histological studies. The explants were fixed in formalin-acetic acid (FAA-95% ethanol; 2:1:17, *v/v/v*) for 24 h, dehydrated using serial grades of ethanol, and embedded in paraffin wax using the methods of Dam *et al.* (2010). Thin sections (~10 μm) were cut using a rotary microtome, mounted on glass slides, and allowed to dry for at least 10 min before staining. Finally, sections were stained with 0.025% toluidine blue O or safranin O and mounted in Di-n-butyl phthalate in xylene (DPX); BDH®, Mumbai, India). The prepared slides were examined through a light microscope (Leica®, Switzerland) and photographed. For scanning electron micrography, samples were frozen in liquid nitrogen and scanned in a low vacuum liquid scanning electron microscope (SEM; Hitachi® S 3400, Tokyo, Japan) with chamber pressure of 30 Pa and an accelerated voltage of 15 kV.

Statistical analysis All experiments were carried out three times, each time with at least 15 explants. All data were subjected to one-way ANOVA. Data are presented as mean, or mean±standard error (SE). Mean separations were determined by Duncan's multiple rage test at a significance level of $P<0.05$ (IBM® SPSS statistics 19).

Results

Development of somatic embryos After 2 wk of culture on SIM 1 containing 2.24 μM 2,4-D and 1.11 μM BA, the leaf sheath explants became swollen and soft (Fig. 1*A*). The explants eventually became crimped, and white clusters of small translucent spherical structures were formed on SIM 2 medium containing cytokinin (BA, Kn, TDZ) alone or in combination with NAA (Fig. 1*B*). Later, these structures differentiated into globular and elongated stages (Fig. 1*C, D*). In contrast, culture of leaf sheath explants on SIM 1 containing 4.49 μM 2,4-D and 1.11 μM BA for more than 3 wk led to a reduction in the percentage of explants forming somatic embryos and an increase in callus formation.

The one-way ANOVA revealed that the SIM 2 media had significant effects on the percentage of explants with somatic embryo induction ($P<0.05$). Somatic embryo induction was observed after 3 wk of culture. In general, Kn was least effective for direct somatic embryogenesis. The best responses occurred using SIM 2 media supplemented with TDZ, with up to 73.3% of explants showing induction of somatic embryos (Table 1). Higher concentrations of BA or TDZ (8.88; 9.10 μM) reduced the induction of somatic embryos (Table 1). Further experimentation confirmed this,

and showed also that BA or TDZ more effectively induced somatic embryos when used in combination with NAA (Table 2). The highest percentage (93.3% of explants responding) of somatic embryo induction was obtained on medium supplemented with 9.10 μM TDZ in combination with 1.33 μM NAA.

Somatic embryos did not germinate on the embryo induction medium. After transfer to 1/2 strength MS medium containing GA_3, somatic embryos germinated within 10 d (Fig. 1*E*). GA_3 concentration had a significant effect on somatic embryo germination (Fig. 1*F, G*). Germination was significantly better from embryos cultured in the dark (Fig. 2). The highest rate of germination (86.7%) was obtained from somatic embryos cultured in the dark on medium containing 1.44 μM GA_3 (Fig. 2).

The small plantlets obtained on the germination medium were further transferred to culture bottle containing 1/2 strength MS basal medium under light (Fig. 1*H*). After 3 wk, the plantlets had formed a vigorous root system and 3–4 leaves. Plantlets were than transplanted to plastic cups containing the potting mixture (Fig. 1*I, J*) and, after 1 mo of primary hardening, 87.43% of plants survived. Randomly selected primary-hardened plants were transferred for secondary hardening (Fig. 1*K*).
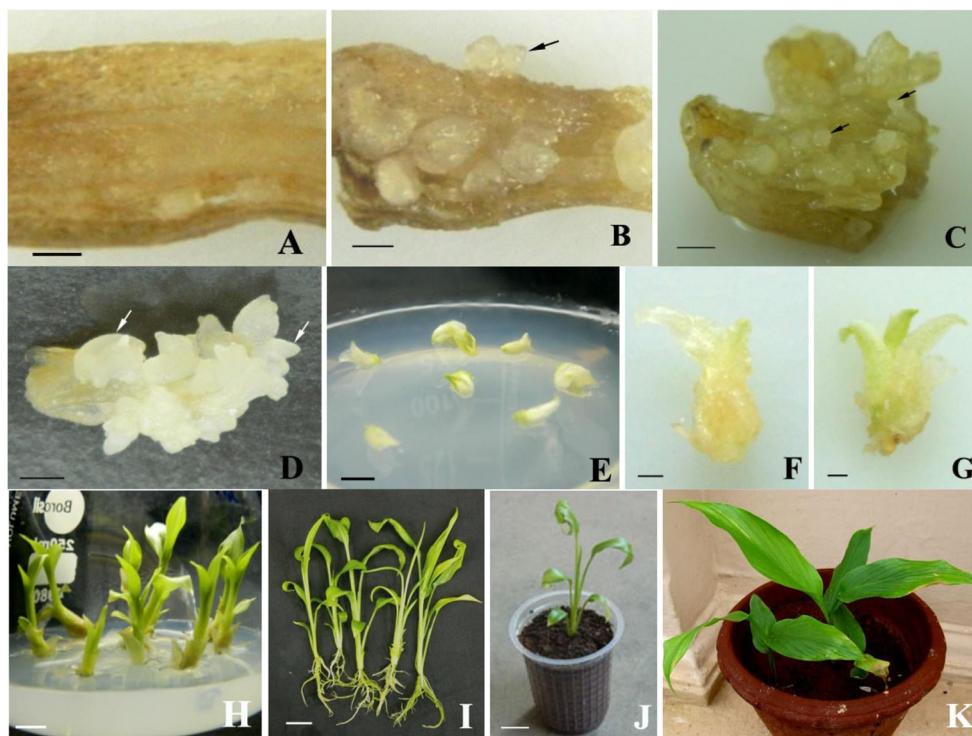

Figure 1. Morphological stages of direct somatic embryogenesis: (*A*) General view of leaf sheath explant after 2 wk of culture on SIM 1 medium containing 2.24 μM 2,4-D in combination with 1.11 μM BA; (*B*) Development of translucent spheres (*arrow*) on SIM 2 medium containing 9.10 μM TDZ in combination with 1.33 μM NAA; (*C*) Early stages of globular embryo development (*arrows*); (*D*) Elongated stages of somatic embryos (*arrows*); (*E*) Germination of somatic embryos on 1/2 strength MS medium containing 1.44 μM GA_3 under dark condition; (*F, G*) Germinated somatic embryo; (*H*) Plantlets regenerated on 1/2 strength MS basal medium under light condition; (*I*) Plantlets showing shoot and roots just before transfer to mixture of sand, soil, and vermiculite (1:2:1); (*J, K*) Plantlets during secondary hardening to mixture of sand, soil, and cattle manure. *Bars*: (*A*) 100 μm; (*B, C*) 500 μm; (*D*) 700 μm; (*E*) 1.0 mm; (*F, G*) 1.5 mm; (*H, I*) 1.0 cm; (*J*) 1.2 cm.

Table 1 Effects of plant growth regulators on the induction of direct somatic embryogenesis from leaf sheath explants of *C. amada*

| Plant growth regulators (μM) | | | | | Explants responding with embryo induction (%)[a] | Number of somatic embryos per explant (mean±SE)[b] |
| SIM 1 | | SIM2 | | | | |
2,4-D	BA	BA	Kn	TDZ		
2.24	1.11				0.0[i]	0.0[f]
4.49	1.11				0.0[i]	0.0[f]
2.24	1.11	4.44			48.8[c]	7.04±1.10[d]
		8.88			64.4[ab]	12.28±1.39[bc]
4.49	1.11	4.44			24.4[gh]	2.46±0.66[e]
		8.88			37.7[def]	5.06±1.00[de]
2.24	1.11		4.64		28.8[fgh]	4.71±1.12[de]
			9.29		39.9[cde]	9.02±1.75[cd]
4.49	1.11		4.64		19.9[h]	2.22±0.73[e]
			9.29		33.3[efg]	4.73±1.02[de]
2.24	1.11			4.55	59.9[b]	16.06±1.99[b]
				9.10	73.3[a]	20.22±1.95[a]
4.49	1.11			4.55	35.5[ef]	8.08±1.65[cd]
				9.10	46.6[cd]	12.42±2.09[bc]

Mean within a *column* followed by same *letter* are not significantly different according to Duncan's multiple range test (*DMRT*) (*P*<0.05)

[a] Data represented as mean

[b] Data represented as mean±SE

Histological and microscopic analysis Histological and microscopic analyses gave insight into the cellular origin of somatic embryos and morphological changes that occurred during the induction of somatic embryogenesis. Somatic embryos seemed to form from either a single cell or small group of cells in the leaf sheath explant (Fig. 3A). Cells in both epidermal and subepidermal regions of the explant that appeared competent for embryogenesis contained dense cytoplasm with

Table 2 Effects of cytokinins in combination with NAA on direct somatic embryogenesis of *C. amada*

| Plant growth regulators (μM) | | | | | | Explants responding with embryo induction (%)[a] | Number of somatic embryos per explant (mean±SE)[b] |
| SIM 1 | | SIM 2 | | | | | |
2,4-D	BA	BA	Kn	TDZ	NAA		
2.24	1.11	4.44			1.33	73.3[bc]	17.31±1.59[cd]
		8.88			1.33	86.6[a]	28.31±1.85[b]
4.49	1.11	4.44			1.33	37.7[gh]	10.13±1.96[ef]
		8.88			1.33	55.5[def]	16.77±2.26[cde]
2.24	1.11		4.64		1.33	48.8[efg]	13.08±2.02[cdef]
			9.29		1.33	59.9[de]	19.55±2.41[c]
4.49	1.11		4.64		1.33	31.1[h]	8.35±1.87[f]
			9.29		1.33	39.9[gh]	11.82±2.18[def]
2.24	1.11			4.55	1.33	82.2[ab]	32.48±2.28[b]
				9.10	1.33	93.3[a]	49.42±2.01[a]
4.49	1.11			4.55	1.33	46.6[fg]	17.48±2.28[cd]
				9.10	1.33	64.4[cd]	30.64±3.43[b]

Mean within a *column* followed by same *letter* are not significantly different according to Duncan's multiple range test (*DMRT*) (*P*<0.05)

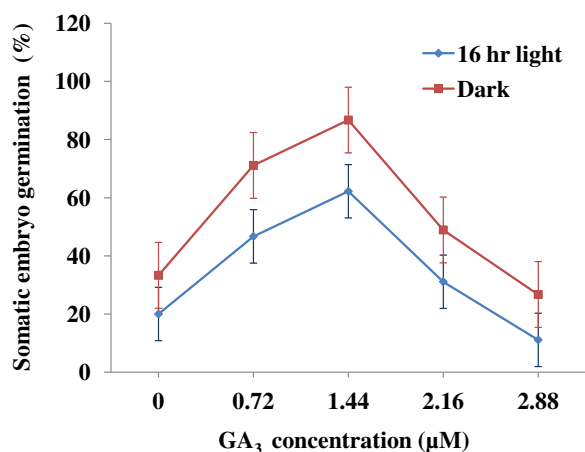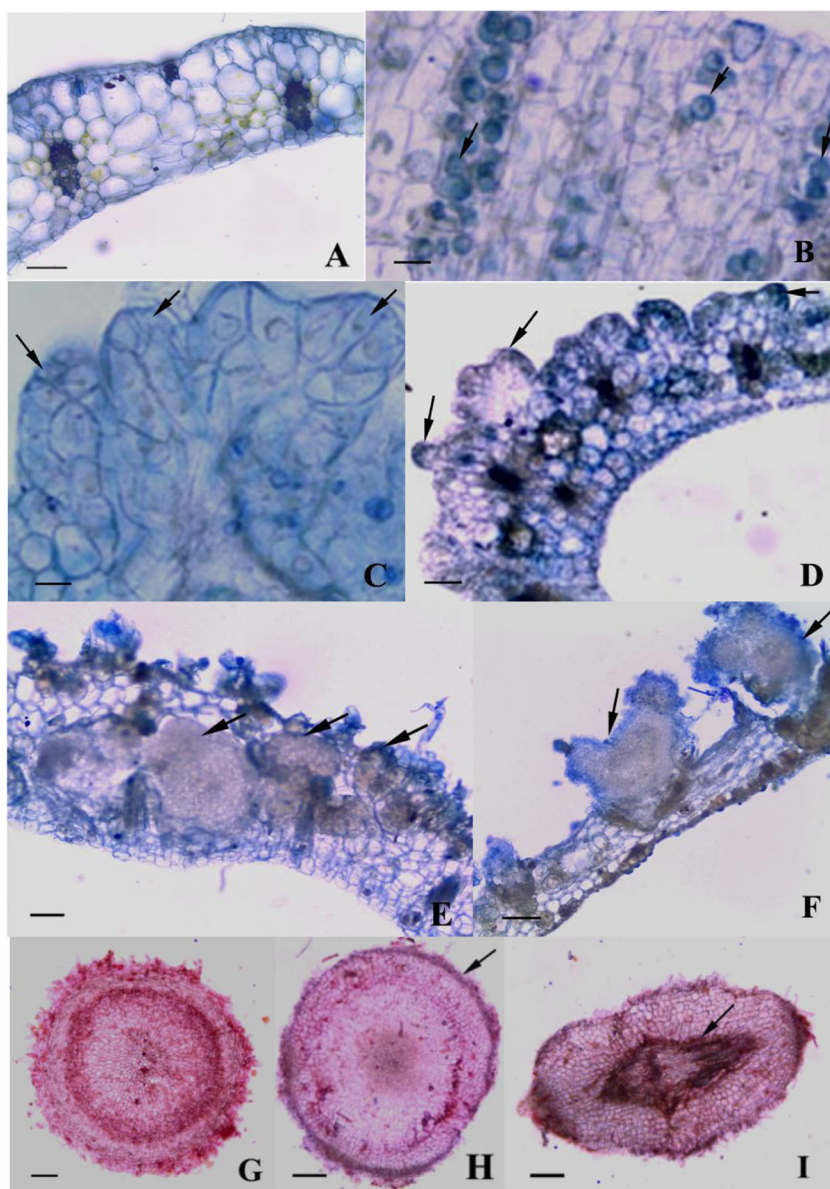[a] Data represented as mean

[b] Data represented as mean±SE

Figure 2. Effects of GA₃ and light condition on *C. amada* somatic embryo germination. Data are shown as mean±SE.

small vacuoles and evident nucleus (Fig. 3*B*), and were obtained within 14 d of culture on SIM 1. These embryogenic cells divided rapidly, and the embryos ultimately protruded from the surface of the explant after 7 d of culture on SIM 2 (Fig. 3*C, D*). Further differentiation of these embryos resulted in the formation of globular and elongated embryos after 15–21 d of culture (Fig. 3*E–G*). The clear presence of the protoderm (the outer most layer of a developing embryo) seen in the globular-stage embryos and the procambial strand was seen in the elongated-stage embryo confirms these to be somatic embryos directly formed from leaf sheath explants (Fig. 3*H, I*). The SEM analysis further confirmed the direct origin of the embryo from the leaf sheath explant (Fig. 4*A, B*) and development of globular embryos with no callus intermediate (Fig. 4*C, D*).

Figure 3. Histological evidence of direct somatic embryogenesis from leaf sheath explant of *C. amada*: (*A*) Transverse section of 3-mo-old *in vitro*-grown leaf sheath explant; (*B*) Embryogenic cells with prominent nucleus, small vacuole, and dense cytoplasm on 14 d of SIM 1 culture (*arrows*); (*C*) Active cell division and formation of embryo observed after 7 d of SIM 2 culture (*arrows*); (*D*) Histology of explant showing direct appearance of embryos (*arrow*); (*E, F*) Direct appearance of globular somatic embryos (*arrows*) from epidermal and subepidermal regions on 15 d of culture; (*G, H*) Globular-shaped somatic embryos with protoderm (*arrow*); (*I*) Elongated stage somatic embryo with procambial strand observed after 21 d of culture (*arrow*). *Bars*: (*A, B*) 100 μm; (*C, D*) 300 μm, (*E–H*) 500 μm, (*I*) 700 μm.

Figure 4. SEM showing direct somatic embryogenesis from leaf sheath explant of *C. amada*. (*A*, *B*) SEM images showing cell clusters on the surface of the explant observed after 7 d of SIM 2 culture (*arrows*), (*C*) Surface of the explant with globular somatic embryos during 15 d of culture (*arrows*), (*D*) Enlarged view of globular somatic embryo (*arrow*).
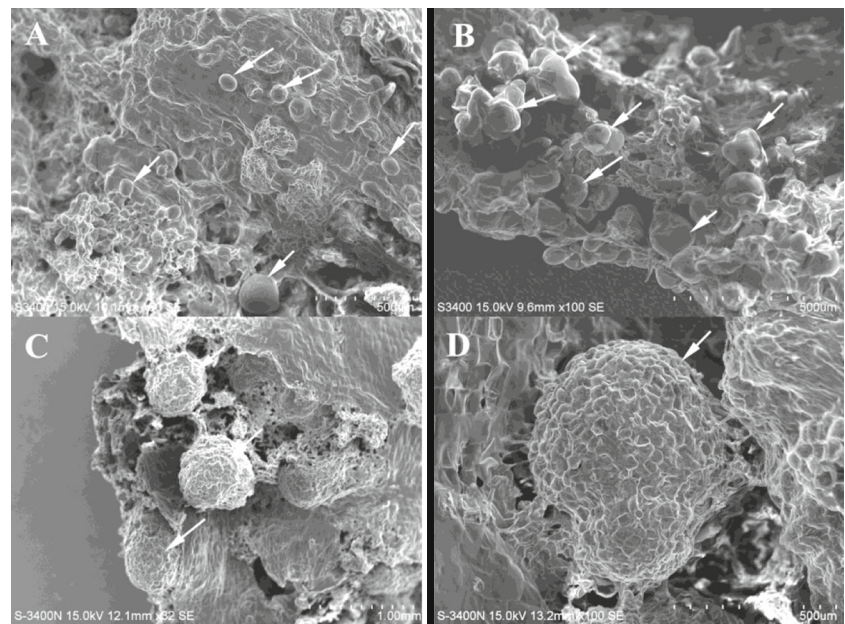
Discussion

We previously reported indirect regeneration system of somatic embryogenesis from callus-derived cell suspension cultures of *C. amada* (Soundar Raju *et al.* 2013). However, direct somatic embryogenesis is a more desirable approach to obtain regenerated plants, similar to the parent plants, since callus formation may cause somaclonal variation (Mizukami *et al.* 2008). Direct embryogenesis has been also reported to be useful for the regeneration of transgenic plants (Manoharan *et al.* 1998; Tokuji and Fukuda 1999). In this study, a high percentage of somatic embryo induction directly from leaf sheath explants was observed, without formation of an intermediate callus. In addition, this method is easier and requires less time than the previously described method of indirect regeneration. Therefore, direct somatic embryogenesis from leaf sheath explants could prove to be another effective regeneration system for more rapid propagation of mango ginger.

2,4-D is highly effective for initiation of somatic embryos in many plant species (Ammirato 1983; Fitch and Manshardt 1990; Haider *et al.* 1993; Anandan *et al.* 2012). Prolonged culture in high concentration of 2,4-D lead to rapid cell division, which can subsequently result in genetic variation among the *in vitro* propagated plants (Venkov *et al.* 2000). Our results showed that the primary treatment in SIM 1 containing a low concentration of 2,4-D and BA for 2 wk is enough to trigger direct somatic embryogenesis. Nhut *et al.* (2000) reported successful direct somatic embryogenesis in rice using explants pretreated with a low concentration of 2,4-D and BA, as their combination seemed to trigger the morphogenetic competency of the explant, leading to the reception of the signals for embryo development in several species (Franklin *et al.* 2006; Dam *et al.* 2010).

Among the three cytokinins, TDZ was better at inducing somatic embryos in SIM 2 than was BA and Kn. TDZ has been used for regeneration studies in many monocots, including banana (Srangsam and Kanchanapoom 2003), bamboo (Lin *et al.* 2004), and *Dendrobium* orchid (Chung *et al.* 2005). TDZ stimulated the conversion of cytokinin nucleotide to more biologically active nucleotides (Laloue and Pethe 1982) and to purine cytokinins. It also promoted the conversion of adenine to adenosine (Capelle *et al.* 1983).

In this study, the combination of 9.10 μM TDZ and 1.33 μM NAA was the most effective at inducing direct somatic embryogenesis than was TDZ alone. This result is consistent with other reports where TDZ in combination with auxins such as 2,4-D and indole-3-acetic acid produced significantly more somatic embryogenesis in plants like orchid sp. *Phalaenopsis* (Kuo *et al.* 2005) and *Digitalis trojana* (Verma *et al.* 2012).

In somatic embryos of some species, particularly of plants that undergo dormancy in natural seeds, the germination and growth of embryos into plants can be stimulated by the application of GA_3 in the culture medium (Gaj 2004; Manrique-Trujillo *et al.* 2013). The germination of somatic embryo of some monocots was enhanced by dark conditions (Nhut *et al.* 2000). In the present study, somatic embryos cultured on 1/2 strength MS medium containing GA_3 under dark condition showed enhanced germination. Similar results on stimulatory effects of GA_3 were reported for *Sesamum indicum* (Xu *et al.* 1997), *Eleutherococcus senticosus* (Choi *et al.* 1999), and *Panax notoginseng* (You *et al.* 2012).

The histological study showed that epidermal and subepidermal cells were the source of somatic embryos in *C. amada*. The initial cell divisions occurred in the epidermal and subepidermal cells of the explant. This regeneration pathway is common in several monocots including river lily (Slabbert *et al.* 1995) and açaí palm (Scherwinski-Pereira *et al.* 2012). Further, the formation of embryogenic cells with prominent nuclei, small vacuoles, and dense cytoplasm was common for several plant species (Paul *et al.* 2011). Globular and elongated embryo stages were regarded as key stages in the identification of somatic embryos (Godbole *et al.* 2002). The presence of protoderm and procambial strands in the developing embryos are the additional key features, which could be used as indicators for somatic embryo formation (Quiroz-Figueroa *et al.* 2002; Sharma and Millam 2004; Jalil *et al.* 2008). Histological study of globular and elongated structure in the present study showed clear evidence of protoderm and procambial strands in the developing somatic embryos of *C. amada*.

Conclusion

This is the first report of direct somatic embryogenesis from leaf sheath explants in mango ginger. This protocol can be used for the multiplication of valuable germplasm on a large scale at a much faster rate, and is easier, compared to the previous method of indirect somatic embryogenesis. Furthermore, these tissues should also be useful for the introduction of genes conferring resistance to pathogens using genetic engineering. Although mango ginger somatic embryo-derived plantlets could be acclimatized and grown under *ex vitro* condition, the degree to which they display a normal appearance and genetic fidelity needs to be confirmed.

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