Plant regeneration in *Chlorophytum borivillianum* Sant. et Fernand. from embryogenic callus and cell suspension culture and assessment of genetic fidelity of plants derived through somatic embryogenesis

Mohd. Zahid Rizvi · Arun Kumar Kukreja ·
Narendra Singh Bisht

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Abstract Efficient *in vitro* propagation of medicinally important endangered plant *C. borivillianum* has been achieved through somatic embryogenesis. Solid embryogenic medium [Murashige and Skoog medium containing 1.79 mM NH_4NO_3 , 10.72 mM KNO_3 , 1.13 μM 2,4-dichlorophenoxyacetic acid, 7.38 μM 2-isopentenyladenine and 0.76 mM proline] supplemented with polyethylene glycol and sucrose (3 % each), exhibited 1.88-fold increase in embryo maturation compared to embryogenic medium containing 3 % sucrose. Liquid embryogenic medium supported better somatic embryo production and maturation. Highest total (79) and mature (cotyledonary stage) somatic embryos (38) as well as highest germination (57.5 %) was observed at inoculum density of 0.4 g/40 ml of liquid medium. 5.86 pH level exhibited optimal growth, maturation and germination of somatic embryos. Random amplified polymorphic DNA (RAPD) analysis of *C. borivillianum* plants regenerated through somatic embryogenesis revealed that they were genetically similar to the mother plant. The protocol established in the present study can be used for rapid mass

multiplication of *C. borivillianum* in bioreactor employing liquid medium.

Keywords *Chlorophytum borivillianum* · Genetic fidelity · Maturation · Polyethylene glycol · Somatic embryos

Introduction

Tuberous roots of monocotyledonous plant safed musli (*Chlorophytum borivillianum* Sant. et Fernand.) possess immunomodulatory, adaptogenic, aphrodisiac and diuretic properties due to the presence of steroidal saponins (Kirtikar and Basu 1975; Ramawat et al. 1988; Tandon and Shukla 1995). Saponins are used in the industrial production of sex hormones, corticosteroids and steroid derivatives. The roots are used in the Indian system of medicine for the treatment of general debility, weakness and impotency. They are also used as cardiac and brain tonic; as a curative agent in various diseases like piles, diabetes and as anti-pyretic, diuretic and hemostatic. *C. borivillianum* has been used in many herbal and Unani formulations in India along with other plants such as *Asparagus adscendens*, *A. racemosum*, *Curculigo orchoides* and *Withania somnifera* (Kirtikar and Basu 1975; Ramawat et al. 1988). Because of its medicinal properties and diversified uses, there is demand for safed musli especially *C. borivillianum* in India. Many countries in Gulf, Europe and USA have been major importers of the dry roots of safed musli for preparation of various herbal products in pharmaceutical, phytopharmaceutical and nutraceutical industries. While the global demand for dry safed musli roots has been estimated in the order of 35,000 tonnes per annum, the supply stands

M. Z. Rizvi · A. K. Kukreja (✉)
Plant Tissue Culture Division,
Central Institute of Medicinal and Aromatic Plants (CIMAP),
Lucknow, P.O. CIMAP-226015, Uttar Pradesh, India
e-mail: akkukreja53@gmail.com

N. S. Bisht
Department of Botany,
Hemwati Nandan Bahuguna Garhwal University (Pauri Campus),
Garhwal-246174, India

Present Address:

M. Z. Rizvi
Department of Botany, Shia Post Graduate College,
Sitapur Road,
Lucknow-226020, India

at 5,000 tonnes per year (www.farmwealthbiotech.com, 2010). The plant regenerates in nature sexually by seeds or more frequently by vegetative propagation through shoot buds from perennating root tubers in the soil. It has low seed set, poor seed germination and long dormancy period leading to scarcity of tuberous roots of the plant in nature (Bordia et al. 1995). The slow growth rate in conventional vegetative propagation is not sufficient for meeting demand of this plant (Bordia et al. 1995). Excessive collections from natural habitats and destructive harvesting methods coupled with poor seed germination and low vegetative multiplication ratio have made the species endangered (Gupta and Chadha 1995). The plant has been enlisted as an endangered plant species in Red Data Book of India (Nayar and Sastry 1988) and National Medicinal Plant Board (NMPB), India has categorized it as one of the prioritized plant species to be promoted for conservation and large-scale cultivation and export of this herb is being actively encouraged by Govt. of India through NMPB. The commercial cultivation has been limited due to scarcity of elite and characterized planting material and non-availability of improved agrotechnological practices. Therefore to fill the gap between demand and supply of its tuberous roots to meet industry's requirement and to provide cost-effective genetically uniform planting material for conventional propagation methods at a rapid rate in a short span of time, alternate propagation strategies such as tissue culture are urgently needed which will lead towards conservation and mass production of the plant.

Somatic embryogenesis has become more frequently used technique for large-scale *in vitro* propagation of plants because it is less labour intensive and thus commercially more viable. The embryogenic suspension culture has great potential for large-scale clonal propagation at rapid rate. Earlier attempts have been made for *in vitro* propagation of *Chlorophytum borivilium* (family Liliaceae) through somatic embryogenesis by Purohit et al. (1994) and Rizvi et al. (2010) but precocious germination of somatic embryos, low germination rate and profuse rooting were observed. Genetic uniformity and stability of the tissue culture raised progeny is an important parameter of a micro-propagation protocol. Although micropropagated plants maintain the genetic fidelity still the possibility of generating somaclonal variations exists (Bindiya and Kanwar 2003). A number of molecular markers can be used to assess the genetic fidelity of *in vitro* derived plants. However random amplified polymorphic DNA (RAPD) is efficient and cheapest among these markers (Jayanthi and Mandal 2001). In the present investigation, culture conditions were established for improved maturation and germination of somatic embryos and plantlet development in *C. borivilium* from embryogenic suspension culture. The genetic fidelity of plants regenerated through somatic embryogenesis was also reported using RAPD markers.

Materials and methods

Maintenance of mother plants

Mother plants were obtained from natural habitat (Rajsa-mand District in the State of Rajasthan). Amongst these mother plants, a high yielding line of *C. borivilium* selected on the basis of root yield in a CIMAP project, was maintained in Botanical Garden of the Institute. This selected high yielding line was used as the initiation plant material. Seeds obtained from this line of *C. borivilium* were used for callus induction.

Culture initiation and maintenance

Seedlings were obtained from surface sterilized seeds (0.1 %, w/v aqueous mercuric chloride solution for 5–6 min) germinated *in vitro* on Murashige and Skoog (MS) medium (1962) supplemented with 57.74 μ M gibberellic acid (GA_3). Hypocotyls from seedlings were implanted on optimal callus induction medium i.e. MS medium supplemented with 1.16 μ M kinetin (Kn) and 1.13 μ M 2,4-dichlorophenoxy-acetic acid (2,4-D), earlier standardized amongst different levels of Kn (0.47–2.32 μ M) and 2,4-D (0.45–4.52 μ M) tested in *C. borivilium* (Rizvi et al. 2010).

Induction and maintenance of embryogenic callus

Callus obtained on callus induction medium showed optimal embryogenic response on modified MS medium containing 1.79 mM NH_4NO_3 , 10.72 mM KNO_3 , 1.13 μ M 2,4-D, 7.38 μ M 2-isopentenyladenine (2-iP) and 0.76 mM proline, hereinafter designated as embryogenic medium. This medium was standardized in earlier experiments by testing effects of NH_4NO_3 (1.79–10.70 mM yielding 50–300 mg nitrogen), KNO_3 (3.56–21.45 mM yielding 50–300 mg nitrogen), 2,4-D (0.45–4.52 μ M), 2-iP (0.98–7.38 μ M) and proline (0.38–2.29 mM) on somatic embryogenesis in *C. borivilium* (Rizvi et al. 2010).

Somatic embryo maturation and germination

In the present study, culture conditions were standardized for efficient maturation and germination of somatic embryos. Experiments were conducted to observe the effect of different carbon sources, osmotica and abscisic acid (ABA) in solid embryogenic medium on somatic embryo maturation and germination. Subsequently effect of different physical states (solid or liquid) of standardized medium on somatic embryogenesis was observed. Experiments were also conducted for observing effect of casein acid hydrolysate (CH), different inoculum density and pH levels in liquid medium on growth, development and germination of somatic embryos.

Carbon sources and osmotic agents

Different carbon sources *viz.* sucrose, glucose, fructose, lactose, galactose, cellobiose, rhamnose, xylose, arabinose, maltose, mannose, soluble starch (each at 3 %, *w/v*) were supplemented to agar-solidified embryogenic medium. Embryogenic medium without any carbon source served as control. Plasmolyzing osmotic agents, D-mannitol or D-sorbitol and non-plasmolyzing osmoticum, polyethylene glycol (PEG, molecular weight 4,000), each at 1–11 % (*w/v*) were supplemented to embryogenic medium with 3 % sucrose. Embryogenic medium containing 3 % sucrose served as control.

Absciscic acid

ABA was supplemented in the range of 0.1–7.0 μM to embryogenic medium containing 3 % sucrose to observe its effect on somatic embryo maturation. ABA was filter sterilized and added to culture media after it was cooled to about 40 °C and stored in dark. Control was embryogenic medium with 3 % sucrose.

Physical state of culture medium

Hypocotyl derived embryogenic callus was inoculated in 40 ml agar-solidified (0.8 %, *w/v*) or liquid (without agar) embryogenic medium containing sucrose and PEG (3 % each). The solid embryogenic medium supplemented with sucrose and PEG (3 % each) served as control.

Casein acid hydrolysate

Embryogenic cell aggregates were inoculated in liquid embryogenic medium having PEG and sucrose (3 % each) and supplemented with (1–4 %, *w/v*) of casein acid hydrolysate (CH). The liquid embryogenic medium supplemented with sucrose and PEG (3 % each) served as control.

Inoculum density and pH levels

To analyze the effect of different inoculum densities, 0.1–1.0 g embryogenic callus was inoculated in 40 ml liquid embryogenic medium containing sucrose and PEG (3 % each). Liquid embryogenic medium having 0.15 g inoculum density was treated as control. Optimal level (0.4 g) of embryogenic cell mass was inoculated in liquid embryogenic medium supplemented with sucrose and PEG (3 % each) and having different pH levels ($3.86\text{--}7.86\pm 0.1$). The liquid embryogenic medium having 5.86 pH level served as control. The liquid cultures were kept on a rotary shaker (New Brunswick Scientific, USA) at 100 rpm agitation.

Germination of somatic embryos

Mature (cotyledonary stage) somatic embryos developed on culture media having different treatments were transferred to optimal embryo germination medium *i.e.* MS medium supplemented with 15.54 μM 6-benzylaminopurine (BAP). In our earlier experiments, amongst a wide range of BAP (6.66–28.86 μM) tested for their efficacy on somatic embryo germination in *C. borivilianum*, BAP at 15.54 μM exhibited optimal germination (Rizvi et al. 2010).

The medium was solidified with 0.8 % (*w/v*) agar (Hi-Media, India). In liquid medium agar was excluded. The pH of the medium was adjusted to 5.8 ± 0.1 (except in case of different pH levels experiment) using 0.1 N HCl or 0.1 N NaOH prior to autoclaving at 121 °C temperature and 15 lb pressure for 20 min. 0.15 g of callus (except where mentioned otherwise) was inoculated per conical flask (100 ml capacity, Borosil) containing 40 ml of liquid or solid medium. Each treatment had fifteen replicates and all experiments were repeated thrice. Cultures were maintained in a culture room having 16 h light/8 h dark photoperiod, $45\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ light intensity provided by cool white fluorescent tubes (Philips, India), 25 ± 2 °C temperature and 50–60 % relative humidity. Observations on callus fresh wt., number of total somatic embryos, number of mature (cotyledonary stage) somatic embryos and number of somatic embryos germinated/20 mature embryos were recorded after 4 weeks.

Hardening and transplantation

Plantlets obtained from *in vitro* germinated somatic embryos after 6 weeks of culture of mature somatic embryos on embryo germination medium were hardened in same culture at room conditions as described above for 2 weeks in $\frac{1}{4}$ strength MS nutrient solution. These plants were transferred to earthen pots containing sand:soil:farmyard manure in 1:1:1 (*v/v*) ratio and covered with transparent polythene bags with small holes. The polythene bags were removed after 2 weeks and the surviving plants were maintained in the glass house.

Histological analysis

Subcultured embryogenic tissues of *C. borivilianum* during 4–6 weeks of culture duration having somatic embryos at different developmental stages were fixed in FAA (formaldehyde:glacial acetic acid:50 % ethyl alcohol in ratio of 5:5:90, *v/v*) solution. The tissues were dehydrated through a graded ethanol series (50, 70, 90 and 100 %), embedded in paraffin wax (congealing point 58–60 °C, Qualigens, India) and serially sectioned (8–10 μM thickness). Sections were stained with safranin and light green, mounted in Canada

Balsom (Thomas Baker, India) and photographed under a photomicroscope (Nikon FX-35 A, Japan).

Genetic fidelity of plants regenerated through somatic embryogenesis: RAPD fingerprinting

Genomic DNA was isolated from fresh and young leaf tissue of 17 *C. borivilianum* plants after 12 weeks of their transplantation in pots along with mother plant following the modified cetyl trimethyl ammonium bromide (CTAB) method (Khanuja et al. 1999). These 17 plants were randomly selected from progeny of 56 plants regenerated *in vitro* through somatic embryogenesis and established in glass house. Quality of the DNA was checked on 0.8 % (m/v) agarose gel by loading an aliquot of samples as well as spectrophotometrically (Nanodrop ND-1000, U.S.A.) by measuring the absorbance at 260 nm. Isolated DNA was amplified through polymerase chain reaction (PCR) using twenty different MAP primers (MAP 01 to MAP 20; Bangalore Genei, India). The PCR amplification reactions were carried out using the thermal cycler [DNA Engine PTC-2000 (M.J. Research, USA)] following the protocol of Khanuja et al. (2000). PCR amplification was carried out in 25 µl volume using 20 decamer primers. The reaction mixture consisted of 25 ng of DNA, 0.6 units of Taq DNA Polymerase, 10 mM of each dNTPs (ATP, GTP, CTP, TTP), 2.5 µl of 10X polymerase buffer (100 mM Tris–HCl; pH 8.3, 500 mM KCl and 15 mM MgCl₂) and 5 pmol of decanucleotide primer. The amplification products were separated electrophoretically on 1.2 % agarose gel containing 0.5 g/ml ethidium bromide using 50 mA current for 3 h. The bands were visualized and photographed using Gel Documentation System (Pharmacia Biotech, USA). Bands were scored as present or absent for each

sample including both micropropagated as well as mother plant. RAPD bands within samples were scored as missing if they were poorly resolved on the gel or if the template DNA did not amplify well. The RAPD experiments were repeated twice to confirm the consistency of results.

Results and discussion

Maturation and germination of somatic embryos

Carbon sources, osmotic agents and abscisic acid

Amongst all carbon sources added to solid embryogenic medium, highest number of total (44) and mature (17) somatic embryos were observed on sucrose (3 %), (Table 1; Fig. 1a) followed by glucose and maltose. Somatic embryogenesis was not observed in control medium (solid embryogenic medium without any carbon source) and medium supplemented with arabinose, galactose, lactose and soluble starch. The mature (cotyledonary) somatic embryos produced on embryogenic medium containing 3 % sucrose showed higher germination (30 %) on transfer to embryo germination medium (MS medium supplemented with 15.54 µM BAP), (Table 1; Fig. 1e). The type of carbon source can play vital role in somatic embryogenesis. In *Panax vietnamensis*, sucrose at 3 % level was most effective for somatic embryogenesis as compared to glucose and fructose (Nhut et al. 2011). In the studies of Gerdakaneh et al. (2009) also, sucrose was the best carbon source for induction and development of somatic embryos. Hong et al. (2008) reported that in somatic embryogenesis of *Oncidium*, maltose was less effective as compared to sucrose

Table 1 Effect of different carbon sources on somatic embryogenesis in *Chlorophytum borivilianum*

Carbon source (3 %)	No. of total somatic embryos	No. of mature (cotyledonary) embryos	No. of embryos germinated/20 mature embryos	Germination percentage (%)
Control ^a	—	—	—	—
Sucrose	44.00±2.21 ^b	17.00±1.24	6.00±1.00	30.00
Glucose	41.00±2.10	14.00±1.05	4.00±0.67	20.00
Fructose	27.00±1.24	3.00±0.66	1.00±0.47	5.00
Lactose	—	—	—	—
Galactose	—	—	—	—
Cellobiose	22.00±1.22	2.00±0.66	1.00±0.47	5.00
Rhamnose	11.00±1.05	—	—	—
Xylose	17.00±1.41	—	—	—
Arabinose	—	—	—	—
Maltose	30.00±1.33	6.00±0.81	3.00±0.81	15.00
Mannose	28.00±1.33	2.00±0.47	—	—
Soluble starch	—	—	—	—

^aAgar-solidified MS medium containing 1.79 mM NH₄NO₃, 10.72 mM KNO₃, 1.13 µM 2,4-D, 7.38 µM 2-iP, 0.76 mM proline (embryogenic medium) without any carbon source

^bAverage value ± Standard Error (SE)

— No response

Fig. 1 Plant regeneration through somatic embryogenesis in *C. borivilianum*. **a.** mature somatic embryo on solid embryogenic medium [MS medium containing 1.79 mM NH_4NO_3 , 10.72 mM KNO_3 , 1.13 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 7.38 μM 2-isopentenyladenine (2-iP) and 0.76 mM proline] with 3 % sucrose after 4 weeks of culture (inset **a.** induction and development of somatic embryos on same medium); **b.** mature somatic embryo after 4 weeks of culture on solid embryogenic medium containing sucrose and PEG (polyethylene glycol), (3 % each); **c.** somatic embryos at different stages of maturation in liquid embryogenic medium after 4 weeks of culture; **d.** a mature somatic embryo in liquid medium; **e.** plantlets regenerated after 4 weeks of culture of mature somatic embryos on solid MS medium supplemented with 15.54 μM 6-benzylaminopurine

(BAP), (somatic embryos matured on solid embryogenic medium containing 3 % sucrose); **f.** plantlets regenerated after 4 weeks of culture of mature somatic embryos on solid MS medium having 15.54 μM BAP, [somatic embryos matured on embryogenic medium containing sucrose and PEG (3 % each)]; **g.** plantlets regenerated after 4 weeks of culture of somatic embryos on liquid MS medium containing 15.54 μM BAP, (somatic embryos matured in liquid embryogenic medium); **h.** somatic embryo derived plantlet with developed root system after 6 weeks of culture; **i.** *C. borivilianum* plant regenerated through somatic embryogenesis in earthen pot after 11 weeks of hardening; **j.** globular somatic embryos appearing from surface of embryogenic cell mass in histological section; **k.** somatic embryos at advanced stage of maturation

while cellobiose was inhibitory to somatic embryogenesis. In the present investigation also sucrose (3 %) was best carbon source for somatic embryogenesis in *C. borivilianum* compared to other carbon sources which inhibited embryogenesis either partially or completely.

The embryogenic callus cultured on solid embryogenic medium containing 3 % sucrose and D-mannitol or D-sorbitol (1–11 %) became necrotic and therefore somatic embryogenesis was inhibited at culture medium containing D-mannitol or D-sorbitol. While on the embryogenic medium supplemented with 3 % each of PEG and sucrose, 1.88-fold increase in number of mature somatic embryos was observed compared to control medium (embryogenic medium with 3 % sucrose) (Table 2; Fig. 1b). Higher embryo germination (50 %) compared to only 30 % on control medium was recorded, (Table 2) when mature somatic

embryos from medium containing PEG and sucrose (3 % each) were transferred to embryo germination medium (Fig. 1f). Amongst different ABA levels (0.1–7.0 μM) tested, mature (cotyledonary stage) embryos were obtained only at 0.5 μM and 1.0 μM but the number of cotyledonary embryos were lower than even obtained on control medium (embryogenic medium with 3 % sucrose only); (Table 3). Somatic embryo germination was observed only at 1.0 μM ABA, recording only 5 % germination frequency which was significantly lower than even on control medium (30 %). On other ABA levels (0.1, 3.0, 5.0 or 7.0 μM) embryo maturation and germination were completely inhibited (Table 3). Embryos became brown and necrotic and did not develop further.

Addition of sorbitol or mannitol to the embryogenic medium inhibited somatic embryogenesis in *C. borivilianum* in the present study consistent with the observations of

Table 2 Effect of different osmotica and 3 % sucrose on somatic embryo maturation and germination in *C. borivilianum*

	Osmoticum concentration (%)	No. of total somatic embryos	No. of mature (cotyledonary) embryos	No. of embryos germinated/20 mature embryos	Germination percentage (%)
	Control ^a	44.00±2.21 ^b	17.00±1.24	6.00±1.00	30.00
	Polyethylene glycol (PEG)				
	1	51.00±3.62	28.00±2.0	8.00±1.05	40.00
	3	56.00±3.05	32.00±3.67	10.00±1.24	50.00
	5	40.00±3.43	18.00±2.16	6.00±0.94	30.00
	7	30.00±3.19	12.00±1.19	4.00±0.66	20.00
	9	26.00±2.35	8.00±1.05	2.00±0.63	12.00
	11	15.00±1.69	4.00±0.81	–	–
	Mannitol (1–11)	–	–	–	–
	Sorbitol (1–11)	–	–	–	–

^aEmbryogenic medium supplemented with 3 % sucrose^bAverage value ± Standard Error (SE)

– Callus cultured on solid embryogenic medium containing D-mannitol or D-sorbitol (1–11 %) became necrotic and therefore somatic embryogenesis was inhibited

Eapen and George (1990) and Walker and Parrott (2001) in peanut and soybean respectively. On the other hand in present investigation, PEG was effective in promoting embryo maturation and germination, reported in *C. borivilianum* for first time. In earlier studies, low somatic embryo germination rates (20 % and 30 %) were reported by Purohit et al. (1994) and Rizvi et al. (2010) respectively, compared to higher germination rate (50 %) recorded in the present study on PEG containing medium. In *Phoenix dactylifera* (Alkhateeb 2006) also addition of PEG to sucrose containing medium improved maturation and germination frequency of somatic embryos.

Physical state of medium

Compared to solid culture medium (embryogenic medium containing 3 % each of sucrose and PEG and 0.8 % agar), liquid medium (without agar) supported better growth and maturation of somatic embryos (Table 4). An average of total 66 somatic embryos were observed in liquid medium compared to only 54 on solid medium. The number of cotyledonary stage (mature) somatic embryos, their fresh weight and

germination rate were also higher in liquid medium (Table 4; Fig. 1g). Somatic embryos induced and developed in liquid medium were translucent, whitish and long (Fig. 1c, d). Therefore in the present study liquid medium was found to be more effective for somatic embryogenesis than agar-solidified medium in *C. borivilianum*. In earlier studies of Martin (2003) and Othmani et al. (2009) liquid suspension culture was reported to be superior for somatic embryogenesis as compared to agar-solidified medium. Regeneration through somatic embryogenesis in *C. borivilianum* using liquid medium has not been reported so far. For scale-up of cultures in bioreactors for mass propagation of plants, liquid culture medium has been recommended (Kim et al. 2004; Paek et al. 2005). The large-scale propagation of plants through somatic embryogenesis in liquid medium has a number of advantages compared to solid medium such as constant availability of embryogenic-competent cells, opportunities for bioprocess automation and reduced manual labour. Liquid medium is more cost-effective than solid medium as agar which is a commonly used solidifying agent adds upto 65 % of the cost of the culture medium (Mitra et al. 1998).

Table 3 Effect of different ABA levels on maturation and germination of somatic embryos in *C. borivilianum*

	ABA concentration (μM)	No. of total somatic embryos	No. of mature (cotyledonary) embryos	No. of embryos germinated/20 mature embryos	Germination percentage (%)
	Control ^a	44.00±2.21 ^b	17.00±1.24	6.00±1.00	30
	0.1	5.00±0.63	–	–	–
	0.5	14.00±1.22	2.00±0.47	–	–
	1.0	20.00±1.41	4.00±0.66	1.00±0.47	5
	3.0	–	–	–	–
	5.0	–	–	–	–
	7.0	–	–	–	–

^aEmbryogenic medium supplemented with 3 % sucrose^bAverage value ± Standard Error (SE)

– No response

Table 4 Effect of different physical states of medium on somatic embryogenesis

Physical state of medium	No. of total somatic embryos	No. of globular somatic embryos	No. of mature (cotyledonary) embryos	Fresh wt. (g) of somatic embryos	No. of embryos germinated/20 mature embryos	Germination percentage (%)
Solid (control) ^a	54.00±3.16 ^b	16.00±1.58	26.00±2.12	0.19±0.02	10.00±1.24	50.00
Liquid	66.00±3.87	20.00±2.23	32.00±2.34	0.28±0.03	10.60±1.34	53.00

^a Agar-solidified (0.8 %) embryogenic medium supplemented with sucrose and PEG (3 % each)^b Average value ± Standard Error (SE)

Casein acid hydrolysate

Casein acid hydrolysate (CH) did not have any stimulatory effect on somatic embryogenesis in *C. borivilianum*. Amongst different CH levels tested (1–4 %), mature (cotyledonary stage) somatic embryos were observed only at 1 % and 2 % CH levels. But their number was significantly lower compared to control medium (liquid embryogenic medium with 3 % each of sucrose and PEG). At other CH levels tested (3 % and 4 %), embryos were in globular stage (Table 5). In liquid medium having different CH levels (1–4 %) root like structures developed from cell aggregates and cultures turned non-embryogenic. Germination of somatic embryos was completely inhibited at all tested levels (1–4 %) of CH (Table 5). In suspension cultures of *Dactylis glomerata*, casein hydrolysate stimulated the root development (Gray and Conger 1985) consistent with the observations in the present study in *C. borivilianum*. In *Picea glauca* somatic embryos matured in the presence of casein hydrolysate while amino acid glutamine was absent from the culture medium (Malabadi et al. 2011). In the studies of Cardoza and D'Souza (1999), supplementation of casein hydrolysate to half strength MS medium increased the somatic embryogenesis and plant regeneration from somatic embryos. Casein hydrolysate enhanced somatic embryo induction and plant regeneration in sugarcane calli also (Nasir et al. 2011).

Inoculum density

Maturation of somatic embryos was promoted at lower inoculum density levels (0.1–0.4 g/40 ml culture medium) while at higher inoculum density levels (0.6–1.0 g/40 ml culture medium), low maturation was observed. The highest average number of total somatic embryos (79), mature embryos (38) and highest fresh weight of somatic embryos were observed at 0.4 g/40 ml inoculum density level (Table 6). Mature somatic embryos produced on 0.4 g/40 ml inoculum density level exhibited highest germination (57.5 %) on transfer to embryo germination medium (Table 6). Embryo germination decreased at inoculum density levels more than 0.4 g/40 ml and was completely inhibited at 1.0 g/40 ml inoculum density level. Highest embryo germination was observed at 0.4 g/40 ml inoculum density level which exhibited highest somatic embryo maturation rate. In embryogenic suspension cultures, cell density could be an important factor that affects somatic embryogenesis. A relatively lower cell density favours the maturation of embryos from embryogenic cells (Fujimura and Komamine 1979) while a high cell density does not promotes the maturation of embryos (Nomura and Komamine 1985). This may be due to the secretion of proteins and/or other cellular factors into the culture medium (Gavish et al. 1992). In the present investigation inoculum density of 0.4 g/40 ml of medium was optimal for maturation of somatic embryos. Higher inoculum densities

Table 5 Effect of different levels of casein acid hydrolysate on somatic embryogenesis

	Casein acid hydrolysate (%)	No. of total somatic embryos	No. of globular somatic embryos	No. of mature (cotyledonary) somatic embryos	Fresh wt. (g) of somatic embryos	No. of embryos germinated/20 mature embryos	Germination percentage (%)
^a Liquid embryogenic medium supplemented with sucrose and PEG (3 % each)	Control ^a	79.00±5.09 ^b	22.00±1.87	38.00±2.34	0.32±0.02	–	–
	1	35.00±2.83	21.00±2.12	4.00±0.70	0.14±0.02	–	–
	2	44.00±3.93	26.00±2.34	7.00±0.80	0.18±0.03	–	–
	3	20.00±2.0	15.00±1.58	–	0.08±0.01	–	–
	4	15.00±2.0	12.00±1.22	–	0.06±0.02	–	–

^b Average value ± Standard Error (SE)

– No response

Table 6 Effect of different inoculum densities on maturation and germination of somatic embryos in *C. borivilianum*

Inoculum density (g of cell aggregates/40 ml of liquid medium)	No. of total somatic embryos	No. of globular somatic embryos	No. of mature (cotyledonary) somatic embryos	Fresh wt. (g) of somatic embryos	No. of embryos germinated/20 mature embryos	Germination percentage (%)
0.10	40.00±3.39 ^b	14.00±1.58	16.00±1.22	0.16±0.02	6.00±0.94	30.00
0.15 (control) ^a	66.00±3.87	20.00±2.23	32.00±2.34	0.28±0.03	10.60±1.34	53.00
0.20	61.00±3.80	17.00±1.87	28.00±2.00	0.24±0.02	10.00±1.24	50.00
0.40	79.00±5.09	22.00±1.87	38.00±2.34	0.32±0.02	11.50±1.22	57.50
0.60	55.00±3.80	23.00±1.87	18.00±1.58	0.21±0.02	7.00±0.89	35.00
0.80	38.00±3.16	19.00±1.58	8.00±1.00	0.15±0.01	2.00±0.63	12.00
1.00	30.00±2.24	21.00±2.12	4.00±1.00	0.13±0.01	–	–

^a Liquid embryogenic medium supplemented with sucrose and PEG (3 % each) and inoculum density of 0.15 g/40 ml of medium ^b Average value ± Standard Error (SE)

– No response

(>0.4 g/40 ml of medium) did not favoured development and maturation of embryos. In banana hybrid cultivar FHIA-18 (AAAB), inoculum density of 0.6 g/25 ml of culture medium was best for somatic embryo growth and multiplication (Kosky et al. 2002). Inoculum density of 3.0 g/20 ml of medium (with 3 % sucrose) showed a 2-fold increase in cell growth in *Lilium x formolongi* Hort. cv. Norikula (Ho et al. 2006) while higher inoculum densities (3.5 and 4.0 g/20 ml) were unfavourable for the growth.

pH levels

Maturation of somatic embryos was promoted between 4.86 and 6.86 pH levels, while at acidic (pH level 3.86) and alkaline (pH level 7.86) media, most of the embryos were in globular stage and their maturation was suppressed. Highest somatic embryo maturation and fresh weight were observed in embryogenic liquid culture medium at pH level 5.86 (control), (Table 7). Mature embryos produced at this

pH level exhibited highest (57.5 %) embryo germination when transferred to embryo germination medium (Table 7). It appears that pH requirements are species specific and developmental stage dependent. In banana hybrid cultivar FHIA-18 (AAAB), acidic medium (pH level 3.8) resulted in inhibition of embryo maturation (87.2 % globular stage embryos), (Kosky et al. 2002). Smith and Krikorian (1990) also reported that in carrot lower pH level (acidic medium) supported embryo development only upto pre-globular stage. The effect of high or low pH on inhibition of embryo development may be subsequently utilized for synchronization of somatic embryos to the initial stages of embryo development. The homogeneity thus obtained in embryo development will be beneficial in handling cultures for plant propagation through somatic embryogenesis at large-scale.

Plants regenerated *in vitro* through somatic embryogenesis and established in glass house were compared for their morphological characters viz. leaf length, leaf width, leaf colour, root length, weight *etc.* after 12 weeks of transplantation in

Table 7 Effect of different pH levels on somatic embryogenesis

pH levels	No. of total somatic embryos	No. of globular somatic embryos	No. of mature (cotyledonary) somatic embryos	Fresh wt. (g) of somatic embryos	No. of embryos germinated/20 mature embryos	Germination percentage (%)
3.86	39.00±2.55 ^b	19.00±1.58	7.00±1.22	0.16±0.02	–	–
4.86	68.00±4.47	21.00±1.41	29.00±2.12	0.27±0.03	7.00±0.71	35.00
5.86(control) ^a	79.00±5.09	22.00±1.87	38.00±2.34	0.32±0.02	11.50±1.22	57.50
6.86	52.00±4.06	15.00±1.00	23.00±2.12	0.21±0.02	7.60±0.89	38.00
7.86	27.00±2.73	13.00±1.22	7.00±1.0	0.11±0.01	–	–

^a Liquid embryogenic medium supplemented with sucrose and PEG (3 % each) and inoculum density of 0.15 g/40 ml of medium with 5.86 pH level

^b Average value ± Standard Error (SE)

– No response

pots. Performance of these plants was uniform and they were morphologically similar as compared to the mother plant (Fig. 1h, i). The average leaf length in regenerated plants was 13.7 cm while average leaf width was 1.10 cm. The shoots of regenerated plants were green. About 4–5 fascicled storage roots with an average length of 3.97 cm and mean fresh weight of 5.10 g could be produced per plant. These roots were also morphologically similar to the mother plant. Out of 70 plantlets, 56 survived after 12 weeks of transfer to pots thus exhibiting 80 % survival of regenerated plants.

Histology

In histological examination, globular embryos were shown to appear from embryogenic cell masses which developed into mature cotyledonary embryos thus initiating bipolar structure (Fig. 1j, k).

Genetic fidelity of *in vitro* regenerated plants: RAPD analysis

In order to confirm genetic fidelity of somatic embryo derived plants, comparison of RAPD band pattern of plants regenerated *in vitro* through somatic embryogenesis and mother plant was carried out. Genetic fidelity of randomly selected 17 micropropagated plants out of a total progeny of 56 plants established in glass house along with mother plant was tested. Out of 20 different MAP primers tested, 15 primers accounting for 75 % of the total primers used, exhibited amplification. Amongst these, MAP 02, MAP 08, MAP 09, MAP 10, MAP 12, MAP 14, MAP 16, MAP

17, MAP 18 and MAP 20 were selected on the basis of their amplification products which revealed clear fragmentation of DNA. The pattern of amplification was reproducible. Although total 51 RAPD bands were produced through amplification with these selected primers but only 47 bands which resolved clearly on the gel, were taken into consideration. Out of these 47 bands, 46 bands were monomorphic and 1 was polymorphic in nature. In the present study 98.33 % monomorphism was exhibited by the randomly selected plants of *C. borivilianum* regenerated through somatic embryogenesis (Table 8). The representative RAPD profile of 17 sample plants raised through somatic embryogenesis and mother plant with three MAP primers (MAP 14, MAP 18 and MAP 20) has been shown in Fig. 2a–c. The number of scorable bands amplified with a given primer ranged from 2 (MAP 02 and MAP 12) to 8 (MAP 08). The size of the DNA fragments produced by selected 10 primers ranged from 125 to 5,148 bp (Table 8).

An important aspect of micropropagation protocols for commercial purposes is the genetic integrity of plants. Long term multiplication may lead to somaclonal or epigenetic variations in the micropropagated plants questioning the fidelity of their clonal nature (Cecchini et al. 1992; Rani and Raina 1998). Therefore, it is important to first establish the suitability of the micropropagation protocol developed with respect to the production of genetically identical and stable plants. The random amplified polymorphic DNA (RAPD), (Williams et al. 1990) is efficient, cost-effective and simple technique. This method often yields large number of markers; does not need either large amounts of DNA or previous information of DNA sequences. Moreover,

Table 8 Description of 10 decamer primers used for RAPD analysis of *C. borivilianum* plants regenerated through somatic embryogenesis and mother plant

S No	Primer	Sequence (5' to 3')	Total no. of bands	Monomorphic bands		Polymorphic bands		Fragment size of scored bands (bp)
				No. of bands	Percentage (%)	No. of bands	Percentage (%)	
1	MAP 02	5'GTCCTACTCG3'	2	2	100.00	–	–	831–1375
2	MAP 08	5'CTATCGCCGC3'	8	8	100.00	–	–	564–3530
3	MAP 09	5'CGGGATCCGC3'	5	5	100.00	–	–	947–2027
4	MAP 10	5'GCGAATTCCG3'	5	5	100.00	–	–	564–1904
5	MAP 12	5'CCCTGCAGGC3'	2	2	100.00	–	–	831–1375
6	MAP 14	5'GTGCAATGAG3'	3	3	100.00	–	–	1904–3530
7	MAP 16	5'AAGATAGCGG3'	4	4	100.00	–	–	125–1375
8	MAP 17	5'GGATCTGAAC3'	6	6	100.00	–	–	1584–5148
9	MAP 18	5'TTGTCTCAGG3'	6	5	83.33	1	16.67	564–2027
10	MAP 20	5'GGACTCCACG3'	6	6	100.00	–	–	125–1584
	Total		47	46	98.33	1	1.67	

– No polymorphic bands were observed

Fig. 2 RAPD profiles of *C. borivilianum* plants regenerated through somatic embryogenesis and mother plant on 1.2 % agarose gel employing MAP primers. **a.** MAP 14; **b** MAP 18 and **c** MAP 20. Samples in gel lanes (from left to right) lane 1 M, molecular size marker (*Hind*III and *Eco*RI restricted λ DNA marker); lane 2 mother plant; lane 3–19 plants raised through somatic embryogenesis after 12 weeks of their transfer in pots

RAPD analysis does not involve radioactive compounds. RAPD has been extensively used for studying genetic fidelity of *in vitro* raised plants in a number of species (Tyagi et al. 2007; Kumar et al. 2010; Nadha et al. 2011). In the present study, the bands amplified through RAPD by responsive primers showed monomorphism with the mother plant and were also similar among the *in vitro* raised plants thus indicating the genetic fidelity of the micropropagated plants. The genetic stability of plants regenerated *in vitro* via somatic embryos has been reported previously (Bennici et al. 2004; Yang et al. 2008; Patel et al. 2011). According to Bennici et al. (2004) the lack of variation in their *Foeniculum vulgare in vitro* regenerants may be due to

developmental constraints which exert selection against variant cells during regeneration. Some investigators have also reported polymorphism in micropropagated plants using RAPD (Bindiya and Kanwar 2003; Elmeer et al. 2009; Peyvandi et al. 2010). Rani et al. (1995) found variations among micropropagated *Populus deltoides* plants originating from the same clone though they were morphologically similar.

In the present study efficient maturation and germination of *C. borivilianum* somatic embryos were established which resulted into production of high quality somatic embryos and subsequently healthy plants with high survival rate. An efficient protocol for high frequency plant multiplication employing embryogenic suspension culture has been scaled-up at shake flask level which may be used for large-scale multiplication of *C. borivilianum* in bioreactor. The observation of 98.33 % monomorphism between plants regenerated through somatic embryogenesis and mother plant lead us to conclude that plants regenerated by somatic embryogenesis in the present study are genetically true to the plant of origin. Therefore suitability of the protocol developed for micropropagation of *C. borivilianum* via somatic embryogenesis with respect to the production of genetically identical and stable plants has been demonstrated which enhances the utility of the protocol developed in the present study.

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