In vitro propagation of an endangered medicinal herb *Chlorophytum borivilianum* Sant. et Fernand. through somatic embryogenesis

Mohd Zahid Rizvi · Arun Kumar Kukreja ·
Narendra Singh Bisht

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Abstract Tuberos roots of *Chlorophytum borivilianum* Sant. et Fernand. which are a source of steroidal saponins, possess immunomodulatory, adaptogenic, aphrodisiac, antipyretic, diuretic, hemostatic and anti-tumour properties. Poor seed setting and germination and slow growth in conventional vegetative propagation are major constraints in the large-scale cultivation of this commercially important medicinal plant. In the present study, a procedure for *in vitro* propagation of this endangered herb through somatic embryogenesis has been established. Seeds of *Chlorophytum borivilianum* were germinated on MS medium supplemented with 57.74 μM gibberellic acid and hypocotyl portion from germinated seedling was used as explant for callus induction. Moderate to good callus induction was observed on MS medium containing 1.16 μM kinetin and 1.13–2.26 μM 2,4-dichlorophenoxyacetic acid. Regular subculturing of callus on kinetin (1.16 μM) and 2,4-dichlorophenoxyacetic acid (1.13 μM) supplemented me-

dium induced somatic embryogenesis. In modified MS medium, 1.79 mM NH_4NO_3 and 10.72 mM KNO_3 was optimal for somatic embryogenesis. 7.38 μM 2-isopentenyladenine supplemented to modified MS medium, showed best response for somatic embryogenesis while proline (0.76 mM) as an amino acid supplement gave better response than glutamine. 30% germination of mature somatic embryos was achieved on MS medium supplemented with 15.54 μM 6-benzylaminopurine. Multiplication of *C. borivilianum* through somatic embryogenesis may offer a better approach compared to organogenesis for developing scale-up technology employing bioreactors for its mass propagation.

Keywords Liliaceae · Safed musli · Saponins · Somatic embryogenesis · Proline

Introduction

Chlorophytum borivilianum commonly known as safed musli (family Liliaceae) is a monocotyledonous plant. Tuberos roots of safed musli contain steroidal saponins (neohecogenin, neotigogenin, stigmaterol, tokorogenin) that are used as tonic and aphrodisiac (Ojha 1987; Ramawat et al. 1988; Tandon et al. 1992; Tandon and Shukla 1993). The active principle, saponins reported to occur in some other species also, are known as stimulants and metabolic enhancers (Li et al. 1990; Tandon and Shukla 1992; Tandon and Shukla 1995). Saponins possess anti-tumour activity (Mimaki et al. 1996). The roots are widely used in the Indian system of medicine for the treatment of general debility, weakness, impotency and sterility; to enhance male potency; as cardiac and brain tonic; as a curative agent in

M. Z. Rizvi (✉) · A. K. Kukreja
Plant Tissue Culture Division, Central Institute of Medicinal and Aromatic Plants (CIMAP), Council of Scientific and Industrial Research,
Lucknow P.O. CIMAP 226015 UP, India
e-mail: zahid682001@yahoo.co.in

N. S. Bisht
Department of Botany, Hemwati Nandan Bahuguna Garhwal University,
Pauri Campus, Srinagar, Garhwal,
Uttarakhand 246174 UP, India

Present Address:
M. Z. Rizvi
Department of Biotechnology, Faculty of Science,
Hamdard University,
New Delhi 110062, India

various diseases like piles, diabetes and as anti-pyretic, diuretic and hemostatic. Generally, *C. borivilianum* has been used along with other plants such as *Asparagus adscendens*, *A. racemosum*, *Curculigo orchoides* and *Withania somnifera* in many herbal and Unani formulations in India (Kirtikar and Basu 1975; Ramawat et al. 1988). Due to its therapeutic activity and diversified uses, there is increased demand for safed musli especially *C. borivilianum*. The annual demand in the country has been estimated to nearly 500 tonnes (Bordia et al. 1995). Safed musli is a rhizomatous herb propagated vegetatively in nature by shoot buds from perennating root tubers in the soil and sexually by seeds. Seeds have poor germination percentage (11–24%), low viability and long dormancy period (Jat and Bordia 1990). The slow growth rate encountered in conventional vegetative propagation methods is not sufficient to meet the present demand (Bordia et al. 1995). There is limited cultivation of this plant in the country. About 95% of the indigenous requirement comes through wild habitats. Due to large-scale and indiscriminate collection of wild material and insufficient attempts to allow its replenishment, *C. borivilianum* is rapidly disappearing. Besides this, the scarcity of elite and characterized planting material and non-availability of improved agrotechnological practices have also limited its commercial cultivation. There is need for conservation and mass multiplication of this plant. 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, India has categorized it as one of the prioritized plant species to be promoted for conservation and large-scale cultivation. To fill the gap between demand and supply of its tuberous roots for commercial gains and to provide cost-effective planting material for conventional propagation methods at a rapid rate in a short span of time, plant tissue culture approach seems to be a profitable alternative. Somatic embryogenesis has recently become much advocated technology for cloning plants through tissue culture. This technique is viewed as having the potential to become the most efficient plant regeneration method because literally millions of plants could be produced from a relatively small amount of cultured cells or tissues. Somatic embryogenesis also offers potential for scaling-up mass propagation of plants. Conventional micropropagation requires intensive labour which often limits its commercial application. Somatic embryos could be easier to handle since they are relatively small and uniform in size and they do not require cutting into segments and individual implanting on media during proliferation. Besides this somatic embryogenesis has great potential to aid plant improvement through *in vitro* selection of mutants and genetic transformation (Finer and Macmullen 1991). Somatic embryos also have potential for long term storage through cryopreservation or dessica-

tion which facilitates flexibility in scheduling production and transportation and therefore fits large-scale production.

In vitro culture of several ornamental plant species of family Liliaceae has been described (Krikorian and Kann 1986) but very few medicinal plants species of this family have so far been established *in vitro* (Jha et al. 1984). Earlier attempts have been made for *in vitro* propagation of an endangered medicinal herb of family Liliaceae, *Chlorophytum borivilianum* through somatic embryogenesis by Kukda et al. (1994); Purohit et al. (1994) and Arora (1999). Somatic embryos were observed in immature zygotic embryo derived callus on MS medium (Murashige and Skoog 1962) supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), (Kukda et al. 1994; Purohit et al. 1994) but precocious germination of somatic embryos and profuse rooting was observed. In the studies of Purohit et al. (1994) plantlets could be recovered only from 20% of somatic embryos on auxin free MS medium and transferred subsequently to light conditions. Arora (1999) used leaf explants for somatic embryogenesis but leaf derived callus could not form a system for continuous production of somatic embryos inspite of use of a large number of combinations of auxins and cytokinins. In the present study somatic embryogenesis from hypocotyl-derived callus and plantlet regeneration is being reported. The protocol can be used for large-scale multiplication of *C. borivilianum* employing liquid culture medium in bioreactors.

Materials and methods

Plant material: maintenance of mother stock plants

Tuberous roots of *C. borivilianum* were collected from natural habitat (Rajsamand District in the State of Rajasthan) and maintained in earthen pots containing mixture of sand: soil: farmyard manure (1:1:1, v/v) under glass house condition. Plants developed from proliferation of shoot buds on stem disc attached to the root tubers. They were subsequently transferred to field in the Botanical Garden of the Institute. These stock plants were used as mother plants for obtaining explants.

Callus induction and maintenance

Seeds of *C. borivilianum* obtained from the mother plants (control plants) maintained in the Botanical Garden of the Institute served as source material for callus induction. Seeds were treated with Teepol 4% (v/v) for 4–5 min and rinsed several times with distilled water, then surface sterilized with 0.1% (w/v) aqueous mercuric chloride for 5–6 min and again rinsed several times with sterile distilled water before inoculation. Seeds were germinated on MS medium supplemented with gibberellic acid GA₃

(57.74 μM) hypocotyl from these seedlings were implanted on two different media i.e. MS (Murashige and Skoog 1962) and B5 (Gamborg et al. 1968) supplemented with different concentrations (0.45–4.52 μM) of 2, 4-D and kinetin (Kn) for callus induction. MS or B5 medium without any phytohormones served as control. Callus induced on the optimal callus induction medium (MS medium containing 1.13 μM 2, 4-D and 1.16 μM Kn) was maintained on the fresh medium of the same composition by regular subculturing after every 3–4 weeks.

Standardization of somatic embryogenesis

Nitrogen levels and cytokinins play important role in somatic embryogenesis. Amino acids have also been reported to affect morphogenetic response especially somatic embryogenesis in many plant species. Therefore experiments were performed to observe the effect of these on somatic embryogenesis in *C. borivilianum*.

(a) Nitrogen sources

About 150 mg callus maintained on optimal callus induction medium was inoculated on MS medium having different concentrations of NH_4NO_3 (1.79–10.70 mM yielding 50–300 mg nitrogen) and KNO_3 (3.56–21.45 mM yielding 50–300 mg nitrogen) and 1.13 μM 2, 4-D and 1.16 μM Kn. The control medium was standard MS medium containing 20.63 mM NH_4NO_3 and 18.81 mM KNO_3 and supplemented with 1.13 μM 2, 4-D.

(b) Cytokinins

To study the effect of different cytokinins 150 mg of seedling derived callus was inoculated on modified MS medium (1.79 mM NH_4NO_3 and 10.72 mM KNO_3) supplemented with 1.13 μM 2, 4-D and different concentrations 2-isopentenyladenine (2-iP, 0.98–7.38 μM) or kinetin (Kn, 0.93–6.97 μM) or thidiazuron (TDZ, 0.45–4.54 μM). The control medium in the present study was modified MS medium supplemented with 1.13 μM 2, 4-D.

(c) Amino acids

For further enhancement of somatic embryogenesis, equal amount (150 mg) of callus was transferred to modified MS medium (1.79 mM NH_4NO_3 and 10.72 mM KNO_3) containing 1.13 μM 2, 4-D, 7.38 μM 2-iP and proline (0.38–2.29 mM) or glutamine (0.34–2.05 mM). Modified MS medium supplemented with 1.13 μM 2, 4-D and 7.38 μM 2-iP served as control.

Germination of somatic embryos

Cotyledonary stage somatic embryos were transferred to MS medium supplemented with different levels (6.66–28.86 μM)

of 6-benzylaminopurine (BAP) or MS medium without phytohormones (control) for germination. Percentage germination rate in the present study was determined as number of mature embryos germinated out of 20 mature embryos taken from their respective treatments and transferred on optimal embryo germination medium (MS medium supplemented with 15.54 μM BAP). Each replicate in embryo germination experiment consisted of 20 mature embryos.

The medium was solidified with 0.8% (w/v) agar (Hi-Media). The pH of the medium was adjusted to 5.8 ± 0.1 using 0.1 N HCl or 0.1 N NaOH prior to steam sterilizing at 121°C temperature and 15 psi pressure for 15 min. Each conical flask (100 ml capacity, Borosil) containing 40 ml of culture medium was plugged with non-absorbent cotton. Three hypocotyls or three seedling derived callus pieces of 150 mg each were inoculated per culture flask. Each treatment was replicated six times and all experiments were repeated twice. Cultures were maintained in a culture room having 16:8 h light:dark alternating regime of 45 $\mu\text{mol m}^{-2} \text{s}^{-1}$ intensity provided by cool white fluorescent tubes, $25 \pm 1^\circ\text{C}$ incubation temperature and 40–50% relative humidity. Observations on fresh wt. of callus, (number of somatic embryos) and number of somatic embryos germinated/20 mature embryos were recorded after 4 weeks.

Hardening and transplantation of *in vitro* raised plants

Plantlets obtained from *in vitro* germinated somatic embryos after 6 weeks of culture of mature somatic embryos on MS medium supplemented with 15.54 μM BAP were washed with water to remove agar without damaging the delicate root system and placed at the top of culture tubes filled with 1/4 strength MS nutrient solution (only the roots were dipped in solution). The culture tubes containing somatic embryo derived plantlets were kept in the culture room for 2 weeks for hardening under similar culture room conditions as described above and then these plants were transferred to earthen pots consisting sand:soil:farmyard manure in 1:1:1 (v/v) ratio. Potted plants were covered with transparent polythene bags with small holes for air ventilation to ensure high humidity in initial stages. The polythene bags were removed after 2 weeks and the surviving plants were maintained in the glass house under 16 h photoperiod at $28 \pm 2^\circ\text{C}$.

Histology

For histological studies embryogenic tissues during 4–6 weeks of culture duration having somatic embryos at different stages of development 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 then serially sectioned (8–10 μM thick sections) on a rotary microtome (Heidelberg, Germany). 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).

Results and discussion

The investigation aimed at developing an efficient protocol for rapid multiplication of *C. borivilianum* via somatic embryogenesis. In the present study culture conditions were established for multiplication of *C. borivilianum* through induction of somatic embryogenesis in seedling derived callus and germination of somatic embryos into plantlets. Experiments were conducted for observing effect of different nitrogen levels, different cytokinins and amino acids on somatic embryogenesis besides selecting optimal medium for germination of somatic embryos.

Seed germination

Seeds cultured on MS medium alone exhibited <10% germination. But addition of GA_3 (57.74 μM) supported about 40–50% seed germination.

Induction of somatic embryogenesis

Hypocotyls were cultured on two different media MS and B5 supplemented with different concentrations of 2,4-D and Kn in the range of 0.45–4.52 μM . Induction of somatic embryogenesis was observed during regular subculturing of calli induced and maintained on MS medium supplemented with different concentrations and combinations of 2, 4-D and Kn. Globular somatic embryos induced regularly at various frequencies (Fig. 1a). Amongst the MS and B5 media, MS medium showed better somatic embryogenesis response than B5 medium and amongst different 2, 4-D and Kn concentrations and combinations tested, MS medium containing 1.13–2.26 μM 2, 4-D and 1.16 μM Kn exhibited better response (Table 1). On the control medium (MS or B5 medium without phytohormones), somatic embryogenesis was not observed. It was also observed that higher concentrations of 2, 4-D proved inhibitory for somatic embryogenesis, therefore, embryogenic cultures were further maintained on lower concentration (1.13 μM) of 2, 4-D.

The basal medium providing different levels of nutrition can affect the frequency of somatic embryogenesis. Varisai et al. (2004) observed that between MS and B5 only MS medium proved responsive in terms of somatic embryogenesis. In the present study also MS medium proved more

efficient than B5 medium. In monocots, in most of the cases primary embryogenesis was induced on auxin-supplemented media. Among the different auxins used to induce somatic embryogenesis, 2, 4-D has been by far the most efficient (Varisai et al. 2004). Therefore 2, 4-D has been used in the majority of embryogenic cell cultures. The influence of exogenously applied auxins particularly 2, 4-D for somatic embryogenesis is well documented (Yeung 1995; Feher et al. 2003). In some of the cases where the exogenous application of auxins has proved to be efficient treatment for the induction of somatic embryogenesis, further development of the existing somatic embryos can be achieved by reducing or removing auxin from the culture media. Rangasamy (1986) observed that optimal development or histodifferentiation requires removal or reduction of the auxin used for embryo induction. In the present study optimal somatic embryogenesis was observed after reducing the concentration of 2, 4-D. Contrarily induction and development of embryos upto the torpedo-cotyledonary stages was achieved on the same auxin medium indicating the relative insensitivity to this inductive hormone in *Macrotyloma uniflorum* (Varisai et al. 2004). Even addition of cytokinins as the sole plant growth regulator has proved to be effective in inducing somatic embryogenesis (Sagare et al. 2000). In most of the cases, however cytokinin addition should be coupled with that of auxins to obtain the desired effect (Merkle et al. 1995). Consistent with our observations, Duque et al. (2006) also reported that cells acquire embryogenic competence when growing in the medium which is supplemented with 2, 4-D and Kn while Varisai et al. (2004) added that addition of BAP and/or Kn to the 2, 4-D supplemented medium suppressed somatic embryogenesis and produced greenish compact callus.

Optimization of somatic embryogenesis

(a) Effect of nitrogen sources

Since nitrogen levels have been shown to affect somatic embryogenesis, optimal level needs to be worked out for different species/genotypes. Manipulations in MS medium constituents (NH_4NO_3 and KNO_3) were made to standardize medium for somatic embryogenesis. Experiments were conducted to study effect of different levels of NH_4NO_3 (1.79–10.70 mM yielding 50–300 mg nitrogen) and KNO_3 (3.56–21.45 mM yielding 50–300 mg nitrogen) in the MS medium containing 1.13 μM 2, 4-D on somatic embryogenesis. It was observed that maximum number of somatic embryos (19) were obtained on MS medium containing 1.79 mM NH_4NO_3 and 10.72 mM KNO_3 and compared to control medium (standard MS medium containing 20.63 mM NH_4NO_3 and 18.81 mM KNO_3 and supplemented with 1.13 μM 2, 4-D), 1.56-fold increase in number of somatic embryos was obtained (Table 2). The levels of NH_4NO_3 and

Fig. 1 Somatic embryogenesis in *Chlorophytum borivilium*. **a.** induction of somatic embryos (globular stage somatic embryos) on MS medium supplemented with 1.13 μM 2, 4-D and 1.16 μM Kn; **b.** growth and development of somatic embryos on modified MS medium (1.79 mM NH_4NO_3 and 10.72 mM KNO_3) supplemented with 1.13 μM 2, 4-D, 7.38 μM 2-iP and 0.76 mM proline; **c.** advanced stage of somatic embryos showing initiation of shoot primordia; **d.** a

somatic embryo germinating on MS medium supplemented with 15.54 μM BAP showing shoot primordia with shoot apex; **e.** plantlet regenerated through somatic embryogenesis with developed root system; **f.** somatic embryo derived *C. borivilium* plant in earthen pot after 8 weeks of hardening; **g.** globular stage somatic embryos appearing from surface of embryogenic cell masses; **h.** somatic embryos at advanced stage of maturation

KNO_3 optimal for somatic embryogenesis proved to be 1.79 mM NH_4NO_3 yielding 50 mg nitrogen and 10.72 mM KNO_3 yielding 150 mg nitrogen. In normal MS medium it is 20.63 mM NH_4NO_3 (yielding 577 mg nitrogen) and 18.81 mM KNO_3 (yielding 263 mg nitrogen). Higher concentrations of NH_4NO_3 or KNO_3 were not favourable to somatic embryogenesis. The effect of different levels of NH_4NO_3 and/or KNO_3 in the culture medium on somatic embryogenesis has also been demonstrated in other plant species (Feng and Ouyang 1988; Menke-Milczarek and Zimny 2001; Choi et al. 2003).

(b) Effect of cytokinins

Effect of different cytokinins on somatic embryogenesis was also studied and it was observed that

amongst different cytokinins tested, highest number of somatic embryos (23) were obtained on modified MS medium (1.79 mM NH_4NO_3 and 10.72 mM KNO_3) containing 1.13 μM 2,4-D and 7.38 μM 2-iP. Observations recorded in the present study revealed that while lower levels of Kn and TDZ were effective for somatic embryogenesis, 2-iP was effective when supplemented at higher levels to culture medium (Table 3). 2-iP at 7.38 μM concentration showed optimal response. Our results are substantiated by the studies of Shibli et al. (2001). On *Olea europea* L. where 2-iP outperformed other growth regulators. In the studies of Kumar et al. (2008), TDZ was more effective amongst different cytokinins tested for embryogenesis on *Dendrobium chingmai* pink. Studies by Shibli and Ajlouni (2000)

Table 1 Effect of different basal media and different concentrations of 2,4-D and Kn on somatic embryogenesis in *C. borivilianum*

MS basal medium					
2, 4-D (μM)	0.45	1.13	2.26	4.52	
Control ^a	–				
Kn (μM)	No. of somatic embryos				
0.47	–	8.00 $\pm$ 1.00 ^b	4.00 $\pm$ 0.71	–	
1.16	6.40 $\pm$ 0.55	12.20 $\pm$ 1.48	10.20 $\pm$ 1.30	3.20 $\pm$ 0.83	
2.32	–	–	5.60 $\pm$ 0.55	1.40 $\pm$ 0.55	
B ₅ basal medium					
2, 4-D (μM)	0.45	1.13	2.26	4.52	
Kn (μM)	No. of somatic embryos				
0.47	–	6.00 $\pm$ 1.00	2.80 $\pm$ 0.71	1.60 $\pm$ 0.55	
1.16	5.00 $\pm$ 1.00	10.00 $\pm$ 1.58	8.00 $\pm$ 1.22	2.80 $\pm$ 0.45	
2.32	–	–	2.40 $\pm$ 0.54	0.80 $\pm$ 0.40	

^a MS or B₅ basal medium without phytohormones^b Average value $\pm$ S.E. (Standard Error)

– No response

indicated that TDZ was not effective and embryogenesis did not occur in endemic black iris (*Iris nigricans*).

(c) Role of amino acids

Of glutamine and proline supplemented to modified MS medium containing 1.13 μM 2, 4-D and 7.38 μM 2-iP in the range of 0.34–2.29 mM, proline at 0.76 mM enhanced somatic embryogenesis response and highest number of somatic embryos (43.2) were obtained at this concentration of proline (Table 4). Supplementing culture medium with glutamine has less stimulating effect on somatic embryogenesis. Present results are consistent with earlier reports of enhancement in somatic embryogenesis by proline in maize (Armstrong and Green 1985) and carrot (Nuti et al. 1984) and pollen embryogenesis in cereals (Sozinov et al. 1981). On the other hand in the studies of Varisai et al. (2004) glutamine enhanced the induction, growth and maturation of somatic embryos in *Macrotyloma uniflorum*. Glutamine also promoted pollen embryogenesis in barley (Xu and Sunderland 1981) while

another amino acid tryptophan favoured somatic embryogenesis in some cultivars of rice (Siriwardana and Nabros 1983). Therefore it is apparent that exogenous supply of amino acids plays a pivotal role in the induction and physiological maturity of somatic embryos. The type and level of amino acid(s) has to be optimized for different species.

Thus a modified MS medium (1.79 mM NH_4NO_3 , 10.72 mM KNO_3) containing 1.13 μM 2, 4-D, 7.38 μM 2-iP and 0.76 mM proline was found optimal for somatic embryogenesis in the seedling derived callus of *C. borivilianum* during present study. Growth and development of somatic embryos was observed on this medium (Fig. 1b) after their induction on MS medium supplemented with 1.13 μM 2,4-D and 1.16 μM Kn.

Somatic embryo germination

Mature cotyledonary stage somatic embryos cultured for 4 weeks on modified MS medium supplemented with

Table 2 Effect of different levels of NH_4NO_3 and KNO_3 on somatic embryogenesis in *C. borivilianum*

No. of somatic embryos						
KNO ₃ (mM)						
NH ₄ NO ₃ (mM)	3.56	7.15	10.72	14.30	17.87	21.45
Control ^a	12.20 $\pm$ 1.30					
1.79	3.00 $\pm$ 0.54 ^b	9.00 $\pm$ 1.00	19.00 $\pm$ 1.58	17.00 $\pm$ 1.87	15.00 $\pm$ 2.00	6.00 $\pm$ 1.00
3.56	12.60 $\pm$ 2.07	16.60 $\pm$ 1.67	14.40 $\pm$ 1.51	17.60 $\pm$ 1.81	9.00 $\pm$ 1.30	14.00 $\pm$ 1.58
5.35	9.60 $\pm$ 1.51	13.40 $\pm$ 1.67	15.40 $\pm$ 2.07	17.00 $\pm$ 2.12	13.60 $\pm$ 1.67	13.00 $\pm$ 2.23
7.14	8.20 $\pm$ 1.48	14.40 $\pm$ 1.14	16.00 $\pm$ 2.16	16.80 $\pm$ 1.78	17.20 $\pm$ 1.51	14.80 $\pm$ 1.64
8.93	7.80 $\pm$ 1.30	11.60 $\pm$ 2.07	12.80 $\pm$ 1.64	14.60 $\pm$ 1.81	11.00 $\pm$ 1.58	4.90 $\pm$ 1.09
10.70	6.00 $\pm$ 1.00	8.40 $\pm$ 1.58	10.80 $\pm$ 1.81	12.60 $\pm$ 1.94	12.10 $\pm$ 1.58	1.00 $\pm$ 0.00

^a Standard MS medium supplemented with 1.13 μM 2, 4-D (Standard MS medium contains 20.63 mM NH_4NO_3 and 18.81 mM KNO_3)^b Average value $\pm$ S.E

Table 3 Effect of different cytokinins on somatic embryogenesis

Cytokinins (μM)	No. of somatic embryos
Control ^a	19.00 $\pm$ 1.58 ^b
Kn	
0.93	20.40 $\pm$ 2.07
2.32	19.20 $\pm$ 2.16
4.65	13.40 $\pm$ 1.34
6.97	6.50 $\pm$ 1.58
TDZ	
0.45	21.60 $\pm$ 2.07
0.91	13.60 $\pm$ 1.81
2.27	9.40 $\pm$ 1.64
4.54	6.80 $\pm$ 1.30
2-iP	
0.98	7.60 $\pm$ 1.14
2.46	11.40 $\pm$ 1.67
4.92	16.20 $\pm$ 1.92
7.38	23.00 $\pm$ 2.54

^a Modified MS medium (MS medium containing 1.79 mM NH_4NO_3 , 10.72 mM KNO_3) supplemented with 1.13 μM 2, 4-D

^b Average value $\pm$ S. E

1.13 μM 2, 4-D, 7.38 μM 2-iP and 0.76 mM proline germinated within 4 weeks upon transfer to MS medium supplemented with BAP (6.66–24.42 μM). Maximum somatic embryos germinated on MS medium supplemented with 15.54 μM BAP (Table 5, Fig. 1c, d). Embryo germination decreased at low (<11.00 μM) or high (>19.98 μM) concentrations of BAP.

Addition of cytokinins to MS medium enhanced development of plantlets from somatic embryos in other

Table 4 Effect of different amino acids on somatic embryogenesis

Amino acids (mM)	No. of somatic embryos
Control ^a	23.00 $\pm$ 2.54 ^b
Glutamine	
0.34	20.40 $\pm$ 2.79
0.68	23.00 $\pm$ 2.16
1.37	22.40 $\pm$ 2.30
2.05	16.80 $\pm$ 3.11
Proline	
0.38	37.00 $\pm$ 3.53
0.76	43.20 $\pm$ 3.36
1.53	32.60 $\pm$ 2.38
2.29	29.00 $\pm$ 3.74

^a Modified MS medium supplemented with 1.13 μM 2, 4-D and 7.38 μM 2-iP

^b Average value $\pm$ S.E

Table 5 Effect of different BAP levels on germination of somatic embryos in *C. borivilianum*

BAP (μM)	No. of embryos germinated/ 20 mature embryos	Percent germination
Control ^a	0.60 $\pm$ 0.55 ^b	3
6.66	1.00 $\pm$ 0.00	5
11.00	2.60 $\pm$ 0.54	13
15.54	6.00 $\pm$ 1.00	30
19.98	3.20 $\pm$ 0.45	16
24.42	2.00 $\pm$ 0.70	10
28.86	–	–

^a MS medium without phytohormones

^b Average value $\pm$ S.E

– No response

studies also. Supplementation of MS medium with BAP supported regeneration of plantlets from two cell lines of dune reed (Wang et al. 2001) while in the studies of Ho et al. (2006) maximum regeneration was observed with Kn supplemented medium in *Lilium x formolongi* Hort. Varisai et al. (2004) reported very low (5%) germination on MS medium alone. In the present study also lowest germination (3%) was observed on MS medium. On MS medium supplemented with 15.54 μM BAP it was 30%. Previously Purohit et al. (1994) reported only 20% germination in *C. borivilianum*. Profuse rooting and precocious germination of somatic embryos was also observed in their studies.

Hardening

Plantlets obtained from *in vitro* germinated somatic embryos after 6 weeks of culture were hardened and maintained in the glass house in earthen pots containing sand:soil:farmyard manure, 1:1:1 (v/v) ratio. The survival was 60–65%. Plants developed through somatic embryogenesis were morphologically similar to the mother plant (Fig. 1e, f).

Histological study

Histological analysis showed different stages of development of somatic embryos in seedling derived embryogenic callus. Globular embryos appeared from embryogenic cell masses which advanced further towards mature cotyledonary stage thus initiating bipolar structures (Fig. 1g, h).

Thus multiplication of *C. borivilianum* through somatic embryogenesis has been standardized by us. Micro-propagation through somatic embryogenesis has proved better than organogenesis due to rapid multiplication rate and easier handling (Debergh and Maene 1981). Somatic embryogenesis therefore can be more favourable for mass-

propagation of *C. borivilianum*. The protocol standardized in the present study may be useful for scale-up of somatic embryogenesis in bioreactors.

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