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Somaclonal Variation in Regenerants from Long-Term Embryonic Cultures of *Chlorophytum borivilianum* Obtained from Agamospermic Seeds of a Triploid Plant

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Summary

Chlorophytum borivilianum is an endangered aphrodisiac plant and most of the plants in nature are triploid. Agamospermic seeds of a triploid plant were used to initiate somatic embryogenesis. Plantlets obtained through seedling–derived embryonic callus showed high level of morphological and cytological variation, which increased with the increase in age of cultures. Variegated leaves were also observed occasionally. High level of variation in the leaf size, stomata number and epidermal cell size was observed. Increase in the variation in chromosome number was observed with the increase in age of cultures. Chromosomal variation was least 2n=3x–3 to 2n=3x+3 in 1 to 4 months-old cultures and increased with the age of cultures with 2n=5x–1 to 7x in cultures older than 6 months. Therefore, an alternative method was developed to reduce somaclonal variation in germinated somatic embryos, by using leaf from genetically stable plants (1–4 months-old cultures) as a source of explants for re-initiation of fresh embryonic cultures. RAPD finger printing of plantlets raised by this method revealed very little variation (6.15 %). Further improvement in somatic embryogenesis is required towards domestication of the plant using biotechnological method of propagation.

Key words. *Chlorophytum borivilianum* – somatic embryogenesis – somaclonal variations – RAPD

Introduction

Chlorophytum borivilianum Sant. & Fern. (*Liliaceae*), commonly known as 'safed musli' (white roots) is an important endangered medicinal plant. Tuberous roots are widely used as tonic and aphrodisiac due to the presence of steroidal saponins (Bordia et al. 1995; Kaushik 2005; Satija and Singh 2005). Steroidal sapogenins like tigogenin, neotigogenin, neohecogenin, stigmasterol and tokorogenin have been isolated from *C. arundinaceum*, a closely related species (Gupta et al. 1979; Tandon and Shukla 1992, 1993). Pharmacological investigations of saponin fractions prepared from dried roots showed significant inhibition of ^{3}H-dopamine up-take in rat striatal syneptosomes thereby, directly stimulating the neuronal activity of dopaminergic system in the brain (our unpublished results).

C. borivilianum has poor seed set, viability and germination. Moreover, this plant has been over-exploited from the wild stands for its pharmacological properties (Bordia et al. 1995). Therefore, the population of *C. borivilianum* is dwindling at an alarming rate (Nayar and Shastri 1988). Attempts made by Bordia and Co-workers to develop field methods of multiplication and propagation through seeds and / or tuberous roots have met with a failure (Bordia et al. 1995). Method of multiplication through organogenesis has been reported (Purohit et al. 1994) and subsequently improved by Suri et al. (1999a). We also reported *in vitro* tuber formation that resulted in 100 % survivability of in vitro raised plants on transfer to soil. Earlier we described a method for somatic embryogenesis in *C. borivilianum* through seedling-derived callus but the embryonic potential declined with the age of cultures (Arora et al. 1999). The immediate task is to conserve and multiply the plant in bulk amount to meet the present demand. Plant tissue culture has been successfully used to micropropagate medicinal plants and several other members of *Liliaceae* (Ramawat et al. 1991, 1998; Famelaer et al. 1996; Suri et al. 1998, 1999b, 2000). Plantlet regeneration has been reported through apical meristem in *C. comosum* var. *folis medio variegatis* (Apollonio et al. 1990). Organogenetic cultures have been maintained in our laboratory during the last seven years. Members of the family liliaceae like onion, garlic, lilies have been cultured *in vitro* and problems like variation in response of different cultivars, limitation of suitable explants, and maintenance of long term embryonic cultures have been experienced (Al-Zahim et al. 1999; Barandiran et al. 1999).

The occurrence of cryptic genetic defects arising via somaclonal variation in the regenerants can seriously limit the utility of micropropagation protocols. Therefore, it is important to establish the suitability of a partic-

ular micropropagation protocol for a particular clone developed with respect to the production of genetically identical and stable plants before it is released for commercial purpose. No such efforts have been made so far for tissue culture-derived clones of *C. borivilianum*. The present work reports the screening of plants raised through long-term embryogenic cultures of *C. borivilianum* and development of an alternative method for the maintenance of genetically stable stock.

Materials and Methods

Somatic embryogenesis

Embryogenic callus was obtained from agamospermic seeds of a triploid plants on B_5 medium (Gamborg et al. 1968) supplemented with 2.25 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.15 μM kinetin, as described earlier (Arora et al. 1999). Cultures were maintained on the same medium by regularly sub-culturing after 4-weeks of growth. Fresh embryogenic cultures were re-initiated from leaf explants obtained by the germination of somatic embryos from 1–4 months-old embryogenic cultures. The medium was gelled with 0.8 % (w/v) bacteriological agar (Himedia). The pH of the medium was adjusted to 5.8 with 0.5 N HCl or NaOH after incorporation of all ingredients of the medium and 33 ml medium was dispensed into each conical flask (100 ml, 'Borosil'). Culture flasks were closed with non-absorbent cotton and autoclaved for 15 min at 121 °C. Cultures were incubated at 26±0.5 °C under white fluorescent light (Philips cool TL 36W) with a total radiance of 36 μmol m^{-2} s^{-1} for 16 hours photoperiod and 60±5 % relative humidity.

Germination of somatic embryos

Somatic embryos formed from seedling-derived callus were isolated under a stereo microscope and transferred onto MS-9 medium (MS medium containing 400 mg l^{-1} NO_3-N and 100 mg l^{-1} NH_4-N containing 10 μM 6-benzyl adenine (BA) as described earlier (Arora et al. 1999). Because the germination of somatic embryos required a cytokinin, root length was reduced. Therefore, such cultures were transferred on B5 medium containing 0.57 μM IAA for the development of roots as described earlier (Arora et al. 1999).

Multiplication of propagule

Shoots obtained by the germination of somatic embryos from 1–4 months-old cultures were used for the micropropagation and maintenance of cultures. A single shoot or cluster of 10 shoots as an inoculum was transferred onto MS-9 medium containing 25 mgl^{-1} adenine, 4.4 to 22.2 μM BA and 0.57 μM indole acetic acid (IAA). Leaves were harvested from these plantlets and were used as an alternative source of explant to initiate somatic embryogenesis on MS-9 medium containing thidiazuron (TDZ, 12.3 μM) and 2,4-D (1.13 μM). All the experiments were repeated twice using 10 replicates per treatment and data were analysed by using ANOVA test with one or two variables, as the case may be. Observations were recorded after 4-weeks growth.

Rhizogenesis and field transfer of miniature plantlets

Shoots produced *in vitro* either by germination of somatic embryos or by organogenesis from stem-disc explants on B5 medium containing 25 mg l^{-1} adenine and 22.2 μM BA (Suri et al. 1999a) were rooted on B_5 medium supplemented with 0.57 μM IAA as described earlier (Suri et al. 1999a). Miniature plantlets were planted in plastic pots (150 ml) containing sterilized garden soil : vermiculite (2:1, v/v) mixture. To maintain high ambient humidity (70–80 %) pots were covered with transparent polyethylene bags containing a few pores to allow gaseous exchange. Plants were irrigated as and when required (25 ml pot^{-1}, tap water). After 15 days growth in soil, polyethylene bags were removed and plants were exposed to sunlight for 2 h day^{-1} and for rest of the period, plants were kept in moist and shady place. One-month-old plantlets were finally transferred in the field, exposed to external environment at the Forest Conservation Research Station, Government of India, Udaipur.

Morphological and cytological variations in plantlets

Plants raised through somatic embryogenesis from seedling-derived callus were screened for morphological variations among the regenerants. Variation in the leaf size, number of stomata per leaf area and their size, epidermal cell size and other unique variation in vigour (data not presented) was scored in a random sample size of 30 miniature plantlets. Control plants were obtained by the germination of seeds.

Further, plants raised through somatic embryogenesis from seedling-derived callus and organogenesis from stem-disc explants (Suri et al. 1999a) were screened for cytological variations. Actively growing root tips of germinated somatic embryos from young (1–4-months old) and aged (18–24 months-old) seedling-derived embryonic cultures, and plantlets raised through direct organogenesis from stem-disc explants, and leaf-derived somatic embryos were pretreated with a saturated aqueous solution of p-dichlorobenzene for 3 h at room temperature, washed in distilled water and fixed in a mixture of acetic acid : absolute ethyl alcohol (1:3, v/v) for 24 h. Metaphase chromosomes were counted in smears as described earlier (Arora et al. 1999). A total of 200 metaphase slides were scored to screen possible chromosomal variation among the regenerants, and data are the average of 50 metaphase preparations from 50 plants in each group.

DNA isolation and RAPD fingerprinting

Genomic DNA was extracted from young leaves of micropropagated plants raised through leaf-derived embryogenic callus by modified CTAB method (Porebski et al. 1997). DNA concentration was determined by both spectrophotometery at 260 nm and gel electrophoresis, using a known quantity of Lambda DNA digested with *Hind*III (Amersham Pharmacia) as a standard.

Random amplified polymorphic DNA (RAPD) fingerprinting was used as a tool to test the performance of an alternative method described above. A total of 25 selected 10-mer primers from kits A, C and K (Operon Technologies Inc., Almeda, California) were used for PCR amplifications from 16 randomly selected plants generated

through leaf-derived somatic embryogenesis. Each 25 µl reaction mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.20 mM each of dNTPs (Promega, Madison), 15 ng of the primer, 1 X *Taq* polymerase buffer, 0.5 units of Taq DNA polymerase (Promega), and 30 ng of genomic DNA template. The reaction mixture was overlaid with an equal volume of mineral oil (Sigma). DNA amplification was performed in Perkin Elmer Cetus 480 DNA thermal cycler (Perkin Elmer Cetus, Norwalk, USA). The thermocycler was programmed for one cycle of initial denaturation at 94 °C for 5 min followed by 45 cycles of denaturation at 94 °C for 1 min annealing at 34 °C for 30 s and polymerisation at 72 °C for 1 min. Final extension was carried out at 72°C for 10 min. Amplified PCR products were electrophorised in 1.2 % (w/v) agarose (Pharmacia) gels with 1X TAE buffer, stained with ethidium bromide, and photographed under UV light. Lambda DNA digested with Hind III (Genei, India) was used as molecular size marker.

Statistical analysis

Data obtained was analysed by using 2 x 3 factorial RBD-ANOVA and single-factorial ANOVA. For RAPD analysis, presence and absence of bands were scored as 1 and 0, respectively and calculated as below:

Simpson's reciprocal index (1/D) = 1/Sum Pi^2

Evenness (E) = Sum Pi

Where, Pi is the proportion of a given category.

Results

Somatic embryogenesis and multiplication of propagule

Somatic embryos were produced from seedling explants on B_5 medium supplemented with 2.25 µM 2,4-D and 1.15 µM kinetin, as described earlier (Arora et al. 1999). These somatic embryos germinated on MS-9 medium containing 22.2 µM BAP but roots remained underdeveloped. In the present investigation, attempts were made to standardize media composition and size of inoculum for mass multiplication of plantlets through organogenesis using germinated somatic embryos as an inoculum. Germinated somatic embryos were inoculated either singly or in a cluster of ten on MS-9 medium supplemented with different concentrations of BAP (4.4–22.2 µM) (Table 1). Increase in the concentration of BAP resulted in increase in shoot proliferation, highest being at 22.2 µM of BAP. The size of inoculum also played an important role in shoot multiplication. When a single germinated somatic embryo was inoculated, an average of 3.8 shoots per culture flask was recovered after 4 weeks of growth. Increase in the inoculum size to 10 germinated somatic embryos, resulted in the recovery of an average of 21.4 shoots per culture flask (i.e., 2.1 shoots per germinated somatic embryo). The effect of BAP, inoculum size and their interactions on shoot proliferation was statistically significant. Therefore, the method developed is efficient enough to mass proliferate shoots through a combination of somatic embryogenesis and organogenesis.

Further, leaf explants from plantlets obtained through somatic embryogenesis from 1–4 months-old seedling-derived cultures were used to re-initiate somatic embryogenesis, which could be an alternative method of retaining genetic stability in regenerated plants. Somatic embryogenesis from leaf explants was initiated on MS-9 medium supplemented with TDZ (12.3 µM) and 2,4-D (1.13 µM). Leaf explants produced profusely growing callus and large number of somatic embryos on this media. Therefore, a continuous system in sequential order of somatic embryogenesis, germination, multiplication and re-initiation of somatic embryogenesis was established by making constant use of genetically stable explants from fresh cultures to re-initiate somatic embryogenesis and further multiplication. Using this method, we have been able to establish genetically stable stock of long-term embryonic cultures (over seven years).

Rhizogenesis and field transfer of plantlets

Shoots produced *in vitro* by germination of somatic embryos were rooted on B_5 medium supplemented with 0.57 µM IAA following the method described earlier (Suri et al. 1999a). Plantlets developed through the germination of somatic embryos were successfully transferred in soil with a high rate of survival (90 %). Starting from 10 g fresh seedling-derived callus, over 3000 somatic embryos could be produced, of which 50 % embryos germinated *in vitro* into plantlets and 1300 plantlets were successfully transferred to the forest. Plants developed through 1–4 months-old embryonic cultures were comparable to *in vivo* material in terms of vigour and yield whereas, some of the plants raised from aged cultures were either abnormal in growth or showed variegated leaves.

Morphological variability in regenerants

One month-old field grown plantlets obtained through seedling-derived somatic embryogenesis and one-month-old control plants (obtained by the germination of agamospermous seeds) were compared for their morphological characters i.e., leaf length, leaf width, size of stomata, number of stomata per unit area and epidermal cell size of 30 randomly selected plants. On the basis of variations recorded in plants raised through seedling-derived embryonic callus, we classified plants into

Table 1. Effect of different concentrations of BAP and inoculum size on shoot proliferation on MS-9 medium containing IAA (0.57 µM) and adenine (25 mg l^{-1}).

BAP (µM)	Shoots/ inoculum	Number of shoots ± S.D.* after 4 weeks of culture	Shoot Length ± S.D.
4.4	1	1.8 ± 0.7	7.0 ± 1.7
11.1	1	2.6 ± 0.5	6.8 ± 1.6
22.2	1	3.8 ± 0.8	7.4 ± 1.7
4.4	10	14.8 ± 0.8	5.6 ± 1.4
11.1	10	16.6 ± 1.1	5.6 ± 1.9
22.2	10	21.4 ± 1.1	6.3 ± 1.7

*Inoculum:Significant at 1 %, SE= 0.48, $CD_{1\,\%}$= 1.53
BAP:Significant at 1 %, SE = 0.59, $CD_{1\,\%}$= 1.91
Inoculum x BAP:Significant at 1 %, SE = 0.83, $CD_{1\,\%}$ = 2.69

Table 2. Morphological variation in one-month old filed grown plantlets generated through somatic embryogenesis from seedling-derived callus.

Group	Leaf		Stomata			Epidermis cell size	
	Length[a] (cm ± S.D.)	Width[b] (cm ± S.D.)	Number[c] mm^{-2} (± S.D.)	Length[d] (µm± S.D.)	Width[e] (µm ± S.D.)	Length[f] (µm ± S.D.)	Width[g] (µm ± S.D.)
Control	4.2 ± 0.4	0.85 ± 0.2	10.0 ± 1.2	25.5 ± 2.3	22.2 ± 0.4	110.5 ± 12.3	31.0 ± 2.1
A	2.5 ± 0.3	0.65 ± 0.1	11.2 ± 0.5	24.8 ± 2.2	21.5 ± 0.8	106.8 ± 39.5	36.2 ± 4.5
B	3.9 ± 0.1	0.68 ± 0.1	8.9 ± 0.5	25.1 ± 1.7	22.5 ± 1.9	107.6 ± 11.7	29.1 ± 3.3
C	6.5 ± 0.3	0.47 ± 0.1	5.3 ± 0.7	39.0 ± 3.9	25.0 ± 1.4	274.7 ± 4.8	33.7 ± 1.9

[a] Significant at 1 %, SE = 0.07, $CD_{1\,\%}$ = 0.19; [b] Significant at 1 %, SE = 0.01, $CD_{1\,\%}$ = 0.03
[c] Significant at 1 %, SE = 0.17, $CD_{1\,\%}$ = 0.47; [d] Significant at 1 %, SE = 0.82, $CD_{1\,\%}$ = 2.26
[e] Significant at 1 %, SE = 0.42, $CD_{1\,\%}$ = 1.17; [f] Significant at 1 %, SE = 7.01, $CD_{1\,\%}$ = 19.38
[g] Significant at 1 %, SE = 1.01, $CD_{1\,\%}$ = 2.80

three group viz., Group A, B and C (Table 2). It is evident from the results that a significant variation was recorded in all the parameters used. There was a significant change in leaf width and leaf length. Plants in group C were 2.6 times longer than that of group A (Fig. 1A). Numbers of stomata per mm^2 also varied significantly, lowest being in group C and the size of stomata was biggest in this group of plants. Epidermal cell size also varied significantly, highest being in plants of group C. Therefore, plants of group C had long narrow leaves with least number of stomata and large epidermal cells (Table 2). These observations clearly indicate that there was a considerable variation in plantlets obtained through somatic embryogenesis. Occasionally variegated plants were also observed in the plants regenerated from long-term cultures (Fig. 1B). Plants in group B were comparable to the control plants.

Cytological variations

The basic chromosome number of the genus *Chlorophytum* is x=7 or 8. Chromosome number reported in *C. borivilianum* is 2n=3x=24 with a basic number of x=8 and occurrence of diploid species is rare (Kumar and Subramanium 1986). Interestingly, a series of polyploidy in the natural population has been reported in the species (Sheriff and Chennayeeraiah 1972; Naik 1976; Kumar and Subramanium 1986; Patil et al. 1987; Bordia et al. 1995). Tremendous morphological, chromosomal, and cytotypic diversity has been also been reported in all the species of the genus *Chlorophytum* (Naik 1976; Bordia et al. 1995). Plants in wild are triploid (2n=3x=24). In the present study, we investigated the cytological variation in the plants regenerated through direct organogenesis from stem-disc explants (Suri et al. 1999a) and somatic embryogenesis through seedling and leaf explants. All plants raised through organogenesis from stem-disc explants showed a stable ploidy level (2n=3x). Decline in embryonic potential with age of cultures was a major problem with seedling-derived callus for its long-term maintenance. Cytological investigations of plantlets raised through somatic embryogenesis from agamospermous seeds (2n=3x=24) showed a wide variation in chromosome number (Table 3, Fig. 1C). A clear tendency of increase in ploidy level with the age of cultures is evident from the results. Least chromosomal variation (2n=3x−3 to 2n=3x+3) was observed in germinated somatic embryos in 1 to 4 months−old cultures. Somatic embryos produced in cultures older than 6 months failed to germinate which may be due to high instability in the chromosome number and increase in ploidy level. These germinated somatic embryos showed a wide variation in the ploidy level, ranging from 2n=5x−1 to 2n=7x. Plantlets raised

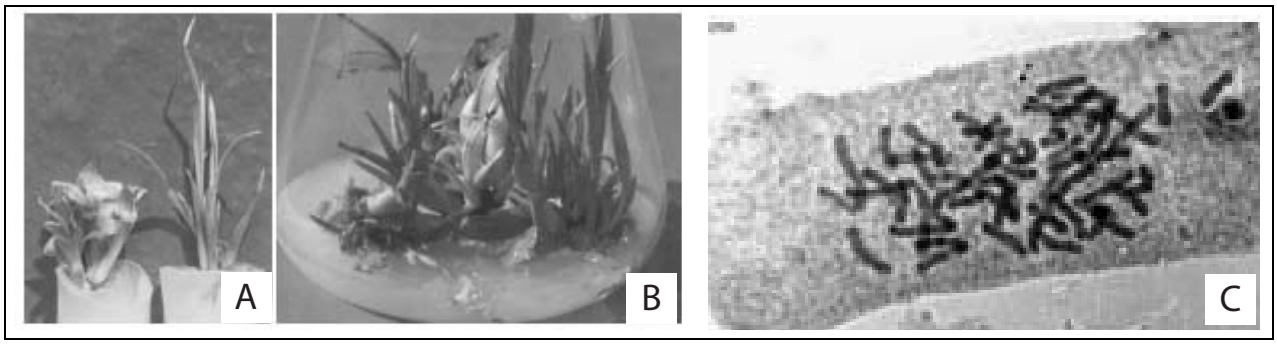


Fig. 1. [A]: Variations in the size of leaves and plants raised through somatic embryogenesis from long-term seedling-derived callus; [B]: Occasional formation of variegated leaves in the plants regenerated from long-term seedling-derived embryonic cultures; [C]: Root tip squash of precociously germinated abnormal somatic embryo showing 56 chromosomes.

Table 3. Chromosomal variation in *in vitro* regenerated plantlets raised through organogenesis from stem-disc explants and somatic embryogenesis (SE) from seedling- and leaf-derived callus.

Material age of cultures (months)	Number of chromosomes	Ploidy level
A: Control plants	24	3x
B: Organogenesis*	24	3x
C_1: Seedlings-derived somatic embryos – SE 1 to 4	21 to 27	3x–3 to 3x+3
C_2: Precociously germinated – SE 18 to 24	39 to 56	5x–1 to 7x
D: Leaf-derived somatic embryos 1 to 4	22 to 26	3x–2 to 3x+2

*Plantlets raised through organogenesis from stem-disc explants (Suri et al. 1999a).

through somatic embryogenesis from leaf explants (from triploid plant), showed comparatively little variation in chromosome number, and the ploidy level varied from 2n=3x–2 to 2n=3x+2. Therefore, the suitability of an alternative method, developed through leaf-derived somatic embryogenesis, in minimizing chromosomal variation is confirmed.

RAPD Fingerprinting

Because of the high level of morphological and cytological variation in plants obtained through seedling-derived embryonic callus, they were not subjected to RAPD fingerprinting. Plants raised through leaf-derived embryonic callus were screened for their genetic stability by RAPD finger printing. Of the primers initially screened, total 25 primers were short-listed for the study. A total of 2080 amplification products were produced by 25 RAPD primers with an average frequency of 5.2 bands per primer. The size of bands ranged from 0.56 to 2.0 kb. Interestingly, 20 out of 25 primers tested produced amplification products that were monomorphic across all 15 accessions and the mother plant (Fig. 2). The remaining 5 primers produced different amount of polymorphic bands that ranged from one, with OPA1 (CAGGCCCTTC), OPA17 (AGGTGACCGT), OPC6 (GAACGGACTC), to 2 with OPA20 (GTTGCGATCC) and 3 with OPC1 (TTCGAGCCAG) (Table 4, Fig. 3). Eight out of the total of 130 scorable bands were polymorphic, indicating low level of polymorphism (6.15 %) in the plants regenerated through leaf-derived somatic embryogenesis. Evenness and Simpson's reciprocal index for each of the primers used is presented in Table 4. The higher the value of Simpson's reciprocal index the greater the diversity. The highest diversity was observed in the results obtained by using the OPC1 primer. Therefore, RAPD fingerprinting revealed that the plant genome amplified by arbitrary primers, did not vary significantly in plants obtained through somatic embryogenesis from single leaf explants. Thus, an alternative method developed to generate plants through leaf-derived somatic embryogenesis could be used to minimize somaclonal variation and to maintain genetic stability in most of the plants.

Discussion

Large-scale propagation is required for the domestication of *C. borivilianum*. Methods of multiplication through or-

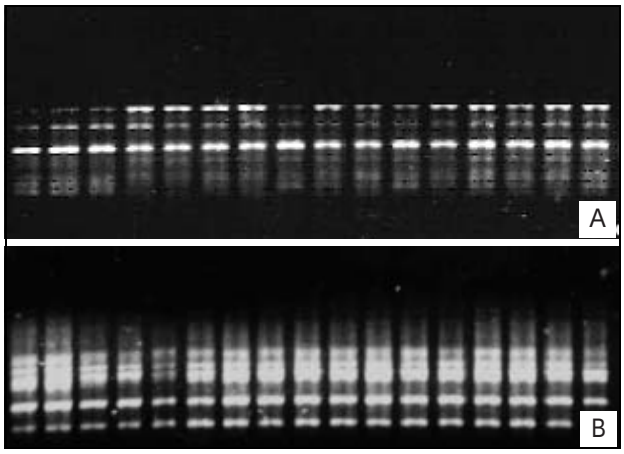


Fig. 2. RAPD fragments obtained from plants raised through somatic embryogenesis from one single leaf explants of *C. borivilianum*. Primer A) OPC02, and B) OPC13 showed monomorphic pattern across all 15 accessions (lane 2–15) and the mother plant (lane 1).

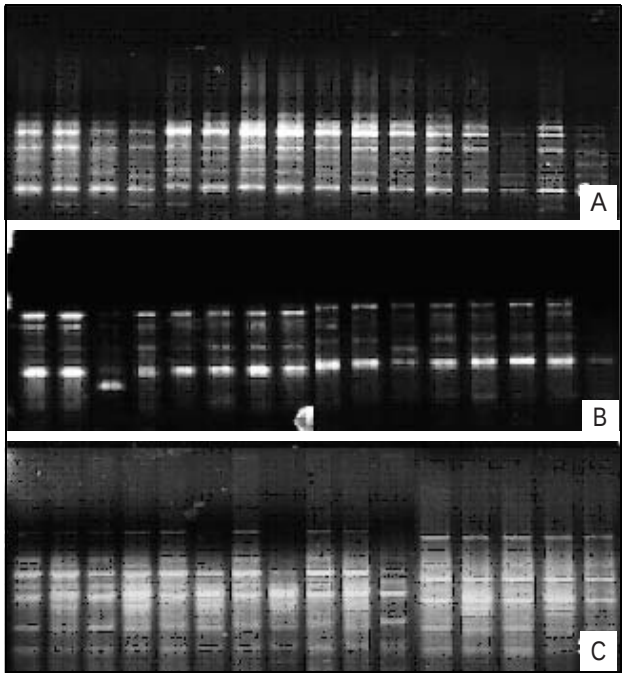


Fig. 3. Polymorphism detected by A) OPA17, B) OPA20 and C) OPC01 RAPD primers in plants raised through somatic embryogenesis (lane 2–15) from single leaf explants of *C. borivilianum*.

Table 4. Attributes of oligonucleotide primers used for RAPD fingerprinting of 15 randomly selected plantlets of *C. borivilianum* raised through leaf-derived somatic embryogenesis.

Primer	Sequence (5'–3')	Total no. of scorable bands per primer	PM (% P)	1/D	E
OPA1	C A G G C C C T T C	6	1 (16.6)	5.46	0.917
OPA9	G G G T A A C G C C	4	0	4	1
OPA11	C A A T C G C C G T	3	0	3	1
OPA12	T C G G C G A T A G	3	0	3	1
OPA13	C A G C A C C C A C	5	0	5	1
OPA16	A G C C A G C G A A	5	0	5	1
OPA17	G A C C G C T T G T	9	1 (11.1)	4.25	0.915
OPA18	A G G T G A C C G T	7	0	7.04	1
OPA20	G T T G C G A T C C	6	2 (33.3)	4.25	0.755
OPC1	T T C G A G C C A G	9	3 (33.3)	8.55	0.928
OPC2	G T G A G G C G T C	6	0	6.02	1
OPC5	G A T G A C C G C C	6	0	6.02	1
OPC6	G A A C G G A C T C	6	1 (16.6)	5.23	0.910
OPC7	G T C C C G A C G A	4	0	4	1
OPC9	C T C A C C G T C C	3	0	3	1
OPC10	T G T C T G G G T G	5	0	5	1
OPC13	A A G C C T C G T C	5	0	5	1
OPC17	T T C C C C C C A G	3	0	3	1
OPK2	G T C T C C G C A A	4	0	4	1
OPK3	C C A G C T T A G G	4	0	4	1
OPK6	C A C C T T T C C C	6	0	6.02	1
OPK10	G T G C A A C G T G	5	0	5	1
OPK13	G G T T G T A C C C	6	0	6.02	1
OPK19	C A C A G G C G G A	6	0	6.02	1
OPK20	G T G T C G C G A G	4	0	4	1
Total		130	8 (6.15)		

PM: No. polymorphic bands; % P: percent of polymorphism; 1/D: Simpson's reciprocal index; E: Evenness

ganogenesis (Purohit et al. 1994; Suri et al. 1999a) have been reported. In an effort to develop a technology for large-scale multiplication of *C. borivilianum*, conditions for organogenesis were optimised and 15,000 plantlets were successfully transferred to the field (Dave et al. 2003, 2004; Purohit et al. 2003). We reported somatic embryogenesis (Arora et al. 1999) in *C. borivilianum* using agamospermous seeds from triploid plants as a source of explants. In the micropropagation system, maintenance of genetic integrity with regard to explant source is of paramount importance and therefore, somatic embryogenesis is generally preferred (Rani and Raina 2000). The present report describes the fate of *C. borivilianum* in long-term embryonic cultures. Most of the plants in nature are triploid; which is therefore, widely available source for the multiplication of this plant. Furthermore, plants generated through seedling-derived embryogenic callus, older than 4 months, showed wide range of morphological and cytological abnormalities and cultures older than 6 months failed to germinate in to a complete plant. Therefore, in the present investigation, we screened plants generated through different means for their genetic stability and an alternative method was developed to reduce somaclonal variation in germinated somatic embryos, by using leaf from genetically stable plants raised from leaf-derived somatic embryogenesis (1–4 months-old cultures) as a source of explants for re-initiation of fresh embryonic cultures.

Gross cytological and morphological variation was observed in plants obtained from long-term seedling-derived callus; therefore, these cultures were not subjected to RAPD analysis. Long-term cultures are not useful for large-scale multiplication at this stage. Establishment of embryogenesis in long-term cultures is a general problem among several members of the family *Liliaceae*, like in *Allium* species (Myers and Simon 1998). We report here high level of somaclonal variation in long-term cultures of *C. borivilianum*. With the age of cultures, callus developed polyploidy and somatic embryos harvested from these cultures showed precocious germination, and these cultures eventually turned non-embryonic. Embryonic cultures remained fairly stable only until for a very short period of growth i.e., 1–4 months. This becomes a major hurdle. Similar results were also observed in several

members of liliaceae like garlic (AL-ZAHIM et al. 1999). Moreover, initially only small amounts of callus were produced even on the optimal medium, which in addition grew slowly. Thus, a very limited time and callus is available for multiplication of embryogenesis mass.

An alternative method of renewed embryogenesis using leaf explants from plantlets raised through fresh embryonic cultures was found to be useful for obtaining genetically uniform material. Plants generated using the method described were distributed to forest departments for conservation of the species. We suggest further work in this line of research to help the "Mother Nature" to save this elite plant in wild stands.

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