Environmental and Experimental Biology (2014) 12: 167–178 *Original Paper*

Direct organogenesis, phytochemical screening and assessment of genetic stability in clonally raised *Chlorophytum borivilianum*

Sautrik Basu[1], Timir Baran Jha[2]*

[1]Department of Botany, Maulana Azad College, 8 Rafi Ahmed Kidwai Road, Kolkata 13, India
[2]P.G Department of Botany, Barasat Govt. College, Barasat, Kolkata 700124, India

*Corresponding author, E-mail: tbjha2000@yahoo.co.in

Abstract

Keeping in mind the immense commercial and pharmacological importance of the rare aphrodisiac herb safed musli (*Chlorophytum borivilianum* Sant et Fern), the present investigation describes the development of a rapid and efficient protocol for direct plant regeneration from leaf explants of two populations of *C. borivilianum* collected from different agroclimatic zones of India. The induction of shoot initials was achieved on both Murashige and Skoog's (MS) and Gamborg's basal media supplemented with varying levels of 6-benzyladenine (1.11 to 8.90 μM) and thidiazuron (1.14 to 9.08 μM). MS basal medium supplemented with 4.54 μM thidiazuron was found ideal for shoot induction and gave rise to numerous shoot primordia within three weeks without any callus phase. Seperation of shoot clumps and their subsequent transfer to MS basal medium supplemented with 4.54 μM thidiazuron resulted in differentiation of more than 90% of the shoot initials into well developed shoots. Healthy shoots were rooted on half strength MS supplemented with varying concentrations of indole-3-acetic acid (2.28 to 11.42 μM), indole-3-butyric acid (1.97 to 9.80 μM) and naphthalene acetic acid (2.7 to 10.8 μM). Regenerated plantlets were transferred to the field with more than 80% success. Comparative cytological, molecular and phytochemical screening of the donor populations and their regenerates revealed genetic and phytochemical stability. The fingerprinting patterns generated in our study using high performance thin layer chromatography can be used effectively for quality screening and checking adulteration among different populations and cultivars of *C. borivilianum*. The micropropagation strategy may be effectively utilized for mass propagation as well germplasm conservation of this rare medicinal herb.

Key words: *Chlorophytum borivilianum*, clonal fidelity, direct organogenesis, randomly amplified polymorphic DNA, thidiazuron.
Abbreviations: BA, 6-benzyladenine; CTAB, cetyl trimethyl ammonium bromide; HPTLC, high performance thin layer chromatography; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; MS, Murashige and Skoog medium; NAA, naphthalene acetic acid; PCR, polymerase chain reaction; TDZ, thidiazuron; RAPD, Randomly Amplified Polymorphic DNA; TCL, total chromatin length.

Introduction

Chlorophytum borivilianum Sant et Fern (Liliaceae), commonly known as safed musli is a rare aphrodisiac herb valued for its dried fasciculated roots. The storage roots of safed musli are widely used for various therapeutic applications in the Ayurvedic and Unani systems of medicine and are also reputed to possess various pharmacological utilities, with saponins as one of the important phytochemical constituents (Oudhia, Tripathi 1999). Saponins exhibit diverse biological activities in animals including membrane-permeabilising, hypocholesterolaemic, anti-carcinogenic, spermatogenic, antidiabetic, antiarthritic and immunostimulatory properties (Francis et al. 2002; Kaushik 2005). In addition to saponins, *Chlorophytum* roots are also major source of sugars, polysaccharides, amino acids, alkaloids, vitamins, steroids, phenol and resins (Thakur et al. 2009).

C. borivilianum is a nationally acclaimed high value medicinal plant. The dried fasciculated roots fetch an attractive price of US $ 30 per kg in the Indian market (Dave et al. 2003). Ever increasing demand in national (~35 000 t) and foreign (~300 to 700 t) markets has led to overexploitation of the species from the wild. In nature, safed musli propagates vegetatively through its fleshy roots and rarely by seeds. Rapid habitat loss, ruinous harvesting practices, prolonged dormancy (nearly 10 months) and poor seed germination rate (14 to 16% only) (Maiti, Geetha 2005) have further enhanced the gap between demand and supply. Thus, for commercialization of this crop, quality planting material is required on a large scale, which in turn requires the development of an easy, rapid and economically viable propagation method.

Application of biotechnological methods can offer an ideal solution for circumventing the preexisting problems. Available reports describing tissue culture-based propagation of *C. borivilianum* (Purohit et al. 1994; Suri et al. 1999; Dave et al. 2003; Basu, Jha 2007; Kumar et al.

2010; Samantaray, Maiti 2010) are mainly based on either shoot multiplication from stem disc/shoot base explants or somatic embryogenesis from stem disc/shoot meristemoid derived calli (Arora et al. 1999). However, *in vitro* propagation of *C. borivilianum* via direct organogenesis has not been reported earlier.

It is also noteworthy to mention that although the saponin rich roots of *C. borivilianum* are the principle material for many herbal formulations and is also often consumed as a raw drug, precise information on the estimation of saponins, sapogenins and other root specific nutritional bioactives has not been established so far from different populations growing in different agroclimatic zones of India. From commercial as well as pharmacological points of view *in vitro* clonal propagation of high yielding cultivars or populations is highly desirable in order to maintain uniformity, ensure quality and efficacy of herbal drug manufacture. Extensive review of literature however reveals a severe paucity of specific quantitative information regarding saponins of different populations of *C. borivilianum,* causing considerable confusion among traders, cultivators as well as researchers. Thus, taking into account the lacunae and inadequacies of the previous research, attempts were made in the present investigation to develop a reliable protocol for direct *in vitro* regeneration coupled with cytological, molecular and phytochemical analysis of the *in vitro* regenerants of two populations of safed musli. To the best of our knowledge, this is the first successful report describing direct regeneration of clonally uniform plantlets from leaf base explants of *C. borivilianum*. The fingerprinting patterns generated in the present investigation using high performance thin layer chromatography (HPTLC) can be effectively used for checking possible adulteration and also for screening and identification of elite populations of *C. borivilianum*.

Materials and methods

Plant material

Samples from two populations of *C. borivilianum* were collected from two different agroclimatic zones of the country: one from Narendrapur, West Bengal (altitude 12 m; 22°34'10" N; 88°22'10" E) and the other from Mt. Abu, Rajasthan (altitude 1220 m; 24°33' N; 72°38' E). The samples maintained *in vitro* for 20 weeks following the previously described methods (Basu, Jha 2007) were used as explant sources. The explants for initiation of micropropagation consisted of 0.5 to 1.3 cm long basal portions of *in vitro* grown mature leaves.

Culture medium and conditions

Full strength Murashige and Skoog (MS; Murashige, Skoog 1962) and B_5 (Gamborg et al. 1968) basal medium supplemented with 3% (w/v) sucrose, 0.8% agar (bacteriological grade) and varying levels of two cytokinins 6-benzyladenine (BA; 1.11 to 8.90 µM) and thidiazuron (TDZ; 1.14 to 9.08 µM) were used for initiation of organogenic cultures. Half strength MS basal medium supplemented with 3% sucrose (w/v), 0.8% agar (w/v) (bacteriological grade) and different concentrations of indole-3-acetic acid (IAA; 2.28 to 11.42 µM), indole-3-butyric acid (IBA; 1.97 to 9.80 µM) and naphthalene acetic acid (NAA; 2.7 to 10.8 µM) were used for induction of roots. Prior to rooting the microshoots obtained via direct organogenesis were maintained further for two weeks on MS basal medium supplemented with 3% sucrose, 0.8% agar and 4.54 µM TDZ for further elongation. The media were adjusted to pH 5.7 ± 0.1 with 1 N NaOH or 1 N HCl before autoclaving at 121 °C for 17 min. All cultures were maintained at 22 ± 2 °C and relative humidity of 50 to 60% under cool white fluorescent tubes (Philips, India) emitting 32 µmol m^{-2} s^{-1} with 16/8 h light/dark period.

Statistical analysis

Each treatment was repeated thrice with five replicates and five explants per replicate. The differences among means of each treatment were analyzed by ANOVA and statistical difference between mean tabulated values were estimated using the Duncan's multiple range test with Statistica software (Version 10.0).

Histological analysis

Histological analysis of the regenerating shoots was performed on explants at different stages of development. The required samples belonging to different stages of organogenesis were removed carefully from culture vessels and fixed in 5 mL formalin + 5 mL acetic acid + 90 mL 70% ethanol according to Sass (1940). Infiltration of the specimens was performed by gradual addition of paraffin wax (melting point 58 to 60 °C) and tertiary butyl alcohol until the solution attained super saturation. The specimens were then cast into paraffin blocks. The paraffin-embedded specimens were sectioned with a rotary microtome. The thickness of the sections ranged from 8 to 10 µm. De-waxing of sections was made by the customary procedure of Johansen (1940). The sections were stained with toluidine blue according to O'Brian et al. (1964). Photographs were taken with a photomicroscope (Carl Zeiss Axiostar plus) fitted with a Canon Power Shot G7 wide zoom digital camera.

Hardening and ex vitro *transfer*

In vitro rooted plantlets were carefully washed under running tap water to remove the agar and then rinsed with 1% solution of carbendazim for 5 min, after which they were planted in plastic containers containing a mixture of soilrite (horticultural grade), sterile sand and garden soil (in 1:1:1 proportion). To maintain high ambient humidity, pots were kept in a mini mist chamber. The temperature and humidity within the chamber ranged from 23 to 25 °C

and 80 to 85%, respectively. The plants were maintained in the mist chamber for 3 weeks before they were transferred to greenhouse and replanted in earthen pots (containing garden soil-sand mixture; 1:1 v/v). At the onset of monsoon (June-July), nearly 100 plants belonging to both populations were transferred from greenhouse to field conditions.

Chromosomal analysis

The material for cytological analysis comprised of *in vivo* donor plants and 25 randomly selected well-established *in vitro* plants. Study of somatic chromosomes were carried out following previously published methods (Basu, Jha 2011a). Healthy root tips of proper sizes (3 to 5 mm) were pretreated with aqueous solution of 0.05% colchicine (Sigma, USA) for 3 h at 12 to 14 °C, fixed in Carnoys' Fixative overnight and stained in 2% propionic orcein/HCl mixture (9:1 v/v) for 2 to 3 h. The root tips were squashed in 45% acetic acid. Well-scattered metaphase plates (with properly condensed chromosomes) were observed under microscope (Carl Zeiss, Axiostar plus) and photographs were taken using a Canon Power Shot G7 wide zoom camera.

Chromosome images were analysed using the software package Axio Vision L.E. 4, Zeiss. Chromosome images were measured from five plates for each material in pixel units that were converted to micrometers with reference to a standard scale (stage micrometer). For detailed karyotype analysis, measurements of long arm (L), short arm (S) and total length of chromosomes were calculated and the arm ratio (r) for each of the homologous pairs was calculated (L/S) following previously published methods (Basu, Jha 2011a). Classification of the chromosomes was made following the system proposed by Levan et al. (1964).

DNA isolation

Total cellular DNA was isolated from fresh young leaves following the method of Doyle and Doyle (1987) with minor modifications. About 3 g of freeze-dried leaf tissue was crushed in a morter and double volume of isolation buffer was added. The isolation buffer consisted of 100 mM Tris-CI (pH 8), 50 mM EDTA, 1.4 M NaCl, 2.5% CTAB (w/v) and 1% polyvinlypolypyrrolidone (w/v). The homogenate was transferred quickly to 50 mL polypropylene centrifuge tubes and incubated at 60 °C for 45 min in order to denature the proteins. After incubation, 5 M potassium acetate was added to the homogenate and mixed thoroughly by gentle inversion. The slurry was incubated at 0 °C for 30 min. After incubation the tubes were centrifuged at 10 000 rpm for 20 min at 4 °C. The supernatant was collected and to it equal volume of chloroform/isoamylalcohol (24:1 v/v) was added. The tubes were again centrifuged at 10 000 rpm for 10 min at 4 °C, after which the aqueous layer was carefully collected. To the collected aqueous layer equal volume of chilled isopropanol was added and the DNA was allowed to precipitate as a pellet. The resultant pellete was washed three times with 70% ethanol, dried under vacuum and resuspended in a volume of sterile double distilled water. After treatment with RNase, the purity of DNA was checked on 0.8% agarose gel and the concentration of DNA was checked spectrophotometrically at 260 nm.

PCR amplification, data scoring and analysis

Isolated DNA from *in vivo* donors and *in vitro* regenerated plants were subjected to polymerase chain reaction (PCR) to generate fingerprinting patterns. PCR amplification was carried out with random decamer primers obtained from Operon Technologies (Alameda, California, USA). Initially 26 random primers were used for Randomly Amplified Polymorphic DNA (RAPD) amplification. Further screening identified eight primers that amplified clear and reproducible bands. DNA amplification was performed in a thermal cycler (Bio-Rad, Mini Gradient Thermal Cycler). Each 25 µL reaction mixture contained 10× PCR buffer (2.5 µL), 25 mM $MgCl_2$ (1.5 µL), dNTP mix (2.5 µL, Sib Enzyme , USA), 0.5 µL of each primer (50 µM), 1 µL (25 ng) template DNA, 16 µL sterile double distilled water and 1 unit of Taq DNA polymerase (Sib Enzyme, USA). PCR conditions included an initial denaturation step at 94 °C for 3 min, followed by 45 amplification cycles: denaturation at 92 °C for 1 min, annealing at 35 °C for 1 min and extension at 72 °C for 2 min, with a final extension step at 72 °C for 10 min.

PCR products were then separated on 1.5% agarose gel in 0.5X TBE buffer (89 mM Tris base, 89 mM boric acid and 2 mM EDTA, pH 8.0) with 0.5 µg mL^{-1} ethidium bromide at 100 V constant voltage for 2 h 30 min. The gels were visualized with a UV trans-illuminator. Photographs were taken using a Gel Doc System (Kodak Gel Logic) and the amplification product sizes were evaluated using Gel Logic image analysis software (Kodak). All PCR reactions were run in triplicate to ensure result consistency and only reproducible and clear bands were scored. Each reproducible band was visually scored '1' for presence and '0' for absence. The data were analyzed using NTSYSpc ver. 2.1 (Rohlf 2002).

Phytochemical analysis

For chemical analysis about 6 g of dry and powdered root tissue were taken from each donor population as well as from the *in vitro* grown regenerants. Marker samples for HPTLC were kindly provided by Prof. S Ghosal of Natreon-Inc Kolkata (India). Extraction of saponins was carried out following previously published methods (Thakur et al. 2007) with minor modifications. For extraction of crude saponins defatted root samples were individually extracted under reflux with 80% methanol (three times). The methanol extracts were pooled and concentrated under reduced pressure to obtain a dark colored residue. The residue of each sample was dissolved in water and then partitioned with n–butanol. The n-butanol layer was separated,

concentrated under reduced pressure and saponins were precipitated with diethyl ether. The precipitated saponins were eventually collected after centrifugation and dissolved in water.

Chromatography and quantification of saponins were carried out from these samples. The HPTLC system consisted of a CAMAG Linomat-5 (Muttenz, Switzerland) automatic sample applicator (with nitrogen flow) and CAMAG TLC Scanner-3 equipped with WINCATS software (version 1.4.4.6337). The stationary phase consisted of pre-coated silica gel 60 F254 plates (20 × 10 cm; with 0.25 mm layer thickness, Merck KGaA; 1.05554. 0007). Samples were applied to the plates as bands 6 mm wide, with 10 mm distance between tracks, by means of a Linomat-5 automatic sample applicator equipped with a 100 µL Hamilton syringe. The mobile phase used for saponins consisted of a mixture of chloroform: methanol: acetic acid: water (60:30:10:8). Extraction, chromatography and quantification of sugars were performed following the method of Di Xin et al. (2003) with minor modifications. For saponins the plates were scanned initially at 210 nm and then at 530 nm after derivatization with vanillin-sulphuric acid reagent and for sugars the plates were scanned at 365 nm after derivatization with a reagent solution consisting of 0.5 g 4-aminobenzoic acid, 9 mL glacial acetic acid, 10 mL water and 0.5 mL of 85% H_3PO_4. The chromatograms and spectra were matched with external standards and the peak areas were evaluated by a CAMAG TLC Scanner 3 equipped with WINCATS software. All experiments were repeated thrice and validated for linearity, precession (intra day and inter day), specificity, limit of detection and limit of quantification following ICH guidelines.

Results and discussion

Establishment of contamination-free cultures of *C. borivilianum* did not pose any problems, as leaf explants were taken from *in vitro* propagated shoots, eliminating the need for disinfection procedures and also reducing the possibility of tissue damage and contamination. Shoot regeneration started within two weeks of culture (Fig. 1A). Nodular shoot masses were clearly visible as protuberances growing directly from the epidermal cell layers of the leaf bases cultured on both MS and B_5 medium fortified with different levels of BA and TDZ (Fig. 1B). Out of the two cytokinins used, TDZ was the most effective, as it significantly affected the shoot number and also the regeneration frequency (Table 1). Maximum number of shoots per explant was obtained at a concentration of 4.54 µM TDZ.

The morphogenic response of the two populations of safed musli cultured on different media and under different hormonal regimes also differed considerably (Table 1). Full strength MS basal medium supplemented with varying levels of BA and TDZ exhibited better response in comparison to B_5 medium with identical levels of growth regulators, thus reconfirming the fact that the type of basal salts in the regeneration medium can also affect the regeneration efficiency and frequency of explants. MS basal medium supplemented with 4.54 µM TDZ produced a maximum of 57.8 shoots (in CB-2 population) after eight weeks of culture (Fig. 1C). Shoot regeneration on different concentrations of TDZ used in combination with both MS and B_5 medium, however, did not follow a definite pattern. Initially the number of shoots increased gradually as the concentration of TDZ increased up to a certain threshold limit (from 1.14 to 4.54 µM) and a steady decline in shoot number was observed as the concentration of TDZ was increased further (6.81 and 9.08 µM). Histological analysis performed on explants at different stages of shoot development clearly revealed continuity of vascular strands from the explant to the shoot buds, thus reconfirming that the shoot buds had emerged from epidermal parenchyma cells with no intermediate callus formation (Fig. 1 F, G, H).

TDZ is the most potent of the diphenylureas that have been evaluated for use in plant tissue culture (Barik et al. 2004). It has been shown to induce adventitious and axillary shoot production in several plant species (Gurel et al. 2011; Van Nieuwkerk et al. 1986; Leblay et al. 1991; Huetteman, Preece 1993; Lane et al. 1998; Pati et al. 2004; Turker et al. 2009a, Turker et al. 2009b) including plants belonging to Liliaceae (Deroles et al. 2010). Taking into account the efficiency and effectiveness of TDZ, the shoot clumps obtained via direct organogenesis (Fig. 1D) were split and cultured further on full strength MS basal medium supplemented with 4.54 µM TDZ, for elongation and differentiation. The split shoot clumps obtained from both populations (CB-1 & 2) exhibited normal and appreciable growth and elongation but no additional shoots were formed after two weeks of culture on TDZ-containing medium (Fig. 1E). It has been reported earlier that, unlike other adenine-type cytokinins (BA or kinetin), TDZ inhibits elongation of shoots (Van Nieuwkerk et al. 1986; Preece, Imel 1991) and this inhibitory effect of TDZ may be attributed to the presence of a phenyl group (Huetteman, Preece 1993). In the present study however no such inhibitory effect of TDZ was observed.

Organogenesis is a complex morphogenetic process that involves the formation of monopolar structures destined to form either shoots or roots. Cytokinins sometimes in concert with auxins are plant growth regulators that induce *in vitro* shoot organogenesis. Although TDZ is structurally totally different from the commonly used auxins and cytokinins, it exhibits both auxin- and cytokinin-like effects even at very low concentrations (10 to 1000 times less than other phytohormones) and has the capacity to provoke regenerative processes like dedifferentiation and redifferentiation in cell and tissue cultures. Organogenesis needs stable regulation of cell proliferation, which are assumed to be confined to plant hormones. It has been

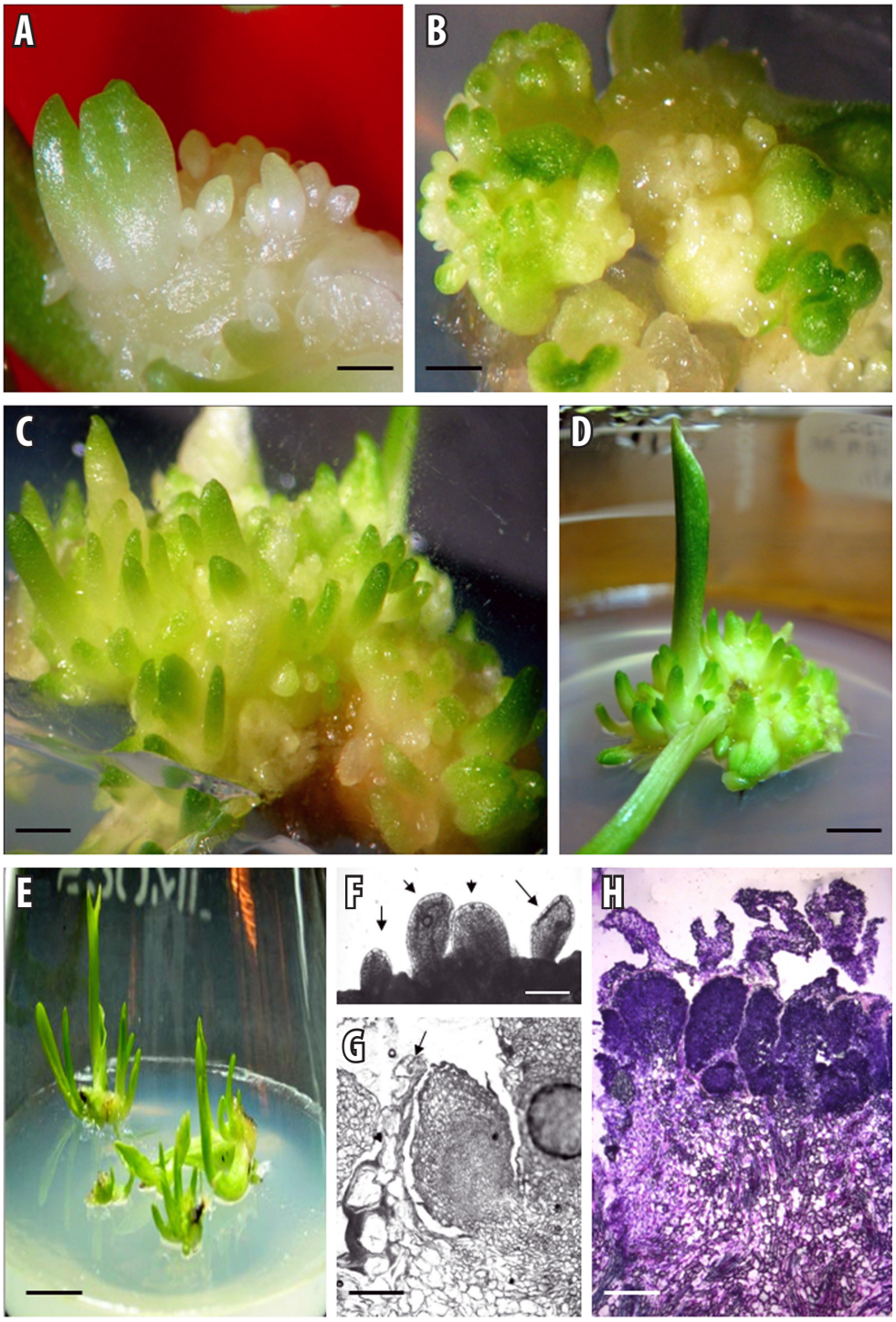

Fig. 1. A, initiation of shoot buds from leaf base explants of *C. borivilianum*. B, appearance of nodular shoot masses directly on leaf bases after four weeks of culture. C, profuse shoot regeneration on MS basal medium supplemented with 4.54 µM TDZ (after eight weeks of culture). D, a single shoot clump obtained via direct organogenesis on TDZ supplemented media (after 8 weeks). E, isolated shoot clumps on MS basal medium supplemented with 4.54 µM TDZ. F, sectional view of the explants showing initiation of shoot buds directly on the epidermal cell layers (indicated by arrows). G, a single shoot bud with two subtending leaves (indicated by arrow). H, sectional view of leaf explant showing a cluster of leaf primordial (after four weeks of culture). Scale bars for A to D 0.5 cm, E 1 cm; F to H 0.1 cm.

reported that the multidimensional growth regulator TDZ helps to establish the optimal internal balance of cytokinin and auxin required for the induction of organogenesis and also for the induction and expression of somatic embryogenesis (Saxena et al. 1992). This promoting effect of TDZ may be attributed to the fact that TDZ is highly

Table 1. Effect of different concentrations of BAP and TDZ on direct shoot organogenesis in *Chlorophytum borivilianum*. Results are mean ± SE of 5 replicates. Means followed by the same letter are not significantly different at 5% level according to Duncan's multiple range test. –, no response; CF, callus formation

Medium	Growth regulator		CB-1			CB-2		
	BA (μM)	TDZ (μM)	Number of explants producing shoots (%)	Mean number of shoots per explant		Number of explants producing shoots (%)	Mean number of shoots per explant	
				4 weeks	8 weeks		4 weeks	8 weeks
MS	0	–	–	–	–	–	–	–
	1.11	–	–	–	–	–	–	–
	2.22	–	22	1.9 ± 0.29 c	7.8 ± 1.64 g	26	2.3 ± 2.3e	8.1 ± 0.97 fg
	4.40	–	48	3.7 ± 1.68 a	9.2 ± 1.92 c	54	4.2 ± 0.81c	8.7 ± 0.34 bc
	6.62	–	63	8.1 ± 1.74 bc	10.4 ± 1.02 ab	67	8.5 ± 0.71a	12.3 ± 0.26 a
	8.90	–	53	7.3 ± 1.04 d	9.8 ± 0.97 d	69	8.2 ± 2.04d	10.1 ± 0.81 b
	–	0	–	–	–	–	–	–
	–	1.14	69	10.8 ± 0.97 h	19.9 ± 1.18 e	73	16.8 ± 1.27d	27.2 ± 1.04 cd
	–	2.27	76	12.6 ± 0.85 fg	27.9 ± 1.49 c	89	14.2 ± 0.91k	31.8 ± 1.16 f
	–	4.54	94	26.8 ± 1.32 de	44.3 ± 1.71 a	100	30.8 ± 0.16de	57.8 ± 2.04 i
	–	6.81	91	15.9 ± 1.06 d	39.6 ± 0.91 ef	95	18.3 ± 1.15f	42.6 ± 1.90 g
	–	9.08	85	13.8 ± 0.75 gh	26.4 ± 1.63 de	92	15.8 ± 2.19cd	28.2 ± 0.61 e
B_5	0	–	–	–	–	–	–	–
	1.11	–	13	4.3 ± 0.59 ef	9.6 ± 1.22 k	16	5.6 ± 0.82g	11.2 ± 1.17 d
	2.22	–	27	7.2 ± 1.41 h	11.5 ± 1.78 hi	32	6.9 ± 0.43e	10.4 ± 2.91 c
	4.40	–	34	6.1 ± 1.26 fg	10.3 ± 1.03 f	41	7.7 ± 1.80cd	11.6 ± 0.94 b
	6.62	–	41	6.9 ± 0.47 de	9.2 ± 0.21 j	49	5.2 ± 1.24f	7.9 ± 1.99 ef
	8.90	–	–	CF	CF	–	CF	CF
	–	0	–	–	–	–	–	–
	–	1.14	71	10.1 ± 0.95 c	21.3 ± 0.48 de	68	11.2 ± 2.01b	21.4 ± 1.36 g
	–	2.27	76	10.8 ± 1.77 a	26.7 ± 1.57 b	74	9.2 ± 1.93a	31.0 ± 0.89 cd
	–	4.54	84	18.6 ± 0.23 b	39.3 ± 1.94 ab	93	21.8 ± 0.67ab	41.6 ± 1.47 a
	–	6.81	87	10.4 ± 1.46 g	28.7 ± 0.58 c	91	12.0 ± 0.29d	33.0 ± 1.23 d
	–	9.08	92	5.8 ± 1.16 cd	17.9 ± 0.95 d	97	7.6 ± 2.04b	20.4 ± 0.32 f

stable in culture media and also persistent in plant tissues, as indicated by a carbon isotope study (Mok, Mok 1985). This high stability and greater efficacy of TDZ may be attributed to its resistance to the enzyme cytokinin oxidase (Meyer, Van Staden 1988).

Of the three auxins used for root induction, IBA was found most suitable. Although rooting occurred in both the absence and presence of IBA, half strength MS basal medium fortified with 3% sucrose and a concentration of 9.80 μM IBA induced maximum number of healthy and thick roots (21.3 ± 0.98) after two weeks of culture (Fig. 2A; Table 2). The effectiveness of IBA in inducing roots has been reported earlier in *Chlorophytum borivilianum* (Purohit et al. 1994; Dave et al. 2003; Basu, Jha 2007), *Chlorophytum arundinaceum* (Lattoo et al. 2006), *Chlorophytum nepalense* (Basu, Jha, 2011b) and also in many other species (Agretious et al. 1996; Verma et al. 2002).

Hardening and *ex vitro* transfer of plants also did not pose any major problems. After three weeks of acclimatization, uniform new leaves were formed and the leaf area also commenced a relatively rapid expansion in more than 85% plants belonging to both populations. When transferred to greenhouse conditions the plants developed normally and exhibited vigorous growth without any morphological aberrations. Of nearly 100 plants belonging to both populations that were transferred to *ex vitro* conditions at the onset of monsoon (June–July), more than 80% survived the monsoon rains and showed appreciable leaf growth and normal fasciculated root development (Fig. 2B).

Cytological analysis carried out from the root tips of donor populations and 25 randomly selected *in vitro* regenerants belonging to both populations revealed a chromosome number of 2n = 28 (Fig. 2 C, D, E, F) in more than 95% cells. Considerable variation was, however, noticed in karyological features, such as karyotype formula, total chromatin length (TCL), arm ratio, total form percentage (TF%) etc. (Table 3). The population collected from West Bengal had six pairs of median chromosomes

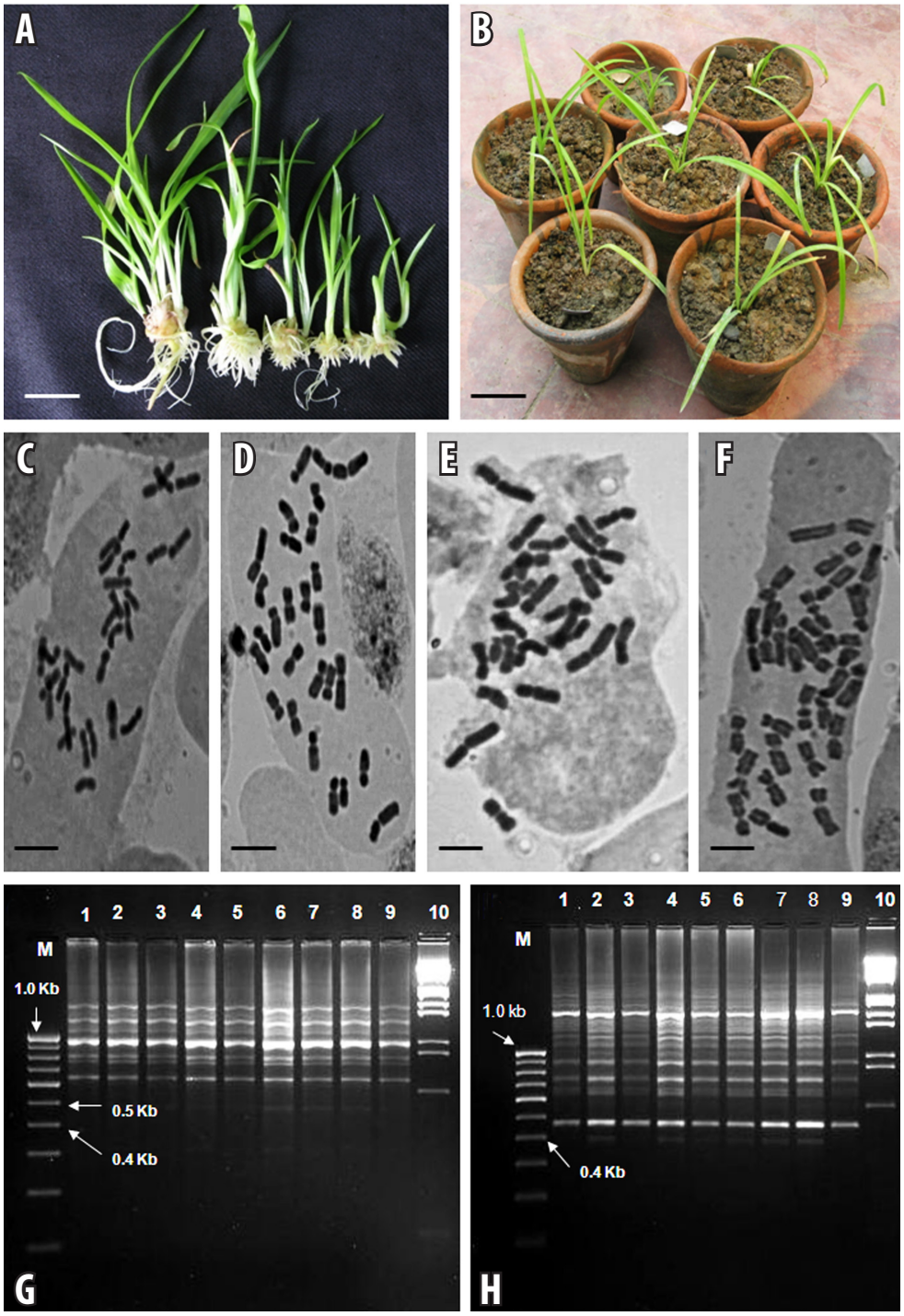

Fig. 2. A, rooted individual shoots of *Chlorophytum borivilianum* (prior to hardening) showing profuse healthy fibrous roots (bar 1 cm). B, field-established plants (30 days old; bar 10 cm). C, a somatic cell of *in vivo* donor plant (population CB-1) showing 2n = 28 chromosomes (bar 5 µm). D, somatic metaphase plate showing 2n = 28 chromosomes from field established plants (belonging to population CB-1; bar 5 µm). E, a somatic cell of *in vivo* donor plant (population CB-2) showing 2n = 28 chromosomes (bar 5µm). F, somatic metaphase plate showing 2n = 28 chromosomes from field established plants (belonging to population CB-2; bar: 5µm). G, DNA amplification profile obtained with primer OPD-5. Lane 1, mother plant (population CB-1); lanes 2 to 9, micropropagated plants; M, molecular mass marker (100 bp DNA ladder); lane 10, λ DNA digested with Eco RI and Hind III. H, DNA amplification profile obtained with primer OPD-1. Lane 1, mother plant (population CB-2); lanes 2 to 9, micropropagated plants; M, molecular mass marker (100 bp DNA ladder); lane 10, λ DNA digested with Eco RI and Hind III.

Table 2. Effect of different auxins on rooting of *in vitro* shoots derived from leaf base explants of two populations of *Chlorophytum borivilianum*. Results are mean ± SE of 5 replicates. Means followed by the same letter are not significantly different at 5% level according to Duncan's multiple range test

Populations (place)	Auxin used			Rooting (%)	Mean number of roots per shoot	Mean root length (cm)
	IAA (µM)	IBA (µM)	NAA (µM)			
CB-1	0	–	–	21	6.9 ± 0.45 d	3.6 ± 1.19 c
(West Bengal)	2.28	–	–	57	9.9 ± 0.87 a	2.4 ± 2.18 h
	5.71	–	–	69	15.8 ± 1.21 cd	2.1 ± 0.93 k
	11.42	–	–	76	18.3 ± 1.54 bc	3.9 ± 0.54 a
	–	0	–	34	3.9 ± 0.03 d	3.7 ± 0.32 op
	–	1.97	–	81	11.4 ± 1.41 ef	4.3 ± 1.34 j
	–	4.90	–	89	17.2 ± 1.28 b	3.5 ± 2.27 k
	–	9.80	–	96	21.3 ± 0.98 ef	2.6 ± 1.97 c
	–	–	0	28	2.6 ± 0.19 f	1.9 ± 0.01 i
	–	–	2.7	42	9.8 ± 1.90 d	2.7 ± 0.80 jk
	–	–	5.4	56	13.6 ± 2.11 b	3.3 ± 0.65 g
	–	–	10.8	73	15.3 ± 1.07 a	4.2 ± 1.72 g
CD-2	0	–	–	14	4.2 ± 0.87 bc	2.8 ± 1.29 hi
(Mt Abu,	2.28	–	–	61	7.2 ± 0.91 d	3.6 ± 2.05 ef
Rajathan)	5.71	–	–	59	11.9 ± 1.99 de	3.8 ± 2.13 fg
	11.42	–	–	67	16.1 ± 2.23 ef	4.1 ± 2.09 i
	–	0	–	24	4.1 ± 1.03 a	3.6 ± 1.25 k
	–	1.97	–	84	10.3 ± 1.14 p	2.9 ± 0.99 c
	–	4.90	–	89	13.9 ± 1.18 d	3.1 ± 0.92 cd
	–	9.80	–	92	19.8 ± 0.12 fg	3.4 ± 0.05 b
	–	–	0	19	3.4 ± 0.05 c	3.8 ± 1.96 c
	–	–	2.7	48	6.8 ± 1.19 bc	2.9 ± 1.19 a
	–	–	5.4	36	13.2 ± 2.09 gh	3.6 ± 0.21 fg
	–	–	10.8	59	11.6 ± 2.25 b	3.9 ± 1.18 j

and eight pairs of submedian chromosomes (12m + 16Sm) and exhibited a TCL of 87.35, an arm ratio of 2.24 and a TF% of 35.7. The population collected from Mt. Abu (Rajasthan) had one pair of metacentric chromosome, five pairs of median chromosomes and eight pairs of submedian chromosomes (2M + 10m + 16Sm). The Mt. Abu population had a TCL of 300, an arm ratio of 2.11 and a TF% of 35.6 (Table 3). When compared with the donor plants, the regenerated plants exhibited similar karyological features, thus confirming their true to type nature. Cytological analysis based on conventionally stained condensed somatic chromosomes is one of the most informative, reliable and cost effective method for the detection of structural/numerical variations occurring in chromosomes of *in vitro* raised plants. Cytological parameters have been used effectively for the assessment of genetic fidelity of a large number of micropropagated plants (Rao et al. 1992; Al-Zakhim et al. 1999; Arora et al. 2006; Lattoo et al. 2006; Chaudhuri et al. 2007; Basu, Jha 2011b). However, taking into account the limitations of conventional cytological techniques, the genetic stability of the regenerated plants was also evaluated through molecular techniques.

Fingerprinting profiles of the regenerants and of the donor populations were generated using a total of 26 primers of which eight generated distinct, reproducible amplified products (Fig. 2 G, H). Eight selected primers used in the present study yielded a total of 60 scorable amplification products ranging from 0.43 Kb (OPB-7 primer) to 1.87 Kb (OPA-9 primer) in size (Table 4). The number of bands for each primer varied from 7 to 9, with an average of 7.5 bands per primer. RAPD fingerprinting generated monomorphic patterns across all the regenerants analyzed and no polymorphism was detected in the micropropagated plants. The results of DNA fingerprinting indicated that the tiny sections of the plant genome that was amplified by arbitrary primers did not vary in plants regenerated via direct organogenesis. Monomorphic RAPD profiles across 60 amplification products indicated clear homogeneity among the culture regenerants and genetic uniformity with that of the donor populations. RAPD based assessment of genetic stability of *in vitro* grown micropropagated plants has been reported earlier in several medicinal, aromatic and horticultural plants (Rout et al. 1998; Rout, Das 2002; Martins et al. 2004; Chaudhuri et

Table 3. Comparative cytological analysis of donor plants of *Chlorophytum borivilianum* with regenerated plants. TCL, total chromatin length; L-S, longest-smallest chromosome; TF%, total form percentage = Σ short arm lengths x 100/ Σ short arm lengths + Σ long arm lengths; L/S, ratio between longest to smallest chromosomes (arm ratio)

Sample	Number of chromosomes	Karyotype formula	TLC (μM)	Longest chromosome (μM)	Smallest chromosome (μM)	L-S (μm)	L/S	TF%
CB-1; donor plants	2n = 28	12m + 16Sm	87.35	4.52	2.02	2.52	2.24	35.7
CB-1; regenerated plants	2n = 28	12m + 16Sm	105.76	5.63	2.66	2.97	2.11	35.98
CB-2; donor plants	2n = 28	2M + 10m + 16Sm	300.0	16.68	7.87	8.81	2.11	35.6
CB-2; regenerated plants	2n = 28	2M + 10m + 16Sm	293.68	16.2	7.18	9.02	2.25	36.6

al. 2007) including medicinally important members of the genus *Chlorophytum* (Lattoo et al. 2006; Kumar et al. 2010; Samantaray, Maiti 2010). Production of genetically uniform plantlets is important for commercial medicinal plants and is also essential for germplasm conservation. It is also well established that regenerated plantlets from organized shoot meristems are less susceptible to genetic variation than those regenerated from disorganized callus tissues (Pierik 1991). Direct regeneration thus can be used effectively for sustainable commercial propagation of safed musli.

The synthesis and concentration of bioactive compounds present in the *in vitro* raised plants have always been the core issue of debate for the Pharma companies and ayurvaidyas. It is a general belief that the compounds responsible for medicinal activity are either absent completely or present in insignificant amounts in tissue culture raised plants. Taking this into account, our study was deeply focused on the comparative phytochemical profiling of the plants collected from two different agroclimatic zones (CB-1&2) (Fig. 3 A, B, C, D) and their *in vitro* raised counterparts (CBT-1 & 2). The fasciculated roots of all the samples were tested for the presence of saponins and sugars. The results are summarized in Table 5. Populations collected from Mt. Abu (Rajasthan) surpassed the other population collected from West Bengal in terms of total saponin content and sugar content. The population collected from West Bengal had the highest percentage of total di- and oligosachharides. *C. borivilianum* is native to the subcontinent and it occurs in habitats comprising several altitudinal zones (up to 1500 meters) which makes it an attractive species to study the variation of metabolic profiles in different populations growing under different climatic conditions. The quantitative analysis of saponins among two populations of *C. borivilianum* over an altitudinal interval of 0 to 1220 m revealed that altitude may have a significant effect on the synthesis of saponins; the highest content of saponins was recorded from the population collected from Mt. Abu (altitude 1220 m). Although *C. borivilianum* is extensively used in many herbal formulations and also as a raw drug, very little work has been done on its biologically active chemicals, particularly saponins, which represent the major bioactive compounds present in *C. borivilianum* roots. Previous reports indicate wide variation in saponin content (2 to 17%) in *C. borivilianum* roots (Bordia et al. 1995). It has also been observed that the content decreased upon cultivation (Bordia, Jat 1990; Bordia et al. 1995). The present investigation, however, reports a much higher amount of spirostane saponins in the Mt Abu and West

Table 4. Details of the different arbitrary primers used for RAPD fingerprinting

Primers	Sequence 5' > 3'	Tm (°C)	%GC	Number of loci	Size range (Kb)	Mean number of bands per primer
OPA-9	GGGTAACGCC	34.0	70	9	1.87- 0.53	7.5
OPB-7	GGTGACGCAG	34.0	70	8	1.48 – 0.43	7.5
OPB-8	GTCCACACGG	34.0	70	7	1.74 – 0.66	7.5
OPB-10	CTGCTGGGAC	34.0	70	6	1.81 – 0.73	7.5
OPD-1	ACCGCGAAGG	34.0	70	8	1.8 – 0.39	7.5
OPD-5	TGAGCGGACA	32.0	60	7	1.54 – 0.47	7.5
OPD-6	GGGGTCTTGA	32.0	60	8	1.7 – 0.7	7.5
OPM-6	CTGGGCAACT	32.0	60	7	1.4 – 0.46	7.5

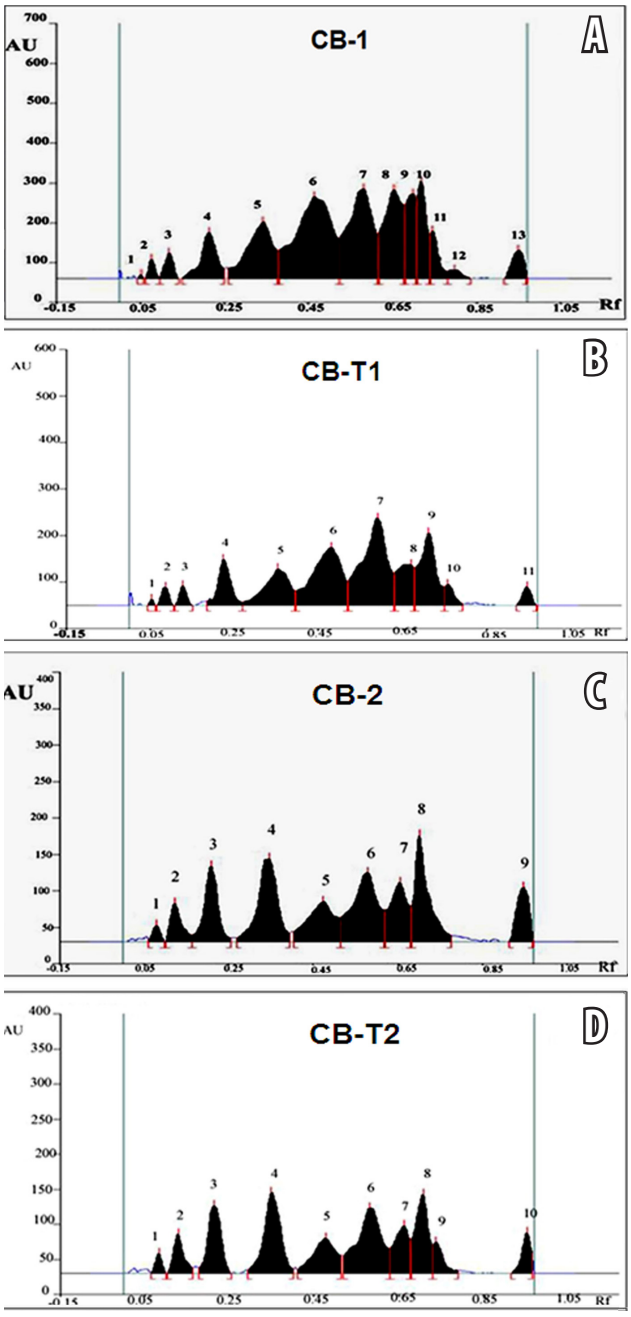

Fig. 3. Saponin profiles of donor population (CB-1; A), tissue cultured plants belonging to population CB-1 (CBT-1; B), donor population (CB-2; C), tissue cultured plants belonging to population CB-2 (CBT-2; D). In all the chromatograms the X axis represents the Rf values of the peaks while the Y axis represents the absorbance of the peaks expressed in absorption units (AU).

Bengal populations as well as in their *in vitro* counterparts. *C. borivilianum* is a rare and expensive plant, as a result of which it is often adulterated/substituted in commercial trade with morphologically similar, easily available, less expensive species. The available literature (Indian Materia Medica, Glossary of Indian Medicinal Plants etc.) also describes few non related species like *Curculigo orchidoides, Asparagus adsendense, Bombax* sp. etc. under the general term 'safed musli', creating considerable confusion among traders and growers regarding the true identity of safed musli. It is therefore important to define specifications that will allow correct identification of the plant that is currently being sold as safed musli. The fingerprinting patterns generated in the present investigation using multiple markers may be useful for quality control, prevention of adulteration and identification of elite populations.

Acknowledgements

The authors are grateful to Prof. S. Ghosal of Natreon Inc. India Ltd. For providing authentic marker samples for HPTLC.

References

Agretious T.K., Martin K.P., Hariharan M. 1996. *In vitro* clonal multiplication of *Alpinia calcarata* Rose. *Phytomorphology* 46: 133–138.

Al-Zakhim A.M., Frod-Lloyd B.V., Newbury H.J. 1999. Detection of somaclonal variation in garlic (*Allium sativum* L.) using RAPD and cytological analysis. *Plant Cell Rep.* 18: 473–477.

Arora D.K., Suri S.S., Ramawat K.G., Merillon J.M. 1999. Factors affecting somatic embryogenesis in long term callus cultures of safed musli (*Chlorophytum borivilianum*), an endangered wonder herb. *Indian J. Exp. Biol.* 9: 37–42.

Arora D.K., Suri S.S., Ramawat K.G. 2006. Assessment of variability in the regenerants from long term cultures of 'safed musli' (*Chlorophytum borivilianum*). *Indian J. Biotechnol.* 18: 473–474.

Barik D.P., Naik S.K., Mohapatra U., Chand P.K. 2004. High frequency plant regeneration by *in vitro* shoot multiplication in cotyledonary node explants of grasspea (*Lathyrus sativus* L.). *In Vitro Cell. Dev. Biol. Plant* 40: 467–470.

Basu S., Jha T.B. 2007. *In vitro* propagation of the rare medicinal plant *Chlorophytum borivilianum* Sant et Fernand. In: Proceedings of the National Symposium on Plant Biotechnology: New Frontiers. P. 285–289.

Basu S., Jha T.B. 2011a. Cytogenetic studies in four species of *Chlorophytum* Ker-Gawl (Liliaceae). *Nucleus* 54: 123–132.

Basu S., Jha T.B. 2011b. *In vitro* propagation of *Chlorophytum nepalense*. *J. Trop. Med. Plants* 12: 33–39.

Table 5. Quantitative data on some important root specific bioactives in different populations of *Chlorophytum borivilianum* and their tissue culture counterparts. Results are mean ± SE of three replicates

Bioactive compounds (% dry mass)	CB-1	CB-2	CBT-1	CBT-2
Steroidal saponins	26.73 ± 1.37	37.36 ± 0.17	24.63 ± 1.19	29.32 ± 0.07
Total sugars	16.83 ± 0.05	18.16 ± 1.92	14.61 ± 0.04	15.36 ± 1.82
Di- and oligosaccharides	0.73 ± 2.13	0.66 ± 0.01	0.71 ± 0.93	0.57 ± 1.43

Chaudhuri R.K., Pal A., Jha T.B. 2007. Production of genetically uniform plants from nodal explants of *Swertia chirata* Buch.-Ham. ex Wall. – an endangered medicinal herb. *In Vitro Cell. Dev. Biol. Plant* 43: 467–472.

Dave A., Bilochi G., Purohit S.D. 2003. Scaling up production and field performance of micropropagated medicinal herb 'Safed Musli' (*Chlorophytum borivilianum*). *In Vitro Cell. Dev. Biol. Plant* 39: 419–424.

Deroles S.C., Seelye J.F., Javellana J., Mullan A.C. 2010. *In vitro* propagation of *Sandersonia aurantiaca* Hook using thidiazuron. *Plant Cell Tissue Organ Cult.* 102: 115–119.

Di X., Chan K.K.C., Leung H.W., Huie C.W. 2003. Fingerprint profiling of acid hydrolyzates of polysaccharides extracted from the fruiting bodies and spores of Lingzhi by high performance thin layer chromatography. *J. Chromatogr. A* 1018: 85–95.

Doyle J.J., Doyle J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf material. *Phytochem. Bull.* 19: 11–15.

Francis G., Kerem Z., Makkar H.P.S., Becker K. 2002. The biological action of saponins in animal system: a review. *British J. Nutr.* 88: 587–605.

Gamborg O.L., Miller R.A., Ojima K. 1968. Nutrient requirements of suspension cultures of sobean root cells. *Exp. Cell Res.* 50: 151–158.

Gurel E., Yucesan B., Aglic E., Gurel S., Verma S.K., Sokmen M., Sokmen A. 2011. Regeneration and cardiotonic glycoside production in *Digitalis davisiana* Heywood (Alanya foxglove). *Plant Cell Tissue Organ Cult.* 104: 217–225.

Huetteman C.A., Preece J.E. 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell Tissue Organ Cult.* 33: 105–119.

Johansen D.A. 1940. *Plant Microtechnique.* Mc Graw Hill Company Inc.

Kaushik N. 2005. Saponins of *Chlorophytum* species. *Phytochem. Rev.* 4: 191-196.

Kumar A., Aggarwal D., Gupta P., Reddy M.S. 2010. Factors affecting *in vitro* propagation and field establishment of *Chlorophytum borivilianum*. *Biol. Plant.* 54: 601–606.

Lane W.D., Iketami H., Hatashi T. 1998. Shoot regeneration from cultured leaves of pear (*Pyrus pyrifolia* L.). *Plant Cell Tissue Organ Cult.* 54: 9–14.

Lattoo S.K., Bamtora S., Sapru Dhar R., Khan S., Dhar A.K. 2006. Rapid plant regeneration and analysis of genetic fidelity of *in vitro* derived plants of *Chlorophytum arundinaceum* Baker – an endangered medicinal herb. *Plant Cell Rep.* 25: 499–506.

Leblay C., Chevreau E., Raboin L.M. 1991. Adventitious shoot regeneration from in vitro leaves of several pear cultivars (*Pyrus communis* L.). *Plant Cell Tissue Organ Cult*. 25: 99–105.

Levan A., Fredga K., Sandberg A.A. 1964. Nomenclature for centromeric position on chromosomes. *Hereditas* 52: 201–220.

Maiti S., Geetha K.A. 2005. Characterization, genetic improvement and cultivation of *Chlorophytum borivilianum* – an important medicinal plant of India. *Plant Gen. Resour.* 3: 264–272.

Martins M., Sarmento D., Oliviera M.M. 2004. Genetic stability of micropropagated almond plantlets, as assessed by RAPD and ISSR markers. *Plant Cell Rep.* 23: 492–496.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant.* 15: 473-497.27.

Meyer H.J., Van Staden J. 1988. *In vitro* multiplication of *Ixia flexuosa*. *Hort. Sci.* 23: 1070–1071.

Mok M.C., Mok D.W.S. 1985. The metabolism of (^{14}C) thidiazuron in callus cultures of *Phaseolus lunatus*. *Physiol. Plant.* 65: 425–432.

O'Brian T.P., Feder N., Mc Cully M.E. 1964. Polychromatic staining of plant cell walls with toluidine blue O. *Protoplasma* 59: 367–373.

Oudhia P., Tripathi R.S. 1999. Scope of cultivation of important medicinal plants in Chattisgarh plains. In: Proceedings of National Conference on Healthcare and Development of Herbal Medicines. P. 71–78.

Pati P.K., Sharma M., Sood A., Ahuja P.S. 2004. Direct shoot regeneration from leaf explants of *Rosa damascene* Mill. *In Vitro Cell. Dev. Biol. Plant* 40: 192–195.

Pierik R.L.M. 1991. Commercial aspects of micropropagation. In: Prakash J., Pierik R.L.M., (eds). *Horticulture – New Technologies and Applications*. Kluwer Academic Publishers Dordrecht, 141–153.

Preece J.E., Imel M.R. 1991. Plant regeneration from leaf explants of *Rhododendron* PJM hybrids. *Sci. Hort*. 48: 159–170.

Purohit S.D., Dave A., Kukda G. 1994. Micropropagation of safed musli (*Chlorophytum borivilianum*), a rare Indian medicinal herb. *Plant Cell Tissue Organ Cult.* 39: 93–96.

Rao S.R., Pandey R., Chandel K.PS. 1992. Genetic stability studies in regenerated plants of *Allium tuberosum* Rottl. ex Spreng. – a cytological approach. *Cytologia* 57: 339–347.

Rohlf F.J. 2002 NTSYS-Pc. Numerical taxonomy and multivariate analysis system version 2.02. Exeter Publishing Ltd.New York.

Rout G.R., Das P., Goel S., Raina S.N. 1998. Determination of genetic stability of micropropagated plants of ginger using Randomly Amplified Polymorphic DNA (RAPD) markers. *Bot. Bull Acad Sin.* 39: 23–27.

Rout G.R., Das G. 2002. An assessment of genetic integrity of micropropagated plants of *Plumbago zeylanica* by RAPD markers. *Biol. Plant.* 45: 27–32.

Samantaray S., Maiti S. 2010. An assessment of genetic fidelity of micropropagated plants of *Chlorophytum borivilianum* using RAPD markers. *Biol. Plant.* 54: 334–338.

Sass J.E. 1940. *Elements of Botanical Microtechnique*. New York: Mc Graw Hill Book Co.

Saxena P.K., Malik C.A., Gill R. 1992. Plantlet formation from isolated protoplasts of *Solanum melongena*. *Planta* 187: 421–424.

Suri S.S., Jain S.K., Arora D.K., Ramawat K.G. 1999. *In vitro* high frequency regeneration of plantlets and tuberous root formation in *Chlorophytum borivilianum*. *Gartenbauwissenschaft* 64: 106–110.

Thakur G.S., Bag M., Sanodiya B.S., Debnath M., Zacharia A., Bhaduriya P., Prasad G.B.K.S., Bisen P.S. 2009. *Chlorophytum borivilianum*: a white gold for biopharmaceuticals and neutraceuticals. *Curr. Pharmaceut. Biotechnol.* 10: 650–666.

Thakur M., Bhargava S., Dixit V.K. 2007. Immunomodulatory activity of *Chlorophytum borivilianum* Sant et Fern. *Evidence-Based Complement. Alternat. Medic.* 4: 419–423.

Turker A., Yucesan B., Gurel E. 2009a. *In vitro* regeneration of *Achillea millefolium* L. from shoot-tips and root segments of seedlings. *J. Plant Biochem. Biotechnol.* 18: 61–69.

Turker A., Yucesan B., Gurel E. 2009b. Plant regeneration via adventitious shoot development from cultured leaf explants of purple loosestrife (*Lythrum salicaria* L.): A medicinal wetland plant. *Biol. Plant.* 54: 750-754.

Van Nieuwkerk J.P., Zimmerman R.H., Fordham I. 1986. Thidiazuron stimulation of apple shoot proliferation *in vitro*. *Hort. Sci.* 21: 516–518.

Verma S., Magotra R., Kaul A.K. 2002. *In vitro* multiplication of *Eremurus oersicus* Boiss. (Liliaceae) – an endangered species. *Phytomorphology* 52: 315–321.

Received 30 August 2014; received in revised form 23 September 2014; accepted 21 November 2014