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MICROPROPAGATION

An efficient multiple shoot induction and genetic fidelity assessment of *Exacum bicolor* Roxb., an endemic and endangered medicinal plant

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Abstract *Exacum bicolor*, an endemic and endangered medicinal plant, belongs to the family Gentianaceae. A rapid protocol has been developed for efficient multiple shoot induction by testing nodal explants on Murashige and Skoog (MS) medium supplemented with various cytokinins. The cytokinins 6-benzyladenine (BA), 6-furfurylaminopurine (Kn), 2-isopentenyladenine (2-iP) and zeatin (Zn) were used individually and in combination at different concentrations (0.5, 1.0, 2.0, 5.0, and 10.0 μM). The maximum number of shoots (19.33±1.09 per explant) as well as their fresh weight (5.1± 0.68 g) and dry weight (216.83±2.84 mg) were obtained with 10.0 μM BA+2.0 μM Kn. After 4 wk, the multiple shoots from agar culture were subcultured into liquid medium containing the same growth regulator combinations. After 8 wk of liquid culture, the best treatment had about tenfold increase in shoot number (199.5±1.14 per explant). The fresh weight (13.76±0.14 g) and dry weight (909.33±1.92 mg) were highest with full strength MS medium supplemented with 3% sucrose and containing 10.0 μM BA+2.0 μM Kn. Maximum root development was observed after 30 d with 0.5 μM indole-3-butyric acid (IBA) supplementation. Regenerated plants were successfully transferred to pots containing coco peat:perlite mixture and showed a 75% survival rate. Genetic fidelity of *in vitro* plantlets compared to mother plant were assessed using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) markers. All the regenerated plants were genetically identical to their mother plant, showing no detectable genetic variation in the regenerated plantlets. Thus, this protocol could be successfully used for mass multiplication and germplasm conservation of *E. bicolor*.

Keywords *Exacum bicolor* · Micropropagation · Nodal explants · Shoot regeneration · Genetic fidelity

Introduction

Exacum bicolor Roxb. (synonyms—*E. tetragonum* Roxb., *E. perrottetii* Griseb.), belonging to the family Gentianaceae, is an endemic, endangered, deciduous perennial herb having attractive flowers (Upadhye *et al.* 1991; Sreelatha *et al.* 2007; Brilliant *et al.* 2012). This species was prevalent throughout tropical and subtropical regions in India, Nepal, Bhutan, China, Malaysia, Java, the Philippines, Sumatra, Borneo, New Guinea, Siam, Sri Lanka, and Burma (Garg 1988). Ethnopharmacologically, *E. bicolor* is used to cure diseases such as diabetes, skin disorders, malaria, fever, inflammation, stomachic, rheumatism, and gout (Megoneitso 1983; Marles and Farnsworth 1995; Khare 2007; Shiddamallayya *et al.* 2010). The leaves also possess anthelmintic, (Ashwini and Majumdar 2014), antioxidant (Ashwini and Majumdar 2015), thrombolytic, and anti-inflammatory activities (Ashwini *et al.* 2015). The major chemical components reported in *E. bicolor* are ursolic acid, apigenin, luteolin, vanillic acid, p-hydroxybenzoic acid, protocatechuic acid, p-coumaric acid, secoiridoids,

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chlorogenic acid, and diosmetin (Das *et al.* 1984; Jeeshna and Paulsamy 2011).

Increased demand, exploitation, improper cultivation practices, deforestation, habitat destruction, and poor seed germination (<5%) (Sreelatha *et al.* 2007) have reduced the populations of medicinal plants and make it difficult to restore populations (Kumar and Sikarwar 2002). Therefore, in recent years, there has been an increased interest in *in vitro* culture techniques, which offer a viable tool for mass multiplication and germplasm conservation of rare, endemic, endangered, and threatened medicinal plants (Anis and Faisal 2005; Baskaran and Jayabalan 2007). Regeneration of explants by *in vitro* propagation is of great interest both for mass propagation and as a mean of producing genetically transformed plants (Kaneyoshi *et al.* 1994). *In vitro* regeneration has been accomplished in many species of Gentianaceae such as *Swertia corymbosa*, *Swertia chirata*, *Exacum travancoricum*, and others (Chaudhuri *et al.* 2007; Janarthanam and Sumathi 2010; Mahendran and Bai 2014). Although indirect regeneration of *E. bicolor* nodal explants has been reported (Jeeshna and Paulsamy 2011), this technique might cause genetic instability in the micropropagated plants. In addition, the report does not describe an optimized protocol, indicate the maximum number of shoots produced, or describe the genetic fidelity of the *in vitro*-propagated plant and the mother plant.

The *in vitro* propagation of plantlets is usually controlled by genetic makeup, stress, plant growth regulators, culture conditions, and other factors which might destabilize the genetic and epigenetic programs of the plant tissue, leading to chromosomal and DNA sequence variation (Larkin and Scowcroft 1981). Therefore, the analysis of genetic fidelity of *in vitro*-micropropagated plants facilitates the management and conservation of genetic resources. It also supports complementary conservation options for rare, endemic, and endangered species (Cenkci *et al.* 2007). Tissue culture methods involving direct shoot regeneration decrease the probability of somaclonal variation among the regenerated plantlets in comparison to callus-mediated regenerants. Several authors have suggested that micropropagation technique cannot be considered as fully developed unless genetic fidelity is maintained (Heinze and Schmidt 1995; Rani and Raina 2000).

Presently, various types of molecular markers are used for genetic studies such as restriction fragment length polymorphism (RFLP), sequence-tagged site (STS), simple sequence repeat (SSR), single-nucleotide polymorphism (SNP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and inter-simple sequence repeat (ISSR) markers (Ateş Sönmezoğlu *et al.* 2012; Phulwaria *et al.* 2013). RAPD and ISSR markers have been applied in the present study to evaluate the genetic fidelity of mother and micropropagated plants. These marker types are favored because of their simplicity, cost-effectiveness, stability, sensitivity, high reproducibility, and reliability (Ray *et al.* 2006). RAPD and ISSR markers have been used to test the genetic fidelity of *S. chirata*, *Citrus jambhiri*, *Lilium orientalis*, and *Viola patrinii*, none of which showed any variation between mother plant and micropropagated plant (Chaudhuri *et al.* 2007; Chalageri and Babu 2012; Liu and Yang 2012; Savita *et al.* 2012).

The aim of this research was to develop and optimize an efficient and reliable protocol to produce genetically stable plantlets of *E. bicolor* by optimizing the Murashige and Skoog (MS) medium strength and the types and concentrations of growth regulators. This appears to be the first report for multiple shoot regeneration capacity obtained in liquid medium and a comparative genetic fidelity study performed by using RAPD and ISSR markers in *E. bicolor*.

Materials and Methods

Plant material. *E. bicolor* Roxb. plants (Fig. 1*a*) were collected from Kumara Parvatha, Western Ghats, Karnataka, India, during the month of November 2010 and was authenticated by the Regional Research Centre, Bangalore, India (Specimen code: SMPU/MADRI/BNG/2010–11/557). A voucher specimen was deposited at the Department of Biotechnology, Center for Post Graduate Studies, Jain University, Bangalore, India.

Surface disinfection. Young disease-free nodal explants (2–3 cm) were treated with 2% (*v*/*v*) Tween 20 (Sigma-Aldrich®, St. Louis, MO) for 10–15 min, followed by 0.04% Bavistin® (BASF India Limited, Mumbai, India) for 20 min and surface-sterilized with 0.1% (*w*/*v*) mercuric chloride (HiMedia®, Mumbai, India) solution for 3 min, followed by washing (four to five times) with sterile distilled water under aseptic conditions. The sterilized explants were trimmed at the ends to about 1–1.5 cm in length and inoculated on to the culture media.

Media and culture conditions. Various strengths (0.25, 0.5, 1.0, 1.5, and 2.0×) of MS medium (Murashige and Skoog 1962) with sucrose (1, 2, 3, 4, and 5%) and 0.8% of Plant Tissue Culture Grade agar (HiMedia®) along with varying concentrations of cytokinins (0.5, 1.0, 2.0, 5.0, and 10.0 μM) were used for initiation of cultures as described in the following section. All growth regulators and media components were from HiMedia®. The media were set to pH 5.7±0.1 and autoclaved at 121°C for 15 min. Nodal explants were placed in culture tubes and incubated under a 16-h photoperiod with irradiance of 40–50 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps (Philips, Kolkata, India) at 75–80% humidity and temperature of 25±2°C.

Figure 1. Micropropagation of *Exacum bicolor* from nodal explants derived from *in vitro* regeneration. (*a*) Mother plant. *Bar*=1 mm. (*b*) Nodal explant. *Bar*=20 mm. (*c*) Shoot induction on MS medium with 10.0 μM BA and 2.0 μM Kn. *Bar*=12 mm. (*d*) Multiple shoot (19.33±1.09 shoots per explant) proliferation after 30 d culture on agar medium (same composition as in (*c*)). *Bar*=4 mm. (*e*, *f*) Shoots transferred into liquid culture from agar medium increased in number of shoots after 8 wk in liquid culture. *Bar*=1.5 mm. (*g*) Complete rooted plantlet. *Bar*=3 mm. (*h*) *In vitro* acclimatized plantlets in plastic pots containing cocoa peat/perlite (70:30 [*v*:*v*]) ratio in growth room. *Bar*=0.1 mm. (*i*) Complete establishment of plantlet after transferring into pots containing garden soil. *Bar*=0.1 mm.

Multiple shoot induction. The nodal explants (Fig. 1*b*) were cultured on MS medium supplemented with different plant growth regulators such as 6-benzyladenine (BA), 6-furfurylaminopurine (Kn), 2-isopentenyladine (2-iP), and zeatin (Z) at various concentrations (0.5, 1.0, 2.0, 5.0, and 10.0 μM), individually and in combination with Kn, indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and α-naphthalene acetic acid (NAA). The control treatment was MS medium without plant growth regulators. The cultures were maintained under the growth conditions described above. After 4 wk, the number of shoots per explant, the shoot length, and the percentage of responsive explants were recorded. The explants that produced the highest numbers of shoots were further transferred to liquid medium (no agar) containing the same growth regulators. After 8 wk, the number of adventitious shoots per explant and the biomass accumulation (fresh and dry weights) were also recorded.

Rooting of in vitro-regenerated shoots. The elongated shoots (>3.0 cm length) with fully expanded leaves were separated and cultured on MS medium supplemented with IBA or IAA at various concentrations (0.5, 1.0, 2.0, 5.0, and 10.0 μM) for rooting. The percentage of rooting and the mean number of roots per plantlet were recorded after 4 wk.

Hardening and acclimatization. Around 60 regenerated plantlets, with 7–10 fully developed leaves on each plantlet, were excised from the medium. Agar was removed from the roots of the plantlets by washing them under tap water. The plantlets were transferred to pots containing autoclaved coco peat:perlite

(70:30 [*w*/*w*]) and covered with transparent plastic bags to ensure high humidity. A solution containing one-tenth of MS basal salts but without sucrose or inositol was poured onto the plantlets every 3 d for 2 wk. The growth chamber was maintained at 26±1°C, 80–85% relative humidity, with light (fluorescent incandescent tubes [Philips]) with an intensity of 50 μmol m^{-2} s^{-1} for a 16-h photoperiod. After 30 d, during which the relative humidity was gradually decreased, the plantlets were transferred to pots containing garden soil and were kept in a greenhouse for another 2–3 wk. The percentage of *in vitro* plantlets acclimatized after hardening was calculated.

DNA extraction. Genomic DNA of *E. bicolor* was isolated using young leaves of *in vitro*-derived field-grown plants and mother plant by a cetyltrimethylammonium bromide (cTAB) procedure (Doyle and Doyle 1987) with the minor modification of adding 10 μL of 2-mercaptoethanol to the extraction. Qualitative and quantitative analysis of DNA was performed by 0.8% agarose gel electrophoresis and UV absorbance at 280 nm, respectively.

Genetic fidelity analysis by RAPD and ISSR markers. Forty arbitrary decamer RAPD primers (OPD-01 to OPD-20 and OPE-01 to OPE-20; Genei Pvt. Ltd, Bangalore, India) were used for DNA amplification by polymerase chain reaction (PCR). Amplification was performed in 25 μL using a PCR mixture consisting of 2.5 μL Taq buffer (10×), 2.5 μL dNTPs (1 mM), 0.5 μL Taq polymerase (1.5 U), 1 μL DNA (approximately 50 ng μL^{-1}), 2.0 μL primer (10 pmol), and 16.5 μL Milli-Q water. The PCR reaction steps included preheating for 4 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at 53°C, and 1.30 min at 72°C; and a final extension step of 5 min at 72°C. Twenty ISSR primers (UBC series, Bioserve, Hyderabad, India) were used for the genetic fidelity analysis. In the case of the ISSR primers, optimal annealing temperature varied with the base composition of the primers. The amplification reaction consisted of an initial denaturation step at 94°C for 4 min, followed by 40 cycles of three steps: denaturation at 94°C for 1 min, annealing at a specified temperature for each primer for 1 min, extension at 72°C for 1.5 min, and a final extension at 72°C for 7 min. After amplification, the PCR products were analyzed in 1.2% agarose gel (HiMedia®) alongside a DNA

Table 1. Effect of concentration of individual plant growth regulators on shoot proliferation from nodal explants of *E. bicolor*

Growth regulator concentration (μM)				Explant response (%)	Average number of shoots/explant	Mean length of shoots (cm)
BA	Kn	2-iP	Z			
0.5				58.33	1.66±0.21^{e}	4.15±0.11^{e}
1.0				66.67	3.50±0.22^{d}	4.53±0.09^{d}
2.0				75.00	5.00±0.26^{c}	5.22±0.14^{c}
5.0				83.33	6.17±0.31^{b}	7.78±0.14^{b}
10.0				91.67	**10.0±0.58^{a}**	**6.37±0.10^{a}**
	0.5			75.00	1.67±0.21^{d}	4.40±0.12^{e}
	1.0			83.33	1.83±0.17^{d}	6.32±0.07^{b}
	2.0			58.33	7.33±0.49^{a}	7.00±0.15^{a}
	5.0			58.33	5.00±0.51^{b}	5.37±0.09^{d}
	10.0			66.67	3.00±0.26^{c}	5.75±0.18^{c}
		0.5		33.33	1.67±0.21^{c}	1.83±0.11^{d}
		1.0		33.33	2.00±0.37^{bc}	2.25±0.09^{c}
		2.0		50.00	2.33±0.21^{abc}	2.33±0.10^{bc}
		5.0		58.33	2.67±0.33^{ab}	2.60±0.09^{b}
		10.0		33.33	3.17±0.40^{a}	3.32±0.11^{a}
			0.5	16.67	2.00±0.26^{b}	2.57±0.07^{b}
			1.0	41.67	1.83±0.31^{b}	3.18±0.13^{a}
			2.0	58.33	3.17±0.31^{a}	3.07±0.10^{a}
			5.0	33.37	2.33±0.33^{b}	3.12±0.09^{a}
			10.0	25.00	1.83±0.31^{b}	3.35±0.07^{a}
MS				41.67	1.33±0.21	2.16±0.34

Values represent mean±SE of three repeated experiments, each with six replications. Means within a *column* followed by the same *letter* are not significantly different according to Duncan's multiple range test ($P \leq 0.05$). Values in bold are the best responses obtained for average number of shoots/explant and mean length of shoots

BA 6-benzyl adenine, *Kn* 6-furfurylaminopurine, *2-iP* 2-isopentenyladenine, *Z* zeatin, *MS* MS medium without plant growth regulators (control)

size marker ladder, and the bands were visualized under a UV illuminator (Wealtec Corp., Sparks, NV).

Statistical analysis. All of the tissue culture experiments were repeated three times with six replicates. The significance of differences among means was assessed using Duncan's multiple range tests at $P \leq 0.05$ (ANOVA). The results were analyzed statistically using SPSS (Version 20, IBM® Corporation, NY).

Results and Discussion

Effect of individual cytokinins. In the present study, nodal explants cultured on MS medium without any plant growth regulators (control) produced 1.33±0.21 shoots per explant, with a shoot length of 2.16±0.34 cm. However, in the presence of various cytokinins (BA, Kn, 2-iP, and Z) at different concentrations (0.5, 1.0, 2.0, 5.0, and 10.0 μM), formation of multiple shoots was observed (Table 1). Hence, the presence of plant growth regulators allowed the explant to respond more quickly than on medium without growth regulators. Varying degrees of shoot growth were observed with different types and concentration of cytokinins. Among the single growth regulator treatments, 10 μM BA produced the maximum number of shoots (10.0±0.58) and shoot length (6.37± 0.10 cm). BA was found to have a greater influence than Kn, 2-iP, or Z on the number of shoots.

According to the literature, nodal explants of *Dendrocalamus strictus* (Poaceae) cultured on MS medium supplemented with 4 mg L^{-1} BA produced 3.68±0.37 shoots per explant, and the length of the shoots was 2.61±0.14 cm after 6 wk (Goyal *et al.* 2015). *Alhagi maurorum* (Fabaceae) cultured on MS medium with BA (2.0 mg L^{-1}) produced shoot bud induction (4.7±

Table 2. Effect of plant growth regulators in combination on multiple shoot induction using nodal explants of *E. bicolor*

Growth regulator concentrations (μM)		Explant response (%)	Average number of shoots/explant	Mean length of shoots (cm)
BA	Kn			
0.5	0.5	33.33	2.67 ± 0.56^{fgh}	4.33 ± 0.59^{ijk}
0.5	1.0	25.00	1.83 ± 0.31^{gh}	4.18 ± 0.28^{k}
0.5	2.0	41.67	1.67 ± 0.21^{h}	4.53 ± 0.38^{ijh}
0.5	5.0	33.33	1.50 ± 0.22^{h}	4.28 ± 0.85^{jk}
0.5	10.0	41.67	2.17 ± 0.54^{fgh}	5.38 ± 0.75^{ghijk}
1.0	0.5	50.00	3.00 ± 0.24^{fgh}	$6.02\pm0.62f^{ghijk}$
1.0	1.0	66.67	2.67 ± 0.21^{fgh}	5.02 ± 0.39^{hijk}
1.0	2.0	58.33	2.00 ± 0.26^{gh}	7.07 ± 0.20^{defgh}
1.0	5.0	66.67	1.83 ± 0.17^{gh}	8.27 ± 0.24^{bcde}
1.0	10.0	66.67	1.67 ± 0.21^{h}	9.30 ± 0.74^{abc}
2.0	0.5	33.33	1.83 ± 0.17^{gh}	4.50 ± 0.44^{ijk}
2.0	1.0	25.00	2.00 ± 0.37^{gh}	7.85 ± 0.81^{bcdef}
2.0	2.0	25.00	3.00 ± 0.58^{fgh}	$5.05\pm0.64h^{ijk}$
2.0	5.0	41.67	4.83 ± 0.95^{de}	4.57 ± 0.52^{ijk}
2.0	10.0	33.33	7.00 ± 0.96^{bc}	6.62 ± 0.98^{efghi}
5.0	0.5	50.00	3.83 ± 0.67^{ef}	5.95 ± 0.74^{ghijk}
5.0	1.0	58.33	3.50 ± 0.34^{efg}	6.32 ± 0.41^{efghijk}
5.0	2.0	83.33	4.83 ± 0.45^{de}	6.52 ± 0.16^{efghij}
5.0	5.0	75.00	3.17 ± 0.34^{efgh}	9.17 ± 0.85^{abcde}
5.0	10.0	41.67	2.00 ± 0.22^{h}	7.42 ± 0.97^{cdefg}
10.0	0.5	66.67	5.83 ± 0.50^{cd}	9.15 ± 0.79^{abcd}
10.0	1.0	91.67	7.67 ± 0.34^{b}	10.78 ± 0.92^{a}
10.0	2.0	100.0	**19.33 ± 1.09^{a}**	**9.85 ± 0.99^{ab}**
10.0	5.0	91.67	6.17 ± 0.79^{bcd}	8.92 ± 0.97^{abcd}
10.0	10.0	83.33	3.50 ± 0.43^{efg}	6.53 ± 0.68^{efghij}

Data recorded after 4 wk of culture. Values represent the mean±SE of three repeated experiments, each with six replicates. Means within a *column* followed by the same *letter* are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test. Values in bold are the best responses obtained for average number of shoots/explant and mean length of shoots

BA 6-benzyl adenine, *Kn* 6-furfurylaminopurine

0.79 per explant) from nodal segments and a shoot length of 3.6±0.43 cm (Agarwal *et al.* 2015). In *Salvadora oleoides* (Salvadoraceae), nodal explants responded optimally with BA (2.0 mg L^{-1}), producing 4.56±0.52 shoots and a mean shoot length of 2.39±0.27 cm (Mahendran *et al.* 2014). In contrast to the above reports, *E. bicolor* produced more shoots (10.0±0.58) even with BA alone, and the number of shoots per responsive explant was significantly ($P \leq 0.05$) affected by the cytokinin type and concentration. According to Otoni and Teixeira (1991), in various medicinal plants, BA was found to be superior to other cytokinins for shoot proliferation from nodal explants, which is in accordance with the present study.

Additive effect of different combinations of plant growth regulators. Nodal explants on MS medium were supplemented with combinations of plant growth regulators (BA+Kn, BA+IAA, BA+NAA, and BA+IBA) (supplementary Table 1). Among these combinations, maximum shoot number (19.33±1.09 per explant; Fig. 1*c*, *d*), length of shoots (9.85±0.99 cm), fresh weight (5.1±0.68 g), and dry weight (216.83±2.84 mg) were obtained with 10.0 μM BA+2.0 μM Kn (Table 2). In contrast, *Swertia bimaculata* produced only 15.6±0.5 shoots per explant on MS medium supplemented with a combination of growth regulators, i.e., 2.22 μM BA+ 2.32 μM Kn+0.54 μM NAA (Dafadar and Jha 2012). An additive effect of BA and Kn in promoting shoot initiation had also been reported earlier in *Acacia catechu* (Rohini 2002). In *E. travancoricum*, explants were cultured on MS with 4.44 μM BA and 1.34 μM NAA, which showed 29.3± 0.3 shoots/explant and had an average length of 4.6±0.1 cm (Janarthanam and Sumathi 2010). Because the number of shoots produced was more than obtained here for *E. bicolor*, additional methods of increasing shoot multiplication were tested using liquid culture.

Strategies to increase the number of shoots and biomass. *Evaluation of different strengths of MS media and sucrose concentrations on shoot multiplication*. Nodal explants placed onto various strengths of MS media (0.25, 0.5, 1.0, 1.5, and 2.0×) and sucrose concentrations (1, 2, 3, 4, and 5%), all containing 10 μM BA+2.0 μM Kn, showed varying degrees of growth (Fig. 2). Among the combinations tested, MS (1×) with 3% sucrose gave the highest level of shoot regeneration and multiplication (20.5±0.76 shoots per explant), which is consistent with the conclusion of other researchers who have observed that optimum sucrose levels support growth (Shatnawi *et al.* 2004). Similarly, *Curcuma aromatica* also exhibited the best growth with 3% sucrose (Sharmin *et al.* 2013). Full-strength MS media was found to be better for shoot multiplication than any other medium strength, which indicated that the plant requires full ionic concentrations of salts for optimum growth. This result is in accordance with a similar study on *Kaempferia galanga* (Shirin *et al.* 2000). Nitrate, ammonia, and carbohydrate are the main medium components involved in the synthesis of nucleic acids, proteins, chlorophyll, and amino acids, all of which are essential for plant growth and development; thus, an excess or deficiency of these major components might cause disturbances in the

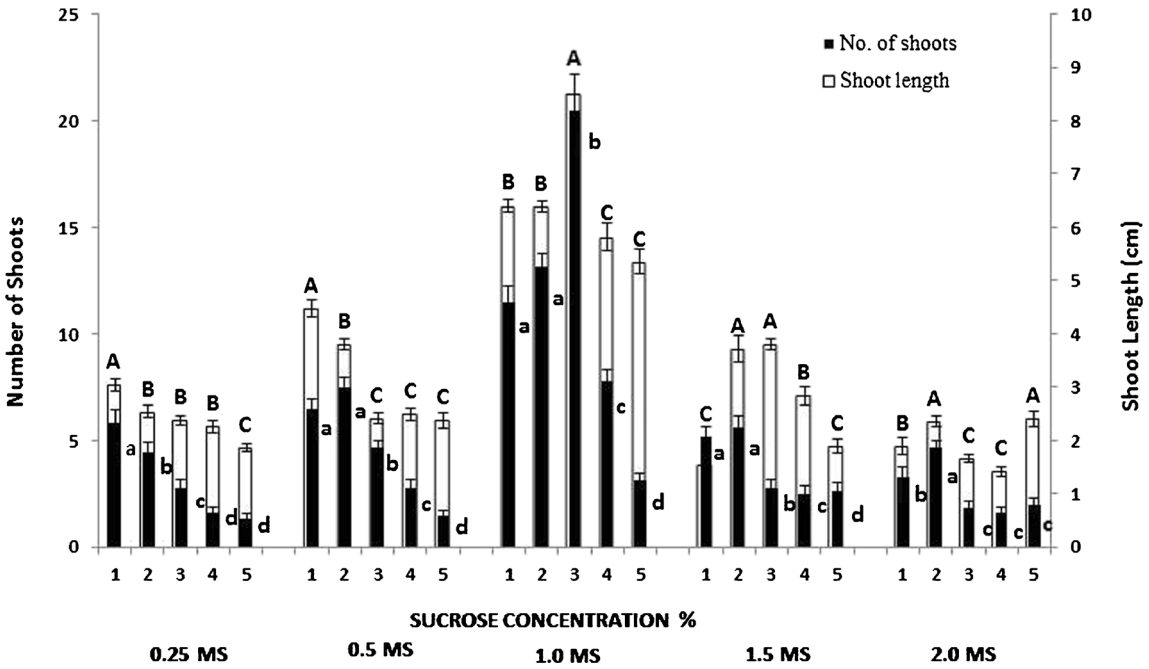

Figure 2. Effect of different concentrations of sucrose and modified MS media with BA+Kn (10.0+2.0 μM) on multiple shoot formation of *E. bicolor* after 4 wk. For each trait measured, mean values within an MS strength level marked with the same *letter* are not significantly different from each other at $P \leq 0.05$ according to Duncan's multiple range test.

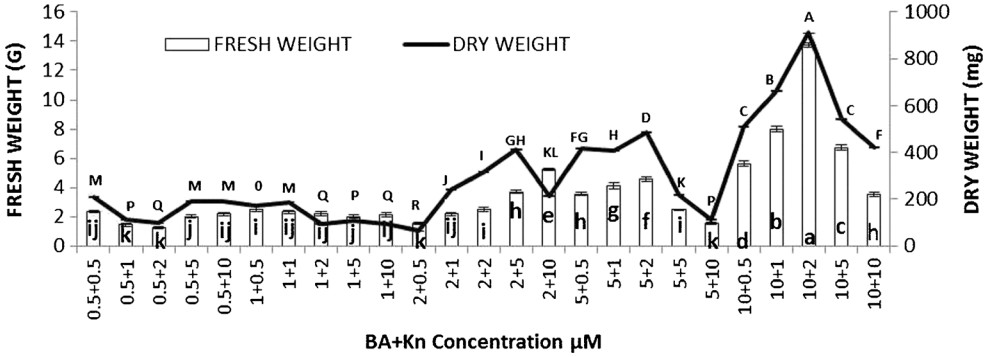

Figure 3. Effect of liquid culture on multiple shoot formation and biomass (plant fresh and dry weights) from *E. bicolor* cultured for 8 wk in MS medium (1×) containing sucrose (3%) and supplemented with various concentrations of BA+Kn. For each trait measured, mean values marked with the same *letter* are not significantly different from each other at $P \leq 0.05$ according to Duncan's multiple range test.

growth of the plant (Tefera and Wannakrairoj 2004). Therefore, the sucrose concentration along with the strength of MS medium directly influences shoot regeneration and multiplication (Naik *et al.* 2010).

Effect of liquid culture on multiple shooting and biomass accumulation. Jo *et al.* (2008) reported that liquid cultures are being efficiently used for multiplication and rapid growth of *in vitro*-propagated plantlets. In the present study, the multiple shoots obtained from agar culture were transferred to liquid culture containing the same growth regulators after 4 wk (Fig. 1*e*), which resulted in an increase in shoot number (199.5±1.14 per explant) of up to tenfold (Fig. 1*f*). By the end of the eighth week after transfer to liquid culture, there was also an increase in shoot biomass in liquid culture (Fig. 3). The fresh weight (13.76 g) and dry weight (909.33 mg) were the highest with 3% sucrose and MS (1×) containing 10.0 µM BA+2.0 µM Kn. In *Eucomis autumnalis*, there was an increase in both fresh and dry weights at 4% sucrose concentration and a decrease at lower sucrose concentrations (Taylor and van Staden 2001). According to Naik *et al.* (2010), in *Bacopa monnieri*, the number of shoots in liquid cultures increased (155.6 shoots per explant), and there was an increase in biomass accumulation (8.60 g fresh and 0.35 g dry biomass). Similar findings were observed in the present study, where liquid cultures were found to promote shoot multiplication and also favored higher biomass. An important aspect of *in vitro* production of medicinal plants is efficient biomass accumulation with higher production of bioactive molecules (Savio *et al.* 2012). Thus, the use of liquid cultures for large-scale production may help to increase the number of shoots and accumulation of biomass that can be utilized as herbal raw material for pharmaceutical industries.

Table 3. Effect of different auxins on *in vitro* rooting of micropropagated shoots

Auxin concentration (µM)[z]		Explant response (%)	Average number of roots/explant	Mean length of roots (cm)
IAA	IBA			
0.5		0	0	0
1.0		58.33	2.00±0.26^{d}	1.90±0.15^{d}
2.0		75.00	3.17±0.17^{c}	2.93±0.15^{c}
5.0		75.00	4.17±0.31^{b}	4.18±0.18^{b}
10.0		91.67	5.33±0.33^{a}	4.58±0.13^{a}
	0.5	91.67	**8.33±0.42^{a}**	**5.48±0.11^{a}**
	1.0	83.33	7.00±0.26^{b}	4.92±0.09^{b}
	2.0	50.00	4.17±0.31^{c}	4.52±0.20^{c}
	5.0	66.67	2.00±0.26^{d}	2.80±0.12^{d}
	10.0	0	0	0

Data recorded after 4 wk of culture. Values represent the mean±SE of three repeated experiments, each with six replicates. Means within a *column* followed by the same *letter* are not significantly different at $P \leq 0.05$ according to Duncan's multiple range test. Values in bold are the best responses obtained for average number of roots/explant and mean length of roots

IAA indole-3-acetic acid, *IBA* indole-3-butyric acid

Table 4. Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) primers used to screen for genetic fidelity in micropropagated plants of *E. bicolor* and the number of amplicons generated by each primer

Primer code	Primer sequence (5′–3′)	Annealing temperature (°C)	No. of bands
OPD-09	CTCTGGAGAC	37.0	7
OPD-14	CTTCCCCAAG	37.0	4
OPD-16	AGGGCGTAAG	37.0	7
OPE-02	GGTGCGGGAA	37.0	3
OPE-05	TCAGGGAGGT	37.0	3
OPE-09	CTTCACCCGA	37.0	4
OPE-13	CCCGATTCGG	37.0	3
OPE-15	ACGCACAACC	37.0	3
OPE-16	GGTGACTGTG	37.0	3
UBC-814	CTCTCTCTCTCTCTCTA	50.4	4
UBC-816	CACACACACACACACAT	50.4	4
UBC-823	TCTCTCTCTCTCTCTCC	52.8	4
UBC-849	GTGTGTGTGTGTGTGTYA	53.0	4
UBC-853	TCTCTCTCTCTCTCTCRT	53.0	5
UBC-855	ACACACACACACACACYT	53.0	4

OP RAPD primers, *UBC* ISSR primers

Rooting of in vitro-regenerated shoots. Successful micropropagation requires efficient rooting of the regenerated shoots and survival of plantlets under greenhouse conditions. The most efficient and effective auxins for rooting are IAA, IBA, and NAA (Bhojwani and Razdan 1996). IBA is a common auxin used for inducing rooting in several Gentianaceae plant species (Chaudhuri *et al.* 2007). In the present study, IAA and IBA were tested for their effects on rooting of the regenerated shoots. IBA (0.5 μM) gave the highest number (8.33±0.42) of roots (Fig. 1*g*) and the greatest root length (5.48±0.11 cm) per plantlet (Table 3) after 25–30 d of culture. Similar results were obtained with IBA in rooting of *Ocimum basilicum* L. (Sahoo *et al.* 1997) and *Murraya koenigii* (Bhuyan *et al.* 1997). In *E. travancoricum*, roots were induced after transfer to half-strength MS supplemented with 2.46 μM IBA, which produced 4.8±0.62 roots per explant with an average height of 3.6±0.10 cm (Janarthanam and Sumathi 2010). In the present study, better root induction was achieved in *E. bicolor* with a lower concentration (0.5 μM) of IBA than was optimum for *E. travancoricum*.

Hardening of in vitro-regenerated shoots. The acclimatization and hardening process usually increases the capacity of plantlets to withstand water loss and will also allow them to survive in the same environmental conditions as the mother plant. In *A. maurorum*, Soilrite was used for acclimatization (Agarwal *et al.* 2015). *S. bimaculata* was hardened in soil, where the survival rate was 80–90% (Dafadar and Jha 2012). In *E. travancoricum*, the rooted plantlets were transferred into a mixture of soil, vermiculite, and farmyard manure (1:1:1 *v*/*v*/*v*) for hardening, and 80% of the plantlets survived (Janarthanam and Sumathi 2010). *S. corymbosa* plantlets were successfully transferred to hardening medium containing vermiculite, with an 87% survival rate (Mahendran and Bai 2014). In the present study, a mixture of coco peat:perlite (70:30 [*w*/*w*]) was tested for the acclimatization process of *E. bicolor* (Fig. 1*h*),

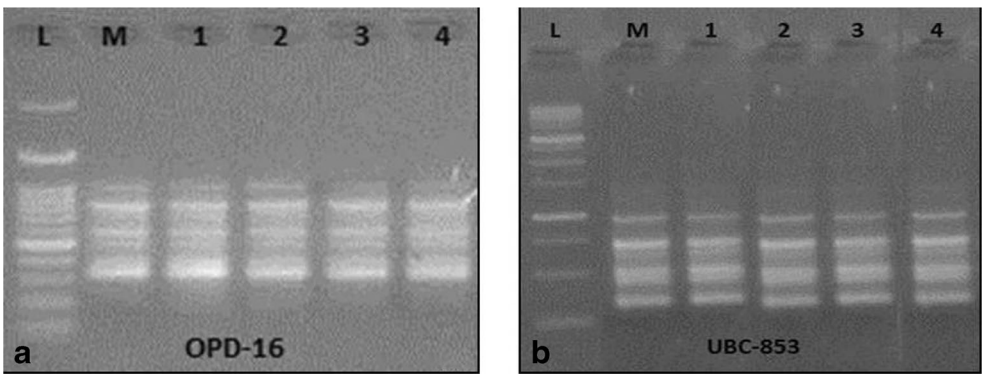

Figure 4. Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) profiles of *in vitro* plantlets and mother plants as shown by RAPD primer OPD-16 (*a*) and ISSR primer UBC-853 (*b*). *L* DNA size marker ladder, *M* mother plant, *lanes 1–4* regenerated acclimatized plants derived from mother plant.

which was in accordance with Fira and Clapa (2009). The hardened plants (Fig. 1*i*) were then grown in greenhouse conditions and showed a 75% survival rate.

Assessment of genetic fidelity of micropropagated plants. RAPD- and ISSR-based DNA molecular markers have been extensively used for the detection of polymorphism among micropropagated medicinal plants because these techniques are simple and cost-effective (Martins *et al.* 2004). Assessment of genetic fidelity of the micropropagated *E. bicolor* plants was performed through RAPD and ISSR analysis after acclimatization. Of the 40 RAPD primers tested, only 9 produced visible and reproducible bands (Table 4). These 9 selected RAPD primers gave a total of 37 scorable bands ranging from 250 to 1250 bp. The number of bands for each primer varied from 3 to 7. The highest number of bands obtained was 7 in the case of primers OPD-09 and OPD-16. The lowest number of bands was 3, obtained for most of the OPE series primers. Out of the 20 ISSR markers screened, only 6 primers resulted in 4–5 scorable bands. These 6 ISSR primers generated 25 scorable bands ranging from 100 to 750 bp in size. Thus, a total of 9 RAPD and 6 ISSR primers generated 62 distinct amplicons. The micropropagated plants had similar banding patterns to each other and to the mother plant (Fig. 4), implying that they are likely to be genetically identical. No polymorphic bands were observed in the mother plant or in micropropagated progenies raised in the present study. The results were in accordance with Ray *et al.* (2006), in which *in vitro*-raised banana shoots were tested for genetic fidelity using RAPD and ISSR markers. In *Gentiana straminea*, the regenerants maintained high genetic fidelity, as shown by using ISSR markers (He *et al.* 2011). Thus, the present study provides evidence of monomorphic bands in mother and micropropagated *E. bicolor*. From an extensive literature survey, this is the first report to demonstrate genetic fidelity between mother and micropropagated *E. bicolor* by using molecular markers.

Conclusions

Since *E. bicolor* is an endemic and endangered species with medicinal potential, there is a need to conserve it. This report describes a method for efficient and rapid large-scale multiplication through direct generation without the callusing phase, and no somaclonal variation was detected. This appears to be the first report on genetic fidelity in *E. bicolor* that demonstrates the true-to-type nature of micropropagated plantlets compared with the mother plant. The information gained here can be applied to the establishment of a unique mass propagation system for the production of genetically stable and identical plantlets of this endemic and endangered medicinal plant.

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