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In vitro plant regeneration via petiole callus of *Viola patrinii* and genetic fidelity assessment using RAPD markers

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Abstract: *Viola patrinii* DC. (Violaceae) petiole explants were used for inducing calli. Significant callus proliferation was observed on MS medium supplemented with 16.12 µM NAA and 13.33 µM BA. Shoot regeneration was achieved upon transferring the green friable calli to MS medium with a 2-fold dilution of potassium dihydrogen phosphate supplemented with 23.25 µM Kn and 2.68 µM NAA. Maximum shoot regeneration was achieved in 4 weeks. Multiple shoots were separated and further cultured in half-strength MS medium supplemented with 9.85 µM IBA with 2% sucrose. Profuse root development was observed after 20-25 days of culturing. Regenerated plants were successfully transferred to soil, showing an 85% survival rate. The genetic fidelity of the micropropagated plants was analysed using random amplified polymorphic DNA (RAPD) markers. The RAPD profile of the regenerated plants showed similar banding patterns to that of the mother plant thus demonstrating the homogeneity of the micropropagated plants. This protocol could be successfully used for the mass multiplication and germplasm conservation of this medicinal plant.

Key words: *Viola patrinii*, regeneration, micropropagation, leaf explants, RAPD, genetic fidelity

Introduction

Viola patrinii DC. (Violaceae), commonly known as China violet, is an important medicinal herb, mentioned in traditional medicine for a variety of therapeutic applications including the purification of blood and the treatment of bruises and ulcers (Kirtikar & Basu, 1995); in the Chinese system of medicine it is recommended for use against cancer disorders. The dried flowers are used as a purgative and for cough and cold. It is also used in Unani recipes, such as Joshanda and Rogan Banafshah (CSIR, 1976). *Viola* spp. are also used for ornamental purposes. The roots of *Viola* spp. contain saponins, glucosides, methyl salicylate, and an emetic principle called violin (Chopra et al., 1958).

Viola patrinii is a perennial herb without an elongated stem and it lacks significant thickening by secondary woody growth. Leaves are glabrous, triangular, usually narrowly elongated, and not deeply chordate. Flowers are usually lilac in colour. In India, *V. patrinii* is distributed in the temperate Himalayan region, extending from the hills of Arunachal Pradesh, Meghalaya, and Manipur in the east southwards to the hills of the Eastern and Western Ghats, at an altitude of 900-2400 m (CSIR, 1976). The plants do not survive in all seasons,

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requiring cool, moist, well-drained humus-rich soil with partial or dappled shade.

In vitro studies on this plant are scanty although a few reports are available. Callus induction through petiole segment was reported by Han et al. (1990) and the development of plantlets from petiole derived calli was reported by Sato et al. (1995). However, these reports failed to explain the successful rooting, acclimatisation process, and genetic fidelity of this species.

Maintaining genetic fidelity is one of the major concerns of tissue culture studies because variations within the progeny can result in serious losses to the end-users. Several approaches, such as karyotyping and isoenzyme profiling, can be used to assess the genetic fidelity of the in vitro derived clones, but most of these methods have their own limitations. Karyotyping does not reveal the alterations in specific genes or small chromosomal rearrangements (Isabel et al., 1993) whereas isoenzyme markers are subject to ontogenic variations. Polymerase chain reaction (PCR) techniques using random amplified polymorphic DNA (RAPD) markers are considered to be sensitive enough to detect the variations or genetic relationship among individuals between and within species (Carlson et al., 1991; Roy et al., 1992; Tripathi et al., 2007). RAPD markers have been successfully used to assess genetic stability and quality among micropropagated plants, thus ensuring the quality of tissue cultured plantlets.

The micropropagation of *V. patrinii* is important because the ferocity of this species is gradually decreasing. Due to the limited distribution of this plant, sustainable conservation is essential. The present study aims to develop a reliable protocol for the mass multiplication of this plant and to analyse the genetic fidelity of the micropropagated plants using RAPD markers.

Materials and methods

Mother plants of *Viola patrinii* were collected from a wild population from Western Ghats of Karnataka, at an altitude of 1700 m. The collected specimens were identified using *The Flora of the Tamilnadu Carnatic* (Matthew, 1981) and compared with herbarium specimens in the Centre for Ecological Studies at the Indian Institute of Science in Bangalore, India. The collected plants were established in the greenhouse of the Research and Development Centre of the The Himalaya Drug Company in Bangalore, India. The actively growing petiole was used as an explant.

The healthy petiole was separated from the mother plant and washed with running water 2-3 times before receiving a pre-treatment with liquid detergent (Pril 5% v/v) for 5-10 min. Washed explants were immersed in 80% ethanol for 2-3 min followed by washing with sterile distilled water 2-3 times. Further surface sterilisation was done by aqueous solution of 0.1% mercuric chloride for 2-3 min and washed with sterile distilled water. The sterilised petiole was cut into 2-3 cm pieces and directly inoculated on to the MS medium.

Media preparation and culture conditions

Murashige and Skoog (1962) (MS) medium was gelled with 0.8% w/v agar (Himedia, India) supplemented with 30 g/L (w/v) sucrose (Himedia, India). The media were adjusted to a pH of 5.8 prior to autoclaving by using 0.1 N HCl/NaOH. The inoculated tubes were incubated in a temperature controlled room at 25 °C ± 1 °C under continuous light (3000 lux intensity) for a photoperiod of about 16 h; a relative humidity level of 70% was maintained.

Initiations of callus, multiple shoots, and rooting

The MS medium was supplemented with different concentrations of cytokinin and auxin used for the differentiation of calli (Table 1). For shoot regeneration, the successfully subcultured calli were transferred on to full-strength MS medium (data not shown) and 2-fold diluted potassium dihydrogen phosphate monobasic (KH_2PO_4) in MS media incorporating the previously mentioned concentrations of α-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BA), and kinetin (Kn) (Table 2). The shoots obtained from calli were maintained through regular subculturing at 4-week intervals on fresh medium with the same composition in order to obtain more shoots. After sufficient growth, shoots (6-7 cm length) were excised from the parent culture and transferred on to MS rooting media (Table 3). The percentage of root formation and the number of roots per shoots were examined periodically up to 4 weeks of culture.

Table 1. The effect of cytokinin and auxin on callus formation from petiole segments of *Viola patrinii* after 3 weeks.

Sl. no.	Growth regulator concentration in MS media (μM)				Degree of callus development	% of callus development after 1st subculture	Type of callus
	BA	NAA	Kn	2,4,D			
1	4.44	5.37	0	0	--	--	----
2	8.88	8.06	0	0	--	--	----
3	13.33	10.75	0	0	--	--	----
4	8.88	10.75	0	0	++	18	Yellowish white
5	13.33	13.44	0	0	++++	60	Greenish white
6	11.11	13.44	0	0	++	29	Yellowish
7	13.33	16.12	0	0	+++++	91	Greenish white friable
8	15.55	16.12	0	0	+	10	Greenish white
9	22.22	26.88	0	0	--	--	----
10	0	5.37	4.65	0	--	--	----
11	0	10.75	13.95	0	--	--	----
12	0	8.06	9.30	0	--	--	----
13	0	10.75	9.30	0	--	--	----
14	0	13.44	13.95	0	+	5	Pale yellowish
15	0	13.44	11.62	0	+	7	Pale yellowish
16	0	16.12	13.95	0	+	7	Creamish white
17	0	16.12	16.27	0	++	17	Greenish white
18	0	26.88	23.25	0	--	--	----
19	4.44	0	0	4.52	--	--	----
20	8.88	0	0	9.04	--	--	----
21	8.88	0	0	11.31	--	--	----
22	11.11	0	0	13.57	--	--	----
23	8.88	0	0	15.83	--	--	----
24	17.77	0	0	22.62	--	--	----
25	0	0	4.65	4.52	--	--	----
26	0	0	9.30	9.04	--	--	----
27	0	0	9.30	11.31	++	5	Yellowish white
28	0	0	11.62	13.57	--	--	----
29	0	0	9.30	15.83	--	--	----
30	0	0	18.60	22.62	--	--	----

The number of plus signs (+) denotes the degree of callusing, -- denotes no response.
Scoring of callus response: + = 5%-10%; ++ = 11%-30%; +++ = 31%-50%; ++++ = 51%-70%; +++++ = 71%-100%.

Table 2. The effect of plant growth regulators on multiple shoot regeneration from the callus of *Viola patrinii* after 4 weeks. (Values are mean ± SE, 40 cultures per treatment, 3 replications).

Sl. no.	Modified MS media	Plant growth regulator (μM)			Mean number of shoots/culture ± SE	Mean length of shoots (cm) ±SE	Frequency of shoot regeneration (%)
		NAA	BA	Kn			
1	MS1	2.68	8.88	0	3.33 ± 0.33^{cd}	2.05 ± 0.11^{ef}	12
2	MS2	2.68	13.33	0	6.00 ± 0.44^{b}	4.45 ± 0.27^{b}	67
3	MS3	2.68	17.77	0	3.50 ± 0.22^{cd}	2.45 ± 0.14^{def}	10
4	MS4	2.68	22.22	0	4.50 ± 0.43^{c}	2.55 ± 0.16^{de}	12
5	MS5	2.68	0	9.30	3.50 ± 0.22^{cd}	2.40 ± 0.15^{def}	12
6	MS6	2.68	0	13.95	3.80 ± 0.30^{cd}	2.31 ± 0.11^{def}	14
7	MS7	2.68	0	18.60	4.16 ± 0.30^{c}	2.80 ± 0.12^{d}	22
8	MS8	2.68	0	23.25	8.83 ± 0.79^{a}	6.95 ± 0.26^{a}	88
9	MS9	5.37	0	13.95	4.00 ± 0.36^{c}	2.21 ± 0.10^{ef}	12
10	MS10	5.37	0	18.60	4.16 ± 0.30^{c}	3.68 ± 0.14^{c}	10
11	MS11	5.37	13.33	0	3.66 ± 0.33^{cd}	2.76 ± 0.14^{d}	8
12	MS12	5.37	17.77	0	2.66 ± 0.33^{d}	1.95 ± 0.11^{f}	7

Means within a column having the same letter did not reach a statistically significant ($P < 0.05$) level according to Duncan's multiple range test (SPSS v. 10.0).

Hardening and acclimatisation

The plantlets with well developed roots and shoots were removed from tubes and planted in 2.5 cm deep plastic pots containing a mixture of sterile soil, sand, and dry cow dung manure (1:1:1 w/v), and were kept in the greenhouse for acclimatisation. The plants were watered at 2-day intervals and maintained at 70%-75% relative humidity. Their survival rate was observed for a period of 4 weeks before the plants were transferred to open field for further growth.

Molecular characterisation of regenerated plants

Genomic DNA was extracted from the fresh aerial parts of hardened plants, calli, shoots raised from calli, and mother plants growing in the greenhouse using a GenElute™ Plant Genomic DNA kit (Sigma Aldrich, St. Louis, MO, USA). Approximately 100 mg of fresh material was ground to a powder in liquid nitrogen using a mortar and pestle. The powder was transferred to a sterile 2-mL microfuge tube and extracted with the aid of the GenElute kit. The quality and quantity of the DNA preparation were checked by gel electrophoresis and spectrometric assay.

PCR amplification was carried out in 25 μL volume using 25 different decamer primers. The reaction mixture consisted of 1U of Taq DNA polymerase (Bangalore Genei), 0.25 μL each of dNTP (10 mM), 5 pmole of decanucleotide primer (Sigma Aldrich), 1× polymerase buffer (2.5 μL), 0.25 mM $MgCl_2$, and 50 ng of DNA sample. The amplification was performed using a thermal cycler (PTC-100™, MJ Research. Inc., USA). The program consisted of initial denaturation at 93 °C for 4 min followed by 40 cycles of denaturation at 93 °C for 1 min, annealing at 37 °C for 1 min, extension at 72 °C for 2 min, and at a final extension cycle of 8 min at 72 °C. The amplified products were checked in a 1.5% agarose gel stained with 0.5 μg/mL of ethidium bromide and documented by a gel documentation system (Pharmacia Biotech).

Table 3. The effect of different concentrations of auxins on the rooting of *Viola patrinii* after 4 weeks. (Values are mean ± SE, 40 cultures per treatment, 3 replications).

Sl. no.	MS media + growth regulators (μM)	Mean number of roots/shoot (cm ± SE)	Mean length of the roots/shoot (Cm ± SE)	Frequency of rooting (%)
1	MS Basal media	5.33 ± 0.71^{b}	3.75 ± 0.28^{b}	63
2	2-fold diluted MS base	4.83 ± 0.60^{b}	3.03 ± 0.31^{bc}	36
3	MS + IBA 4.93	3.16 ± 0.30^{c}	1.73 ± 0.17^{de}	15
4	MS + IAA 5.71	1.83 ± 0.30^{c}	0.60 ± 0.08^{f}	17
5	2-fold diluted MS + IBA 9.85 + 2% sucrose	7.50 ± 0.42^{a}	4.71 ± 0.32^{a}	83
6	2-fold diluted MS + IAA 11.42 + 2% sucrose	2.66 ± 0.33^{c}	1.05 ± 0.19^{ef}	12
7	2-fold diluted MS + IBA 14.77 + 2% sucrose	2.50 ± 0.42^{c}	2.28 ± 0.37^{cd}	21

Means within a column having the same letter did not reach a statistically significant ($P < 0.05$) level according to Duncan's multiple range test (SPSS v. 10.0).

Data analysis

All of the cultures were examined periodically, each experiment was repeated 3 times, and subculturing was carried out at 4-week intervals. The mean number of shoots per culture and their lengths were recorded. The percentage frequencies of rooting and the mean number of roots per shoot were also recorded. The data were analysed statistically using Duncan's multiple range test (ver. 10.0). Means followed by the same letter within columns were not significantly different at $P < 0.05$ levels.

Results

Effect of various combinations of auxin and cytokinin on the development of callus

The enlarged petiole with clusters of cells on the margin and the cut surface of the explant was observed morphologically within 2 weeks of culture. Low concentrations of NAA (13.44 μM) and BA (13.33 μM) showed an optimum production of callus. Although callusing frequency was observed in medium containing 10.75 μM NAA and 8.88 μM BA, the rate of growth was moderate. Notable morphogenic friable callus was observed on MS medium containing 16.12 μM NAA and 13.33 μM BA (Figure 1). No response was seen with higher concentrations of NAA and BA. MS medium augmented with Kn and NAA at a level of 16.27 μM and 16.12 μM respectively showed less callus formation, but further growth was not found in this combination. However, the addition

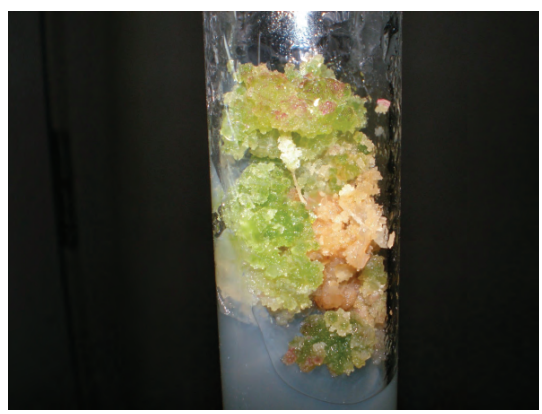

Figure 1. Compact greenish white callus of *Viola patrinii* in MS medium containing NAA (16.12 μM) + BA (13.33 μM), within 4 weeks.

of 2,4-dichlorophenoxy acetic acid (2,4 - D) with Kn, at levels of 11.31 µM and 9.30 µM respectively, promoted a similar growth rate to that seen with the Kn and NAA combination; in later stages, the calli turned brown and gradually the death of the callus was observed. The further addition of BA with 2,4- D did not show any encouraging results with regard to the further development of callus.

Shoot regeneration from callus

The primary calli were subcultured 4-5 times to obtain compact calli. In the present study, it was possible to obtain shoot buds from calli when full strength MS medium was used with different combinations of NAA, BA, and Kn (data not shown). In these combinations the callus exhibited the tendency to regenerate shoots rather than the callus (Shrivastava & Rajani, 1999). However, it was observed that the shooting response was less (8%-10%) and stunted shoots with small leaves were observed 4 weeks after subculture whereas MS medium containing ½ KH_2PO_4 (Himedia, India) with growth regulators in different combinations was found to be successful in obtaining well developed shoots. Among the different concentrations tried, NAA (2.68 µM) with Kn (23.25 µM) showed 88% shoot regeneration, which was achieved in 4 weeks (Figures 2, 3). A combination of BA (13.33 µM) with NAA (2.68 µM) also led to shoot proliferation (67%).

Rooting of shoots

Initially, elongated shoots (6-7 cm in length) were rooted on MS basal medium and on 2-fold diluted

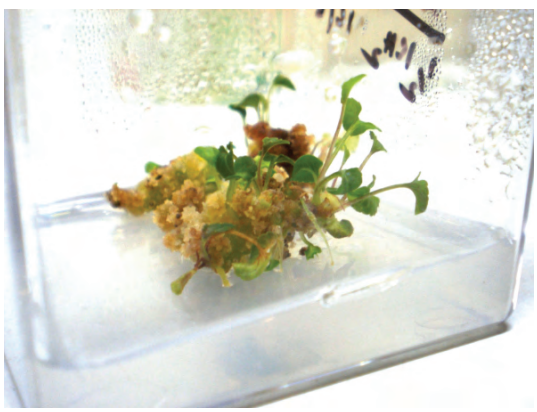

Figure 2. Multiple shoot development on MS medium containing half-strength diluted KH_2PO_4 with NAA (2.68 µM) and Kn (23.25 µM), within 4 weeks.

Figure 3. Regeneration of multiple shoots in the same medium.

MS medium with a combination of IAA or IBA in different concentrations of sucrose. Both the growth and the number of roots were moderate in the MS basal medium (63%) as well as in the 2-fold diluted MS basal medium (36%), but a significant induction of roots was observed in 2-fold diluted MS with 9.85 µM IBA and 2% (w/v) sucrose within 20-25 days of subculture (83%) (Figure 4). The percentage of root formation was significantly reduced in other

Figure 4. The development of roots in 2-fold diluted MS medium with 2% sucrose and IBA (9.85 µM).

combinations of IBA (4.93 and 14.77 µM). Similarly, MS medium with IAA at a level of 5.71 and 11.42 µM did not induce notable root formation.

Hardening of plantlets

The rooted plantlets were transferred to pots containing sterile soil, sand, and dry cow dung manure in a ratio of 1:1:1 (w/v) and kept in the greenhouse for hardening (Figure 5). Four weeks after being transferred, 85% of the plants survived in the greenhouse. Later the plants were transferred to a normal environment (Figure 6).

RAPD analysis of regenerated plants

RAPD markers are useful tools for the genetic identification of micropropagated plants. In order to confirm the homogeneity of the plantlets, the RAPD pattern of the cultured plantlets was compared with that of the mother plant. Out of 25 different decamer Oligo primers tested (HD1-HD25), 24 primers gave good amplification and products were monomorphic across all of the tested plants. The representative RAPD profiles of the callus, in vitro plant, hardened plant, and mother plant with 2 different primers are

Figure 5. Hardened plantlets of *Viola patrinii* after 6 weeks in the growth chamber.

Figure 6. Re-established plantlets of *Viola patrinii* in a normal environment.

shown in Figure 7. The numbers of amplification products obtained using different primers are summarised in Table 4. The primers HD-3, 19, and 22 gave single monomorphic bands whereas HD-13 gave 7 monomorphic bands (Table 4). The size of the monomorphic DNA fragments produced by the tested primers ranged from 250 bp to 2100 bp.

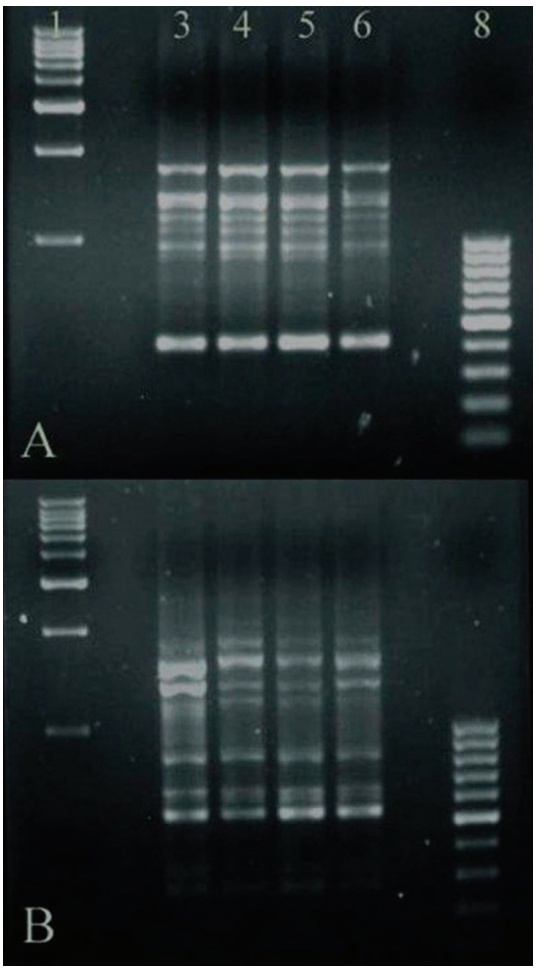

Figure 7. A RAPD profile generated by Oligo primer HD10 (A) and HD18 (B).
Lane 1 - marker 1 kb; 3 callus; 4 in vitro plant; 5 mother plant; 6 in vitro-raised hardened plant; 8 marker 100 bp.

Discussion

The present study investigated the effect of various concentrations of auxin and cytokinin on callus formation. In this context, 9 different concentrations of NAA and BA were investigated for callus induction. The results showed that the combination of 16.12 µM NAA with 13.33 µM BA resulted in maximum callus induction in terms of the percentage of calli. On the other hand, when the NAA concentration was reduced to 8.06 µM, the callus formation was hampered. It was interesting to note, however, that combining 8.88 µM of BA with 10.75 µM NAA was beneficial in invigorating the callus induction. These findings revealed that a combination of NAA with BA is good for the development of callus; although other hormonal combinations, such as Kn with NAA and 2,4-D with BA or NAA, promoted callus formation, induction was poor. The present findings differ from the studies reported by Han et al. (1990) in that callus induction was possible in low concentrations of growth regulator.

Some of the reports have shown a proliferation of shoots from calli by adding cytokinin or auxin or by altering the mineral concentration in the medium. The uneven diffusion of growth regulators leads to polarity in mature dividing cells and forms shoot buds (Yao & Wang, 1987). In the present study, a stimulatory effect of shoots from callus was observed in NAA (2.68 µM) with Kn (23.25 µM) in ½ KH_2PO_4 MS medium (88%) while a moderate response was observed in BA (13.33 µM) with NAA (2.68 µM) in the same medium (67%). Similarly, some previous reports have concluded that altering the macronutrients present in MS medium can achieve more shoot regeneration (Araki et al., 1992; Nahako & Shizufumi, 1990; Prakash et al., 1999). The findings of the present investigation were in agreement with those of Kobayashi (1982), who noted that callus formation was suppressed in the potato by reducing the mineral concentration of MS basal medium to half-strength. Separately, the addition of low concentrations of auxin along with cytokinin was suitable for increasing the rate of multiplication (Sharma et al., 1993; Sharma & Singh, 1997; Rout, 2005). The present deviating response could be due to the use of growth regulator in the medium or the genotype and/or explant type studied (Samantaray et al., 1995; Erişen et al., 2011). This could be a similar conclusion for obtaining effective and healthy shoots in this altered medium.

The significant development of roots was observed on 2-fold diluted MS medium containing IBA (9.85 µM) with 2% sucrose (83%). This observation was not found in the other combinations tried. The effectiveness of IBA in rooting has been reported for many medicinal plants, including *Clitoria ternatea* L. (Barik et al., 2007), *Murraya koenigii* (L.) Spreng (Bhuyan et al., 1997), and *Ocimum basilicum* L. (Sahoo et al., 1997). This is due to an imbalance between endogenous auxin and exogenously supplied

Table 4. The number of amplification products generated with the use of RAPD primers in the analysis of *V. patrinii* regenerants.

Sl. no.	Sequence (5'-3')	Oligo primers name	No. of bands obtained	Size of the amplified bands (bp)
1	5'AGAGGCTCAC 3'	HD-1	3	450-780
2	5'GCGTTGACTG 3'	HD-2	2	280-800
3	5' AGGGCAAACC 3'	HD-3	1	350
4	5' CCCGTTACAC 3'	HD-4	2	660-1400
5	5'CATACGCCTC 3'	HD-5	3	300-820
6	5'CCGAAGACAC 3'	HD-6	3	300-1300
7	5'GCAGTACGAG 3'	HD-7	3	550-1200
8	5'GCAGTACGTG 3'	HD-8	3	690-1300
9	5'CGAGGAGACC3'	HD-9	4	300-780
10	5'GTTCCCAGAC3'	HD-10	6	410-1800
11	5'TCGCGCAAAG3'	HD-11	6	280-1400
12	5'AAACCGCAGG3'	HD-12	6	300-1920
13	5'CCGATAGGAC3'	HD-13	3	930-1550
14	5'CTGGTGTCTC3'	HD-14	7	480-1720
15	5'GGTCACGTAG3'	HD-15	3	300-1050
16	5'ATCACGCAGG3'	HD-16	4	650-1100
17	5'TCTCTCGCTC3'	HD-17	3	700-1650
18	5'GTGTCCGGTG3'	HD-18	7	250-1910
19	5'GCGATGGTGG3'	HD-19	1	900
20	5'ACGCAGTTCC3'	HD-20	5	610-1650
21	5'AAGGACAGGG3'	HD-21	2	560-910
22	5'AGATTGCACC3'	HD-22	1	380
23	5'AGGAGCAGAG3'	HD-23	4	420-900
24	5'CAACGGAGTG3'	HD-24	---	No amplification
25	5'CACGTGCTAC3'	HD-25	4	560-2100

auxin IBA (Manickam et al., 2000). However, a similar conclusion was also seen in the work of Anand et al. (1997) and Usha and Swami (1998). This method and type of rooting in *V. patrinii* proved to be quite successful for acclimatisation. In the present report, the plantlets were acclimatised with an 85% survival rate on the basis of the total number of shoots removed from the in vitro culture.

The RAPD technique, being simple and cost effective, has been used to assess the genetic variations of tissue culture plants in numerous studies (Salvi et al., 2001; Martins et al., 2004). Micropropagated plants derived from axillary buds/ organised meristems are generally considered to be at low risk for genetic instability (Pierik, 1991; Gupta & Varshney, 1999) because the organised meristems

are generally less prone to genetic changes than unorganised calli under in vitro conditions (Rani & Raina, 2000).

Somaclonal variation has been reported in some micropropagated plants such as *Populus deltoides* Marsh. (Rani et al., 1995) and *Begonia* L. (Bouman et al., 1992). Since callus-derived plantlets are highly prone to somaclonal variation, we used RAPD markers to ensure the genetic fidelity by comparing the banding patterns with that of the mother plant. The results of the RAPD analysis showed no evidence of polymorphism between the mother plants and the micropropagated plants. This study provides the first information on the molecular basis of polymorphism detection using RAPD markers in micropropagated plants of *V. patrinii*.

Conclusions

The present study details an efficient protocol for the mass multiplication of *Viola patrinii*. Earlier research reported successful induction and shoot regeneration through callus culture. However, these reports failed to explain the successful rooting of the plantlets, their hardening, and the genetic fidelity of the micropropagated plants. Our findings establish an efficient protocol for callus development and its subsequent shoot and root proliferation. Well developed plantlets were successfully propagated in a normal environment. The genetic fidelity of the micropropagated plants were successfully analysed using RAPD markers. This protocol could be an important source for plant breeders and for germplasm conservation studies of *Viola patrinii*.

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