Rapid in vitro propagation, conservation and analysis of genetic stability of *Viola pilosa*

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Abstract A protocol for in vitro propagation was developed for *Viola pilosa*, a plant of immense medicinal value. To start with in vitro propagation, the sterilized explants (buds) were cultured on MS basal medium supplemented with various concentrations of growth regulators. One of the medium compositions MS basal+0.5 mg/l BA+0.5 mg/l TDZ+0.5 mg/l GA₃ gave best results for in vitro shoot bud establishment. Although the problem of shoot vitrification occurred on this medium but this was overcome by transferring the vitrified shoots on MS medium supplemented with 1 mg/l BA and 0.25 mg/l Kn. The same medium was found to be the best medium for further in vitro shoot multiplication. 100 % root induction from in vitro grown shoots was obtained on half strength MS medium supplemented with 1 mg/l IBA. In vitro formed plantlets were hardened and transferred to soil with 83 % survival. Additionally, conservation of in vitro multiplying shoots was also attempted using two different approaches namely slowing down the growth at low temperature and cryopreservation following vitrification. At low temperature retrieval rate was better at 10 °C than at 4 °C after conservation of in vitro multiplying shoots. In cryopreservation–vitrification studies, the vitrified shoot buds gave maximum retrieval of 41.66 % when they were precooled at 4 °C, while only 16.66 % vitrified shoots were retrieved from those precooled at 10 °C. Genetic stability of the in vitro grown plants was analysed by RAPD and ISSR markers which indicated no somaclonal variation among in vitro grown plants demonstrating the feasibility of using the protocol without any adverse genetical effects.

Keywords *Viola pilosa* · Medicinal plant · In vitro propagation · In vitro conservation

Abbreviations

BA or BAP	6-benzyladenine or 6-benzyladenine amino purine
TDZ	Thidiazuron
GA ₃	Gibberellic acid
IBA	Indole-3-butyric acid
Kn	Kinetin
DMSO	Dimethyl sulfoxide
RAPD	Randomly amplified polymorphic DNA
ISSR	Inter simple sequence repeat

Introduction

Viola pilosa Blume (family Violaceae) is a small rosette like perennial herb having short tufted root stocks and stem covered with scales. Green leaves are ovate-cordate or crenate-serrate. Fruit is globose capsule and contains very few seeds. The flowering and fruiting occur between the months of February and March (Manander 2002).

Medicinal importance is attributed to glycoside- methyl salicylate and an alkaloid-violine present in the roots and leaves. It also contains secondary metabolites namely rutin, sapoinin. The flowers are credited with demulcent properties and are used for preparation of squashes. The flowers are also used as remedies in many disorders and are supposed to be especially serviceable to the eyes (Sharma and Mishra 2009).

The harvest of medicinal plants on mass scale from their natural habitat is leading to depletion of plant resources. Plant tissue culture is recognised as one of the major areas of biotechnology because of its significant role in multiplying and conserving plant germplasm. Micropropagation is an alternative to the conventional method of propagation with the objective of enhancing the rate of multiplication and conserving the medicinal plants (Kaur et al. 2010).

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Viola pilosa being a medicinal plant of great value has been over exploited by pharmaceutical industries without skilled efforts for its cultivation and conservation. Flowers in addition, to leaves and roots of this plant species are used up for herbal preparations. So this plant species requires immediate attention for its protection, large scale systematic cultivation and conservation. The present study being the first one is aimed to develop a reliable protocol for in vitro propagation along with its conservation. Additionally, the communication documents the use of ISSR and RAPD markers to assess genetic similarity of micropropagated and in vitro conserved plants.

Material and methods

Plant material

The plants of *Viola pilosa* Blume were collected from fields of Department of Forest Products of Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan, India.

Explant preparation

The explants were prepared from the uprooted plants. Explants i.e. axillary buds were excised with the help of sharp blade and collected in a beaker containing water. The explants were washed under running tap water for about 30 min and then treated with 5 % tween-20 solution (a detergent) for 5 min for surface cleaning from waxy layer. There after washed thrice with distilled water. The explants were then surface sterilized under aseptic conditions using 0.1 % solution (w/v) of bavistin (carbendazim- a fungicide) for 10 min and finally washed for 3 to 4 times with autoclaved distilled water to remove all the traces of sterilants.

In vitro propagation

The sterilized explants were transferred to 100 ml glass flasks containing MS (Murashige and Skoog 1962) basal medium supplemented with different concentrations and combinations of growth regulators i.e. BA @ 0.25 to 1 mg/l, Kn @ 0.25 to 2 mg/l, TDZ @ 0.5 to 2 mg/l and GA₃ @ 0.5 to 2 mg/l for the establishment of shoot buds. The pH of the medium was adjusted at 5.8 before gelling and then autoclaved at 121 °C temperature and 15 psi pressure for 15 min. The cultures were incubated at 25±2 °C under 16/8 photoperiod with 50 µmol/m²/s photosynthetic photon flux (PPF), obtained from cool fluorescent tube lights (Philips, Kolkata, India).

The effect of different plant growth regulators such as BA, Kn, GA₃ and TDZ was studied on initiation and in vitro shoot multiplication. Five explants were cultured in each culture flask. Number of shoots per sprouted buds were recorded after

5 weeks. The effect of three gelling agents, agar-agar (0.8 %), agarose 0.5 % and gelrite gellan gum 0.25 % was recorded on growth of cultures.

In vitro rooting

The plant species follows its natural 'rosette' habit of growth even in in vitro cultures, therefore the in vitro shoots were excised on the basis of length of leaf petiole. When the petiole gained a length of 3–4 cm the shoots were separated from the rosette and transferred to culture tubes (15 cms×150 cm) containing either MS basal medium of different strengths (full strength and half strength) alone or supplemented with IBA @ 0.5 to 1.0 mg/l for in vitro root induction.

Acclimatization and hardening

For acclimatization and hardening, in vitro regenerated plants were removed from test tubes and agar medium attached was washed off completely. Plants were kept dipped in 0.2 % solution of carbendazim for 30 min before transferring to plastic pots (6.0 cm diameter) containing autoclaved potting mixture of sand: soil: FYM (Farm Yard Manure) in the ratio of 1:1:1, perlite and cocopeat singly or in combination of 1:1. The pots were covered with perforated polythene bags and watered at 1 day interval and supplied with ¼ strength salts of MS medium twice a week by spraying. Subsequently polythene bags were removed and plants were transferred to earthen pots (25 cm diameter) containing FYM: soil 1:1 and watered as and when required. The survival rate of plantlets was recorded after 1 month of transfer to plastic pots.

In vitro conservation

In vitro maintained shoots subcultured at every 4–5 week interval were the source of explants for in vitro conservation.

Two approaches have been followed as given below:

i.) Slowing down the growth by reducing culture temperature

An attempt has been made to conserve in vitro multiplying shoots at low temperature. In vitro shoots were incubated for their performance at two lower temperatures i.e. at 4 °C and 10 °C. The other culture conditions and medium composition remained same as were for in vitro culture of shoots.

ii.) Cryopreservation following vitrification of in vitro shoots

In vitro raised shoots of *Viola pilosa* were divided into two sets and precooled separately at 4 °C and 10 °C in incubator for 1 month to slow down the growth by reducing culture temperature. The medium used, henceforth was supplemented with various cryoprotectants such as sucrose, glycerol, ethylene glycol etc. (Engelmann 1997). The pre-cooled in vitro shoot

segments were placed on V_1 (MS salts +0.5 M sucrose) medium in petriplates and parafilm sealed petriplates were kept overnight in dark at 4 °C temperature inside a refrigerator. The shoot segments were then transferred to pre-cooled cryovials containing V_2 (MS salts +0.5 M sucrose +3 M glycerol) solution and cryovials were again incubated at 4 °C for 20–30 min. After this V_2 solution was pipetted out from cryovials leaving only shoot segments behind and 1.5–2 ml of vitrification solution V_3 (MS salts+30 % glycerol+13 % DMSO +15 % ethylene glycol) was added to them. The cryovials were ice incubated in laptop cooler for 60 min. Finally, the cryovials were directly immersed in liquid nitrogen. For retrieval the cryovials were taken out thawed rapidly in water bath at 40 °C for 2–3 min.

Than V_3 solution the was discarded and buds were washed with V_4 (MS salts+1.2 M sucrose) solution to completely eradicate the effects of cryoprotectants and then the shoots were placed on an autoclaved filter paper inside a petridish containing 1 ml of liquid shoot multiplication medium i.e. MS basal+0.25 mg/l Kn+1.0 mg/l BA. The petridish was sealed with parafilm and kept overnight in dark at 25±2 °C.

After this, the shoot buds were transferred to fresh, solid shoot multiplication medium in conical flasks and incubated in diffused light for 7 to 10 days. Finally the shoot tips were transferred to normal culture conditions as is given for in vitro propagation. Retrieval was recorded by counting number of shoot tips regaining growth on multiplication medium.

Experimental design and statistical analysis

The data recorded was analyzed according to CRD (completely randomized design) analysis using standard statistical software. All experiments were replicated five times. The data of 'percent' was converted to arc sine value for the analysis of variance (ANOVA). The critical differences between the treatments were calculated and used to compare the means of treatments and interpretations of results were made accordingly. Each treatment throughout the experimentation consisted of five replicates and the experimental unit was five explant per treatment. The results summarise the data of five independent experiments.

Assessment of trueness-to-type

Genetic fidelity of in vitro-raised clones was assessed using RAPD and ISSR markers. For this purpose, 23 in vitro raised plants were chosen randomly from the population (in vitro propagated and in vitro conserved) and compared with the mother plant from which the explants were taken. Total genomic DNA of the mother plant and in vitro-raised clones was extracted from young leaf tissue by using CTAB method

(Doyle and Doyle 1987) with some modifications. 4 RAPD and 4 ISSR primers were used for initial screening. PCR amplifications were carried out in a total volume of 20 µl containing 25–35 ng genomic DNA, 1X PCR Buffer A, 2.2 mM $MgCl_2$, 1 mM dNTP, 0.2 µM of primer, 1 U Taq DNA polymerase. PCR amplification was performed in a DNA thermal cycler, which was programmed for initial DNA denaturation at 95 °C for 4 min, followed by 40 cycles of 1 min denaturation at 95 °C, 1 min annealing (temperature specific to the primer) and 2 min extension at 72 °C, with a final extension at 72 °C for 10 min for RAPD markers and one initial denaturation cycle of 2 min at 94 °C, 40 cycles each of 10 s at 94 °C, annealing of 30 s at primer specific annealing temperature, extension of 65 s at 72 °C and a final extension of 10 min at 72 °C for ISSR markers. Amplified products were resolved by electrophoresis on 1.2 % agarose gel in TAE buffer stained with 0.5 µg/ml ethidium bromide and photographs were taken by using the gel documentation system.

Results

In vitro establishment and multiplication

The initial establishment of axillary buds was observed in all the medium compositions used. Both BA and Kn promoted bud establishment. However, BA in combination with TDZ and GA_3 TDZ each @ 0.5 mg/l gave the highest percentage (77.50 %) of bud sprouting (Table 1). No bud sprouting was obtained on any of the medium supplemented alone or in combination with higher concentration of BA and GA_3 (Table 1). In a few of the cases, the sprouted buds became vitrified i.e. showed uneven buds, stunted growth of shoots and hypertrophy after about 1 week of proliferation. However, after transferring the vitrified shoots on culture medium having BA and Kn without TDZ, normal growth of vitrified shoots was resumed. Subsequently, in vitro shoot multiplication was continued on BA (1.0 mg/l) and Kn (0.25 mg/l) supplemented medium since this proved to be the best composition giving an average of 10.16 shoots after 5 weeks of culturing.

Average number of shoots were recorded after each subculture of 5 weeks ranged between 6.12 and 6.25. No significant difference was recorded in the average number of shoots on successive subculturing till sixth subculture though the growth of shoots with respect to colour and leaves was recorded to be luxuriant. An average number of shoots was recorded to be 10.16 after sixth subculture (data not shown). Out of three solidifying agents viz. Agar-agar, gelrite gellan gum and agarose, gelrite gellan gum proved to be best for holding proper growth, multiplication and rooting of shoots.

Table 1 Effect of different growth regulator combinations on establishment of cultures from shoot buds of *Viola pilosa* Blume

Kn mg/l	BA mg/l	GA ₃ mg/l	TDZ mg/l	Percentage of buds proliferated
0.25	0.25	–	–	25.33
0.25	0.50	–	–	9.83
0.25	0.75	–	–	26.16
0.25	1.0	–	–	68.50
0.50	0.25	–	–	20.00
0.50	0.50	–	–	14.33
0.50	0.75	–	–	16.50
0.50	1.0	–	–	43.65
0.75	0.25	–	–	26.00
0.75	0.50	–	–	31.16
0.75	0.75	–	–	21.50
0.75	1.0	–	–	43.16
1.0	0.25	–	–	33.33
1.0	0.50	–	–	6.16
1.0	0.75	–	–	31.66
1.0	1.0	–	–	61.16
2.0	1.0	–	–	9.50
–	0.50	0.50	0.50	77.50
–	2.0	1.5	1.0	0.00
–	2.0	2.0	2.0	0.00
–	–	–	0.50	0.00
–	–	–	1.0	0.00
CD _{0.05} (critical difference)				11.95

In vitro rooting

Overall, MS basal medium of ½ strength proved better for root induction as compared to full strength MS basal medium whether with or without IBA of any concentration (Table 2). The rooting characters like percentage of shoots producing roots, average number of roots per shoot and root length have been influenced by the MS strength and IBA concentration. Medium having ½ MS fortified with 1.0 mg/l IBA recorded to be the best medium giving 100 % root induction with an

Fig. 1 Various stages of in vitro propagation and acclimatization of *Viola pilosa*. **a** In vitro shoot bud establishment after 7 days of culturing. **b** In vitro established shoot buds **c** In vitro multiplying shoots after 5 weeks of culturing **d** Fully formed plantlet **e** Acclimatized plantlets **f** Micropropagated plantlets performing in fields

average number of 10.5 roots per cultured shoot and an average root length of 2.75 cm.

Acclimatization and hardening

Amongst different hardening mixtures used, sand: soil: FYM (1:1:1) was best for plant survival. 100 % survival was achieved on this mixture. When different hardening environments were

Table 2 Effect of different medium compositions on in vitro root induction in microshoots of *Viola pilosa* Blume

Composition	Days taken for root initiation	Rooting percentage	Average number of roots/shoot	Average root length (cm)
MS full strength	–	0.0	–	–
MS full strength+0.5 mg/l IBA	10	32.91 (34.94)	4.50	1.60
MS full strength+1.0 mg/l IBA	10	35.00 (35.90)	4.75	2.02
MS half strength	6	80.18 (63.67)	6.75	2.62
MS half strength+0.5 mg/l IBA	7	83.09 (65.67)	7.25	2.46
MS half strength+1.0 mg/l IBA	5	100 (90.00)	10.5	2.75
CD _{0.05}		6.926	2.812	4.75

Table 3 Primers used to assess genetic stability of in vitro propagated plants of *Viola pilosa*

Primer code	Primer sequence (5' to 3')	Annealing temperature	Total number of amplified products
RAPD primer used			
RAPD-G	GACCGCTTGT	35 °C	7
RAPD-H	AGGTGACCGT	35 °C	5
RAPD-I	CAAACGTCGG	35 °C	2
RAPD-J	GTTGCGATCC	35 °C	3
ISSR primers used			
ISSR-A	CTCTCTCTCTCTCTT	55 °C	3
ISSR-B	CACACACACACACAT	53 °C	2
ISSR-C	TCTCTCTCTCTCTCA	54 °C	2
ISSR-D	TCTCTCTCTCTCTCG	55 °C	2

compared, it was observed that the plants which were kept in glass house covered with green net under controlled light and temperature conditions showed best growth and survival. All the stages of in vitro propagation are shown in Fig. 1a–d.

In vitro conservation

Slow growth by reducing temperature

In vitro shoots multiplying on MS+1.0 mg/l BA+0.25 mg/l Kn medium were incubated at two different temperatures i.e. 4 °C and 10 °C at normal photoperiod of 16/8 h for checking the effect of low temperature on their growth. After 30 days of

incubation, the shoots were brought back to normal culture conditions. No enhanced growth was observed in the shoots cultured both at 4 °C as well as 10 °C, though the shoots cultured at 4 °C remained seemingly normal. As much as 85.7 % shoots grown at 4 °C were retrieved and the retrieval percentage was as high as 100 % in case of shoots grown at 10 °C when recorded after 5 weeks of culturing at normal culture conditions. No morphological variations were observed in those shoots which had been retrieved after incubating at 4 °C. However, the microshoots incubated at 10 °C became etiolated and distorted i.e. without proper shape during incubation. However, normal growth was obtained in these shoots when brought to normal culture conditions.

Cryopreservation following vitrification technique

The in vitro shoot pre-cooled at 4 °C for 1 month showed multiplication within 3 weeks of culturing. The retrieval rate was 41.66 %. However, they attained their normal growth rate after subculturing. While the per cent retrieval of shoot tips with prior incubation at 10 °C was very less i.e. 16.66 as compared to that of shoots with prior incubation at 4 °C and they showed multiplication after 4 weeks of culturing.

Assessment of trueness-to-type

In order to confirm the homogeneity of the plantlets, the RAPD and ISSR pattern of the cultured plantlets was compared with that of the mother plant. A total of 4 RAPD and 4 ISSR primers were used for initial screening with the mother

Fig. 2 RAPD, and ISSR pattern. 1–23: 23 randomly selected micropropagated plants. P: Parent plant. M: Known Molecular weight ladder 100 bp

plant of *Viola* and all primers gave clear and reproducible bands (Table 3). All RAPD and ISSR primers produced distinct and scorable bands. Negligible polymorphism was detected during the marker analysis of micropropagated and in vitro conserved clones. All banding profiles from micropropagated plants were monomorphic and similar to those of the mother plant (Fig. 2a and b).

Discussion

In the present investigations, a standard protocol for in vitro establishment of axillary buds and in vitro shoot multiplication has been developed using MS basal medium (Murashige and Skoog 1962) supplemented with different concentrations of growth regulators like BA, Kn, GA₃ and TDZ.

Cultures were established using axillary bud explants. Plant habit being 'rosette' it is not possible to excise out nodal explants as in case of strawberry gerbera (Kaur et al. 1999; Mahajan et al. 2001). So in such case axillary buds are used as explants.

The TDZ enhanced shoot multiplication, however, the sprouting shoots initially became vitrified i.e. yellow in colour and wilted. It has been demonstrated in earlier studies that overdose of cytokinin leads to vitrification (Ault 1992; Miroshnichenko et al. 1998; Tsay 1998; Drazeta 1997; Martin et al. 2006; Bhatia et al. 2008). Eliminating TDZ and lowering down the concentration of Kn proved beneficial in devitrifying the shoots. After about 10 days of culturing, the shoots became normal in appearance and there was no further decline in growth of shoots.

In vitro shoot multiplication from the established shoot buds is very important as only this can lead to mass propagation in less time. Role of cytokinins is obvious in bud proliferation and shoot multiplication (Aggarwal et al. 1999; Pereira et al. 1999; Kaur et al. 2000; Sharma et al. 2004; Kaloo et al. 2013) and the stimulating effect of BA on multiple shoot formation has been reported for several medicinal and aromatic plant species (Pattnaik and Chand 1996; Saxena et al. 1997; Khalafalla and Hattori 1999; Faisal et al. 2006; Kaur et al. 2007).

One hundred percent in vitro root induction was recorded on ½ strength MS fortified with 1 mg/l IBA. Earlier studies have also demonstrated the role of IBA in root induction in *Viola patrinii* (Gururaj and Uddagiri Venkanna 2012). Number of roots per rooted shoot, rooting percentage, rooting length were also recorded significantly higher on this medium.

Autoclaved mixture of soil: sand: FYM in the ratio of 1:1:1 proved to be best for hardening of micropropagated plantlets. The survival rate was 83 %. Hence, it was recommended that this standardised hardening medium for this plant species can

be used before transplanting the plantlets to the natural conditions for their better survival in the field. In vitro regeneration is an efficient means of ex situ conservation of plant diversity, especially medicinal plants which are over exploited without disciplined efforts for their cultivation. In vitro shoot multiplication on reduced temperature has been found to be effective for medium term conservation of this plant species.

The assessment of the genetic fidelity of in vitro derived clones is an essential step for carrying out micropropagation of germplasm, conservation relies on micropropagation. However phenotypic and genotypic variations have been reported to occur during plant tissue culturing give rise to somaclonal variations. Two PCR-based techniques, RAPD and ISSR, were used to test clonal fidelity because of their simplicity and cost-effectiveness. The use of two markers, which amplify different regions of the genome, allows better chances for the identification of genetic variations in the clones (Martin et al. 2004). No polymorphism was detected during the RAPD and ISSR analysis of in vitro-raised clones. The absence of genetic variation using RAPD and ISSR marker has been reported in micropropagated shoots of *Pinus thunbergii* (Goto et al. 1998) and swertia (Joshi and Dhawan 2007), in vitro-regenerated turmeric (Salvi et al. 2001), and in vitro-raised bulblets of *Lilium* (Varshney et al. 2001).

The present study confirms the fact that axillary multiplication is a safest mode of in vitro multiplication and conservation. The record of very low level of polymorphism between clones supports the conclusion that the micropropagation and in vitro conserved plantlets are likely to be true to the type of mother plant.

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