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Micropropagation of *Vanasushava pedata* - An Endangered Medicinal Plant of South India

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Abstract

An efficient *in vitro* propagation of an endangered medicinal plant *Vanasushava pedata* (Apiaceae) by axillary shoot proliferation from nodal segments of mature plants was designed. The medium type and growth regulators markedly influenced *in vitro* regeneration of *V. pedata*. An *in vitro* plantlet production system has been investigated on MS with the synergistic combination of BA (5.0 mg/l), IAA (0.1 mg/l) and 3 % sucrose which promoted the maximum number of shoots (8.6) as well as enhanced shoot lengths. Subculturing of nodal segments from *in vitro* derived shoots on a similar medium enabled continuous production of healthy shoots with a similar frequency. Rooting was highest (100%) on half strength MS containing IAA (2.0 mg/l). Micropropagated plants established in garden soil and forest humus (1 : 1) were uniform and identical to the donor plants with respect of growth characteristics as well as floral features. These *in vitro*-raised plants grew normally in greenhouse and natural habitat without showing any morphological variation.

Introduction

Vanasushava pedata (Wight) Mukh. & Const. (Apiaceae), a small, diffuse branched herb with small white flowers in umbel, is endemic to the South Western Ghats of Peninsular India. The seeds contain coumarins - a potential anti-carcinogenic natural product (Rastogi and Mehrotra 1993). This species commonly grows under the shola floor of high elevation *ca.* 2500 m (msl) forests in the Palni hills and high wavy mountains. Due to biotic pressure, intense deforestation activities, and absence of pollinators, population of *V. pedata* dwindled in its original habitat in the recent past. Only a countable number of individuals were recorded from the Palni hills in 1976 (Mukherjee 1983). This species is included in Indian Red Data book (Nayar and Sastry 1988).

Fast forest clearing activities of Western Ghats is leading to a depletion of valuable plant resources; the conservation of these valuable genotypes is

imperative. 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, endangered and threatened medicinal plants (Arora and Bhojwani 1989, Vishwanath and Jayanthi 1997, Anis and Faisal 2005, Uppendra et al. 2005). Further, genetic improvement is another approach to augment the drug-yielding capacity of the plant (Mulabagal and Tsay 2004). Therefore, it is important to conserve *V. pedata* and save it from extinction. There have been a few reports to date (Fraternale et al. 2002; Uei-Chern et al. 2006), where nodal explants were used for micropropagation of endangered Apiaceae members.

The purpose of this study was to develop an *in vitro* propagation method from nodal segments of *V. pedata*. The present work reports, for the first time, a reproducible method for axillary shoot proliferation from nodal explants. We have also examined the morphological characteristics, growth characteristics, and floral features of *in vitro*-raised plants.

Materials and Methods

V. pedata was collected from the Berijam shola of the Palni hills of Western Ghats during August 2004 and was grown in Sri Krishnadevaraya University Botanical Garden under greenhouse condition. The garden-grown plants were collected and washed thoroughly in running tap water for 30 min followed by treatment with a solution of 3 % Tween 20 (v/v) for 15 min and thereafter washed three to five times with sterile distilled water. The explants were then treated with 0.1 % (w/v) aqueous mercuric chloride solution for 5 - 7 min and finally rinsed thoroughly with sterile distilled water under Laminar airflow cabinet. The nodal segments were trimmed at both ends (1.5 - 2.0 cm) prior to inoculation on the culture media.

Single disinfected nodal segments were cultured on MS basal medium supplemented with 3 % (w/v) sucrose (Sd-fine Chemicals, India) for culture initiation which also served as explant sources for subsequent experiments. The pH of the medium (supplemented with respective growth regulators) was adjusted to 5.7 with 1N NaOH or 1N HCl before gelling with 0.8 % (w/v) agar. In all the experiments, the chemicals used were of analytical grade (Merck and Sd-fine Chemicals, India). The medium was dispensed into culture vessels (Borosil, Mumbai, India) and autoclaved at 105 kPa and 121 ^{o}C for 15 min. The surface sterilized explants were implanted vertically on the culture medium [test tubes (150 × 25 mm) containing 15 ml medium] and test tube mouth tightly covered with aluminium foil. All the cultures were incubated at 25 ± 2 ^{o}C under 16 h photoperiod of 45 - 50 μ mol/m^2/s irradiance provided by cool white

fluorescent tubes (Philips, India) and with 60 - 65 % relative humidity. All subsequent subcultures were done at four weeks intervals.

Three different media including MS, B_5 (Gamborg et al. 1968) and Woody Plant Medium (WPM: Lloyd and McCown 1981) were evaluated for their effects on *in vitro* growth and development of *V. pedata.* All the basal media contained 3 % (w/v) sucrose and were solidified with a 0.8 % (w/v) agar using different concentrations of cytokinins, including 1.0 - 7.0 mg/l BA, 1.0 - 7.0 mg/l Kn and 1.0 - 7.0 2iP. Nodal segments were cultured on solidified MS containing 3% (w/v) sucrose. The supplements added to MS were: different combinations and concentrations of cytokinins, including 2.0 mg/l BA + 1.0 - 3.0 mg/l Kn and 2.0 mg/l BA + 1.0 - 3.0 mg/l 2iP. The other combinations used were: 2.0 mg/l BA + 0.1 - 2.0 mg/l IAA, 2.0 mg/l BA + 0.1 - 2.0 mg/l IBA and 2.0 mg/l BA + 0.1 - 2.0 NAA.

Elongated shoots were excised from each culture passage and transferred to full- and half-strength MS containing 1 % (w/v) sucrose and 0.7 % (w/v) agar. The medium was further supplemented with 0.5 - 3.0 mg/l IAA, 0.5 - 3.0 mg/l IBA and 0.5 - 3.0 mg/l NAA individually.

Plantlets with well-developed roots were removed from the culture medium and after washing the roots gently under running tap water, plantlets were transferred to plastic pots (10 cm diameter) containing autoclaved chopped peatmoss and farmyard manure (1 : 1). Each was irrigated with half-strength MS basal salt solution devoid of sucrose and inositol every fourth day for two weeks. The potted plants were covered with porous polythene sheets for maintaining high humidity and were maintained inside the culture room conditions. The relative humidity was reduced gradually, and after 30 days the plantlets were transplanted to pots (25 cm diameter) containing forest humus and garden soil (1 : 1). The pots were transferred to greenhouse for further growth and development. Well acclimatized *in vitro*-raised plants were transferred finally to its original habitat for its survivalability. The morphological characteristics, growth characteristics, and floral features were examined.

Experiments were set up in a randomized block design and each experiment usually had 15 replications and was repeated at least three times. Ten to 15 explants were used per treatment in each replication. Observations were recorded on the frequency (number of cultures responding for axillary shoot proliferation and root development) and number of shoots per explant, shoot length, roots per shoot, and root length, respectively. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means. The treatment means were compared using Tukey test at a 5 % probability level.

Results and Discussion

Nodal explants of *V. pedata* were cultured on three types of media supplemented with various concentrations of BA, Kn and 2iP individually for shoot regeneration. Among the three different media and growth regulators tested, MS medium was found to support a better response for shoot regeneration than B_5 and WPM media (data not shown). MS medium was more effective than other media for other Apiaceae members (Suchitra et al. 1999, Sakia et al. 2001, Shashikala et al. 2005). In WPM and B_5 media smaller number of vitrified shoots were produced.

Among the three different media used, the explants in MS medium appeared healthy and grew vigorously. On MS supplemented with 2.0 mg/l BA, 93.6% of nodal explants produced shoots after 45 days with an average of 6.7 shoots per explant (Fig. 1a). Uei-Chern et al. (2006) reported that the best response was observed on MS with 0.25 mg/l BA alone for shoot proliferation in *Bupleurum kaoi*. A similar phenomenon was observed in other medicinal plants (Jeyakumar and Jayabalan 2002, Baskaran and Jayabalan 2005). In all concentrations of Kn-containing medium single healthy shoots were produced (Fig. 1b).

In the present study, higher concentrations of cytokinin reduced the shoot number as well as shoot length. Higher concentrations of BA (5.0 - 7.0 mg/l) reduced the number of shoot bud induction (Table 1). A similar response was observed in *Centella asiatica* (Nath and Buragohain 2003). Kn and 2iP also moderately increased the shoot number up to 2.0 mg/l, but concentrations of 4.0 mg/l and above reduced the shoot number and shoot length. In all media, shoot sprouting frequency was higher in BA than Kn and 2iP.

In each medium, BA was more effective than Kn and 2iP for shoot length. The highest shoot length of 7.9 cm was obtained on MS fortified with 2.0 mg/l BA attained after 45 days of culture. All the media and hormones except BA induced little white compact callus at the base of the explants after 30 days of culture. The induction of plantlets directly from the plant tissue without intervening callus is of potential value for the *in vitro* multiplication and storage of a given genotype (Shashikala et al. 2005).

Various concentrations of Kn and 2iP with 2.0 mg/l BA were tried for shoot induction and to determine the multiplication potential of nodal explants (Table 2). In combination 2.0 mg/l Kn and 2.0 mg/l BA induced slightly increased number (7.1) and shoot length (8.1 cm) than BA (2.0) alone. The combined effect of BA and Kn enhanced the shoot bud regeneration in *Arachis hypogaea* (Venkatachalam and Jayabalan 1997), whereas BA in combination with 2iP was not found to enhance significant shoot multiplication rate.

Addition of exogenous auxins to the medium promoted axillary shoot proliferation from nodes of explants and enhanced the growth of culture. One of the advantages of adding auxins at low concentration on the culture media is to nullify the effect of the higher concentrations of cytokinin on axillary shoot elongation (Hu and Wang 1983). In the present study, BA (2.0 mg/l) combined with different concentrations of IAA, IBA and NAA to determine the shoot proliferation potential of *V. pedata* nodal culture. The maximum shoot number (8.6) and shoot length (9.1 cm) were produced from the combination of 2.0 mg/l

Table 1. Effect of cytokinins on shoot regeneration from nodal explants of *V. pedata*.

Growth regulators (mg/l)	Shoot sprouting frequency (%)	Number of shoots per explant (Mean ± SE)	Shoot length (cm) (Mean ± SE)
BA			
1.0	80.1^{c}	3.5 ± 0.28^{ab}	3.2 ± 0.23^{c}
2.0	93.6^{a}	6.7 ± 0.26^{a}	7.9 ± 1.52^{a}
3.0	92.1^{a}	5.8 ± 0.21^{a}	6.4 ± 1.94^{a}
4.0	91.5^{a}	4.9 ± 0.20^{a}	5.2 ± 1.92^{b}
5.0	86.4^{b}	2.7 ± 0.21^{b}	5.1 ± 1.83^{b}
7.0	82.6^{bc}	2.5 ± 0.19^{b}	4.5 ± 0.75^{bc}
Kn			
1.0	73.8^{d}	1.0 ± 0.02^{c}	2.5 ± 0.05^{d}
2.0	80.4^{c}	1.4 ± 0.10^{c}	3.1 ± 0.20^{c}
3.0	82.3^{bc}	2.0 ± 0.40^{b}	4.6 ± 1.29^{b}
4.0	74.2^{d}	1.6 ± 0.47^{c}	2.6 ± 1.10^{c}
5.0	71.5^{e}	1.0 ± 0.01^{c}	2.0 ± 0.05^{c}
7.0	70.1^{e}	1.0 ± 0.01^{c}	2.0 ± 0.05^{c}
2iP			
1.0	76.3^{d}	1.5 ± 0.12^{c}	2.9 ± 0.12^{d}
2.0	82.1^{bc}	2.1 ± 0.35^{b}	3.2 ± 0.07^{c}
3.0	81.0^{c}	1.9 ± 0.20^{b}	4.4 ± 1.20^{b}
4.0	75.8^{d}	1.2 ± 0.10^{c}	3.0 ± 0.04^{c}
5.0	72.2^{d}	1.2 ± 0.10^{c}	2.2 ± 0.05^{c}
7.0	70.4^{e}	1.0 ± 0.01^{c}	2.1 ± 0.05^{cd}
BA (2.0) + Kn			
1.0	92.9^{a}	4.2 ± 0.20^{a}	5.2 ± 1.23^{b}
2.0	95.6^{a}	7.1 ± 0.52^{a}	8.1 ± 1.46^{a}
3.0	93.2^{a}	6.3 ± 0.42^{a}	7.3 ± 1.02^{a}
BA (2.0) + 2iP			
1.0	82.1^{bc}	3.9 ± 0.21^{ab}	5.1 ± 1.20^{b}
2.0	86.8^{b}	6.9 ± 0.28^{a}	7.8 ± 1.28^{a}
3.0	87.1^{b}	4.2 ± 0.19^{a}	6.5 ± 1.00^{b}

Means followed by the same letter was not significantly different by the Tukey test at 0.05 % probability level.

BA with 0.1 mg/l NAA. When increasing the concentrations of auxins, shoot proliferation affected significantly. In all the three types of auxin combinations with BA (2.0 mg/l) showed that higher concentrations of auxins with cytokinin produced comparatively less shoot proliferation frequency, but in this condition produced moderate and massive callus at the basal portion of the nodal explants and which greatly inhibited the growth of multiple shoots in culture. Similar observation was reported in *Rauvolfia serpentina* (Ahmad et al. 2002).

Fig. 1. Micropropagation of *V. pedata* from nodal explant. (a) Multiple shoots on 2.0 mg/l BA. (b) Single axillary shoot on 2.0 mg/l Kn. (c) *In vitro* rooting on ½ MS + 1.0 mg/l IBA. (d) One month old acclimated plant.

Proliferated shoots were excised from the culture tubes and cultured on half strength of MS containing different concentrations and combinations of IAA, IBA and NAA. The percentage of root frequency, number of roots per shoot and length of roots were recorded after four - five weeks of culture. The rooting response to different auxin treatments is shown in Table 3. No rooting was found at auxin free medium. Percentage of root induction and number of roots per shoot were noticeably influenced by the combination, concentration, and type of auxin. Among three types of auxins IBA was found to be comparatively more effective than other two auxins NAA and IAA at different concentrations tested for producing roots. IBA (1.0 mg/l) produced 100 % rooting frequency with 4.8 roots per shoot within 40 days (Fig. 1c). IBA was reported as best rooting

Table 2. Effect of different auxins with BA (2.0 mg/l) combination on shoot regeneration of *V. pedata*.

Growth regulators (mg/l)	Shoot sprouting frequency (%)	Number of shoots per explant (Mean ± SE)	Shoot length (cm) (Mean ± SE)
BA (2.0) + IAA			
0.1	80.00^{b}	4.3 ± 0.42^{c}	4.2 ± 0.51^{bc}
0.2	83.33^{ab}	5.9 ± 0.63^{b}	5.4 ± 0.91^{b}
0.5	53.40^{c}	4.3 ± 0.59^{c}	4.9 ± 0.47^{bc}
1.0	40.00^{d}	2.2 ± 0.17^{d}	3.2 ± 0.29^{c}
2.0	-	-	-
BA (2.0) + IBA			
0.1	86.66^{ab}	4.6 ± 0.32^{c}	3.1 ± 0.44^{b}
0.2	73.33^{b}	3.6 ± 0.64^{c}	3.7 ± 0.67^{b}
0.5	33.26^{d}	2.9 ± 0.43^{d}	2.1 ± 0.47^{c}
1.0	26.22^{d}	2.0 ± 0.29^{d}	2.0 ± 0.41^{c}
2.0	-	-	-
BA (2.0) + NAA			
0.1	96.33^{a}	8.6 ± 0.91^{a}	9.2 ± 1.10^{a}
0.2	95.40^{a}	7.3 ± 0.84^{a}	7.2 ± 1.01^{a}
0.5	74.66^{b}	3.2 ± 0.55^{c}	4.3 ± 0.63^{bc}
1.0	66.22^{c}	3.2 ± 0.61^{c}	4.2 ± 0.61^{bc}
2.0	20.00^{d}	2.0 ± 0.32^{d}	3.8 ± 0.83^{c}

Means followed by the same letter was not significantly different by the Tukey test at 0.05 % probability level.

auxin for Apiaceae members *Anethum graveolens* (Sharma et al. 2004) and *Thapsia garganica* (Makunga et al. 2006). On the other hand, among the auxin combination 1.0 mg/l IBA + 1.0 mg/l NAA was found to be the best combination

of auxins for proper rooting in which 100 % shoots rooted within four - five weeks of culture and the highest average number of roots was found to be 4.9 with root length 2.6 cm (Table 3). Similar combination and concentration of auxins was reported for effective root induction in *Rauvolfia serpentina* (Ahmad et al. 2002). The use of NAA and IBA to promote rooting has been reported by others (Nair and Seeni 2001, Nalawade et al. 2003, Rout 2004). In higher concentration of auxins profuse callus was produced at the basal end of microshoots which inhibits the growth and elongation of roots. In the present study NAA alone and in combination with IBA (1.0 mg/l) produced more basal calli and minimum number of roots per shoot.

Table 3. Effect of auxins in half-strength MS with 1 % sucrose on root formation *in vitro* from *V. pedata* microcuttings cultured for five weeks.

Growth regulators (mg/l)	Rooting frequency (%)	Number of roots per shoot (Mean ± SE)	Root length (cm) (Mean ± SE)	Days to root formation	Basal callus formation
Without auxins	-	-	-	-	-
IAA					
0.1					
0.2	53.22^{cd}	1.1 ± 0.12^{c}	1.0 ± 0.12^{c}	25-30	+++
0.5	60.00^{c}	1.2 ± 0.12^{c}	1.1 ± 0.11^{c}	25-30	+++
1.0	62.42^{c}	1.2 ± 0.12^{c}	1.0 ± 0.12^{c}	26-35	+++
2.0					
IBA					
0.1	20.00^{d}	1.8 ± 0.28^{c}	1.2 ± 0.18^{c}	18-20	-
0.2	26.66^{d}	2.1 ± 0.32^{b}	2.2 ± 0.32^{b}	15-20	-
0.5	73.33^{ab}	4.2 ± 0.45^{a}	2.5 ± 0.42^{b}	15-20	-
1.0	100.0^{a}	4.8 ± 0.58^{a}	3.2 ± 0.48^{a}	12-18	-
2.0	66.66^{c}	3.0 ± 0.47^{b}	2.1 ± 0.33^{b}	15-20	+
NAA					
0.1	40.00^{d}	1.1 ± 0.10^{c}	1.2 ± 0.16^{c}	20-15	+
0.2	52.73^{cd}	1.2 ± 0.12^{c}	1.2 ± 0.16^{c}	20-25	+
0.5	60.00^{c}	1.8 ± 0.24^{c}	1.4 ± 0.18^{ab}	20-25	+
1.0	66.22^{c}	2.0 ± 0.31^{b}	1.4 ± 0.18^{ab}	20-25	++
2.0	26.66^{d}	1.2 ± 0.10^{c}	1.2 ± 0.16^{c}	20-25	++
IBA (1.0) + NAA					
0.2	35.55^{d}	1.8 ± 0.29^{c}	1.4 ± 0.18^{c}	18-20	+
0.5	81.22^{b}	2.5 ± 0.14^{b}	2.4 ± 0.36^{b}	15-20	++
1.0	100.0^{a}	4.9 ± 0.45^{a}	2.6 ± 0.48^{b}	15-20	++
IBA (1.0) + IAA					
0.2	30.33^{d}	1.2 ± 0.26^{c}	1.1 ± 0.12^{c}	20-25	++
0.5	79.32^{b}	2.1 ± 0.12^{b}	1.2 ± 0.16^{c}	20-25	++
1.0	82.28^{b}	2.5 ± 0.14^{b}	2.3 ± 0.33^{b}	20-25	+++

- = No response; + = Slight callusing; ++ = Considerable callusing; +++ = Profuse callusing. Means followed by the same letter was not significantly different by the Tukey test at 0.05% probability level.

The ultimate success of *in vitro* propagation lies in the successful establishment of plants in soil. The rooted *in vitro* plantlets were transferred to polythene cups containing autoclaved peatmoss and farmyard manure (1 : 1) for acclimation at 25 ± 2°C for ten days during which elongation and growth of leaves were observed. Later, these plantlets were transferred to pots containing forest humus and garden soil (1 : 1). The potted plants were finally transferred to original habitat after four weeks. The *in vitro*-grown plants established at survival rate of 81.28 and 54.12% in polythene cups and field conditions, respectively (Fig. 1d). The growth characteristics of *in vitro*-raised plants did not show any significant morphological variations from those of the natural habitat. All *in vitro* regenerated plants grew normally and produced multiple branching with roots at nodes and also with umbellate small flowers eight weeks after their transfer to the original habitat In conclusion, a reproducible, quick and large-scale *in vitro* propagation protocol was established from nodal explants of *Vanasushava pedata*. The protocol reported here may prove suitable for conservation of this valuable endangered medicinal herb from extinction.

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