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In Vitro Propagation of *Parrotiopsis jacquemontiana* (Decne) Rehd. Using Mature Tree Explants

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ABSTRACT

The vegetative propagation of *Parrotiopsis jacquemontiana* (Decne) Rehd., an endemic species of the Hamamelidaceae family growing wild in Kashmir, is still considered to be difficult owing to its difficult-to-root stem cuttings. The present study reports, for the first time, an *in vitro* propagation technique for this species using shoot apex and nodal stem segments of a mature tree as explants. The initial shoot cultures were established in agarified Murashige and Skoog (1962) basal medium supplemented with a cytokinin (6-benzylaminopurine (BA) or kinetin at 1-10 µM). The explants secreted a large amount of phenolic substances which frequently led to tissue browning; this problem could be overcome by washing the explants for at least 30 min before inoculation and frequently transferring them to fresh medium during the culture establishment phase. The initial shoots were subcultured in a multiplication medium having a combination of an auxin (1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA) or indole-3-acetic acid (IAA) at 2 µM) and a cytokinin (BA) at 2.5-10 µM. Shoot multiplication was best (5-6 shoots/culture) on MS medium having BA and IAA at 5 and 2 µM, respectively. *In vitro* shoots 2.5 cm or longer were successfully rooted in auxin-supplemented ½-MS medium within 8 weeks. IAA, NAA or IBA at 2.5 and 5 µM were the most effective concentrations for inducing rooting. The plantlets were acclimatized with 40% survival.

Keywords: nodal stem segments, rooting, shoot apex, shoot multiplication
Abbreviations: BA, 6-benzyladenine; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **Kn**, 6-furfuryl aminopurine; **MS**, Murashige and Skoog (1962) medium; **NAA**, 1-naphthaleneacetic acid

INTRODUCTION

Parrotiopsis jacquemontiana (Decne) Rehd. is a member of witch hazel family (Hamamelidaceae), known locally as *pohu*, *posh* or *hatab*. The species is found as a small deciduous tree up to 7 m high or as a large shrub in association with *Cedrus deodara* trees in North West Himalaya from Yamuna westwards at altitudes of 900-2700 m above sea level. The species is a good soil improver and also plays role in the economy of rural people as the wood of this species is the source of good fuel and also provides wicker for making baskets and *kangri* (heating pots). The wood of the species is very hard and is considered to be best for making tool handles, agricultural implements, etc. (Jan 1992).

The species has been ruthlessly exploited for decades for its multidimensional utility which has drastically reduced its presence in the wild and which could threaten its existence in the future. The species is now confined to only some areas of Kashmir valley (Wadoo 1988). Since the species is difficult-to-root, vegetative propagation is difficult. A tissue culture technique for the propagation of this species would thus be a valid method to propagate this plant at a large scale and also to save its germplasm.

Although some members of the Hamamelidaceae have been subjected to *in vitro* studies such as *Liquidambar styraciflua* (Sommer and Brown 1980; Sutter and Baker 1985; Brand and Linesberger 1988, 1991; Vendrame *et al.* 2003; Dai *et al*. 2004; Su 2005) and *Hamamelis* sp. (Marks and Simpson 1990), to date no attempt has been made to micropropagate *P. jacquemontiana*. This study is the first report of the *in vitro* propagation of this species.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents were purchased from Himedia (Mumbai, India), unless specified otherwise and were of the highest possible grade available.

Explant source and preparation

Fresh and healthy twigs of a plant growing in the Botanical Garden of Kashmir University were cut during March-June and immediately swabbed with cotton moistened with 70% alcohol to cleanse the outer surface. From these twigs small shoot apices and nodal segments 2.5 cm in length were cut and immediately washed in running tap water using lab detergent 'Labolene' (Qualigens, Mumbai, India) for at least 30 min. This was followed by surface sterilization of explants in 0.1% $HgCl_2$ for 20 min containing one or two drops of Tween 20 (a wetting agent) followed by a rinsing in autoclaved double distilled water 3-5 times.

Culture establishment and plantlet regeneration

The surface-sterilized explants were cultured in agarfied Murashige and Skoog (1962) basal medium fortified with a cytokinin (6-benzylaminopurine; BA) and kinetin (Kn) at various concentrations (1-10 µM) to encourage bud burst and shoot growth ultimately to obtain aseptic shoot cultures. The pH of the medium was adjusted to 5.8 and was autoclaved at 121°C and 1.05 kg/cm^2 (15-20 psi) for 25 min. The cultures, which were incubated under controlled conditions (25 ± 5°C, 70% relative humidity, a 16-h photoperiod, light intensity of 3000 lux), were observed every 4 weeks.

The initially established shoot cultures were subcultured in multiplication medium comprising MS medium containing a combination of an auxin (1-naphthaleneacetic acid (NAA), indole-3-

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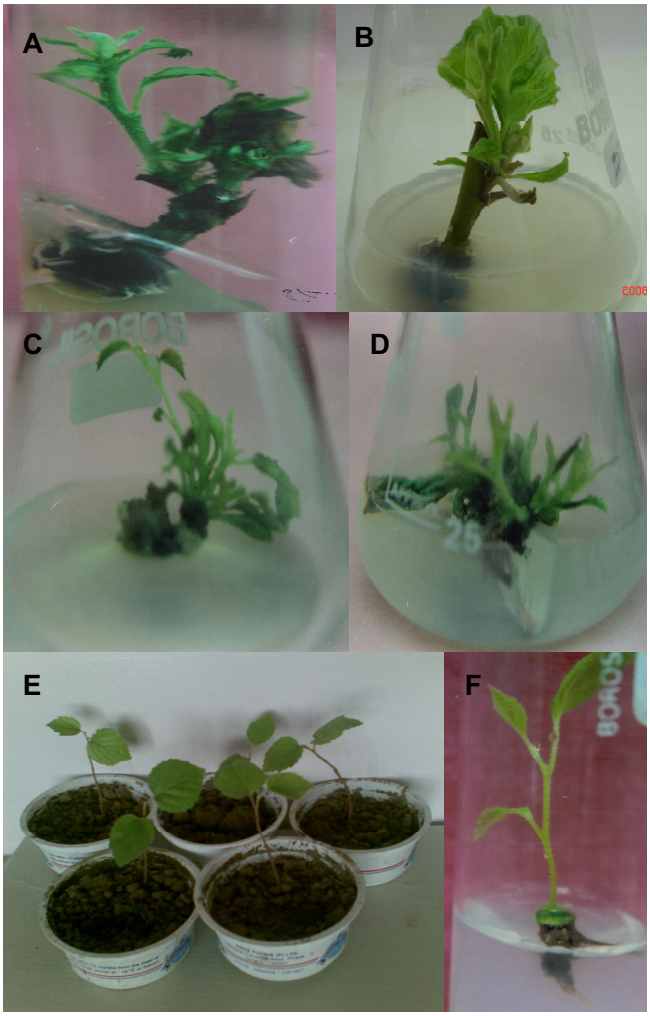

Fig. 1 (**A**) Shoot apex growth on MS + 5 µM BA. (**B**) Nodal segment growth on MS + 2.5 µM BA. (**C**) Multiple shoot formation on MS + 5 µM BA + 2 µM IAA. (**D**) Multiple shoot formation on MS + 5 µM BA + 2 µM NAA. (**E**) Rooting of shoot in MS + 2.5 µM IBA. (**F**) Acclimatized plantlets in vermicompost and sand (after 8 weeks).

butyric acid (IBA)) at 2 µm and a cytokinin (BA) at various concentrations (2.5-10 µM). Individual shoots at least 2.5 cm long from proliferating cultures were subcultured on rooting medium (½-MS basal medium with an auxin (either IBA, NAA or indole-3-acetic acid (IAA)) at 0.5-7.5 µM) for inducing roots.

Hardening

Rooted *in vitro* plantlets were not acclimatized. They were washed in running tap water to remove all remaining culture medium and planted in pots (one plant/pot) with sterilized vermicompost (produced by the earthworm, *Pheretima posthuma* on vegetable waste/ cow dung and soil in the ratio of 2: 1 for 30 days) under controlled conditions of temperature (25°C, 16-h photoperiod, light intensity of 3000 lux, 70% relative humidity.

Statistical analyses

All experiments were carried out in a completely randomized block design (CRD). 20-25 replicates were used for each treatment and observations were recorded after 8 weeks. The data were subjected to one-way multifactorial analysis of variance (ANOVA) using SPSS software (version 13.0) and significance between treatment means was determined using Duncan's multiple range test ($\alpha \leq 0.05$) for the mean number/length of shoots/roots.

RESULTS

The establishment of initial cultures was difficult as the explants exuded large volumes of phenolic compounds which lead to browning of the medium and tissue and eventual death of the explants. To overcome this problem, explants were frequently (3-4 times) transferred to fresh medium until all the phenolic substances were leached out into the medium.

Table 1 Culture establishment: Effect of BA/Kn at varying strengths on the shoot formation percentage of shoot apex and nodal stem segment explants cultured on MS medium*.

Plant growth regulator	Concentration µM	Shoot apex % of cultures	Nodal stem segment % of cultures
BA	1	16	24
	2.5	60	92
	5	100	100
	7.5	28	24
	10	16	12
Kn	1	-	12
	2.5	20	24
	5	40	44
	7.5	12	16
	10	12	8
Control	-	-	-

* Recorded after 8 weeks; 25 replications/treatment;
- no response; BA, 6-benzyladenine; Kn, kinetin

The shoot apex and nodal segment explants were initially recalcitrant and it was only after 4 weeks that the buds started to grow. In the case of shoot apices, 100% culture response was achieved with 5 µM BA; however, a high concentration of BA (i.e., 12 µM) was phytotoxic. Kn also encouraged shoot growth, particularly at 5 µM (**Table 1; Fig. 1C, 1D**). Nodal segments were more responsive than the shoot apex and bud burst initiated after 10 days and a 100% culture response was observed with 5 µM BA (**Table 1**).

Axenic shoot cultures, after subculture onto multiplication medium, showed varying results (**Table 2**). Initially, shoots failed to proliferate in the first week. However, multiple shoots formed in almost all the phytohormonal combinations tried. Use of BA and IAA at 5 and 2 µM, respectively resulted highest mean shoot number i.e. 5.6/culture. Moreover, shoots elongated on multiplication medium containing IAA. Shoots grew at a multiplication rate of 1.5 after every two weeks on multiplication medium.

Individual shoots from the shoot multiplication medium were subcultured on rooting medium. The rooting response of shoots was very slow and difficult (**Table 3**). The first root initial was observed only after 4 weeks in culture and complete rooting was noticed after 8 weeks (**Fig. 1E**). The rooting response with different auxins at 2.5 µM could be ranked: IBA > IAA > NAA. Concentrations of auxins above 5 µM were deleterious and cultures died.

Plantlets showed 40% survival 8 weeks after transfer (**Fig. 1F**).

DISCUSSION

The establishment of cultures from mature tree explants has always been difficult for two main factors viz. phenolic exudation and a high degree of contamination. In our studies on *P. jacquemontiana*, the explants secreted large quantities of phenolic substances leading to browning of medium and death of explants. In addition, contamination also adversely affected the establishment of shoot cultures. The death of explants due to phenolic exudation has also been noticed in a wide range of species (e.g., Ziv and Halevy 1983; Hildebrandt and Harney 1988). Loomis and Battaile (1996) noted that phenolic compounds polymerize with proteins in the plant tissue causing growth inhibition or death of explants. To overcome browning in this study, explants were washed in running tap water for at least 30 min before surface sterilization; in addition, explants were frequently sub-cultured onto fresh medium 3-4 times during the establishment phase. Sutter and Barker (1985) also noted that *Liquidambar styraciflua* mature tree explants (shoot tips/

Table 2 Shoot multiplication and elongation response of subcultured shoots to various combinations of plant growth regulators in MS medium*.

Plant growth regulator (µM)		Shoot number Mean ± SD**	Shoot length (cm) Mean ± SD**	% Culture***
BA	**NAA**			
2.5	2	2.9 ± 0.2 b	1.5 ± 0.3 a	56
5	2	4.4 ± 0.5 e	2.5 ± 0.4 d	100
7.5	2	3.4 ± 0.5 c	2.2 ± 0.4 c	100
10	2	2.8 ± 0.3 b	2.1 ± 0.4 bc	72
BA	**IAA**			
2.5	2	2.2 ± 0.4 a	3.9 ± 0.1 h	100
5	2	5.6 ± 0.4 f	3.9 ± 0.4 h	100
7.5	2	4.1 ± 0.3 d	2.9 ± 0.1 ef	100
10	2	2.9 ± 0.2 b	2.7 ± 0.2 e	64
BA	**IBA**			
2.5	2	2.9 ± 0.2 b	3.2 ± 0.4 g	92
5	2	4.1 ± 0.3 d	3.1 ± 0.2 fg	100
7.5	2	4.2 ± 0.4 de	2.8 ± 0.2 e	100
10	2	2.4 ± 0.5 a	1.9 ± 0.3 b	68
Kn	**NAA**			
2.5	2	-	-	-
5	2	-	-	-
7.5	2	-	-	-
10	2	-	-	-
Kn	**IAA**			
2.5	2	-	-	-
5	2	-	-	-
7.5	2	-	-	-
10	2	-	-	-
Kn	**IBA**			
2.5	2	-	-	-
5	2	-	-	-
7.5	2	-	-	-
10	2	-	-	-
Control (basal medium)		-	-	-

* Data recorded after 8 weeks; 25 replicates/treatment

** Means followed by different letters are significant at the level $\alpha \leq 0.05$ using Duncan's multiple range test (*F* and *P* values of ANOVA are depicted in **Table 2.1**)

*** % Culture was calculated by recording the successful cultures showing a response out of 25 replicates in one treatment

- = no response; BA, 6-benzyladenine; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; Kn, Kinetin; NAA, 1-naphthaleneacetic acid

Table 2.1 ANOVA.

		Sum of squares	df	Mean square	*F* value	*P* value
Shoot number	Between treatments	257.664	11	23.424	139.754	0.000
	Within treatments	42.070	251	0.168		
	Total	299.734	262			
Shoot length	Between treatments	121.734	11	11.067	90.633	0.000
	Within treatments	30.648	251	0.122		
	Total	152.382	262			

nodal buds) became chlorotic and eventually died if they were not transferred to fresh medium during the first month of culture. A combination of cytokinins and auxin in the multiplication medium effectively induced shoots, particularly BA and IAA at 5 and 2 µM, respectively. Laksamee (1996) observed most shoots in MS medium with BA and IBA at 1.0 and 0.1 mg/l, respectively in *Liquidambar formosana*. Aftab (2009) also observed the formation of multiple shoots from nodal buds of *Tectona grandis* in MS medium containing 10 µM BA and 5 µM NAA. Morphogenesis of multiple shoots from axillary bud explants using a high BA and low IBA regime has also been reported by Shoenweiss and Meier-Dinkel (2005) in *Fraxinus excelsior* and Battut et al (1993) in *Sorbus torminalis*. The necessity of BA for axillary shoot formation from nodal buds has also been demonstrated by Tripathi and Kumari (2010) in *Spondias mangifera* and by Phulwari *et al*. (2011) in *Salvadora persica*. Our studies regarding inefficiency of Kn are akin to the findings of Gokhale and Bansal (2009) who observed that BA at 4.43 µM was better than Kn with highest frequency of shoot initiation and maximum number of shoots initiated in apical nodal bud tissue culture in *Oroxylum indicum*.

The rooting response of *in vitro* shoots varied depending on the auxin concentration used. IBA at 2.5 µM was the most effective. Brand and Linesberger (1991) and Sutter and Barker (1985) also found IBA to effectively root *Liquidambar styraciflua* at 3.7 µM and 0.5 mg/l, respectively.

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Table 3 Rooting response of *in vitro* shoots cultured in different auxins at varying strengths in ½-MS basal medium*.

Auxin (µM)		Root number Mean ± SD**	Root length Mean ± SD**	% Culture***
IBA	0.5	1.2 ± 0.5 ab	1.3 ± 0.4 ab	20
	1.0	1.4 ± 0.5 bcd	1.5 ± 0.3 ab	90
	2.5	1.8 ± 0.5 d	2.1 ± 0.2 d	100
	5.0	1.2 ± 0.4 ab	1.9 ± 0.4 cd	100
	7.5	-	-	-
NAA	0.5	1.1 ± 0.3 ab	1.6 ± 0.3 abc	45
	1.0	1.3 ± 0.4 ab	1.6 ± 0.4 abc	65
	2.5	1.5 ± 0.5 bcd	1.7 ± 0.4 bcd	100
	5.0	1.2 ± 0.4 ab	1.6 ± 0.4 abc	100
	7.5	-	-	-
IAA	0.5	1.2 ± 0.4 ab	1.2 ± 0.2 a	60
	1.0	1.4 ± 0.5 bc	1.7 ± 0.3 bcd	85
	2.5	1.7 ± 0.4 cd	1.9 ± 0.5 cd	100
	5.0	1.0 ± 0.0 a	1.3 ± 0.9 ab	100
	7.5	-	-	-
Control (basal medium)		-	-	-

* Data recorded after 8 weeks; 25 replicates/treatment

** Means followed by different letters are significant at the level $\alpha \leq 0.05$ using Duncan's multiple range test (*F* and *P* values of ANOVA are depicted in **Table 3.1**)

*** % Culture was calculated by recording the successful cultures showing a response out of 25 replicates in one treatment

- = no response; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; NAA, 1-naphthaleneacetic acid

Table 3.1 ANOVA.

		Sum of squares	df	Mean square	*F* value	*P* value
Root number	Between treatments	11.120	11	1.011	5.080	0.000
	Within treatments	36.020	181	0.199		
	Total	47.140	192			
Root length	Between treatments	13.053	11	1.187	5.070	0.000
	Within treatments	42.366	181	0.234		
	Total	55.420	192			

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