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Micropropagation of an Elite Line of *Picrorhiza scrophulariiflora*, Pennell, an Endangered High Valued Medicinal Plant of the Indo-China Himalayan Region

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ABSTRACT

An elite genotype of *Picrorhiza scrophulariiflora* Pennell was multiplied *in vitro* for its conservation. Rhizomes of mature plants collected from various locations of the eastern Himalayan region of Indo-China border were characterized morphologically and analyzed by HPLC to determine the content of marker compounds, namely picroside I and II. Amidst the genotypes, one from Ha, Bhutan was found to contain the highest amount of total picroside (7.33% dw). Subsequently, a rapid and highly reproducible method of micropropagation from rhizome or shoot tips was developed. While 100% bud break from rhizomes was achieved on Woody Plant Medium (WPM) containing 0.44 µM BAP (6 benzyl amino purine), 40-fold multiplication was achieved on WPM fortified with 2.3 µM Kn (kinetin) within 12 weeks. The multiplied shoots were elongated on WPM supplemented with 0.44 µM BAP. Around 90% of *in vitro* shoots were rooted without basal callus formation on WPM supplemented with 5.3 µM NAA (α-naphthalene acetic acid) within 4 weeks. Following this protocol, 1100 micropropagated plantlets of an elite line (Ha, Bhutan) were hardened in their natural habitat. The present study illustrates the usefulness of additives for mass propagation and germplasm conservation and is, to the best of our knowledge, the first report of *in vitro* propagation of *P. scrophulariiflora.*

Keywords: *in vitro* regeneration, herb, HPLC, herbal medicine, picroside, Scrophulariacea

INTRODUCTION

Picrorhiza scrophulariiflora (Pennell), *Scrophulariacea*, is an endangered small herbaceous plant found in the sub-alpine as well as alpine zone of the eastern Himalayas comprising Sikkim, Nepal and China (Hara *et al*. 1982). The rhizomes are used in Tibetan and Chinese traditional medicines to treat various ailments such as liver disorders, fever, asthma, jaundice and have pharmaceutical value for hepato-protective, immunomodulator and antiasthamatic activities (Ghisalberti 1998; Smit *et al*. 2000). Though both *Picrorhiza* species i.e. *P. kurroa* and *P. scrophulariiflora*, are a rich source of irridoid glycosides such as picroside I and II, and kutkoside (Rastogi *et al.* 1949; Kitagawa *et al*. 1969; Weinges *et al*. 1972; Jia *et al*. 1999), *P. scrophulariiflora* contains an additional phenylethanoid glycoside and plantamajoside which are absent in *P. kurroa* (Li *et al.* 1998). Thus *P. scrophulariiflora* is a better substitute for *P. kurroa* (Smit *et al*. 2000).

Several reports indicate the need for its conservation, sustainable utilization and cultivation (Ohba and Akiyama 1992; Olsen 1998; Manandhar 1999; Subedi 2000). This plant is not only heavily exported by local traders but also natural regeneration is hampered due to intentional fires set by local shepherds for making grazing area for their yaks which ultimately leads to unsustainable management and depletion of the species (Bantawa *et al.* 2009). As a result, this species was enlisted in a red data book around 20 years ago (Anon. 1987). Additionally, seed setting and seedling survival has been reported to be generally poor in alpine plants (Pandey 2000).

An extensive literature survey revealed that though the genus *Picrorhiza* is well characterized chemically as well as pharmacologically, except for few reports of the micropropagation of *P. kurroa* (Lal *et al.* 1988; Upadhyay *et al.* 1989; Chandra *et al.* 2006), no attempts either to identify elite lines of any kind or *in vitro* culture of this species have been made. Thus the present study was undertaken to identify chemically superior plants among the existing population and mass scale propagation of this line through tissue culture for sustainable management.

MATERIALS AND METHODS

Plant material

Detailed accounts of plant material (**Fig. 1A**) collected from different locations of the eastern Himalayas during September-November are given in **Table 1**. Morphological parameters of 10 dried rhizomes per ecotype in three independent experiments were recorded which were then used for chemical analysis.

Thin Layer Chromatography (TLC)

A Camag HPTLC system equipped with an automatic TLC sampler ATS4, TLC scanner 3 and an integrated software Win-CATS version 1.2.3 was used for the analysis. The entire matured rhizome (~6 cm long) of an individual plant was oven-dried, powdered and out of that, 100 mg was extracted with water: ethanol (50: 50) 2-3 times. The combined percolations were dried under vacuum at 45°C and dissolved in 2 ml of HPLC grade methanol. Samples and standards were applied to a pre-coated silica gel 60 F_{254} TLC plate (Merck, Darmstadt, Germany) as 10 mm bands, 10 mm from the bottom, 10 mm from the side, 6 mm between two spots with a Camag automatic TLC applicator (ATS4), equipped with a 25 µl syringe under N_2 gas flow. Ascending development of

Received: 22 January, 2009. Accepted: 25 December, 2009.

Original Research Paper

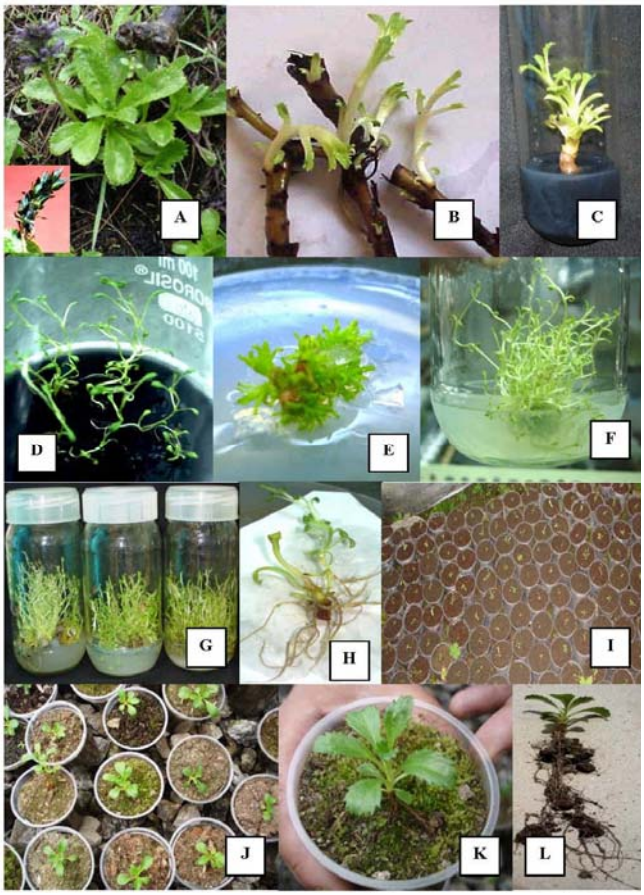

Fig. 1 ***In vitro*** **propagation of** ***P. scrophulariiflora*****.** (**A**) The plants are in its natural habitat of Sikkim (mature spike). (**B**) Adventitious buds induced at laboratory. (**C**) Aseptic culture initiation from apical shoots WPM with 0.44 µM BAP. (**D**) Germinated seeds on MS. (**E**) Multiple shoot formation at WPM with 2.3 µM Kn at initial stage after 4 weeks. (**F**) After 8 weeks. (**G**) After 16 weeks. (**H**) The *in vitro* multiplied shoots rooted on WPM with 5.3 µM NAA. (**I**) Transferred plantlets on plastic pots containing 9: 1 (virgin soil: sand). (**J**) Well hardened plants after 6 month. (**K**) Closed up view of acclimatized plantlets after 1 year and (**L**) Before field transfer.

the plate, migration distance 90 mm, was performed at 25 ± 2°C in choloroform: methanol (82: 18) as the mobile phase in a saturated Camag twin-trough chamber. After development, TLC plates were dried with the help of an air drier in a wooden chamber of appropriate ventilation. Densitometric scanning was performed at λ = 270 nm with Wincats Software, using the deuterium light source with a slit width of 6 × 0.4 mm, scanning speed of 20 mm/s, and data resolution with 100 µl/step.

High performance liquid chromatography (HPLC)

For quantifying the picrosides of different genotypes, we adopted the HPLC protocol of Singh *et al.* (2005). Briefly, 100 mg of the same sample which was prepared for TLC analysis from dried rhizome was used for HPLC analysis on a Shimadzu Prominence HPLC system, equipped with an LC-20AT quaternary gradient pump, dual wavelength SPD-20A UV-VIS detector, CBM-20A communication bus module, CTO-10AS VP column oven, 7725i rheodyne injector and Shimadzu CLASS-VP software. The chromatography was carried out on a Luna C_{18} (2) column (250 mm × 4.6 mm, 5 µm particle size) from Phenomenex (Torrance, CA, USA). Desired resolution of picroside I and II with symmetrical and reproducible peaks was achieved using isocratic elution of water: acetonitrile (70: 30) as mobile phase with a 0.7 ml min^{-1} flow rate, for a run time of 30 min and detection wavelength of 270 nm.

For preparation of a calibration curve, standard stock solution prepared in methanol of picroside I and II (Life Technologies India Pvt. Ltd., India, 99.90% purity). (0.2 mg/ml) was serially diluted to appropriate concentrations (0.8, 1.6, 2.4, 3.2 µg/ml) and injected with 7725i rheodyne injector in triplicate.

Explant preparation for micropropagation

For standardization of the micropropagation protocol, explants of Kuppup origin were used due to the availability of a large number of plants, after which the protocol was employed for mass-scale micropropagation using the elite lines i.e., from Ha in Bhutan. Two different types of explants were used in this study, 1) shoot tips and rhizomes immediately after collection, 2) two-weeks old sprouted buds that emerged from collected rhizomes, kept in the laboratory (**Fig. 1B**) under moist-dark conditions with fungicide Bavistin (BASF India Pvt. Ltd., India) solution (0.2% w/v) at room temperature. For inoculation, explants were washed thoroughly under running tap water for 10-15 min to reduce the surface dirt. Thereafter, the terminal or single nodes of the rhizomes were cut into small pieces (1-1.5 cm), washed with Tween-80 (Himedia) for 10 min followed by a wash under running tap water for 30 min. Immediately after the wash, once again they were treated together with a mixture of fungicide Bavistin (0.5% w/v) and Master (Tata Chemicals Ltd., India) (0.2%) as well as rifampicin (Himedia) (50 mg/l) for 4 h. Subsequently they were placed under a laminar hood and treated with mercuric chloride (Himedia) (0.1% w/v) for 3 min and washed 3 times with autoclaved sterile water each with 10 min.

All explants, one per test tube (25 × 200 mm) were inoculated in an upright position with the 5 mm basal portion embedded in 5 ml of MS (Murashige and Skoog 1962) medium solidified with 0.8% (w/v) agar (Himedia) in the presence of activated charcoal (AC, Himedia) (0.2% w/v) and fortified with 3% (w/v) sucrose (Himedia). The pH of the medium was adjusted with 0.1 N KOH to 5.8 ± 0.1 before autoclaving the medium at 15 psi for 15 min. Cultures were then kept at 24 ± 2°C under a 12 h photoperiod at a light intensity of 2000 lux from cool florescent light tubes (Model LIFEMAX-A 73, Phillips India Ltd., India). Sub-culturing was done at 4-week intervals. Subsequently for various experiments, the basal medium, either MS or WPM (Woody Plant Medium; Lloyd and McCown 1980) were used along with different combinations of cytokinin such as Kn (kinetin, Himedia), BAP (6 benzyl amino purine, Himedia) and TDZ (thidiazuron, Sigma-Aldrich) (alone or in combination with NAA (α-naphthalene acetic acid). After bud break, the shoots were cut at the base and subcultured onto multiplication medium consisting of WPM with various concentration of either Kn or BAP with different auxins such as IAA (indole-3-acetic acid, Himedia), NAA (Himedia) and subsequently onto elongation medium (WPM with various concentration of 0.44 µM BAP). For rooting, the individual shoots of 3-4 cm length were segregated from the clumps and subcultured on media containing various concentrations of NAA, IBA (indole-3-butyric acid, Himedia and IAA.

Effect of activated charcoal (AC) on multiplication

To check the effect of AC on the multiple shoot formation, two combinations that induced high multiplication i.e. Kn at 1.8 and 2.3 µM in MS were fortified with 0.2% AC. The data were recorded after 4 weeks of subculture.

Hardening

The rooted explants (about 3 cm) from any treatment were transferred directly to potting mixture containing virgin soil (top layer of black jungle soil collected from deep forest area) and sand (9: 1) in Hikko trays under a poly-shade house at Kyungnosla nursery, Department of Forest and Wild Life, Govt of Sikkim, Changhu (3758 msl), Sikkim, India. The survival percentage was recorded after 60 d of transfer.

Statistical analysis

The experiments were set up in a randomized block design. Data were analyzed using analysis of variance (ANOVA) to detect significant differences between means (Sokal and Rohlf 1987). Means differing significantly were compared using Duncan's Mul-

Table 1 Morphological descriptions and Picroside I and II contents in different *Picrorhiza* rhizomes.

Picrorhiza sp.	Altitude	Rhizome			Picroside I (%)	Picroside II (%)	Total (%)
		Diameter (cm)	Length (cm)	Dry weight (g/rhizomes)			
P. kurroa	Palampur, Himachal Pradesh (3000 m)	0.45 ± 04 d	7 ± 0.23 c	1.45 ± 0.16 c	0.55 ± 0.23 d	1.34 ± 0.24 d	1.89 ± 0.47 c
P. scrophulariiflora	Thangu, North Sikkim (4000 m)	0.67 ± 0.05 b	9.74 ± 0.52 b	1.56 ± 0.14 b	0.95 ± 0.05 c	5.40 ± 0.56 a	6.35 ± 0.61 b
P. scrophulariiflora	Kuppup, East Sikkim (4200 m)	0.64 ± 0.05 c	6.43 ± 0.41 d	1.41 ± 0.05 d	2.99 ± 0.12 a	4.17 ± 1.02 c	7.16 ± 1.14 a
P. scrophulariiflora	Ha, Bhutan (4200 m)	0.7 ± 0.12 a	11.89 ± 0.4 a	2.12 ± 0.31 a	2.21 ± 0.56 b	5.12 ± 0.12 b	7.33 ± 0.68 a

*Data (mean ± SE) pooled from three independent experiments; Means followed by the same letter does not differ significantly according to Duncan's Multiple Range Test

tiple Range Test (DMRT) at $P \leq 0.05$ with STATISTICA software ver. 5.0 (INC StatSoft 1995). Data is expressed as the mean ± standard error (SE).

RESULTS AND DISCUSSION

Rhizome morphology

In general, the rhizomes of Ha (Bhutan) plants are found to be the thickest (**Fig. 2**) and the longest (**Table 1**). This is very important as rhizomes are the only economic part of this species, thus higher biomass production has a direct link with the profitability for a commercial cultivation. However, no colour or texture differences were noticed among the collected rhizomes.

Quantification of picrosides

The identification of picrosides was confirmed by TLC (**Fig. 3**) and later by comparison of their retention times, UV spectrum with standard compounds and by spiking the samples with standard stock solution (**Fig. 4**). Although different techniques such as spectrophotometry (Narayanan and Akamanchi 2003) and HPTLC (Sharma and Ramamurthy 2000) have been standardized for determining picroside content, HPLC has been the most successful (Dwivedi *et al.* 1997; Sturm and Stuppner 2000, 2001; Drasar and Moravcova 2004). The analytical data revealed that percentage mean values of total picroside content varied from a minimum of 6.35% (dw) of Thangu, North Sikkim, to a maximum of 7.33% (dw) of the plants from Ha, Bhutan (**Table 1**), which is higher than an earlier report of Smit *et al.* (2000), who also found that the total picroside content of *P. scrophulariiflora* was 6.2% (dw). Under the same experimental conditions, we also compared and found that the total picroside content of *P. kurroa* was 1.89%. Thus in the present study, picroside content is clearly higher in *P. scrophulariiflora* than in *P. kurroa*, which is in agreement with an earlier report of Singh *et al.* (2005) who found that total picroside content of *P. kurroa* varied from 0.021 to 3.1% among the different genotypes collected from the western Himalayas. In the present study, a wide range of variability in terms of rhizome morphology and picroside content has been detected and the best genotype was subsequently used for micropropagation using a range of explants. Such variability in chemical content has already been reported in a number of other medicinal plants (Hisiger and Jolicoeur 2007), including *Picrorhiza* (Singh *et al.* 2005). The difference in chemical contents among the accessions of *P. scrophulariiflora* could be explained by abiotic and biotic factors (Echeverrigaray *et al.* 2003; Kamarainen *et al.* 2003; Jayram and Prasad 2008). The chemical diversity determined in the present investigation will open further opportunities for varietal improvement through conventional breeding.

Micropropagation

Being a high altitude plant, the climate was wet due to high rainfall and humidity which favour microbial and algal growth (Martin and Pradeep 2003) and thus establishment of an aseptic culture was a big challenge due to high percentage of microbial contamination. To avoid this, different types of available explants were tested. Plants with forcibly sprouted buds under laboratory conditions registered a low (30%) level of contamination whereas these levels in shoot tips as well as rhizomes used as explants immediately after collection reached as much as 60%. Some contaminations, even after 4-5 months of sub-culture, were noticed. Of importance, buds that emerged from shoot tips were stronger and healthier (diameter of the shoots < 5 mm) (**Fig. 1C**) than those that emerged from rhizomes.

Fig. 2 Mature rhizomes of different genotypes of *P. scrophulariiflora*. (**A**) Bhutan, (**B**) North Sikkim, (**C**) East Sikkim.

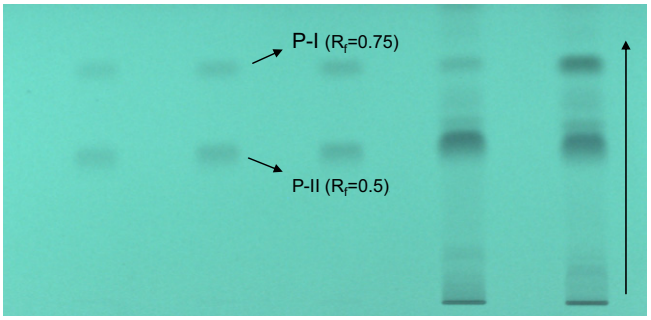

Fig. 3 TLC plate of picrosides I and II. Arrow indicates the migration of the sample.

Evaluation of basal medium, growth regulators on establishment and bud break

A different degree of bud break occurred between MS and WPM medium under a wide range of PGR concentrations. In general, though, bud break was achieved within 4 weeks in all combinations, the lowest being 50 to 55% on basal media and highest 100% with BAP (0.44 µM) alone in both media (**Table 2**). However, increasing or decreasing the concentration of either BAP alone or in combination with NAA not only did not improve the percentage of bud break but also a tendency to produce long comparatively thinner (< 2 mm diameter) shoots was observed (**Fig. 1D**). Occasional rooting and lower bud break (60%) were also observed with 0.26-0.53 µM NAA alone, a typical effect of NAA normally found during *in vitro* rooting. Although both media resulted in 100% bud break, subsequent sub-culturing on MS medium lead to sudden death of explants due to the secretion of some unknown, jelly-like substances at the basal portion of the explants. Therefore we decided to restrict all subsequent experiments to WPM medium only. This may have been caused by a high salt concentration in MS more than in WPM resulting in a stress which led to exudation of some secondary metabolites from the explants.

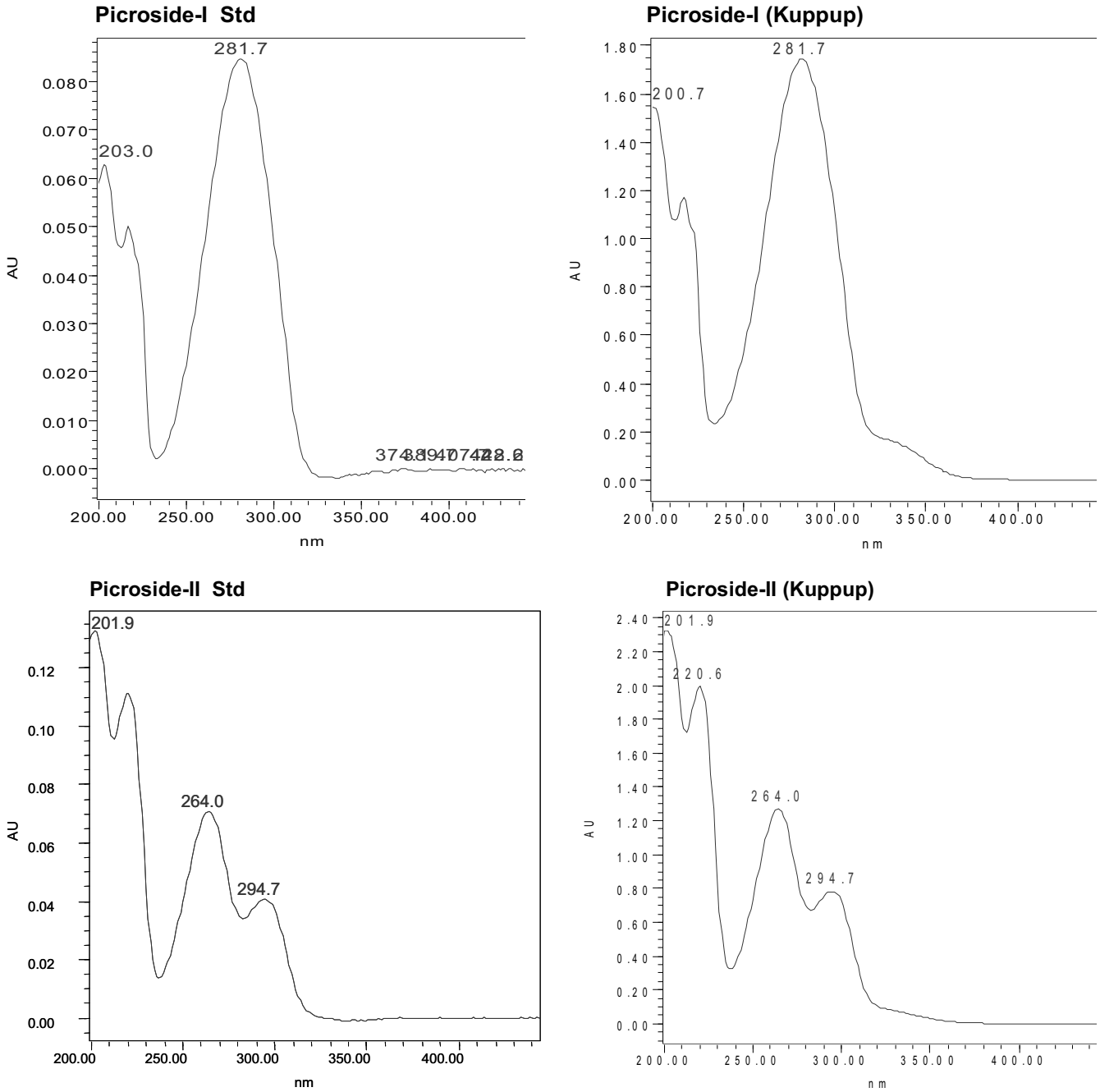

Fig. 4 Comparison of UV spectrum of picrosides extracted from *P. scrophulariiflora* and standard.

Effect of PGRs on multiplication and elongation

Further for multiplication, a wide range of PGR combinations was used (**Table 3**). All combinations of Kn alone (0.46-9.2 µM) induced shoot multiplication but the maximum of 33 shoots per explant was observed only at 2.3 µM Kn (**Fig. 1E-G**). Multiple-shoot induction (i.e. >1) was observed within 12-15 days of incubation at all concentrations of PGRs tested. However, when Kn was used in combination with either IAA (0.28-5.7 µM) or NAA (0.28-5.3 µM), the multiplication rate did not improve as the maximum of 21% only of the multiplication rate was achieved in 0.46 µM Kn- and 0.26 µM NAA-containing media (**Table 3**).

BAP alone (0.44-8.8 µM) or in combination with IAA (0.28 and 0.57 µM) or NAA (0.26 and 5.3 µM), produced multiple shoots from a minimum of 1/explant (8.8 µM, BAP) to a maximum of 13/explant (0.88 µM BAP). Shoots at lower concentrations of BAP (0.88–1.76 µM) were normal and had the tendency to elongate while at a higher concentration (2.2-8.8 µM), they became thinner and weaker in subsequent sub-cultures (**Table 3**). Thus, we decided to use 0.44 µM BAP for elongating individual shoots. In contrast, Upadhyay *et al.* (1989) found that BAP (0.88 µM) was best for shoot multiplication for *P. kurroa*. Although the reason is not clear at present, this observation may be attributed to the difference in species, a phenomenon which often occurs in plant tissue culture. Additionally, Kn, being a mild cytokinin, is perhaps suitable for tender herbs such as *Stevia rebaudiana* (Ahmed *et al.* 2007), *Alpinia galangal* (Borthakur *et al.* 1999) and *Asparagus adscendens* (Mehta and Subramanian 2005).

Effect of AC on multiplication and elongation

Further, to improve the multiplication rate, the effect of AC was evaluated in two ideal media formulations. Although we found that AC was required for initial bud break, later it hindered shoot bud multiplication significantly. In contrast, AC-free media enhanced shoot bud multiplication (**Fig. 5**). Therefore, in all subsequent multiplication and elongation steps, media was devoid of charcoal. Ebert *et al.* (2005) de-

Table 2 Effect of BAP and NAA in MS/WPM on bud break response.

	Percentage of bud break							
	BAP (μM)							
	MS				WPM			
NAA (μM)	0	0.44	1.33	2.22	0	0.44	1.33	2.22
0	50 ± 0.6 m	100 ± 1.2 a	98 ± 0.9 ab	60 ± 0.85 k	55 ± 0.78 l	100 ± 0.55 a	100 ± 0.5 a	76 ± 0.56 h
0.13	55 ± 1.2 l	98 ± 0.4 abc	88 ± 1.05 f	60 ± 1.2 k	57 ± 0.89 l	97 ± 1.56 cd	92 ± 0.8 e	80 ± 0.23 g
0.26	60 ± 0.9 k	92 ± 0.2 e	96 ± 0.2 d	68 ± 0.6 i	61 ± 1.12 k	96 ± 0.56 cd	98 ± 0.64 abc	88 ± 0.52 f
0.53	60 ± 1.3 k	95 ± 0.8 d	92 ± 0.6 e	64 ± 0.6 j#	60 ± 0.7 k	95 ± 0.12 d	98 ± 0.78 abc	88 ± 0.56 f#

All values represents the mean ± SE. Means followed by the same letter does not differ significantly according to Duncan's Multiple Range Test ($P \leq 0.05$) *= thin shoots; # = stunted growth

Table 3 Effect of different PGRs in the shoot multiplication of *Picrorhiza scrophulariiflora*.

PGR name and concentration (μM)	№ of shoots/explant*	№ of leaves/shoot*	Shoot length (in mm)*
0 (control)	1.2 ± 0.22 j	8.0 ± 0.26 j	5.2 ± 0.19 jkl
0.46 Kn	2.1 ± 0.65 j	13.43 ± 0.12 a	7.4 ± 0.15 a
0.93 Kn	5.1 ± 0.30 i	11.36 ± 0.21 e	6.56 ± 0.17 de
1.4 Kn	7.26 ± 0.20 h	9.9 ± 0.15 fg	6.46 ± 0.23 ef
1.8 Kn	21.1 ± 1.23 c	8.56 ± 0.23 ij	6.13 ± 0.08 ef
2.3 Kn	33.13 ± 1.13 a	12.56 ± 0.08 bc	6 ± 0.15 fg
4.6 Kn	20.76 ± 0.32 c	11.76 ± 0.14 de	5.7 ± 0.15 gh
9.2 Kn	11.16 ± 0.31 f	9.76 ± 0.08 fg	5.06 ± 0.06 kl
0.46 Kn + 0.28 IAA	1.7 ± 0.26 j	12.56 ± 0.37 bc	7 ± 0.15 fg
0.93 Kn + 0.28 IAA	3.33 ± 0.49 j	12.26 ± 0.26 bcd	6.2 ± 0.10 fg
1.4 Kn + 0.28IAA	6.6 ± 0.20 h	11.33 ± 0.13 e	5.56 ± 0.17 hij
1.8 Kn + 0.28 IAA	13.83 ± 1.01 e	9.6 ± 0.20 g	4.96 ± 0.03 kl
2.3 Kn + 0.57 IAA	19.76 ± 0.68 b	9 ± 0.47 hi	4.86 ± 0.14 kl
0.46 Kn + 0.26 NAA	21.06 ± 0.62 c	8.7 ± 0.5 ij	4.63 ± 0.18 l
0.93 Kn + 0.26 NAA	17.06 ± 0.18 d	8.16 ± 0.2 j	4.16 ± 0.14 l
1.4 Kn + 0.26 NAA	2.7 ± 0.49 j	7.6 ± 0.44 j	3.56 ± 0.08 l
1.8 Kn + 0.26 NAA	1.16 ± 0.08 j	9.56 ± 0.26 gh	5.1 ± 0.11 jkl
2.3 Kn + 0.53 NAA	2.13 ± 0.27 j	13.3 ± 0.11 a	6.03 ± 0.14 fg
0.44 BAP	11.83 ± 0.95 f	9.46 ± 0.13 a	7.96 ± 0.12 d
0.88 BAP	13.63 ± 0.78 e	12 ± 0.30 d	4.93 ± 0.14 kl
1.32 BAP	2.60 ± 0.97 j	7.9 ± 0.20 j	4.93 ± 0.13 kl
1.76 BAP	2.2 ± 0.05 j	6.96 ± 0.16 j	4.73 ± 0.08 l
2.2 BAP	1.8 ± 0.24 j	6.6 ± 0.21 k	3.3 ± 0.20 l
4.4 BAP	1.6 ± 0.41 j	6.0 ± 0.45 k	3.0 ± 0.08 l
8.8 BAP	1.15 ± 0.36 j	4.2 ± 0.22 k	1.8 ± 0.28 l
0.44 BAP + 0.28 IAA	1.63 ± 0.16 j	13.43 ± 0.12 ij	6.8 ± 0.20 abc
0.88 BAP + 0.28 IAA	10.8 ± 0.36 f	10.2 ± 0.23 f	5.83 ± 0.06 g
1.32 BAP + 0.28 IAA	5.9 ± 0.20 hi	9.9 ± 0.10 fg	5.23 ± 0.06 ijkl
1.76 BAP + 0.28 IAA	2.73 ± 0.44 j	10.03 ± 0.12 fg	5.06 ± 0.18 kl
2.2 BAP + 0.57 IAA	2.1 ± 0.55 j	8.53 ± 0.17 ij	4.73 ± 0.13 l
0.44 BAP + 0.26 NAA	1.63 ± 0.16 j	13.43 ± 0.12 a	6.8 ± 0.20 d
0.88 BAP + 0.26NAA	1.77 ± 0.44 j	13.22 ± 0.20 a	6.84 ± 0.18 bcd
1.32 BAP + 0.26NAA	2.08 ± 0.42 j	13.40 ± 0.38 a	6.98 ± 0.42 abc
1.76 BAP + 0.26 NAA	2.26 ± 0.37 j	13.63 ± 0.23 a	7.3 ± 0.22 ab
2.2 BAP + 0.53 NAA	9.06 ± 0.38 g	12.6 ± 0.20 b	6.6 ± 0.20 de

*Each value represents the mean ± SE. Each mean value followed by the same letter does not differ significantly according to Duncan's Multiple Range Test ($P \leq 0.05$)

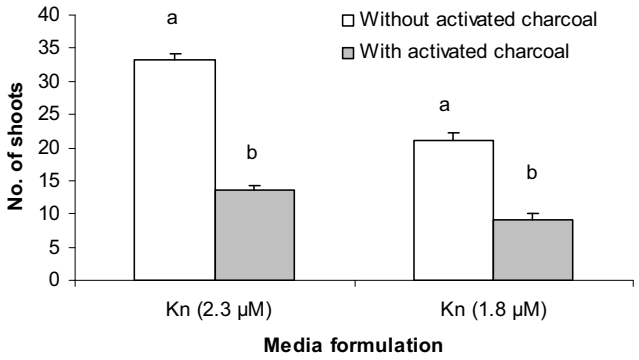

Fig. 5 Effect of shoot multiplication rate of AC in MS media. Bar represent mean ± SE. Means followed by the same letter does not differ significantly according to Duncan's Multiple Range Test.

monstrated that activated AC absorbs plant growth regulators (PGRs) such as BAP and 2,4-D, which lowered the concentration of those PGRs in the medium and subsequently reduced the multiplication rate. Similarly, Sharma and Ramamurthy (2000) as well as Chagas *et al.* (2003) found that AC inhibited the multiplication rate of *Eucalyptus tereticornis* and sweet orange (*Citrus sinensis*) in *in vitro* cultures.

Rooting and hardening of the plantlets

Well developed shoots (3 cm) from *in vitro* culture growing on WPM with 0.44 μM BAP were excised and transferred to rooting medium containing WPM salt augmented with NAA (0.53-10.6 μM), IAA (0.51-10.2 μM) or IBA (0.49-9.8 μM) alone. While basal medium induced minimal *in vitro* rooting, a maximum of 97% shoots formed an average of 7 roots/shoot on WPM with 5.3 μM NAA. Roots were also found to be longest at this concentration (**Table 4**). Rooting was, however, also observed at all the concentrations of IAA and IBA but the maximum only of 1-2 and 4-5 roots/shoot were produced by 56% (11.4 μM IAA) and 75% (10.7 μM IBA) of shoots, respectively. Both increasing or decreasing the concentration of either IAA or IBA did

Table 4 Rooting response of micropropagated shoots of *Picrorhiza scrophulariiflora*.*

PGRs (μM)	Response		
	30 days		Rooting % after 30 days
	No. of roots	Root length (cm)	
0 (control)	0.77 ± 4.22 g	0.7 ± 6.21 g	24.02 ± 4.88 i
IAA 0.5	1.66 ± 1.00 cdefg	1.8 ± 1.22 ef	25.28 ± 1.88 hi
IAA 2.8	1.8 ± 0.86 cdefg	2.0 ± 1.42 de	28.08 ± 1.08 h
IAA 5.7	1.89 ± 1.26 cdefg	2.2 ± 1.22 de	34.74 ± 1.42 g
IAA 11.4	1.45 ± 1.68 fg	0.9 ± 1.26 fg	56.24 ± 2.22 d
NAA 0.5	3.43 ± 1.0 bcd	4.1 ± 0.34 c	54.60 ± 1.80 d
NAA2.6	5.07 ± 1.34 b	4.3 ± 0.12 bc	68.88 ± 2.08 c
NAA 5.3	7.0 ± 2.96 a	5.1 ± 0.71 ab	97.28 ± 2.22 a
NAA 10.7	7.1 ± 1.56 a	5.8 ± 0.33 a	75.06 ± 1.66 b
IBA 0.4	3.34 ± 0.26 bcde	2.2 ± 1.33 de	38.22 ± 1.88 f
IBA 2.6	3.68 ± 1.46 b	2.9 ± 0.96 d	47.22 ± 1.66 e
IBA 5.3	3.92 ± 1.86 b	4.2 ± 0.88 bc	68.28 ± 2.02 c
IBA 10.7	4.55 ± 2.22 b	4.4 ± 1.94 c	75.08 ± 0.98 b

*Each value represents the mean ± SE. Each mean value followed by the same letter does not differ significantly according to Duncan's Multiple Range Test ($P \leq 0.05$). MS was used as basal media

not improve *in vitro* rooting. However, NAA as a better choice for *in vitro* rooting has been well reported in a number of plants such as in *Picrorhiza kurroa* (Upadhyay *et al.* 1989), *Vitis labrusca* (Lewandowski 1991), *Berberis trifoliate* (Mackay *et al.* 1996), *Actinidia polygama* (Tanaka *et al.* 1997), *Stevia rebaudiana* (Ahmed *et al.* 2007), and *Dioscorea oppositifolia* (Poornima and Ravishankar 2007). Although root initiation started within 15-18 d, maximum rooting occurred at 30 d (**Fig. 1H**). Following this protocol, ~1100 *in vitro*-rooted shoots were transferred from culture tubes into plastic cups (**Fig. 1I-J**) containing virgin soil: sand (9: 1) with a 90% survival after 60 d. The acclimatized, well-rooted plantlets (**Fig. 1K-L**) were successfully established in the field after 12 weeks.

CONCLUSION

In the present study, we have identified a genotype of *P. scrophulariiflora* which is superior to any other inter and intra species genotypes for picroside content. Additionally, a highly reproducible micropropagation protocol has been developed that will be an immense help for producing large number of plantlets. Presently works are also in progress (1) to develop a composite cultivation package for large scale cultivation at their natural habitat, (2) to know the reason for variation of picroside content in different genotypes through molecular markers. Thus demonstration of propagation techniques and distribution of elite plantlets among the interested farmers for large scale cultivation will pave the way for *in situ* conservation of this endangered species.

ACKNOWLEDGEMENTS

The authors are thankful to Department of Biotechnology and Department of Science and Technology, Govt. of India for financial assistance, Mr. Bijoy Gurung, Department of Wildlife and Forest, Govt. of Sikkim, India for his help to conduct the survey, Mr. Kamal Das of this laboratory for his assistance.

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