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Full Length Research Paper

In vitro embryogenesis and marker guided analysis of podophyllum hexandrum: An endangered medicinal plant

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For *in vitro* embryogenesis, excised embryos germinate within a week of inoculation on Murashige and Skoog basal medium supplemented with different concentrations of Plant growth regulators, BA (1.0 - 4.0 mg/l) and IAA (0.5 - 2.0 mg/l). Basal medium with BA concentration (0.5 mg/l) and IAA (1.0 mg/l) showed better results than other combinations and was therefore adopted for further studies. The combination of MS + BA (0.5 mg/l + IAA 1.0 mg/l) supplemented with activated charcoal (0.5 - 1.0%) result in optimum growth of *Podophyllum hexandrum* plantlets. Comparative chemotaxonomic studies were done to investigate the phylogenetic relationship of different accessions within the Podophyllum species. Chemical profiles demonstrated that all *Podophyllum hexandrum* accessions collected from different geographical regions are chemically diverse. Chemotaxonomic data showed that chemical characters of the investigated species were able to generate essentially the same relationship as revealed by RAPD analysis. The study has revealed that maximum amount of the podophyllotoxin (5.97%) and podophyllotoxin β-D glycoside (5.72%) was present in the Podophyllum population collected from Keller (Shopian) and Khilanmarg (Gulmarg) area of Jammu and Kashmir, respectively.

Key words: Podophyllum, podophyllotoxin, quantitative, metabolite, chemotaxonomic.

INTRODUCTION

Himalayan mayapple (*Podophyllum hexandrum* Royle) grows wild in the interior himalayan ranges of India. Rhizomes contain antitumour lignans such as podophyllotoxi n and podophyllotoxin-β-D-glycoside (Figure 1) (Tyler et al., 1988; Bromhead and Dewick, 1990). Among the lignans, podophyllotoxin is most important for its use in the semisynthesis of anticancer drugs, etoposide and teniposide (Issel et al., 1984). A number of plant products have been evaluated for protection against lethal dose of radiation including *P. hexandrum* (Goel et al., 1998). Pre-radiation administration of the extracts of *P. hexandrum* mitigated radiation induced postnatal and physiological alterations (Goel et al., 2002). Traditionally dried rhizomes of the plant are mixed with liquid and taken as a laxative or to get rid of intestinal worms as a powerful purgative. Powder of the rhizome is also used as a poultice to treat warts and tumorous growth on skin. Physicians in Missouri, Mississpi and Lousiana used resin from rhizomes of *P. hexandrum* for treatment of genital warts. Podophyllotoxin is the major lignan present in the resin and is dimerized product of the intermediates of the phenylpropanoid pathway (Panda et al., 1989). The Indian *P. hexandrum* is superior to its American counterpart, Podophyllum peltatum in terms of its higher podophyllotoxin content (4%) in dried roots in comparison to only 0.25% of Podophyllum peltatum (Panda et al., 1992).

The podophyllotoxin derivatives etoposide, etopophos (etoposide phosphate), and teniposide are successfully utilized in the treatment of a variety of malignant conditions. Also it is used as a precursor for the chemical synthesis of anticancer drug etoposide, teniposide and etopophos (Farkya et al., 2004).These compounds have been used for the treatment of lung and testicular cancers as well as certain leukemias (Stahelin and Wartburg, 1991; Imbert, 1998). The patent for the use of etoposide in such therapies expired in 1995 and since then, etoposide has been tested in 167 clinical trials for the use as new investigative cancer treatment or as positive con-

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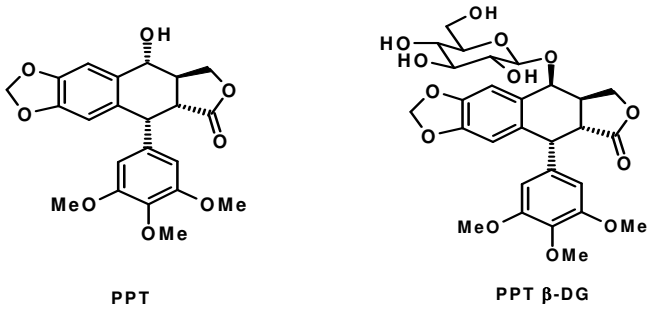

Figure 1: Structure of podophyllotoxin and podophyllotoxin β - D- glycoside.

trol (Ekstrom et al., 1998; Holm et al., 1998; Ajani et al., 1999). In addition, podophyllotoxin is also the precursor to a new derivative CPH 82 that is being tested for rheumatoid arthritis in Europe (Lerndal and Svensson, 2000). Podophyllotoxin preparations are also in the market for dermatological use to treat genital warts (Beutner, 1996) and recently, immune-stimulatory activities of podophyllotoxin have been reported (Pugh et al., 2001). Total synthesis of podophyllotoxin is an expensive process and availability of the compound from natural resources is an important issue for pharmaceutical companies that manufacture these drugs (Canel et al., 2000). Although some efforts have done for production of podophyllotoxin by plant cell cultures using bioreactors but the quantity is not sufficient to meet the demand (Saurabh et al., 2002). Podophyllotoxin, its congeners and derivatives exhibit pronounced biological activity mainly as strong antiviral agents and as antineoplastic drugs. Bioactive molecules of mayapple (Podophyllum spp.) are of interest to the pharmaceutical industry as cheapest source of podophyllotoxin. The traditional medicinal uses of *P. hexandrum* is the treatment of colds, constipation, septic, wounds, burning sensation, erysipelas, mental disorders, rheumatism, plague, allergic and inflammatory condition of the skin, cancer of the brain, bladder and lung, veneral waste, Hodgkin's disease and non-Hodgkins lymphoma (Singh et al.,1994; Beutner et al.,1990).

Since worldwide demand for *P. hexandrum* in particular has far exceeded the capacity for sustainable root harvesting. Effective cultivation of Podophyllum species is imperative. Growers of Podophyllum species must take an interest in genetic preservation to protect their native sources of germplasm diversity for the consequences of over exploitation. Taking into consideration present status of Podophyllum population in the region, it needs immediate attention for conservation, studies of taking appropriate actions for safeguarding this overexploited species, its population biology and genetic diversity, important for successful development of conservation strategies by improving propagation techniques and encouraging its cultivation.

The main aim of the present study was to determine quantitative evaluation of phytochemical component and to assess the diversity in *P. hexandrum* populations collected from different geographical zones of north western Himalayas including Himachal Pradesh. Accumulation of the quantity and the type of marker compounds in both rhizomes and leaves of *P. hexandrum* has been evaluated.

MATERIALS AND METHODS

Collection and authentication of plant material

Live plant material of *P. hexandrum* Royle (syn. *Podophyllum emodi* Wall) was collected from high altitude regions of Kashmir Himalayas (>3500 m asl), J and K, India and transplanted in gene bank layout (Table 1) for developing agro- technological protocols (Figures 2 and 3). The plant material was identified by Centre of Plant Taxonomy, University of Kashmir, Srinagar, J and K. Voucher specimen were deposited in the repository of IIIM, Srinagar.

Isolation and characterization of marker compounds

Air dried plant material (1 kg) was extracted with 100% methanol in a soxhlet for 6 hours. The extract was crystallized with benzene-methanol to give a mixture consisting of podophyylotoxin and podophyllotoxin β-D glycoside. The light brown colored residue obtained after solvent evaporation was dissolved in methanol (400 ml) and filtered. The methanol extract on removal of solvent gives a solid which is recrystallized by ethyl acetate to give white amorphous solid (9.5 g). The solid was subjected to column chromatography using silica gel and the fraction got eluted with hexane: EtOAc (2:8) yielded podophyllotoxin β-D- glycoside. The compound was characterized by various analytical techniques like ^{1}H-NMR, ^{13}C-NMR, DEPT and MS spectral data (Bastos et al., 1995; Yossi et al., 2001 and Mudasir et al., 2007).

Preparation of phytoextracts and HPLC analysis

The rhizome samples of P. hexandrum were collected from 12 different accessions grown at three different altitudinal locations viz. gene bank, IIIM-Srinagar, field Station Bonera (Pulwama) and Yarikha (Gulmarg), Jammu and Kashmir. The dried samples were powdered and subjected to methanolic extraction in a soxhlet by hot extraction (two washes for 3 h each). Phytoextracts were prepared by removing solvents under vacuum. HPLC analysis was performed on a ThermoFinnigan HPLC machine with pump system with the detection wavelength set at 240 nm. Satisfactory separation was obtained with reverse phase column utilizing E. Merck RP-

Table 1. Layout of *Podophyllum hexandrum* plants grown in gene bank of IIIM, Srinagar and its associated field stations.

Population	Location	No. of plants	Acc. code	Altitude (feets)
1	Gurez	65	PGr-A	12,000
2	Veerinag (Sector-15)	640	PV15-A	9,000
3	Gulmarg	300	PG-A	13,000
4	Phalagam	60	PP-A	10,500
5	W. Khrew	131	PW-A	6,000
6	Haftnar	150	PHh-A	9,500
7	Keller	200	PKs-A	10,500
8	Khilanmarg	250	PK-A	11,500
9	Veerinag(Sector-16)	1100	PV16-A	7,500
10	Yousmarg	85	PY-A	9,500
11	Sonamarg	220	PS-A	10,000
12	Himachal	200	PH-A	12,000

Acc; accession

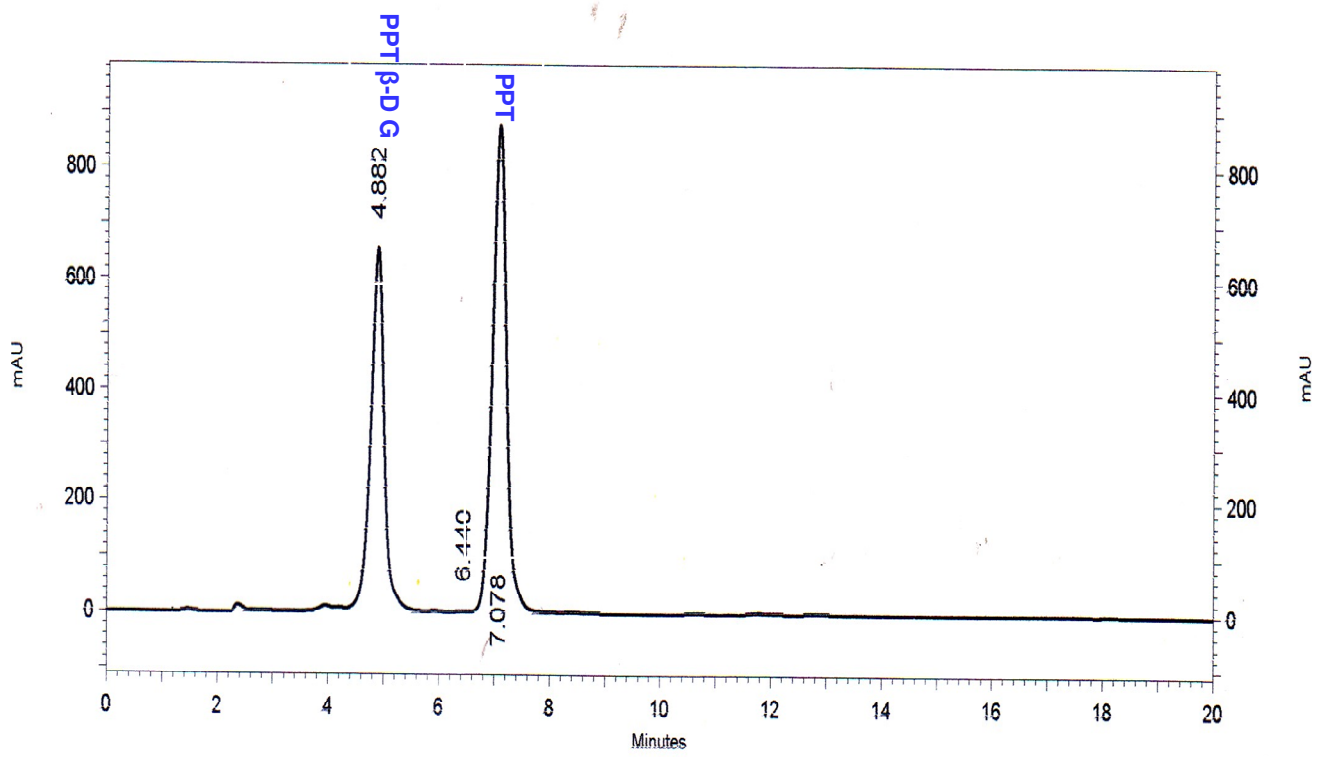

Figure 2. Chromatograms of Standard compounds showing podophyllotoxin and PPT β-D glycoside.

18 column (250 × 4 mm, 5 µm) with a diode array detector (SPDM-10 A VP/RF -10 AXL fluorescent detector) and auto-injector STL-10 AD VP. Elution was done with the mobile phase (MeoH: H2O; 60:40) for 30 min at a flow rate of 0.8 ml/min. A standard mixture of two marker compounds with known concentration of podophyllotoxin and podophyllotoxin β-D glycoside were used to create calibration curves (percentage area with respect to the quantity). Both the marker compounds exhibited enough differences in their retention times, which made their quantification easier (Figure 2).

Culture Initiation

In vitro propagated plantlets of *P. hexandrum* were obtained from excised zygotic embryos of surface disinfected seeds with a solution of mercuric chloride (0.1% w/v for 3 min) followed by 5 min washing with sterile distilled water. The seeds were imbibed for 72 h in double distilled water prior to the excision of zygotic embryos. The zygotic embryos were carefully excised with a sharp needle and excised embryos were transferred to a tissue culture medium

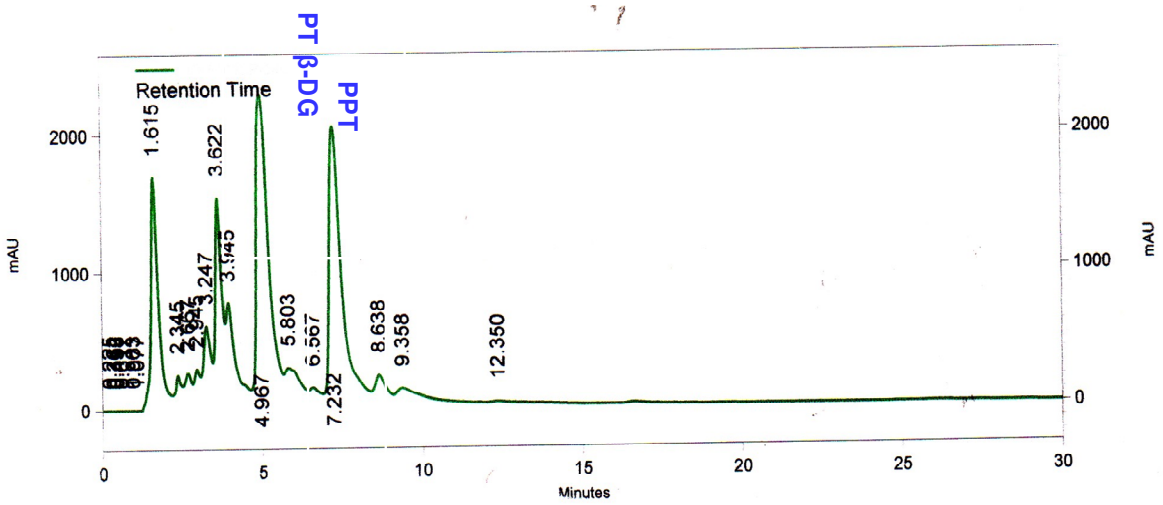

Figure 3. Chromatograms of *P. hexandrum* extract showing two major peaks at the corresponding retention time of the authentic samples. PPT; podophyllotoxin.

medium composed of Murashige and Skoog (1962) containing 3% sucrose and activated charcoal (0.5 - 1%), 0.8% agar and various combinations of growth regulators (cytokinin: BA, 0.5 - 4.0 mg/l), (auxins: IAA, 1.0 - 4.0 mg/l, NAA, 1.0 - 3.0 mg/l and 0.5 - 1.0% activated charcoal. The medium was dispensed in 100 ml glass culture vessels which were steam sterilized at (15psi, 121℃ for 20 min).The experiment was repeated more than once and ten flasks were used for each treatment. The culture vessels were incubated at 25 ± 2℃ under 16 h light with cool fluorescent tubes (Philips TI 40/54). The cultures were transferred to fresh medium after 5 - 7 week interval.

Hardening of rooted plantlets

The rooted plants of *P. hexandrum* were washed in running tap water and finally transferred to jiffy pots containing sand, soil and vermiculite in the ratio of 1:1:1. Before transferring to soil, plants were exposed to higher humidity and gradually reduced that is from 90 - 65% during first 20 days. The potted plants were finally grown under ex vitro conditions as shown in Figure 6 and 7.

RESULTS AND DISCUSSIONS

The mean levels of major marker compounds in 12 different Podophyllum populations showed significant quantitative variation (ANOVA, $p < 0.05$). In order to determine potential variation in concentration of these marker compounds in *P. hexandrum* samples, a detailed chemoprofiling was done for three years and twice in a year. The highest content of podophyllotoxin was found in the accession PKs-A, location, Keller, Shopian, while the highest content of podophyllotoxin β-D glycoside was found in accession PK-A, Location, Khilanmarg (Baramulla).There were significant differences in the amount of marker compounds in all the samples belonging to different ecozones (Table 2).

The total podophyllotoxin content varied from 2.28 - 5.97% on dry wt. basis and PPT β-D glycoside range from 1.81 - 5.72% (Table 2). The differences were significant for most of the comparisons, as determined by ANOVA ($p < 0.05$). To assess the precision of the method, we injected standard solution of PPT, PPT β-D glycoside on the same day of analysis. The precision as well as reproducibility of the method was satisfactory. The results of the recoveries of PPT, PPT β-D-glycoside ranged from 97.6 to 102.3% (Table 3). The relative standard deviations (R.S.D) of recoveries of these two constituents ranged between 1.35 and 2.64%.

For establishment of cultures under in vitro conditions, excised embryos germinate within a week of inoculation on basal medium supplemented with different concentrations of PGR's BA (1.0 - 4.0 mg/l) and IAA (0.5 - 2.0 mg/l). Basal medium with BA concentration (0.5 mg/l) and IAA (1.0 mg/l) showed better results than other combinations and was therefore adopted for further studies. Higher concentrations of IAA and BA (4.0 mg/l) resulted in wilting of tissues after 5 - 7 days of inoculation. Green friable callus was obtained on MS+NAA (1.0mg/l) along with embryogenic root formation (Figure 4 and 5). Media composition for shoot elongation and growth were evaluated. The combination of MS+BA (0.5 mg/l + IAA 1.0 mg/l) supplemented with activated charcoal (0.5 - 1.0%) result in optimum growth of *P. hexandrum* plantlets. A better response was obtained on MS+BA+IAA (0.5 and 1.0 mg/l) respectively for shoot elongation (Table 4). The optimal growth conditions were obtained by incorporating these chemicals at various concentrations. The various growth regulators tested included IAA (0.5 - 3.0 mg/l), NAA (0.5 - 2.0 mg/l) and 2, 4-D (1.0 - 2.0 mg/l), cytokinins 6-BAP (0.5 - 4.0 mg/l) and kinetin (0.5 -1.0 mg/l). Maximum number of shoots was obtained when the zygotic embryos were placed in media supplemented with BA (0.5 mg/l) and IAA (1.0 mg/l). The base position of the germinating embryos with cotyledonary leaves give rise

Table 2. HPLC Analysis of Podophyllum samples in different seasons.

Location	Year analysis	Percentage of Marker compounds			
		PPT (%)		PPT β-D Glycoside (%)	
		Podophyllotoxin	Mean ± RSD	Podophyllotoxin β-D-glycoside	Mean ± RSD
Phalgam	2004	2.56	2.53 ± 0.18	5.74	5.70 ± 0.40
	2005	2.29		6.18	
	2006	2.74		5.20	
Verinag-15	2004	2.51	2.19 ± 0.28	3.70	3.19 ± 0.37
	2005	2.25		2.79	
	2006	1.81		3.10	
Khrew	2004	4.29	4.29 ± 0.26	2.14	2.15 ± 0.16
	2005	4.62		2.37	
	2006	3.98		1.96	
Khilanmarg	2004	5.52	5.41 ± 0.68	6.15	5.72 ± 0.33
	2005	4.53		5.68	
	2006	6.19		5.34	
Yousmarg	2004	2.94	3.23 ± 0.20	5.09	4.91 ± 0.17
	2005	3.34		4.68	
	2006	3.42		4.96	
Sonamarg	2004	3.24	3.60 ± 0.31	2.33	3.65 ± 0.98
	2005	3.55		3.93	
	2006	4.01		4.69	
Gurez	2004	2.95	3.12 ± 0.15	2.47	2.92 ± 0.33
	2005	3.10		3.04	
	2006	3.33		3.27	
Himachal	2004	2.01	2.32 ± 0.24	1.92	1.91 ± 0.10
	2005	2.35		1.78	
	2006	2.61		2.03	
Haftnar	2004	2.60	2.29 ± 0.21	2.12	1.82 ± 0.37
	2005	2.20		1.30	
	2006	2.09		2.05	
Gulmarg	2004	2.09	2.39 ± 0.49	2.63	2.30 ± 0.37
	2005	2.0		1.78	
	2006	3.1		2.50	
Veerinag-16	2004	1.97	2.28 ± 0.29	2.82	1.81 ± 0.72
	2005	2.20		1.18	
	2006	2.67		1.43	
Keller	2004	4.60	5.97 ± 1.11	1.69	1.92 ± 0.77
	2005	5.98		2.96	
	2006	7.33		1.11	

to multiple shoots after 5 weeks of inoculation. The cultures remain greenish on medium supplemented with activated charcoal (0.5 - 1.0%) and long roots developed in this combination. Basal medium with MS+NAA (0.5 mg/l) resulted in 60% rooting of shoots (Table 5). Callus formation was obtained in combinations of MS+NAA (1.0mg/1) and MS+IAA+BA (2.0+1.0 mg/l each) respectively from the basal end of germinating embryos along with embryogenic root formation without any shoot elongation. The number of roots were 1 - 4 from each germinated embryo and resulted in green callus formation after inoculating separated segments of such roots on MS + NAA (0.5 mg/l). The browning of tissues occurs due to release of phenolics from the leaves of germinating embryos which was overcome by adding activated charcoal in the medium.

In present study excised embryos show 95% germination on basal medium containing activated charcoal (0.5 - 1.0%) and give rise to maximum 4 shoots/ embryo and subsequent rooting and remain greenish than any

Table 3. Recovery of podophyllotoxin and podophyllotoxin β -D-glycoside

Standard	Spiked (mg)	Found (mg)	Recovery %	Recovery % (R.S.D)
podophyllotoxin	0.0038	0.0080	105.2	102.3 (2.64)
	0.0046	0.0086	102.9	
	0.1771	0.3944	98.8	
Podophyllotoxin β -D- glycoside	0.0680	0.1333	98.2	97.96 (1.35)
	0.807	0.1423	96.2	
	0.0462	0.1027	99.5	

List of abbreviations: RSD = Relative standard deviation, mg = milligram.

Figure 4. Callus formation from root segments

Figure 5. Established shoots from callus cultures

Table 4. Response of excised embryos of *Podopdyllum hexandrum* cultured on MS medium supplemented with PGR's.

Treatment with PGR,s	Callus intensity	No. of shoots/ embryo	Mean No. of shoots/ embryo ± SD	Mean length of shoots ± SD
Control	-	-	-	-
MS+BA+IAA (1.0+1.0)	-	1 - 5	3.00 ± 0.30	3.5 ± 1.26
MS+BA+IAA (1.0 + 3.0).	++	2 - 6	4.26 ± 0.42	3.4 ± 0.4
MS+BA (1.0)	-	1 - 6	3.80 ± 0.19	5.2 ± 0.79
MS+IAA (0.7)	-	1 - 5	-	5.3 ± 0.43
MS+NAA (1.0)	++	-	-	-
MS+BA+IAA (1.0 + 2.0)	++	1- 3	2.33 ± 0.60	2.5 ± 0.04

*Embryos transferred to MS basal medium supplemented with different conc. Of PGR, s, Control embryos were cultured on PGR-free medium throughout. - mark denotes no response, + mark denotes magnitude of response; SE = standard error, n = 10, 10 embryos per flask. SD = Standard deviation, mg/l = milligram per litre, AC = activated charcoal, MS = Murashighe and Skoog

Table 5. Rooting response in germinated seedlings of *Podophyllum hexandrum* after inoculation on MS medium supplement with auxins (observations recorded after 35 days).

Type of medium supplemented auxins with (mg/1)	Percent Rooting	Rooting in Days	Mean No. of roots ± SD	Mean length of roots ± SD
Control	-	-	-	-
MS	15	18 - 20	1.3 ± 0.30	1.9 ± 0.83
MS1/2	20	16 - 18	1.9 ± 0.41	2.7 ± 0.55
MS+AC (0.8%)	35	21 - 23	2.5 ± 0.44	3.2 ± 0.40
MS+NAA (0.5)	60	15 - 18	2.9 ± 0.50	2.5 ± 0.26
MS+IAA	10	13 - 15	1.9 ± 0.25	2.3 ± 0.43

* The data is based on 5 replicate cultures, while the experiment was repeated thrice. SD; Standard deviation, mg/l; milligram per litre, AC; activated charcoal, MS; Murashige and Skoog.

other combination (Table 4). Rooted plantlets were transplanted in jiffy pots filled with a mixture of sand: soil and vermiculite in the ratio of 1:1:1 for field transfer. The pots have been covered with plastic bag to maintain a high humidity and placed in hardening unit at 23 - 25 ± 2℃ under 16 h photoperiod.

DISCUSSION

The major goal of the present study was to investigate the effect of environmental factors on diversity and chemistry of the important marker compounds of *P. hexandrum* to set a framework for the authentication of these important botanicals. To accomplish this goal, chemical analysis was done on seasonal basis following proper SOP's. Because of the fact that Podophyllum was often sold as rhizomes as whole or powder or an extract. We evalua-ted the utility of chemical characters derived from meta-bolic profiling experiments to reconstruct the phylogeny of the medicinally important Podophyllum species and to distinguish between different accessions. Based on the compounds that were detected or identified from the different samples, we found that more or less both the major marker compounds are present in all the samples in the present study that is; there is no apparent qualitative difference in their HPLC profile chromatogram. Since these plants were not grown under identical conditions, thus role of environmental factors could not be elimi-nated. The collected *Podophyllum* samples appeared to be chemically not very similar. At the biochemical level, clear differences between different accessions could be observed, where both of the compounds were found at different levels. This suggests that genetic factors control not only which specific compounds are produced but at what levels. The phylogenetic trees generated using the chemical profiling and the RAPD data were almost identical in structure, and it was clearly found that PKs-A (Keller, Shopian) was found closely related to PK-A-11 (Khilanmarg, Baramulla) both at metabolic level as well as at molecular level. The analysis of different accessions of *P. hexandrum* on the basis of its two major chemical compounds may serve as biochemical markers for specific Podophyllum species to identify the true Podophyllum species.

Conclusion

The results presented in this paper indicate that HPLC-UV methodology may be useful tool for the quality control of phytoextracts of *P. hexandrum*, since this method showed reproducibility and sensitivity adequate for these extracts. The method is simple and sensitive, and detection and quantification are low enough to analyze podophyllotoxin and PPT β- D glycoside in Podophyllum plants. The method is thought to be ideally suited for rapid routine analysis.

In vitro techniques seem to be an effective alternative in recovery of some rare and endangered medicinal plant species. The suitability of this regeneration protocol for genetic transformation will be investigated. The study also indicates that *P. hexandrum* population in North western Himalayas is highly diverse at least at phytochemical level.

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