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Molecular Characterization of *Valeriana* Species with PCR, RAPD and SDS PAGE

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Abstract

Characterization of plant germplasm using molecular techniques is playing an important role in the management and utilization of plant genetic resources. The present study deals with the standardization of the protocol for isolation of DNA from *Valeriana wallichi* DC. and *Valeriana officinalis* L., to standardize the protocol for isolation of total soluble protein and to standardize the PCR condition for RAPD analysis and SDS-PAGE analysis for total soluble protein. The DNA quality was detected by UV spectrophotometer and Agarose gel electrophoresis. The DNA of leaf tissues from two biotypes of *Valeriana* was analyzed and the amount of DNA in mg per gm was calculated by taking absorbance at 260 nm/290 nm. The ratio of absorbance 260 nm/280 nm was found to be in the range of 1.6 to 1.8 and the DNA yield ranged from 0.59 µg/ml to 0.90 µg/ml. This work shows that the DNA, which was isolated by some modifications in the CTAB method, was of high quality containing very low contamination of terpenoids and polysaccharides. The chemicals, which were used during isolation of DNA by CTAB method, increase DNA purity by removing all impurities. Long-term chloroform isoamylalcohol treatment removes chlorophyll, pigments and dyes. Overnight treatment of RNase degrades RNA. Other precipitates (detergents, protein, polysaccharides etc.) were removed by additional step of phenol:chloroform:isoamylalcohol (25:24:1,v/v/v) and phenol:chloroform (24:1). DNA isolated by this method yielded strong and reliable amplification products showing it's compatibility for RAPD-PCR using AG2 primer. Reproducible amplifiable products were observed in all PCR reactions. Thus the results indicate that the optimized protocol for DNA isolation and PCR was amenable to plant species belonging to different genera which is suitable for further work on diversity analysis. [Nature and Science. 2009;7(7):41-49]. (ISSN: 1545-0740).

Key words: *Valeriana,* DNA, SDS-PAGE, RAPD

Introduction

India, with its tropical climate and varied ecological features, is rich in biodiversity of plants. There are many families, which comprise so many medicinal plants for e.g. Ranunculaceae, Valerinaceae etc. Valerinaceae is a well-known medicinal family in India and contain only 10 genera and about 370 species, mostly distributed in North Temperate region. Some species are reported from high altitude region of tropical zone only. The members of family Valerianaceae have long been used as sedatives in Europe and Asia. Valerianaceae occurs naturally throughout the world except Australia and New Zealand. Among 250 known species, three have commercial importance: *Valeriana wallichi* DC (Indian Valerian), *Valeriana edulis* Nutt., ssp. *procera* F.G. Mayer (the Mexican Valerian) and *Valeriana officinalis* L. The plants have diploid, tetraploid and octaploid forms and therefore display considerable morphological diversity. Indian valerian root yields a volatile oil (0.5-2.12%) and root rhizomes are reported to contain upto 3.82% monoterpene derivatives called 'Valepotriates' used in preparation of medicines (Bajaj, 1999). The dried rhizome and roots of *V. officinalis* L. are used medicinally in certain cardiac ailments. The fresh juice of the rhizomes and roots containing a volatile oil is used against nervous disorders and certain cardiac disease. The efficiency of the drug is lost on drying. To an evolutionary biologist or a breeder, variation among plants species has always fascinated a mind of enquiry and helped to select desirable variants or breed a new from of greater agronomic value.

Taxonomic studies and molecular characterization of medicinal plants play an important role in generating new crop varieties with the high yield potential and resistance to environmental stresses. DNA based markers provide powerful and reliable tools for discerning variation within germplasm and to study evolutionary relationships (Gepts, 1993, Joshi et al., 2009). PCR based techniques have been used successfully in DNA fingerprinting of plant genomes and in genetic diversity studies. These techniques include RAPD (Randomly amplified polymorphic DNA),

RFLP (Restriction Fragment Length Polymorphism), SSR (Simple Sequence Repeat) and AFLP (Amplified fragment Length Polymorphism). So far, the most used techniques seems to be PCR and RAPD (Chong et aI., 1994; Lowe et aI., 1996).These procedures, due to their great sensitivity, constitute a powerful technique widely used for enzymatic amplification of stretches from small amounts of DNA and provide an alternative approach to distinguish genotypic variants. In RAPD technique short oligonucleotides of arbitrary sequences are used singly to support the amplification of the plant genome and amplification products are separated by gel electrophoresis. RAPD technique has also been used in plants for the construction of genetic maps (Reitar *et al*., 1992) and molecular characterization (Mehmood et al., 2008). Very little information exists on the molecular aspects of *Valeriana species, which* requires high quality DNA. Therefore, the present study was planned with following objectives

1. Taxonomic consideration and protocol standardization for isolation of DNA from *Valeriana* species (i.e. *V. officinalis* L. and *Valeriana wallichi DC.*).
2. To standardize protocol for isolation of total soluble protein, PCR conditions for RAPD analysis and SDS-PAGE analysis for total soluble protein.

Material and methods

Plant material

Two species, *V. wallichii* DC., *and V. officinalis L.* were taken for experiment. *V .officinalis L.* was collected from G.B. Pant Institute of Himalayan Environment & Development, Kosi – Katarmal, Almora (Uttarakhand) and grown at glass house of Deptt. of Botany D.S.B. campus Nainital(Kumaun University Nainital). *V. wallichii* DC. was collected from Ayarpata Nainital .

DNA Extraction

Total genomic DNA was extracted using CTAB method (Doyle & Doyle, 1987) with some modification. 1gm freshly harvested leaf whose gel was removed was ground to fine pulp using liquid nitrogen along with 0.1 g PVP. Extraction buffer (pH-8) preheated to 65ºC containing 2% CTAB (w/v), 5.0 M NaCl, 0.5 M EDTA and 0.5 m tris HCl were added to the pulp in a centrifuge tube, shacked and incubated for 1 hour at 65 ºC in a water bath with intermittent shacking and swirling in every half an hour. To this equal volume of Chloroform:Isoamylalohol (24:1) was added and mixed by inversion for 30 min and centrifuged at 12,000 rpm for 15 min. Supernatant was transferred to a new tube and was precipitated with equal volumes of cold Isopropanoal, and gently mixed to produce fibrous DNA and incubated at -20ºC for 30 min. Samples were centrifuged at 12,000 rpm for 15 min. The pellete was washed with 70% ethanol and kept for drying. After drying, the pellete was dissolved in 3 µl of TE buffer (1 mM EDTA and 10 mM Tris HCL pH-8). To remove contaminating RNA 5 µl of RNAs (10 mg/ml) was added. The tubes were incubated over night at 37ºC. Dissolved DNA was extracted with equal amount of Phenol:Choloroform: Isoamylalcohol (25:24:1.v/v/v) and centrifuged at 8000 rpm for 15 min. then aqueous layer was transferred to a fresh 15 ml tube and equal volumes of chloroform:isoamyalcohol (24:1) was add and centrifuged at 12,000 rpm for 15 min. Finally supernatant was transferred to a fresh tube, equal volume of absolute alcohol and 1/10 volume of sodium acetate were added and incubated at -20ºC for 30 min and centrifuged at 12,000 rpm for 15 min. The final pellet was dried and resuspended in TE buffer.

Analysis of *Valeriana* sp.through RAPD

For RAPD analysis, the standards given in **Table 1** are used. In RADP analysis both the species were analyzed and compare simultaneously. For RAPD analysis, a large number of universal primers (decamers) (1,2,3,4,5,6,7 and 8) are used. For the optimization of RAPD reaction from *Valeriana* species, arbitrary oligonucleotide primers were used for amplification to standardize the PCR condition. The reaction was carried out in a DNA Thermocycler (Biometra). Reaction without DNA were used as a negative controls. Protein was extracted from the leaves of *V. wallichii* DC. and V. *officinalis* L. both (1 g each) using Borate Buffer (pH 9.0) and centrifuged at 12000rpm for 30 min.

Table 1 Standards for RAPD amplification

Steps	Temp(°C)	Duration
Lid temp	105	
T1	94	2min
T1	94	30sec
T2	55 (gradient of 10 °C)	45sec
T3	72	30sec

DNA amplification

For polymerase chain reaction 02 oligonucleotide primers (17-30 nucleotide) were used. The analysis conditions and quantification of product are given in **Table 2** and **Table 3**.

Table 2 Analysis of *Valeriana sp.*Through PCR

Contents	Amount (µl)	Conc.
Water	21.9	-
Assay buffer	0.5	10x
dNTPs	1.0	1mM
Primers	0.4	1µM
Taq	0.2	1U
Template (sample DNA)	1.0	25.0ng

Note- Primer used is AG21.

Table 3 Thermocycler programme

Steps	Temp(°C)	duration
Lid temp	105	-
T1	94	2min
T1	94	30sec
T2	55	45sec
T3	72	30sec

Agarose gel electrophoresis

PCR products were electrophorased on 1.5 %(w/v) agarose gels, in 1x TAE Buffer at 50 V for 3 h and than stained with ethidium bromide (0.5 µi/ml). Gels with amplification fragments were visualized and photographed under UV light (260 and 280 nm).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS – PAGE)

Glass slabs, spacers and spacers were washed with water and wiped with tissue paper. Plates are set in the gel caster and sealed with the help of sealing agar (1% agarose). Constituents of the resolving gel were mixed in the given ratio as per given in table. Resolving gel was poured down from one edge of the spacer, leaving a gap of two to three cm. on top. Water saturated n-butanol was pipetted above it. The gel was allowed to polymerize for 45 – 90 min. After polymerization the gel surface is rinsed several times with triple distilled water and drained well. Comb was placed keeping a distance of 1 - 1.5 cm. from separating gel, and the constituents of stacking gel were mixed and were poured from one side of comb. It was allowed to polymerize for 45 – 60 minutes. After polymerization the comb was removed carefully and the plates were fixed on to the electrophoresis apparatus. 10 – 20 µg samples were loaded in each well. The gel was run at 15µA constant current until the dye front reached the bottom of the gel. The protein bands were detected by silver staining.

Data analysis

Concentration of protein were determined by Lawry's method (1951) and comparing the concentration of the samples with standard curve value of BSA. Bands with same mobility were

treated as identical fragments. The positions of PCR bands were compared with molecular weight standards.

Results and Discussion

Taxonomic consideration

Most taxonomists place the family Valerinaceae along with the Caprifoliaceae in the order Dipsacales or Rubiales. Hutchinson treats it as a member of Valerinales. From the studies undertaken and data collected its correct position is the Dipsacales among with the Caprifoliaceae and other families.

Valeriana species

Valerian is a common name given to the genus *Valeriana*. The genus *Valeriana* (family – Valerinaceae) comprises large number of species, around 350, which are throughout the world (Bantly et al., 1983). Out of these 20% have been recorded as used for medicinal purposes. This herb may have been named Valerian after the Roman Emperor Valerian (Pubhus Licinius Valerianus, 253-260 A.D.), who first used it in medicine. The other sources mention the name derived from Latin word "Valere" means to be strong and healthy.

About 12 species have been reported to occur in India. Three species viz. *V. wallichii, V. hardwickii, and V. pyrolaefolia* are found in the temperate Himalayas from Kashmir to Bhutan and Khasia hills at 1200-1800m. *V. officinalis* is native to Europe and north and south – west Asian countries. *Valeriana wallichi* (commonly known as tagar, samao) is a perennial herb. The roots and rhizome with or without stolons are used in unani and ayurvedic system of medicine.

Valeriana officinalis L. (Common Valierin or Garden Heliotrope) (Plate 1)

A common glabrous herb; attain a height up to 1 m. Stem solitary, erect. Furrowed and hollow. Leaves opposite, pinnate, lower leaves with long petioles and upper small. Flower appears in panicle, corymb, and white or dull white in colour, fragrant, small calyx many toothed and corolla 5-lobed. Fruit oblong, ovate, small, smooth, without hairs, one seeded. Rootstock is thicker, short with many fasciculate rootlets. This species is native to Europe and Kashmir in India at 2500 m altitude. Now a day it is cultivated in many parts of India and world. The plant is propagated by seeds or through rootstocks. It grows in temperate climate and in India cultivated in Kashmir, H.P., U.P. and Uttarakhand. Medium fertile soil, rich in humus is suitable for this crop. Seeds are sown in well-drained raised beds in the nursery during April-May or July- August.

The plant consists of rhizome, stolons and roots, which constitute the drug. Roots are harvested after two years during their dormancy in November. Roots are washed thoroughly, washed properly and dried. The root contains epoxy-iridoid esters called valepotriares, which include valtrate didrovaltrate, acevaltrate, isovaltrate and isovaleroxyhydroxy didrovaltrate. It also contains chatinine and valerine alkaloids and volatile oil.

The drug is said to be sedative, hypertension, antispasmodic, and stomachic. The juice of fresh rhizome is used in treatment of hysterical fits and other nervous disorders and flatulence. The root preparation is also known as tonic and stimulant. The fresh juice is administered in case of insomnia and in certain cardiac preparations. The essential oil of root is used as a flavour in food products and beaverages.

Plate 1 *Valeriana officinalis* L.

***Valeriana wallichi* DC. *(syn V. jatamansi jones)* (Plate 2)**

It is another species of similar properties as that of *V. officinalis* L. it is a perennial herb upto 45 cm tall and stems tuffed. Flowers are white tinged with pink. Rootstock thick and horizontal, aromatic and nodular. This species is native to Himalayan region of India, Nepal and South Western China. Roots of this plant are useful in diseases of eye, blood, liver and spleen enlargement. They are useful in clearing of voice. The crushed leaves are rubbed in extreme forehead. The root preparations are used as cosmetic and hair oil.

Plate 2 *Valeriana wallichi DC*.

Extraction of Genomoic DNA

The extracted protein of *Valeriana* sp. was of high quality as it showed a reading between 1.6 and 1.8 after calculating the ratio 260/290 nm absorbances. The DNA yield obtained ranged from 0.59µl/ml to 0.90µl/ml.

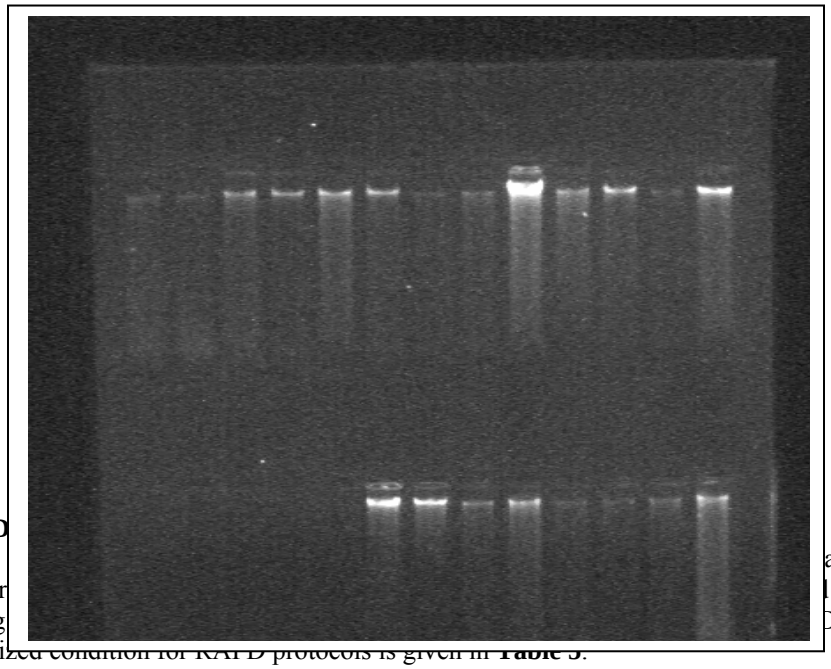
Total genomic DNA

The quality and quantity of DNA isolated was checked in UV spectrophotometer and agarose gel electrophoresis (**Fig. 1.**). The DNA bands extracted with modified CTAB method did not show any smearing or RNA contamination. The concentration of genomic DNA ranged between 1 to 2µl/ml. DNA isolated by this method yielded strong and reliable amplification products showing it's compatibility for RAPD-PCR using AG2 primer. DNA isolated by this method was of high quality and there was very low contamination of terpenoids and polysaccharides. The presence of terpenoids reduces the yield and purity of extracted DNA since it is a strong oxidizing agent and binds to DNA covalently, making it useless for research applications (Perterson et al. 1997; Porebaski et al. 1977). Tannins, terpenes and resins are considered as secondary metabolites, are also difficult to separate from DNA (Ziegenhagen and Scholz, 1998). Certain polysaccharides are known to inhibit RAPD reactions. They distort results in many analytical applications and therefore, lead to wrong interpretations (Kotchoni et al. 2003).

Co precipitation of polysaccharides is avoided by adding a selective precipitant of nucleic acid i.e.CTAB. Additions of PVP along with CTAB may help in removal of impurities because it forms a complex with polyphenols through hydrogen bonds. Long – term chloroform isoamyl alcohol treatment removes chlorophyll and other coloring substances such as pigments and dyes.

Many DNA isolation producers also yield large amounts of RNA (Doyle and Doyle, 1987). RNA in samples can chelate Mg^{+2} and can reduce the yield of PCR. An overnight treatment of RNAse degraded RNA and yielded RNA free pure DNA. Other precipitates (detergents, proteins, polysaccharides) were removed by additional precipitation step of phenol; chloroform; isoamyl alcohol (25:24:1,v/v/v) and phenol:chloroform(24:1) (Khanka et al., 2009).

W W W W W W M O O O O O O



RAPD ation of $MgCl_2$, primer time intervals during D protocol. The optimized condition for RAPD protocols is given in **Table 5**.

Table 5 Optimization of RADP-PCR reaction parameters for analysis of *Valeriana* sp.

PCR parameters	Tested range
$MgCl_2$ (mM)	1,2,3,4
DNTPs (mM)	0.1, 0.2, 0.3, 0.4
Primer concentration (µM)	0.2, 0.3, 0.4
Taq polymerase	0.2, 0.3, 0.4
Initial denaturation time interval at 94ºC (min)	2,3,4,5
Annealing temperature (ºC)	30, 35, 38, 40, 42
Time interval (sec)	20, 30, 40
Reaction volume (µl)	5, 10, 15
Number of cycles	30, 35, 40, 45, 50

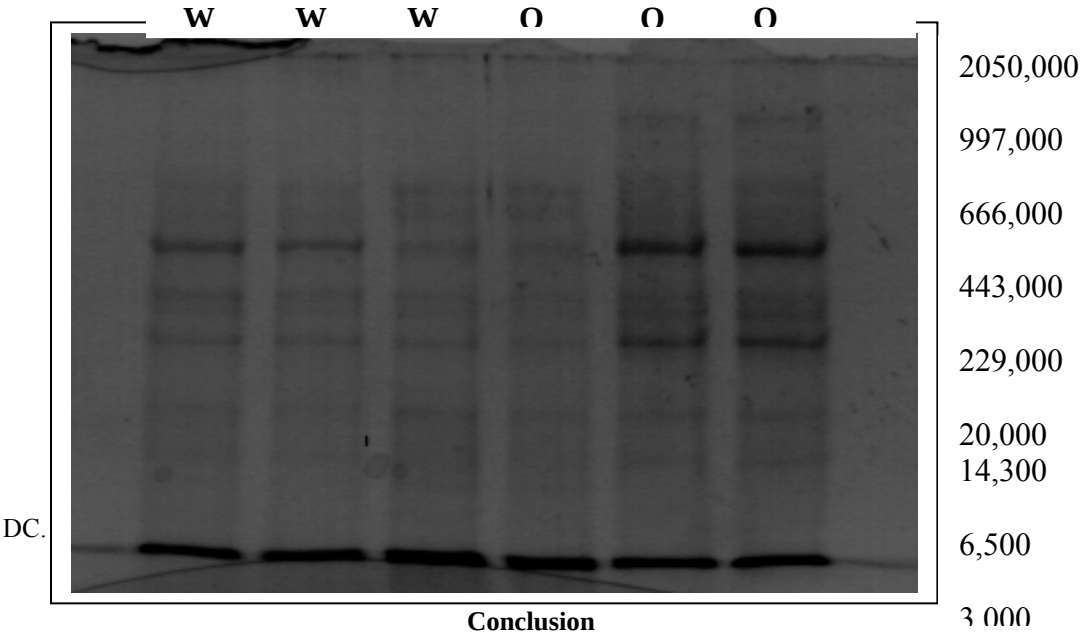
PCR amplification of DNA extracted from two species of *Valeriana* i.e. *V.* wa*llichi* DC. and *V. officinalis* L. Out of these, not any random decamer showed any positive result (**Table 6**). PCR product was run on 1.5 % agarose gel.

Table 6 Sequence of primers which gave negative results during RAPD analysis of DNA extracted from *V.* wa*llichi* DC. and *V. officinalis* L.

Sl. No.	Primers	*V.* wa*llichi* DC.	*V. officinalis* L.
1.	5'-GCAGGGATAGC-3'	-	-
2.	5'-GTCCTCAAACG-3'	-	-
3.	5'-GTCCTACTCG-3'	-	-
4.	5'CTACTACCGC-3'	-	-
5.	5'CTACACAGGC-3'	-	-
6.	5'-CCTGATGACC-3'	-	-
7.	5'-GTCCTTAGCG-3'	-	-
8.	5'-TGCCGAGCTC-3'	-	-

Protein profiling

SDS-PAGE (**Fig. 2**) analysis revealed 9 and 8 bands in *V.* wa*llichi* DC. and *V. officinalis* L. respectively, where molecular weight ranged from 3kDa to 43kDa. Silver stained revealed 12, 11 bands in *V.* wa*llichi* DC. and *V. officinalis* L. respectively, where molecular weight ranged from 3kDa to 205 kDa. This dissimilarity shows than composition of the gel differs and some proteins are present in very low concentration in one species, some are absent while distinct proteins are present in each species.



Conclusion

The study include optimize a protocol for isolation of total genomic DNA and PCR condition for RAPD analysis of *Valeriana spp*. which have high level of terpenoids, poyphenols and secondary metabolites. DNA extraction was done by modifying some steps of CTAB method originally develop for other plants (Doyle and Doyle, 1987). The DNA quality and quantity were detected by UV spectrophotometer and agarose gel electrophoresis. The DNA yield obtained ranged from 0.59µl/ml to 0.90µl/ml. The concentration of genomic DNA ranged between 1µl/ml to 2 µl/ml. This work shows that the DNA, which was isolated by some modifications in the CTAB method, was of high quality containing very low contamination of terpenoids and polysaccharides. The chemicals, which were used during isolation of DNA by CTAB method, increase DNA purity by removing all impurities.

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