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Modified method of DNA isolation and optimization of PCR protocol for RAPD analysis of endangered medicinal plant *Nardostachys jatamansi* D C

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ABSTRACT

An important but limiting step in any molecular biological work is the reliable method of DNA isolation and suitable protocol for PCR analysis. Due to abundance of phenols, polysaccharides, terpenoids and other secondary metabolites in the rhizome of Jatamansi, it is a major problem to get high quality DNA. To isolate quality DNA for PCR analysis modified protocol developed in our lab to overcome such serious problems. We tried to optimize the concentration of PVP, β-mercaptoethanol and NaCl. Upon gel documentation of isolated DNA by modified method evinced single discrete band of genomic DNA and yielded significantly superior, 30-50 µg/g DNA from dry rhizome. The composition of PCR master mix was also modified for RAPD analysis which includes changing the concentration of $MgCl_2$ and observed a good amplification. Reproducible amplifiable products were observed in all accessions.

INTRODUCTION

Nardostachys jatamansi D C is a critically endangered rhizome bearing medicinal plant which belongs to Valerianaceae family, grows in steep, moist, rocky, undisturbed grassy slopes up to 5000 m above sea level (asl) randomly. It has a long history of used in ethno medicine, perfume, incense and modern medicine. The plant valued for its antispasmodic and stimulant properties, useful in the treatment of hair loss, heart palpitations, constipation, urination, menstruation and digestion (Singh et al., 2013a). Most of the alkaloids of medicinal value are present in its rhizome. Its rhizome is also used in various rituals by local people. Due high medicinal value of its rhizome plant were over exploited from its natural habitat. A recent conservation Assessment and Management Plan (CAMP) review identified *N. jatamansi* as a critically endangered species of North West Himalaya.

Taxonomist used morphological characters to classify the genus *Nardostachys* while some says that *N. grandiflora*, *N. chinensis* are synonyms of *Nardostachys jatamansi* (Olsen, C.S. 1999). Morphological polymorphism is common in the genus, and discrimination of many of the taxa depends on characters that could be influenced by environmental conditions. To provide solution to these types of problems, in combination with morphological studies, polymerase chain reaction (PCR) – based molecular markers are widely used at molecular level in different genus because they are not influenced by changing conditions of environment.

Though studies have been conducted on different aspects of *N. jatamansi*, but very limited study has been reported at the molecular level with the genus Nardostachys. Before starting of an extensive molecular taxonomic study, a reliable DNA isolation method is required. The molecular analysis is not so easy because the dried rhizome of *N. Jatamansi* contains high amounts of polyphenols, polysaccharides, protein contents, tannins, hydrocolloids and other secondary metabolites such as alkaloids, terpenes and quinines which would interfere with the DNA isolation procedures. Thus, an efficient protocol for isolation of DNA as well as PCR conditions is required. Current studies indicate that extraction of DNA is not always routine and simple, and conventional methods are not necessarily reproducible for all plant, especially for dry material (Doyle and Doyle, 1987). Hence, this study was aims to standardize the protocol for DNA isolation suitable for dry rhizomes of *N. jatamansi* and utilization of DNA in PCR amplification.

MATERIALS AND METHODS

Plant material & DNA isolation

Dry rhizome of seven accessions of *N. jatamansi* collected from different location of Uttarakhand, India were stored at room temperature and used for study (Table 1). For genomic DNA isolation cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987) with slight modification (Table 2) was used for the present study. Samples were washed, dried and grinded with liquid N_2. Eight ml of pre-chilled DNA extraction buffer (1.0 M Tris-Cl (ph 8.0), 25mM EDTA, 1.5 M NaCl, 2.5 % CTAB, 0.5% β- mercaptoethanol (v/v) and 1% PVP (w/v) were added in centrifuge tube containing 1 g of dried sample. PVP and β-mercaptoethanol were added in extraction buffer immediately before use. Tissue lysate was mixed by gentle shaking for 3 minute and incubated for 2-3 hrs at 65 °C in shaking water bath keeping rpm 100. The mixture was centrifuged at 10,000 rpm for 10 minutes at room temperature and supernatant was transferred in another tube. Two ml of NaCl (5M) was added in supernatant and mixed by gentle shaking and sequentially, 0.7 volume of ice cold isopropanol was added in the supernatant and incubated at -20ºC for 1-2 hrs. Incubated sample were centrifuged at 10,000 rpm for 10 min at 4 °C. Pellets were washed with 70 % ethanol and dried in laminar flow for 30 minutes. Dried pellet was dissolved in TE buffer followed by addition of 5µl of ribonuclease A and kept at 37 °C for 30 minutes. Equal amount of phenol: chloroform was added in the mixture and after gentle shaking mixture was centrifuge at 12,000 rpm for 10 minute at room temperature. Supernatant was taken and 2 volume of chilled ethanol was added and incubated for 1 hrs. The incubated mixture was centrifuged at 12,000 rpm for 10 minutes at 4 °C. Pellet was washed with 70% ethanol and after dry, resuspended in 200 µl Millipore water.

Quantification of genomic DNA

The genomic DNA dissolved in Millipore water was taken for quantification by UV spectrophotometer (Eppendorf Biophotometer, US). Reference was set against ultrapure water and then after thorough rinsing of the quartz cuvette, the absorbance of the sample was measured at 260 nm and 280 nm. The dilution factor and wavelength at which optical density was measured were entered into Biophotometer. The concentration was recorded ranges from 20-50 µg/g. The quality of genomic DNA was also checked on 0.8% agarose gel electrophoresis with ethidium bromide stain and 1 Kb ladder as the DNA size marker (Fig 1).

Table 1: List of different population of *N. jatamansi* with its geographical locations (Singh et al., 2013b)

S. No	Sample Code	Location	District	State/Country
1	PK	Panwali Kantha (3200 m asl)	Tehri Garhwal	Uttarakhand, India
2	JM	Jumla (2562 m asl)	Jumla	Karnali, Nepal
3	KN	Kedarnath (3584 m asl)	Rudraprayag	Uttarakhand, India
4	HKD	Har Ka Doon (3400m asl)	Uttarkashi	Uttarakhand, India
5	TN	Tungnath (3600m asl)	Rudraprayag	Uttarakhand, India
6	MS	Munsiyari (2380 m asl)	Pithoragarh	Uttarakhand, India
7	DR	Dayara (3500 m asl)	Uttarkashi	Uttarakhand, India

Table 2: Variation tried out for the optimization of DNA extraction in *N. jatamansi*

Method	Variation	Results
Doyle and Doyle CTAB method (1987)	Without any modification	Shearing in bands
	Crushing in liquid nitrogen	Reduced shearing
	Addition of PVP in extraction buf fer	Prevent oxidation of DNA
	Increased concentration of CTAB (2.5%) , NaCl (5M) and β- mercaptoethanol	2.5 % CTAB with 5M NaCl provide efficient removal of major polysaccharides and deactivate enzymatic activity
	Extraction with phenol: chloroform an d chloroform	Remove the protein contaminations
	RNase A treatment	Improved RAPD reproducibility

RAPD analysis

Different parameters were detected for optimization of PCR reactions. The extracted DNA (20ng) was used for PCR –RAPD, analysis in 25µl reaction mixture containing 1.0 mM $MgCl_2$, 1.2 mM dNTP's, 2.5 U Red Taq DNA polymerase, 30 ng primers (Bangalore Genei) and 1µl genomic DNA. DNA amplification cycles were performed in a thermal cycler (Eppendorf, USA). After initial cycle of denaturation at 94°C for 5 min., 40 cycles of denaturation at 94°C for 1 min., annealing at 35°C for 1 min., and extension at 72°C for 1.5 min., were provided, followed by a final extension of 72°C for 7 min. Reaction mixture, wherein template DNA replaced with Millipore water were used as negative control. A total of 24 primers were used to check the fidelity of amplification (Table 3). Amplified product were resolved on 1.6% agarose gel (1X TBE) followed by ethedium bromide staining (5µl/100 ml TAE). The results of RAPD were documented using Gel documentation system (Alfa InfoTech, USA).

RESULTS AND DISCUSSION

Isolation of DNA from dry tissue like rhizome is difficult due to high level of polyphenol, polysaccharide, proteins, tannins and other secondary metabolites. DNA extraction was improved by modifying some of the steps in the original CTAB DNA isolation protocol (Doyle and Doyle, 1987). The main problems seen during isolation and purification of DNA from dry rhizome of *N. jatamansi* include degradation of DNA due to endonucleases, co- isolation of highly viscous polysaccharides, inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions (data not shown).

We have tested previously established protocol (Doyle and Doyle, 1987) but this method resulted in DNA with lot of impurities and not very suitable for PCR analysis. Therefore, we reported here a total genomic DNA isolation protocol derived from a method originally developed for other plants. Several modifications were introduced to Doyle and Doyle (1987)-CTAB method for removal of impurities (Table 2). Combination of high concentrations of polyvinylpyrolidone (1%) and sodium chloride (5M) in the extraction buffer along with treatment of Phenol:Chloroform, chloroform alone and final precipitation with cold ethanol proved very effective.

During homogenization, polyphenols released from vacuoles and interact irreversibly with DNA (Porebski et al., 1997). Treatment with polyvinyl pyrolidone, prevent DNA from oxidation. Polysaccharide contaminations are particularly problematic (Scott and Playford, 1996) as they can inhibits the activity of many commonly used molecular biological enzymes, such as polymerases. This is due to nucleic acids form tight complexes with polysaccharides creating a gelatinous pellet and the embedded DNA is inaccessible to the enzymes (Sharma et al., 2002). CTAB and sodium chloride helpes in removal of polysaccharides (Khanuja et al., 1999; Paterson et al., 1993; Murray and Thompson, 1980), while treatment of phenol:chloroform and chloroform removes protein impurities (Chakraborti et al., 2006).

However, in comparison with protein, polysaccharide contamination was more difficult to remove as they remained in trace amount until the end and co-precipitated with the DNA sample in a complex way finally concentrating the nucleic acids to a semi soluble gelatinous pellet, ineffective for any application (Crowley et al., 2003). Several methods on removal of polysaccharides from DNA have been extensively reviewed (Lodhi et al., 1994; Maryam Sarwat et al., 2006; Crowley et al., 2003) of which salt precipitation has been recommended to be most effective. Salts, when used in precipitation increase the solubility of polysaccharides thus preventing its coprecipitation with DNA (Lodhi et al., 1994). Many DNA isolation procedures also yield large amounts of RNA, especially 18S and 25S rRNA (Doyle and Doyle, 1987; Mejjad et al., 1994). Large amounts of RNA in the sample can chelate Mg^{2+} and reduce the yield of the PCR. Treatment by RNase for 30 minutes degraded RNA into small ribonucleosides that do not contaminate the DNA preparation, and yield RNA- free pure DNA. The purity of the extracted DNA was reconfirmed by subjecting the isolated DNA to gel electrophoresis and spectrophotometer. The reproducibility of the protocol was tested in seven different accessions of *N. jatamansi*, wherein the protocol resulted, in all the samples, distinct, sharp and clear DNA bands with yield ranging from 30-50 µg per gram of dry rhizome tissues.

For the optimization of RAPD reaction using DNA, extracted from different accessions of *N. jatamansi*, oligonucleotide primers (Bangalore Genei, India) were used for amplification to standardize the PCR condition. The protocol for PCR-RAPD was optimized by introducing several modifications to the original Williams et al. (1990) protocol in both PCR components such as template DNA, primer, magnesium chloride, Taq DNA polymerase, dNTPs, as well as in amplification cycles including number of cycles, temperature and time intervals for denaturation, annealing and extension steps. The optimized reaction conditions produced clear, scorable amplified products suitable for RAPD applications in all 24 primers tested (Fig. 2). The present study on development of modified protocol for isolation of high purity DNA and optimization of RAPD conditions is the successful report for jatamansi. This will form a strong beginning for future molecular characterization and genetic improvement works in this promising medicinal plant.

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Table 3: Profile of polymorphism among eight accession of *N. Jatamansi* resulting from twenty RAPD Markers (Singh et al., 2013b)

S. No.	Primer name	Total no. of bands	Number of polymorphic bands	Polymorphism (%)
1.	NJ-1	19	17	89.4
2.	NJ-3	18	14	77.7
3.	NJ-4	13	10	76.9
4.	NJ-5	14	11	78.5
5.	NJ-10	12	8	66.6
6.	NJ-11	13	10	76.9
7.	NJ-12	11	5	45.4
8.	NJ-13	17	13	76.4
9.	NJ-14	13	9	69.2
10.	NJ-17	12	10	83.3
11.	NJ-19	5	4	80.0
12.	NJ-20	12	10	83.3
13.	NJ-30	13	11	84.6
14.	NJ-33	10	7	70.0
15.	NJ-34	10	7	70.0
16.	NJ-37	10	8	80.0
17.	NJ-41	18	14	77.7
18.	NJ-42	16	11	68.7
19.	NJ-43	20	16	80.0
20.	NJ-45	21	18	85.7
21.	NJ-46	19	16	84.2
22.	NJ-47	16	12	75.0
23.	NJ-49	20	16	80.0
24.	NJ-53	14	10	71.4

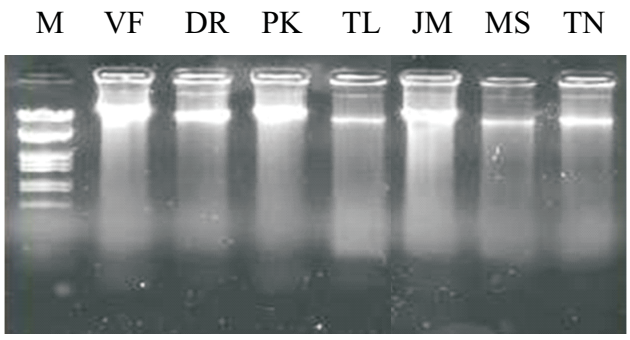

Fig.1. Genomic DNA isolated by modified method from seven accession of *N. jatamansi*

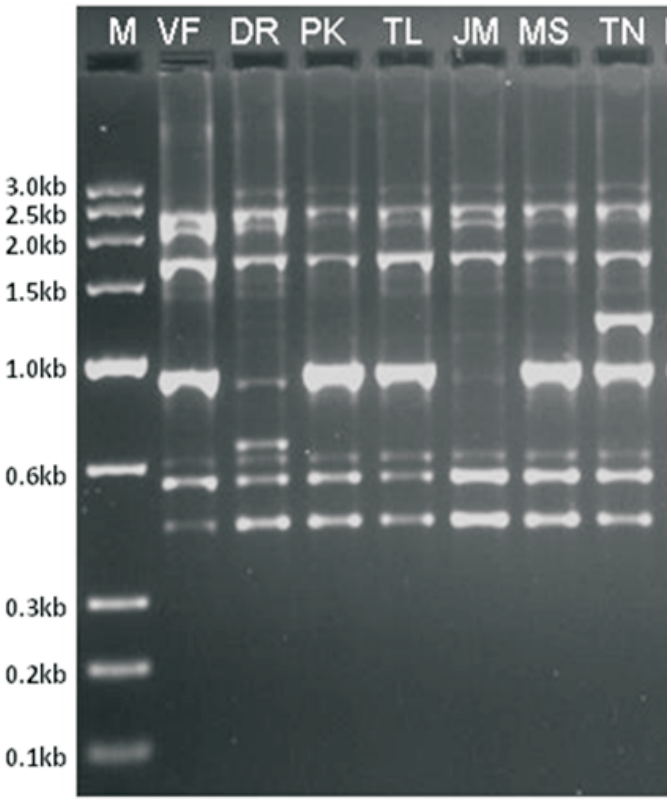

Fig 2: RAPD profile of different accessions of *N. jatamans*i: Marker (M) NJ-12 ; (1Kb) (Singh et al., 2013b)

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