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Full Length Research Paper

Genomic DNA isolation from *Artemisia* species grown in cold desert high altitude of India

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Artemisia **which produces a large number of secondary metabolites is naturally found in cold desert high altitude environment of India. Secondary metabolites such as alkaloids, flavonoids, phenols, polysaccharides and terpenes represent a significant barrier to the extraction of pure genomic DNA. Thus, in this study, the DNA extraction protocol to extract pure genomic DNA from different** ***Artemisia*** **species was tailored. The protocol was based on the CTAB method with slight modifications. In the study, 1.6 M NaCl, 2% cetyltrimethyl ammonium bromide (CTAB), 3% polyvinylpyrrolidone (PVP) and 0.5% β-mercaptoethanol was used in the extraction buffer. The incubation period was kept for 1 h at 65°C with one-tenth of the volume of warm (55°C) 10% CTAB solution during the extraction process. This study described a reliable protocol for extracting good quality and optimum amount of DNA from** ***Artemisia*** **species suitable for PCR analysis.**

Key words: *Artemisia*, genomic DNA isolation, PCR amplification, secondary metabolites.

INTRODUCTION

Artemisia (family Asteraceae) species is a source of highly valuable phytochemicals and essential oils that are used in the treatment of different diseases found naturally growing in Ladakh, the Indian Trans-Himalayan region with an altitude of 2700 to 6100 m amsl. It is situated between latitudes 30° and 36° N and longitudes 76° and 79° E. The region is characterized by harsh and different climatic conditions and very complex soil formation patterns. Annual temperature varies from +40 to -40°C and annual precipitation is less than 100 mm, only in the form of snow along with a relative humidity between 20 to 40%. It is one of the significant and unique places with nominal exploitation of their resources in the world from the point of view of plant genetic resources and plant genetic diversity (Chaurasia et al., 2007).

Artemisia annua is used for the production of antimalarial and possible antibacterial agents as well as serving as a natural pesticide. Artemisinin extracted from the leaves and flowers act as an antimalarial agent against *Plasmodium falciparum* and *Plasmodium vivax* including the multidrug resistant strains (Abdin et al., 2003). *Artemisia tourenefortiana* produces aromatic oil that is used against intestinal worms. Leaves and flowers extracts of *Artemisia gmelinii* are used as medicine for headache, cold, cough and abdominal upsets. As this extracts contain flavonoids, scopoletin, monoterpenes and sesquiterpene, they are also used in the treatment of hepatitis. *Artemisia sieversiana* leaves contain secondary metabolites like sieversinin and siersin which have anti-microbial properties. *Artemisia dracunculus* is commonly known as tarragon and its leaves and flowers extracts are used against toothache and urinary problems, while the roots are useful in treating pharyngitis and lung diseases. They are also used for the treatment of intestinal worms and to relieve toothache (Chaurasia et al., 2007). Most of the *Artemisia* germplasm conserved naturally in cold desert high altitude regions of India are rarely exploited commercially as there is a dearth of references for its genetic variability. Furthermore, DNA extraction from *Artemisia* species is a very difficult task as it is known to be highly rich in polysaccharides and secondary metabolites (Sangwan et al., 1998). Extraction of the best quality DNA and in optimum quantity is the first step in

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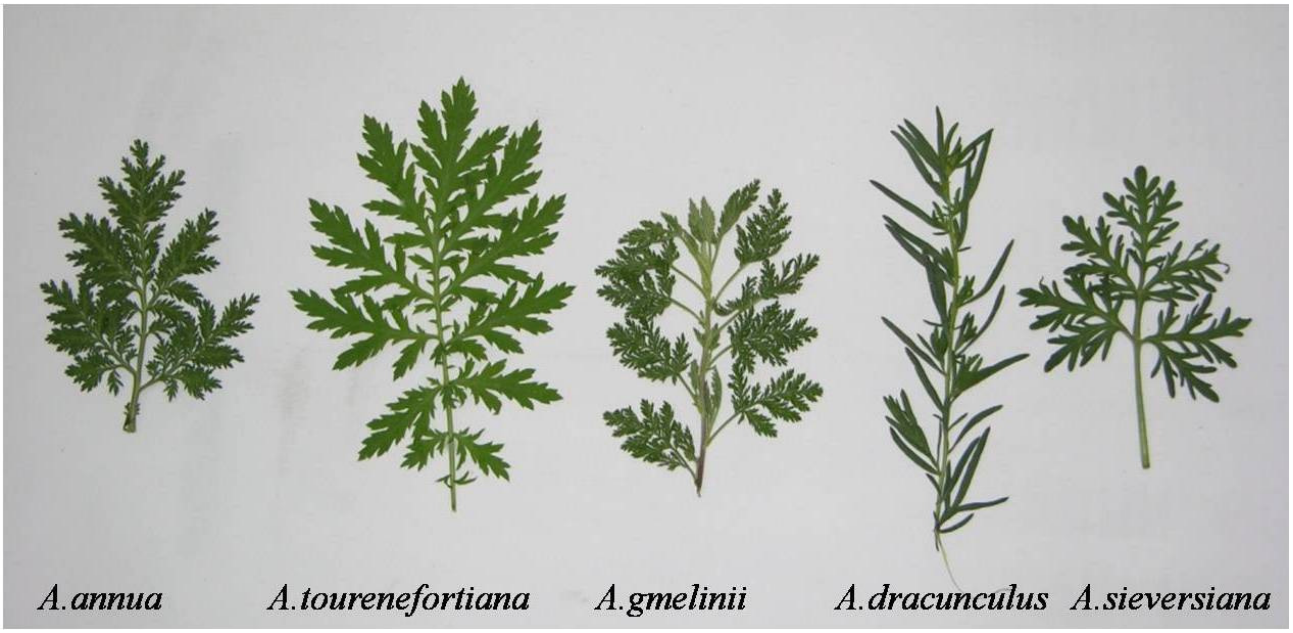

Figure 1. *Artemisia* species selected for the study.

molecular analyses. There are so many DNA purification methods available to enhance the DNA quality. These are CsCl ultracentrifuge method (Sosa and Oliveira, 1992), agarose gel-electrophoresis purification (Saunders, 1993) and hydroxyapatite column purification (Dutcher et al., 1991). Although, these methods improved the DNA quality, they are complex, time consuming and expensive methods. Many protocols are used for DNA isolation but fail to obtain pure genomic DNA from *Artemisia* species (Sayed, 2008) and commercially available genomic DNA extraction kits and solutions (TRI reagent, Sigma) also give poor results. By using one DNA isolation protocol, the optimal DNA yield may not be possible from different plant taxa. For example, some closely related species of the same genus require different isolation protocols (Jena et al., 2010). Therefore, standardization of an efficient protocol for DNA isolation and optimization of the PCR conditions are imperative. Thus, the objective of this study was to modify CTAB protocol and minimize the increased level of interference with secondary metabolites and polysaccharides during the extraction of pure genomic DNA in *Artemisia* species.

MATERIAL AND METHODS

Plant materials

Fresh young leaves samples of *A. annua*, *A. sieversiana*, *A. dracunculus, A. gmelinii* and *A. tourenefortiana* (Figure 1) were collected from different valleys lying at different altitudes in the Ladakh region of India (Table 1). Leaf samples of the plants were brought to the laboratory in icebox and stored in a deep freezer at –20°C for further analysis. The young leaves were taken for DNA extraction.

Reagents and chemicals

These included 10% CTAB (w/v), 1 M Tris-HCl (pH 8.0), 0.5 M EDTA (pH 8.0), 4 M NaCl, 3 M sodium acetate, isopropanol, ethanol (70, 100% AR grade), chloroform-isoamyl alcohol (24:1, v/v), polyvinylpyrrolidone (PVP) (40,000 MW), β-mercaptoethanol and ribonuclease A (10 mg/ml).

Extraction buffer

The buffer contained 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.6 M NaCl, 2% CTAB (w/v), 3% PVP (w/v) and 0.5% β-mercaptoethanol (v/v).

TE buffer

The TE buffer contained 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA (pH 8.0).

Taq DNA polymerase enzyme and nucleotides

This included dNTP mix (A T G C), PCR buffer A (Bangalore Genei, India) and RAPD primers (Integrated DNA Technologies, USA).

DNA isolation protocol

The harvested leaves were rinsed with autoclaved distilled water and blot dried. Leaves (2 g) were crushed into a pre-chilled mortar and pestle to a fine powder in liquid nitrogen. Using 3 volumes of freshly prepared extraction buffer, the extract was incubated at 65°C for 1 h with intermittent mixing. The tubes were cooled at room temperature. Equal volume of chloroform : isoamyl alcohol (24:1) was added followed by invert mixing. The content was centrifuged at 11000 rpm for 20 min and supernatant was collected carefully. The one-tenth volume of warm (55°C) 10% CTAB solution

Table 1. Sampling sites of different *Artemisia* species located in different valleys of Ladakh.

S/N	*Artemisia* sp.	Location and altitude (m, amsl)
1	*A. gmelinii*	Zanskar Valley (5091), Leh Valley (3450)
2	*A. annua*	Leh Valley (3400), Nubra Valley (3050)
3	*A. dracunculus*	Suru Valley (3850), Khaltse (3350)
4	*A. tourenefortiana*	Leh Valley (3450)
5	*A. sieversiana*	Leh valley (3400), Nubra valley (3050)

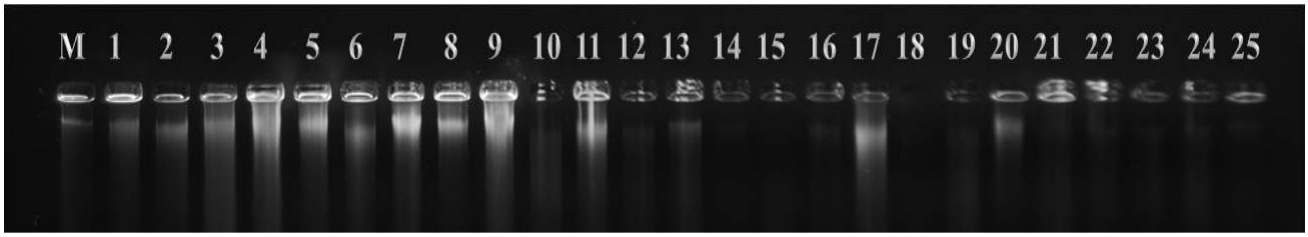

Figure 2. Agrose gel electrophoresis showing the genomic DNA of *Artemisia species* before the modification was resolved on 0.8% agarose gel. Lane 1, 100 ng uncut λ DNA; Lanes 2 to 6, *A. gmelinii* genomic DNA; lanes 7 to 11 *A. annua*; lanes 12 to 16, *A. dracunculus*; lanes 17 to 21, *A. sieversiana*; lanes 22 to 26, *A. tourenefortiana*.

was added with chloroform-isoamyl alcohol and equal volume of chilled isopropanol. The precipitate was collected as pellets after centrifugation. The supernatant was discarded and the pellet was washed with 70% ethanol followed by drying of the pellet at 30°C. The pellet was dissolved in double distilled water. Ribonuclease A 10 µg/100 µl (v/v) was added and the content was incubated at 37°C for 1 h and then equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) was added and mixed followed by centrifugation at 10,000 rpm for 5 min at room temperature. The aqueous phase was separated into a fresh tube and extracted in equal volume of chloroform : isoamyl alcohol (24:1) and the upper aqueous phase was collected and extracted again in chloroform : isoamyl alcohol (24:1) with repeated centrifugation. The separated upper aqueous phase was mixed with one-tenth volume of 3 M sodium acetate and DNA was precipitated by adding two volumes of chilled absolute alcohol and was pelleted by centrifugation at 5000 rpm for 3 min. The pellet was dried in vacuum and dissolved in double distilled water. Primarily, the analysis of genomic DNA banding pattern was analysed using agarose gel electrophoresis and was compared with standard uncut lambda DNA (100 µg MW) (Figure 2).

PCR amplification

Genomic DNA of *Artemisia* species were analysed on 0.8% agarose gel at 70 V for 1 h and stained with ethidium bromide. PCR reaction were conducted in 25 µl of a mixture containing 50 ng of genomic DNA, 2.5 µl of 10 x PCR buffer, 0.2 mM dNTPs, 2 µM primers and 1 unit of *Taq* DNA polymerase. Errors were minimized by making master mix of all the reagents, except template DNA for each primer. The Biometra Gradient Thermal Cycler was programmed as follows: An initial cycle of 3 min at 94°C, followed by 40 cycles each consisting of 45 s at 94°C, 30 s at 35°C, 1 min at 72°C and final extension of 5 min at 72°C. PCR products were stored at 4°C before analysis. Amplified PCR products were separated on 1.5% (w/v) agarose gel in Tris–acetate–EDTA (TAE) buffer containing 0.5 µg/ml ethidium bromide (Figure 4). The PCR products were visualized under UV light by Gel Documentation System (Alfa Innotech). The 100 bp ladder was used as a molecular weight size marker.

RESULTS AND DISCUSSION

The agarose gel electrophoresis separated high molecular weight *Artemisia* genomic DNA of the different genotypes (Figure 3) and ~200 ng/µl quantity of the genomic DNA was obtained. The electrophoresis of the different genotypes showed high molecular weight band without smearing and fragmentation. It was also found that the age and quality of the plant material were influenced by PCR amplification.

In the plants grown at the cold desert high altitude environment, having medicinal and aromatic importance, difficulties were encountered from the stage of cell lysis to DNA separation and subsequent reactions when the procedures described by Doyle and Doyle (1987), Murray and Thompson (1980), Dellaporta et al. (1983) and Porebski et al. (1997) were followed. Major problems encountered were low DNA yield, poor PCR amplification reactions and restriction endonuclease digestion for hybridization based DNA fingerprinting. The plants contain high amounts of polysaccharides, polyphenols and other secondary metabolites that are entangled to nucleic acid during DNA isolation which interfere with subsequent isolation procedure (Zidani et al., 2005). The secondary compounds may hamper DNA isolation as well as any further reaction to be carried out on DNA preparations for example; restriction enzymes may be inhibited in the presence of unusual substances. Sometimes, the contaminants were not detectable but were sufficiently high to interfere in the analysis of polymorphisms. Therefore, in this attempt, the amount of β-mercaptoethanol and PVP in the extraction buffer and centrifugation time that removed polysaccharides, high essential oils and polyphenolic content, were modified.

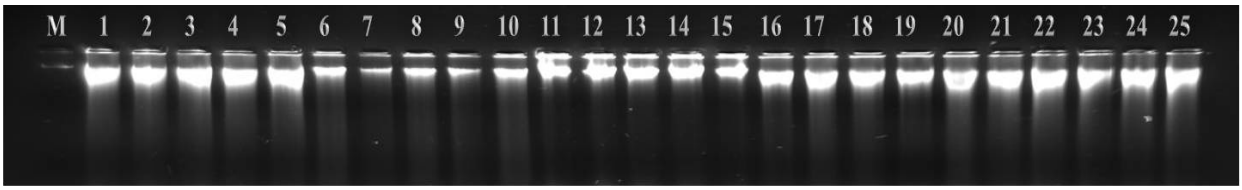

Figure 3. Agrose gel electrophoresis showing the purified high MW genomic DNA of *Artemisia* species. More than 200 ng of genomic DNA from each species was resolved on 0.8% agarose gel. Lane 1, 100 ng uncut λ DNA; lanes 2 to 6, *A. gmelinii* genomic DNA; lanes 7 to 11, *A. annua*; lanes 12 to 16, *A. dracunculus*; lanes 17 to 21, *A. sieversiana*, lanes 22 to 26, *A. tourenefortiana*.

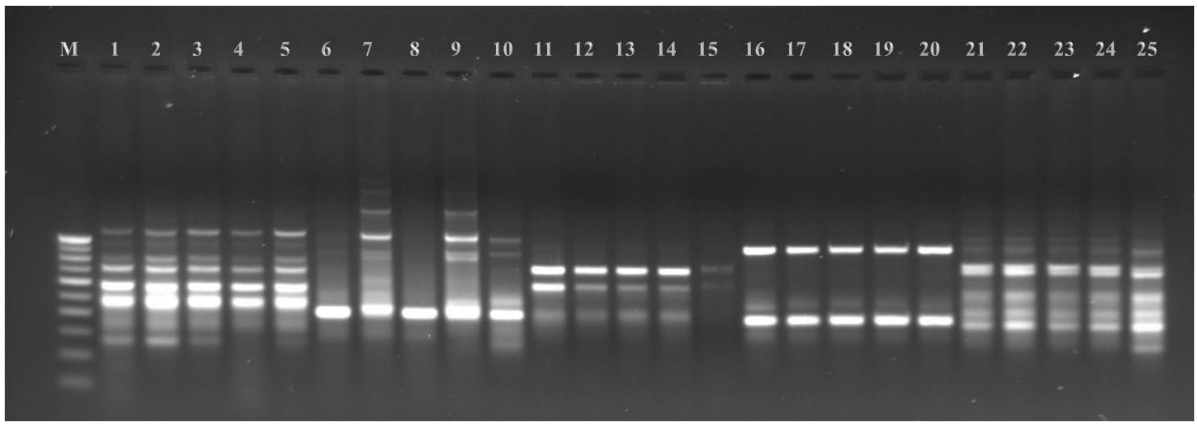

Figure 4. RAPD fingerprints generated for the different *Artemisia* sp. using S-21 primer. Amplified products were resolved on 1.5% agarose gel and were detected by ethidium bromide staining.

PVP formed a complex with polyphenolics through hydrogen bonding and thus helped in the removal of polyphenolics from the homogenate. The concentration of β-mercaptoethanol avoided polyphenol oxidation. Finally, good yield of DNA for PCR based assays and other applications was obtained (Figure 4). Young leaves contain comparatively low levels of polysaccharides, polyphenols and other secondary metabolites than the mature leaves (Zhang and Stewart, 2000).

Conclusion

The modified protocol proved very efficient and reliable for the isolation of pure genomic DNA from the leaves of different *Artemisia* species which contain large amounts of secondary metabolites and polysaccharides. The collecting conditions and preservation of samples which are important for the quality of DNA (Ribeiro and Lovato, 2007), were also substantiated in this study. The storage treatments where the samples were either frozen or rapidly dried with silica gel gave higher DNA yields of better quality than oven dried or air-dried leaves. Therefore, in the *Artemisia* species, the methods of storage after leaf sample collection and the type of DNA extraction reagents had a major influence on the DNA quality and on the success of the molecular studies.

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