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Polyphenolics Free DNA Isolation from Different Types of Tissues of *Aconitum heterophyllum* Wall-endangered Medicinal Species

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Abstract: The present study is aimed to isolate DNA from three types of tissues of *Aconitum heterophyllum* Wall using previously reported methods after modifications. *Aconitum heterophyllum* Wall is a vital and critically endangered herb, which needs to have its fingerprinting data for its shrewd use. Use of DNA markers with the techniques like Polymerase Chain Reaction (PCR) and molecular cloning can provide every minute detail about the subject sample. Good quality DNA is always a prerequisite in all molecular biology experiments especially PCR. As this plant contains high amounts of secondary metabolites that interfere with DNA isolation. The two methodologies described in the present study yielded good-quality, high-molecular weight DNA that is free of contaminants and colored pigments and is suitable for PCR amplification.

Key words: *Aconitum heterophyllum* Wall, DNA isolation, polyphenols, PCR, tissue culture

INTRODUCTION

The demand for medicinal plant species has been increased globally due to the resurgence of interest in herbal medicine due to which high value medicinal plant species are threatened their status ranging from low risk, near threatened to critically endanger. *Aconitum heterophyllum* Wall (Ranunculaceae), commonly known as Atis, occurring in alpine and sub-alpine regions of temperate Himalayas, is reputed for its medicinal and pharmaceutical value since long. The alkaloids of the tuberous roots of the *Aconitum* have been reported to possess significant antipyretic and analgesic properties and a high therapeutic index (Jabeen *et al*., 2006). The alkaloids from the roots of *A. heterophyllum* have been reported to have considerable antibacterial and enzyme inhibition activities (Ahmad *et al*., 2008: Nisar *et al*., 2009). The large scale demand of this medicinal herb in herbal pharmaceutical industries has attracted its market and collectors to extract it unsustainably as well as illegally making it to be identified as critically endangered (Nayar and Sastry, 1987; IUCN, 1993; Nautiyal *et al*., 2002; CAMP, 2003). In recent years

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tissue culture techniques has been emerged as a key component in the conservation and mass multiplication of these plants. The only shortcoming of the technique is there may be a chance of genetic variation in the clonally propagated plants due to stress imposed by tissue culture components. The best way to overcome this situation is use of DNA based molecular marker technology to confirm the clonal fidelity of the stock. Molecular marker technology is important in spices, medicinal and aromatic plants for identifying varieties/accessions, germplasm organization, identifying market samples and gene cloning. As *A. heterophyllum* is conserved through *in-vitro* propagation (Giri *et al*., 1993; Jabeen *et al*., 2006) and also it is frequently adulterated by *A. kashmiricum* (Shah, 2005), its authentication through DNA based markers is the need of the time. Thus isolating pure DNA of high molecular weight is essential. The isolation of pure nucleic acids from plants presents special challenges. The methods used for growing, harvesting, storing and preparing plant tissue can influence subsequent nucleic acid purification. DNA based molecular markers have proved their utility in fields like taxonomy, physiology and embryology. For genetic and molecular studies there is a prerequisite of good quality DNA.

A. heterophyllum Wall is considered to be difficult plant for DNA isolation due to its high polyphenolic content, which may interfere with the DNA purity especially for subsequent manipulations. Several plant metabolites have chemical properties similar to those of nucleic acids, making contaminating metabolites and are difficult to remove from nucleic acid preparation (Bench Guide). Some common problems associated with contamination of nucleic acids prepared from plants are inaccurate spectrophotometric quantification, siltered electrophoretic mobility, degradation during storage and inhibition of enzymatic and PCR reactions (restriction digestion, reverse transcription, DNA amplification). To overcome these difficulties, we modified available DNA isolation methods. Till date no efficient protocol is available for the isolation of DNA from *A. heterophyllum* Wall and the present method yields good quality DNA that is free of contaminants and pigments and can be amplified by means of PCR.

MATERIALS AND METHODS

Plant Material

The present study is the part of project funded by Uttarakhand Council of Science and Technology (UCOST) carried out at Sardar Bhagwan Singh P.G. Institute of Biomedical Sciences and Research, Balawala, Dehradun from 01-01-2008 till 01-01-2011. Fresh leaves of *A. heterophyllum* Wall were collected from Forest Nursery, Milam Village, Munsiyari (alt-2290 m, Lat.-30°13'12", Long.-80 °25'12"), Uttarakhand, India. The tissues were brought to the laboratory in an icebox and stored at -80° C (New Brunswick) until further analysis. Leaves from tissue culture raised plants on full strength MS medium (Murashige and Skoog, 1962) supplemented with 0.25 Kinetin for six months were also used for DNA isolation. Seeds of *A. heterophyllum* Wall (IC-567646, NBPGR, Pusa Campus, New Delhi) were also used for DNA isolation.

Reagents and Chemicals

- Extraction buffer (autoclave): 2% Hexadecyltrimethyl-ammonium bromide (CTAB) (w/v), 100 mM Tris-HCl (pH 8), 20 mM EDTA (pH 8), 1.4 M NaCl:
    - 15 mM Ascorbic acid add 3% polyvinylpyrrolidone (PVP) (w/w) in case of leaf tissue and 20 mM Ascorbic acid add 3.5% polyvinylpyrrolidone (PVP) (w/w) added in the mortar while crushing the tissue

Three percent β-mercaptoethanol (v/v) was added in the extraction buffer immediately before preheating

- Chloroform-isoamyl alcohol (IAA) (24:1 v/v)
- Isopropanol, -20°C
- 80% ethanol, -20°C
- 76% ethanol -20°C
- TE buffer: 1 M Tris-Cl buffer (pH 8.0), 1 mM EDTA (pH 8.0)

DNA Isolation
Methodology I

- Preheat 1.5 mL CTAB isolation buffer containing 3% β-mercaptoethanol (v/v) in a 2 mL microtube at 60°C in a water bath
- Grind leaf tissue/seeds (500 mg) into fine powder by adding liquid nitrogen (-196°C) and PVP in a chilled mortar pestle and transfer it into preheated extraction buffer
- Incubate the contents at 65°C for 30 min with intermittent shaking. Check it for the lysis of tissue
- After complete lysis of the tissue, add 500 μL volume of chloroform-IAA (24:1) to the microtube and emulsify the contents at 25°C for 10 min
- Centrifuge at 13000 rpm for 10 min at 4°C to pellet the debris
- Carefully pipette out the clear aqueous phase out of three layers obtained and transfer it to a fresh 1.5 mL microtube
- Precipitate DNA from aqueous layer by adding equal volume of chilled iso-propanol (-20°C) and incubate at 4°C for 1½ h (to avoid binding of polyphenols to the precipitated DNA)
- As soon as the threads get precipitated loop out the DNA threads with the sterilized round head pin in a fresh microtube
- Wash it with 80% ethanol (400 μL) and centrifuge at 10000 rpm for 5 min at 4°C
- Again wash the pellet with 76% ethanol (400 μL) and centrifuge at 10000 rpm for 5 min at 4°C
- Air-dry the pellet. Suspend the pellet in 100 μL TE buffer
- Store at 4°C or -20°C for further use

Methodology II

- Perform the step 1-12 of methodology I
- Add 400 μL of chloroform-IAA (24:1) and gently inversion of contents is performed. Centrifuge at 10000 rpm for 10 min at 4°C
- Carefully pipette out the clear aqueous layer and transfer it to a fresh microtube. Precipitate DNA from aqueous layer by adding equal volume of chilled iso-propanol (-20°C) and incubate at 4°C for 12-24 h
- Centrifuge at 12000 rpm for 10 min at 4°C. Discard the supernatant
- Wash the pellet with 76% ethanol (400 μL) and centrifuge at 10000 rpm for 5 min at 4°C. Air-dry the pellet
- Suspend the pellet in 50 μL TE buffer. Store at 4°C or -20°C for further use
- Visualise DNA samples on 0.8% (w/v) agarose gel and quantify the samples in Biophotometer (eppendorf)

Some important instructions are to be followed during the isolation process. It should be properly taken care that the powder should be fine and not allowed to be thaw at any point before adding the isolation buffer. By keeping the temperature below 0°C, the oxidizing enzymes are inactivated during this step. The microtube should not be shaken vigorously during any step because the DNA is very vulnerable to fragmentation. Pellet should not be dried excessively because it poses difficulty to dissolve.

RESULTS AND DISCUSSION

The success of biotechnological tools such as molecular markers and genetic engineering are critically dependent on development of reliable protocol for isolating superior quality DNA and hence PCR analysis. It has been widely reported in many plant species such as chickpea (Chakroborthy *et al*., 2006), *Pinus radiate* (Stange *et al*., 1998) etc. We successfully isolated DNA from field grown *A. heterophyllum* Wall, tissue cultured plants and seeds using the methodologies described above. High quality PCR compatible DNA was isolated from *A. heterophyllum* Wall using Methodology I with each kind of sample (Fig. 1a), while methodology 2 failed to produce usable DNA from the first two sources but yielded good quality DNA with seed samples (Fig. 1b). Methodology II can be considered preferable for field grown plants, but the DNA yield was low. The yields of the DNA isolated from each sample using two methodologies have been listed in Table 1. The yield of the DNA from all the three samples was found be best with Methodology I when compared with Methodology II and to that of Doyle and Doyle (1987). Although, we considered 2 extractions adequate, the extractions may be continued if a low amount of DNA is required, as in our experience, the quantity of DNA decreases with the number of extractions performed. *A. heterophyllum* Wall contains several polyphenolic component and also interfere in the *in vitro* propagation (Giri *et al*., 1993). When DNA isolation methods described by Doyle and Doyle (1987) and Murray and Thompson (1980) were followed, DNA of desired quality and quantity could not be isolated. Taking clue from the initial steps the methodology was modified. Polyphenols and polysaccharides bind firmly to nucleic acids during DNA isolation and interfere with subsequent reactions. So we decreased the time of

Table 1: Yield of DNA isolated from different types of samples of *A. heterophyllum* Wall using two methodologies

	Yield of the DNA samples (ng μL^{-1})		
Types of samples *A. heterophyllum* Wall	Method I	Method II	Doyle and Doyle 1987
Leaves from field grown plants	957.33±1.52	633±3.6	447.66±7.57
Seed samples	751±1.73	621±5.56	223±11
Leaves from tissue culture raised plants	934.66±4.04	556.33±3.05	325±5.56

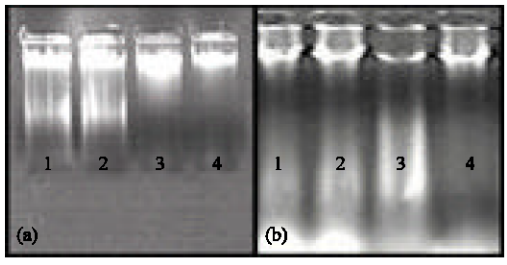

Fig. 1: DNA isolated by (a) Methodology I and (b) Methodology II. Lane 1: DNA from field grown plants; Lane 2: DNA from seeds Lane 3-4: DNA from tissue culture raised plants

incubation with Iso-propanol and soon as DNA get precipitated, they are looped out quickly as longer incubation may allow binding of remaining polyphenolic content. A number of methods are available and are being developed for the isolation of nucleic acids from plants. Modified CTAB method for DNA extraction has also been found suitable by Nazhad and Solouki (2008). Because plants contain high amounts of many different substances, it is unlikely that just one nucleic acid isolation method suitable for all plants can ever exist (Loomis, 1974). It has been seen that CTAB and sodium chloride helped removal of majority of polysaccharides (Murray and Thompson, 1980; Paterson *et al*., 1993; Khanuja *et al*., 1999). *Aconitum heterophyllum* Wall is endangered medicinal plant of Himalayas, so different conservation programmes (cultivation and micropropagation) are being used but authentication of these protocols are necessary which can be done through DNA based markers. Although, protocols for PCR (RFLP/RAPD/ SSR/ISSR) have been reported for some other species of the genus Aconitum (Fico *et al*., 2003; Mitka *et al*., 2007; Cadre Le *et al*., 2005), however, no appropriate methodology has been reported for DNA isolation from *Aconitum heterophyllum* Wall. As polyphenolics and other inhibitors are the main hindrance in the molecular biology experiments, using present method their level can be reduced considerably. Also, complete removal of these components is desirable due to their interference problems that include failure of DNA amplifications during PCR due to inhibition of *Taq* polymerase activity (Fang *et al*., 1992), inhibition of DNA modifying enzymes and in the quantitation process involving spectrophotometers (Wikie *et al*., 1993). In the present study each step of the DNA isolation protocol was monitored by precipitating DNA and running it on agarose gel. Adding high concentrations of PVP and β-mercaptoethanol was helpful to remove tannins and other polyphenolics from the tissues. Our protocol consistently produced a good yield of high-quality DNA. This work represents an efficient method that does not require high time ultracentrifugation to isolate DNA from medicinal and aromatic plants. Because we found the methods described in this paper functional for plants for which DNA isolation was difficult, we believe that these methods will be of help for molecular biological studies of many other aromatic and medicinal plants.

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