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Research Article

Genomic DNA Extraction Protocol for *Artemisia Annua* L. Without Using Liquid Nitrogen and Phenol

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Abstract

Artemisia annua is an important medicinal plant, used for curing various diseases especially malaria. It secretes verities of secondary metabolites which hinders in the DNA extraction. This investigation describes an efficient DNA extraction protocol for *A. annua* based upon the Cetyl Trimethyl Ammonium Bromide (CTAB) extraction method without using hazardous chemicals i.e. liquid nitrogen and phenol. The developed protocol is simple, reliable and operative in normal laboratory condition yielding intact DNA having good quantity (502.7 to 1288.5 ng/µl) and quality ($A_{260/280}$ ratio - 1.82 to 1.85) in two working days. Proper amplification of extracted DNA indicates its suitability for the various molecular biological applications.

Keywords: Cetyl Trimethyl Ammonium Bromide (CTAB); DNA Extraction; *Artemisia annua;* artemisinin; Malaria

Introduction

Artemisia anuua L. is a traditional Chinese medicinal plant well known for its bio-active compound artemisinin–an antimalarial drug. Artemisinin based Combination Therapy (ACT) is most commonly used for the curing of malaria including cerebral malaria (WHO, 2015), but low and fickle production of artemisinin does not justify the total requirement of ACT worldwide (WHO, 2011). Emphases are being made to enhance the economic commercial production of artemisinin and the molecular techniques will play a major role. Good quality, quantity and intact DNA extraction is very crucial for PCR based applications, cloning, sequencing and other molecular research. Plants which synthesize and accumulate a large numbers of bio-active compounds like *A. annua* (Bhakuni *et al.*, 2001) their DNA extraction became a tedious process for the molecular biologist. Presence of polysaccharides in the extracted DNA shown to inhibit the Taq polymerase activity while the oxidized form of polyphenols covalently binds to DNA giving a brown colour and reduces maintenance time, making it useless for molecular studies (Katterman and Shattuck, 1983; Fang *et al.*, 1992).

Although there are many DNA extraction protocol for *A. annua* has been developed by using liquid nitrogen and/or phenol (Kumar *et al.*, 2011; Lakshmi *et al.*, 2012). Sangwan *et al.*, (1998) isolated The DNA form *A. annua* by using CTAB method that includes a rapid micro-column chromatography through DE-52 ion- exchange resin. Many researchers also used DNA extraction kits for the DNA extraction which is less time consuming and eliminate the use of toxic chemicals, however they used liquid nitrogen. But there is a need for development of DNA extraction method for normal laboratory conditions, which avoid the use of liquid nitrogen and phenols. Keeping these views, the present investigation was intent to develop a simple, efficient genomic DNA extraction protocol for *A. annua* without using liquid nitrogen and phenol.

Material and Methods

Fresh, young and disease free leaves of *A. annua* variety Jeevanraksha (Kumar *et al.*, 1999) was taken from the medicinal plants collection of Prof. T. S. Murthy Science and Technology Station, MP Council of Science and Technology, Obaidullaganj, Raisen, Madhya Pradesh, India. Leaves were washed in a series of Millipore water, 70% ethanol and again two times in Millipore water. Fresh weight of the leaves was taken and they were chopped into fine pieces and subjected to genomic DNA isolation with following steps.

1. The chopped leaves (500 mg) were ground into pre-chilled mortar and pestle (at refrigerator for 1 hour) and homogenized with 700 µl of pre warmed (at 65ºC) extraction buffer (Haque *et al.*, 2008) consisting, 150 mM Tris Buffer (pH 8.0), 25 mM EDTA (pH 8.0), 1.5 M NaCl, 3.5% CTAB, 3% PVP and 0.3% β-mercaptoethanol.
2. The suspension was collected in 1.5 ml Eppendorf tubes and mixed with 5 µl of RNAse (stock 20 mg/ml).
3. The Eppendorf tubes were incubated for 1 hour at 65°C in water bath with 2-3 times gentle inversions.
4. Equal amount of chloroform: isoamyl alcohol (24:1) was added into the room temperature (RT) cooled eppendorf tube, missed gently mixed to form a emulsion and centrifuged at 10000 rpm for 10 minutes at RT.
5. The upper aqueous layer was taken and the step 4 (treatment of chloroform: isoamyl alcohol) was repeated again.
6. Isopropyl alcohol (0.6 volumes) was added into the upper aqueous layer, mixed well by gentle inversion and incubated at -20 °C for 30 minutes.
7. The tubes were centrifuged at 10000 rpm for 10 minutes at 5 ^{0}C and pellet was washed with 70% ethanol.
8. The pellet was air dried, dissolved in 500 µl of TE buffer (10 mM Tris buffer, pH 8.0 and 0.1mM EDTA, pH 8.0) and stored at 4 °C for overnight. (First day extracted DNA)
9. On the subsequent day, DNA dissolved in TE buffer was treated with equal volume of chloroform: isoamyl alcohol (24:1) added into the Eppendorf tube and centrifuged at 10000 rpm for 10 minutes at RT.
10. Isopropyl alcohol (0.6 volumes) was added into the supernatant aqueous layer, incubated at -20 °C for 30 minutes and centrifuged at 10000 rpm for 10 minutes at 5 °C.
11. The pellets were washed with 70% alcohol, air dried pellet was dissolved in 50 µl of TE buffer and stored at 4 °C for overnight (Second day extracted DNA).

Quantification of first and second day extracted DNA was performed in a Nanodrop spectrophotometer at 260 nm and the purity was checked from A_{260}/A_{280} ratio. The DNA quality was also tested for PCR amplification with using EST-SSR primer (AAESP19) derived from EST sequence of *A. annua*. PCR reaction mixture (20 µl) includes of 1X Red Taq PCR mix (Sigma), 1 µl of forward and reverse primers (20 pm), 1 µl of genomic DNA (25 ng) and DNAse, RNAse free water and following PCR conditions of Agrawal, (2013) i.e. 3 min at 94 °C; 10 cycles of 30 sec at 94 °C, 30 sec at 60 °C minus 0.5 °C/cycle, 30 sec at 72 °C; 25 cycles of 30 sec at 94 °C, 30 sec at 55 °C, 45 sec at 72 °C; and 7 min at 72 °C for final extension.The PCR products were separated electrophoretically on 3 % agarose gel (with 8 µl/100 ml EtBr, in 1 X TAE buffer). The EST-SSR bands on gel were visualized in gel documentation system (Alfa Inotech) under UV light.

Results and Discussion

In the present study, genomic DNA of *A. annua* was isolated without using liquid nitrogen and phenol in two subsequent days. The first day extracted DNA was brown in colour having DNA concentration from 742.6 to 1556.9 ng/µl with A_{260}/A_{280} ratio of 0.83 to 0.95, while the DNA concentration of second day extracted DNA was varied from 502.7 ng/µl to 1288.5 ng/µl with A_{260}/A_{280} ratio of 1.82 to 1.85 (Table 1). A ratio of A_{260}/A_{280} is used to assess the purity of nucleic acid. A_{260}/A_{280} ratios of good quality DNA are commonly ~1.8 and ratio below this indicates the contamination of chemical constituents (Thermo Scientific). Thus the first day extracted DNA had contaminants of proteins, polysaccharides and other secondary metabolites, which was eliminated in second day by the another treatment of chloroform: isoamyl alcohol (24:1). Although The DNA concentration of first day extracted DNA was higher than second day extracted DNA, might because of residual chemical contamination present in the first day extracted DNA which result in the overestimation of DNA concentration (Thermo Scientific) or loss of DNA along with chemical contamination during the treatment of chloroform: isoamyl alcohol (24:1) on second day.

Table 1: Quantity and quality of isolated genomic DNA of *A. annua*

Genomic DNA	First Day		Second Day	
	DNA yield (ng/µl)	$A_{260/280}$ ratio	DNA yield (ng/µl)	$A_{260/280}$ ratio
AAD1	1556.9	0.95	1288.5	1.85
AAD2	824.6	0.87	528.9	1.84
AAD3	798.2	0.89	527.8	1.83
AAD4	742.6	0.85	502.7	1.82
AAD5	974.5	0.83	699.4	1.84

AAD = *Artemisia annua* DNA

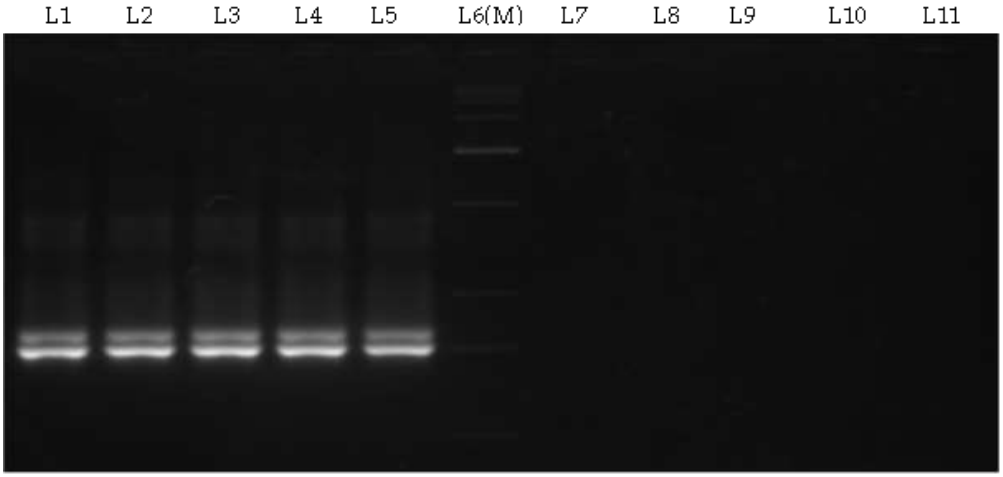

Fig.1: PCR amplification of isolated genomic DNA of *A. annua* by EST-SSR primer (AAESP19) in 3% agarose gel. (AAESP – *Artemisia annua* EST-SSR primer). Lane 1 to 5 (L1 to L5)-Second Day extracted DNA sample AAD1 to AAD5; Lane 6 (L6M)-Low Range DNA Ruler (3000, 2500, 2000, 1500, 1000, 600, 300, 200 and 100 bp); Lane 7 to 11 (L7 to L11)-First Day extracted DNA sample AAD1 to AAD5.

Use of liquid nitrogen and phenol is very crucial in the DNA extraction procedure. Liquid nitrogen is used for grinding plant tissue to break down the cell wall of plants, while phenol used for the elimination of protein contaminants from the genomic DNA. However both the chemicals are highly hazardous in nature and thus require proper handling during the procedure, which may be not possible in the normal laboratory conditions. Therefore, efforts have been made for the isolation of genomic DNA without using these chemicals, which leads into the development of DNA extraction protocols for plants avoiding the use of liquid nitrogen and/or phenol (Sharma *et al.*, 2003, 2010; Biswas and Biswas, 2011; Ferdous *et al.*, 2012; Sahu *et al.*, 2012; Sairkar *el al.*, 2013). As for as *A. annua* plant, there are many DNA extraction protocols had been developed, although they use either liquid nitrogen or phenol or both (Kumar *et al.*, 2011; Lakshmi *et al.*, 2012). In the present investigation, genomic DNA of *A. annua* was isolated without using liquid nitrogen and phenol and yielded good quantity and quality DNA.

The extracted DNA was further checked for its proper PCR amplification through EST-SSR primer (AAESP19). The first day extracted DNA did not amplified, while the second day extracted DNA give proper amplification (Fig. 1). This confirms that the second day extracted DNA was good enough to be used for the molecular biological experiments of *A. annua*.

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