DNA isolation from fresh leaf tissue of *Tylophora indica* and *Bacopa monnieri*

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Abstract

Tylophora indica and *Bacopa monnieri* are two important medicinal plants containing several secondary metabolites of pharmaceutical value. To understand the biosynthetic pathways of these metabolites, molecular studies will be helpful. A prerequisite for these studies is good quality DNA that is free of contaminants. A number of DNA extraction protocols are available, but many of them require expensive and harmful chemicals. Thus, a protocol was developed to isolate genomic DNA from fresh leaf tissue of these plant species using minimum chemicals. The quantity of the isolated DNA was found to be 6200 μg mL^{-1} and 4925 μg mL^{-1} for *T. indica* and *B. monnieri,* respectively, with their $A_{260/280}$ ratios ~1.80. For both, the sample $A_{260/230}$ ratios were ~2.0 and $A_{260/270}$ ratios were ~1.2. Spectroscopic analysis, agarose gel electrophoresis and polymerase chain reaction revealed that the isolated DNA was suitable for carrying out genomic studies.

Key words: *Bacopa monnieri*, DNA extraction, *Tylophora indica*.
Abbreviations: **A**FLP, amplified fragment length polymorphism; CTAB, cetyl trimethyl ammonium bromide; dNTP, dinucleotide triphosphate; EDTA, ethylenediamine tetraacetate; EtBr, ethidium bromide; NFW, nuclease-free water, PCR, polymerase chain reaction; PVP, polyvinyl pyrollidone; RAPD, random amplified polymorphic DNA; TAE, Tris acetic acid EDTA.

Introduction

Tylophora indica (Burm. F.) Merrill (Asclepiadaceae) and *Bacopa monnieri* (L.) Wettst (Scrophulariaceae) are important medicinal plants of India. They contain a variety of secon-dary metabolites, which are used in several formulations. A prerequisite to carry genomic studies with, for example, random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP), is good quality of the DNA free of contaminants. The development of rapid and cost-effective genomic DNA extraction protocols is very important, as the quality of template DNA influences the success of subsequent downstream experiments (Wang et al. 2011). In the case of plants the extraction of genomic DNA has a number of obstacles due to interfering substances like secondary metabolites, polyphenols, polysaccharides and other organic compounds (Porebski et al. 1997; Varma et al. 2007). They affect the quality of the DNA sample and also inhibit enzymatic reactions, such as polymerase chain reaction (PCR) when DNA is used as a template (Moore, Dowhan 2012).

In the present study, a cetyltrimethyl ammonium bromide (CTAB) based extraction procedure was used but it differed from other methods by eliminating the use of polyvinyl pyrollidone (PVP), ribonuclease, proteinase K, phenol, ammonium acetate or any other salts. In the case of plant material usually liquid nitrogen is used for isolation (Sharma et al. 2003) but it is not easily available and hence in our procedure the DNA was isolated directly from fresh leaf tissue. A simple genomic DNA extraction protocol for two different plant species is described below, which is cost-effective and also found to be suitable for performing experiments like PCR.

Materials and methods

Healthy leaves (1 g) of *T. indica* and *B. monnieri* were collected from the Botanical garden of the M.S. University of Baroda, Vadodara, India and used immediately for DNA extraction.

For genomic DNA isolation and its analysis, the following chemicals were used: CTAB isolation buffer (2% CTAB, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris HCl), chloroform:isoamylalcohol (24:1), isopropanol, ethanol (85 and 75%), 1X Tris EDTA buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.4), 50X Tris acetic acid EDTA buffer (24.2 g Tris base, 5.71 mL glacial acetic acid, 0.5 M EDTA, pH 8.0), agarose, ethidium bromide (EtBr), and Bromophenol blue.

For PCR, the reaction mixture of total volume of 20 μL included 2 μL (100 ng) of genomic DNA, 12 μL nuclease-

free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 2 µL of 10 µM primer (Eurofins, Bangalore, India) and 0.2 µL (5 U μL^{-1}) Taq Polymerase (Bangalore Genei, Bangalore, India).

The procedure for the genomic DNA isolation was a modification of Doyle and Doyle (1987) method, as follows.

Preheated 5 mL CTAB isolation buffer in a centrifuge tube (15 mL) at 65 °C for 15 min was added to fresh healthy leaves (1 g) along with 2-mercaptoethanol (0.2%). The leaves ground into a paste, put into the centrifuge tube (15 mL) and kept in a water bath at 65 °C for 1.5 h. After that the paste was distributed equally into Eppendorf tube and equal volume of chloroform / isoamylalcohol (24:1) was added, mixed well and centrifuged at 6000 rpm for 15 min at 15 °C. The aqueous phase was collected carefully using a wide bore tip and transferred to a new Eppendorf tube. Equal volume of cold isopropanol was added, well mixed and kept overnight at –20 °C. On the next day, it was centrifuged at 12 000 rpm for 15 min at 15 °C. Supernatant was discarded and 500 µL of ethanol (85%) was added. The pellet was mixed by gentle tapping and centrifuged at 10 000 rpm for 10 min at 15 °C. This step was repeated with 500 µL of ethanol (75%) and supernatant was discarded. The pellet was allowed to air dry (45 min to 1 h). Then 30 µl TE buffer (1X) was added and the mixture was kept in a hot air oven (65 °C) for 10 min. Then it was allowed to cool at room temperature and kept in refrigerator at 4 °C. After isolation of DNA, its quality and quantity were checked by different methods,

The spectrophotometric analysis of the DNA samples was mainly done to check the quality and quantity of the isolated DNA sample. Quantification of the isolated DNA samples was determined by taking the absorbance at 260 nm (1 O.D. = 50 µg mL^{-1} of double stranded DNA). Also $A_{260/280}$, $A_{260/230}$ and $A_{260/270}$ were determined to identify the impurities of proteins, polysaccharides and polyphnolics respectively (Peterson et al. 1997; Chen et al. 1999; Singh et al. 1999; Ahmad et al. 2004). For pure DNA, the $A_{260/280}$ ratios should be 1.8, $A_{260/230}$ should be greater than 1.8 and $A_{260/270}$ should be between 1.2 to 1.3 (Varma et al. 2007).

For agarose gel electrophoresis, 0.8% agarose gel was prepared and placed into an electrophoretic unit containing 1X TAE buffer. Undiluted DNA samples (2 µL) mixed with bromophenol blue were loaded into the well and 150 V voltage was applied for 20 to 45 min. The intact band of genomic DNA was observed in the gel by staining with ethidium bromide.

A qualitative test to check whether the isolated DNA was PCR amplifiable, was conducted to test if any contaminating material that could inhibit the PCR reaction. Stock genomic DNA was diluted to a final concentration of 50 ng μL^{-1} and random primers of sequence: 5'-GTGATCGCAG-3' (Eurofins Genomics, Bangalore) were used in the PCR reaction mixture.

Amplification was performed using a PTC-100 thermal cycler (MJ Research, Watertown, Massachusetts, USA) according to the following protocol. First, 94 °C for 5 min followed by 45 cycles of 94 °C for 1 min, 37 °C for 1 min, 72 °C for 2 min and final extension at 72 °C for 10 min. The amplified products were checked by electrophoresis on 1.2% agarose gel stained with ethidium bromide.

Results and discussion

Spectrophotometric analysis revealed that the amount of isolated DNA sample was 6200 µg mL^{-1} and 4925 µg mL^{-1} for *T. indica* and *B. monnieri*, respectively (Table 1). Also, the ratios of $A_{260/230}$ and $A_{260/270}$ were found to be within the range (Table 1).

The isolated DNA for *T. indica* (Lane1) and *B. monnieri* (Lane 2) showed a single intact band of the genomic DNA and no bands of RNA on agarose gel (0.8%) were observed (Fig. 1A). This indicated that these samples were free of RNA.

Further, qualitative analysis of the samples was done by PCR using random primers. After completion of 45 cycles, the products (Lane 2 and 3) were checked on 1.2% agarose gel along with the 100 bp DNA ladder (Lane 1, Fig. 1B), which confirmed that the isolated DNA was PCR amplifiable.

In the present protocol, the Doyle and Doyle's method (1987) for DNA isolation was used with certain modifications as follows. The incubation time of plant tissues with CTAB extraction buffer was increased from 30 – 60 min to 1.5 h. In the original method the ratio of cold isopropanol to sample was 2:3 without incubation, which was changed to 1:1 and incubation was done overnight at –20 °C. Ammonium acetate was not added to the sample as it remains as an impurity within it (Riahi et al. 2010). Due to its high affinity with DNA, its removal from the sample becomes difficult. Even though the RNase treatment was not given to the samples, we were able to isolate pure DNA without any RNA contamination.

Most of the DNA isolation protocols for plants use PVP in the extraction buffer (Maliyakal 1992; Giniwal, Mittal 2010), but in our study we eliminated this step along with a number of routinely used reagents but still a good quality and quantity of DNA could be obtained.

Table 1. Qualitative and quantitative analysis of genomic DNA

Plant species	Concentration	$A_{260/280}$	$A_{260/230}$	$A_{260/270}$
Tylophora indica	6200 µg mL^{-1}	1.75	2.14	1.21
Bacopa monnieri	4925 µg mL^{-1}	1.78	2.00	1.18

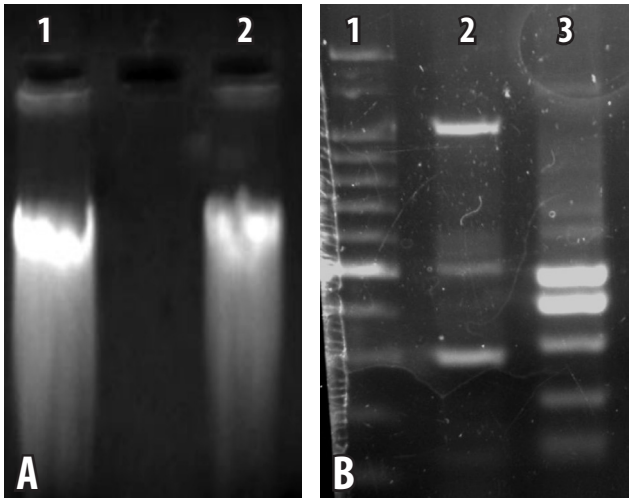

Fig. 1. A, bands of genomic DNA from *T. indica* (Lane 1) and *B. monnieri* (Lane 2); B, amplified products of isolated DNA using random primer. Lane 1 represents marker of 100 bp, Lane 2 and 3 are PCR products for *T. indica* and *B. monnieri*, respectively.

The two plants under study are important medicinally and are now placed under the threatened category (Faisal et al. 2005; Pandey et al. 1993) as they are being lost from the wild due to demand by pharmaceutical industries. Hence, conservation of these species is imperative and *in vitro* propagation methods are found to be suitable in rapidly multiplying the species. As *in vitro* methods may generate off types, it is essential to analyze the genetic fidelity of these plants, which can be done by using RAPD technique. As our further studies will be on analyzing the genetic integrity of *in vitro* plants, a standardized protocol for DNA isolation became mandatory. In the present method we were able to obtain a good quality DNA which could be utilized for different studies. There are reports on RAPD analysis of these plants (Jayanthi, Mandal 2001; Ceasar et al. 2010; Manikandan et al. 2010) using conventional DNA isolation protocols, where a large number of chemicals have been used as compared to the method developed by us.

In summary, the present method is efficient in extracting an ample amount of pure genomic DNA from plants belonging to different families with varied chemical constituents, and hence it can potentially be also used for the other species.

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