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Original Research Article

Standardization of Isolation of Genomic DNA from *Senna obtusifolia*

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A B S T R A C T

In molecular biology, there are many procedures that require the isolation of high quality genomic DNA which is difficult in plant species like *Senna obtusifolia* because of the presence of secondary metabolites that interfere with DNA isolation. The downstream application such as DNA restriction, amplification and cloning is affected by such poor quality of DNA. This plant has potential to provide highly nutritious supplementary food to rural population (Paster *et al.*, 2007). As this plant has high amount of secondary metabolites, polysaccharides and phenolic compounds. We describe a modified procedure based on exadecyl trimethyl ammonium bromide (CTAB) to isolate DNA from leaf tissues containing high levels of polysaccharides, polyphenols and secondary metabolites. This modified protocol of CTAB (2%) for isolation of genomic DNA includes the use of polyvinyl pyrrolidon (PVP), 0.2% β-mercaptoethanol and 2M NaCl with an initial prolonged Chloroform: isoamyl alcohol (24:1) step (30 min) shaking repeated twice, RNAse treatment for 1 hr and avoiding incubation and precipitation steps in isopropanol. The yield of DNA was approximately 19µg DNA/200 mg of initial fresh leaf tissues. This method solved the problem of DNA degradation and co-precipitation of the secondary metabolites. The isolated DNA was docile for downstream applications. This method is fast, reproducible and can be applied for further molecular approaches.

Keywords

Isolation of Genomic, DNA, *Senna obtusifolia*

Introduction

Tarota is an annual plant considered as a serious weed. It heights about 1.5-2.5 m. The seed pod is 10-15 cm long and 3-5 mm wide. Although it is regarded as one of the indigenous leafy vegetable, it has potential to provide food to the rural population. The plant contains some antinutrients therefore it is thought of as a poisonous to humans and livestock. The high level of most antinutrients indicates the potential of interfering with the utilization of nutrients. This therefore creates a need for detoxification of it before using. The seeds and leaves samples of *Senna obtusifolia* were collected from Yavatmal, Wardha, Washim, Akola and Chindwada (MP). Advances in plant molecular research will help in finding the elite genotype of this plant which can yield nutritional, medicinal and commercial compounds at enhanced level for which isolation of plant DNA for use in Southern blotting, Restriction Fragment Length Polymorphisms (RFLPs), Polymerase Chain Reaction (PCR), Arbitrary primed DNA amplification (RAPD, SSR-PCR) and Genomic Library

construction is one of the most important step. The presence of polyphenols, which are powerful oxidizing agents present in many plant species, can reduce the yield and purity of extracted DNA (Loomis 1974). The commonly encountered problems in the isolation of high molecular weight DNA from certain medicinal plants is difficult due to degradation of DNA because of endonuclease, co-isolation of highly viscous polysaccharides, inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions (Weishing *et al.*, 1995).

We followed the protocols given by Dellaporta *et al.,* (1983) and Doyle and Doyle (1987) but we found that it is necessary to develop a standardized protocol for isolation of genomic DNA from *Senna obtusifolia* as this plant contains polyphenols, polysaccharides etc. which interfere in the yield of isolated DNA.

The protocol described here is relatively simple, efficient, rapid, provides higher yield and is consistently restrictable and amplifiable by PCR. The yield of the genomic DNA using this protocol is higher than that obtained by other protocols given above. Several experiments were carried out using different methods, however only the optimized protocol is described here.

Materials and Methods

Plant source: The plant leaves samples were collected from various districts of Vidarbha region as Yavatmal, Akola, Washim, Buldhana and Chindwada, a district of Madhyapradesh. The young leaves were harvested fresh before DNA isolation.

Stock solution 10% Hexadecyltrimethyl-ammonium bromide (CTAB) (w/v)(Himedia), 1M Tris-HCl (pH 8.0) (Himedia), 0.5 M EDTA (pH 8.0) (Himedia), 5M NaCl, Chloroform: isoamylalcohol (24:1), 80% ethanol (Himedia) and TE buffer having 1mM Tris (pH 8.0) and 0.5 mM EDTA (pH 8.0) (Himedia). All the solutions were sterilized by autoclaving.

Extraction buffer: An extraction buffer consisting of 2% CTAB (w/v), 100 mM Tris (pH 8.0), 25mM EDTA (pH 8.0), 2 M NaCl, 2% polyvinyl pyrrolidone (PVP-Mr 10,000) (added before grinding) and 1% β-mercaptoethanol (v/v), (added immediately before grinding) was prepared. Several standard protocols (Doyle and Doyle, 1987) were followed. However, only the optimized protocol for isolation of genomic DNA from *Senna obtusifolia* is described here.

DNA isolation protocol

Fresh leaf samples of 200 mg were collected and ground in liquid nitrogen along with PVP. The pulverized leaves were quickly transferred to microcentrifuge tubes (2ml), containing prewarmed (65°C) extraction buffer (600 µl) with β-mercaptoethanol (20 µl) and shaken thoroughly to form a slurry. The tube was incubated at 65°C for 60 min with frequently swirling every 10 min.

An equal volume of chloroform: isoamylalcohol (24:1) was added and mixed gently for 30 min and centrifuged at 12,000 rpm for 15 mins at room temperature (RT) to separate the phases. The supernatant was carefully decanted and transferred to a new micro centrifuge tube. The same step was repeated twice. To the supernatant an equal volume of chilled isopropanol was added and DNA in the form of fibres was spooled and transferred into another tube containing 70% ethanol (300µl) and washed by centrifuging at 8000 rpm for 10 min.

The pellet was air-dried and resuspended in Tris EDTA (TE) 500 µl and 2µl of RNAse (HiMedia) was added and incubated at 37°C for 1 hour.

To the sample equal volume of phenol: chloroform: isoamylalcohol (24:25:1, v/v/v) was added and centrifuged at 8000 rpm for 12 min.

The pellet was discarded carefully and the supernatant was taken in new tubes. To the supernatant an equal volume of chilled isopropanol was added and mixed gently.

The spooled DNA were washed in 70% ethanol by centrifuging at 8000 rpm for 8 min. the pellet was air dried and the DNA was dissolved in TE depending on size of the pellet.

Amount and purity of DNA

A UV- Visible Spectrophotometer (Cintra 5) was used to measure the yield of DNA per 200 mg of leaf tissue at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm (Sambrook *et al.*, 1989). For this, 5 µl of stock DNA was added to 1 ml of double distilled water (a dilution factor of 1:200). The nucleic acid concentration was calculated using the formula A260 × 50µg/ml × 0.001 µl/ml × dilution factor (1000/5). This provides the concentration of DNA in µl/ml which is diluted to 50 ng/µl using sterile distilled water and used for RAPD reaction.

DNA analysis

PCR

The genomic DNA were analyzed for amplification by using operon primers OPA-07, OPOA-14, OPB-11 and OPB-15. The reactions were carried out in a thermocycler. The RAPD protocol was followed according to Padmalatha and Prasad (2006).

Results and Discussion

The protocol developed here is the modification of the CTAB method of Khanuja *et al.*, (1999) and Pirttila *et al.*, (2001) for isolation from fresh samples of plants producing large amounts of secondary metabolites.

The species of *Senna obtusifolia* is rich in secondary metabolites. Moreover, many impurities like tannins, gums, polysaccharides etc., interfere with the DNA and makes it difficult to isolate. But the procedure here can be used to obtain consistently high quality, low polysaccharide genomic DNA. Other standard protocols from Dellaporta *et al.*, (1983) and Doyle and Doyle (1987) also resulted in large quantity of DNA but the DNA was contaminated with high levels of polysaccharides which interfered, making it difficult to dissolve.

Secondary compounds such as polysaccharides or polyphenols are released when the cell disrupts leading to either embedding od DNA in a sticky gelatinous matrix or brown coloured products (Guillemaut and Marachel-Drouard, 1992). The presence of contaminants or inhibitors in the DNA might lead to appearance of a smear or absence of amplification during PCR reaction.

Hence young leaves were used to test the effects of various modifications in DNA extraction protocol wherein nucleic acid contamination by plant metabolites that interfere with solubilisation and precipitation is reduced to some extent. We first observed the effects of detergents in

DNA extraction buffer. Detergents such as sodium dodecyl sulphate (SDS), CTAB and a combination of both were added in extraction buffer and it was found that CTAB was comparatively a better option. Inclusion of PVP 2% instead of 1% improved the colour of nucleic acid. Use of β-mercaptoethanol prior to incubation helped in the removal of brownish colour at some extent.

Addition of pre warmed CTAB buffer to the frozen leaf tissues saves precious time in bringing the tissues from -80°C to 65°C as rapidly as possible resulting in DNA of higher quality (Puchooa, 2004). During incubation at 65°C, it was found that incubating for 120 min gives better yield of DNA as compared to 60 min and overnight incubation. In our protocol the extraction buffer contained high amount of PVP and β-mercaptoethanol, helpful in removal of polyphenols and also to prevent oxidation of secondary metabolites in disrupt plant material. The extraction of DNA was repeated twice with chloroform: isoamylalcohol because of the presence of many pigments, gums and polyphenolics, which enter into the organic layer. Once the nucleic acids were precipitated in isopropanol, incubation and centrifugation steps were avoided because of interference of gums and resins which precipitate along with the DNA. The spooled DNA was very carefully washed in 70% of ethanol, since CTAB is soluble in ethanol, and most of residual impurities like polysaccharides were removed (Demeke and Adams, 1992; Fang *et al.*, 1992). The purification of DNA from proteins was achieved by using equal volumes of phenols, chloroform and isoamylalcohol.

The yield was approximately 15-20 µg DNA/200 mg of initial fresh leaf tissues. The DNA quality was estimated by measuring the 260/280 UV absorbance ratio, which varied between 1.8 and 2.0. Isolation of genomic DNA using described method was quite easy and amenable for future works. To evaluate the suitability of the isolated DNA in the downstream application, we subjected the genomic DNA to amplification through PCR. Figure 1 shows the results of the extracted DNA run on a 0.8% agarose gel, stained with ethidium bromide and visualised under UV light wherein there are no impurities like polysaccharides, proteins and RNA on the gel visually. The PCR amplification of genomic DNA using Operon primer OPA-07, OPA-14, OPB-11 and OPB-15 resulted in clear amplification as indicated by ethidium bromide staining of a 2% agarose gel (Fig. 2, 3, 4 and 5). It shows that the PCR products from successful amplification consist of 2, 3 and 6 major bands with the Operon primer and some minor bands which are not seen visually. However minor differences in band size were also present which may be due to slight sequence length differences in the amplified spacer regions. Therefore, it demonstrates that the isolated DNA is suitable for any diagnostic purpose employing PCR as a technique.

Even though there is a wealth of protocols for optimization of DNA extraction in several plant species (Teixeira da Silva and Tanaka, 2006) to our knowledge this is the first report of DNA isolation from leaves of *Senna obtusifolia* species which is simple, rapid and efficient for PCR amplification and further diagnostics.

Moreover we found that the method described in this paper is functional for plants that were otherwise recalcitrant to DNA isolation hence we believe that this method will be helpful for molecular biological studies of many wild plant species of socio-economic importance.

Fig.1 Electrophoresis of total genomic DNA of *Senna obtusifolia* on 0.8% agarose gel stained with ethidium bromide visualized under UV light

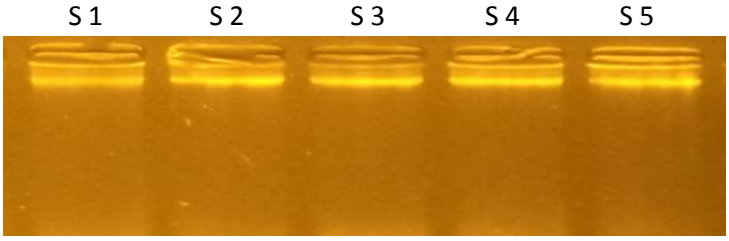

Fig.2, 3, 4, 5 RAPD profile of *Senna obtusifolia* genomic DNA samples using the OPA-07, OPB-11, OPB-15 and OPA-14 respectively on 2% agarose gel

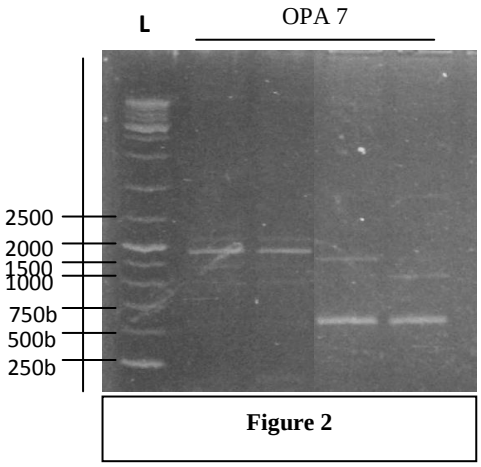

Figure 2

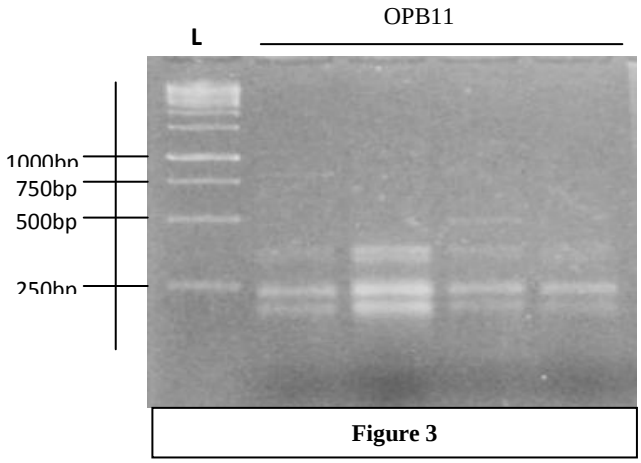

Figure 3

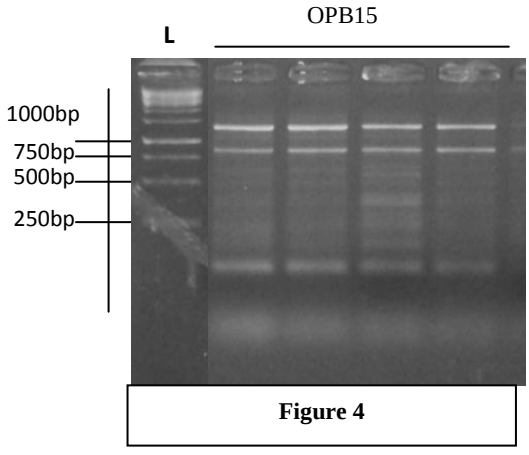

Figure 4

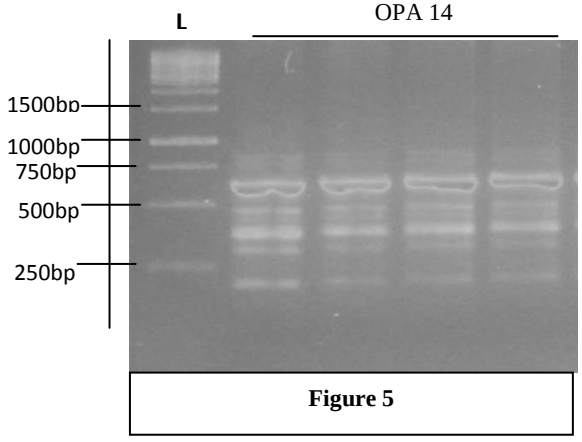

Figure 5

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