academicJournals

Vol. 15(22), pp. 1035-1040, 1 June, 2016
DOI: 10.5897/AJB2016.15232
Article Number: 295D03958676
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Standardization of DNA extraction from invasive alien weed *Parthenium hysterophorus*

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Received 20 January, 2016; Accepted 23 March, 2016

DNA isolation from the weed, *Parthenium hysterophorus* is complicated due to the presence of high amount of allelochemicals in the form of secondary metabolites that causes hindrance in extraction and enzymatic reactions. A modified and efficient DNA extraction from *P. hysterophorus* leaf has been developed. The present protocol is a modified version of cetyltrimethylammonium bromide (CTAB) method constituting high salt concentration to remove polysaccharides. The increased concentration of β-mercaptoethanol, polyvinylpolypyrrolidone (PVPP), and phenol/chloroform/isoamyl alcohol extractions eliminated protein and phenolic compounds well. Good amount and quality DNA was obtained by this method. The resulted genomic DNA showed fine random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR) banding pattern, polymerase chain reaction (PCR) amplification of actin gene and restriction digestion confirm the efficiency of modified procedure.

Key words: Allelochemicals, DNA extraction, *Parthenium hysterophorus*, stress tolerance.

INTRODUCTION

Parthenium hysterophorus is an annual invasive weed of asteraceae. It spreads rapidly worldwide in a short period due to its great structural and physio-chemical flexibility, vegetative multiplication, effective dispersal of seeds, longer seed dormancy, stress tolerance and presence of secondary metabolites (Patel, 2011). *P. hysterophorus* exhibited wide adaptability towards various environmental conditions which enabled this plant to grow successfully in drought and heat prone areas (Hedge and Patil, 1982; Shubneet et al., 2014; Kumar, 2014). Apart from these properties, *P. hysterophorus* also displayed antibacterial, antioxidant, and cytotoxic activities (Kumar et al., 2013). The occurrence of diverse alkaloids, terpenoids, flavonoids, hydrocarbons and fatty acids in its tissues may execute these properties (Panwar et al., 2015). However, no molecular information is available on *P. hysterophorus*. Keeping the above facts in mind, *P. hysterophorus* can be used as a good source for identification of valuable genes, proteins and metabolites that is responsible for diverse properties of this weed. Therefore, molecular characterization of this weed is necessary for further use in transgenic approach. The quality as well as quantity of DNA is a matter of great concern in functional genomics. Fine quality DNA is an

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essential requirement in all the manipulations related to recombinant DNA technology. A fast, simple and reliable DNA isolation method is immensely required for this purpose since similar DNA isolation protocols may not be suitable for all plants (Akhtar et al., 2013), because size, content and organization of genome and metabolic setup of different plant systems differ from each other (Sangwan et al., 1998). Many problems were faced in the isolation and purification of DNA which include degradation of DNA, contamination with highly viscous polysaccharides, polyphenols, tannins, terpenoids, flavonoids, quinines and other allelochemicals which affects the downstream application (Kotchoni et al., 2011). Therefore, a genomic DNA isolation protocol was modified from the earlier method (Doyle and Doyle, 1990) to simplify and optimize the procedure for *P. hysterophorus.*

MATERIALS AND METHODS

Collection of plant sample

Seeds from authenticated *P. hysterophorus* were collected from Jamia Millia Islamia University campus, New Delhi, India (Latitude 28.6° 4'N and Longitude 77.2°) growing in the alluvial soil. The plant was further identified with authentic specimen and deposited with accession no. 115597 at Botanical Survey of India, Dehradun, India. Seed sterilization was done by soaking of seeds in 0.3% $KMnO_4$ for 10 min, and then rinsed ten times with sterilized distilled water. Germinated seed (five days old seedlings) were transferred to Soilrite™ (Keltech Energies Ltd., India) containing pots (6"× 6", 300 g/pot) and cultured in 14 h/10 h light/dark at 25°C with 250 µmol photons m-2 s -1 for 55 days. Thus, two months old plants were used in the present study.

Solution used

Extraction buffer was 2.5% cetyl trimethyl ammonium bromide (CTAB), 100 mM Tris HCl (pH 8.0), 1.5 M NaCl, 25 mM ethylenediamine tetraaceticacid (EDTA), 3% polyvinylpolypyrrolidone (PVPP), 0.4% β-mercaptoethanol (added just before use). Isopropanol 70% ethanol (v/v); Phenol: Chloroform: Isoamyl alcohol (25: 24: 1, v/v); Chloroform: Isoamyl alcohol (24:1, v/v); 0.3 M sodium acetate (pH 5.4); TE (Tris/EDTA) buffer: 10 mM Tris-HCl (pH 8.0); and 1 mM EDTA (pH 7.4).

DNA extraction procedure

(1) 1.0 g of plant material (*P. hysterophorus* leaves) was immediately placed in pre-chilled (-80°C) mortar and pestle and ground thoroughly. The ground sample was transferred to 30 ml sterile centrifuge tube containing 5 ml preheated extraction buffer and mixed by inversion. The slurry was formed and tube incubated at 65°C for 60 min in water bath with occasional gentle swirling.
(2) Next, incubated sample was extracted with equal volume of chloroform: isoamyl alcohol (24:1) and mixed gently by inversion thoroughly for about 30 min at room temperature. The mixture was centrifuged at 12,000 rpm (10 min, 25°C) for phase separation.
(3) After the two phases were separated visibly of which top clear aqueous phase was carefully transferred to a new sterile centrifuge tube and equal volume of chilled isopropanol was added to precipitated nucleic acid. The whole preparation was mixed gently and kept at -20°C for 1 h.
(4) The mixture was centrifuged at 10,000 rpm for 15 min. The supernatant was removed carefully and pellet was washed with 70% ethanol, air dried at room temperature for 1 h and dissolved in 400 µl TE buffer. Next, 5 µl RNase A (1 µg/µl) was added and incubated at 37°C for 1 h.
(5) For further purification, mixture was extracted with an equal volume of phenol: chloroform: isoamyl alcohol.
(6) The upper aqueous layer was transferred to another sterile 2.0 ml micro-centrifuge tube and further extracted with equal volume of chloroform: isoamyl alcohol. It was then centrifuged at 10,000 rpm for 10 min
(7) In the next step, the top aqueous layer was picked carefully and transferred to fresh sterile 2.0 ml micro-centrifuge tube and precipitated the DNA by addition of 1/10th volume of sodium acetate (3 M pH 5.2) with equal volume of chilled isopropanol. The tube was inverted thoroughly and kept at -20°C for at least 1 h. Later, the mixture was centrifuged at 10,000 rpm for 15 min.
(8) The supernatant was decanted and pellet was washed with 70% (v/v) ethanol. Resulted air dried pellet was dissolved in 100 µl TE buffer.

Quantification of DNA

The DNA (5 µl) was added to 495 µl TE, and mixed by vortexing. The absorbance was read in spectrophotometer (SICAN 2301, India) by setting the blank against TE. The observations were taken at 260 and 280 nm. The ratio of $OD_{260\ nm}/OD_{280\ nm}$ provides an estimate of purity of nucleic acid. Pure preparation of DNA have OD_{260}/OD_{280} ratio of 1.8 to 2.0 (Sambrook et al., 1989). The DNA concentration (µg/µl) was calculated by using the following formula:

$$\text{DNA concentration (µg/)} = \frac{OD_{260} \text{ x Dilution factor x } 50}{1000}$$

Qualitative analysis of DNA

Qualitative analysis of DNA isolated from fresh leaves was done by 0.8% agarose gel electrophoresis. Gels were stained with ethidium bromide and documented using gel documentation system (Bio-Rad, USA).

Restriction digestion

The composition of reaction mixture and procedure used for the restriction digestion is shown in Table 1. Genomic DNA (4 µg) was used in restriction digestion mediated by *Eco* R I (Figure 2).

RAPD amplification

Random decamer primers synthesized from Life Technologies (India) were used in random amplified polymorphic DNA (RAPD) amplification. The amplification of template DNA was carried out by using a total volume of 25 µl containing 2.5 µl reaction buffer (10X), 2.0 µl dNTPs mix (200 µM each), 30 ng of decamer primer, 50 ng of genomic DNA and 0.5 µl (3U μl^{-1}) *Taq* DNA polymerase. Thermal cycler (VeritiR, Applied Biosystem, USA) was used to perform PCR reactions having an initial denaturation at 95°C for 5 min, denaturing at 94°C for 1 min, annealing at 40°C for 30 s and polymerization at 72°C for 2 min and final extension 72°C for 5 min. After amplification, the PCR product was resolved on 1.5% agarose gel in 1X TAE buffer. DNA was stained with ethidium bromide and visualized under UV light using gel documentation system (Bio-Rad, USA) (Figure 3).

Table 1. Composition of reaction mixture and procedure used in restriction digestion.

Reaction mixture	Quantity
DNA	2 µl
Buffer	1 µl (10 X)
Enzyme	1 µl (20 units/µl)
Sterilized doubled distilled water	6 µl
Total volume	10 µl
Procedure	
The reaction mixture was prepared as above in an Eppendorf tube	
The mixture was mixed gently	
Then it was incubated at 37°C for 2 h	
The mixture was taken out after 2 h and the digestion was monitored on 1.5% agarose gel running at 50 V for 45 min	

ISSR amplification

Six inter simple sequence repeat (ISSR) primers were employed with genomic DNA of *P. hysterophorus* for the amplification. The PCR reaction was performed by using mixture that consisted of 50 ng genomic DNA, 2.0 mM $MgCl_2$, 1X PCR buffer, 100 mM of dNTP, 25 ng primer and 1.5 U *Taq* polymerase in 25 µl volumes. PCR programme had an initial denaturation for 5 min at 95°C, followed by 40 cycles for 1 min at 94°C denaturation, annealing at 50°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 7 min. The amplification was conducted in a thermal PCR (VeritiR, Applied Biosystems, USA). After amplification, 10 µl of PCR products were loaded on the 1.5% agarose gel and electrophoresed in 1X TAE buffer. The image was taken under UV-light using gel documentation system (Bio-Rad, USA).

PCR amplification of actin gene

The gene specific primer for actin gene was used in PCR amplification. A reaction tube mixture contained 40 ng of DNA, dNTP mixture (100 mM each), Taq DNA Polymerase (1.0 U), 10X reaction buffer and gene specific primers (30 ng). The thermal cycling of DNA was performed with program as follows 95°C for 5 min (Initial denaturation), 35 cycles at 95°C for 1 min (denaturation), 52°C for 1 min (annealing), 72°C for 1 min (extension) and final extension at 72°C for 5 min. The amplified PCR product was resolved on 1.2% agarose gel in 1X TAE buffer. The gels were examined under UV light and photographed by documentation system (Bio-Rad, USA).

RESULTS AND DISCUSSION

In the present study, optimized CTAB method was used for isolation of DNA from parthenium leaf. The main complications of plant DNA isolation were associated with the presence of polyphenolic compounds, terpenoids and polysaccharides (Kotchoni and Gachomo, 2009). Weed plant such as *P*. *hysterophorus* showed strong allelopathic character which is associated with secondary metabolites (Bhadoria, 2011; Mawal et al., 2015). Thus, quality and amount of DNA were greatly affected by these interfering compounds which co-precipitate during the DNA isolation from *P. hysterophorus* leaf. Extraction using high salt CTAB buffer was done to remove polysaccharides. CTAB also acts as a good detergent that solubilizes cell wall, lipid membrane and denatures protein (Clarke, 2009). Polyphenols associated with DNA cause hindrance in polymerase chain reaction and restriction digestion. DNA molecules are damaged by reactive oxygen species produced by oxidized product of phenolics (Li and Trush, 1994). Plant cells were effectively lysed on increasing the temperature (65°C) of heat treatment. Increased amount of PVPP (3%) was used to support elimination of phenolic compounds mediated by hydrogen bond formation with them. Oxidation of polyphenol as well as browning of the DNA is effectively prevented by PVPP (Guillemaut and Maréchal-Drouard, 1992; Borse et al., 2011). On the other hand, increased concentration of β-mercaptoethanol (0.4%) in modified protocol also improves the quality of DNA because β- mercaptoethanol works as a strong reducing agent which contributes in removal of polyphenols from the crude extract of plant and facilitates the linearization of proteins by disruption of disulphide bonds between cysteine residues (Nalini et al., 2004). Elimination of protein was carried out by phenol: chloroform mixture followed by centrifugation steps. Long term treatment using chloroform: isoamyl alcohol was done to get rid of pigments. Isolation of RNA free DNA was achieved by 1 h RNase treatment at 37°C. The use of liquid nitrogen was substituted by prechilled mortar and pestle stored overnight at −80°C. The shearing of DNA was not visible in the present method (Figure 1), which proved the good quality of extracted DNA.

Finally, white pellet of DNA was obtained. The value of A260/280 ratio was 1.79±0.005 (Table 2) showing the absence of contaminants. Suitability of *Eco*RI mediated restriction digestion (Figure 2) of DNA isolated by modified method indicating the efficiency of this method. Clear, reproducible and consistent banding patterns yield demonstrated its compatibility for polymerase chain reaction using RAPD (Figure 3) and ISSR primers (Figure 4). A distinct PCR amplification of actin gene (Figure 5) was also obtained, which further proved that a good

Table 2. Quantification of DNA extracted by Doyle and Doyle's (1990) and modified methods from leaf of *Parthenium hysterophorus*.

Protocol used	Concentration of DNA (µg/µl)	Concentration of DNA (µg/g FW)	A_{260}/A_{280}	A_{260}/A_{230}
Doyle and Doyle's (1990)	1.23±0.005	122±0.578	1.64±0.005	1.86±0.008
Modified	2.1±0.057	199±0.577	1.79±0.005	2.01±0.006

n=3, P ≤ 0.05; FW, fresh weight.

Table 3. Comparison of components of two DNA extraction methods.

Doyle and Doyle (1990)	Modified protocol
2% CTAB	2.5% CTAB
100 mM, Tris HCl (pH 8.0)	100 mM, Tris HCl (pH 8.0)
1.4 M NaCl	1.5 M NaCl
0.2%, β-mercaptoethanol	0.4%, β-mercaptoethanol
20 mM, EDTA	25 mM, EDTA
PVP (1%)	PVPP (3%)
60°C/60 min (Heat treatment)	65°C/60 min (Heat treatment)
2.5 M, Ammonium acetate (pH 7.7)	0.3 M, Sodium acetate (pH 5.4)
Tissue/ Buffer (w/v), 1:10	Tissue/ Buffer (w/v), 1:5

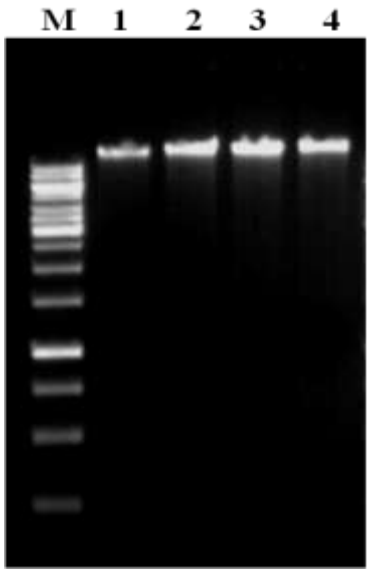

Figure 1. Total DNA (Lanes 1, 2, 3 and 4) extracted from *P. hysterophorus* leaf using present modified method visible on 1% agarose gel stained with ethidium bromide. Lane M is 1Kb ladder.

Figure 2. Restriction enzyme digestion of *P. hysterophorus* genomic DNA with *Eco* RI (Lanes 4, 5, 6).

quality of DNA was obtained. Several independent extractions and replicates proved the reliability of the modified method over Doyle and Doyle's (1990) (Table 3).

Conclusion

Conclusively, a modified CTAB method for DNA isolation from weed plant *P. hysterophorus* was described. It is reliable and cost-effective and does not require liquid

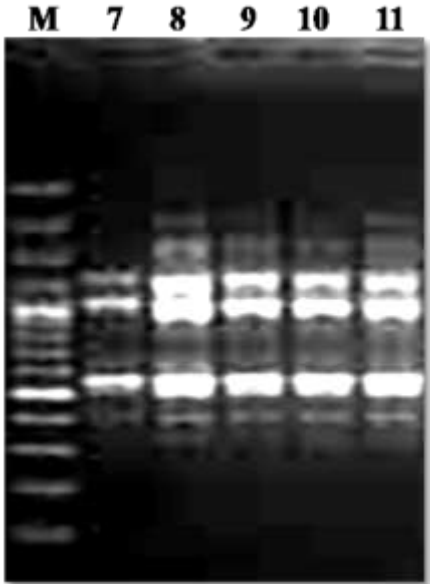

Figure 3. RAPD profile (lanes 7, 8, 9, 10 and 11) of *P. hysterophorus* obtained with primers LC-71, 72, 73, 78 and 93. Lane M is 100 bp ladder.

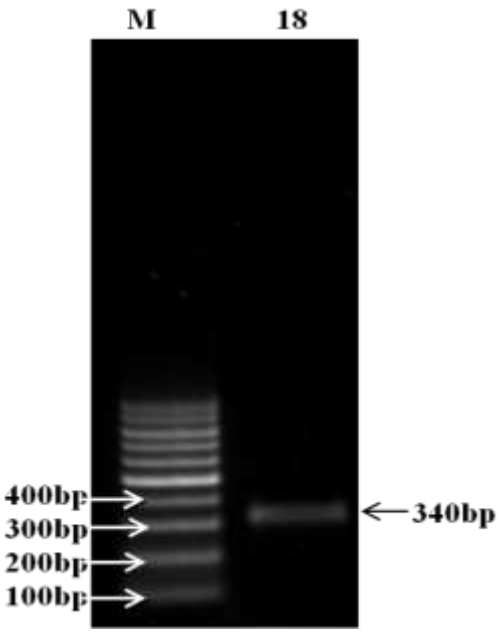

Figure 5. PCR amplification of actin gene on 1% agarose gel stained with ethidium bromide with arrow indicates the actin gene product (lane,18); DNA ladder (M: 100 bp ladder).

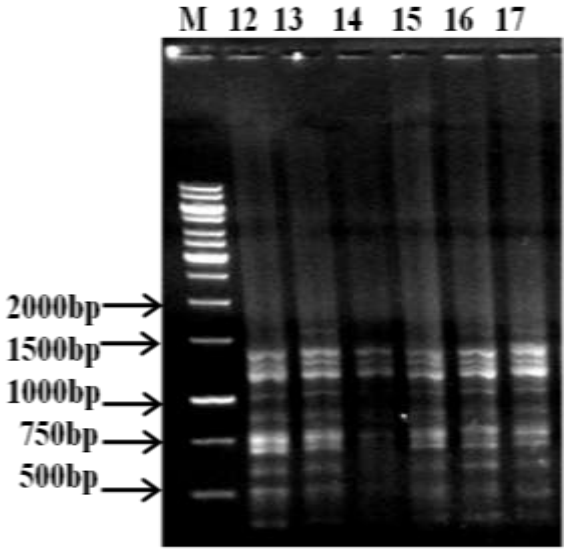

Figure 4. ISSR profile (lanes 12, 13, 14, 15, 16, and 17) of *P. hysterophorus* obtained with primers IR-1, 2, 3, 4, 8, 9. Lane M is 1Kb ladder.

nitrogen. The quality as well as yield of extracted DNA is also not compromised. Absorbance ratio in purity range and sharp band intensity on agarose gel electrophoresis indicated the effectiveness of the optimized method for DNA isolation. The extracted DNA product is suitable for molecular studies such as RAPD, ISSR, PCR amplification and restriction digestion analysis.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for supporting this work through research group no. RGP-297.

REFERENCES

Akhtar M, Qureshi MI, Singh, NK (2013). Standardization of DNA isolation protocol from *Chenopodium album* and *Lepidium latifolium*. Bioinfolet 10(2b):612-615.

Bhadoria PBS (2011). Allelopathy: a natural way towards weed management. Am. J. Exp. Agric. 1(1):7-20.

Borse T, Joshi P, Chaphalkar, S (2011). Biochemical role of ascorbic acid during the extraction of nucleic acids in polyphenol rich medicinal plant tissues. J. Plant Mol. Biol. Biotechnol. 2(2):1-7.

Clarke JD (2009). Cetyltrimethyl ammonium bromide (CTAB) DNA miniprep for plant DNA isolation. Cold Spring Harbor Protocols 2009(3):pdb-prot 5177.

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12(1):13-15.

Guillemaut P, Maréchal-Drouard L (1992). Isolation of plant DNA: A fast, inexpensive, and reliable method. Plant Mol. Biol. Rep. 10(1):60-65.

Hedge BA, Patil TM (1982). Effect of salt stress on the structure and carbon flow mechanism in a noxious weed *Parthenium hysterophorus* L. Weed Res. 22(1):51-56.

Kotchoni SO, Gachomo EW (2009). A rapid and hazardous reagent free protocol for genomic DNA extraction suitable for genetic studies in plants. Mol. Biol. Rep. 36(6):1633-1636.
Kotchoni SO, Gachomo EW, Jimenez-Lopez JC (2011). A plant cocktail amenable for PCR-based genetic analysis in *Arabidopsis thaliana*. Mol. Biol. Rep. 38(8):5281-5284.
Kumar A (2014). *Parthenium hysterophorus* L. and its impact on living world. Indian J. Sci. Res. 4(1):8-14.
Kumar S, Pandey S, Pandey, AK (2014). *In Vitro* antibacterial, antioxidant, and cytotoxic activities of *Parthenium hysterophorus* and characterization of extracts by LC-MS analysis. BioMed Res. Int. Vol. 2014, Article ID 495154, 10 p.
Li Y, Trush MA (1994). Reactive oxygen-dependent DNA damage resulting from the oxidation of phenolic compounds by a copper-redox cycle mechanism. Cancer Res. 54:1895-1898.
Mawal SS, Shahnawaz M, Sangale, MK, Ade AB (2015). Assessment of allelopathic potential of the roots of *Parthenium hysterophorus* L. on some selected crops. Int. J. Sci. Res. Knowl. 3(6):145.
Nalini E, Bhagwat SG, Jawali N (2004). A simple method for isolation of DNA from plants suitable for long term storage and DNA marker analysis. BARC Newsl. 249:208-214.
Panwar R, Sharma, AK, Dutt D, Pruthi V (2015). Phenolic Acids from *Parthenium hysterophorus*: evaluation of bioconversion potential as free radical scavengers and anticancer agents. Adv. Biosci. Biotechnol. 6(1):11.
Patel S (2011). Harmful and beneficial aspects of *Parthenium hysterophorus*: an update. 3 Biotech. 1(1):1-9.
Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning. New York: Cold Spring Harbor Laboratory Press. 2:14-29.
Sangwan NS, Sangwan, RS, Kumar S (1998). Isolation of genomic DNA from the antimalarial plant *Artemisia annua*. Plant Mol. Biol. Rep. 16(4):365-365.
Shubneet M, Gurmeen R, Amrit B, Dev SA (2014). Analysis of hydrophilic antioxidant enzymes in invasive alien species *Parthenium hysterophrus* under high temperature abiotic stress like conditions. J. Stress Physiol. Biochem. 10:2.