Available online at www.pelagiaresearchlibrary.com

Pelagia Research Library

European Journal of Experimental Biology, 2013, 3(6):33-38

ISSN: 2248 –9215
CODEN (USA): EJEBAU

Optimization of DNA isolation and RAPD-PCR protocol of *Acanthus volubilis* wall., a rare mangrove plant from Indian Sundarban, for conservation concern

Surya Shekhar Das, Swati Das (Sur) and Parthadeb Ghosh*

Cytogenetics and Plant Biotechnology Research Unit, Department of Botany, University of Kalyani, Kalyani, West Bengal, India

---

ABSTRACT

Extraction of pure and high molecular weight genomic DNA is a prerequisite for molecular biological studies of an organism. However, the presence of polysaccharides and polyphenols in plants upset the isolation of pure DNA and downstream reactions like PCR amplification. Here we present the optimization of DNA isolation protocol and PCR conditions for RAPD analysis of Acanthus volubilis, as available standard protocols do not produce high quality PCR amplifiable DNA. The method involves a modified CTAB extraction employing 2M NaCl, 2.5% CTAB, 3.0 % β-mercaptoethanol, 4.0% PVP and 0.13% sodium sulphite. The yield of DNA was 82.14 µg per gram of leaf tissue and the A_{260}/A_{280} value was 1.79 indicating minimal levels of contamination. RAPD protocol was optimized based on the use of 3mM $MgCl_2$, 200 µM dNTP mix, 0.2 unit of Taq DNA polymerase, 5 picomoles of single random decamer primer and 25ng of template DNA. An annealing temperature of 38^0C resulted in optimal amplification. In all PCR reactions reproducible amplified products were observed. High intensity amplification with random decamer primers during PCR also indicates that the DNA was of good quality and free from interfering compounds. Thus the results indicate that the optimized protocol for DNA isolation and RAPD-PCR will aid in further work on genetic diversity analysis, phylogenetic studies and most importantly in developing conservation strategies of this very rare mangrove plant from Indian Sundarban.

Key words: *Acanthus volubilis*; conservation; DNA isolation; polysaccharides and polyphenols; RAPD.

INTRODUCTION

The Sundarban, shared between India and Bangladesh, is the largest mangrove forest in the world and a UNESCO world heritage site. The western part of this gigantic delta belongs to West Bengal, India. In the Indian Sundarban, the genus *Acanthus* is represented by *A. ilicifolius* Linn. and *A. volubilis* Wall.. *A. volubilis* was once regarded as extinct from India, but has been recorded again with its very limited population existing in confined locations of Sundarban [1]. This species has not been reported from any other mangrove habitat in India [1]. *A. volubilis*, locally known as “Lata Hargoja”, occurs in areas with freshwater inputs especially on mud lobster mounds, as a bush-like, sprawling herb, or when there are other taller structures around, as a climber. It has simple opposite leaves that has pointed tips when they are growing in areas exposed to the sun, but become more rounded when they are growing in the shade. The leaf blades are mostly without spines. The leaves often appear moist, especially in the morning before it gets too hot, as they excrete excess salt through their leaves. Sometimes, tiny salt crystals can be seen on the leaf surfaces. The flowers are white. In folklore medicine, the leaves of *A. volubilis* are used for dressing boils and wounds whereas powdered seeds are taken with water as a blood cleansing medicine and against ulcers.

Phenylethanoid glycosides, benzoxazinoid glucosides and aliphatic alcohol glycosides, reported from *A. volubilis* have analgesic and anti-inflammatory actions [2].

Due to overexploitation, habitat destruction and global warming mangroves are being destroyed at an alarming rate and has resulted in the loss of genetic diversity [3]. To overcome these losses, conservation and sustainable management is, thus, a major priority. However, the genetic structure of plant species within the mangrove ecosystem of Sundarbans is poorly understood. Studying the genetic diversity of mangrove plants is important in taking effective measures to protect these species [4]. Genetic studies are, therefore, aimed at providing the information needed for afforestation, domestication and for the conservation of genetic resources. Molecular markers can be used to assess polymorphism in this mangrove species to identify and detect distinct genotypes for long-term conservation. Development of molecular methods has provided opportunities to take mangrove research in new directions and to address unresolved issues in mangrove studies [5].

DNA fingerprinting offers various applications, especially in the field of genotype identification, genetic diversity analysis, systematics, phylogenetic relationship construction and most importantly in conservation biology [4]. Developing a suitable genomic DNA extraction protocol is a pre-requisite in DNA fingerprinting studies, and the integrity, purity and quantity of extracted DNA influence the success of subsequent experiments. The problems encountered in the isolation and purification of DNA from plants include degradation of DNA by endonucleases, co-isolation of highly viscous polysaccharides, inhibitor compounds like polyphenols and RNA which interfere with further enzymatic reactions like PCR amplification [6]. Chemotypic heterogeneity among plant species may not permit optimal DNA yields from one isolation protocol, and perhaps even closely related species may require different isolation strategies [7]. Although several successful DNA extraction protocols for plant species containing polyphenols and polysaccharides have been developed, none of these are universally applicable to all plants [8]. Therefore, researchers often modify a protocol or blend two or more different procedures to obtain DNA of the desired quality [8]. Commercial genomic DNA extraction kits have also been developed. However, these kits are generally either expensive or not readily available, especially for researchers in developing and under-developed countries worldwide [9]. In addition, the use of such kits is limited to certain organisms, and they are not for universal organism DNA extraction [10].

We have tested previously established DNA isolation protocols but these methods resulted in DNA with impurities and not very suitable for RAPD analysis. Therefore, the purpose of the present study is to optimize the DNA isolation and PCR protocol for RAPD analysis of *A. volubilis* and to aid in downstream molecular studies which in turn will help in genetic diversity analysis, phylogenetic studies and most importantly in developing conservation strategies of this very rare mangrove species from Indian Sundarban.

MATERIALS AND METHODS

Plant Material

Young, fresh and healthy leaves of *A. volubilis* Wall. were collected from different sites in the mangrove forest of Sundarban, West Bengal and stored with silica gel in separate zip-lock plastic bags. From each of the sites, three individuals were randomly selected and leaf samples of small quantity were harvested. Leaves were collected and bulked from different plants and replicated three times for DNA isolation. 1 gm of leaf tissue from each replica was subsequently used for each DNA isolation experiment.

Reagents/ Chemicals used

An extraction buffer consisting of 100 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 2 M NaCl, 2.5% CTAB(w/v), 3.0 % β-mercaptoethanol (v/v), 4.0% PVP (w/v) and 0.13% sodium sulphite was prepared. TE buffer (10 mM Tris-HCl at pH 8 and 1 mM EDTA at pH 8), chloroform: isoamylalcohol (24:1,v/v), ethanol (70 % and 100 %), sodium acetate (3M) solution (pH 8.0), pure cold (-20°C) isopropanol, PCR buffer (10mM Tris-HCl pH 8; 50mM KCl), *Taq* DNA polymerase (Genei, India), ribonuclease A (Genei, India), 3mM $MgCl_2$, all four dNTPs (Genei, India), decamer primers (Genei, India), agarose gel (0.8% and 1.5%), 1X TBE buffer (1L 5X stock : 54 g Tris, 27.5 g boric acid, 20 ml 0.5 M EDTA) and ethidium bromide were the additional solutions and chemicals required.

Genomic DNA isolation protocol

a) Silica gel-dried young and healthy leaf samples (1 g) were ground in liquid Nitrogen using a pre-chilled mortar and pestle.
b) The ground powder was quickly transferred to a centrifuge tube and then 10 ml of pre-warmed (65^{o}C) extraction buffer was added and shaken gently to form slurry.

c) The mixture was incubated at 65°C in water bath for 75 minutes with intermittent shaking for every 10 minutes with occasional inversion and cooled to normal temperature.
d) An equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed properly for 30 minutes by inverting the tubes 30-35 times to form an emulsion and centrifuged at 13000 rpm for 15 minutes at RT to separate the phases.
e) The supernatant was carefully decanted and transferred to a new tube and the second chloroform: isoamyl alcohol (24:1) extraction was performed.
f) The aqueous phase (supernatant) was pipetted out in a fresh polypropylene tube and two volume of ice cold isopropanol was added and mixed by quick gentle inversion for about 5 minutes. The DNA was precipitated by incubating the mixture at -20°C for 75 minutes.
g) The samples were centrifuged at 10,000 rpm for 15 minutes at RT. The supernatant was discarded gently and the pellet was washed with 1000 µl 70% chilled ethanol. The centrifugation and subsequent ethanol wash steps were repeated 3 times. The pellet was air dried for about 30 min at room temperature and was re-suspended in 300 µl of TE buffer.
h) 7µl of RNAase (10 µg/µl) was added and incubated at 37°C for three hours. To this 600 µl of Ice chilled absolute ethanol and 90 µl of 3M sodium acetate was added and incubated at -20°C for 30 minutes to re-precipitate DNA.
i) The solution was centrifuged at 12000 rpm for 10 minutes; DNA pellet was dried at 37°C and resuspended in 100 µl of Tris–EDTA (TE) buffer.

Checking yield, purity and quality of the extracted DNA

The yield of DNA per gram of leaf tissue was estimated following [11] from the value of absorption at 260 nm (A_{260}) measured by a UV spectrophotometer (Perkin-Elmer USA). The purity or cleanliness of DNA was determined by estimating the ratio of absorbance at 260 nm to that of 280 nm and 230 nm. The first ratio (A_{260}/A_{280}) was to check any contamination by RNA or protein whereas the second ratio (A_{260}/A_{230}) was to check any contamination by polysaccharide and / or polyphenol. The quality or integrity of the extracted DNA was tested by running the extracted DNA samples on 0.8 % agarose gel stained with ethidium bromide in 1×TBE buffer at 100 V for 90 minutes. The gel was visualized under ultraviolet trans-illuminator and photographed using Gel Doc (Bio Rad). Presence of a highly resolved high molecular weight band indicates good quality DNA whereas a smeared band indicates DNA shearing or degradation.

Optimization of RAPD reaction

To find out the perfect conditions for PCR reaction, almost all the tested parameters like the concentration of primer, Taq polymerase, dNTPs, magnesium chloride, template DNA and temperature and time intervals during denaturation, annealing and elongation were optimized. Random decamer primers were used to amplify parts of genomic DNA extracted from *Acanthus volubilis*. The reactions were carried out in a DNA thermocycler (Gene Amp PCR system 2400, Perkin Elmer**).** Each 25 µl reaction volume contained about 2.5 µL of 10X PCR Buffer (10mM Tris-HCl pH 8; 50mM KCl), 3mM $MgCl_2$, 200 µM dNTP mix, 0.2 unit of Taq DNA polymerase (Bangalore Genei, India), 5 picomoles of single random decamer primer (Bangalore Genei, India) and 25ng of template DNA. The thermocycler was programmed for an initial denaturation at 94°C for 4 minutes followed by 36 cycles at 94°C for 1 min, annealing at 38°C for 1 minute and extension at 72°C for 2 minutes, followed by one final extension at 72°C for 6 minutes and at last the hold temperature was of 4°C. PCR products were electrophoresed on 1.5% agarose gel containing 0.5 µg ml^{-1} elthidium bromide, in 1 x Tris Borate-EDTA (TBE) buffer at 70 V for four hours. Gels with amplification fragments were visualized under ultraviolet trans-illuminator and photographed using Gel Doc (Bio Rad**).** For each experiment the reproducibility of the amplification products was tested twice using similar reaction conditions at different times.

RESULTS AND DISCUSSION

A sufficient amount of clean and unsheared genomic DNA was obtained using the optimised protocol described in "Materials and Methods". The yield range was 82.14 µg per gram of fresh weight of leaf tissue (Table 1). Spectrophotometric analyses revealed that the A_{260}/A_{280} value was 1.79, while the A_{260}/A_{230} value was 2.12 (Table 1). The value of the first ratio (A_{260}/A_{280}) confirmed us that the isolated DNA was almost free from any contamination by RNA or protein whereas the second value (A_{260}/A_{230}) indicated little or no contamination by polysaccharide and / or polyphenol. The quality or integrity of the extracted DNA was tested by running the extracted DNA samples on 0.8 % agarose gel stained with ethidium bromide. Presence of a highly resolved high molecular weight band (Figure 1) indicates good quality DNA. For RAPD analysis, it is more important to have good quality DNA samples (unsheared / undegraded DNA), than high quantities of DNA. After optimization of RAPD conditions, DNA isolated by this method yielded strong and reproducible amplification products (Figure 2 and 3) showing its compatibility for RAPD-PCR. High intensity amplification with arbitrary RAPD primers during

PCR also indicates that the DNA was of good quality, free from interfering compounds, and it would be suitable for further downstream molecular analysis.

Table 1—Yield and purity index of extracted genomic DNA

Plant Species	Organ from which DNA was extracted	A_{260}/A_{280} *	A_{260}/A_{230} *	DNA yield* [µg/g of fresh weight]
Acanthus volubilis Wall.	young and healthy leaf	1.79 ± 0.01	2.12 ± 0.03	82.14 ± 0.56

**Results are expressed as mean of 3 extractions with standard errors (SE). Means followed by different letters are significantly different at P ≤ 0.05* [12].

Fig.1. Genomic DNA, isolated from two samples of young and healthy leaf tissue of *Acanthus volubilis*, resolved on 0.8% agarose gel stained with ethidium bromide (*lanes 1* and *2*)

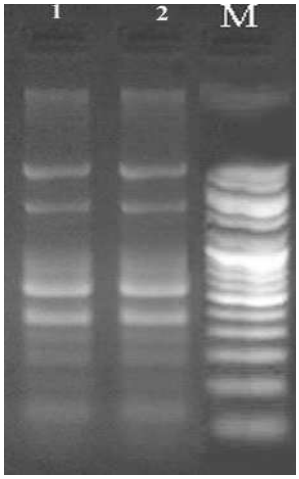

Fig. 2. Ethidium bromide-stained 1.5% agarose gel showing PCR-amplified products of *Acanthus volubilis* genomic DNA (*lanes 1, 2)* generated by a random primer (5'AGGTGACCGT3'). M= Marker, λ DNA digested with *EcoRI* and *Hind-III*

Mangrove plants contain exceptionally high amount of polysaccharides and secondary metabolites such as alkaloids, flavonoids, terpenes, resins, tannins and other polyphenols which would interfere with the DNA isolation procedures [13]. Such compounds interfere by precipitating along with the DNA, thus degrading its quality and reducing yield. The polyphenols, a very powerful oxidizing agent, present in many plant species can reduce the yield and purity by binding covalently with the extracted DNA making it useless for most research applications [14].Tannins, terpenes and resins are also difficult to separate from DNA [15]. Many polysaccharides interfere with RAPD reactions and thus distort the results in many analytical applications leading to wrong interpretations [16]. Moreover, the contaminating RNA that precipitates along with DNA causes many problems including suppression of PCR amplification [17].

In our experiments we encountered difficulties from the stage of cell lysis to DNA separation in the supernatant and subsequent reactions when following the steps of CTAB protocol [18]. DNA obtained was dirty yellow in appearance and with high viscosity probably due to high endogenous levels of phenolics and polysaccharides, respectively. Such DNA could not be subjected to even agarose gel electrophoresis [17]. Polyphenol contamination of DNA makes it

resistant to DNA manipulating enzymes and interacts irreversibly with proteins and nucleic acids. The end result was low DNA yield and poor PCR amplification reactions. So, modifications were necessary to standardise an efficient protocol. We performed several experiments with one parameter tested at a time to find one that yielded a good amount of high quality, pure genomic DNA of *Acanthus volubilis*. A number of experiments were set up to close down the polysaccharide contamination of the DNA by using a range of concentrations of sodium chloride (concentrations 1.0- 3.0 M) and for removing the polyphenol content from the isolated genomic DNA we used a range of concentrations of β-mercaptoethanol (2-5%), PVP (2-6%) and sodium sulfite (0.06-0.16%). An optimized protocol was designed on the basis of results obtained from the above experiments. Use of 3% β-mercaptoethanol, 4% PVP and 0.13% sodium sulfite was found to be most appropriate. The addition of high concentration of PVP and β-mercaptoethanol were helpful in removing the polyphenols and to prevent oxidation, respectively. Mixing of Poly Vinyl Pyrrrolidone (PVP) along with CTAB may bind to the polyphenolic compounds by forming a complex with hydrogen bonds and may help in removal of impurities to some extent [19]. As the use of sodium sulfite is also recommended to prevent oxidation, it was included in the extraction buffer. The problem arising from the presence of high levels of polysaccharides was overcome by adding NaCl at a higher concentration (2M) as it serves to remove polysaccharides. Polysaccharide co-precipitation is avoided by using higher concentration (2.5%) of cetyl trimethyl ammonium bromide (CTAB), a selective precipitant of nucleic acids. 25 mM EDTA at pH 8.0 was used in the extraction buffer as a chelating agent that binds Mg. By binding Mg with EDTA, the activity of the present DNase can be decreased. Tris-HCl (100mM, pH 8.0) provided the solution a pH buffering capacity (a low or high pH damages DNA). Elimination of protein is performed through chloroform : isoamyl alcohol treatment [20]. Long term chloroform-isoamyl alcohol treatment for 30 minutes also ensures removal of chlorophyll and other pigments.

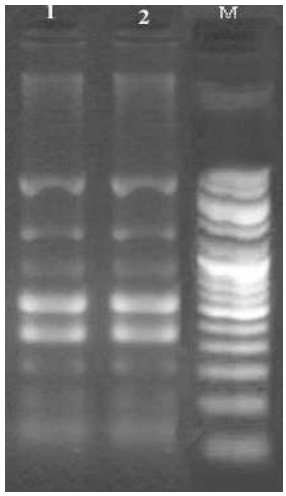

Fig. 3. Ethidium bromide-stained 1.5% agarose gel showing PCR-amplified products of *Acanthus volubilis* genomic DNA (*lanes 1, 2)* generated by a random primer (5'GTGAGGCGTC3'). M= Marker, λ DNA digested with *EcoRI* and *Hind-III*

Increasing the number (3 times) of washing in 70% chilled ethanol gave better DNA because it helped to remove the residual NaCl and/ or CTAB (CTAB is soluble in ethanol). Using isopropanol and sodium acetate was also found to be efficient in removing polysaccharides and secondary metabolites from DNA. Many DNA isolation procedures also yield large amount of RNA, especially 18S and 25S rRNA [21]. In this modified CTAB method RNA contamination was removed by the enzyme RNase. A prolonged RNase treatment degraded RNA into small ribonucleotides that did not contaminate the DNA preparations and yielded RNA-free DNA. The extraction buffer to tissue ratio and incubation time also plays an important role in the removal of contaminants and improving the quality of DNA [22]. Increased buffer to tissue ratio with increased 75 min incubation at 65^0C came up with considerably good yield of DNA. All the centrifugation steps were carried out at RT to avoid precipitation with CTAB, DNA degradation and to obtain good quality of DNA. Moreover, the procedure also eliminates the necessity of phenol, which makes the method less hazardous. Additional centrifugation steps, modified speed and time removed large amounts of precipitates like protein and polysaccharides. We found these modified steps necessary to isolate high quality genomic DNA.

For RAPDs almost all the tested parameters like the concentration of primer, Taq polymerase, dNTPs, magnesium chloride, template DNA and temperature and time intervals during denaturation, annealing and elongation were optimized which also had an effect on amplification, banding patterns and reproducibility. The described conditions in the present work, modified for use in RAPD analysis, consistently amplified DNA fragments of *Acanthus volubilis*. The small amount of genomic DNA, primers, Taq polymerase, dNTPs, and the small reaction volume used in the RAPD protocol has made it inexpensive.

The protocol described here is simple, inexpensive, reliable, rapid, less hazardous and yields appreciable levels of high quality, clean genomic DNA. Moreover, DNA isolated by this efficient method yielded strong and reproducible amplification products showing its compatibility for RAPD-PCR. High intensity amplification with arbitrary RAPD primers during PCR also indicates that the DNA was of good quality, free from interfering compounds. We anticipate that this protocol will be adequate for extracting high-molecular weight DNA suitable for further molecular analysis from some other plant species containing large amounts of secondary metabolites and polysaccharides. The optimized DNA isolation and RAPD-PCR protocol will aid in further downstream applications like genetic diversity analysis, phylogenetic studies and most importantly in developing conservation strategies of this very rare mangrove species from Indian Sundarban.

Acknowledgements
Authors are grateful to the Head, Department of Botany, University of Kalyani, West Bengal for providing central equipment facility funded by DST-FIST.

REFERENCES

[1] R.N. Mandal & K.R. Naskar, Tropical Ecology, **2008**, 49(2): 131-146.
[2] T. Kanchanapoom, P. Noiarsa, H. Otsuka, S. Ruchirawat, *Biochem. Syst. Ecol*., **2006**, *34*, 442-445.
[3] Q.F. He, H.Q. Fan and Zh. Ch. Mo., *Wetlands Science & Management*, **2009**, 5(3): 44-46.
[4] Shao-bo Chen *et al.*, *Pak. J. Boy*., **2010**, 42(6): 3755-3764.
[5] A. E. Schwarzbach, and L. A. McDade. *System Botanique*, **2002**, 27: 84-98.
[6] T. Dey, S. Saha, T. N. Dhar, S. Adhikary, P. Ghosh. General and *Applied Plant Physiology*, **2010**, 36 (3–4), pp. 232–238.
[7] K. Weising, H. Nybom, K. Wolff & G. Kahl, *DNA fingerprinting in plants : Principles, methods, and applications*, 2nd edn (CRC Press, Taylor & Francis Group, Boca Raton), Appendix 1, 311-321, **2005**
[8] A.H. Varma and N. Padh, Biotechnology Journal, **2007**, 2, 386-392.
[9] S. O. Kotchoni, E. W. Gachomo, E. Betiku and O. O. Shonukan, Afr. J. Biotechnology, **2003**, 2: 88-90.
[10] V.M. Margam, E.W. Gachomo, J.H. Shukle, O.O. Ariyo, *Mol. Biol. Rep.*, **2010**, 37: 3631-3635.
[11] J. Sambrook, D.W. Russed, *Molecular Cloning*. A Laboratory Mannual 3rd edition. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, Yew York, USA, **2001**.
[12] D.B. Duncan, *Biometrics*, **1955**, 11: 1–42.
[13] W.M. Bandaranayake, Bioactives, *Wetland Ecol. Manage*., **2002**, 10: 421-452.
[14] B. Singh, R. Yadav, H. Singh, G. Singh and A. Punia, *Aust. J. Basic Applied Sci.*, **2010**, 4: 6570-6574.
[15] M. Mazid, T. A. Khan and F. Mohammad, *Biol. Med.*, **2011**, 3: 232-249.
[16] X. Beatriz, F. Ortega, L. Elenes and R. Ruiz-Medrano, *American Journal of plant physiology*, **2010**, 5, 241-256.
[17] K. Padmalatha and M.N.V. Prasad, *African Journal of Biotechnology*, **2006**, 5, 230-234.
[18] J.J. Doyle, L.J. Doyle, *Phytochem. Bull.*, **1987**, 19: 11–15.
[19] A Sahashrabudhe and M. Deodhar, *Int. J. Bot.*, **2010**, 6: 293-298.
[20] A. Dehestani and S. K. Kazemi Tabar, *Asian J. Plant Sci.*, **2007**, 6: 977-981.
[21] N. Joshi, R. A. Rawat, B. Subramanian and K. S. Rao, *Indian J. Sci. Technol.*, **2010**, 3: 1214-1217.
[22] H. S. Ginwal, N. Mittal, S. S. Maurya, S. Barthwal and P. Bhatt, *Indian Journal of Biotechnology*, **2011**, 10, 33-38.