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OPTIMIZATION AND COMPARISON OF EFFICIENCY BETWEEN TWO DNA ISOLATION PROTOCOLS IN *CYMBOPOGON* SPECIES

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Summary. The isolation of pure and high molecular weight genomic DNA is a pre-requisite for many molecular biology applications including the polymerase chain reaction. Various protocols are available for the isolation of DNA from plant materials. Two protocols: cetyl trimethyl ammonium bromide protocol and benzyl chloride protocol, have been optimized for isolation of genomic DNA in essential oil of *Cymbopogon* sp. as available standard protocols do not produce high quality PCR amplifiable DNA. Spectrophotometric and electroporetic analyses indicated that the isolated DNA according to both methods was highly pure and could be amplified by using RAPD primers.

***Key words*:** BCl_2; CTAB; *Cymbopogon sp.*; DNA isolation; RAPD.

INTRODUCTION

Medicinal and aromatic plant utilization and conservation have attracted global attention (Parrotta, 2001). Several of these medicinal and aromatic plant species contain exceptionally high amounts of polysaccharides, polyphenols, tannins, hydrocolloids (sugars and carragenans) and other secondary metabolites which will interfere with DNA isolation procedures. The procedures encounter in the isolation and purification of DNA especially from medicinal and aromatic plants include degradation of DNA by endonucleases, co-isolation of highly viscous polysaccharides, inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions. Moreover, the contaminating RNA that precipitates along with DNA causes many problems including suppression of PCR amplification (Pikkart and Villeponteau, 1993). Often different plant taxa may not permit optimal DNA yields from one isolation protocol, for example some closely related species of the same genus require different isolation protocols. Then an efficient protocol for DNA isolation is required. We have tested previously established DNA isolation protocols but these methods resulted in DNA with a lot of impurities and not very

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suitable for RAPD analysis.

Therefore, we have standardized here the total genomic DNA isolation protocol based on CTAB and BCl_2 methods originally developed for other plants (Murray and Thompson, 1980; Zhu et al., 1993). Modifications were made to minimize polysaccharide co-isolation and to simplify the procedure for processing a large number of samples. We also compared the yield and quality of the isolated DNA of the two methods (mod. CTAB method and BCl_2 method) used and their acceptability on the basis of their efficiency.

MATERIALS AND METHODS

Plant Material.

Sample slips of the field grown superior somaclones along with mother clone (control) of two species of *Cymbopogon* (*C. winterianus* and *C. flexuosus*) and field grown natural plants of *C. martinii* were used for DNA isolation.

Extraction buffer.

Method 1: 100 mM Tris-Cl (pH 8.0), 20 mM EDTA, 1.5 M NaCl, 2%CTAB,0.2% β-mercaptoethanol (v/v) (added immediately before use) and 1% PVP (w/v) (added immediately before use).

Method 2: Tris-Cl pH 8.0 (100 mM), EDTA pH 8.0 (40 mM).

DNA Extraction Protocol.

Method 1: Modified CTAB method.

Extraction Step. 1 g Young fresh leaf tissue in liquid nitrogen and rhizome tissues without liquid nitrogen was ground with mortar and pestle. To the homogenate 5 ml extraction buffer was added and mixed by inversion to slurry before incubating at 65°C for 1 h in a water bath with intermittent shaking. An equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed by inversion for about 1 min and centrifuged at 15000 rpm for 10 min at 4°C. The aqueous phase was pipetted out in a fresh polypropylene tube and 2/3 volume of ice cold isopropanol was added and mixed by quick gentle inversion for about 2 min. The DNA was precipitated by incubating either 30 min or overnight (12h) at -20°C and centrifuged at 6000 rpm for 10 min at 4°C. The supernatant was discarded gently and the pellet was washed with 1000 µl 70% chilled ethanol (v/v). Centrifugation was performed at 10000 rpm for 5 min at 4°C and steps were repeated 3-4 times. The pellet was air dried for about 30 min at room temperature and the pellet was re-suspended in 1 ml of sterile millii Q water or TE buffer.

Purification step. 10 µl of 10 mg/ml DNase free RNase was added and incubated at 37°C for 1 h. Then an equal volume of phenol: chloroform (1:1) was added and mixed by gentle inversion for 2 min. Centrifugation was performed at 11000 rpm for 10 min at 4°C and transferred the upper phase to a sterile micro centrifuge tube. The extraction was repeated with chloroform: isoamyl alcohol (24:1) twice followed by centrifugation at 5000 rpm for 5 min at 4°C. To the supernatant, 0.1 vol. of 3 M sodium acetate and 2/3 vol. of isopropanol was added and mixed by gentle inversion and incubated at -20°C for 15 min to precipitate DNA. After centrifuging at 10000 rpm for 5 min at 4°C the pellet was washed with 1ml of 70% chilled ethanol 3 times. The sample was air dried and the pellet was dissolved in 100 µl sterile milli-Q water or TE buffer.

Method 2: Benzyl Chloride Method.

1 g Of fresh young leaves was cut into small pieces of approximately 1cm^2 each. To each sample 5 ml of extraction buffer, 1 ml 10% SDS and 3 ml benzyl chloride was added. The tube was vortexed and incubated at 60°C for 30 min in a water bath with mixing by gentle swirling or repeated vortexing at 5-min intervals to keep the two phases thoroughly mixed. 3 ml 3M sodium acetate (pH 5.0) was added and the tube was kept on ice for 5 min. Centrifugation was carried out at 6000 rpm at 4°C for 15 min and the supernatant was collected. The DNA was precipitated by adding an equal volume of cold isopropanol and incubated on ice for 12 h or overnight. After centrifugation at 6000 rpm at 4°C for 15 min, the supernatant was discarded. The pellet was washed with 1000 µl 70% chilled ethanol and centrifuged at 5000 for 10 minutes at 4°C. The step was repeated 3-4 times and the pellet was dried. Finally the dried pellet was resuspended in TE buffer and after spinning at 2500 g the pellet was stored at -20°C.

Estimation of DNA quality and quantity.

The amount of DNA present in the solution was calculated from the absorption at 260 nm (A_{260}) and the purity of DNA was calculated by $A_{260/A280}$ (Sambrook et al., 2001). The quality of the extracted DNA was tested by running the DNA on 0.8% agarose gel. The gel was examined under ultraviolet transilluminator and photographed using Gel Doc (Bio Rad).

RAPD analysis.

The PCR of the isolated DNA was carried out using random decamer primers obtained from Bangaloe Genei Pvt. Ltd, Bangalore, India. The RAPD reaction was performed in 25µl reaction volume according to the procedure of Bhattacharya et al. (2008). The amplified products were separated on 1.5% agarose gel, visualized by staining the gel in 0.5 µg ml^{-1} ethydium bromide, and documented with the gel documentation system (Bio Rad).

RESULTS AND DISCUSSION

A sufficient amount of clean genomic DNA was obtained using the optimized CTAB protocol described in the Material and Methods. However, the quantity and quality of the isolated DNA from rhizome (91.31µg/g) tissue was significantly higher compared to leaf tissue (80.13 µg/g). Mean $A_{260/280}$ ratio of leaf DNA was 1.76, which was slightly less than rhizome DNA (1.81), indicating the presence of some proteins in leaf DNA (Table 1). This high purity and amount of rhizome DNA may be due to the meristematic nature of

Table 1. Comparison of DNA quantity and quality obtained from leaf and rhizome tissues of *Cymbopogon sp*. using a modified CTAB method. Results are expressed as means of 3 samples with SE (m). Means followed by different letters are significantly different at $P \leq 0.05$ (Duncan 1955).

Method	Organ	A_{260}/A_{280}	DNA yield [µg/g of fresh weight]
CTAB (mod.)	Leaf	$1.76^b \pm 0.01$	$80.13^b \pm 0.49$
CTAB (mod.)	Rhizome	$1.81^a \pm 0.01$	$91.31^a \pm 0.49$

the rhizome tissue. Another advantage of isolating DNA from rhizome tissue was that grinding with liquid nitrogen was not required as in leaf tissue grinding. The amount of DNA from leaf was four times (80.13 µg/g) higher using the modified CTAB compared to the BCl_2 method (21.81 µg/g) (Table 2). The spectrophotometric analysis at A_{260}/A_{280} revealed a higher ratio (1.80) when BCl_2 method was used compared to the modified CTAB method (1.76). Thus, among the two methods the modified CTAB method was more efficient with respect to yield, but benzyl chloride method was better when the objective was to isolate pure DNA. The CTAB method of extraction without modifications gave poor DNA yield, so we made several modifications to get better yield. Higher CTAB concentration (2%) was maintained in the extraction buffer as lysis of the membrane was accomplished by the CTAB detergent. 20 mM EDTA at pH 8.0 was used in the extraction buffer as a chelating agent that among other metals binds Mg. By binding Mg with EDTA, the activity of the present DNase can be decreased. Tris-Cl (100mM, pH 8.0) provided the solution a pH buffering capacity (a low or high pH damages DNA). Contamination of isolated DNA with polysaccharides hinders enzymatic reactions such as *Taq* DNA polymerase amplification (Pandey et al., 1996) and restriction endonuclease digestion (Abdulova et al., 2002). High concentration of NaCl in the modified CTAB method was maintained to overcome this problem and to separate DNA from the CTAB-DNA complex (Murray and Thompson, 1980; Paterson et al., 1993). Long term chloroform-isoamyl alcohol treatment ensures removal of chlorophyll and colouring substances such as pigments, dyes etc. On the other hand, in BCl_2 method benzyl chloride itself reacted with the -OH residues of polysaccharides (including cellulose, hemi-cellulose) and removed polysaccharides. So, we successfully amplified the isolated DNA using RAPD primers in all tested samples which clearly indicated the purity of DNA obtained by the two methods (Fig. 2 a, b). The DNA obtained following the two methods was unshared, showing little or no contamination with RNA (Fig. 1 a, b, c). In the modified CTAB method 60 min incubation at 65°C and in BCl_2 method 30 min incubation at 60°C were found to be necessary to obtain optimum results. Moreover, addition of high concentration of PVP and β-mercaptoethanol gave positive results in removing the polyphenols from *Cymbopogon* species in CTAB method (Khanuja et al., 1999; Puchooa and Khoyratty, 2004). Increasing the number (3-4 times) of washing in 70% chilled ethanol gave better DNA because it helped to remove the residual NaCl and/

Table 2. Comparison of DNA quantity and quality obtained from leaf tissues of *Cymbopogon sp.* using modified CTAB and BCl_2 methods. Results are expressed as means of 3 samples with SE (m). Means followed by different letters are significantly different at $P \leq 0.05$ (Duncan 1955).

Method	Organ	A_{260}/A_{280}	DNA yield [µg/g of fresh weight]
CTAB (mod.)	Leaf	$1.76^b \pm 0.01$	$80.13^a \pm 1.14$
BCl_2	Leaf	$1.80^a \pm 0.01$	$21.81^b \pm 1.14$

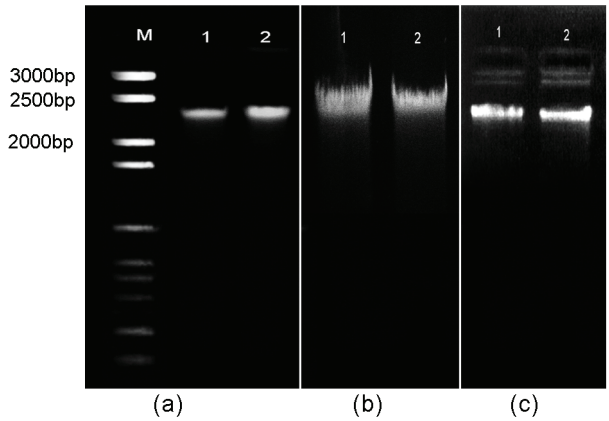

Fig. 1. Genomic DNA isolated from *Cymbopogon* species resolved on 0.8% agarose gel (Lanes 1 and 2: Propagated plant and somaclonal variant of *Cymbopogon*). (a) Genomic DNA isolated from rhizome tissues by a modified CTAB method. M= Marker, λ DNA digested with *EcoRI* and *Hind-III*; (b) Genomic DNA isolated from leaf tissues by a modified CTAB method; (c) Genomic DNA isolated from leaf tissues by the BCl_2 method.

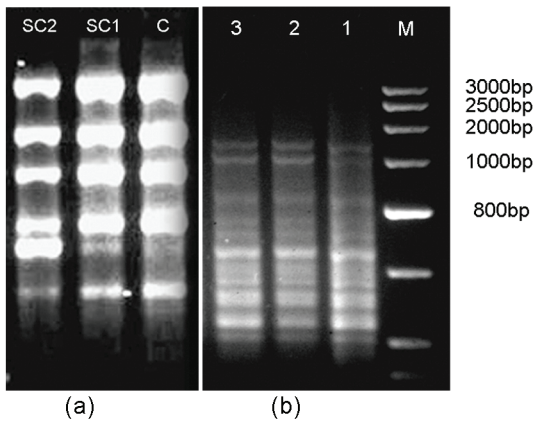

Fig. 2. PCR amplification of *Cymbopogon* DNA. (a) RAPD profile generated by MS10G8 (5'CGGGATCCGC3') primer from genomic DNA isolated using a modified CTAB method from control plants (C) and somaclones (SC1, SC2) of *C. winterianus*; (b) RAPD profile generated by MS10G1 (5'AAATCGGAGC3') primer from genomic DNA isolated by the BCl_2 method from field grown plants (lane 1-3) of C. *winterianus*. M= Marker, λ DNA digested with *EcoRI* and *Hind-III.*

or CTAB (CTAB is soluble in ethanol) in both methods. Using isopropanol and sodium acetate was also found to be efficient in removing polysaccharides and secondary metabolites from DNA in both methods (Xu et al., 2004). Many DNA isolation procedures also yield large amounts of RNA (Doyle and Doyle, 1987). Large amounts of RNA in the sample can chelate Mg^{++} and reduce the yield of PCR. In the modified CTAB method RNA contamination can be removed by digesting the sample with RNase, followed by phenol extraction and precipitation (Keb-llanes et al., 2002). This prolonged RNase treatment degraded

RNA into small ribonucleotides that did not contaminate the DNA preparations and yielded RNA-free DNA. However, RNA did not interfere with amplification of DNA during RAPD in our laboratory. Additional precipitation steps removed large amounts of precipitants like proteins and polysaccharides by centrifugation. DNA degradation and precipitation were avoided due to this modification. We found that these modified steps in CTAB method were absolutely necessary to standardize and increase quality and quantity of genomic DNA. Though the yield of DNA in BCl_2 method was less compared to the modified CTAB method, it had some advantages over other methods of DNA isolation. Firstly, this method can be efficiently used in laboratories where availability of liquid nitrogen is an additional impediment because freezing of samples in liquid nitrogen is not required. Secondly, this method avoids tedious homogenizing of each sample with mortar and pestle or a mechanical homogenizer. Thirdly, extraction with phenol-chloroform or chloroform-isoamyl alcohol is not necessary here. Fourthly, this method does not require gradient sedimentation with cesium chloride or precipitation with CTAB. Moreover, it requires minimal amounts of reagents and expensive laboratory materials. In additon, this method substantially saves labour, cost and time relative to some other methods. Thus, it can be concluded that both methods described here can be used to isolate DNA from aromatic plant species (*Cymbopogon*) depending on the laboratory setup and objectives of isolation. The isolated DNA can be used for characterization of crops, screening and selection of varieties and identification of somaclonal variants by molecular markers like RAPD, RFLP, ISSR, etc.

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