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RAPID DNA ISOLATION AND ISSR-PCR OPTIMIZATION FOR FIBROUS LEAF TISSUES OF WILD PALMS OF SOUTHERN PENINSULAR INDIA

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Abstract

Isolation of pure DNA is a significant component for PCR amplification and DNA fingerprinting analysis of plant species. Leaves of wild palms are generally hard, enormously fibrous, and are difficult to grind. The 25 species belong to 9 genera such as Areca (one species), Arenga (one species), Bentickia (one species), Borassus (one species), Calamus (fifteen species), Caryota (one species), Corypha (one species), Phoenix (three species) and Pinanga (one species) of the family Arecaceae and were collected from various parts of Southern India. An experiment was done to isolate the high molecular weight DNA from fibrous young leaves of twenty five wild palm species followed by several modifications in the novel CTAB DNA isolation method. DNA was isolated with slight modification in the CTAB method with liquid nitrogen and the quantification of obtained DNA was measured using a spectrophotometer and 0.8% agarose gel electrophoresis. DNA was further diluted with $T_{10}E_1$ buffer and optimized for ISSR-PCR amplification. Hence, the described protocol has proven to be advantageous due to its simple, efficient, affordable reagents resulting in a high molecular weight DNA of good quality from leaf fibrous tissues.

Key words: *Arecaceae, CTAB, genomic DNA isolation, ISSR, Western Ghats.*

INTRODUCTION

Palms are woody monocotyledons belongs to family Arecaceae or Palmae. They are one of the prominent biotic components of the forest ecosystem. Oftenly they fascinate with graceful architecture; they usually dominate the landscape of tropical habitats by providing wide range of utility for human life. Palms have been extensively exploited by the local communities for food, fodder, handicrafts and construction purpose. Food and allied products have gained importance due to its nutritional quality and also the fibrous tissue present in palm species is very flexible, strong and has a significant role in furniture industry. Thus, a large number of inhabitants depend upon the palms and palm byproducts for their livelihood, which ultimately leads to the consumption of land areas for supplying physical space and expanding population and industry. Globally, there are 211 genera consists of 3000 palm species, whereas in India there are about 22 genera and 105 species present in three major geographical regions such as Peninsular India, Northeastern India and Andaman and Nicobar Islands (Takhtajan, 1987). Palms are less distributed in few regions of India, especially in Gangetic plains and in the lower hill valleys of North India.

Various molecular biology tools were utilized for isolation of genomic DNA to attain appropriate purity. Several conventional methods have been established for isolation of pure and integral DNA from plant tissues (Saghai Maroof et al., 1984; Doyle & Doyle, 1990; Scott & Playford, 1996; Sharma et al., 2000; Pirttilä et al., 2001; Drábková et al., 2002; Shepherd et al., 2002; Mogg & Bond, 2003; Haymes, 1996). Thus, plant species comprises of same or related genera may exhibit tremendous variability in the complex pathways of expendable functions. Due to variability among species or genera the same DNA extraction procedure cannot be utilized for all the plant species (Porebski et al., 1997; Ribeiro & Lovato, 2007; Fatemeh et al., 2018). Whereas, cetyltrimethylammonium bromide

(CTAB) process and its modifications have proved as one of the prominent methods for good quality DNA for amplification based on polymerase chain reaction (PCR) downstream applications. Numerous commercial extraction kits are available such as DNeasy Plant Mini Kits but due to high cost price, sensitivity for certain temperature, the constituent of the buffers is unknown to the user, and the lysis step is not always sufficient for some types of plant material (Ahmed et al., 2009). So using such commercially available kits hence proved to be laborious, time consuming and expensive. Certain limitations necessitate the advancement of universally accepted protocol for isolating DNA from diverse plant species. Hence, in the present study genomic DNA isolation protocol was optimized for 25 species that belong to 9 genera such as *Areca* (one species), *Arenga* (one species), *Bentickia* (one species), *Borassus* (one species), *Calamus* (fifteen species), *Caryota* (one species), *Corypha* (one species), *Phoenix* (three species) and *Pinanga* (one species) of the family Arecaceae. This method does not require any hazardous chemicals. Based on the quantity and quality of the DNA obtained by this modified protocol is well enough to perform thousands of PCR-based reactions and also used for DNA manipulation techniques, such as restriction digestion, AFLP, Southern blotting and cloning. Due to its low cost price, consumption of short time period makes this method one of the phenomenal technique for isolating genomic DNA in wild palms. The main objective to develop this protocol was to make this technique readily available for isolating highly pure genomic DNA from fibrous leaves of wild palm and to optimize the DNA concentration. The isolated high quality genomic DNA is amenable to ISSR (Inter-Simple Sequence Repeats).

MATERIALS AND METHODS

Collection of Samples

Young leaves of twenty-five wild palm belong to 9 genera which are basically an endemic, endangered and rare species which possess limited distribution range and only available in selected regions with the elevation of 400 m to 2600 m were collected from KFRI (Palmetum), Peechi, Kerala and some of them were also collected from wild habitats of Western Ghats region of Chikmagalur, Shivamogga, Dakshina Kannada and Uttara Kannada districts of Karnataka. Soon after collection, leaf samples were transferred to the laboratory and washed in 70% alcohol, blotted with tissue paper, quickly frozen by dipping in liquid nitrogen and kept at -80°C until further use.

Reagents, Chemicals and Laboratory Materials

CTAB extraction buffer consisted of 2% CTAB, 5 M NaCl, 0.5 M EDTA and 1 M Tris-HCl. Stock solutions for the different CTAB buffer components were prepared to homogenize the following protocol. Other reagents and materials such as 3% β-mercaptoethanol, 15% PVP-4.0, propanol, ethanol (absolute), Tris-EDTA ($T_{10}E_1$) buffer solutions were prepared. Laboratory pestle and mortar, 1.5 and 2 mL microcentrifuge tubes, micropipettes, microtips, pair of scissors, and a 40-well holding racks were used for the experiments. Centrifugation was done by Centrifuge 5415-R at maximum speed of 14000 rpm.

DNA Extraction and Quantification

CTAB method was followed for the extraction and isolation of DNA as prescribed by Doyle and Doyle (1987). Several modifications were made to the original protocol in order to fit with the current experimental conditions. The CTAB buffer was prepared using 2% cetyltrimethylammonium bromide, 1 M Tris-HCl pH 8.0, 0.5 M EDTA pH 8.0, 5 M NaCl, 3% β-mercaptoethanol and 2% PVP. About 0.5-1 g leaf sample was ground into fine powder using liquid nitrogen in a mortar and pestle and 4 ml of preheated 2× CTAB lysis buffer at 65 to make the paste. The mixture is incubated at 65°C for 90 minutes for lyses and lysate was extracted with phenol: chloroform: isoamyl alcohol (25: 24: 1) upon cooling to room temperature. The DNA is precipitated by adding an equal quantity of prechilled isopropanol. The DNA pellet is washed twice in 70% alcohol, air dried, dissolved in $T_{10}E_1$

and stored at -20°C until further use. Gel electrophoresis of genomic DNA was done with 0.8% agarose gel in 1× TAE buffer (Mini Sub System, BioRad, India). The image of the gel obtained is shown in Figure 1.

ISSR-PCR based Amplification using Extracted DNA Samples

An experiment was conducted to optimize DNA concentration by Polymerase Chain Reactions (PCR). A total volume of 10 µl PCR reaction mixture consisted of Taq buffer, $MgCl_2$, primers (UBC 834 AGAGAGAGAGAGAGAGYT), dNTPs, Milli Q water and Taq polymerase (3 U/µl), along with DNA sample. PCR amplification was carried out in a 10 µl reaction volume containing 1 µl genomic DNA, Taq Buffer 1.5 µl, $MgCl_2$ 1 µl, UBC 834 1 µl, dNTPs 1 µl, Milli Q water 3.75 µl and Taq Polymerase 0.25 µl. Amplification was carried out in initial denaturation step at 95°C for 3 min, followed by 32 cycles denaturation 95°C for 30 sec. UBC 834 Primer annealing temperature was 59.1°C, for 45 sec, 72°C for 2 min (primer extension) and a final extension at 72°C for 10 min. Electrophoresis was carried out to perform ISSR-PCR products on 1.8% agarose gel with 1× TAE and a standard 1 kb ladder.

Agarose Gel Electrophoresis

The PCR products were visualized on a 1.8% agarose gel with a standard 50 bp ladder. Agarose gels were prepared using 1× TAE buffer. A concentration of 0.6 µl of ethidium bromide was added to the gel for visualization of the bands. The PCR product mixture was loaded along with loading dye 0.0042 g bromophenol blue in 0.0607 g of sucrose solution. The gel profile of each ISSR primer amplicons was visualized in a UV Trans-illuminator Gel Documentation System (Vilber Lourmat Infinity-1000/26 M).

RESULTS AND DISCUSSIONS

High yield pure DNA was obtained for twenty-five different species of Arecaceae members followed by few modifications in the novel CTAB DNA isolation protocol (Doyle and Doyle, 1987) (Table 1). Fresh leaf samples are recommended for genomic DNA isolation. If any tissues are immersed in liquid nitrogen it will be brittle hard to ease crushing into powder, the main purpose is to maintain low temperature of plant material. This step can be skipped for spongy tissues and easy to grind material like flower petals. Cellular DNA extraction from hard, fibrous leaves of palm is very complex when compared to flower petals and soft leaves. Most of the developing countries still facing problem of unavailability of liquid nitrogen as its storage and maintenance also quite difficult. Cetyltrimethylammonium bromide (CTAB) is one of the phenomenal methods for DNA extraction from a variety of plant materials (Sambrok et al., 1989). Generally, there will be certain contaminants associated with plant DNA interfere PCR reactions such as polyphenolic compounds and polysaccharides (Krishna et al., 2012).

Table 1. Qualitative Analysis of Genomic DNA Isolated from Wild Palms of Southern Peninsular India

Palm Species	OD Ratio	DNA ng/µL
Areca triandra Roxb.	1.85	763.7
Arenga wightii Griff.	1.85	470.6
Bentinckia condapanna Berry ex Roxb.	1.75	297.6
Borassus flabellifer Linn.	1.96	797.7
Calamus brandisii Becc.	1.98	532.8
Calamus delessertianus Becc.	1.97	381.1
Calamus dransfieldii Renuka	1.99	225.2
Calamus hookerianus Becc.	1.99	512.4
Calamus karnatakensis Renuka & Lakshmana	1.78	209.3
Calamus lacciferus Lakshmana & Renuka	2.02	437.5
Calamus lakshmanae Renuka	1.89	398.0
Calamus metzianus Schltdl.	1.89	215.9
Calamus nagbettai R.R. Fernald & Dey	1.98	195.8
Calamus prasinus Lakshmana & Renuka	1.97	235.7
Calamus stoloniferus Renuka	1.91	352.3
Calamus thwaitesii Becc.	1.83	228.8
Calamus travancoricus Bedd.	1.88	202.6
Calamus vattayila Renuka	1.78	672.4
Calamus viminalis Willd.	1.97	816.3
Caryota urens L.	2.01	254.8
Corypha umbraculifera L.	1.69	748.3
Phoenix loureiroi Kunth	1.88	280.4
Phoenix pusilla Gaertn.	1.78	249.8
Phoenix sylvestris (L.) Roxb.	1.96	286.5
Pinanga dicksonii (Roxb.) Blume.	1.81	507.1

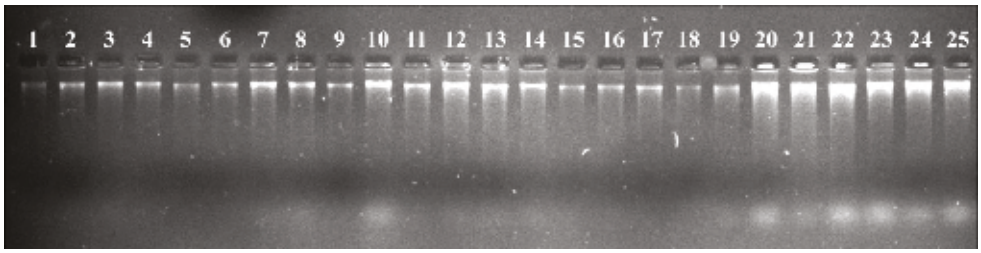

Figure 1. Genomic DNA Isolated from Twenty Five Wild Palms: 1 – *Areca triandra*; 2 – *Arenga wightii*; 3 – *Bentinckia condapanna*; 4 – *Borassus flabellifer*; 5 – *Calamus brandisii*; 6 – *C. delessertianus*; 7 – *C. dransfieldii*; 8 – *C. hookerianus*; 9 – *C. karnatakensis*; 10 – *C. lacciferus*; 11 – *C. lakshmanae*; 12 – *C. metzianus*; 13 – *C. nagbettai*; 14 – *C. prasinus*; 15 – *C. stoloniferus*; 16 – *C. thwaitesii*; 17 – *C. travancoricus*; 18 – *C. vattayila*; 19 – *C. viminalis*; 20 – *Caryota urens*; 21 – *Corypha umbraculifera*; 22 – *Phoenix loureiroi*; 23 – *P. pusilla*; 24 – *P. sylvestris*; 25 – *Pinanga dicksonii*

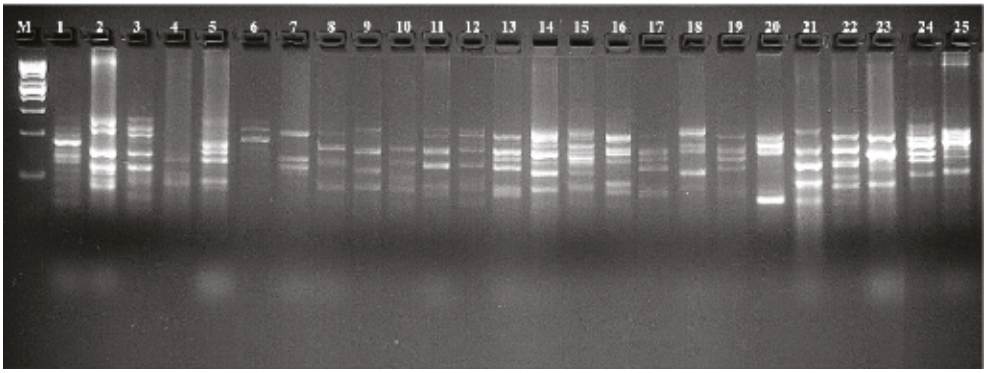

Figure 2. Optimization of DNA Concentration for ISSR-PCR: M-1kb DNA Ladder: 1 – *Areca triandra*; 2 – *Arenga wightii*; 3 – *Bentinckia condapanna*; 4 – *Borassus flabellifer*; 5 – *Calamus brandisii*; 6 – *C. delessertianus*; 7 – *C. dransfieldii*; 8 – *C. hookerianus*; 9 – *C. karnatakensis*; 10 – *C. lacciferus*; 11 – *C. lakshmanae*; 12 – *C. metzianus*; 13 – *C. nagbettai*; 14 – *C. prasinus*; 15 – *C. stoloniferus*; 16 – *C. thwaitesii*; 17 – *C. travancoricus*; 18 – *C. vattayila*; 19 – *C. viminalis*; 20 – *Caryota urens*; 21 – *Corypha umbraculifera*; 22 – *Phoenix loureiroi*; 23 – *P. pusilla*; 24 – *P. sylvestris*; 25 – *Pinanga dicksonii*

Polyphenols are potent oxidizing agent present in various plant species which are capable of decrease in the yield and purity of the DNA (Katterman & Shattuck, 1983; Peterson & Boehm, 1997; Porebski et al., 1997). Avoidance of contamination is made by adding PVP along with CTAB which promotes to bind with polyphenolic compounds by forming a compound with hydrogen bonds (Maliyakal, 1992).

Secondary metabolites such as phenolics, terpenes and alkaloids are very difficult to separate from DNA (Ziegenhagen & Scholz, 1998). For removal of polysaccharides and to avoid damage in leaf tissue, NaCl is used in DNA extraction buffer (Fang et al., 1992). Phenol: chloroform: isoamyl alcohol is used for exclusion of chlorophyll and various coloring agents such as pigments and dyes. To remove detergents and proteins precipitation steps were increased by increasing the speed and time of centrifugation. The modification necessitates amplifying high quality and quantity of genomic DNA. The purity and quantity may differ among applications (Hussein et al., 2005; Zidani et al., 2005).

The minimum OD 1.69 and maximum of 2.02 was observed (Table 1). High quality DNA was obtained from all the wild palm leaf samples and amount of DNA isolated between 195.8 µg/ml and maximum 816.3 µg/ml. Reading was taken using spectrophotometer. Both the nucleic acids (DNA and RNA) absorb at 260 nm, so evaluation of concentration of DNA

sample is done with same spectrophotometric conditions.
A protein also absorbs light at this wavelength so it is possible to obtain contamination of protein in high concentration which gives a false result in sample. Though, protein, also absorb light at 280 nm by evaluating both A260 and A280 it is easy to measure the ratio of nucleic acid to protein in the solution and thus estimate the accuracy of DNA concentration. The absorbance ratio obtained was (A260/A280) 1.8-2.0 is considered acceptable (Sambrook & Russell, 2001; Iruela et al., 2002; Wang et al., 2011; Wang et al., 2012). Lower values comprise of high level of protein contamination and estimation of the DNA concentration will not be accurate.

It is necessary to obtain high quality DNA that is proportionately free from the various contaminants found in plant cells (Shiv et al., 2017). Naturally large amount of proteins are found in most of the plant species and several other components are rectified which can bind firmly to nucleic acids during isolation of DNA and can interfere in DNA amplification (Angeles et al., 2005; Ribeiro & Lovato, 2007). Storing of isolated DNA is a significant factor, generally at -20°C gave positive percentage. This is an expected result because room temperature is certainly not a suitable condition for storing isolated DNA (Supriya et al., 2019).
The DNA obtained was used for PCR analysis and possess high intensity amplification. PCR amplification also showed that the DNA was of high quality, free from several interfering compounds and that it would be capable for various other DNA analyses such as ISSR (Lavanya et al., 2008; Sunil Kumar et al., 2012; Shafiei-Astani et al., 2015). The present study was carried out to optimize the concentration of total genomic DNA for the PCR using ISSR primers. The amplification of DNA from PCR analysis with ISSR primer was clear as shown in Figure 2.
The absence of RNA, polysaccharides and the amplification of molecular bands are evident of a good DNA quality with modified Doyle and Doyle protocol. The satisfactory quality and DNA yield was obtained from this protocol and hence considered as a simple, cost effective and time saving protocol can further be used for extraction of DNA from large populations of wild palm.

CONCLUSIONS

Here we have illustrated a very efficient, simple, cost-effective and less time consumable modified CTAB DNA extraction method that provides high-quality DNA from wild palms containing an elevated concentration of polyphenolic compounds and polysaccharides. For removal of polysaccharides and to avoid damage in leaf tissue, 5 M NaCl is used in DNA extraction buffer. This method eliminates the need to use environmentally hazardous chemicals to obtain high-quality genomic DNA. The resulting optimized CTAB protocol facilitates the isolation of high quality genomic DNA amenable to ISSR and various other processes such as enzyme digestion and cloning techniques. A fibrous leaf of palm is very complex when compared to flower petals and soft leaves, this method proved to be phenomenal especially the plant with high fibrous tissue. The CTAB percentage was increased though there will be certain contaminants associated with plant DNA interfere PCR reactions such as polyphenolic compounds and polysaccharides. Prevention of contamination is made by adding PVP along with CTAB which promotes to bind with polyphenolic compounds by forming a compound with hydrogen bonds. Therefore this method can be recommended for low-technology laboratories for high-throughput sample preparation suitable for various molecular analytical techniques.

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