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OPTIMIZATION OF DNA ISOLATION AND RAPD TECHNIQUE IN ARECANUT (*Areca catechu* L.)

Rajesh M. K.[1], Bharathi M.[2], Nagarajan P.[2]

[1] Division of Crop Improvement, Central Plantation Crops Research Institute, Kasaragod 671124, Kerala, India.
[2]Department of Plant Molecular Biology and Biotechnology, CPMB, Tamil Nadu Agricultural University, Coimbatore 641003, TN, India.

A simple and efficient protocol for extracting high quality DNA from arecanut (*Areca catechu* L.) leaves is presented. DNA yield and purity were monitored by gel electrophoresis and by determining absorbance at UV (A_{260}/A_{280}). The ratio was between 1.7 to 1.9 indicating that the presence of contaminating metabolites was minimal. The quantities of DNA obtained were 100- 400 ìg/g starting material. The isolated DNA proved amenable to PCR amplification and restriction digestion. DNA was completely digested with the three restriction enzymes (*Eco*R I, *Eco*R V and *Hin*d III) confirming the purity of the extracted DNA. Using the isolated DNA, the parameters for randomly amplified polymorphic DNA (RAPD) protocol was standardized.

Key words: Arecanut palm, gel electrophoresis, enzimes, EcoR I, EcoR V, Hind III

Otimização de isolado de DNA da arequeira (*Areca catechu L.)* e a técnica do RAPD. Se apresenta um protocolo simples e eficiente para extrair DNA de folhas da palmeira Arequeira (*Areca catechu L.)*. A produção e qualidade do DNA foi monitorada por eletroforese em gel e determinada a absorvância UV (A_{260}/A_{280}). A proporção extraída de DNA esteve entre 1.7 e 1.9, indicando que a presença de matabolitos foi mínima. As quantidades de DNA obtidas variaram entre 100 e 400 µg/g do material inicial. O DNA isolado provou ser apropriado para amplificação via PCR e digestão restrita. O DNA foi digerido em sua totalidade por três enzimas de restrição (Eco RI, Eco RV e Hind III) confirmando-se a pureza do DNA. Usando-se o DNA isolado foram uniformizados os parâmetros do protocolo para a amplificação polimórfica ao acaso do DNA (RAPD).

Palavras-chave: Palma arequeira, eletroforesis, emzimas, EcoR I, EcoR V, Hind III

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Introduction

Molecular techniques require isolation of genomic DNA of suitable purity for PCR and restriction enzyme digestion. The problem often encountered during DNA extraction is to separate DNA from naturally occurring plant cell contaminants such as polysaccharides and polyphenolic compounds (Porebski *et al*., 1997). Palms contain inhibitor compounds like polyphenols and other secondary metabolites, which directly or indirectly interfere with enzymatic reactions (Reynolds & Murashige, 1979). Presence of polyphenols, which are powerful oxidizing agents, can also decrease the yield and purity of extracted DNA. Various protocols have been developed for extraction of DNA from palms such as oil palm (Jack *et al*., 1995), coconut (Rhode *et al*., 1995; Upadhyay *et al*., 1999) and date palm (Aitchitt *et al*., 1993; Ouenzar *et al*., 1998). No work has been carried out so far in arecanut.

In the present work, a rapid protocol for isolation of high quality and quantity of DNA from mature leaves of *Areca catechu* L. is presented. Conditions were also optimized for RAPD analysis using the isolated DNA.

Materials and methods

Collection of plant materials

Mature leaves were collected from yielding palms of arecanut cultivars from India *viz*., *Sreemangla*, *Sumangla*, *Mohitnagar* and *Hirehalli Dwarf*. Fresh samples were used for extraction immediately or refrigeration (4ºC) allows extraction to be delayed for several days.

DNA extraction protocol

A modified protocol of Doyle and Doyle (1990) was carried out, which is described below:

1. Weigh out 1g of starting leaf material. Cut into small bits just prior to grinding.

2. Cool the pestle and mortar and grind the leaves to a fine powder in the presence of liquid nitrogen.

3. Add PVPP (5%) to the powdered leaf sample.

4. Transfer the powdered sample to 50 ml polypropylene tube containing 4.5 ml extraction buffer (100mM Tris-HCl, pH 8.0, 1.5 M NaCl, 25 mM EDTA, 2% SDS)

5. Incubate the mixture for one hour at 60ºC, mixing 2-3 times during incubation by inverting the tube.

6. Add 5 ml of chloroform: isoamyl alcohol (24:1) and mix gently by swirling the tubes for 15 minutes.

7. Centrifuge the tubes for 15 minutes at 10,000 rpm at 4 ºC.

8. Transfer the supernatant to a new tube.

9. Add 0.7 volume of ice-cold isopropanol and gently mix the tube by inverting.

10. Pool the precipitated DNA with the help of a microtip and transfer to a 1.5 ml microfuge tube.

11. Wash the DNA twice with 70% alcohol.

12. Dry the pellet and dissolve it in 500 ìl TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA).

13. Add 5 ìl RNase solution (10 mg/ml) and incubate for 30 minutes at 37 ºC.

14. Repeat chloroform: isoamyl alcohol extraction.

15. Precipitate DNA by addition of ice-cold isopropanol.

16. Pool the precipitated DNA with the help of a microtip and transfer to a 1.5 ml microfuge tube.

17. Wash the DNA thrice with 70% alcohol.

18. Dry the pellet and dissolve it in 500 ìl TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA).

Determination of DNA quantity and quality

The DNA was quantified by measuring absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) in a UV spectrophotometer and A_{260}/A_{280} ratio was taken to evaluate the purity of DNA. The quality of extracted DNA was also checked by electrophoresis in 0.8% agarose gel.

Restriction analysis

The quality of the extracted DNA was further confirmed by digestion with *Eco*R I, *Eco*R V and *Hin*d III restriction enzymes (M/s Bangalore Genei, India). Digestion was performed under the conditions recommended by the manufacturers. Reagents were mixed, incubated for overnight at 37ºC, after which were loaded in 0.8% agarose gel. Electrophoresis was performed in a horizontal gel electrophoresis system at 75 V. Gels were run at 75 V for 2 hours in a BIO-RAD sub-cell electrophoresis unit, stained with ethidium bromide and visualized on a UV-transilluminator.

Optimization of RAPD reaction for arecanut DNA

For the optimization of RAPD reaction (Williams *et al*., 1990) using the DNA extracted from arecanut, the decamer primer OPB6, from M/S Operon Technologies, was used. The assays for optimization tested:

1. Template DNA: Five levels *viz*., 10, 20, 30, 40, 50 ng

2. *Taq* polymerase concentration (M/s Bangalore Genei, India): Five levels *viz*., 0.3, 0.5, 0.7, 1.0, 1.2 Units.

3. Magnesium chloride concentration: Five levels *viz*., 1.5, 2.0, 2.5, 3.0, 3.5 mM.

4. Primer concentration: Five levels *viz*., 2.5, 5.0, 7.5, 10, 12.5 pmoles.

5. dNTPs concentration (M/s Bangalore Genei, India): 100, 150, 200, 250, 300 ìM.

5. Annealing temperature: Eight levels *viz*., 37, 40, 42, 45, 47, 50, 53, 55 ºC.

DNA amplification was performed in a 10µl volume. To determine the effect of a single parameter the given parameter was varied, keeping the rest constant. A negative control (blank) containing all components of typical PCR reaction except the template DNA was used in every

experiment. The PCR amplification of arecanut DNA was performed on an Eppendorf Gradient Master Cycler programmed for 4 minutes initial denaturation at 94°C followed by 1 minute denaturation at 94°C, 1 minute annealing at the different temperatures tested and 2 minutes primer extension at 72°C for a total of 40 cycles. A final extension at 72°C was given for 7 minutes. The PCR products were stored at -20°C until electrophoresis.

10 µl of each PCR reaction, together with 2 µl of 6 X loading dye were separated by gel electrophoresis in 1.2% agarose with 1x Trizma base- boric acid-EDTA buffer (TBE) as the running buffer. Gels were run at 100V for 2 hours in a BIO-RAD sub-cell electrophoresis unit, stained with ethidium bromide and visualized on a UV-transilluminator.

Results and Discussion

DNA yield and purity

Large quantities of high molecular weight DNA was extracted from arecanut leaves by using the simple DNA extraction method. DNA yield ranged from 100- 400 ìg/g starting material. The ratios from absorbency at A_{260}/A_{280} ranged from 1.7 to 1.9 showing that the DNA was of high purity. DNA quality was also estimated by agarose gel electrophoresis of the genomic DNA. High molecular weight, intact DNA was visualized (Figure 1).

DNA was completely digested with the three restriction enzymes (*Eco*R I, *Eco*R V and *Hin*d III) further confirming the purity of the extracted DNA (Figure 2).

Optimization of RAPD parameters

The parameters for RAPD technique for arecanut were optimized:

Annealing temperature

The selection of the annealing temperature is possibly the most critical component for optimizing the specificity of a PCR reaction. The annealing temperature is a function of the length and base composition of the primer as well as the ionic strength of the reaction buffer. Eight levels of annealing temperatures were tested viz., 37, 40, 42, 45, 47, 50, 53 and 55°C. Amplification at 40ºC gave good scorable bands (Figure 3).

Template DNA

All the five levels of template DNA tested, viz., 10 ng, 20 ng, 30 ng, 40 ng and 50 ng were amplified but DNA concentration of 30 ng gave good scorable bands.

Primer concentration

Optimal primer sequences and appropriate primer concentrations are essential for maximal specificity and efficiency in PCR. Of the five levels of primer concentration tested *viz.*, 2.5, 5.0, 7.5, 10, 12.5 pmoles, all the levels gave amplification but 2.5 pmoles gave optimum amplification products (Figure 4). As the primer concentrations were increased, the number of amplicons decreased. Higher primer concentration may promote mis-priming and accumulation of non-specific products and increase the probability of generating primer-dimer artifacts (Innis and Gelfand, 1990).

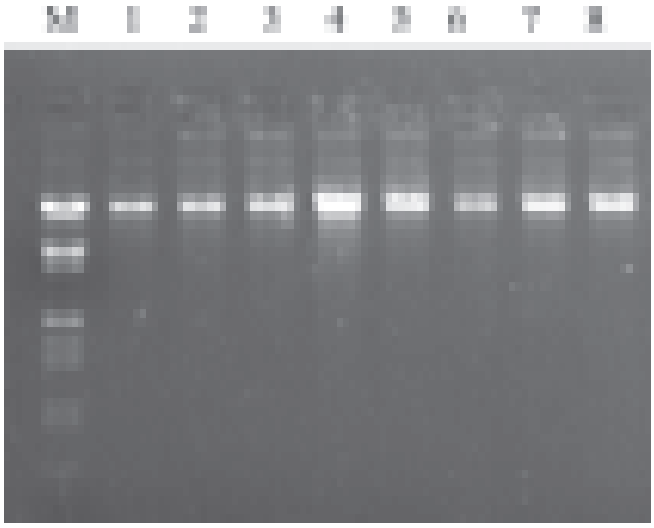

Figure 1. Agarose gel analysis of genomic DNA isolated from arecanut leaves. Lane 1, 2: *Sreemangla;* Lane 3, 4: *Mohitnagar;* Lane 5, 6: *Sumangla;* Lane 7, 8: *Hirehalli Dwarf;* M: Molecular weight marker (λ DNA *EcoR* I/*Hind* III double digest).

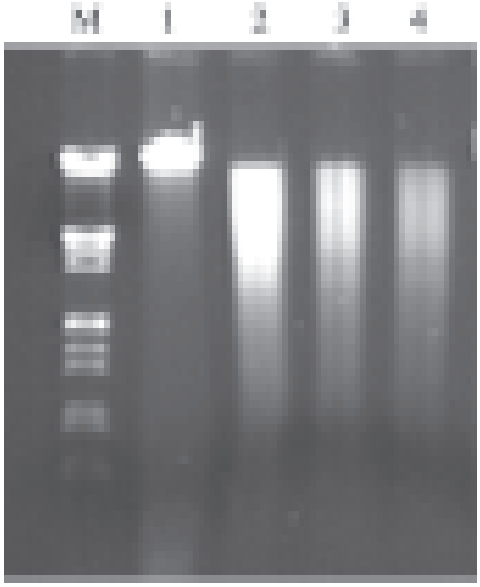

Figure 2. Restriction digestion of genomic DNA from arecanut leaves. Lane 1: Intact arecanut DNA; Lane 2: Arecanut DNA restricted with *Eco*R I; Lane 3: Arecanut DNA restricted with *Eco*R V; Lane 4: Arecanut DNA restricted with *Hin*d III; M: Molecular weight marker (l DNA *EcoR* I/*Hind* III double digest).

Concentration of Taq polymerase

The amount of Taq polymerase, which gave optimum amplification products, was 0.7 Units per reaction. When

the Taq polymerase concentration was reduced, insufficient amounts of desired products were produced. Non-specific products were formed and the resolutions of the bands were decreased at higher enzyme levels.

Concentration of $MgCl_2$

Lower $MgCl_2$ concentrations yielded more number of amplification products while higher $MgCl_2$ concentrations failed to yield visible bands. The optimum $MgCl_2$ concentration in the reaction mix was 2.5 mM. The $MgCl_2$ concentration may affect one or all of the following: primer annealing, strand association temperature of both template and PCR product, product specific formation of primer-dimer artifacts and enzyme activity and fidelity (Innis and Gelfand, 1990).

Concentration of dNTPs

Of the five levels of dNTPs concentration tested, 300 µM gave good and scorable bands. Lower levels (100 µM and 150 µM) failed to yield visible bands.

Conclusion

The DNA extraction protocol described here is rapid and technically easy. Sufficient quantities of pure and high-molecular weight DNA could be extracted from arecanut leaves. The DNA was suitable for PCR-based assay (RAPD) and digestion with restriction enzymes. For RAPD, best results were obtained with 30 ng DNA, 2.5 pmoles of primer, 0.7 Units of Taq polymerase, 2.5 mM of $MgCl_2$, 300 µM of dNTPs and an annealing temperature of 40°C. This is the first report of DNA extraction and RAPD analysis in arecanut.

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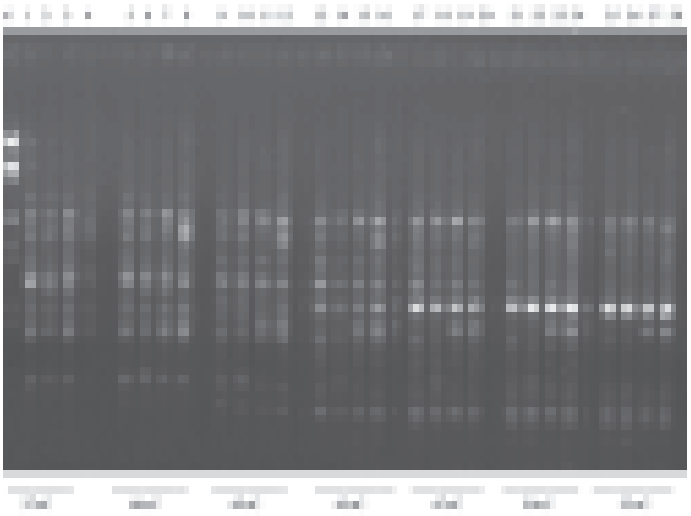

Figure 3. RAPD profile of arecanut cultivars using different annealing temperatures. Lanes 1, 5, 9, 13, 17, 21, 25: Sreemangla; Lanes 2, 6, 10, 14, 18, 22, 26: Mohitnagar; Lanes 3, 7, 11, 15, 19, 23, 27: Sumangla; Lanes 4, 8, 12, 16, 20, 24, 28: Hirehalli Dwarf; M: Molecular weight marker (λ DNA EcoR I/Hind III double digest).

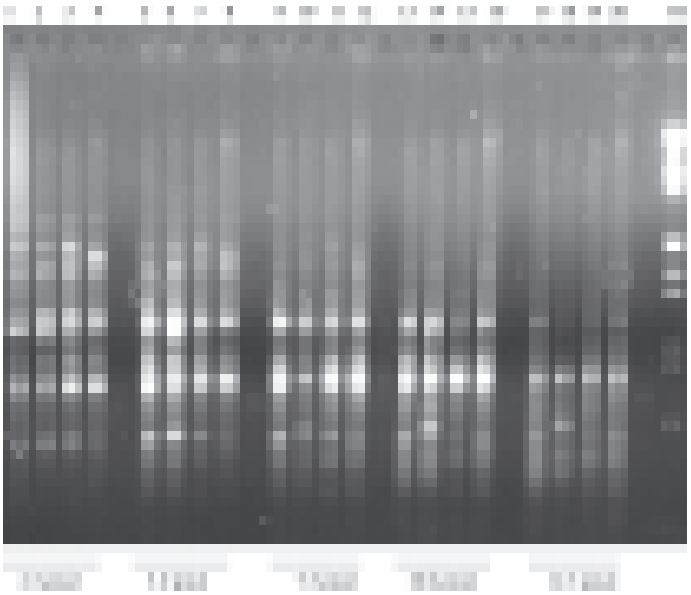

Figure 4. RAPD profile of arecanut cultivars using different primer concentrations. Lanes 1, 5, 9, 13, 17: Sreemangla; Lanes 2, 6, 10, 14, 18: Mohitnagar; Lanes 3, 7, 11, 15, 19: Sumangla; Lanes 4, 8, 12, 16, 20: Hirehalli Dwarf; M: Molecular weight marker (l DNA EcoR I/Hind III double digest).

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