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An Alternative Method of Plant DNA Extraction of Cashew (*Anacardium occidentale* L.) for Randomly Amplified Polymorphic DNA (RAPD) analysis

Eine Alternativmethode zur Extraktion von Pflanzen-DNA aus Cashew (*Anacardium occidentale* L.) für die RAPD-Analyse

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Summary

The extraction of DNA from plants has been described by numerous authors. Each author described a different method to overcome the problems for isolation of genomic DNA. Some woody plants like cashew have certain metabolites like polyphenolic compounds, polysaccharides, tannin and RNA which interfere with DNA isolation and polymerase chain reaction (PCR) amplification. An alternative protocol was developed for rapid isolation of genomic DNA with reproducible PCR amplification of cashew. The protocol described the use of extraction buffer (boric acid, CTAB, EDTA, NaCl and β-mercaptoethanol) and subsequently two steps chloroform extraction to bind chlorophyll and proteins. Addition of 5 M NaCl and ethanol increased the solubility of polysaccharides thereby effectively decreasing co-precipitation of the polysaccharides and DNA. DNA purification was made by using sodium iodide and washing buffer . The purified DNA was fairly reproducible in all varieties of cashew. Amplification of DNA fragments was determined by PCR reactions. This method is also helpful for isolation of DNA and PCR amplification in other woody plants.

Zusammenfassung

Die Extraktion von DNA aus Pflanzen wurde von zahlreichen Autoren beschrieben. Dabei führt jeder Autor eine andere Methode an, um die Probleme bei der Isolierung der Genom-DNA zu überwinden. Einige holzige Pflanzen wie Cashew haben zahlreiche Metaboliten (z.B. Polyphenole, Polysaccharide, Tannin und RNA), die bei der DNA Isolierung und Polymerase-Kettenreaktionen (PCR) Amplifikation stören. Im Rahmen dieser Untersuchung wurde eine Methode zur schnellen Isolierung der Genom-DNA gekoppelt mit reproduzierbarer PCR von Cashew protokolliert. Das Verfahren setzt sich aus dem Gebrauch von Extraktionspuffern (Borsäure, CTAB, EDTA, NaCl und β-Mercaptoethanol) und der daran anschließenden zweistufigen Extraktion mittels Chloroform zur Bindung von Chlorophyll und Proteinen zusammen. Die Zugabe von 5 M NaCl und Ethanol erhöht die Löslichkeit der Polysaccharide, wodurch sich das gleichzeitige Ausfällen von Polysacchariden und DNA vermindert. Gereinigte DNA wurde durch den Anwendung von Natriumjodid und Waschpuffer gewonnen. Die gereinigte DNA konnte bei allen Cashew-Varietäten gewonnen werden. Die Amplifikation von DNA Fragmenten wurde durch PCR erreicht. Diese Methode ist auch für die Isolierung von DNA und die PCR Amplifikation bei anderen holzigen Pflanzen geeignet.

Introduction

A reliable method for plant DNA isolation is required for polymerase chain reaction analysis in plant breeding, plant gene mapping and plant genetic diversity. Classical approaches to plant DNA isolation have aimed to produce larger quantities of DNA and more highly purified DNA than that needed for polymerase chain reaction (PCR) (DELLAPORTA et al 1983; BENDICH et al 1980). Many simple procedures have been developed for use in preparing plant DNA for PCR (BERTHOMIEU et al. 1991; DOYLE and DOYLE 1990; LUO et al 1992). However, these procedures may only work for a limited number of tissue or plant species, and most are still multi step procedures. In many cases, the presence of polysaccharides (e.g. starch) and secondary metabolites (e.g. phenolics) often inhibited restriction endonuclease digestion and/or PCR amplification (GUILLEMAUT and MARECHAT-DROUARD 1992; RICHARDS et al 1994; WEISHING et al 1995; POREBSKI et al 1997). Many DNA isolation procedures also yielded large amounts of RNA, especially 18 s and 25 s rRNA (ROGERS and BENDICH 1985; DOYLE and DOYLE 1990; MEJJARD et al 1994). RNase treatment is the generally accepted method to remove RNA; however, degradation is often incomplete. The contaminated RNA that precipitates along with DNA causes many problem including suppression of PCR amplification (PIKBART and VILLEPONTEAU 1993).

Plant species belonging to the same or related genera or variety can exhibit enormous variability in the complexity of pathways of dispensable functions. Thus, the biochemical composition of plant tissue of different species or varieties is expected to vary considerably. The chemotypic heterogeneity among species or vari-

eties may not permit optimal DNA yields from one isolation protocol, and perhaps even closely related species may require different isolation protocols (WEISHING et al 1995). Cashew belongs to family Anacardiaceae and genus *Anacardium* having high amount of secondary metabolites, orthohydroxyphenols and polysaccharides (NAGARAJA 2000), which are powerful oxidising agents to interfere with genomic DNA . Some varieties are recalcitrant to inhibit the PCR amplification. There is no report on isolation and purification of genomic DNA from leaf tissues of cashew suitable for randomly amplified polymorphic DNA (RAPD) analysis. Here, we report an efficient protocol for DNA extraction and purification from fresh leaves of different varieties of cashew for RAPD analysis.

Materials and Methods

Plant Materials

Fresh leaves of *Anacardium occidentale* vars. BPP-2, BPP-3, BPP-4, BPP-8 (H-2/16), Vridhachalam-2 (M-44/3), Vridhachalam-3 (M-26/2), ULLAL-2, ULLAL-3, Vengurla–2, Vengurla-3, Vengurla-4, Vengurla-7, H-320, Madakkathara-1 (BLA-39-4), Dhana (H-1608), Kanaka (H-1598), Priyanka (H-1591), Jhargram-1, NRCC-1 and NRCC-2 were collected in an ice box from the cashew orchard maintained by the Orissa University of Agriculture and Technology, Bhubaneswar, Orissa, India.

DNA isolation

About 2.5 g of fresh young leaves were collected and powdered by using liquid nitrogen. The powered material was transferred to 50 ml polypropylene tube containing 10 ml of pre-warmed (60 °C, 1 hr) extraction buffer (1 M Boric acid (pH - 8.0), 2 mM EDTA, 1.4 M NaCl, 1.5 % [w/v] Cetyltrimethyl ammonium bromide [CTAB], 0.2 % (v/v) β-mercaptoethanol) and incubated at 60 °C in a water bath for 2 hours with gentle shaking at every 15 minutes interval. Equal volume of chloroform was added after it came down to room temperature, mixed thoroughly and kept for at least 10 minutes at room temperature. It was centrifuged at 8000 x g for 15 minutes at room temperature. The supernatant was collected and equal volume of chloroform was added, mixed gently for 10 minutes and centrifuged at 5000 x g for 10 minutes at room temperature. The supernatant was collected and 1.5 ml of 5 M NaCl was added and then mixed properly. Equal volume of chilled ethanol was added and kept at 4 °C overnight. Then it was centrifuged at 5000 x g for 10 minutes at room temperature; the supernatant was decanted and the pellet was washed with 80 % (v/v) ethanol. The pellet was air dried and dissolved in 1 ml of TE buffer. Three to four micro litre of RNase was added and incubated at 37 °C for 1 hr. Further, equal volume of chloroform was added and mixed thoroughly for 5 min and spinned at 5000 x g for 10 min. The aqueous layer was transferred to a sterile 2 ml eppendorf tube and 2 volumes of chilled ethanol was added. It was kept at 4 °C for 15 minutes and spinned at 5000 x g for 10 minutes at room temperature; the supernatant was discarded and the pellet was washed with 80 % ethanol. The pellet was air dried and dissolved in 200 µl of sterile distilled water. Subsequently, 2.5 volumes of Sodium Iodide solution (M/S Bangalore Genei, India) was added and mixed thoroughly and incubated at 50 °C for 2–3 minutes. 15 µl of glass solution (M/S Bangalore Genei, Bangalore, India) was added and mixed properly at room temperature for 5 min. Then it was spinned at 10,000 x g for 30 sec and the supernatant was removed. Subsequently, 200 µl of wash buffer (Bangalore Genei, India) was added, mixed thoroughly and spinned at 10,000 x g for 30 sec and again the supernatant was removed. Further, 200 µl of wash buffer was added and mixed thoroughly and the tube was kept in dry hot bath at 37 °C for 10 min to remove any trace of wash buffer. Then 40 µl of sterile distilled water was added to the pellet, the pellet resuspended by mild vortexing and then incubated at 50 °C for 5 min. It was spinned at 10,000 x g for 30 sec and again the supernatant was collected in a fresh eppendorf tube. The quality and quantity of DNA content in the supernatant were determined.

Quantification of genomic DNA

DNA concentration was determined spectrophotometrically by measurement of optical density. For this, 10 µl of DNA sample was diluted with 100 µl of sterile distilled water (10 X dilution) and the optical density was measured at 260 nm against a distilled water blank in a UV-Visible Spectrophotometer (V-530, JASCO, JAPAN). The DNA concentration was then calculated according to the known method. Optical density (O.D) values were also taken at 280 nm (corresponding to protein), 230 nm (corresponding to RNA) and 320 nm (contamination). Total DNA purity was tested by a ratio of O.D values at 230: 260: 280.

Quality of the isolated DNA

Two microliter of the isolated DNA, 7 µl of sterile distilled water were mixed with 1.0 µl of 10 X loading dye and was loaded in a lane of 1 % (w/v) agarose gel containing 0.05 $\mu g\,ml^{-1}$ of ethidium bromide for checking the quality of the DNA. Hind III digested lambda DNA served as a size marker. The agarose gel electrophoresis was carried out for ~1 hr at 50 V. The gel was visualized and photographed under UV light . The intact double standard DNA forming a thick single band of high molecular weight confimed the good quality of the extracted DNA.

PCR amplifications

A set of thirty random decamer oligonucleotides purchased from Operon Technologies Inc. (Alameda, California, USA) was used as single primers for the amplification of RAPD fragments. Polymerase Chain Reactions (PCR) were carried out in a final volume of 25 µl containing 20 ng template DNA, 100 µM each deoxynucleotide triphosphate, 20 ng of decanucleotide primers (M/S Operon Technology, Inc., Alameda, CA 94501, USA), 1.5 mM $MgCl_2$, 1 x Taq buffer ([10 mM] Tris-HCl [pH -9.0], 50 mM KCl, 0.01 % gelatin) and 0.5 U Taq DNA polymerase (M/S Bangalore Genei, India). Amplification was achieved in a PTC 100 thermal

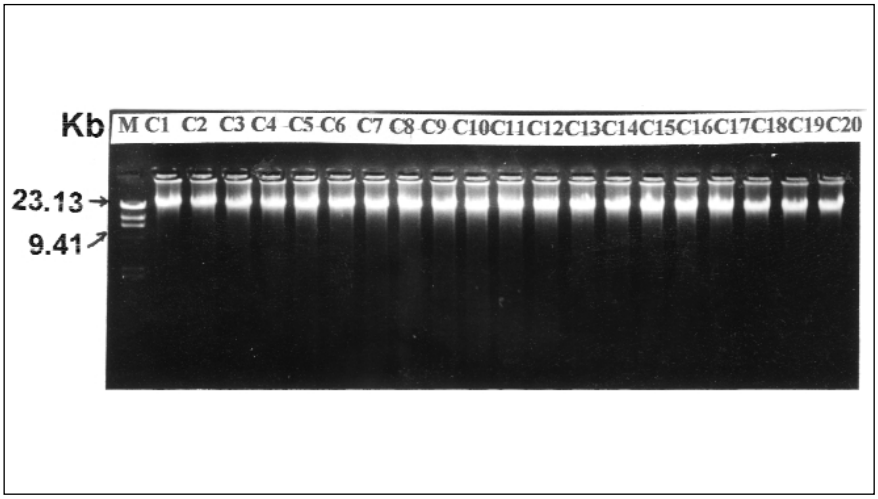


Figure 1. Genomic DNA profile of 20 varieties of cashew in 1.0% (w/v) agarose gel.
DNA-Profile von 20 Cashewnuss-Varietäten mittels 1.0% (w/v) Agarosegel.
M = Kb molecular weight λ-DNA; C1 = BPP-2; C2 = BPP-3; C3 = BPP-4; C4 = BPP-8 (H-2/16); C5 = Vridhachalam-2 (M-44/3); C6 = Vridhachalam-3 (M-26/2); C7 = ULLAL-2; C8 = ULLAL-3; C9 = Vengurla-2; C10 = Vengurla-3; C11 = Vengurla-4; C12 = Vengurla-7; C13 = H-320; C14 = Madakkathara-1 (BLA-39-4); C15 = Dhana (H-1608); C16 = Kanaka (H-1598); C17 = Priyanka (H-1591); C18 = Jhargram-1; C19 = NRCC-1 and C20 = NRCC-2.

cycler (MJ Research, USA) programmed for a preliminary 4 min denaturation step at 94 °C, followed by 45 cycles of denaturation at 94 °C for 1 min, annealing at 37 °C for 1 min and extension at 72 °C for 2 min, finally at 72 °C for 10 min. Amplification products were separated alongside a molecular weight marker (1 Kb ladder, MBI Fermentas Inc., USA) by electrophoresis on 1.0% agarose gels run in 0.5 X TAE (Tris Acetate EDTA) buffer, stained with ethidium bromide and visualized under UV light. Gel photographs were scanned through Gel Doc System (Gel Doc 2000, BioRad, USA) and the amplification product sizes were evaluated by using the software (Quantity one, BioRad, USA).

Results and Discussion

In case of cashew (*Anacardium occidentale*), the isolation of genomic DNA and subsequent PCR amplification were complicated due to abundance of polyphenolics, polysaccharides, RNA and other secondary products (THIMMAPPIAH 1997; NAGARAJA 2000). Polyphenols and other secondary plant compounds, cause damage to DNA and/or inhibit restriction enzymes and Taq polymerases. These compounds bind to DNA when cells are lysed (DOYLE and DOYLE 1990). Cell disruption by grinding in liquid nitrogen and incubation in extraction buffer results in lysis of both the cells containing the phenolics and the organelles. In a considerable number of plant species, DNA preparation tend to be brown coloured due to the oxidation of polyphenols to quinonic compounds. These are powerful oxidising agents that damage DNA and protein (LOOMIS 1974). As a consequence, yield of high molecular weight DNA from plants is often very poor. Even in the presence of soluble-polyvinylpyrrolidone (e.g. PVP-40) and polyphenol absorbants such as bovine serum albumin (BSA) in the isolation buffer at concentrations of 1–4%, the phenolics adhere to DNA in solution, forming a coloured matrix around the DNA. Further, the inclusion of phenoloxidase inhibitors such as diethyldithiocarbonic acid (DIECA), antioxidants (such as sodium bisulfite, cystine, ascorbic acid) or β-mercaptoethanol in the isolation buffer helps to reduce browning (HOWLAND et al 1991; PATERSON et al 1993). WEISHING et al (1995) reviewed the various methodologies on isolation and purification of DNA for DNA profiling. HONEYCUTT et al (1992) succeeded in the isolation of nuclear DNA in sugarcane by using modified CTAB method. Further, SUL and KORBAN (1996) described a highly efficient method for isolating genomic DNA from leave tissue of *in vitro* raised plants of *Malus domestica* and *Nicotiana tabaccum* by using modified CTAB protocol which includes reduced centrifugation runtimes for both separation of two-phase layers and precipitation of genomic DNA, and the addition of lithium chloride during extraction for restriction digestion analysis. By using various methods described by WILLMITZER and WAGNER (1981), DELLAPORTA et al. (1983), ROGERS and BENDICH (1988), DOYLE and DOYLE (1990), COUCH and FRITZ (1990), SUL and KORBAN (1996) and POREBSKI et al. (1997) indicated very low DNA yield and no PCR amplification reactions. The modified protocol described in this paper was used for isolation and purification of DNA. The result indicated that the quantity of the genomic DNA was about 300–400 ng μl^{-1} (Fig. 1) and the total DNA yield about 12–14 $\mu g\, g^{-1}$ of fresh young leaf. Further, the isolated genomic DNA was tested for PCR amplification reactions. The results showed that the PCR amplification lead to reproducible amplification with primer OPA-02 and OPA-03 (Figs. 2a-b). There was no interference with PCR amplification reactions. Our protocol involves two step use of chloroform initially because of precipitation of chlorophyll and proteins. The addition of 5 M NaCl and ethanol help in precipitation of DNA. FANG et al. (1992) observed that the addition of high concentration of NaCl increased the solubility of polysaccharides in ethanol, effectively decreasing co-precipitation of the polysaccharides and DNA. LODHI et al. (1994) indicated that higher concentration of NaCl was more effective for removing polysaccharides in *Vitis* species. Contamination with RNA occurs when isolating genomic DNA, copious amounts of cellular RNA precipitated concomitantly with DNA. Several reports have indicated that the presence of RNA can suppress PCR amplification and lead to non-reproducible and unreliable DNA amplification patterns in RAPD analysis (YOON and GLAWE 1993; MEJJARD et al 1994). The addition of RNase reduces the amount of RNA present in the genomic DNA. Apart from RNA, because of presence of large amounts of tannin in woody genera, the purification of DNA is

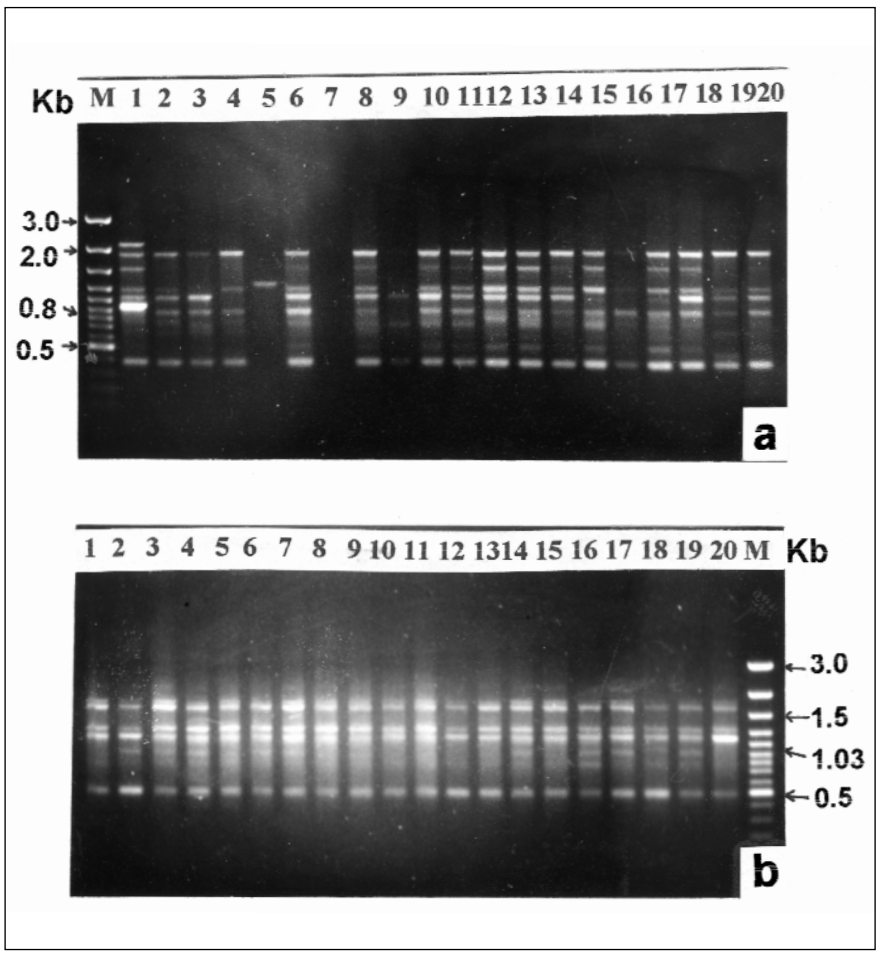


Figure 2a-b. RAPD profiles of the 20 varieties of cashew (*Anacardium occidentale*) amplified with primer (a) OPA- 02 (5'-TGCCGAGCTG-3') and (b) OPA–03 (5'-AGTCAGCCAC-3'). *RAPD-Profile von 20 Cashewnuss-Varietäten (*Anacardium occidentale*) angereichert mit den Primern (a) OPA-02 (5'-TGCCGAGCTG-3') und (b) OPA-03 (5'-AGTCAGCCAC-3').*
M = Kb molecular weight ladder; C1 = BPP-2; C2 = BPP-3; C3 = BPP-4; C4 = BPP-8 (H-2/16); C5 = Vridhachalam-2 (M-44/3); C6 = Vridhachalam-3 (M-26/2); C7 = ULLAL-2; C8 = ULLAL-3; C9 = Vengurla–2; C10 = Vengurla-3; C11 = Vengurla-4; C12 = Vengurla-7; C13 = H-320; C14 = Madakkathara-1 (BLA-39-4); C15 = Dhana (H-1608); C16 = Kanaka (H-1598); C17 = Priyanka (H-1591); C18 = Jhargram-1; C19 = NRCC-1 and C20 = NRCC-2.

very difficult (LINDER et al 2000). The use of sodium iodide and washing buffer in subsequent step help in the purification of high quality DNA for PCR amplification. The proposed protocol was found to have advantages for isolation of DNA from different varieties of cashew leaves. The amount of DNA recovered per gram of leaf tissue material was sufficiently high enough for PCR amplification. This method described here is efficient for the isolation of purified DNA from woody plant like cashew having high composition of tanin, polyphenols and polysaccharides. It will also help in other woody plant system.

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Literature

BENDICH, A. J., R. S. ANDERSON and B. L. WARD 1980: Plant DNA: long, pure and simple. In: LEAVER, C. J (Ed.): Genome organization and expression in plants. Plenum Press, New York, 31–33.

BERTHOMIEU, P., P. BERTHOMIEU and C. MEYER 1991: Direct amplification of plant genomic DNA from leaf and root pieces using PCR. Plant Mol. Biol. **17**, 555–557.

COUCH, J. A. and P. J. FRITZ 1990: Isolation of DNA from plants high in polyphenolics. Plant Mol. Biol. Rep. **8**, 8–12.

DELLAPORTA, S. L., J. WOOD and J. B. HICKS 1983: A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep **1**, 19–21.

DOYLE, J. J and J. L. DOYLE 1990: Isolation of plant DNA from fresh tissue. Focus **12**, 13–15.

FANG G, S. HAMMAR and R. REBECCA 1992: A quick and inexpensive method for removing polysaccharides from plant genomic DNA. Biotechniques **13**, 52–56.

GUILLEMAUT, P. and L. MARECHAL-DROUARD 1992: Isolation of plant DNA: A fast, inexpensive and reliable method. Plant Mol.. Biol. Rep **10**, 60–65.

HOWLAND, D. E., R. P. OLIVER and A. J. DAVY 1991: A method of extraction on DNA from birch. Plant Mol. Biol. Rep. **9**, 340–344.

HONEYCUTT, R., B. W. S. SOBRAL, P. KEIM and J. E. IRVINE 1992: A rapid DNA extraction method for sugarcane and its relatives. Plant Mol. Biol. Rep. **10**, 66–72.

LINDER, C. R., L. A. MOORE and R. B. JACKSON 2000: A universal molecular method for identifying underground plant parts to species. Mol. Ecol. **9**, 1549–1559.

LODHI, M. A., G. N. YE, N. F. WEEDEN and B. I.

REISCH 1994: A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species. Plant Mol. Biol. Rep. **12**, 6–13.

LOOMIS, M. D 1974: Overcoming problems of phenolic and quinones in the isolation of plant enzymes and organelles. Methods Enzymol. **31**, 528–544.

LUO G, A. G. HEPBURN and J. M. WIDHOLM 1992: Preparation of plant DNA for PCR analysis: a fast, general and reliable procedure. Plant Mol. Biol. Rep. **10**, 319–323.

MEJJARD, M., F. VEDEL and G. DUCREUX 1994: Improvement of DNA preparation and of PCR cycling in RAPD analysis of marine macroalgae. Plant Mol. Biol. Rep. **12**, 101–105.

NAGARAJA, K. V 2000: Biochemical composition of cashew (*Anacardium occidentale* L.) kernel testa. J. Food Sci. and Technol. **37**, 554–556.

PATERSON, A. H., C. L. BRUBAKER and J. F. WENDEL 1993: A rapid method for extraction of cotton (*Gossypium species*) genomic DNA suitable for RFLP or PCR analysis. Plant Mol. Biol. Rep. **11**, 122–127.

PIKBART, M. J. and B. VILLEPONTEAU 1993: Suppression of PCR amplification by high levels of RNA. Biotechniques **14**, 24–25.

POREBSKI, S., L. G. BAILEY and B. R. BAUM 1997: Modification of a CTAB extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Mol. Biol. Rep. **15**, 8–15.

RICHARDS, E., M. REICHARDT and S. ROGERS 1994: Preparation of genomic DNA from plant tissue. In: AUSUBEL, F. M., R. BRENT, R. E. KINGSTON, D. D. MOORE, J. G. SEIDMAN, J. A. SMITH and K. STRUHL (Eds.): Current protocols in molecular biology. New York, Pp. 2.3.1–2.3.7.

ROGERS, S. O and A. J. BENDICH 1985: Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissue. Plant Mol. Biol. **5**, 69–76.

ROGERS, S. O. and A. J. BENDICH 1988: Extraction of DNA from plant tissues. Plant Mol. Biol. Manual A6, Kluwer Acad. Publ., Dordrecht, The Netherlands, 1–10.

SUL, I. W. and S. S. KORBAN 1996: A highly efficient method for isolating genomic DNA from plant tissues. Plant Tissue Culture and Biotechnology **2**, 113–116.

THIMMAPPIAH 1997: Cashew improvement, Ph. D Thesis, Mangalore University, India.

WEISHING, K., H. NYBOM., K. WOLFF and W. MEYER (Eds.) 1995: DNA isolation and purification. In: DNA fingerprinting in plants and fungi. CRC Press, Boca Raton, Florida, USA, 44–59.

WILLMITZER, L. and K. G. WAGNER 1981: The isolation of nuclei from tissue-cultured plant cells. Exp. Cell Res. **135**, 69–77.

YOON, C. S. and D. A. GLAWE 1993: Pretreatment with RNAse to improve PCR amplification of DNA using 10-mer primers. Biotechniques **14**, 908–910.

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