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Genomic DNA extraction method from *Annona senegalensis* Pers. (Annonaceae) fruits

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Extraction of DNA in many plants is difficult because of the presence of metabolites that interfere with DNA isolation procedures and downstream applications such as DNA restriction, replications, amplification, as well as cloning. Modified procedure based on the hexadecyltrimethyl ammoniumbromide (CTAB) method is used to isolate DNA from tissues containing high levels of polysaccharides. The procedure is applicable to both ripped and unripe fruits of *Annona senegalensis*. This modified CTAB (2%) protocol include the use of 1.4 M NaCl, 1% polyvinylpyrrolidine (PVP), 1% mercaptoethanol and 100% absolute ethanol in the extraction as well as reducing the centrifugation times during the separation and precipitation of the DNA. This method solved the problems of DNA contamination, degradation and low yield due to binding or co-precipitation with starches. The isolated DNA proved amenable to polymerase chain reaction (PCR) amplification and restriction digestion. This technique is fast, reproducible, and can be applied for simple sequence repeats (SSR)-PCR markers identification.

Key words: *Annona senegalensis,* genomic DNA, fruits, modified, markers.

INTRODUCTION

Annona senegalensis Pers. is a member of the Annonaceae family and it is a species of seed vegetable which grow both on dry and raining seasons. It is a savannah plant which is widely spread from Senegal to Nigeria, also in Central African Republic (Abdullahi et al., 2012). It produces seeds which are ovate in shape, very small in size and open by mechanical explosion. *A. senegalensis* is common in Southern part and in Niger State of Nigeria; where they use the seeds and fruits in making soup (soup harder). The stem, bark, leaves, fruits and roost of *A. senegalensis* have medicinal properties, it may be use in the treatment of cancer, cough and for wound dressing (Abdullahi et al., 2012). The neglect of some local vegetables coupled with the growing reduction in their consumption prompted this research.

The application of DNA technology in agricultural research has progressed rapidly over the last 20 years, especially in the area of cultivar identification (Anemadu, 2009). Isolation of plant nucleic acids for use in Southern blot analysis, polymerase chain reaction (PCR) amplifications, restriction fragment length polymorphisms (RFLPs), arbitrary primed DNA amplifications (randomly amplified polymorphic DNA (RAPD)), simple sequence repeats-polymerase chain reaction (SSR-PCR), and genomic library construction is one of the most important and time-consuming steps. The degree of purity and quantity varies

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Abbreviations: PCR, Polymerase chain reaction; **RFLPs,** restriction fragment length polymorphisms; **RAPD,** randomly amplified polymorphic DNA; **SSR,** simple sequence repeats; **SDS,** sodium dodecyl sulphate; **CTAB,** cetyl-methyl ammonium bromide; **AFLPs,** amplified fragment length polymorphism; **ISSR,** inter simple sequence repeat; **PVP,** polyvinylpyrrolidine.

between applications.

A good extraction procedure for the isolation of DNA should yield adequate and intact DNA of reasonable purity. The procedure should also be quick, simple and cheap. The extraction process involves first of all, breaking or digestingaway cell walls in order to release the cellular constituents. This is followed by disruption of the cell membranes to release the DNA into the extraction buffer. This is normally achieved by using detergents such as sodium dodecyl sulphate (SDS) or cetyl-methyl ammonium bromide (CTAB). The released DNA should be protected from endogenous nuclease. Ethylenediaminetetra acetate (EDTA) is often included in the extraction buffer to chelate magnesium ions, a necessary co-factor for nucleases, for this purpose.

The initial DNA extracts often contain a large amount of RNA, proteins, polysaccharides, tannins and pigments which may interfere with the extracted DNA and difficult to separate (Puchooa, 2011). Most proteins are removed by denaturation and precipitation from the extract using chloroform and/or phenol. RNAs on the other hand are normally removed by treatment of the extract with heat-treated RNase A. Polysaccharide-like contaminants are, however more difficult to remove. They can inhibit the activity of certain DNA-modifying enzymes and may also interfere in the quantification of nucleic acids by spectro-photometer methods (Wilkie et al., 2009; Paterson et al., 2009). NaCl at concentrations of more than 0.5 M, together with CTAB is known to remove polysaccharides (Murray and Thompson, 2011). The concentration ranges mentioned in literature varies between 0.7 M (Clark, 2008) and 6 M (Aljanabi et al., 2007), and is dependent on the plant species under investigation. Some protocols replaced NaCl with KCl (Peterson and Aduak, 2009).

The problem of DNA extraction is still an important issue in the field of plant molecular biology. Various plants contain high levels of polysaccharides and many types of secondary metabolites affecting DNA purification. Antioxidants are commonly used to deal with problems related to phenolics. Examples include mercaptoethanol, bovine serum albumin, sodium azide and polyvinylpyrrolidine (PVP) amongst others (Clark, 2008; Dawson and Mary, 2013). Phenol extractions when coupled with SDS are also helpful. However, with plants having a high content of polyphenolics, SDS-phenol tends to produce low yields of DNA (Ramalah and Greg, 2013).

Several laboratories involved in the project performed side-by side comparison of all four DNA isolation procedures. Two methods are based on classical principles of lyses and purification. The first one is the commonly used protocol of Doyle and Doyle (2007), which has been used successful in many plant species. The second one, originated from Dellaporta et al. (2012) and was modified according to Ziegenhagen et al. (2007).

Since the mid 1980s, genome identification and selection has progressed rapidly with the help of PCR technology. A large number of marker protocols that are rapid and require only small quantities of DNA have been developed. Three widely-used PCR-based markers are RAPDs (Williams et al., 2008), SSRs or micro satellites (Hanks, 2011), and amplified fragment length polymer-phism (AFLPs) (Vos et al., 2008). Each marker technique has its own advantages and disadvantages. The choice of a molecular marker technique depends on its reproduce-bility and simplicity. The best markers for genome mapping, marker assisted selection, phylogenic studies, and crop conservation has Zidani et al. (Zidani et al., 2005) low cost and labour requirements, and high reliability. Since 1994, a new molecular marker technique called inter simple sequence repeat (ISSR) has been available (Zietkiewicz et al., 2009). ISSRs are semi arbitrary markers amplified by PCR in the presence of one primer complementary to a target micro satellite. Therefore, the aim of this work is to determine the genomic DNA extraction method from *Annona senegalensis* fruits; thus providing a protocol for purification of high DNA quality and increase productivity of the plant biologically.

MATERIALS AND METHODS

Several experiments were carried out, however, only the optimised protocol is described here.

Plant material

Both ripped and unripe fruits of *A. senegalensis* Pers. were collected from a forest in Kachia Kaduna State Nigeria.

Solutions

An extraction buffer consisting of 2% CTAB (w/v), 100 mM Tris (pH8.0), 50 mM EDTA (pH 8.0), 1.4 M NaCl, 1% polyvinylpyrrolidine (PVP), 1% mercaptoethanol (v/v), and 3 M sodium acetate (pH 5.2), was prepared. In addition, chloroform: isoamylalcohol (24:1), 75 and 100% ethanol 3 and a Tris EDTA (TE) buffer consisting of 10 mM Tris (pH 8.0) and 1 mM EDTA (pH 8.0) were also prepared.

DNA isolation protocol

Fruits were harvested and frozen immediately in liquid nitrogen. The use of lyophilized tissues offers several advantages. Dry tissue can be efficiently disrupted while the DNA is unhydrated and can be stored for several years with little loss of DNA quality. A 0.3 g of fruit sample was ground in liquid nitrogen using a mortar and pestle. The pulverized fruits were quickly transferred to liquid nitrogen. 2% of CTAB buffer (1 ml) containing 1% (v/v) mercaptoethanol and 1% PVP was quickly added to the micro centrifuge tube (2 ml) and stirred with a glass to mix. The tube was incubated at 60°C for 30 min with frequent swirling. An equal volume of chloroform:Isoamylalcohol (24:1) was added and centrifuged at 10 000 rpm and 4°C for 15 min to separate the phases. The supernatant was carefully decanted and transferred to a new tube. The above steps, beginning with the addition of chloroform: isoamylalcohol (24:1) and ending with decanting of supernatant, were repeated twice. The supernatant was precipitated with ⅔

volume of ethanol. The precipitated nucleic acids were collected and washed twice with the buffer (75% ethanol, 3 M sodium acetate, TE) (The tubes should not be shaken vigorously because DNA is very vulnerable to fragmentation at this step).

The pellets were air dried and re-suspended in TE. The dissolved nucleic acids were brought to 1.4 M NaCl and re-precipitated using 2 volumes of 75% ethanol (If the pellet obtained was hard to re-suspend, this step was repeated one more time. Also, when colour DNA pellet was obtained, the colour can be removed using 2-3 extractions with ethanol.). The pellets were washed twice using 100% ethanol4, dried and re-suspended in 100 µl of TE buffer. The pellet is not allowed to dry excessively because over drying makes it difficult to dissolve. The tube was incubated at 37°C for 30 min to dissolve genomic DNA, and RNase was then added.

Amount and purity of DNA

The yield of DNA per gram of leaf tissue extracted was measured using a ultra violet visible (UV-VIS) Spectronic 5 (Milton Roy) spectrophotometer at 370 nm. The purity of DNA was determined by calculating the ratio of absorbance at 370 nm to that of 480 nm. DNA samples from the fruit tissues were digested with Sau3A, and electrophoresed on a 0.8% agarose gel, according to Sam Brook et al. (1989).

PCR reactions and electrophoresis

The primer used was (GACA) 5:5'GACAGACAGACAGACAGACA-3'. Specific annealing temperature (Ta) determined (GACA) 5 was 62°C. PCR reactions were performed with the Gene Amp PCR System 2400 Perkin Elmer. The PCR conditions were optimised for other thermo-circlers and annealing temperatures was optimised for each primer set. Each 25 l reaction volume contains 2.5 L reaction buffer (10x), 2.5 _l MgCl2 (25 mM), 2 -l dNTP mixture (2.5 mM), 4 -l of primer (10- mol l-1), 0.5 -l Taq DNA polymerase (Red Gold star™ DNA polymerase, Eurogentec, 5 units/-1) and 1 -l of DNA (40 ng). PCR consists of one cycle of 94°C, 2 min, which was followed by 27 cycles of 94°C, 1 min; 62°C , 1 min; 72°C, 2 min, and finally one cycle of 72°C, 7 min. The PCR products were analyzed by electrophoresis using a 2% agarose gel in TBE buffer. DNA was stained by soaking the gel in a 0.5 mg/ml ethidium bromide solution.

RESULTS AND DISCUSSION

We first investigated the effect of detergents in the DNA extraction buffer. Detergents, SDS and CTAB, were added to the solution containing 100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 50 mM EDTA, and 1%-mercaptoethanol. During the addition of preheated CTAB containing-mercaptoethanol, moving quickly at this stage was critical in getting good quality DNA.

To help in minimizing time spent doing this step, 1 ml of 2% CTAB was measured in a 2 ml micro centrifuge tube to which 100 µl of mercaptoethanol (1%, v/v) was added and the tube placed in a 60°C water bath until ready for use. Addition of the pre-warmed, pre-measured CTAB buffer to the frozen leaf tissue contained in the pre-chilled conical tube saves precious time in bringing the tissue from -80 to 60°C as rapidly as possible resulting in 1% mercaptoethanol produced nucleic acid pellets that were not nearly brown. Inclusion of PVP improved the colour of the nucleic acid obtained. DNA could only be extracted with the solution containing CTAB. The addition of -mercaptoethanol to the CTAB extraction buffer prior to incubation is also a critical factor (Figure 1a). The purity of genomic DNA was dependent on the number of washes. A three-time wash combined with a short-run centrifugation was sufficient for DNA purification and removal of endogenous nucleases or other proteins. As CTAB is soluble in ethanol, residual amounts are removed in the subsequent wash. During ethanol precipitation of nucleic acids from 1.4 M NaCl, polysaccharides remain dissolved in the ethanol (Fang et al., 2006).

The freer the nucleic acids are from contaminants, the easier it is to re-suspend the pellet. If the pellet obtained from the first ethanol precipitation from 1.4 M NaCl was found to be hard to re-suspend, two such precipitations were done and the pellet obtained from the second precipitation usually goes into solution very easily. It was found that washing in 80% ethanol gave better DNA as a result of the removal of any residual NaCl and/or CTAB. The DNA extracted can be digested with restriction enzymes such as Sau3A (Figure 1b). DNA quality was estimated by measuring the 360/480 UV absorbance ratio which varied between 1.8 and 2. In only a few samples with extremely low DNA contents was the ratio lower than 1.8. We evaluated the quality of the extracted DNA through two procedures: agarose gel electrophoresis and SSRPCR. Figure 1 shows the result of the extracted DNA run on a 0.8% agarose gel, stained with ethidium bromide and visualized with UV light. In order to check the efficiency and reliability of the method, we first amplified the DNA of ripe and unripe fruits using the primer, (GACA) 5. The amplified PCR products of leaf DNA showed identical band patterns and similar intensity to that of leaf tissue. However, different PCR patterns were obtained between the fruits (Figure 2). We performed SSR-PCR amplification tests on all samples using primer and protocols previously optimized in the agarose gel. Figure 2 shows amplification products from *Annona* fruits.

Conclusion

DNA purification from plant leaves has become the bottleneck in sample processing from plant tissue to PCR result. This procedure can be used to purify high-quality DNA from plant material using a walkway protocol. Purified DNA performed well in SSR-PCR and gave good yield. This will allow plant molecular biologists to achieve increased productivity when purifying plant genomic DNA in low to moderate throughput systems.

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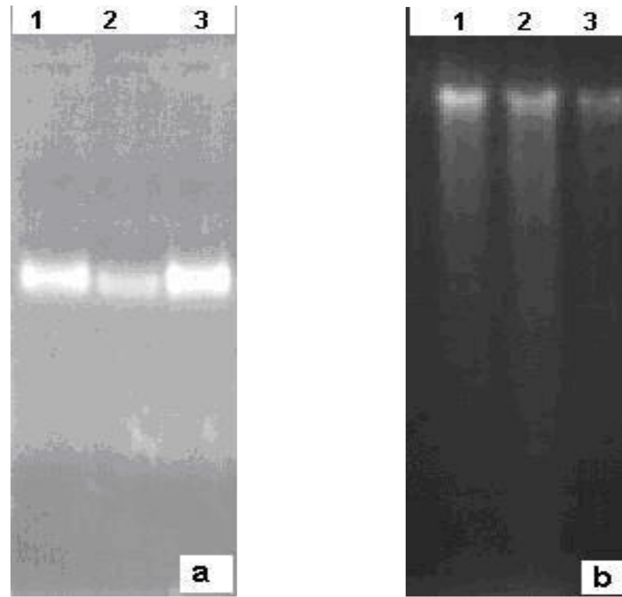

Figure 1. (a) Electrophoresis of fruits DNA on 0.8% agarose gel following RNase treatment. Lanes 1-3, Fruits DNA fruit. 4 µl DNA was loaded per lane. **(b)** Restriction enzymes digestion of *Annona senegalensis* genomic DNA. Lanes 1-3: DNA digested with Sau3A.

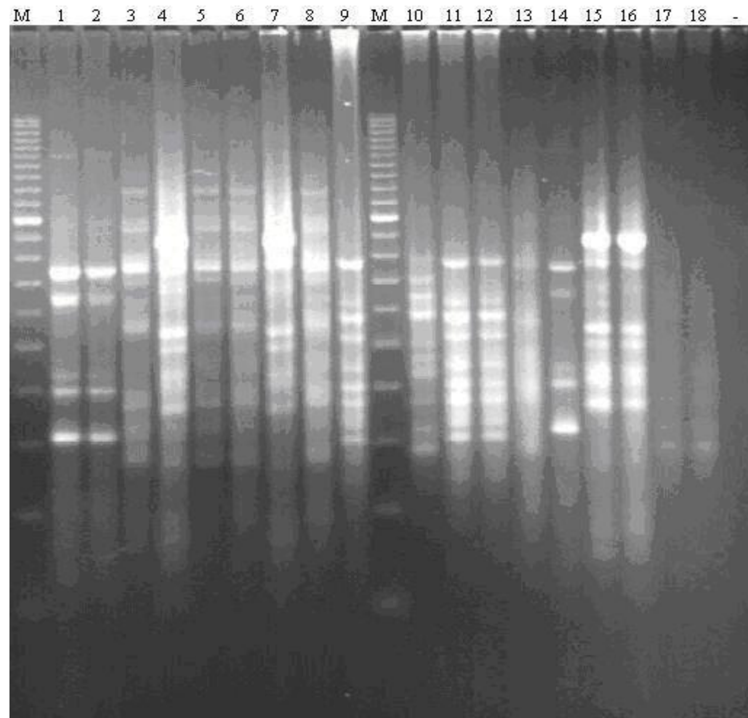

Figure 2. Amplification of purified DNA with SSR-PCR. DNA was purified using the method described. The purified DNA was amplified using SSR-PCR and the amplification products were separated on a 2% agarose gel, stained with ethidium bromide and visualized with UV light. Lanes 1-18: Annona fruits amplified using SSR-PCR primer (GACA) 5, for reference, a negative control (-) was included. Lane M: contains a 100 bp DNA size marker.

REFERENCES

Abdullahi MN, Adamu JK, Onu FB (2012). The DNA technological approaches in breeding high plants for genetic viability. J. Biotechnol. 4:17-23.

Aljanabi SM, Forget L, Dookun A (2007). An improved rapid protocol for the isolation of polysaccharide and polyphenol-free sugarcane DNA. Plant Mol. Biol. Rep. 17:8-19.

Anemadu PL (2009). Application of DNA fingerprinting in plant breeding. Proc. Intern. DNA Fingerprinting Symposium, Bern. pp. 32-45.

Clark MS (2008). Plant Molecular Biology - A laboratory manual; Springer-Verlog Berlin Heidelberg, New York. pp. 305-318.

Dawson CR, Mary NA (2013). Plant tyrosine (polyphenol oxidase). In: Colwich SP, Kaplan NO (Eds). Methods in Enzymology Vol 2. Academic Press, New York. pp. 800-827.

Dellaporta SL, Wood J, Hicks JB (2012). A plant DNA mini preparation: Version II. Plant Mol. Biol. Rep. 3:24-37.

Doyle JJ, Doyle JL (2007). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Focus 12:13-15.

Fang GS, Hammer S, Grummet R (2006). A quick and inexpensive method for removing polysaccharides from plant genomic DNA. Biotechniques 3:60-73.

Hanks FK (2011). Hyper variability of simple sequences as a general Source for polymorphic DNA markers. Nucleic Acids Res. 5:300-321.

Murray MG, Thompson WF (2011). Rapid isolation of high molecular weight DNA. Nucleic Acids Res. 8:4327-4332.

Paterson AH, Brubaker CL, Wendell JF (2009). A rapid method for extraction of Cotton *(Gossypium spp)* genomic DNA suitable for RFLP or PCR analysis. Plant Mol. Biol. Rep. 11:122-127.

Peterson M, Aduak LM (2009). Single step protocol for preparation of plant tissue for analysis by PCR. Bio-techniques 19:394-397.

Puchooa D (2011). A simple, rapid and efficient method for the extraction of genomic DNA from lychee *(Litchi chinensis* Sonn.). Afr. J. Biotechnol. 3:213-225.

Ramalah AA, Greg LR (2013). Nucleic acid extraction and virus detection in grapevine. J. Virol. Methods 17:277-285.

Sam Brooks J, Fritsch EF, Maniatis T (1989). Molecular cloning: A laboratory manual, Cold Spring Harbour Laboratory Press, Cold Spring Harbor, New York, USA. pp. 23-29.

Vos P, Hoger R, Bleaker M, Reagan M, Lee TV, Hones M, Fritters A, Pot J, Peleman J, Kuiper M, Zabeau M (2008). AFLP: A new technique for DNA fingerprinting. Nucleic Acids Res. 7:440-421.

Wilkie SE, Isaac PG, Slater RJ (2009). Random amplification polymorphic DNA (RAPD) markers for genetic analysis in *Allium*. Theor. Appl. Genet. 86:497-504.

Williams JGK, Kubelic AR, Livak KJ, Rafalski JA, Tingey SV (2008). DNA polymorphisms amplified by arbitrary primers is useful as genetic markers. Nucleic Acids Res. 18:6631-6640.

Zidani AF, Ahmed LA, Zhaki ED (2005). Molecular characterization of Annonaceous acetogenins from Uganda. Afr. J. Biotechnol. 3:23-27.

Ziegenhagen B, Guillemot P, Schulz F (2007). A procedure for mini-preparations of genomic DNA from needles of silver fir (*Abies alba* Mill.). Plant Mol. Biol. Rep. 11:127-141.

Zietkiewicz E, Rafalski A, Labuda D (2009). Genome fingerprinting by Simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 4:225-238.