Isolation and Amplification of Genomic DNA from Recalcitrant Dried Berries of Black Pepper (Piper nigrum L.)—A Medicinal Spice

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HINTS AND TIPS

Isolation and Amplification of Genomic DNA from Recalcitrant Dried Berries of Black Pepper (*Piper nigrum* L.)—A Medicinal Spice

K. Dhanya · Jaleel Kizhakkayil · S. Syamkumar · B. Sasikumar

Abstract Black pepper is an important medicinal spice traded internationally. The extraction of high quality genomic DNA for PCR amplification from dried black pepper is challenging because of the presence of the exceptionally large amount of oxidized polyphenolic compounds, polysaccharides and other secondary metabolites. Here we report a modified hexadecyl trimethyl ammonium bromide (CTAB) protocol by incorporating potassium acetate and a final PEG precipitation step to isolate PCR amplifiable genomic DNA from dried and powdered berries of black pepper. The protocol has trade implication as it will help in the PCR characterization of traded black peppers from different countries.

Keywords Black pepper · DNA isolation · PEG · Geographical Indication · Molecular markers

Black pepper (*Piper nigrum* L.), 'King of Spices' or 'black gold' is valued as a medicinal spice all over the world. Black pepper of commerce is the dried mature berries of black pepper and its value added products like white pepper, dehydrated green pepper, freeze dried green pepper, oleoresin, oil and black pepper powder. India, Brazil, Malaysia, Indonesia, Vietnam, Sri Lanka, China, Thailand and Micronesian countries are the major black pepper producing countries in the world. Liberalization of trade envisaged in the WTO gives scope for Intellectual Property Rights (IPR) of reputed commodity. Geographical Indication (GI) appellation is one such IPRs. Eligibility of Geographical Indication protection of a commodity is based on certain specific quality, reputation or other tangible and intangible properties including the appearance of the commodity, which are exclusively and essentially attributable to the geographical limits. Apart from the accurate physical and quality description of the commodity, development of DNA based molecular techniques will help to protect the commodity through GI by restricting unfair trade practices. Isolation and amplification of DNA from such traded produces are the first and foremost step in molecular characterization of the commodity. Dried black pepper berries are recalcitrant storage tissues containing high level of polyphenolic compounds in their oxidized forms along with polysaccharides, which present a major contamination problem in purification of plant DNA. Here we describe a simple and efficient protocol for isolation of PCR amplification of genomic DNA from black pepper berries.

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Materials and Methods

Export grade dried berries of Indian (Malabar), Indonesian and Vietnam black pepper used in the study were procured through Spices Board, Cochin, India. The berries were powdered using Cyclotech 1093 sample mill and used for DNA extraction as per the protocol given below:

1. Grind one gram of powdered sample to a finer form using mortar and pestle.
2. Suspend the powder thoroughly in 6 ml of extraction buffer (3%CTAB, 100 mM Tris-HCl (pH 8), 20 mM EDTA (pH 8), 1.5 M NaCl and 1%PVP) taken in a 30-ml Oakridge tube.
3. Incubate the tube at 65°C in a water bath for 30 min with intermittent shaking.

4. Cool to room temperature, add one-third volume of 5 M potassium acetate, gently mix the contents and incubate on ice for 1 h.
5. Add equal volume of chloroform—isoamylalcohol (24:1) and mix by inversion for 5 min.
6. Centrifuge at 10,000*g* for 15 min at 4°C using an Eppendorf centrifuge, 5804R.
7. Transfer the aqueous phase to a fresh Oakridge tube and add equal volume of 30% polyethylene glycol 6000, gently mix and incubate the tubes on ice for 30 min to 1 h.
8. Centrifuge at 12,000*g* for 20 min at 4°C and discard the supernatant.
9. Wash the pellet with 70% ethanol.
10. Dry and dissolve the DNA pellet in nuclease free water.
11. Store the DNA sample frozen at –20°C.

The concentration and quality of the DNA were determined spectrophotometricaly by calculating the 260/280 ratio and also quantified by means of agarose gel electrophoresis.

To check the quality and suitability of the DNA for high throughput analysis restriction digestion was carried out using three restriction enzymes (Eco RV, Mse I, and Hae III) along with standard DNA (Human genomic DNA, Bangalore Genei, India) as control. The digested products were resolved in 1% agarose gel.

PCR analysis: Four random decamer primers (Operon Technologies, Almada, USA) were used for PCR amplification following the procedure of Williams et al. [1] with a few modifications. Amplifications were performed in 25 µl reaction volume with 35 ng genomic DNA, 0.2 mM dNTPs, 10 picomole primer, 2 mM $MgCl_2$ and 1U *Taq* DNA polymerase using a PTC-100 Programmable Thermal Controller (MJ Research, Inc., USA). After a pre denaturation step of 3 min at 94°C, amplification reactions were cycled 35 times at 94°C for 1 min, 37°C for 1 min and 72°C for 1 min. A final amplification was allowed for 10 min at 72°C. Two ISSR primers from IDT, USA (IDT-01 $(GACA)_4$ and IDT-12 $(CA)_8$ T were tested. For ISSR reaction the primer concentration used was 60 picomoles reaction^{-1} and the annealing temperature in PCR condition was raised to 55°C and number of cycle repeats was 32.

The amplified products were loaded and visualized by running in 2% agarose gel containing 0.5 µg ml^{-1} of ethidium bromide and documented by a gel documentation system (Alpha Imager 2220, USA).

Results and Discussion

The preferred method of DNA extraction for a given recalcitrant tissue can be determined only through trial and error methods [2, 3]. Even though we tried several DNA isolation methods [4–12] to isolate high-quality DNA suitable for PCR amplification from dried and powdered black pepper berries, most of them did not yield DNA. However, the two methods proposed by Aras et al. [11] and Syamkumar et al. [12] yielded DNA that was completely sheared, dark brown or almost black in colour and was not amplifiable (Fig. 1a). Hence, the basic CTAB DNA extraction protocol of Doyle and Doyle [10] was modified by increasing the concentration of CTAB to 3%, NaCl concentration to 2 M, addition of PVP to extraction buffer, addition of potassium acetate (Step 4) and precipitation of DNA using PEG 6000 (Step 7) instead of isopropanol. The polyphenol present in the recalcitrant tissues, in their oxidized form covalently bind to DNA giving brown colour making it unusable for PCR [13]. Since we have used dried and powdered berries, this would have caused further problems as already reported by Do and Adams [14].

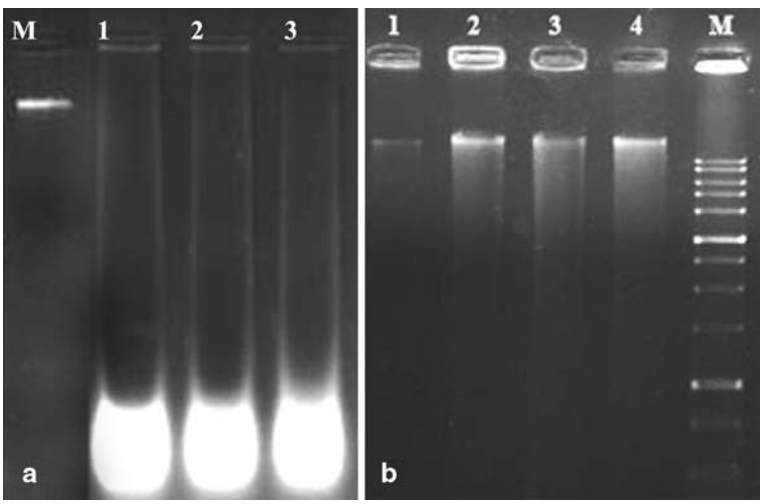

Fig. 1 DNA isolated from dried and powdered berries of black pepper from different sources. (**a**) DNA isolated using Doyle & Doyle method. M-Human genomic DNA, Lane 1-Indian pepper (Malabar), Lane 2-Indonesian pepper, Lane 3-Vietnam pepper. (**b**) DNA isolated using the modified method. Lane 1-Human genomic DNA, Lane 2-Indian pepper (Malabar), Lane 3-Indonesian pepper, Lane 4-Vietnam pepper, M-1Kb DNA ladder (Biogene, USA)

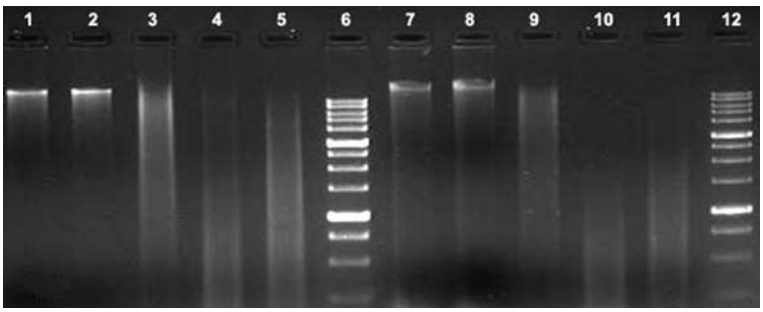

Fig. 2 Restriction digestion of genomic DNA isolated from dried and powdered berries of black pepper. Lane 1—Standard DNA, Lane 2—Standard DNA incubated without restriction enzyme, Lane 3—Standard DNA digested with Eco RV, Lane 4—Standard DNA digested with Mse I, Lane 5—Standard DNA digested with Hae III, Lane 6—1 KB ladder, Lane 7—Black pepper DNA, Lane 8—Black pepper DNA incubated with out restriction enzyme, Lane 9—Black pepper DNA digested with Eco RV, Lane 10—Black pepper DNA digested with Mse I, Lane 11—Black pepper DNA digested with Hae III, Lane 12—1 KB ladder

In the new protocol it was observed that 1-g sample tissue per 6 ml of modified CTAB extraction buffer was needed for extracting PCR amplifiable genomic DNA instead of 4 ml per gram of tissue usually followed. It is reported that increase in CTAB and NaCl concentrations increase the yield of cellular DNA [15]. CTAB is generally used as a detergent to separate out polysaccharides. Similarly NaCl concentration greater than 1.5 M removes the polysaccharides [16]. Though it is also reported that increase in the concentration of secondary metabolite chelators or antioxidants like PVP, PVPP, β-mercaptoethanol are also useful for increasing the yield and quality of DNA [9], we did not find it useful in the present study. But the addition of potassium acetate (Step 4) in our protocol was found efficient in removing most of secondary metabolites and polysaccharides from the DNA resulting in better yield of high-molecular weight DNA to some extent. Dark coloured viscous and degraded DNA with poor yield can also be due to the polyphenolics co-extracted during the DNA extraction and co-precipitated with DNA during the ethanol or isopropanol precipitation [2, 15]. Though we have tried repeated chloroform extraction or ethanol or isopropanol precipitations, we could not remove these contaminants and it adversely affected the yield and quality of DNA. Hence in our new protocol we have used PEG for precipitating DNA instead of isopropanol. The precipita-

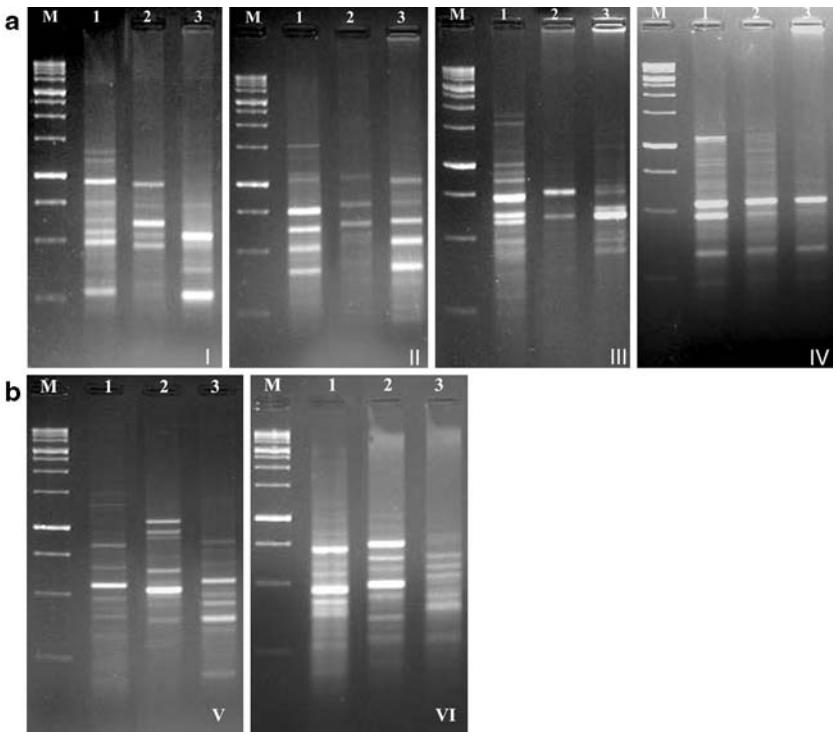

Fig. 3 (**a**) RAPD profile of the DNA isolated from dried and powdered berries of black pepper amplified with primers OPC-05 (I), OPC-11(II), OPC-16(III) and OPA-18(IV) respectively. (**b**) ISSR profile of the DNA isolated from dried and powdered berries of black pepper amplified with primers IDT-01(V) and IDT-12(VI). M-Marker (1Kb DNA ladder), Lane 1-Indian pepper (Malabar), Lane 2-Indonesian pepper, Lane 3-Vietnam pepper

tion of DNA with PEG resulted in comparatively purified homogenous DNA preparations devoid of proteins and polysaccharides as reported [17, 18]. Already PEG is a poly phenol adsorbent and it removes the coloured pigment in the samples during DNA extraction [12].

The spectrophotometric measurements of the DNA at 260 and 280 nm gave an absorbance ratio (A260/A280) of 1.75–1.82 indicating the purity of DNA. The yield of DNA isolated from the samples ranged from 10 μg g^{-1} to 15 μg g^{-1} of dried tissue. The quality of DNA isolated from dried berries of black pepper was also checked by agarose gel (0.7%) electrophoresis. We have observed a conspicuous band of high molecular weight DNA with some shearing (Fig. 1b). The quality of the isolated DNA was also checked by restriction digestion. The sample DNA was completely digested with three restriction enzymes (frequent cutters), indicating the good quality of DNA so also the standard DNA (Fig. 2).

The DNA isolated by this modified protocol was also consistently amplified with random decamer primers and inter simple sequence repeats primers used for PCR amplification. RAPD and ISSR banding patterns of DNA isolated from dried powdered berries of Indian, Indonesian and Vietnam black peppers using the RAPD primers OPC-05, OPC-11, OPC-16, and OPA-18 and ISSR primers IDT-01 and IDT-12 are shown in Fig. 3a and b.

The absorbance ratio of the isolated DNA at 260/280 (1.7–1.8) and restriction digestion pattern of the DNA compared to the standard DNA along with the consistent amplification pattern in PCR using RAPD and ISSR markers revealed that the genomic DNA isolated using the modified protocol from the recalcitrant berries of black pepper is of good quality. The protocol may be also applicable to other dry plant tissues rich in polysaccharides and polyphenolic compounds.

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