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A method for isolation and reverse transcription of high quality RNA from *Piper* species

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Abstract

A rapid and efficient protocol for isolation of RNA from *Piper* leaves is described. RNA was extracted from two *Piper* species namely, *P. nigrum* and *P. colubrinum*, using strong denaturing buffer containing guanidinum thiocyanate and polyvinylpyrollidone followed by precipitation with pre-cooled sodium acetate and extraction with phenol:chloroform: isoamylalcohol (25:24:1). RNA was precipitated with isopropanol. The extraction procedure reliably yielded high quality RNA suitable for reverse transcription experiments.

Key words: *Piper* spp., reverse transcription, RNA isolation.

Abbreviations: cDNA: complementary DNA, DEPC: diethylpyrocarbonate, ESTs: expressed sequence tags, GTC: guanidinum thiocyanate, LiCl: lithium chloride, MOPS: 3-(N-morpholino)-propanesulphonic acid, PVP: polyvinylpyrollidone, SDS: sodiumdodecylsulphate

Introduction

Black pepper (*Piper nigrum* L.) (Piperaceae) is one of the oldest and most important spice widely used across the world. *Piper colubrinum* Link. is a South American species distantly related to *P. nigrum* and is the only species reported to be resistant to major diseases and pests of black pepper namely, foot rot caused by *Phytophthora capsici* Leonian, slow decline caused by *Radopholus similis* Cobb. and *Meloidogyne incognita* (Kofoid & White) and pollu beetle *Longitarsus nigripennis* Mots. (Nambiar & Sarma 1977; Ramana & Mohandas 1987; Devasahayam 2000).

RNA based approaches have become more popular in the genomic era for development of ESTs based on cDNA, identification of genes, etc. Extraction of high quality, high molecular weight RNA is therefore essential for cDNA related investigations in *Piper* spp. Several methods are commonly used for isolation of RNA (Chirgwin *et al.* 1979; Chomczynski & Sacchi 1987; Han *et al.* 1987). However, extraction of RNA from plant tissue is often tedious because of high amount of polysaccharides and polyphenolics. Several articles published over the last few years for RNA isolation describe the methods and tools to overcome the afore-mentioned problems using strong protein denaturants, detergents such as SDS and LiCl precipitation (Logemann *et al.* 1987; Guena *et al.* 1998; Yolanda *et al.* 2002). However, such methods

may not yield good quality RNA from all kinds of plant tissues. The large number of published procedures for plant RNA isolation reflects these difficulties and each plant tissue appears to have specific requirement for the isolation of RNA (Baker *et al.* 1990; Lay-Yee *et al.* 1990; Schneiderbauer *et al.* 1991; Levi *et al.* 1992; Schultz *et al.* 1994; Guena *et al.* 1998).

Success of RNA isolation depends upon successful disruption of cells, denaturation of nucleoprotein complex, inactivation of endogenous RNase activity and purification of RNA from contaminating DNA and proteins (Sambrook *et al.* 1989). The ability to isolate good quality RNA, free of contaminants such as proteins, genomic DNA and secondary metabolites is crucial for reverse transcription (RT-PCR), cDNA library construction and northern hybridization (Jun-Jun *et al.* 1998). In this study, we report for the first time, a simple and efficient method for isolating high quality RNA from leaves of *P. nigrum* and *P. colubrinum*. RNA was extracted based on the modified GTC-phenol extraction protocol (Chomczynski & Sacchi 1987).

Materials and methods

Plant materials

P. nigrum and *P. colubrinum* plants were maintained in a green house at Indian Institute of Spices Research, Calicut. Before RNA isolation, the leaves were thoroughly washed with DEPC treated water, and transferred to sterile plastic bags, freezed immediately using liquid nitrogen and stored at -80°C.

Reagents

Denaturing buffer: 4 M GTC, 25 mM sodium citrate (pH 7), 1% sarcosyl, 0.6% α-mercaptoethanol (v/v) and 4% PVP (v/v) added prior to use; water saturated phenol (pH 7); 2 M sodium acetate (pH 4); isopropanol; chloroform:isoamyl alcohol (24:1); DEPC treated water; 75% ethanol; nuclease free water; 100% formamide.

Protocol

Pulverise 2 g of the frozen leaves in the presence of liquid nitrogen in a pre-cooled mortar and pestle.

Transfer the ground leaf material to a polypropylene tube containing 5 ml of denaturing buffer.

Add 0.5 ml, 2 M sodium acetate (pH 4) and mix by inverting the tube.

Add 5 ml of water saturated phenol and mix the tubes gently by inverting.

Add 1 ml of chloroform:isoamyl alcohol (24:1) and mix the tubes.

Incubate the tubes on ice for 20 min.

Centrifuge the tubes at 10,000 g for 20 min at 4°C.

Carefully transfer the supernatant to a fresh polypropylene tube.

Add equal amount (5 ml) of cold isopropanol, mix well and incubate the tubes at -20°C for 1 h to precipitate RNA.

Centrifuge the tubes at 10,000 g for 20 min at 4°C.

Dissolve the pellet containing total RNA in 1.5 ml of denaturing buffer and distribute 0.5 ml in three microfuge tubes.

Add equal volumes of cold isopropanol to each tube, mix well and incubate at -20°C for 1 h.

Centrifuge at 10,000 g for 15 min at 4°C and discard the supernatant.

Resuspend the RNA pellet in 75% ethanol and incubate at room temperature for 15 min.

Centrifuge the tubes for 10 min at 10,000 g at 4°C and aspirate the supernatant.

Vacuum dry the RNA pellet for 15 min and dissolve in 50 µl of nuclease free water or 100% formamide (for long time storage).

Keep overnight at 4°C for dissolving the pellet.

Aliquot the RNA in 1.5 ml tubes and store at -80°C until use.

Denaturing gel electrophoresis

To confirm the integrity of RNA samples from *Piper* spp. extracted by the above protocol, they were resolved in 1% denaturing agarose gel (Sambrook *et al.* 1989). RNA samples (4.5 µl) were pre-treated by adding 10 x MOPS buffer (2 µl), formaldehyde (3.5 µl) and formamide (10 µl). The samples were then heated at 60°C for 10 min and immediately cooled in ice for 5 min. The RNA samples were loaded to the gel, run at appropriate voltage and visualized by staining with ethidiumbromide.

Estimation of RNA purity

Purity of the RNA samples was estimated spectrophotometrically at wave lengths of 260 and 280 nm (Sambrook *et al.* 1989).

Reverse transcription and PCR amplification

Reverse transcription was performed using oligo $dT_{(18)}$ primer. After annealing, the RNA strand was reverse transcribed with reverse transcriptase enzyme. The synthesized cDNA was used for subsequent PCR following conventional reaction procedures.

First strand cDNA synthesis

In a 0.2 ml tube, 1.5 µl of oligo $dT_{(18)}$ primer (5 µM) and 1 µg of RNA were combined and adjusted the volume to 10 µl with DEPC treated water. RNA and primer were denatured by incubating at 65° C for 2 min and then kept on ice. To the above tube, 4 µl of cDNA synthesis buffer (5x) (GenHunter Corporation, USA), 1 µl of dNTPmix (10 mM) (Finnzymes, Finland), 1 µl of RNase inhibitor (40 U μl^{-1}) (Bangalore Genei, India), 0.75 µl of $MgCl_2$ (50 mM) (Finnzymes, Finland) were added and the volume was made up to 19 µl using DEPC treated water. The thermocycler was then programmed at 37°C for 60 min. After incubating the tubes at 37°C for 10 min, the thermocycler was paused and 1 µl of MMLV reverse transcriptase (100 U μl^{-1}) (GenHunter Corporation, USA) was added and mixed before continuing incubation. At the end of reverse transcription, the enzyme denaturation was done by incubating the tubes at 75°C for 5 min. The tubes were then spun briefly and stored at -20°C for later use.

PCR amplification

Only 10% of the cDNA synthesized (2 µl), was used for subsequent PCR amplification. To the tube containing 2 µl of the first strand product, 2 µl of DNA polymerase buffer (10x) (Finnzymes, Finland), 1 µl of dNTP mix (10 mM), 0.75 µl of $MgCl_2$ (50 mM),1 µl Oligo $dT_{(18)}$ (5 µM), 2 µl of arbitrary 13 mer primer (2 µM) (GenHunter Corporation, USA) and 1.5 µl of DyNAzyme EXT DNA Polymerase (1U μl^{-1}) (Finnzymes, Finland) were added and the volume was made up to 20 µl using nuclease-free water. PCR amplification was carried out using the following thermo- cycling conditions: 94°C for 2 min, followed by 40 cycles of denaturing at 94°C for 30 sec, annealing at 40°C for 2 min and extension at 68°C for 3 min. The 40th cycle was followed by a final extension step at 68°C for 10 min.

Results and discussion

Several established and modified methods for RNA isolation were initially tried to isolate RNA from *P. colubrinum* and *P. nigrum* leaves. These included standard LiCl precipitation, non phenolic extraction, and precipitation with various salts (Schuler & Zielinski 1989; Chang *et al.* 1993; Bugos *et al.* 1995). The success of the RNA isolation procedure was judged by quantity, quality and the integrity of RNA recovered. All these methods failed to render good quality RNA and the yield was very poor. The poor yield may be due to polysaccharide contamination, oxidation of phenolic compounds, increased amount of RNases and the presence of genomic DNA as contaminant. The RNA isolated from the afore-said protocols was partially degraded, with no distinct rRNA bands, and was not amplifiable (Fig. 1a).

The protocol described in the present study rendered a rapid and simple procedure for isolating good quality RNA from *Piper* leaves. Addition of α-mercaptoethanol (0.6%) and PVP (4%) to the denaturing buffer was the major modification from the original protocol described by Chomczynski & Sacchi (1987). Using this protocol, 100 µg total RNA from 1 g (fresh weight) of *P. nigrum* and *P. colubrinum* leaves were obtained (Fig. 1b). This yield was comparable to those protocols reported from other plant materials, which gives good quality of amplifiable RNA (Logemann *et al.* 1987; Schultz *et al.* 1994). In addition, the spectrophotometric reading A_{260}/A_{280} ratio ranged between 1.8 and 2.0 indicating little or no protein contamination. The integrity of RNA was judged by denaturing agarose gel electrophoresis which showed clear, discrete ribosomal RNA with no apparent RNA degradation. Formamide (100%) was used as a storage buffer in this study to keep the RNA stable for a longer duration. The RNA resuspended in formamide could be directly used for RT-PCR.

The quality of RNA was tested by RT-PCR. Oligo $dT_{(18)}$ was used for the first strand cDNA synthesis followed by arbitrary 13 mer primers for the second strand synthesis. Two different primers were used for second strand synthesis and amplification of cDNA products from *P. nigrum* and *P. colubrinum* samples. Amplification products ranging from 200 bp to 1100 bp size could be resolved in agarose gels (2%) stained with ethidium bromide in *P. colubrinum* sample (Fig 2a). Amplification products ranging from 250 bp to 1200 bp was observed in case of *P. nigrum* sample (Fig 2b).

The protocol described above will be useful to isolate high quality RNA from different *Piper* species for cDNA related investigations.

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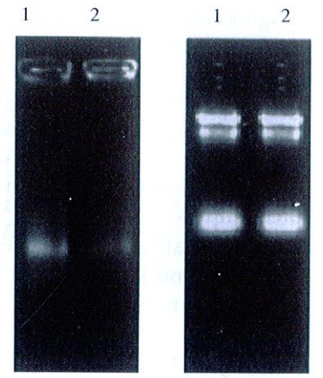

Fig 1. Denaturing 1% agarose gel of total RNA extracted from the leaves of *Piper* species.
(a) RNA isolated from *P. nigrum* (1) and *P. colubrinum* (2) using LiCl method. (b) RNA isolated from *P. nigrum* (1) and *P. colubrinum* (2) using the modified GTC-buffer system.

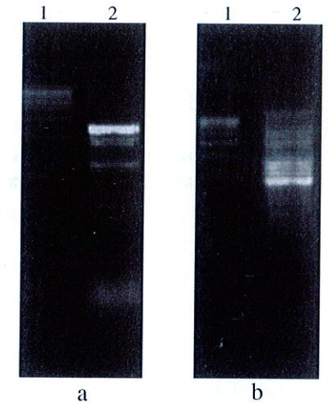

Fig 2. RNA amplification products from *Piper* spp. using oligo $dT_{(18)}$ and random 13 mer primers.

(a) (1) 500 bp ladder. (2) RT-PCR products obtained from RNA sample of *P. colubrinum* using random 13 mer (5′-AAGCTTCATTCCG-3′). (b) (1) 100 bp ladder (2) RT-PCR products obtained from RNA sample of *P. nigrum* using random 13 mer (5′-AAGCTTGATTGCC-3′)

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