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An Improved Method for the Isolation of Total RNA from *Avicennia germinans* Leaves

Daniel Gonzalez-Mendoza[a,c], Adriana Quiroz Moreno[b], and Omar Zapata-Perez[a,*]

[a] Departamento de Recursos del Mar, Cinvestav Unidad Merida, Merida, Yucatan, Mexico. Fax: 52 99 99 81 23 34. E-mail: ozapata@mda.cinvestav.mx
[b] Unidad de Biotecnología, CICY, Merida, Yucatan, Mexico
[c] Departamento de Bioquimica, Instituto Nacional de Caldiología, Merida, Yucatan, Mexico

* Author for correspondence and reprint requests

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Isolation of high-quality RNA of *Avicennia germinans* L. tissue is difficult due to high levels of phenols and other substances that interfere when using conventional procedures for the isolation. These substances not only decrease the yield but also the quality of RNA is almost poor. We present here a simple RNA protocol and fast methodology that effectively removes these contaminating substances without affecting the yield. The protocol developed is based on the SDS/phenol method with modifications including β-mercaptoethanol to prevent oxidation of phenolic complexes, and phenol/chloroform extraction is introduced to remove proteins, genomic DNA, and secondary metabolites, and co-precipitated polysaccharides. Both A_{260}/A_{230} and A_{260}/A_{280} absorbance ratios of isolated RNA were around 2 and the yield was about 0.3 mg g^{-1} fresh weight. Good-quality total RNA from leaves of *Avicennia germinans* could be easily isolated within 2 h by this protocol which avoided the limitation of plant materials and could provide total RNA for all kinds of further molecular studies.

Key words: Avicennia germinans L., RNA Isolation, SDS-TRIS

Introduction

Mangroves represent a critical ecological habitat in the coastal environment of tropical and subtropical areas. The black mangle (*Avicennia germinans*) and others species of mangle are shoreline stabilizers that protect adjacent land from wave and storm erosion. Other functions include sediment stabilization, flood regulation, nutrient supply and regeneration, treatment of dissolved and particulate wastes, and wildlife habitat. Considering their significant value, mangroves have been a new hotspot in the fields of biodiversity, phylogeny, and evolution (Duke *et al.*, 1998) and have become a mine of salt-tolerance-related genes (Yamada *et al.*, 2002). This characteristic has motivated studies focused on exploring the physiological mechanisms of tolerance to saline stress at molecular level. Nevertheless, for studying the gene expression of black mangle the isolation of pure RNA it is crucial because is a pre-requisite of diverse molecular techniques such as reaction of polymerase (PCR), Southern blot and construction of genic libraries. Isolation of RNA from plant tissues can be difficult and often requires modifications of existing protocols. Although there have been some reports on total RNA isolation from mangroves (Fu *et al.*, 2004) an optimized protocol to provide high quantities of pure total RNA for gene expression studies was lacking. In the present study, an easy and rapid protocol was developed for the isolation of enough good-quality total RNA from foliar tissues of wild native *Avicennia germinans.*

Materials and Methods

Plant material

Leaves of *A. germinans* were collected from seedlings grown at 26 °C with a 12-h light/12-h dark period. The photosynthetic photon flux density of light for seedlings' growth was 400–450 μmol m^{-2} s^{-1}. Samples were snap-frozen in liquid N_2 and stored at –80 °C until use.

RNA extraction buffer and solutions

The tubes and bottles were treated with 0.1% DEPC solution at 37 °C overnight, autoclaved twice at 121 °C for 20 min, and then dried at 100 °C before use. The tips used in RNA extraction were RNase-free and DNase-free and purchased from Axygen, USA. The extraction buffer

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was 3% SDS (w/v) containing 0.5 mM EDTA, 0.1 mM Tris-HCl/pH 8.0), and 0.1% DEPC (v/v) at pH 8. 0. Additionally, a mixture of chloroform/phenol (1:1, v/v) was also prepared. All the buffers and solutions were incubated at 37 °C overnight before autoclaving (121 °C, 20 min) twice. A solution of 2.5% β-mercaptoethanol was added into the extraction buffer when used.

RNA extraction procedure

The leaves tissue (500 mg of black mangle tissue) was ground to a fine powder and added to 10 ml pre-warmed extraction buffer and 2.5 μl β-mercaptoethanol shaken vigorously for 15 s. Then 500 μl of chloroform/phenol (1:1) were added carefully and the mixture was incubated at 65 °C for 5 min. Later the homogenate was cooled to room temperature and centrifuged at $12,000 \times g$ at 4 °C for 5 min. The supernatant was transferred to a new tube, and an 1-fold volume of cool isopropanol was added and mixed thoroughly for precipitating total RNA at −20 °C for 30 min. RNA was pelleted at $12,000 \times g$ at 4 °C for 30 min, washed with 75% ethanol twice, dried in vacuum, re-dissolved in 200 μl DEPC-treated MiniQuantum water, and stored at −80 °C for further use. Concentration, yield, and quality control indices based on the absorbance at 230, 260, and 280 nm ($A_{260/280}$ ratios and $A_{260/230}$ ratios) were performed with 2 μl of re-suspended total RNA. 5 μl of the total RNA solution were loaded on 1% agarose gel, electrophoresed to separate the RNA, stained with ethidium bromide, and visualized under UV light to check the size distribution of the total RNA and the integrity of ribosomal bands.

Reverse transcription PCR

Equal amounts of total RNA (1 μg) were converted to first-strand cDNA by using reverse transcriptase, and specific cDNA was amplified by PCR with Taq DNA polymerase according to the manufacturers (Invitrogen, California, USA) instructions. The synthesized cDNA was used for PCR amplifications of *18S* primers (5′-TGTTCACCACCACAGCAGAGCG-3′ and 5′-CACCTGTCCGTCGGGTAACTCG-3′). PCR program parameters were: 94 °C for 2 min, followed by 35 cycles of 1 min at 94 °C for DNA denaturing, 1 min at 45 °C for primer annealing, and 1 min at 72 °C for extension. The program was terminated with 5 min extension at 72 °C. The amplified products were separated on 1.5% agarose gel and visualized by ethidium bromide staining. 2 μl of cDNA were used as PCR template.

Results and Discussion

In the present study, a new protocol was developed to isolate enough good-quality total RNA from the leaves of *Avicennia germinans* plants. In this protocol, higher-strength SDS (3%) was used as lysis buffer and β-mercaptoethanol (2.5%) was added to the SDS buffer, which ensured the rapid removal of phenolic compounds and prevented oxidation and binding to RNA. In this study, the RNA was extracted without degradation (Fig. 1). In all lanes, 2 distinct bands corresponding to 28S and 18S rRNA are clearly shown with no apparent degradation. A smeared background is also visible which probably corresponds to mRNA. The absorbance $A_{260/280}$ was 1.87, suggesting that the RNA was pure and could be used for further analysis. In all samples, the $A_{260/230}$ ratios were more than 1.9, suggesting less contamination by polyphenols and polysaccharides (Table I). These results are important because the procedures for RNA isolation are usually evaluated by quantity, quality, and integrity of the RNA obtained (Asif *et al.*, 2000).

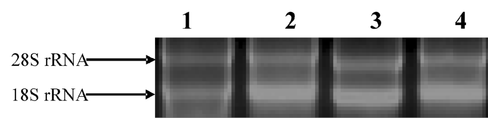

Fig. 1. Electrophoresis gel showing the total RNA extracted from *Avicennia germinans* leaves. Lines 1 to 4, different individual leaves.

Table I. Absorbance ratios and yield of total RNA isolated from *Avicennia germinans* leaves[a].

$A_{260/230}$	1.90 ± 0.048
$A_{260/280}$	1.87 ± 0.028
Yield [μg g^{-1} fresh weight]	308 ± 43.8

[a] Values are means ± SD (n = 4).

On the other hand, the SDS-TRIS extraction buffer contained a high concentration of β-mercaptoethanol. In this way, β-mercaptoethanol was used to inhibit the RNase activity by means of reduction of the disulfide bonds to sulfhydryl groups (these disulfide bonds are essential for the activity of RNases) and to prevent sample oxidation as well, since phenolic compounds are readily oxidized to form covalently linked quinines and av-

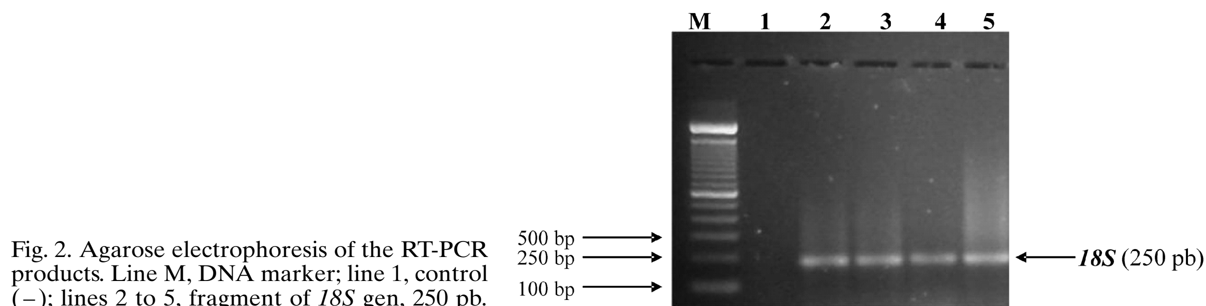

Fig. 2. Agarose electrophoresis of the RT-PCR products. Line M, DNA marker; line 1, control (–); lines 2 to 5, fragment of *18S* gen, 250 pb.

idly bind nucleic acids which cause irreversible damage to RNA (Wang *et al.*, 2004; Chirgwin *et al.*, 1979). The purification by phenol/chloroform extraction was introduced to remove polysaccharides and polyphenols, because the phenol/chloroform extraction denatures RNases and removes proteins much more effectively than chloroform-only extraction (Wang *et al.*, 2005). Additionally, the use of 100% isopropanol has facilitated the effective precipitation of RNA and rendered it more stable in the precipitated form. LiCl precipitation was not used because polymers such as polysaccharides and polyphenols from extracts of some plant tissues limit the concentration of nucleic acids attainable, with unsatisfactory consequences for the quantitative recovery of RNA by LiCl precipitation (Manning, 1991).

On the other hand with the purpose to evaluate the suitability of the isolated RNA in downstream molecular procedures, total RNA was used for RT-PCR analysis (reverse transcription is highly sensitive to impurities) and as expected, a fragment of the *18S* gene was amplified (Fig. 2). Owing to the chemical and biological diversities of plants, until now, there is no universal RNA extraction method available (Jones *et al.*, 1997). In this way, the current protocol is efficient and recommended for the isolation of good-quality total RNA from mangrove plants like *A. germinans* in less than 2 h.

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