

An Efficient Method for the Isolation of Good Quality RNA from Bark Tissues of Mature Rubber Trees

M.B. MOHAMED SATHIK^{*#}, K.N. NATARAJA^{**}, M. THOMAS^{*},
J. JACOB^{*} AND M. UDAYAKUMAR^{**}

In this study, an attempt has been made to extract good quality RNA from soft bark tissues of Hevea which generally contain plenty of interfering compounds such as phenolic compounds, tannins, lignins and carbohydrates. The method explained here involves removal of phenolic compounds using polyvinyl pyrrolidone and selective precipitation of RNA using lithium chloride. The purity of RNA prepared by this method was tested for its ability to get a good quality cDNA prepared. The cDNA produced from total RNA preparation was further confirmed by amplification of specific house keeping genes using quantitative real time PCR method. This protocol appears best suited for Hevea bark that is rich in several contaminating factors.

Key words: *Hevea brasiliensis*; RRII 105; recalcitrant bark tissue; tapping panel dryness; total RNA isolation; cDNA; real time PCR

Isolation of RNA from bark of *Hevea* is a prerequisite to study the expression of different genes associated with rubber biosynthesis, tapping panel dryness, biotic and abiotic stress responses, etc. A good quality RNA preparation is essential to perform experiments such as northern hybridisation, cDNA library construction, *in vitro* translation, differential display RT-PCR, subtractive hybridisation, etc. In tree species containing high levels of phenolic compounds, carbohydrates and other unidentified compounds, isolation of good quality RNA in large quantities becomes very difficult. During tissue homogenisation phenolic compounds get immediately oxidised to form covalently linked quinines¹ that readily bind with nucleic acids making the RNA

unusable for many of the above mentioned experiments. Moreover, the higher levels of endogenous RNase activity in *Hevea* make isolation of good quality RNA a tough task².

There have been many methods of RNA isolation reported in *Hevea*^{3,4}. Except for one protocol³, all the others are meant for RNA isolation from either leaf or latex which is comparatively easier than isolating RNA from bark tissues. Isolation of RNA from bark has been very difficult as they are rich in phenolic compounds, terpenoids and tannins which may bind to RNA after cell lysis³ or compounds that co-precipitate with RNA. For extracting good quality total RNA from highly recalcitrant tissues such as the bark of mature *Hevea* trees

*Plant Physiology Division, Rubber Research Institute of India, Kottayam 686 009, India

**Department of Crop Physiology, University of Agricultural Sciences, GKVK, Bangalore, India

#Corresponding author (e-mail: sathik@rubberboard.org.in)

rich in phenolic compounds, use of PVP in the extraction buffer could prove to be incompatible with phenol extraction^{5,6}. When PVP is added in the RNA extraction buffer, the homogenate has to be treated with phenol only after removing the PVP. This is well explained in the protocol described by Salzman and co-researchers⁶. By this method, the phenolic compounds are bound to soluble PVP in the extraction buffer, which are later eliminated by ethanol precipitation of the RNA. The protein and carbohydrates precipitated along with the RNA in this preparation are subsequently removed by phenol extraction and lithium chloride precipitation, respectively. The commercial kits available for the RNA isolation such as RNeasy Plant Mini kit from Qiagen (Qiagen Inc, GmbH) and TRI reagent from Sigma (Sigma, USA) were also used for RNA isolation from bark. But they also did not yield satisfactory results.

Considering the phenol incompatibility with PVP, an alternate approach to isolate RNA using Salzman *et al.* method has been attempted with a few modifications in the present study. This method has been reported as effective for getting good RNA preparation from other recalcitrant plant tissues also⁶. The high quality RNA obtained by this method from the soft bark tissues of mature *Hevea* was used to make cDNA which was further checked with real time PCR for its amplifiability of known genes using heterologous primers. The results confirmed the good quality of RNA obtained by this method from bark tissues of mature *Hevea*.

EXPERIMENTAL

Sample Collection

The bark samples of clone *Hevea* RR11 105 (16 year-old-trees) were collected from the RR11

campus. Bark samples of about two square inch size were cut from each tree with the help of a chisel. The exuding latex was wiped with tissue paper. The inner soft bark was shaved as quickly as possible with a sterile scalpel on a sterile cutting board, packed in aluminium foil and dipped in liquid N₂ before it was transported to the laboratory to store at -80°C.

RNA Extraction Protocol

All operations explained below were performed with sterile gloves and the reagents and other preparations were made from DEPC treated water unless otherwise specifically mentioned. All glasswares, scalpel holder, pestle and mortar were treated with DEPC water before they were autoclaved. The pipettes, centrifuge rotors and other accessories were wiped clean with alcohol before the operation. The centrifuge tubes were kept in ice throughout the procedure unless otherwise specifically mentioned.

About 1 g bark was homogenised in liquid N₂ to a fine powder. The powder was transferred into an oak ridge centrifuge tube containing 10 mL of RNA extraction buffer (4 M guanidine thiocyanate, 100 mM Tris HCl, pH 8.0, 25 mM sodium citrate, pH 8.0, 0.5% N-lauryl sarcosine) with 100 mg of PVP (soluble) and 200 µL β-mercaptoethanol freshly added. The contents were mixed by shaking for a few minutes and were added with equal volume of chloroform:isolamyl alcohol mixture (24:1 v/v). The tubes were again mixed for another 10 min to 15 min before they were spun at 16 000 × g for 10 min at 4°C. Though a swinging bucket rotor would be best suited for this step, this was performed using a fixed angle rotor only. The upper aqueous phase was carefully transferred into a fresh tube with a wide bore tip and the chloroform extraction was

repeated once again. The aqueous phase was transferred to a fresh tube and was mixed with $2 \times$ homogenate volume of chilled absolute ethanol and $0.1 \times$ homogenate volume of 5 M NaCl. The tubes were stored at -20°C for 3 h or overnight after which they were spun at $16\,000 \times g$ for 10 min at 4°C . The supernatant was carefully discarded and the pellet was resuspended in 10 mL DEPC treated distilled water. If resuspension was turbid, an optional spin for 5 min at $20\,000 \times g$ was performed and the supernatant was transferred to a fresh tube.

An equal volume of Tris saturated phenol (pH 8.0): chloroform: isoamylalcohol (25:24:1 v/v) was added to the tubes and the contents were mixed by shaking continuously for 10 min to 15 min at room temperature. The tubes were spun at $13\,000 \times g$ for 10 min at room temperature after which the upper phase was transferred to another tube. This extraction was repeated once again. The upper phase was transferred to another tube and was again ethanol precipitated as above ($2 \times$ volume of ethanol and 0.1 volume of 5 M NaCl at -20°C for 3 h or overnight). After this precipitation, the tubes were spun at $16\,000 \times g$ for 10 min at 4°C . The supernatant was discarded and the pellet was resuspended in $800 \mu\text{L}$ DEPC water. If the resuspension looks cloudy, it can be centrifuged again to pellet down the insoluble material. The supernatant was transferred to a microcentrifuge tube in which the volume was finally made up to 1 mL. The RNA was precipitated by adding $333 \mu\text{L}$ of 8 M lithium chloride (2 M final concentration) and by keeping the tubes at 4°C for 3 h or overnight. Later, the tubes were centrifuged at $12\,000 \times g$ for 15 min at 4°C . The pellet was rinsed with $400 \mu\text{L}$ of chilled 80% ethanol and then spun again for 10 min. The supernatant was carefully removed till the final drop using a pipette tip and the tubes were dried for a few minutes under vacuum. The pellet was dissolved in $100 \mu\text{L}$ DEPC treated distilled

water. Quality of RNA was checked on gel and quantitated spectrophotometrically. For long-time storage, the RNA was again added with $2 \times$ volume of absolute ethanol and 0.1 volume of 5 M NaCl and stored at -80°C .

To quantitate and to check the quality of the RNA being prepared, absorbance was measured at 260 nm and 280 nm. The RNA was also run on an agarose gel electrophoresis. RNA (around $10 \mu\text{g}/10 \mu\text{L}$) was mixed with equal volume of gel loading buffer (100 mL of this buffer contained 54.7 mL formamide, 18.25 mL of 37% formaldehyde, 10 mL of $20 \times$ MOPS buffer, 6.6 mL of glycerol, 10 mL of DEPC treated water, 0.1% bromophenol blue and 0.1% xylene cyanol) containing $1.0 \mu\text{g}$ per mL ethidium bromide. The tubes were kept at 65°C for 15 min and were transferred to ice. After 5 min, the samples were loaded on a 1.4% agarose gel (containing $1 \times$ MOPS buffer and 2.2 M formaldehyde) and were run in a tank filled with $1 \times$ MOPS buffer at a speed of 5 V per cm for an hour. The gel was visualised on a UV transilluminator.

Reverse Transcription and Real-time PCR Conditions

To test the quality of RNA extracted from the bark tissue, cDNA was synthesized and a quantitative real-time polymerase chain reaction (RT-PCR) was performed. About $15 \mu\text{g}$ of total RNA was reverse transcribed using 200 U Moloney Murine Leukemia Virus reverse transcriptase (M-MuLV-reverse transcriptase⁷) at 42°C for one hour. The reaction was primed with $2.5 \mu\text{M}$ oligo dT primers in the presence of 10 mM dNTPs mixture in a total volume of $20 \mu\text{L}$. Transcript levels of two most abundant RNAs, β -Actin⁸ and 18S rRNA⁹ were tested in the cDNA pool. Oligonucleotide primers were designed with the DNASTAR software

(PrimerSelect, DNASTAR, Inc.) with care to avoid the primer-dimer, and hair-pin formation (Table 1).

The cDNA pool was used as template to perform quantitative real-time PCR in the presence of SYBR-green dye. The optimisation of the real time PCR conditions was performed according to the manufacturer's instructions (MJ Research, USA & MJ Bioworks, Inc.). The PCR was performed using the DyNAmo SYBR-Green qPCR master mix containing *Tbr*-DNA polymerase, SYBR-green 1, 5 mM MgCl₂ and optimised PCR buffer with dNTPs (DyNAmo SYBR-Green qPCR Kit FINNZYMES, Finland). The conditions for the PCR were as follows: 95°C for 7 min, 30 cycles of denaturation (at 94°C for 1 minute), annealing for 30 sec (50°C and 58°C for actin and 18 S gene, respectively), polymerisation for 1 min (72°C) followed by plate reading at 72°C for 5 min, estimation of melting curve from 50°C to 95°C and incubation at 72°C for 7 min. The amplification plots of the PCR reaction were used to determine the threshold cycle (C_T).

RESULTS AND DISCUSSION

In this study, an attempt has been made to isolate total RNA from bark of mature trees of healthy and TPD affected *Hevea brasiliensis* trees by using modified Salzman *et al.*⁶ protocol. It is

known that the bark tissues from TPD trees would generally have more tannins, lignins and phenolic compounds than the bark from normal trees^{10,11}. Hence, bark materials from TPD trees have also been included in this study to check if the protocol used here could yield good quality RNA in spite of the presence of high levels of phenolic compounds, stone cells and RNases in the TPD bark samples. The quality of total RNA isolated using this protocol appears good on agarose gel (Figure 1). The A₂₆₀ and A₂₈₀ ratios of the RNA prepared was in the range of 1.7 to 1.9 indicating its good purity. The quantity of RNA obtained ranged from 400 µg/g to 550 µg/g fresh tissue. To further check the quality of the isolated RNA, a first strand cDNA was synthesized using RevertAid™ H Minus M-MuLV-Reverse Transcriptase. The success of a good quality cDNA synthesis largely depends upon the quality of RNA. The quality of cDNA being synthesised was confirmed by PCR amplification of specific maintenance gene sequences using heterologous primers under real time PCR conditions.

SYBR-Green, a minor-groove DNA binding dye was used for assessing the amplification of gene of interest under real time conditions. This dye has high affinity for double stranded DNA (dsDNA) and exhibits enhanced fluorescence upon binding to a dsDNA¹². The SYBR-Green dye is excited at 485 nm wavelength, and the emission is measured at 520 nm wavelength. Fluorescence of the SYBR-Green dye was

TABLE 1. GENE SPECIFIC PRIMERS USED FOR REAL TIME PCR TO CHECK THE QUALITY OF cDNA

Oligo name	Sequence (5'-3')
Actin forward primer	CCCAGAGCAAGAGAGGTA
Actin reverse primer	GCGTATCCTTCGTAGATGGG
18S rRNA forward primer	GCTGCGATGATTTCATGATAAC
18S rRNA reverse primer	GAAGGGATACCTCCGCATAGC

monitored at the end of each PCR cycle, which is required to detect the product of interest during the linear phase of amplification. The increase of fluorescence above background is dependent on the initial template concentration¹³. The specificity of the product was measured by its melting curve as reported earlier¹⁴. A single dissociation peak with a T_m within variation of 2°C of the expected temperature indicated the amplification of gene of interest. The C_t value (the fractional cycle number at which the fluorescence passes the fixed threshold) of the genes tested were 17.4 ± 0.35 and 15.2 ± 0.71 for actin and 18S rRNA, respectively (Figure 2). These results suggest that the quality of the RNA extracted from the bark tissue was good and could be used for all types of applications.

The results confirm the good quality of total RNA extracted from the bark of *Hevea* which is supposed to be recalcitrant with plenty of phenolic compounds, terpenoids and tannins^{10,11}. Similar kind of results could not be obtained when several other methods were tried including commercial kits from Qiagen (RNeasy kit) and Sigma (TRI reagent). Applying routine methods for RNA extraction from bark tissues³ employing phenol/SDS may result in poor yield and quality of total RNA due to PVP-phenol incompatibility^{5,6}. Incorporation of PVP in the extraction buffer contributed effectively to removing the phenolic compounds from the preparation. Subsequent elimination of PVP from the extraction buffer by ethanol precipitation and the phenol extraction of the

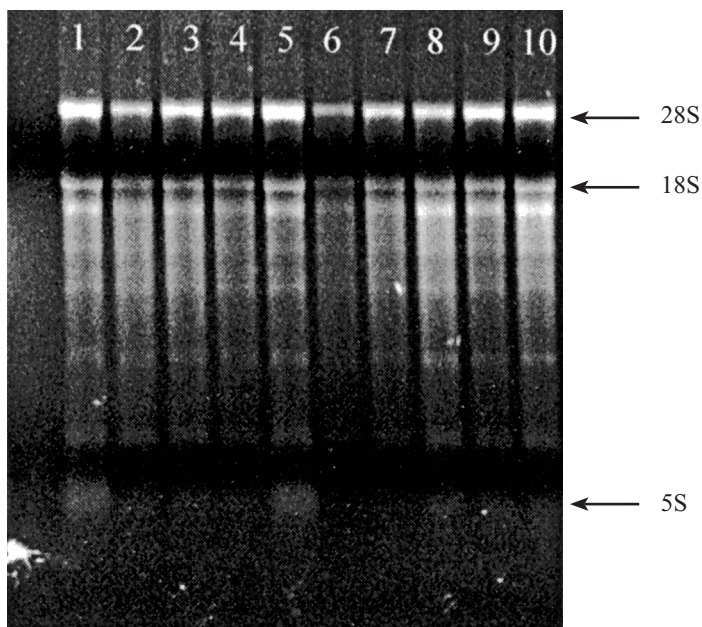


Figure 1. Visualisation of total RNA (10 μ L) isolated from *Hevea* bark tissue of normal (Lanes 1 to 5) and TPD trees (Lane 6 to 10) on 1.4% denaturing agarose gel.

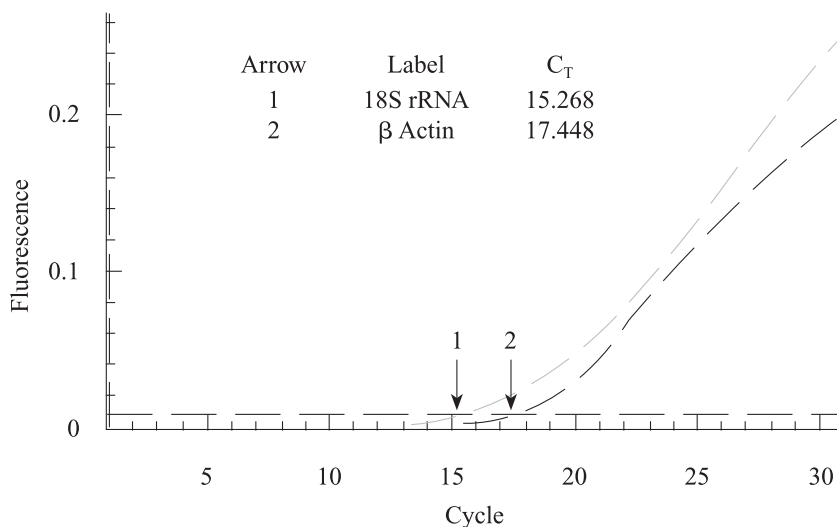


Figure 2. Amplification of a segment of the 18S rRNA and beta-actin gene from the cDNA pool of normal bark tissue of *Hevea*. Arrow 1 indicates the C_i (the fractional cycle number at which the fluorescence passes the fixed threshold) value of 18S and arrow 2 indicates C_i value of beta-actin gene on the amplification plot.

crude RNA improved the effective removal of phenolic compounds as well as protein in the sample. The other contaminating materials such as polysaccharides, genomic DNA and unidentified materials that absorb strongly at 230 nm were effectively removed by the final lithium chloride precipitation. When the RNA was examined on gel, the 28S rRNA band was more abundant than 18S rRNA band indicating the good quality of the preparation. Typical yields of RNA by this method was about 500 $\mu\text{g/g}$ fresh weight of bark tissue, which compares on par with yields reported for other methods designed for high-phenolic tissues^{3,6,15}. The quantitative real time PCR results also confirmed the good quality cDNA obtainable by this method, which in turn proved the good quality of RNA obtainable by the present method. This method therefore appears best suited for *Hevea* bark that is rich in several contaminating factors.

CONCLUSIONS

The present protocol incorporates steps to remove phenolic compounds, tannins, carbohydrates, unidentified contaminating materials and DNA from the extraction buffer. Our results show that, this method works well with a recalcitrant tissue like *Hevea* bark. The RNA extracted by this method also worked well for the subsequent steps like reverse transcription (cDNA) and real time PCR of known genes like actin and 18S rRNA. The present protocol may be highly suitable for extracting RNA from other highly recalcitrant tissues that contain interfering molecules.

ACKNOWLEDGEMENT

This study is part of an RRII-UAS collaborative project (No. PPD/D-601/2004). UAS is

indebted to DST, Government of India for the Real Time PCR equipment (No. SR/FST/LSI-084/2002). Thanks are due to Dr. N.M. Mathew (Director of Research, RRII) and Dr. Rama, N. (UAS, Bangalore) for their help and assistance.

Date of receipt: November 2004

Date of acceptance: June 2005

REFERENCES

1. LOOMIS, W.D. (1974) Overcoming Problems of Phenolics and Quinines in the Isolation of Plant Enzymes and Organelles. *Meth. Enzymol.*, **31**, 528–545.
2. KUSH, A., GOYVAERTS, E., CHYE, M.L. AND CHUA, N.H. (1990) Laticifer-specific Gene Expression in *Hevea brasiliensis*. *Proc. Natl. Acad. Sci. USA*, **87**, 1787–1790.
3. VENKATACHALAM, P., THANSEEM, I. AND THULASEEDHARAN, A. (1999) A Rapid and Efficient Method for Isolation of RNA from Bark Tissues of *Hevea brasiliensis*. *Curr. Sci.*, **77(5)**, 635–637.
4. THOMAS, M., RAGHOTHAMA, K.G. AND JACOB, J. (2002) A Simple and Efficient Method for the Isolation of Total RNA and mRNA from Mature Leaves of *Hevea brasiliensis*. *Indian J. nat. Rub. Res.*, **15(1)**, 93–95.
5. VAN DRIESSCHE, E.S., BEECKMANS, R., DEJAEGERE, R. AND KANAREK, L. (1984) Thiourea: The Antioxidant of Choice for the Purification of Proteins from Phenolrich Tissues. *Anal. Biochem*, **141**, 184–188.
6. SALZMAN, R.A., FUJITA, T., ZHU-SALZMAN, K., HASEGAWA, P.M. AND BRESSAN, R.A. (1999) An Improved RNA Isolation Method for Plant Tissues Containing High Levels of Phenolic Compounds or Carbohydrates. *Plant Mol. Biol. Reporter*, **17**, 11–17.
7. KOTEWICZ, M.L., SAMPSON, C.M., D'ALESSIO, J.M. AND GERARD, G.F. (1998) Isolation of Clonal Moloney Murine Leukemia Virus Reverse Transcriptase Lacking Ribonuclease H Activity. *Nucleic Acids Res.*, **16**, 265–277.
8. KREUZER, K. A., LASS, U., LANDT, O., NITSCHKE, A., ELLERBROK, H., PAULI, G., LASER, J., HUHN, D. AND SCHMIDT, C. A. (1999) A Highly Sensitive and Specific Fluorescence RT-PCR Assay for the Pseudogene Free Detection of β -actin Transcripts as Quantitative Reference. *Clin. Chem.*, **45**, 297–300.
9. PAUL, M.R. AND WHITE, R.J. (2000) Survey and Summary: Transcription by RNA Polymerases III and I. *Nucleic Acids Res.*, **28**, 1283–1298.
10. DE FAY, E. AND JACOB, J.L. (1989) Symptomatology, Histological and Cytological Aspects, *Physiology of Rubber Tree Latex (d'Auzac, Jacob, J. and Chrestin, H. eds.)*, pp. 407–430. Boca Raton, Florida: CRC Press.
11. KRISHNAKUMAR, R., SREELATHA, S., THOMAS, M., GOPALAKRISHNAN, J., JACOB J. AND SETHURAJ, M.R. (1998) Biochemical Composition of Soft Bark Tissues in *Hevea* Affected by Tapping Panel Dryness. *Indian J. nat. Rub. Res.*, **12(1 & 2)**, 92–99.
12. WITTER, C.T.G., HERRMANN, M.G., MOSS, A.A. AND RASMUSSEN, R.P. (1997) Continuous Fluorescence Monitoring of Rapid Cycle DNA Amplification. *Bio-Techniques*, **22**, 130–138.
13. DHAR, A.K., ROUX, M.M. AND KLIMPEL, K.T. (2001) Detection and Quantification of Infectious Hypodermal and Hematopoietic

- Necrosis Virus and White Spot Virus in Shrimp Using Real-time Quantitative PCR and SYBR Green Chemistry. *J. Clin. Microbiol.*, **29(8)**, 2835–2845.
14. RIRIE, K.M., RASMUSSEN, R.P. AND WITTEWER, C.T. (1997) Product Differentiation by Analysis of DNA Melting Curves during the Polymerase Chain Reaction. *Anal. Biochem.*, **270**, 154–160.
15. WOODHEAD, M., TAYLOR, M.A., DAVIES, H.V., BRENNAN R.M. AND MCNICOL, R.J. (1997) Isolation of RNA from Blackcurrant (*Ribes nigrum L.*) Fruit. *Mol. Biotechnol.*, **7(1)**, 1–4.