

Full Length Research Paper

Co-extraction of high-quality RNA and DNA from rubber tree (*Hevea brasiliensis*)

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Accepted 10 April, 2012

High-quality nucleic acids are the basic requirement for performing genomic research. A reliable and efficient method was developed for co-extracting high-quality DNA and RNA from rubber tree (*Hevea brasiliensis*) in this study. Polyethylene glycol (PEG) and cetyltrimethylammonium bromide (CTAB) extraction buffer with high concentrations of polyvinylpyrrolidone (PVP) and β -mercaptoethanol was used in this study. The results show that 3.2% polyethylene glycol 8000 is the optimal concentration for successful separation of DNA and RNA. Spectrophotometric determination (A_{260}/A_{280} and A_{260}/A_{230} ratios), agarose electrophoresis analysis and reverse transcription (RT-PCR) of isolated nucleic acids indicate that high-quality DNA and RNA were extracted by this method. The general applicability of this method was also evaluated, and the results show that it was suitable for a variety of plants.

Key words: *Hevea brasiliensis*, polyethylene glycol (PEG), nucleic acid, co-extraction, higher plants.

INTRODUCTION

Rubber tree (*Hevea brasiliensis*) is indigenous to the Amazon basin of South American. More than 98% of commercial source of nature rubber (NR) comes from *H. brasiliensis*. Today, rubber tree is extensively cultivated in Southeast Asia, where it covers more than 90% of the world's nature rubber production (Priyadarshan and Goncalves, 2003). Due to the increasing demands of NR in the world market, scientists began to pay more and more attention to rubber tree genomic research. Therefore, quality of nucleic acids isolated from rubber tree tissues is essential for future molecular studies of

rubber tree. Rubber tree contains high levels of polysaccharides and polyphenols. Thus, it is difficult to extract high-quality DNA and RNA from multiple tissues of rubber tree. Nowadays, although several alternative methods have been developed for the extraction of total RNA or DNA from rubber tree, the procedures for DNA extraction are complicated (Bease et al., 1994; Lespinasse et al., 2000; Lekawipat et al., 2003), and most RNA methods can only be used for rubber tree latex (Kush et al., 1990; Oh et al., 1999; Ko et al., 2003; Tang et al., 2007) with only one exception for the bark (Venkatachalam et al., 1999).

Polyethylene glycol (PEG) can fractionate different molecular mass DNA (Lis and Schleif, 1975). Under the conditions of high PEG and salt concentration, DNA would bind to the surface of carboxyl coated magnetic particles, and the DNA-bead complex could be extensively washed and finally eluted with water to yield purified DNA (Lis, 1980). PEG has not been only used to isolate DNA from bacterium and viruses (Lewis and Metcalf, 1988; Griffiths et al., 2000; Roose-Amsaleg et

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Abbreviations: PEG, Polyethylene glycol; CTAB, cetyltrimethylammonium bromide; PVP, polyvinylpyrrolidone.

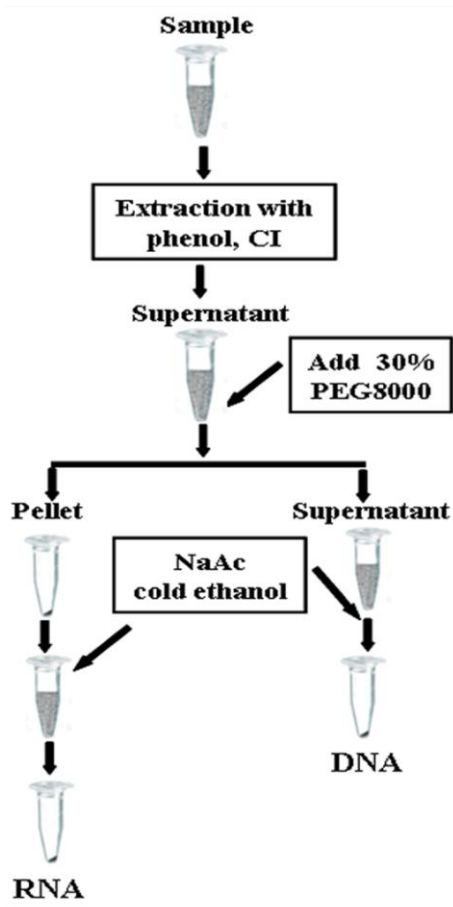


Figure 1. Overview of the procedure for co-extracting RNA and DNA.

al., 2001), but also it was demonstrated that PEG 15,000 to 20,000 at 1 to 2% (w/v) was effective to isolate RNA from succulent plant species rich in polyphenols and polysaccharides (Gehrig et al., 2000).

We herein report a method developed for co-extracting DNA and RNA from rubber tree. Firstly, a total nucleic acid preparation was yielded by cetyltrimethylammonium bromide (CTAB) extraction buffer. Subsequently, differential precipitation with polyethylene glycol 8000 (PEG8000) buffer was used to isolate high molecular-weight RNA, and the supernatant was used to recover genomic DNA by absolute ethanol. By using this protocol, high-quality RNA and DNA were co-extracted and separated from *H. brasiliensis*, rice (*Oryza sativa* L.) and soybean (*Glycine max*), respectively.

MATERIALS AND METHODS

Plant materials

H. brasiliensis used in this study was maintained in Rubber Research Institute, Chinese Academy of Agricultural Sciences. *O. sativa* L. and *G. max* were provided by College of Agriculture,

Hainan University.

Solutions and equipment

All reagents were purchased from Sangon Biotech (Shanghai) Co., Ltd. Solutions used in this study were as follows: water-saturated phenol (WP); chloroform:isoamyl alcohol (CI) (24:1); 3 M sodium acetate (NaAc) (pH5.2); 30% (W/V) PEG8000; TE (10 mM Tris, 1 mM EDTA) (pH 8.0); absolute ethanol; 70% ethanol; distilled diethylpyrocarbonate (DEPC) -treated autoclaved water. Extraction buffer contained 2% CTAB, 25 mM EDTA, 2 M NaCl, 4% polyvinylpyrrolidone-40 (PVP-40) (W/V), 100 mM Tris-HCl (pH 8.0), 0.5 g/L spermidine and 4% β -mercaptoethanol (V/V) (being added right before use). All solutions were prepared with DEPC-treated water at 37°C overnight, and autoclaved for 20 min at 121°C before use. Glassware, mortars and pestles were baked for 8 h at 180°C.

Selective precipitation of RNA and DNA by PEG8000

Samples (0.8 g) were ground to a fine powder with a mortar and pestle in liquid nitrogen. The powder was transferred to a clean 15 ml-tube with 5 ml of extraction buffer preheated at 65°C, and homogenized by mixing gently followed by incubation for 5 min in a 65°C water bath. When the homogenate was cooled to room temperature, an equal volume of WP was added. Then, the mixture was vigorously vortexed and centrifuged at 12000 $\times g$ for 20 min at 15°C. The supernatant was carefully transferred to a clean 15 ml-tube containing an equal volume of CI (24:1). After vortexing, the mixture was centrifuged at 12000 $\times g$ for 10 min at 4°C. In order to improve the purity of nucleic acids, the supernatant was collected and re-extracted with one volume of CI.

After the re-extraction, the supernatant was transferred to a clean 15 ml-tube containing varying volumes of 30% (w/v) PEG8000 solution and incubated on ice for 30 min before centrifugation at 12000 $\times g$ for 10 min at 4°C. The pellet and supernatant were collected respectively for total RNA or genomic DNA isolation (Figure 1).

Total RNA isolation

The pellet was washed twice with 70% ethanol. After air drying for 10 min, it was resuspended in 400 μ l RNase-free water and transferred to a clean 1.5 ml microcentrifuge tube. To recover total RNA, 0.1 volume of 3 M NaAc and 2.5 volumes of cold absolute ethanol were added and mixed gently. After incubation for 1.5 h at -20°C and centrifugation at 12000 $\times g$ for 10 min at 4°C, the pellet was washed and air-dried as previously indicated. Finally, it was resuspended in 30 μ l TE buffer, and stored at -80°C.

Genomic DNA isolation

The supernatant was transferred to another new tube containing 0.1 volume of 3 M NaAc and 2.5 volumes of cold absolute ethanol and mixed gently followed by incubation for 1.5 h at -20°C and centrifugation at 12000 $\times g$ for 10 min at 4°C. The pellet was washed and air-dried as previously indicated and resuspended in 30 μ l TE buffer and stored at -20°C.

Evaluation of integrity, purity, and yield of RNA and DNA

The integrity of RNA and DNA were evaluated by running 1 μ l of extraction nucleic acid sample on 1.2% agarose-formaldehyde gel a

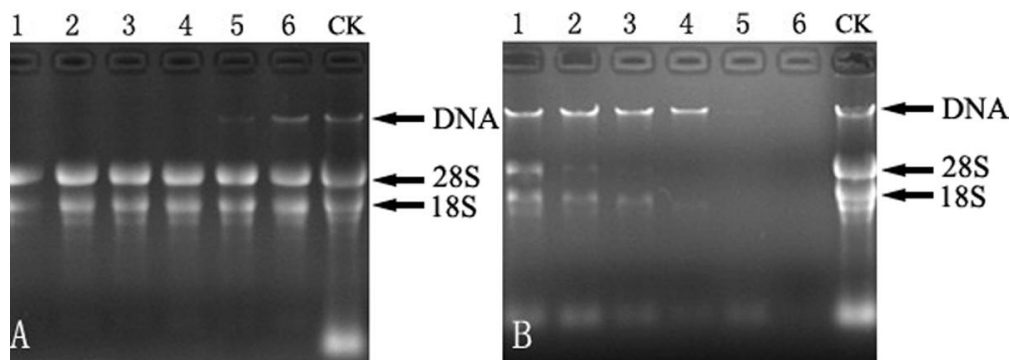


Figure 2. Effects of different PEG8000 concentrations on precipitation of nucleic acid in *H. brasiliensis*. 1 μ l of the extracts was separated on 1.2% agarose-formaldehyde gel. Lanes 1 to 6 indicate that PEG8000 final concentration was 1, 2, 3, 4, 5 and 6%, respectively. CK, Nucleic acid precipitated with 0.1 volume of 3 M NaAc and 2.5 volumes of cold ethanol. (A) Nucleic acids in the PEG8000 deposit; (B) residual nucleic acids in the supernatant.

spectrophotometer (Ultrospec 3300 pro, GE Healthcare) at The purity and concentration of RNA and DNA were assessed with and 0.8% agarose gel, respectively. The gel was stained with Gelview (BioTeke Corporation, China) and visualized with UV light. wavelengths of 230, 260, 280 and 320 nm respectively.

Reverse transcription (RT)-PCR analysis

Total RNA was treated with DNase I (Fermentas). cDNA synthesis was conducted using the RevertAid™ Premium First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. 2 μ g of total RNA was used in each reaction. To examine the quality of RNA, 1 μ l of the RT mixture diluted 20 fold was used as template for amplifying 18S rRNA and *Hbactin* gene (GU270586) in 20 μ l reaction volume, respectively. The primer pairs were as follows: 5'-GGT CGC AAG GCT GAA ACT-3' and 5'-ACG GGC GGT GTG TAC AAA-3' for 18S rRNA; 5'-GAT TCC GTT GCC CAG AAG TC-3' and 5'-CAC CAC TCA GCA CAA TGT TAC C-3' for *Hbactin*. The PCR program was set as an initial denaturation for 5 min at 94°C, followed by 35 cycles of 94°C for 30 s, 58°C for 40 s, 72°C for 90 s, and a final extension for 7 min at 72°C.

Digestion analysis of genomic DNA

Genomic DNA was digested for 20 min with FastDigest *Hind* III (Fermentas) and FastDigest *Eco*R I (Fermentas), respectively. The reactions were performed according to the manufacturer's instructions.

RESULTS

Determination of the effective concentration of PEG8000

To optimize the concentration of PEG8000, varying volumes of 30% (w/v) PEG8000 were added to the supernatant after the homogenization using CI. A gradient of final concentration of PEG8000 was 1, 2, 3, 4, 5 and 6%. The results show that nucleic acids in PEG8000 deposit increased gradually with increasing

final concentration of PEG8000, whereas residual nucleic acids in the supernatant were gradually decreased (Figure 2). When the final concentration of PEG8000 reached 4%, DNA began to appear in the pellet, whereas when it increased to 6%, there was no residual nucleic acid in the supernatant (Figure 2). It indicates that nucleic acids in the supernatant can be completely precipitated at 6% PEG8000 concentration.

According to the above results, a PEG8000 final concentration gradient (3.0, 3.2, 3.4, 3.6, 3.8, 4.0, 4.2, 4.4, 4.6, 4.8 and 5.0%) was set. The results show that there was no DNA contaminant in 3, 3.2 and 3.4% PEG8000 deposit (Figure 3). DNA began to appear in the PEG8000 pellet when the PEG8000 concentration increased to 3.6% (Figure 3). When the concentration reached 4.6%, there was a thimbleful of the residual DNA in the supernatant. Therefore, in order to obtain high-quality RNA, we chose 3.2% as the optimal PEG8000 concentration.

Extraction of RNA and DNA from *H. brasiliensis*, *O. sativa* L. and *G. max*

Based on the above results, nucleic acids were isolated at 3.2% PEG8000 from leaves, bark and latex of *H. brasiliensis*, respectively. High-quality DNA and RNA were obtained from each tissue (Figure 4). In order to test wide application of the method described in this paper, DNA and RNA were co-extracted from *O. sativa* L. and *G. max* and it showed that DNA and RNA were completely separated by this method (Figure 5). All of the electrophoresis results indicate that the bands of RNA and DNA were clear, and the brightness of the 28S rRNA band was approximately twice that of the 18S rRNA bands, demonstrating that the RNA was intact (Figures 4 and 5). In addition, no DNA smear was observed around DNA band, indicating that DNA was not degraded (Figures 4 and 5).

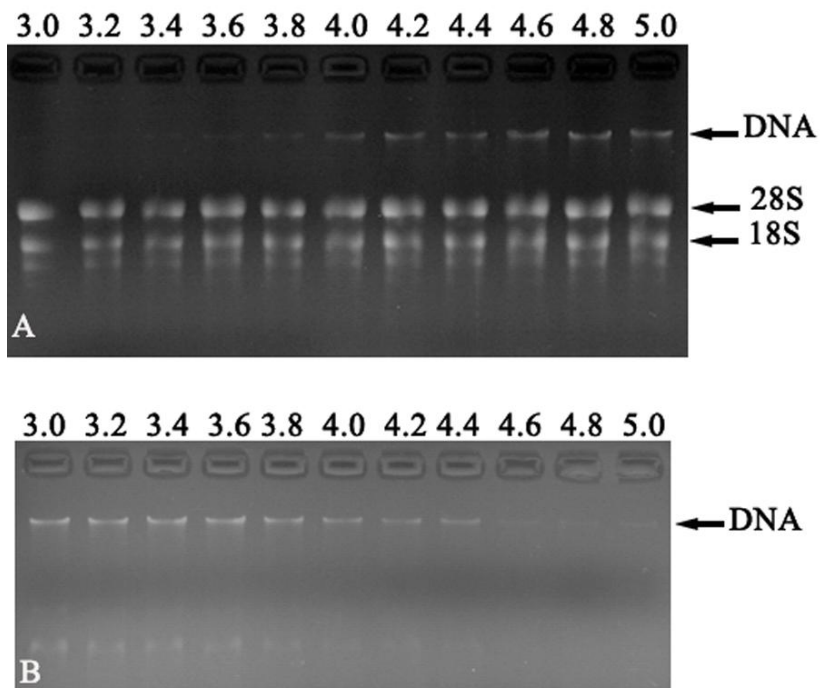


Figure 3. Further optimization of PEG8000 concentration by a fine gradient in *H. brasiliensis*. The concentrations were set at 3.0, 3.2, 3.4, 3.6, 3.8, 4.0, 4.2, 4.4, 4.6, 4.8 and 5.0%. 1 μ l of the extracts was separated on 1.2% agarose-formaldehyde gel. (A) Nucleic acids in the PEG8000 deposit; (B) residual nucleic acids in the supernatant.

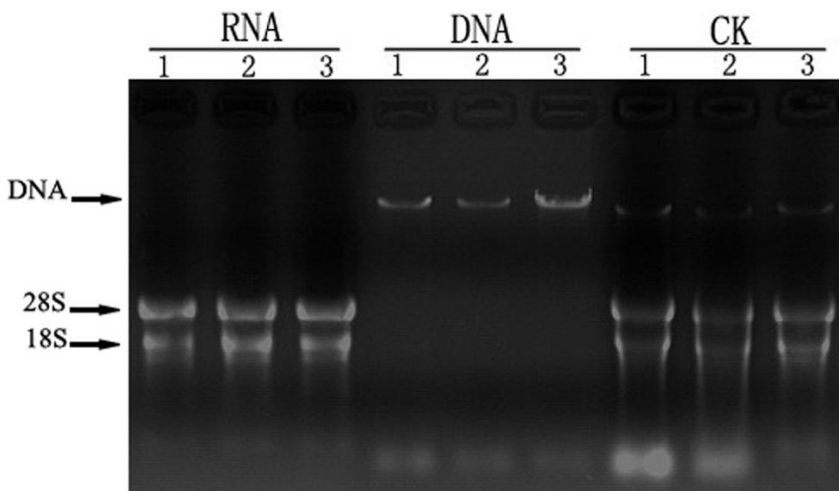


Figure 4. Nucleic acids extracted from different tissues of *H. brasiliensis*. 1 μ l of the extracts was separated on 1.2% agarose-formaldehyde gel. Lanes 1 to 3, Nucleic acids isolated from leaves, bark and latex, respectively; CK, nucleic acids precipitated with 0.1 volume of 3 M NaAc and 2.5 volumes of absolute ethanol.

Yield and purity of RNA and DNA isolated from *H. brasiliensis*, *O. sativa* L. and *G. max*

All of the RNA and DNA isolated from *H. brasiliensis*, *O. sativa* L. and *G. max* had a good purity and high yields

(Table 1). For RNA samples, A_{260}/A_{280} and A_{260}/A_{230} ratios were 1.84 ± 0.05 to 2.14 ± 0.00 and 2.00 ± 0.04 to 2.42 ± 0.03 , respectively and for DNA samples, they were 1.80 ± 0.00 to 1.96 ± 0.00 and 2.03 ± 0.05 to 2.10 ± 0.05 , respectively. The yields for RNA were 349.2 ± 37.9 to 1222.2 ± 138.6

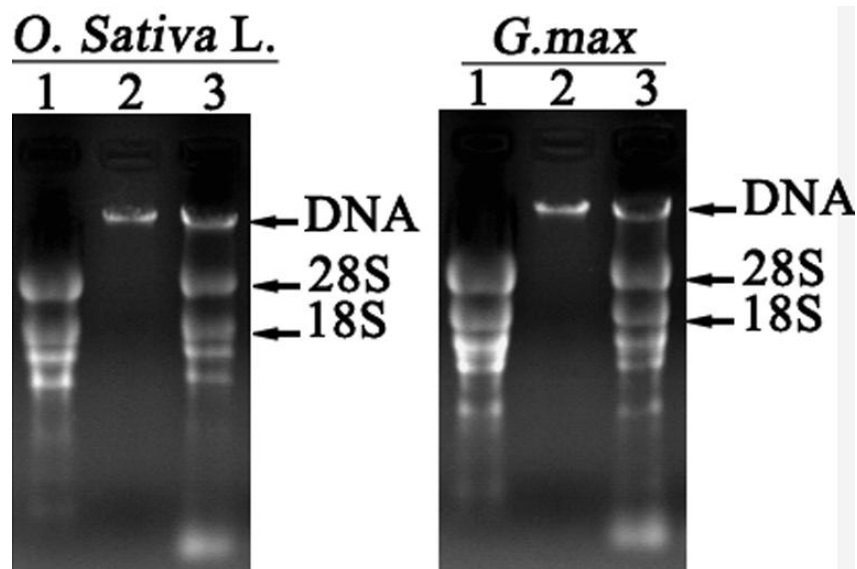


Figure 5. Nucleic acids extracted from *O. sativa* L. and *G. max*. 1 μ l of the extracts was separated on 1.2% agarose-formaldehyde gel. Lane 1, total RNA; lane 2, genomic DNA; lane 3, nucleic acids precipitated with 0.1 volume of 3 M NaAc and 2.5 volumes of absolute ethanol.

Table 1. Yields and purities of nucleic acid isolated from different materials^a

Material	RNA			DNA		
	A _{260/280}	A _{260/230}	Yield (μ g/g) ^b	A _{260/280}	A _{260/230}	Yield (μ g/g) ^b
<i>Hevea</i> leaves	2.09 \pm 0.01	2.07 \pm 0.06	381.9 \pm 39.0	1.92 \pm 0.03	2.07 \pm 0.08	159.6 \pm 5.5
<i>Hevea</i> bark	1.84 \pm 0.05	2.00 \pm 0.04	349.2 \pm 37.9	1.89 \pm 0.03	2.05 \pm 0.07	115.2 \pm 17.4
<i>Hevea</i> latex	2.05 \pm 0.06	2.09 \pm 0.09	433.8 \pm 67.2	1.96 \pm 0.00	2.03 \pm 0.05	252.3 \pm 17.9
<i>O. sativa</i> L.	2.12 \pm 0.01	2.35 \pm 0.02	1188 \pm 157.1	1.80 \pm 0.00	2.05 \pm 0.04	231.7 \pm 15.1
<i>G. max</i>	2.14 \pm 0.00	2.42 \pm 0.03	1222.2 \pm 138.6	1.80 \pm 0.00	2.10 \pm 0.05	225.9 \pm 10.2

^aResults represent the mean \pm standard deviation of three separate samples. ^bYields were determined spectrophotometrically and are presented as microgrammes RNA or DNA/gram fresh weight.

μ g/g and for DNA, 115.2 \pm 17.4 to 252.3 \pm 17.9 μ g/g. Although it showed that RNA and DNA isolated from *Hevea* bark had the lowest yields (349.2 \pm 37.9 and 115.2 \pm 17.4 μ g/g, respectively), their purities still were good enough for various molecular assays.

Further evaluation of RNA quality by RT-PCR

Reverse transcription is sensitive to impurity of RNA samples. Therefore, effective cDNA synthesis is a suitable indicator of high-quality RNA (Kolossova et al., 2004). To test the quality of the RNA on a functional basis, RT-PCR was carried out. The first strand cDNA products of the RNA samples consistently ranged in size from 0.5 to 3 kb and higher (Figure 6A), which is indicative of a very high-quality RNA substrate. *Hbactin* and 18S rRNA were subsequently successfully amplified

(Figures 6B and C). All these results show that RNA isolated by this method is suitable for genomic research.

Restriction endonuclease analysis

To determine DNA quality, DNA was digested with *EcoR* I and *Hind* III, which are easily inhibited by contaminants (Claire and Herrin, 1997). A perfect migration pattern was observed on agarose gel (Figure 7) indicating that DNA isolated from *H. brasiliensis*, *O. sativa* L. and *G. max* were completely digested. Therefore, genomic DNA isolated by this method has a good quality to meet the genomic research.

DISCUSSION

Rubber tree contains high levels of secondary metabolites

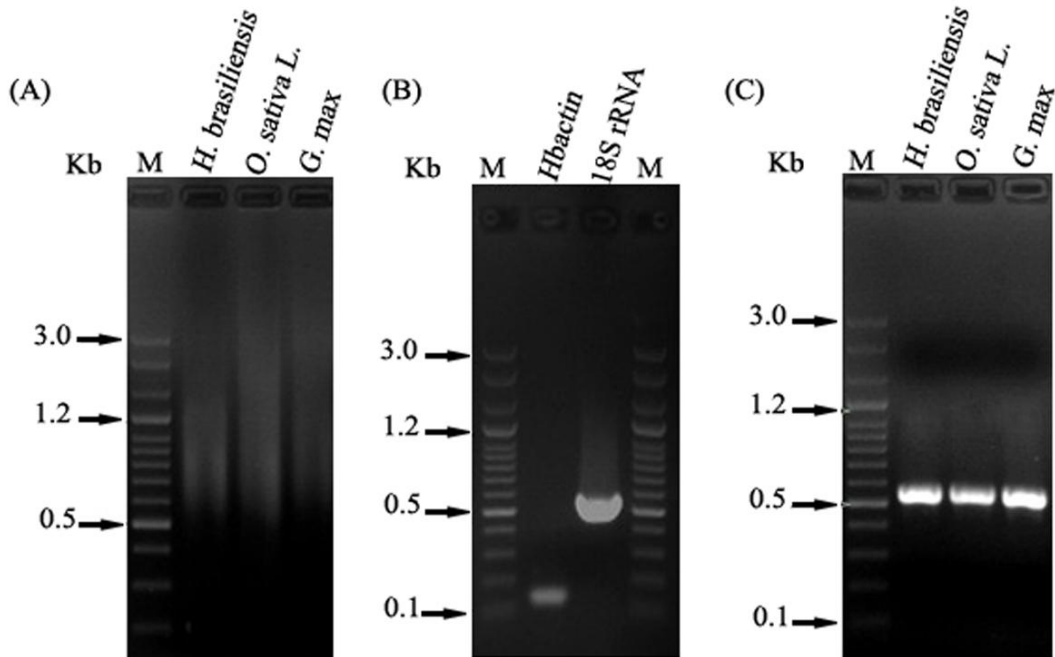


Figure 6. Assessment of RNA extracted from *H. brasiliensis*, *O. sativa L.* and *G. max*. (A) The first strand cDNA synthesis; (B) amplification of *Hevea* housekeeping genes. The amplified products of *Hbactin* and 18S rRNA are 144 and 529 bp, respectively; (C) amplification of 18S rRNA. The products of 18S rRNA are 529 bp in the species used in this study; M, DNA marker.

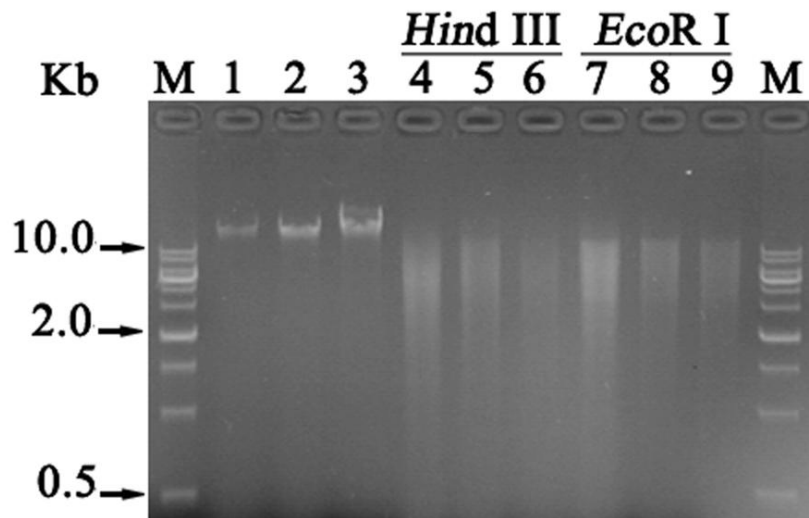


Figure 7. Electrophoresis of undigested and digested genomic DNA. The samples were separated on 0.8% agarose gel. M, DNA marker; lanes 1, 4 and 7, DNA extracted from *H. brasiliensis*; lanes 2, 5 and 8, DNA extracted from *O. sativa L.*; lanes 3, 6 and 9, DNA extracted from *G. max*.

such as phenolic compounds in a high level. The phenolics are rapidly oxidized to form covalently linked quinones (Loomis et al., 1974), which vigorously bond nucleic acids resulting in brown and insoluble RNA or DNA pellets. Besides this, polysaccharides may be

problematic due to their co-precipitation with nucleic acids in buffers under low ionic strength (Pandit et al., 2007). Such samples may have negative effects on the activity of enzymes such as reverse transcriptase, restriction enzymes and polymerases.

Trizol reagent (Invitrogen) has been used to isolate RNA from a lot of live materials, however we obtained brown pellets from *H. brasiliensis* using this reagent (data not shown). To obtain much cleaner samples, we added the contaminant absorbents such as PVP in combination with a powerful reductant (β -mercaptoethanol) into the extraction buffer in this method. In our experiments, the presence of high concentration of β -mercaptoethanol (4%) and PVP (4%) in the extraction buffer could effectively prevent oxidation of phenolic compounds which were removed in the following steps using WP and CI. The concentrations of PVP and β -mercaptoethanol added in our extraction buffer was higher than that of other reports (Pandit et al., 2007; Asif et al., 2000; Gasic et al., 2004), and the yield of nucleic acids isolated by this method was also higher than previous reports (Kush et al., 1990; Tang et al., 2007). Our results show high-quality DNA and RNA samples with minimal polysaccharide, and polyphenol and protein contaminations were obtained using this method (Table 1, Figures 4 and 5).

Although more DNA can be obtained by using PEG8000 precipitation than using ethanol, residual PEG in the pellet will interfere with the following experiments and need to be removed (Cullen and Hirsch, 1998). Griffiths et al. (2000) re-purified the PEG6000 pellets, and used them for downstream research. In this study, PEG8000 was removed by re-precipitating RNA pellet with absolute ethanol, and the purified RNA was suitable for RT-PCR (Figure 6).

The overall results show that high-quality RNA and DNA samples were obtained by our protocols, and they can be used for various molecular biology researches. The method developed in this study is reliable, efficient and cost-effective.

ACKNOWLEDGEMENTS

We thank Dr. Zheng Liu for correcting the manuscript. This work was partly supported by the National Natural Science Foundation of China (30860221, 30960319), the Fundamental Research Funds for Rubber Research Institute, CATAS (1630022011002) and Promotive Research Fund for Excellent Young and Middle-aged Scientists of Shandong Province (BS2009NY014). Chinese Academy of Tropical Agricultural Sciences and Hainan University contributed equally to this work.

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