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ISOLATION OF HIGH-QUALITY TOTAL RNA FROM LEAVES OF *Myrciaria dubia* "CAMU CAMU"

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ISOLATION OF HIGH-QUALITY TOTAL RNA FROM LEAVES OF *Myrciaria dubia* “CAMU CAMU”

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□ *Myrciaria dubia* is a main source of vitamin C for people in the Amazon region. Molecular studies of *M. dubia* require high-quality total RNA from different tissues. So far, no protocols have been reported for total RNA isolation from leaves of this species. The objective of this research was to develop protocols for extracting high-quality total RNA from leaves of *M. dubia*. Total RNA was purified following two modified protocols developed for leaves of other species (by Zeng and Yang, and by Reid et al.) and one modified protocol developed for fruits of the studied species (by Silva). Quantity and quality of purified total RNA were assessed by spectrophotometric and electrophoretic analysis. Additionally, quality of total RNA was evaluated with reverse-transcription polymerase chain reaction (RT-PCR). With these three modified protocols we were able to isolate high-quality RNA ($A_{260nm}/A_{280nm} > 1.9$ and $A_{260nm}/A_{230nm} > 2.0$). Highest yield was produced with the Zeng and Yang modified protocol ($384 \pm 46 \mu\text{g ARN/g}$ fresh weight). Furthermore, electrophoretic analysis showed the integrity of isolated RNA and the absence of DNA. Another proof of the high quality of our purified RNA was the successful cDNA synthesis and amplification of a segment of the *M. dubia actin 1* gene. We report three modified protocols for isolation total RNA from leaves of *M. dubia*. The modified protocols are easy, rapid, low in cost, and effective for high-quality and quantity total RNA isolation suitable for cDNA synthesis and polymerase chain reaction.

Keywords genetic studies, purification protocols, tropical fruit, vitamin C

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INTRODUCTION

Myrciaria dubia (Kunth) McVaugh “camu camu” is recognized as one of the most important natural sources of vitamin C for people in the Amazon region. This plant species is characterized by fruit with more than 2000 mg vitamin C per 100 g pulp,^[1] and various substances with potential medical use.^[2,3] Nevertheless, remarkable variation in fruits yield and vitamin C content exists,^[3,4] affecting fruit quality and consequently bringing a decrease in market demand.

To improve the yield and fruits quality of *M. dubia* is necessary to carry out genetic and molecular biology studies. For these studies, is essential to isolate high-quality total RNA from leaves and other tissues with an appropriate method. This should be inexpensive, be easy to do, and allow us to obtain intact genetic material free of contaminants.

However, total RNA purification from plant tissues is difficult due to copurification with polysaccharides, polyphenols, and secondary metabolites.^[5] These contaminants often affect quality, yield, and downstream applications.^[6,7] Although a considerable number of protocols for RNA isolation from leaves of a variety of plant have been reported,^[7–9] these could be inappropriate for isolation RNA from leaves of *M. dubia*, given that plant species differ in their chemical composition in both quantity and type of substances. These characteristics are unique in each species and difficult to establish by a universal purification method, so a protocol developed for one species or tissue may not be suitable for purifying the genetic material of other species or different tissues from the same species. Consequently, purification methods established for some species or tissues need to be modified to adapt to the particular characteristics of the material under study.^[10]

To date, molecular studies on *M. dubia* are lacking and no method has been reported for the isolation of total RNA from leaves of this species. Although a method for partially purified RNA from a mixture of pulp and peel of this species has been reported,^[11] this method is tedious, time-consuming, and unsuitable for total RNA purification from leaves. The reason for this is that each plant tissue and cell is differentiated and specialized in its metabolic activities, producing various chemicals that may interfere with the purification process.

In this study we present three modified protocols for the isolation of total RNA from leaves of *M. dubia* with high quality and yield.

MATERIALS AND METHODS

Plant Material

Young *M. dubia* leaves were collected from the *M. dubia* germ-plasm bank (03°57'17" S, 73°24'55" W), of the Instituto Nacional de Innovación

Agraria of Peru, department of Loreto, during the 2011 field season (in March and April 2011). All tissues were stored at -80°C until further use.

Solutions and Reagents

The following reagents were used:

Extraction buffer 1 (modified from Zeng and Yang, 2002): 300 mM Tris-HCl (pH 8.0), 25 mM ethylenediamine tetraacetic acid (EDTA), 2 M NaCl, 2% cetyltrimethylammonium bromide (CTAB), 3% polyvinylpyrrolidone (MW 40,000), and 2% β -mercaptoethanol.

Extraction buffer 2 (modified from Silva, 2006): 300 mM Tris-HCl (pH 8.2), 25 mM EDTA, 2 M NaCl, 2% CTAB, 3% polyvinylpyrrolidone (MW 40,000), and 2% β -mercaptoethanol.

It should be noted that β -mercaptoethanol was added just before use. Other reagents and solutions included chloroform:isoamyl alcohol (24:1), phenol:chloroform:isoamyl alcohol (25:24:1), 10 M LiCl, 3 M sodium acetate (pH 5.2), 10% sodium dodecyl sulfate (SDS), isopropanol, absolute ethanol, 70% ethanol, and TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Before operation, all the nondisposable plastic materials were first treated with 0.1% diethylpyrocarbonate (DEPC)-treated water and autoclaved. Glass materials as well as the mortars and pestles were baked overnight at 180°C . All the reagents (except Tris-HCl) were prepared with DEPC-treated water and autoclaved.

The modifications to the protocols are shown in Table 1.

Protocol 1

Modified from Zeng and Yang,^[8] steps were:

1. Transfer 450 mg of leaves to a mortar containing 4.5 mL extraction buffer 1 (preheated at 65°C) and completely grind in a mortar.
2. Quickly transfer samples to sterile centrifuge tubes and then incubate at 65°C for 10 min with gentle inversion every 2 min.
3. Add an equal volume of chloroform:isoamyl alcohol (24:1), mix vigorously in a vortex for 30 s, and centrifuge at $21,380 \times g$ for 10 min at 4°C .
4. Transfer the aqueous supernatant to a new tube, and repeat the chloroform:isoamyl alcohol extraction.
5. Transfer the upper phase to a new tube, add 0.25 volumes of 10 M LiCl (final concentration 3 M), mix well, and incubate at -20°C for 1 hr.
6. Centrifuge at $21,380 \times g$ for 10 min at 4°C ; completely discard the supernatant.

TABLE 1 Comparison of the Original and Modified Protocols for Total RNA Isolation from Leaves of *M. dubia*

	Zeng and Yang (2002)		Silva (2006)		Reid et al. (2006)	
	Original	Modified	Original	Modified	Original	Modified
Grind tissues with liquid nitrogen	Yes	No	No	No	Yes	No
Extraction buffer components						
Tris HCl	100 mM, pH 8.0	300 mM, pH 8.0	200 mM, pH 8.2	300 mM, pH 8.2	300 mM, pH 8.0	300 mM, pH 8.0
CTAB	2%	2%	2%	2%	2%	2%
PVP mol wt 25,000	2%	—	—	—	—	—
PVP mol wt 40,000	—	3%	—	3%	—	3%
PVPP	—	—	—	—	2%	—
EDTA	25 mM	25 mM	20 mM	25 mM	25 mM	25 mM
NaCl	2 M	2 M	1.4 M	2 M	2 M	2 M
Spermidine trihydrochloride	0.05%	—	—	—	0.05%	—
β -Mercaptoethanol	2%	2%	0.1%	2%	2%	2%
Ratio extraction buffer:sample	21–30	10	10	10	20	20
Incubation temperature and time	65°C for 10 min	65°C for 10 min	65°C for 1 hr	65°C for 10 min	65°C for 10 min	65°C for 10 min
Conditions of centrifugation for aqueous and chloroform-isoamylalcohol phases separation	10,000 \times g for 10 min	21,380 \times g for 10 min	13,173 \times g for 25 min	21,380 \times g for 10 min	3500 \times g for 15 min	21,380 \times g for 10 min

Centrifugation for pellet and discard the insoluble material	30,000 × g for 20 min	-	-	-	30,000 × g for 20 min	-
Total nucleic acids precipitation with sodium acetate and isopropanol	-	-	-	-	Yes	Yes
Centrifugation for recovery total nucleic acids					21380 × g for 10 min	3 M
LiCl final concentration for RNA precipitation	2.5 M	3 M	3 M	3 M	3500 × g for 30 min	2.4 M
Addition of the carrier tRNA	No	No	Yes	No	No	No
Time incubation for RNA precipitation	12 hr at 4°C	1 hr at -20°C	12 hr at 4°C	1 hr at -20°C	12 hr at 4°C	1 hr at -20°C
Centrifugation for recover the RNA	30,000 × g for 30 min	21,380 × g for 10 min	9681 × g for 40-60 min	21,380 × g for 10 min	20,000 × g for 30 min	21,380 × g for 10 min
RNA resuspension in SDS 0.5% and reextraction with phenol:chloroform and chloroform:isoamylalcohol	-	-	Yes	Yes	-	-
RNA precipitation with sodium acetate and absolute ethanol	-	-	3 h at -70°	1 h at -20°C	-	-
% ethanol to wash the RNA pellet	75	70	70	70	70	70
DNase treatment	No	Yes	No	Yes	Yes	Yes
Approximate time required	14 hr	3 hr	19 hr	4 hr	15 hr	4 hr

7. Wash the pellet with 1 mL of 70% ethanol 3 times, and air dry at room temperature for 10 min.
8. Dissolve the total RNA pellet in 89 μ L of DEPC-treated water. Store the total RNA sample at -80°C until use.

Protocol 2

Modified from Silva,^[11] steps were: Follow protocol 1, steps (1) to (6), but use extraction buffer 2, and resuspend the pellet with 100 μ L of 0.5% SDS. Add 100 μ L of phenol:chloroform:isoamyl alcohol (25:24:1) and shake the tube vigorously in a vortex for 30 s. Separate the phases by centrifuging at $21,380 \times g$ for 10 min at 4°C . Transfer the aqueous phase to a new tube. Add 100 μ L of a mixture of chloroform:isoamyl alcohol (24:1), shake the tube vigorously in a vortex for 30 s, then separate the phases by centrifuging the tubes at $21,380 \times g$ for 10 min at 4°C . Transfer the aqueous phase to a new tube, mix with 0.1 volumes of 3 M sodium acetate (pH 5.2) and 3 volumes of absolute ethanol, and incubate at -20°C for 1 hr. Follow protocol 1 steps (6) to (8).

Protocol 3

Modified from Reid et al.,^[9] steps were: Follow protocol 1, steps (1) and (2), but use 9 mL of extraction buffer 1. Follow protocol 1, step (3), but centrifuge the tubes at $21,380 \times g$ for 10 min at 4°C . Transfer the aqueous phase to a new tube and mix with 0.1 volumes of 3 M sodium acetate (pH 5.2) and 0.6 volumes of isopropanol. Shake the tube in a vortex for 30 s and incubate at -80°C for 30 min. Separate precipitated nucleic acids by centrifuging at $21,380 \times g$ for 10 min at 4°C . Eliminate the supernatant, and then resuspend the pellet of nucleic acids in 750 μ L of TE (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA). Add 250 μ L of 10 M LiCl (final concentration 3 M), mix well, and incubate at -20°C for 1 hr. Follow protocol 1 steps (6) to (8).

DNase Treatment

To 89 μ L of total RNA sample add 10 μ L of buffer $10 \times$ TURBO DNase and 1 μ L of TURBO DNase (2 U/ μ L), mix well, and incubate at 37°C for 10 min. Add 100 μ L of phenol:chloroform:isoamyl alcohol (25:24:1), mix in vortex for 30 s, and centrifuge at $21,380 \times g$ for 5 min at 4°C . Transfer the aqueous phase to a new tube, add 0.1 volumes of 3 M sodium acetate (pH 5.2) and 3 volumes of absolute ethanol, and incubate at -20°C for 1 hr. Centrifuge at $21,380 \times g$ for 10 min at 4°C , completely discard the supernatant, wash the pellet with 0.5 mL of 70% ethanol, and air-dry it

for 10 min. Dissolve the total RNA pellet in 50 μ L of DEPC-treated water. Store the total RNA sample at -80°C until use.

RNA Analysis

RNA quality and quantity were assessed by standard OD measurement^[12] by spectrophotometry (Spectronic; Genesys 6.0) with 20 \times dilution in TE buffer. Contamination due to phenol/carbohydrates and proteins was determined by recording the OD ratios A_{260}/A_{230} and A_{260}/A_{280} , respectively. In order to verify RNA integrity, samples were resolved by standard formaldehyde denaturing gel electrophoresis in 1.2% agarose gel,^[12] stained with ethidium bromide, and visualized under ultraviolet (UV) light. RNA quantity was estimated according to Sambrook et al.^[12]

Degenerate Primer Design

Full-length mRNA sequences of the actin 1 genes of *Arabidopsis thaliana* (NM_179953.2), *Populus trichocarpa* (XM_002298674.1), and *Vitis vinifera* (XM_002282480.1) were obtained from the Gene Bank database of the National Center for Biotechnology Information (NCBI). These sequences were aligned with the Clustal W2 software and degenerate primers designed with the SC Primer software^[13] in a manner such that at least one of the primer pairs spanned an intron–exon junction. Where this was not possible, primers were designed to lie on either side of an intron splice site. Primer design specifications were: an amplicon size of 200 to 400, optimum primer length of 20 to 22 bp, primer T_m of 59 to 65 $^{\circ}\text{C}$, allowed T_m difference 3 $^{\circ}\text{C}$, and primer GC% of 45% and 55%, with all other parameters left at default. The primers selected from the results primer table were *MdACT1f*: 5'-TYGTYTGYGACAATGGAAC-3' and *MdACT1r*: 5'-ATTGTAGAAWGTGTGATGCCAA-3', which hybridized in exon 2 and 3, respectively, of the homologue gene of *A. thaliana* and flanking intron 2 (Figure 1). Expected product size was 254 bp for cDNA and 390 bp for genomic DNA.



FIGURE 1 ACT1 gene structure of *Arabidopsis thaliana* homologue showing its exons (filled boxes) and introns (lines). Likewise, the areas of hybridization of the degenerate primers (arrows). The image was designed based on the coded sequence NC_003071.1 from the NCBI gene bank.

Reverse Transcription and PCR

Single-stranded cDNA was obtained from 1.5 µg of total RNA using MuLV reverse transcriptase and oligo(dT)₁₆, following the manufacturer's instructions (Applied Biosystems). Reaction conditions were as follows: 25°C for 10 min, 42°C for 1 hr, enzyme denaturing at 95°C for 5 min. Products were stored at -20°C. The synthesized cDNA was used for polymerase chain reaction (PCR) in order to estimate the expression level of the actin gene. The PCR reaction components were: 1 × buffer, 3 mM MgCl₂, 0.8 mM dNTPs mix, 0.75 µM of each of the degenerated primers *MdACT1f* (5'-TYGTYTGYGACAATGGAAct-3') and *MdACT1r* (5'-ATTGTAGAAWGTGTGATGCCAA-3'), 0.4 U Taq polymerase, 2 µL cDNA, and water. The PCR conditions were as follows: DNA denaturing at 95°C for 5 min, followed by 40 cycles of 45 s at 95°C for DNA denaturing, 30 s at 60°C for primer annealing, and 1 min at 72°C for extension. The program was terminated with a 10-min extension at 72°C. The amplified products were resolved on a 1.5% agarose gel and visualized after ethidium bromide staining.^[12]

Sequencing

PCR products were gel purified using the Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen, Carlsbad, CA) and cloned into pCR 2.1-TOPO cloning vector (Invitrogen, CA) according to the manufacturer's instructions. Gene sequences were confirmed by sequencing the cloned products with M13 Forward (-20) and M13 Reverse primers and verifying their identity with BLAST analysis. The gene sequence was deposited in GenBank database with accession code JX569368.1.

Data Analysis

Absorbance, quality (A_{260}/A_{280} and A_{260}/A_{230} ratios) and yield data (µg RNA/g leaves) were analyzed using the program IBM SPSS Statistics 19. Mean and standard deviation were determined and one-way analysis of variance (ANOVA) with Tukey's HSD test, at $\alpha = 0.05$, was performed.

RESULTS AND DISCUSSION

The utility of a total RNA isolation protocol is commonly assessed by the quality, quantity, and integrity of RNA obtained. With the three modified

protocols used we were able to obtain total RNA of adequate quality and quantity from the leaves of *M. dubia* (Table 2). The $A_{260\text{nm}}/A_{280\text{nm}}$ means were >1.9 , indicating that total RNA obtained with the three modified protocols was not contaminated by proteins. ANOVA did not detect significant differences between $A_{260\text{nm}}/A_{280\text{nm}}$ ratios ($F=0.95$, $df=2$, $p=0.480$). Furthermore, $A_{260\text{nm}}/A_{230\text{nm}}$ means were above 2.0, showing that the total isolated RNA was not contaminated by polysaccharides and polyphenols. For this quality parameter, the modified protocol from Reid et al.^[9] was most efficient, producing total RNA with $A_{260\text{nm}}/A_{230\text{nm}}$ values >5 , which were significantly higher than those from the Silva^[11] protocol (the difference to the Zeng and Yang method was not significant; $F=11.85$, $df=2.0$, $p=0.041$). According to Shultz et al.^[14] and Salzman et al.^[15], the failure to obtain total RNA of appropriate quality and quantity from plant tissues is due to the presence of phenolic compounds, polysaccharides and complex secondary products. Consequently, the results show that the three modified protocols used were able to remove these contaminants present in the leaves of *M. dubia*.

With respect to the yield, it is clear that the modified protocol of Zeng and Yang produced 1.4 times more than the modified protocol of Silva and up to 2.3 more than the modified protocol of Reid et al. (Table 2). These differences were significant between the modified protocols of Zeng and Yang and Reid et al. ($F=9.30$, $df=2.0$, $p=0.046$).

Furthermore, electrophoresis under denaturing conditions showed the high quality and integrity of total RNA obtained (Figure 2). For the three modified protocols it showed that the ribosomal RNA bands 28 S and 18 S were unbroken, with the first band (28 S) being up to 1.5 times more intense than the second (18 S). The electrophoretic analysis also showed that genomic DNA is not a contaminant of the total RNA purified with the three modified protocols.

Differences in quality and yield of total RNA obtained with the modified protocols listed can be attributed to various aspects that differ among procedures. First, the ratio extraction buffer:sample was 10 in the modified

TABLE 2 Quality and Yields of Total RNA Extracted from Leaves of *M. dubia* Using Modified Protocols

Modified Protocol	OD Ratio		Yield ($\mu\text{g/g}$ Fresh Weight)
	A_{260}/A_{280}	A_{260}/A_{230}	
Zeng and Yang (2002)	2.01 ± 0.07	2.63 ± 0.46	384 ± 46
Silva (2006)	1.94 ± 0.05	2.14 ± 0.09	273 ± 31
Reid et al. (2006)	2.05 ± 0.02	5.77 ± 0.87	168 ± 26

Data are shown as the mean \pm SD ($n=3$).

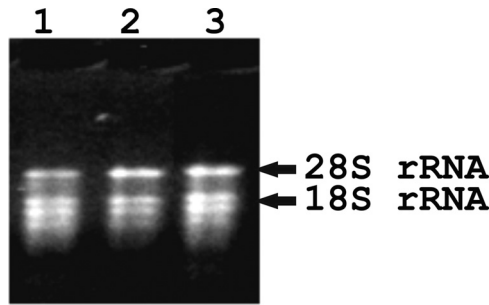


FIGURE 2 Electrophoretic analysis of total RNA isolated from leaves of *M. dubia* with three modified protocols used: 1 (Zeng and Yang, 2002), 2 (Silva, 2006) and 3 (Reid et al., 2006). The arrows indicate the 28S and 18S units of rRNA.

protocols of Zeng and Yang and Silva, but this ratio was 20 in the modified protocol of Reid et al. (Table 1). With the latter method, we have obtained total RNA with the highest quality, because contaminants were eliminated more efficiently by using a larger volume of extraction buffer. However, the modified protocols of Silva and Reid et al. showed lower yield of total RNA compared to the Zeng and Yang protocol (Table 2). This low yield may be attributable to the fact that both protocols require three stages of total RNA precipitation (Table 1).

The failure in the generation of amplicons from total RNA purified with the three modified protocols and amplicon synthesis of cDNA obtained from the total RNA are further evidence for the high quality of RNA obtained (Figure 3). In lanes corresponding to the PCR products of the total RNA (1, 2, and 3), no DNA band of 390 bp was observed (intron plus flanking exons of actin 1 gene). This indicates that the RNA was not contaminated with genomic DNA. However, when performing PCR using a template of cDNA synthesized from the total RNA purified with each protocol, a PCR product of 254 bp is generated (lanes 5, 6, and 7), corresponding to the regions' flanking exons of the actin 1 gene.

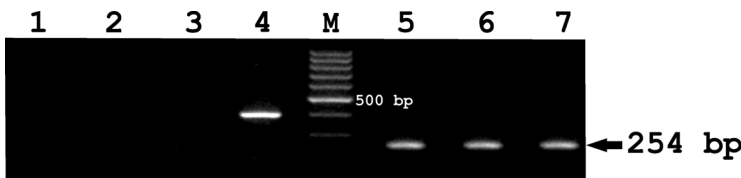


FIGURE 3 Electrophoresis of PCR products from the ACT1 gene of *M. dubia* obtained from total RNA purified with the three modified protocols: 1 (Zeng and Yang, 2002), 2 (Silva, 2006), and 3 (Reid et al., 2006); from genomic DNA (4); and from cDNA synthesized from total RNA purified with the three modified protocols from Zeng and Yang (5), Silva (6) and Reid et al. (7). M: molecular weight marker 100-1000 bp.

This reveals that the total RNA purified with the three modified protocols is free of reverse transcriptase or Taq DNA polymerase inhibitors, again indicating high purity.

It is worth mentioning that the three modified protocols used lithium chloride to precipitate the RNA. Although RNA precipitation with alcohol and monovalent cations, such as sodium and ammonium, are widely used, precipitation with lithium chloride is more advantageous, because it does not efficiently precipitate DNA, proteins, or carbohydrates,^[16] and it is the preferred method for removing cDNA synthesis inhibitors.^[17]

CONCLUSION

In this research we report three modified protocols for isolation total RNA from leaves of *M. dubia*. The modified protocols are easy, rapid, low in cost, and effective for high-quality and quantity total RNA isolation suitable for cDNA synthesis and polymerase chain reaction.

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