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A MODIFIED PROTOCOL FOR TOTAL RNA ISOLATION FROM FORAGE PEANUTS

UM PROTOCOLO MODIFICADO PARA ISOLAMENTO DE RNA TOTAL DE AMENDOIM FORRAGEIRO

[1]Jônatas Chagas de Oliveira, [2]Carla Cristina da Silva, [2]Anete Pereira de Souza, [*3]Tatiana de Campos
[1]Universidade Federal do Acre – UFAC,
[2]Universidade Estadual de Campinas – Unicamp,
[3]Empresa Brasileira de Pesquisa Agropecuária – Embrapa Acre

*Autor correspondente: tatiana.campos@embrapa.br

ABSTRACT

The development of specific protocols for extraction of high-quality RNA are key advances in molecular techniques for elucidating biological processes and mechanisms. The forage peanut has significant economic value for use in mixed pastures. However, it is difficult to extract high-quality RNA from forage peanuts due to high contents of tannins, polysaccharides, and other secondary metabolites. Here, it is described an efficient method for obtaining high-quality, high-yield total RNA that is usable in downstream transcriptome analysis. This modifications helped eliminate the problems associated with the presence of high concentrations of secondary metabolites in forage peanut leaves, and lacked signs of degradation.
Keywords: Total RNA extraction, high yield, high quality, secondary metabolites.

RESUMO

O desenvolvimento de protocolos específicos para extração de RNA de alta qualidade são avanços chave em técnicas moleculares para elucidar mecanismos e processos biológicos. O amendoim forrageiro tem significante valor econômico para uso em pastagens consorciadas. Entretanto, é difícil extrair RNA de alta qualidade de amendoim forrageiro devido aos altos conteúdos de taninos, polissacarídeos e outros metabólitos secundários. Aqui, descreve-se um eficiente método para obtenção de RNA total de alta qualidade e alto rendimento que é necessário para análises posteriores do transcriptoma. Essas modificações ajudaram a eliminar os problemas associados com a presença de altas concentrações de metabólitos secundários em folhas de amendoim forrageiro e sem sinais de degradação.
Palavras-chave: Extração de RNA total, alto rendimento, alta qualidade, metabólitos secundários.

The forage peanut (*Arachis pintoi* Krapov. and W.C. Greg.) is a tropical herbaceous legume with attributes facilitating use in mixed pastures due to its desirable dry matter and high protein contents; it can improve weight gain in cattle and provide biological nitrogen fixation, thus contributing to soil fertility and recovery of degraded areas [1,2]. In 2018, it was reported that forage peanut had a positive economic impact of approximately USD 20 million in Acre State, located in the Brazilian Amazon [3]. Despite its economic importance, molecular studies of *A. pintoi* are still lacking, particularly due to difficulty in the extraction of high-quality RNA.

A. pintoi contains a large quantity of secondary metabolites such as phenolic compounds, tannins, and polysaccharides [4], which directly interfere with the quality of extracted RNA [5]. Thus, a protocol to obtain RNA of high purity and quality is desirable.

Herein, we developed a method modified from the lithium chloride (LiCl) precipitation-based method described by [6] for use in *Arabidopsis thaliana*, *Avicennia schaueriana*, *Theobroma*

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cacao, *Paspalum notatum*, and *Sorghum bicolor*. LiCl was effective at solving problems related to the large number of secondary metabolites present in *A. pintoi* leaves.

Incompletely expanded young leaves were harvested from healthy forage peanut plants. Approximately two leaflets (100 mg of fresh weight) were sampled into 2.0 mL microfuge tubes and placed in liquid nitrogen. After cooling the mortar and pestle with liquid N_2, add leaf materials, frozen in liquid N_2 and grind. Add 2 mL of cold extraction buffer (0.465 M Tris-HCl (pH 8.0), 0.23 M LiCl, 0.0215 M EDTA (pH 8.0), 2.33% SDS, 4.7% β-mercaptoethanol, H_2O DEPC) into the mortar, then homogenize, and transfer 1 mL to 2 mL microfuge tubes, totaling two tubes per sample which will be processed separately until 12 M LiCl precipitation step. Add 600 µL of cold acid phenol, vortex for 1 minute and centrifuge for 15 minutes at 4 °C and 12,000 rpm. Transfer the supernatant (450 – 500 µL) to a 2 mL RNAse-free microfuge tube and add an equal volume of cold acid phenol. Then vortex for 1 minute and centrifuge for 15 minutes at 4 °C and 12,000 rpm. Repeat twice.

Transfer the supernatant (approximately 200 to 400 µL) to a 2 mL tube and add the same volume of chloroform. Vortex for 1 minute and centrifuge for 15 minutes at 4 °C and 12,000 rpm. Repeat 4 times. Transfer the supernatant (approximately 400 µL) in the two 2 mL tubes of each sample to a 1.5 mL RNAse free microfuge tube. Add half a volume of 12 M LiCl and gently mix. Incubate for 2 h at -80 °C. Centrifuge for 50 min at 4 °C and 12,000 rpm. Discard the supernatant and wash the pellet with 500 µL of 6 M LiCl. Do not vortex. Centrifuge for 10 min at 4 °C and 12,000 rpm. Repeat twice.

Discard the supernatant and resuspend the pellet in 300 µL DEPC H_2O. Vortex for 1 minute. Add 100 µL of 3 M sodium acetate (pH 5.2) and 800 µL ice-cold absolute ethanol. Homogenize by inversion. Incubate overnight at -20 °C or for 2 h at -80 °C. Centrifuge for 50 min at 4 °C and 12,000 rpm. Discard the supernatant and add 500 µL of ice-cold 70% ethanol. Centrifuge for 10 min at 4 °C and 12,000 rpm. Repeat twice. The pellet is dried for approximately 20 min at room temperature in a laminar flow cabinet, then resuspended in 30 – 50 µL DEPC H_2O and stored at -20 °C.

A spectrophotometric analysis was performed to assess the purity of the extracted total RNA. Ratios for UV absorption at A_{260}/A_{280} were recorded using a Nano-Drop ND-2000 (Thermo-Scientific, USA). Total RNA degradation was assessed by 1% agarose gel electrophoresis and the RNA integrity number (RIN) was obtained using an Agilent BioAnalyzer 2100 (Agilent Technologies Inc., USA). RIN ranges from one to ten, with one representing degraded RNA and ten intact RNA.

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The protocol described here overcame the problem caused by severe contamination with high concentrations of secondary metabolites present in *A. pintoi* leaves, thus avoiding a loss of quality in extracted RNA [5]. Although an extraction test with the protocol developed by [6] was able to obtain RNA from *A. pintoi*, the quality and yield were lower than those obtained using our modified protocol (Table 1), demonstrating the efficiency of the modifications.

Table 1. Yield, purity, and integrity of RNA extracted from *A. pintoi* leaves (± SD, n = 10).

Protocol	**RNA yield (µg)**	**A260/280**	**RIN**
Original protocol [6]	1.09±0.53	1.8±0.14	2.37±0.35
Modified	21.55±3.85	1.92±0.06	6.04±2.17

The amount of RNA obtained in our study was similar to that found in *A. thaliana* [6] but lower than that obtained in *Arachis hypogaea* by other methods [5,7]. However, the protocols developed for *A. hypogaea* require the addition of proteinase K or DNase to obtain contaminant-free RNA. Our particular modifications are important because they reduce the cost and work time of researchers in extracting RNA.

The integrity of the RNA extracted from the samples using our protocol was higher than that reported by [6], with no apparent signs of degradation (Figure 1). RIN values ranged from 1.70 to 7.60 (Table 1). Samples with RIN less than six are not recommended for analysis because high quality is important for analysis and saves time and costs of researchers. However, low to medium RIN scores or other RNA quality metrics do not necessarily indicate that the samples are not suitable for analysis [8]. We observed a small quantity of DNA in the samples, but this didn't interfere with downstream analysis [6].

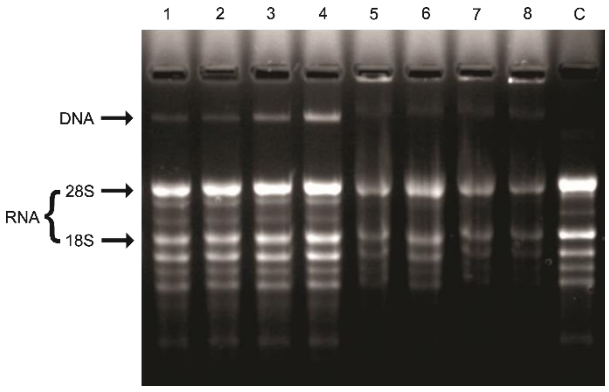

Figure 1. Integrity of RNA extracted from *A. pintoi* leaves on 1% agarose denaturing gel. Lanes 1 to 4: total RNA extracted using the modified protocol. Lanes 5 to 8: total RNA extracted using the original protocol [6]. Lane C: positive control (total RNA extracted from *Arabdopsis* sp. using the original protocol [6]).

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In conclusion, adjustments in RNA extraction protocols are common in plant species because of variations in the concentrations of phenolic compounds, polysaccharides, and other secondary metabolites between species. This method allowed us to obtain pure intact nuclei, which is necessary for most transcriptomics studies. In addition, this optimized protocol may be useful for other species that have similar secondary metabolite profiles and concentrations.

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