

Full Length Research Paper

Extraction of high quality RNA from hard tissues of adult coconut palms

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An experimental protocol originally designed to isolate plant DNA was modified to obtain high quality total RNA from organs of adult coconut palms collected *in situ*. With this protocol, high quality RNA was extracted from leaves, inflorescences, primary and secondary roots, zygotic embryos and solid endosperm, with no carbohydrate or protein contamination. Reverse transcription-polymerase chain reaction (RT-PCR) amplification of a 470 bp cDNA, corresponding to a highly conserved domain of the eukaryotic mitogen-activated protein kinases, demonstrated the integrity of the RNA samples. Isolation of intact RNA from coconut palms growing under wild conditions facilitates the study of gene regulation *ex vivo*.

Key words: Coconut palms, RNA extraction, secondary metabolites.

INTRODUCTION

Mangrove and coconut palms are key elements that protect the coastal zones and tropical wetlands against the environmental impacts. Cultivation of coconut represents a major income for the people in the tropical and subtropical zones of the world (Mathew, 1986; Hyman, 1990); however, plantations are continuously threatened by different pests and diseases that reduce crop productivity (Zizumbo-Villarreal et al., 2006; Magalhães et al., 2008). Breeding of the coconut palms to generate stress-resistant varieties by means of biotechnological methods has been delayed due to their

recalcitrance to cultivation *in vitro* (McCown, 2000). Also, their organs contain secondary metabolites that potentially interfere with the RNA isolation. The leaves are rich in lignin and their surfaces have a dense coat of epicuticular waxes (Escalante-Erosa et al., 2007); the inflorescences contain high amounts of lignin fibers, which have been found to reinforce epoxy composite materials (Sapuan et al., 2005); the embryos are specialized in the accumulation of lipids and carbohydrates (Sugimura and Murakami, 1990; López-Villalobos et al., 2001); and the roots possess a complex

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structure and composition that allow the palm to struggle with the high saline environment found in the coast soils (Nainanayake et al., 2000). High polyphenol contents in adult tissues become evident because activated charcoal must be added to the culture media when adult tissues are used as explants for somatic embryogenesis *in vitro* (Gupta et al., 1984; Chan et al., 1998).

The isolation of coconut ribonucleic acid (RNA) from soft tissues, young seedlings and calli cultivated can be performed by the use of standard protocols that are based on the use of guanidine thiocyanate (Chomczynski and Sacchi, 1987) or cetyltrimethylammonium bromide (CTAB) detergent (Xiao et al., 2012; Gao et al., 2014; Liang et al., 2014; Yuan et al., 2015). Lizama et al. (2007) analyzed the molecular regulation of disease responses by comparing transcript populations isolated from chitosan-elicited *in vitro* coconut calli. In a different study, Pérez-Núñez et al. (2009) quantified transcript levels of a gene encoding a receptor-like kinase during the development of coconut embryogenic calli. Rajesh et al. (2015) isolated total RNA from embryogenic calli and characterized the global transcriptome of coconut palm (*Cocos nucifera* L.) during somatic embryogenesis. In this study, the isolation of high quality RNA was performed by the use of Trizol® reagent (Invitrogen). Other commercial protocols the like RNeasy™ Plant Kit (Qiagen) or the Plant Total RNA Miniprep Purification Kit™ (Gmbiolab Co., Ltd.) have also been used to isolate RNA from embryonic tissues (Bandupriya et al., 2014) or seedling leaves (Huang et al., 2013), to determine the expression of a coconut homeotic gene in zygotic and somatic embryos and during germination, and to analyze the chloroplast genome of the coconut palm, respectively.

An effort had been made in the laboratory to isolate total RNA from different organs of adult palms growing in the coasts. However, neither of the protocols reported above nor other protocols designed to isolate RNA from woody or secondary metabolite-rich plants yielded RNA from several organs of adult palms (Jaakola et al., 2001; Valenzuela-Avendaño et al., 2005), with the minimum quality even visualized in agarose gels. Thus, a specific protocol for the isolation of high quality total RNA from adult coconut palms was established, by complementing reported protocols with modifications devoted to eliminate interfering contaminants during the isolation of RNA.

In the present work, the isolation of high quality total RNA of different organs from adult coconut palms collected *in situ* was reported, by the modification of a CTAB method designed to extract RNA from plant tissues with high phenolic compounds, polysaccharides and elevated levels of RNases (Jaakola et al., 2001). The purity and integrity of the RNA samples was evaluated spectrophotometrically and by electrophoretic fractionation in agarose gels. The integrity of the isolated RNAs samples was confirmed by the successful reverse transcription-polymerase chain reaction (RT-PCR)

amplification of a complementary deoxyribonucleic acid (cDNA) fragment corresponding to a coconut mitogen-activated protein kinase (MAPK) transcript.

MATERIALS AND METHODS

Plant

The coconut immature inflorescences (physiological state (PS) = -4, numbered regressively from the last open inflorescence), the pine group of the last emitted leaf (flag leaf), and the meristematic zone of primary and secondary roots were collected from adult palms of the "Atlantic Tall" variety, cultivated in the San Crisanto town in the north coast of the Yucatan Peninsula, Mexico (21° 21' 00.74" N; 89° 11' 32.49" O). All tissues were immediately frozen in liquid nitrogen and transported to the laboratory, and then they were stored at -80°C until processed. Mature nuts were collected *in situ* and transported to the laboratory to dissect the zygotic embryos and the solid endosperm by the method reported by Chan et al. (1998).

RNA extraction

RNA extraction was attempted following different methodologies. Trizol® reagent, TRI Reagent® and Concert® were used accordingly to the manufacturer's instructions. The CTAB method reported by Jaakola et al. (2001) and the method reported by Valenzuela-Avendaño et al. (2005) were followed as reported. The CTAB modified method presented here was performed as follows. 250 mg of each coconut tissue were ground to powder in liquid nitrogen. Then 1 mL of the CTAB solution (2% cethyl-trimethylammonium bromide; 2 M NaCl; 20 mM EDTA, pH 8; 100 mM Tris-HCl, pH 8, with freshly added β-mercaptoethanol and polyvinylpyrrolidone-40 (PVP-40) to 2% final concentration each) was added and the solution was thoroughly homogenized. The crude extracts were halved into two 1.5 mL Eppendorf tubes and then they were mixed vigorously for 10 min at room temperature. The samples were centrifuged at 12,000 ×g for 15 min at room temperature. The supernatants were transferred to new tubes and then they were extracted twice with one volume of a chloroform: isoamyl alcohol solution (49:1) and centrifuged at 12,000 ×g for 10 min at room temperature. The last supernatants were transferred to clean tubes and mixed perfectly with 0.5 volumes of ice-cold isopropanol; then, 0.5 volumes were added of the saline solution (0.8 M sodium citrate/ 3 M NaCl) and the tubes were gently mixed for 10 min. The solution was centrifuged at 12000 ×g for 10 min at 4°C. The pellets were washed twice with 75% ethanol and centrifuged at 12000 ×g for 10 min at 4°C and they were air-dried for 10 min at room temperature. The pellets were dissolved in 100 μL of H₂O-DEPC, and then they were mixed with 264 μL of ice-cold 4 M LiCl (2.85 M final concentration) and stored for 1 h on ice. After a centrifugation at 12000 ×g for 10 min at 4°C, the RNA pellets were washed twice with 75% ethanol and they were air-dried for 10 min at room temperature. The pellets were dissolved in 30 μL of H₂O-DEPC free (SIGMA). The integrity and purity of the RNA preparations were assessed by electrophoretic fractionation in agarose gels, and by measuring the optical density at 230, 260 and 280 nm, respectively.

Reverse transcription-polymerase chain reaction (RT-PCR)

To evaluate the functionality of the RNA preparations, 0.8 μg of

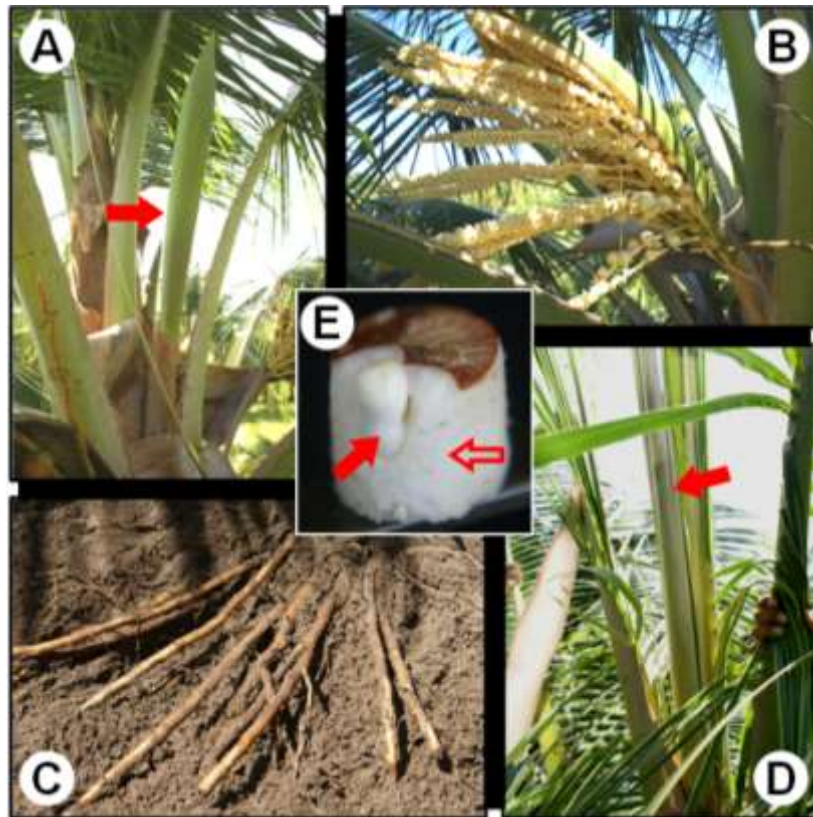


Figure 1. Organs of the coconut plant used as source of RNA. Organ samples were collected *in situ*, immersed immediately in liquid nitrogen and transported to the laboratory. A: Immature inflorescence (solid arrow); B: open immature inflorescence as reference; C: secondary roots; D: flag leaf (solid arrow); E: zygotic embryo (solid red arrow) and solid endosperm (open red arrow) were isolated from mature nuts in the laboratory.

total RNA isolated from each organ sample were used as template for the RT-PCR amplification of a 470 bp cDNA fragment, corresponding to the conserved domain of mitogen-activated protein kinases. The Superscript One-Step RT-PCR kit with Platinum *Taq* polymerase® (Invitrogen) was used as recommended by the manufacturer, using the degenerate primers 5'-GGNGCYTACGGHATYGTTTGYTCK-3' (forward) and 5'-GGNGCYTACGGHATYGTTTGYTCK -3' (reverse), under the following cycling conditions: one cycle at 42°C, 2 min; 48°C, 30 min; 94°C, 2 min; then, forty cycles at 94°C, 15 s; 50°C, 30 s; 72°C, 1 min, followed by a final extension step at 72°C for 10 min. 3 µL aliquots of each RT-PCR product were fractionated by agarose gel electrophoresis, and then the gel was stained with 1 µg ml⁻¹ ethidium bromide and visualized under UV light.

RESULTS AND DISCUSSION

The isolation of high quality RNA is an essential step to carry out molecular studies in plants; however, the extraction of RNA is compromised in plant tissues rich in secondary metabolites and complex carbohydrates (Jaakola et al., 2001). This is the case of the coconut

palm; indeed, the breeding of elite coconut varieties has been hampered because of several adverse factors inherent to the adult palm, including its size and long life cycle. In addition, the coconut palm is a perennial monocot woody plant with high recalcitrance to *in vitro* cultivation (McCown, 2000). Furthermore, the difficulty of isolating biological molecules from coconut can be evidenced by the existence of extremely few reports in this area.

In the laboratory, investigation of the regulation of the molecular responses of coconut cells to the presence of pathogenic signals in the environment was done. While, the use of coconut tissues growing *in vitro* has been suggested as an alternative to study the gene regulation (Chakraborty et al., 2009; Lizama et al., 2007), the analysis of gene function must be done in whole palms, requiring the extraction of RNA from the organs of interest.

Adult organs of coconut palms growing in the field was collected (Figure 1) and tested different commercial methods to isolate total RNA; however, their use yielded

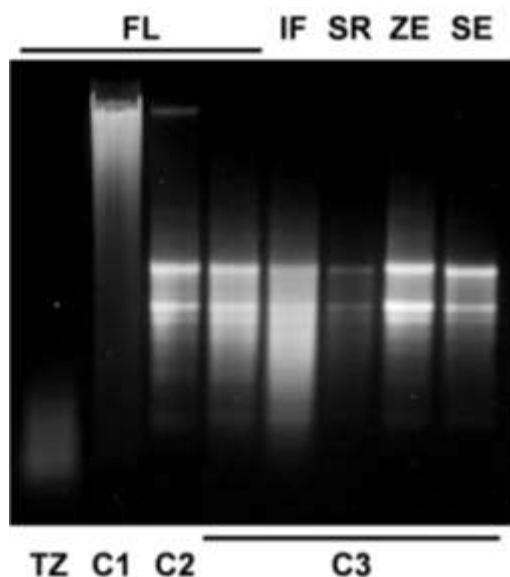


Figure 2. Integrity of the RNA samples isolated from coconut organs. 1 μg of total RNA isolated from different organs of the adult coconut palms were fractionated by gel electrophoresis in 1.3% agarose and stained with 1 $\mu\text{g}\cdot\text{mL}^{-1}$ ethidium bromide. RNA samples were extracted from the following organs: FL: flag leaf; IF: immature inflorescence; SR: secondary root; SE: solid endosperm; ZE: zygotic embryo. Different methods were applied: TZ: Trizol®; CN: Concert®; TR: Triagent®; C1: CTAB original method; C2: CTAB modified method I; C3: CTAB modified method II.

RNA samples that could not be visualized [Trizol® (Invitrogen), TRI Reagent® (SIGMA-ALDRICH)] or appeared as smears in agarose gels [Concert® (Invitrogen)] (Figure 2, TZ, TR and CN, respectively). Conversely, the use of CTAB method as described by Jaakola et al. (2001) yielded only DNA (Figure 2, lane C1).

It has been proposed that the CTAB effect during the extraction of nucleic acids from plants resides in its capacity to bind anionic polymers (that is glucuronoarabinoxylans) (Kiefer et al., 2000). The authors decided to modify the CTAB protocol to increase its capacity to eliminate phenolic compounds and complex carbohydrates by the addition of polyvinylpyrrolidone and a further precipitation step in the presence of a high concentration of salts. The addition of PVP during the extract preparation, and the extraction of the cleared crude extract with a mixture of chloroform: isoamyl alcohol (49:1) followed by the precipitation of carbohydrates from the aqueous phase with a saline solution (0.8 M sodium citrate/3 M NaCl), and a final precipitation step with isopropanol were determinant to

Table 1. Purity and yield of the RNA samples isolated with the CTAB-II method.

Tissue	Absorbance ratio		RNA yield ¹ (ng·mg ⁻¹)
	260/230	260/280	
Flower	2.22	2.04	235.4
Inflorescence	1.95	2.08	89.0
Secondary R	2.09	1.94	42.8
Zygotic Embryo	2.14	2.01	82.9
Solid Endosperm	2.06	2.04	28.3

RNA yield is expressed as nanograms of RNA per milligram of fresh weight of tissue.

precipitate integral RNA from all samples (Figure 2, lane C2). The soluble nature of PVP could extend its capacity to form complex with phenolic compounds, preventing their union and the further oxidation of the RNA samples (Bekesiova et al., 1999). In this protocol, it was not necessary to heat the CTAB extracts at 65°C. It has been reported that precipitation of aqueous extracts with high concentrations of salts (1 M NaCl) favours the elimination of polysaccharides from genomic DNA (Fang et al., 1992) and from RNA (Valenzuela et al., 2005). However, with these concentrations of salt, the spectrophotometric measurements gave 260/230 absorbance ratios ≤ 1.5 (data not shown). The increment of the NaCl concentration to 3 M (0.8 M sodium citrate/3 M NaCl) during the precipitation step produced RNA samples with 260/230 absorbance ratios $\cong 2$. The contaminating DNA was eliminated from the salt-cleared RNA samples by a further precipitation with ice cold LiCl (2.85 M final) (Figure 2, lanes C3). Also, it was found that incubation of the LiCl-RNA mix at -20°C was not necessary because incubation on ice for 1 h produced high RNA yields. All preparations obtained with the new modifications yielded RNA with high integrity, as estimated by the band integrity of the major ribosomal RNAs (Figure 2).

The yield and purity of the RNA samples were evaluated by spectrophotometric absorbance at 230, 260 and 330 nm. Table 1 shows that both 260:230 and 260:280 absorbance ratios were around the value of 2.0, implicating no significant contamination of the RNA samples with carbohydrates and proteins, respectively. The modified CTAB method gave good RNA yields, especially from the palm flag leaf (235.4 ng mg⁻¹). The smaller yield was obtained from solid endosperm secondary roots (28.3 ng mg⁻¹).

The quality of the RNA populations isolated from *in situ* collected adult organs was assessed by their capacity to function as template for reverse transcription *in vitro*. As can be seen in Figure 3, a single band was obtained by reverse transcription coupled with the polymerase chain reaction, using a pair of DNA oligonucleotides flanking a

Table 2. Key modifications to the CTAB original method.

Method	Phenol extraction	T (°C)	Salt concentration (NaCl/NaCit)	LiCl ²
CTAB ¹	+	65	--	--
CTAB-I	-	Room temperature	1 M/0.8 M	Overnight -20°C
CTAB-II	-	Room temperature	3 M/NaCit	1 h, Ice

The CTAB method as originally described by Jaakola et al. (2001). Ultracentrifugation in 2.85 M LiCl.

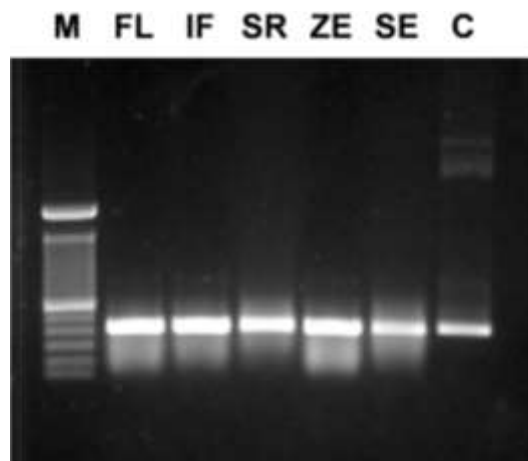


Figure 3. Quality of the RNA isolated from coconut organs. 2 μ l aliquots of the RT-PCR products synthesized with degenerate primers from RNA samples isolated by different protocols were fractionated by gel electrophoresis in 1.3% agarose and stained with 1 μ g ml⁻¹ ethidium bromide. FL: flag leaf; IF: immature inflorescences; SR: secondary roots; SE: solid endosperm; ZE: zygotic embryos; M: 100 bp DNA ladder (Invitrogen); (-): RT-PCR assay with no reverse transcriptase as negative control; (+): PCR product from the coconut MAPK-2 cDNA cloned in the pGEM-T-Easy® vector (PROMEGA) as positive control; TZ: Trizol® method; CN: Concert® method; TR: TriReagent® method.

470 bp fragment of the ribonucleotide sequence encoding part of catalytic domain of the universally-conserved eukaryotic mitogen-activated protein kinases (MAPK). This result confirmed that the improved CTAB method yielded RNA extracts from adult palms cultivated in their natural ecosystems, with the quality required to perform molecular biology experiments required in modern biotechnological breeding programs. It is interesting to note that although the RNA samples obtained with the Concert® protocol served as templates to obtaining an RT-PCR amplicon of the expected size (Figure 3, lane CN), they appeared smeared after electrophoretic fractionation in agarose gels (Figure 2, lane CN), and their absorbance ratios 260/230 nm were below 1.0 units

(data not shown). These results suggested contamination of the Concert® RNA sample with carbohydrates, which did not interfere with RT-PCR experiments, but affected electrophoretic mobility.

Recently, few protocols to isolate RNA populations from soft tissues of coconut palms have been reported (Xiao et al., 2012; Bandupriya et al., 2014; Gao et al., 2014; Liang et al., 2014; Rajesh et al., 2015; Yuan et al., 2015), however, they have not been applied to hard tissues of coconut palm, like inflorescences or roots. The protocol presented here utilizes economic and easy-to-find chemical ingredients; it could be applied to small amounts of tissue, reducing the cost of transportation and the amount of liquid nitrogen required to preserve samples collected in the field. It does not employ toxic chemicals (guanidine isothiocyanate) or organic solvents (phenol) (Table 2), and it could be applied, with simple modifications, to the isolation of both DNA and RNA from the same coconut tissue.

Conflict of interests

The authors have not declared any conflict of interest.

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Abbreviations

CTAB, Cethyl-trimethyl-ammonium bromide; **PVP**, polyvinylpyrrolidone; **FL**, flag leaf; **IF**, immature inflorescence; **SR**, secondary root; **ZE**, zygotic embryo; **SE**, solid endosperm, **MAPK**, mitogen-activated protein kinase.

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