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Full Length Research Paper

Isolation of total RNA from ripe and unripe soursop (*Annona muricata* L.) fruit

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Soursop fruit tissue is known by its acidic pH and high levels of polysaccharides, polyphenolics and secondary metabolites. These conditions are recognized to interfere unfavorably with conventional methodologies for RNA isolation. We describe here a rapid and simple method for the isolation of total RNA from soursop fruit. RNA was extracted in less than 4 h through a combination of SDS/potassium acetate precipitation and selective binding on a silica-gel-based membrane (Qiagen) through microspin speed technology. In comparison to other methods applied for RNA extraction from soursop fruit, our protocol improved substantially RNA quality as well as RNA yield. The isolated RNA served as a robust template for RT-PCR analysis. Comparable RNA quality and yield per dry weight were obtained from unripe and ripe fruits. This makes the method appropriate to being used in studies on differential gene expression in post-harvest behavior.

Key words: *Annona muricata* L., RNA isolation, tropical fruit.

INTRODUCTION

The ability to isolate RNA with good quality and free of contaminants like protein, genomic DNA and secondary metabolites, is crucial for cDNA library construction and molecular analysis, e.g. northern hybridization and reverse transcription-polymerase chain reaction (RT-PCR) (Liu et al., 1998). Several methods have been routinely used for isolation of total RNA (Chirgwin et al., 1979; Chomczynski and Sacchi, 1987; Logemann et al., 1987) and are still being developed due to the fact that plant species from the same genus or related genera may contain variable amounts of diverse substances, like polysaccharides, polyphenolics and secondary metabolites. Therefore, it is not expected to find a unique nucleic acid isolation method adequate for all plants (Loomis, 1974; Weishing et al., 1995; Sharma et al., 2003).

The extraction of RNA from fruit tissue can be affected by several factors, mainly by high levels of RNases and alcohol insoluble substances (AIS) (Romani et al., 1975). Many compounds in the fruit pulp, such as polysaccharides, and phenolic compounds, are known to interfere with nucleic acid extraction (Ikoma et al., 1996; Woodhead et al., 1997; Jaakola et al., 2001). Concerning fruit pulp tissue, several protocols have been described for the isolation of RNA (Podivinsky et al., 1994; Jones et al., 1997; Liu et al., 1998; Woodhead et al., 1997; Asif et al., 2000; Jaakola et al., 2001; Valderrama-Chairez et al., 2002). However, when protocols are applied to new material without further adaptation, RNA quality and yield can be poor or, in same cases, no RNA could be recovered. In a current study on soursop fruit ripening, we characterized the expression profile of alternative oxidase

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(AOX) at the protein level (Brasil, 2002). Further inve-stigations require a reliable protocol that provides the same quality and quantity of RNA from different stages of ripening. Here, we report a simple and efficient method for isolating total RNA from ripe and unripe soursop fruit tissues. This protocol is based partially on a rapid nucleic acid extraction method (Dorokhov and Klocke, 1997), using SDS and potassium acetate for precipitation of contaminants (polysaccharides and proteins) in combination with steps of the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

MATERIALS AND METHODS

Fruit material

Soursop fruits (*Annona muricata* L. cv. criola) from commercial orchard in Quixeré (Ceará State, Brazil) were harvested in the pre-climacteric stage and immediately transported to the Brazilian Agricultural Research Corporation - EMBRAPA packed house and allowed to ripen at 24,5°C (± 0,5°C) and 80 - 85% relative humidity for 8 days.

RNA extraction procedure

Total RNA from fruit representing two ripening stages (unripe and ripe), according to a subjective assessment of Annonacea fruit color (dark green as a unripe and light green as a ripe fruit) and texture, was isolated separately three times using different fruits.

Methods of RNA extraction according López- Gómez and Gómez-Lim (1992) and the commercially available kit (RNeasy Plant Mini kit, Qiagen) were used to compare the efficiency with the proposed method. Solutions and reagents were used as follows:

Extraction buffer: 0.5 % (w/v) sodium dodecyl sulfate (SDS), 25 mM ethylenediamine tetra-acetic acid (EDTA), 250 mM NaCl, 145 mM β-mercaptoethanol and 250 mM Tris (adjusted to pH 7.5 with HCl).
Pre-cooled (4°C) 5 M DEPC-treated potassium acetate (pH 6.0).
95% (v/v) ethanol.
0.2% (v/v) DEPC-treated autoclaved double-distilled water

Glass ware and plastic ware used were as follows:

Glassware and mortar pestles (baked overnight at 180°C), pipette tips (DEPC treated and autoclaved), gel running apparatus (treated with 3% (v/v) H_2O_2 and incubated overnight with DEPC- treated water), 50 mL polypropylene centrifuge tubes and 15 mL polyethylene tubes (BD Falcon™) (washed and incubated overnight at room temperature with DEPC-treated water).

The RNA extraction protocol

The RNA extraction protocol used in this study was partially based on the method of Dorokhov and Klocke (1997) [steps 3 to 7] and on the RNeasy Plant Mini Kit (http://www1.qiagen.com/Products/RnaStabilizationPurification/RNeasySystem/RNeasyPlantMini.aspx) [steps 8 to 11]. For the ripe pulp fruit we included previous steps for sample concentration [1 and 2], in order to eliminate the excess of soluble polysaccharides and others soluble contaminants.

Preparing the ripe fruit sample

1) Use 5 g fresh weight of pulp tissue and cut in small pieces of approximately 0.5 x 0.5 cm^3 with the help of scissors. Add 4 mL of the extraction buffer and transfer the suspension into a 50 mL polypropylene centrifuge tube.

2) Centrifuge 10 min at 500 x g at room temperature for sample concentration to gain a high number of cells, discharge supernatant.

3) Homogenize 1 g of the pellet with 4 mL of extraction buffer for 1 min using the pestle and mortar.

Preparing the unripe fruit sample

Use 600 mg of pulp and excise in small pieces and homogenize with the same conditions as ripe fruit, using 4 mL of extraction buffer for 1 min.

Both fruits (unripe and ripe)

1) Transfer the homogenate (from ripe or unripe fruits) into a 60°C pre-treated 15 mL polyethylene tube (BD Falcon™), keep in a water bath at 60°C for about 15 min (with intermittent shaking every 3 min) and quickly cool down in an ice bath for 10 min.
2) Add pre-cooled 5 M potassium acetate (2 mL) and mix carefully.
3) Transfer the material into a 50 mL polypropylene centrifuge tube and centrifuge at 9,800 x g at room temperature for 20 min.
4) Distribute the supernatant in 1.5 mL tubes (450 ∝L in each tube).
5) Start with only one tube, add 0.5 volume (225 ∝L) of 95% ethanol and mix by pipetting.
6) Transfer step-by-step the total volume of one extraction into a RNeasy mini column (RNeasy Plant Mini Kit – Qiagen, Hilden, Germany) and centrifuge the columns at 8.000 x g at room temperature for 15 s. Discard the flow-through.
7) Follow the protocol of the kit in order to wash and elute the selective column bound RNA and store at –80°C until required.
8) Quantify the RNA by monitoring the absorbance at 260 nm using quartz cuvettes. Also calculate the A260/A280 ratio.
9) Run 1 ∝g of RNA on 1.5 % denaturing agarose gel to check the integrity according to Sambrook, et al. (1989).

RT-PCR Analysis

To test the quality of obtained RNA for expression profiling at harvest and post-harvest stages, RT-PCR were performed from unripe and ripe fruits. One ∝g of total RNA was reversely transcribed with Moloney marine leukemia virus reverse transcr-iptase ("Ready-To- Go™ RT-PCR Beads" Kit - Amersham Biosciences – Piscataway, NJ, USA) according to the manufacturer's instructions, using oligo $pd(N)_6$ and $pd(T)_{12-18}$ primers contained in the kit. The PCR amplification was carried out using a degenerated primer pair designed by Saisho et al. (1997) [P1: 5' CTGTAGCAGCAGT-VCCTGGVATGGT 3' and P2: 5'
GGTTTACATCRCGRTGRTGWGCCTC 3'] for a gene-specific sequence of AOX under the following therm-ocycling conditions: 95°C for 4 min followed by 35 cycles of amplification, each for 1 min at 92°C, 2 min at 55°C, and 2 min at 72°C. The final cycle was followed by an extra extension step at 72°C for 10 min. The reaction product was electrophoretically separated on 1.5% (w/v) agarose gel. After being stained with ethidium bromide, the gel was visualized and photographed under UV light.

RESULTS AND DISCUSSION

Different methods of RNA extraction have been applied

Table 1. Comparison of yield and purity of RNA isolated by different extraction methods from unripe and ripe fruits pulps. Data are shown as mean values ± standard deviation of three independent experiments.

Procedure	A260 nm		A280 nm		A260nm/A280nm		RNA (∝g/g dry weight)[a]	
	Unripe pulp	Ripe pulp	Unripe pulp	Ripe pulp	Unripe pulp	Ripe pulp	Unripe pulp	Ripe pulp
Rneasy Plant Mini kit (Qiagen)	0.018 ± 0.004	0.016 ± 0.004	0.014 ± 0.003	0.012 ± 0.004	1.29 ± 0.02	1.27± 0.05	21.3 ± 4.6	23.8 ± 5.9
López-Gómez & Gómez-Lim, (1992)	0.064 ± 0.009	0.051 ± 0.006	0.045 ± 0.007	0.039 ± 0.007	1.41 ± 0.05	1.31 ± 0.06	75.8 ± 10.6	75.9 ± 8.9
This study	0.140 ± 0.014	0.126 ± 0.017	0.069 ± 0.011	0.063 ± 0.009	2.01 ± 0.05	2.03 ± 0.04	165.9 ± 16.6	187.5 ± 25.3

(a) The yields were calculated from 0.6g (0.135g dry weight) and 1g (0.107g dry weight) for unripe and ripe fruit pulps, respectively.

to plant material. As indicated in Table 1, the technique of López-Gómez and Gómez-Lim (1992) and the commercially available kit (RNeasy Plant Mini kit, Qiagen) used to isolate RNA from the pulp of soursop fruit resulted in low yield and contaminated RNA. This fact was probably due the presence of protein and/or phenolic compounds (relation A260/A280 ≅ 1.3). The main obstacles for isolating RNA from soursop fruit are the known high acidity of pulp fruit extracts (pH ≅ 4.0) and the great content of carbohydrates (15 - 20%) that undergo a drastic biochemical change during ripening. In addition to marked increase in soluble sugars there are also a noteworthy increase in organic acids, chlorophyll breakdown, and polymerization of phenolic compounds in the ripening fruit (Mattoo et al., 1975). Polysaccharides of the fruit tissues are recognized to form a jelly-like precipitate during extraction affecting the yield and quality of RNA (Sharma et al., 2003). Additionally, the particular composition of soursop fruit cell walls, the presence of great quantities of secondary compounds such as phenolic compounds, among others, in the pulp may contaminate the RNA and probably affect the yield and successful isolation of intact, high-quality RNA. According to Figure1, the RNAs obtained with those methods were highly degraded, suggesting an increase of RNase activity during soursop fruit ripening. It has been shown that various enzyme activities may increase during fruit ripening, and RNases might be one of them (Mattoo et al., 1975). Since the tested routine procedures failed to give good results for soursop, a new method was assayed. Key steps in our protocol were the use of high cell numbers and of a suitable extraction buffer to prevent contaminating substances from binding to nucleic acids that probably interfered during RNA extraction by applying the method of López Gómez and Gómez Lim (1992) or the protocol for the RNeasy plant mini kit. Important features of this method include the high buffering and magnesium chelating capacity (250 mM Tris/HCl, 25 mM EDTA) of the lysis buffer to protect the RNA from being degraded. In addition, SDS/potassium acetate in association to the selective binding properties of mini silica-gel columns and the microspin technology were used to precipitate or wash out high levels of polysaccharides and phenolic compounds, reducing the viscosity of the lysates by disrupting gelatinous material formed without affecting the yield of RNA. The acidity of cells of ripe fruits (pH 4.0) was at first rapidly stabilized in extraction buffer and then cells were concentrated by centrifugation before homogenization as a prerequisite for higher yield and quality. The well known ripe fruit pulp peculiarities as high pectin level and the reduced cell number per tissue gram limit RNA isolation yield requiring higher amount of mature tissue compared to immature one. On the other hand, the higher number of cells per pulp gram in unripe fruits eliminates the necessity of a previous sample concentration. Also, the pool of undesirable contaminants in unripe fruit is less than in ripening fruit. The present rapid and effective nucleic acid extr-action method for soursop fruits at different stages in ripening involved a modification of the method of Dorokhov and Klocke (1997) associated with selective RNA-binding following the RNeasy Plant Mini kit-(Qiagen) procedure. As can be seen in Table 1, this method consistently gave good RNA yield in an average ratio of 175 ∝g per gram of dry weight for unripe and ripe fruits and 20 and 37∝g per gram of fresh weight for unripe and ripe fruits, respectively. These data were similar to peach fruit (Miesel et al., 2005) and apple fruit yields (Asif et al., 2006). However, they were higher than the attained yields for cactus fruit (Valderrama et al., 2002) using others methods. Intact and distinct 28S and 18S rRNA

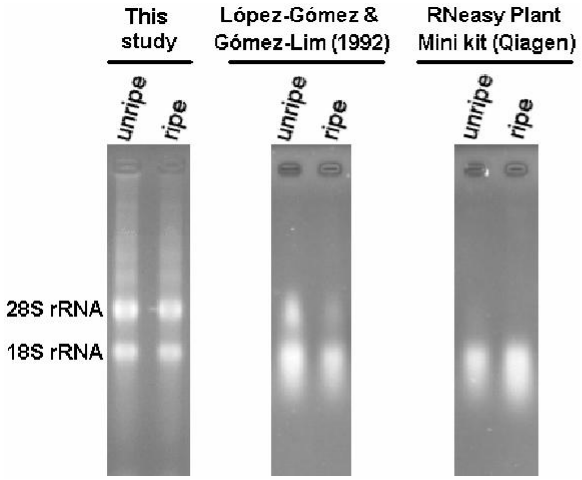

Figure 1. Ethidium bromide-stained 1.5% (w/v) agarose formaldehyde gel of total RNA (1∝g) isolated from unripe and ripe soursop fruit pulp by different extraction procedures: This study; López-Gómez and Gómez-Lim, 1992 and RNeasy Plant Mini kit (Qiagen).

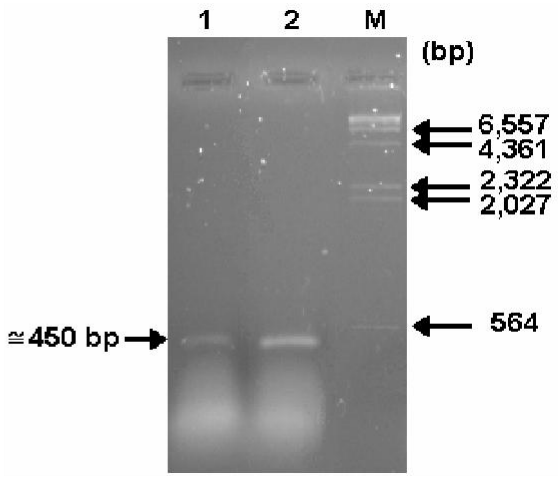

Figure 2. Amplification of a specific AOX fragment by RT-PCR from total RNA (1∝g) extracted from unripe and ripe soursop fruit pulp. 1- unripe soursop fruit pulp RT-PCR product; 2 - ripe soursop fruit pulp RT-PCR product; M - lambda DNA-*Hind* III digest (marker).

bands without visible degradation were observed as compared to the results given by other methods (Figure 1). Values around 2.0 were repeatedly found for the A260/A280 ratios (Table 1), indicating a good quality of the RNA (Asif et al., 2000). Thus, the quality was substantially improved, when compared with RNA extracted from soursop fruits by help of other protocols.

As indicated in Figure 2, RNA prepared with the method described in this study, serves as a good template for reverse transcription. A DNA fragment of the expected length of approximately 450 bp could be specifically amplified by heterologous primers of the AOX gene.

Conclusion

It may be pointed out that the established technique is simple and efficient for the isolation of RNA especially from fruits that possess a wide range of secondary compounds known to interfere with RNA extraction and analysis. Furthermore, this method does not require ultracentrifugation and can be completed in less than 4 h.

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