



## Short Communication

## An easy and robust method for the isolation of high quality RNA from coconut tissues

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## ABSTRACT

**Background:** Coconut tissues consist of a complex network of polysaccharides, proteins, polyphenols, and lipids that can bind to nucleic acids and pose difficulty in isolation. Certainly, a vigorous method is required to isolate high quality and quantity of RNA from such tissues for the purpose of downstream experiments. In this paper, we discuss a newly developed method for the Isolation of RNA from Complex Matrices (IRCM) method from coconut tissues.

**Results:** The method is robust, cheap, and efficient for the extraction of quality RNA in high quantities from the solid endosperm of stored and fresh coconut (150 µg/g FW with A260/280 = 1.89 and 247.5 µg/g FW with A260/280 = 1.91), coconut apple (263.8 µg/g FW with A260/280 = 1.97), and coconut bud (1052.5 µg/g FW with A260/280 = 2.00). The other well established methods, such as Method of RNA Isolation from Palm (MRIP), Cetyl Trimethyl Ammonium Bromide (CTAB), TRIZOL, and RNA plant kit failed to isolate quality RNA in appreciable quantities from the coconut tissues. Furthermore, the resultant RNA performed well in the downstream experiment, that is, RT-PCR for the production and amplification of cDNA.

**Conclusions:** From the study, we concluded that the present method will play a vital role in the extraction of high quality RNA from complex matrices in a short time.

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## 1. Introduction

The tissues from the plant kingdom are usually rich in polysaccharides, lipids, proteins, polyphenols, and some other biomolecules that can interfere with the extraction of high quality RNA. The co-precipitation of macro biomolecules with RNA during the isolation process can greatly affect the quantity and quality of RNA [1,2,3]. Besides, some alkaloids and polyphenols can bind to the RNA, making it difficult to isolate high quality RNA [4,5]. However, an intact RNA with high purity is essential for downstream experiments, including construction of a complementary DNA (cDNA) library, RNA sequencing, reverse transcription real time PCR (RT-PCR), quantitative real time PCR (RT-qPCR), etc. [6,7,8,9]. Therefore, it is of utmost importance to develop a method that can remove all the interfering components to have a clean and high-quality RNA as a final product.

Recently, a number of methodologies and commercial kits were developed to extract high quality RNA from various complex matrices. The commercial kits from Invitrogen TRIZOL Reagents were successfully used for the isolation of RNA from the starch rich tissues of rice [10], oil rich tissues of rapeseed [11], and Arabidopsis [12]. However, the method has failed to extract RNA from the palmaceae that are rich in fat, polysaccharides, and phenolics [7]. Tiangen company has also marketed their own kit (Tiangen RNA plant reagent kit) for active isolation of RNA from potato tubers, banana, apple, and pear tissues that are rich in polyphenols and starch [13]. Similarly, a lithium chloride method was developed for the extraction of RNA from polyphenol-rich tissues [14]. Nevertheless, the most commonly used method for the isolation of RNA from complex plant tissues is CTAB [2]. The method has a low operational cost, but is time-consuming, which can cause the degradation of RNA during the extensive isolation process. Moreover, the co-isolation of DNA is very common and demands additional steps to purify RNA. In recent years, the Coconut Research Institute also developed a Method for RNA Isolation from Palm (MRIP) that showed promising results for leave

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tissues of palm. In this paper, we explain an IRCM method that gave us consistent results. The method was quick, cheap, and reliable for isolating RNA from the very complex matrices of coconut tissues that were rich in polysaccharides, lipids, proteins, and polyphenols. Additionally, the method provided us with highly intact RNA in high quantities, which were actively utilized in downstream experiments. IRCM method was developed primarily for the isolation of RNA from the coconut tissues and can be extended to other plant and animal tissues that are rich in polysaccharides, lipids, proteins, and polyphenols.

## 2. Materials and methods

The materials used included agarose gel, chloroform, chloroform-isoamyl alcohol (24:1, i.e. 24 parts of chloroform mix with 1 part of isoamyl alcohol), acid buffered phenol (pH 4.5), isopropanol, ammonium thiocyanate, guanidine thiocyanate, sodium acetate, glacial acetic acid, glycerol,  $\beta$ -mercaptoethanol, polyvinylpyrrolidone-40 (PVP-40), RNase-free tubes, mortar and pestle, scissor/razor blade, plastic bags, freezer, spatula, pipette, Tris borate EDTA (TBE) buffer, ethidium bromide, DNA ladder, and DPEC water. All the chemicals used were of high analytical purity and were purchased from Real Times and Chemical Reagents, China.

### Cautions!

Phenol, chloroform, and ethidium bromide are potentially hazardous and should be handled with great care in a fume hood. The rest of the chemicals have a hazardous effect to a certain degree, and therefore, eye and skin contact should be avoided.

All the glassware, mortar, pestle, and spatulas should be autoclaved at 121 °C for 20 min before the experiment. They should be then wrapped in an aluminum foil to avoid any contamination with the extrinsic RNase.

### 2.1. Equipments

Agarose gel electrophoresis system.  
Bench centrifuge (Eppendorf Centrifuge 5804 R).  
Vortex (Vortex-BE1, Kylin-Bell Lab Instruments).  
Gel Doc system (Syngene G: Box F3, Gene Company Limited).  
Nanodrop (Nanodrop 2000 spectrophotometer, Thermo Scientific).  
PCR machine/thermocycler (TaKaRa thermocycler dice touch TP350, TaKaRa Bio Incharge).  
Agilent 2100 Bioanalyzer (Agilent Technologies, USA).

### 2.2. Plant materials

The solid endosperm of the stored coconuts and freshly harvested coconuts was collected at the Coconut Research Institute, Chinese Academy of Tropical Agricultural Sciences, Wenchang, Hainan-China. Similarly, coconut apple and coconut leaf bud samples were taken from the freshly sown coconuts at the coconut farm of the same institute. All the samples were cut into small pieces with the help of scissors or a razor blade and transferred to small plastic bags. The plastic bags were then preserved quickly in liquid nitrogen and were kept frozen at -80 °C till further analysis.

### 2.3. IRCM buffer recipe

- i. Ammonium thiocyanate: 0.4 M
- ii. Guanidine thiocyanate: 0.7 M
- iii. 3 M sodium acetate (pH 5): 3.3 ml
- iv. Phenol: 38 ml
- v. Glycerol: 5 ml
- vi. Diethyl pyrocarbonate (DEPC) water: up to 100 ml
- vii.  $\beta$ -mercaptoethanol: 1%
- viii. PVP-40: 1.5%

### 2.4. Preparation

Take 30 ml of (DEPC water) and add the contents to it one by one (except PVP-40). Once dissolved, the volume should be adjusted to 100 ml by adding DPEC water. The buffer should be handled in a fume hood as it contains phenol and should be kept refrigerated in a dark bottle to avoid oxidation. The buffer can be used within six months of preparation.

#### Critical step

Addition of PVP-40 to the IRCM buffer in the presence of ammonium thiocyanate produces a pink color, which tends to significantly decrease the performance of the extraction buffer. Therefore, PVP-40 should be added to the mortar before crushing the sample in liquid nitrogen.

### 2.5. Protocol

#### 2.5.1. Tissue collection and pulverization

1. Transfer the sample-containing bag from the freezer into liquid nitrogen to avoid any thawing before the homogenization of the tissue.

#### Critical step

Thawing of the tissue can allow the RNase to degrade RNA.

2. Pour liquid nitrogen into a clean mortar and pestle (previously autoclaved) to make it cool.
3. Take about 0.08 g of the frozen tissue from the plastic bag and transfer it to the mortar containing liquid nitrogen and PVP-40 (1.5%). Grind the tissue to a fine powder with a pestle and fill the mortar with liquid nitrogen during grinding to avoid any thawing.

#### Critical step

Great care should be taken during the homogenization process:

- a) The addition of liquid nitrogen to the mortar should be slow because splashes can result in sample loss.
- b) Thawing of the sample should be avoided due to the release of RNA from the broken cells, which can be degraded by RNase.
- c) This step should be performed in a fume hood to avoid cross contamination of the sample with RNase.

#### 2.5.2. Extraction of RNA

4. Immediately transfer pulverized tissue with the help of a frozen spatula to a 1.5 ml tube containing 1 ml of extraction buffer (IRCM), vortex for 15 s, and then incubate on ice for 10 min.

#### Critical step

It should be noted that all bits of tissue should be transferred to the RNase-free tube containing IRCM buffer. Failure to do so can result in significant loss of RNA.

#### Caution!

Care should be taken regarding personal safety as the buffer contains phenol. This step should be carried out in a fume hood.

5. Add 200  $\mu$ l of chloroform, vortex thoroughly for 15 s, and then incubate on ice for 10 min. Centrifuge for 7 min at 12,000 rpm at 4 °C.
6. Transfer the supernatant (500  $\mu$ l) to a new RNase-free tube, add an equal volume of chloroform/isoamyl alcohol to it, and centrifuge at 12,000 rpm for 7 min.

#### Critical step

This step is one of the necessary steps as it will isolate RNA from the supernatants left behind after steps 4 and 5. Further, transfer the supernatant with caution and leave some of the supernatant behind to avoid disturbance of the middle phase and thus contamination.

#### Caution!

Care should be taken regarding personal safety as the buffer contains chloroform. This step should be carried out in a fume hood.

### 2.5.3. Precipitation of RNA

- Carefully transfer the upper aqueous phase (400  $\mu$ l) of the solution to a fresh, RNase-free tube, add an equal volume of 99% pure ice-cold isopropanol (500 or 600  $\mu$ l), mix the solution by inverting the tubes, and then incubate for 7 min on ice.

#### Critical step

Carefully transfer the supernatant to a new RNase tube to avoid contamination. The use of ice-cold isopropanol will save time because it has the ability to precipitate the RNA in a short period of time.

- Centrifuge for 7 min at 12,000 rpm and 4°C. After centrifugation, discard the supernatant. The RNA pellet is generally at the bottom of the tube. Remove all residual isopropanol by placing the tube upside down and let the pellet dry for 5 min.

#### Critical step

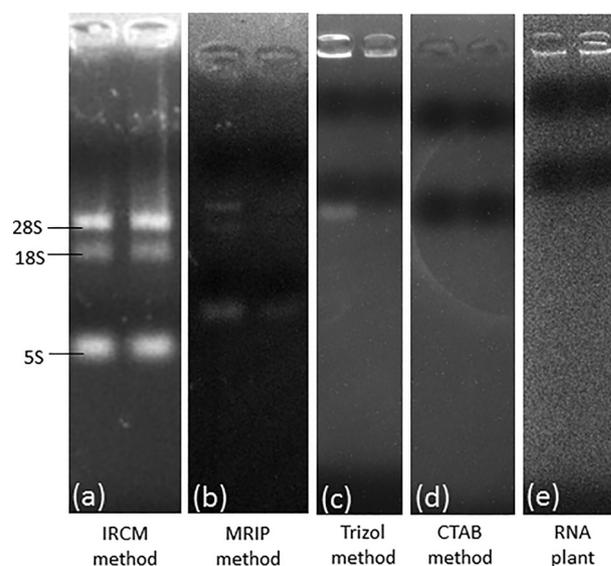
Mark the location of the pellet on the outside of the tube before drying. In most cases, drying can make the pellet transparent and difficult to locate. Further, complete drying of pellet will make it difficult to dissolve.

- Redissolve RNA with 30  $\mu$ l of RNase-free (DEPC) water by pipetting gently for 1–2 min.

### 2.5.4. Qualitative and quantitative analysis of RNA

- The quantity and purity of the RNA were checked by the Nanodrop, where the quality was confirmed by agarose gel electrophoresis.
  - Nanodrop 2000 analysis: The concentration of RNA was determined spectrophotometrically by applying 1  $\mu$ l of sample to the Nanodrop 2000 and measured the absorbance at 260 and 280 nm.
  - Agarose gel electrophoresis: We prepared a 0.8% (w/v) agarose gel by dissolving 0.2 g of RNA grade agarose in 25 ml of 0.5 $\times$  TBE buffer in an RNase-free conical flask. The flask was heated in a microwave oven till the complete dissolution of agarose in the TBE buffer. A quantity of 2  $\mu$ l of ethidium bromide was added to the solution, and the flask was swirled. The mixed content of the flask was then poured into the gel tray with a comb and was left to set for about 20 min. After setting of the gel, RNA loading buffer (2  $\mu$ l) was

## Fresh coconut endosperm



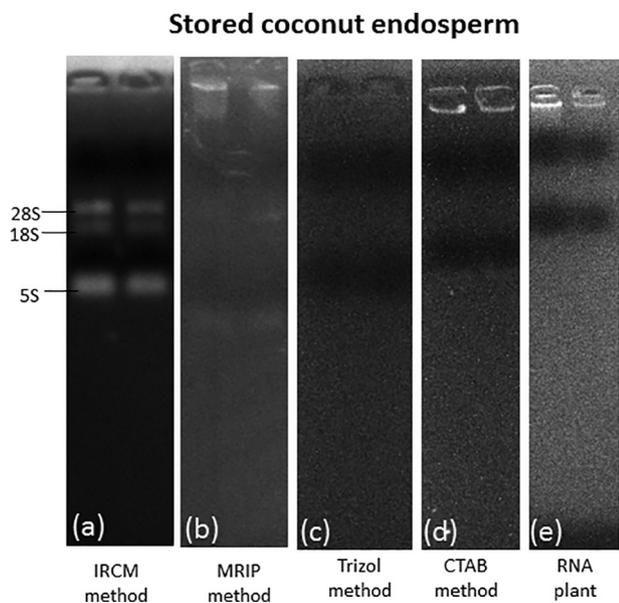
**Fig. 1.** Isolation of RNA from the fresh coconut endosperm by various methods. (a) represents RNA bands that were obtained after loaded 3  $\mu$ l of extracted RNA from the fresh coconut endosperm by IRCM method and run on the agarose gel for 12 min; (b) represents RNA bands that were obtained after ran 3  $\mu$ l of extracted RNA from the fresh coconut endosperm by MRIP method; (c) represents the RNA bands that were obtained after ran 6  $\mu$ l of extracted RNA from the fresh coconut endosperm by Trizol method; (d) represents the RNA bands that were obtained after ran 6  $\mu$ l of extracted RNA from the fresh coconut endosperm by CTAB method; (e) represents the RNA bands that were obtained after ran 6  $\mu$ l of extracted RNA from the fresh coconut endosperm by RNA plant kit method; 28S, 18S, and 5S represent the different bands of rRNA. All the gel photographs were cropped from their respective original photographs using Microsoft Office Picture Manager.

added to the sample (3  $\mu$ l) and the combination was loaded onto the gel. The gel was run for 12 min at 120 V and 240 mA. After development, the bands in the gel were observed in the Gel Doc system, and photographs were taken and saved. All the gel pics were cropped from their respective original pics by using Microsoft Office Picture Manager.

**Table 1**  
Concentration and quality of RNA from different tissues of the coconut by IRCM and MRIP method.

Method	Sample tissue	Conc. ( $\mu$ g/g FW)	A260/280 ratio	A260/230 ratio
IRCM	Endosperm (stored coconut)	150 $\pm$ 1.37	1.89 $\pm$ 0.01	1.88 $\pm$ 0.06
	Endosperm (fresh coconut)	247.5 $\pm$ 2.25	1.91 $\pm$ 0.01	1.90 $\pm$ 0.05
	Coconut Apple	263.5 $\pm$ 12.62	1.97 $\pm$ 0.06	1.99 $\pm$ 0.03
	Coconut Leaf Bud	1052.5 $\pm$ 46.5	2.0 $\pm$ 0.03	1.95 $\pm$ 0.05
MRIP	Endosperm (stored coconut)	67.5 $\pm$ 2.75	1.62 $\pm$ 0.01	0.93 $\pm$ 0.01
	Endosperm (fresh coconut)	146.3 $\pm$ 1.13	1.53 $\pm$ 0.19	1.15 $\pm$ 0.04
	Coconut Apple	122.5 $\pm$ 30.25	1.76 $\pm$ 0.03	1.60 $\pm$ 0.09
	Coconut Leaf Bud	628.8 $\pm$ 63.62	1.75 $\pm$ 0.06	1.73 $\pm$ 0.01
CTAB	Endosperm (stored coconut)	7.5 $\pm$ 0.38	1.05 $\pm$ 0.01	0.69 $\pm$ 0.01
	Endosperm (fresh coconut)	7.5 $\pm$ 1.25	1.09 $\pm$ 0.06	0.81 $\pm$ 0.07
	Coconut Apple	6.25 $\pm$ 0.25	0.81 $\pm$ 0.16	0.99 $\pm$ 0.02
	Coconut Leaf Bud	18.8 $\pm$ 0.38	0.95 $\pm$ 0.06	1.03 $\pm$ 0.06
TRIZOL	Endosperm (stored coconut)	130 $\pm$ 0.63	1.47 $\pm$ 0.12	0.97 $\pm$ 0.05
	Endosperm (fresh coconut)	145 $\pm$ 3.00	1.38 $\pm$ 0.03	1.41 $\pm$ 0.08
	Coconut Apple	103.8 $\pm$ 11.00	1.71 $\pm$ 0.02	1.66 $\pm$ 0.03
	Coconut Leaf Bud	560 $\pm$ 29.50	1.74 $\pm$ 0.01	1.71 $\pm$ 0.08
RNA plant	Endosperm (stored coconut)	38.8 $\pm$ 5.50	1.47 $\pm$ 0.03	1.43 $\pm$ 0.02
	Endosperm (fresh coconut)	57.5 $\pm$ 17.25	1.53 $\pm$ 0.03	1.69 $\pm$ 0.01
	Coconut Apple	11.3 $\pm$ 1.38	2.11 $\pm$ 0.05	1.78 $\pm$ 0.04
	Coconut Leaf Bud	26.3 $\pm$ 3.63	2.09 $\pm$ 0.01	1.81 $\pm$ 0.06

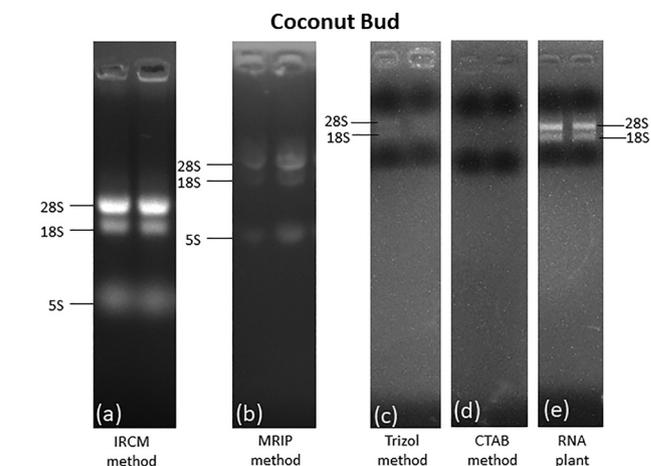
Each data point represents mean of triplicated data of extracted RNA with  $\pm$  standard error. IRCM stands for Isolation of RNA from Complex Matrices, and MRIP stands for Method of RNA Isolation from palms. A 1  $\mu$ l drop was loaded onto the Nanodrop 2000. Each data point represents the mean of triplicated data with  $\pm$  standard error.



**Fig. 2.** Isolation of RNA from the stored coconut endosperm by various methods. (a) represents RNA bands that were obtained after ran 3  $\mu$ l of extracted RNA from the stored coconut endosperm by IRCM method on the agarose gel for 12 min; (b) represents RNA bands that were obtained after ran 3  $\mu$ l of extracted RNA from the stored coconut endosperm by MRIP method; (c) represents the RNA bands that were obtained after ran 6  $\mu$ l of extracted RNA from the stored coconut endosperm by Trizol method; (d) represents the RNA bands that were obtained after ran 6  $\mu$ l of extracted RNA from the stored coconut endosperm by CTAB method; (e) represents the RNA bands that were obtained after ran 6  $\mu$ l of extracted RNA from the stored coconut endosperm by RNA plant kit method; 28S, 18S, and 5S represent the different bands of rRNA. All the gel photographs were cropped from their respective original photographs using Microsoft Office Picture Manager.

**Critical step**

All the parts of the assembly, including the comb and tray, should be free of RNases. Further, the equipment RNA loading buffer and TBE



**Fig. 4.** Isolation of RNA from the stored coconut bud by various methods. (a) represents RNA bands that were obtained after ran 3  $\mu$ l of extracted RNA from the coconut bud by IRCM method on the agarose gel for 12 min; (b) represents RNA bands that were obtained after ran 3  $\mu$ l of extracted RNA from the coconut bud by MRIP method; (c) represents RNA bands that were obtained after ran 6  $\mu$ l of extracted RNA from the coconut bud by Trizol method on the agarose gel for 12 min; (d) represents RNA bands that were obtained after ran 6  $\mu$ l of extracted RNA from the coconut bud by CTAB method; (e) represents RNA bands that were obtained after ran 6  $\mu$ l of extracted RNA from the coconut bud by RNA plant kit method; 28S, 18S, and 5S represent the different bands of rRNA. All the gel photographs were cropped from their respective original photographs using Microsoft Office Picture Manager.

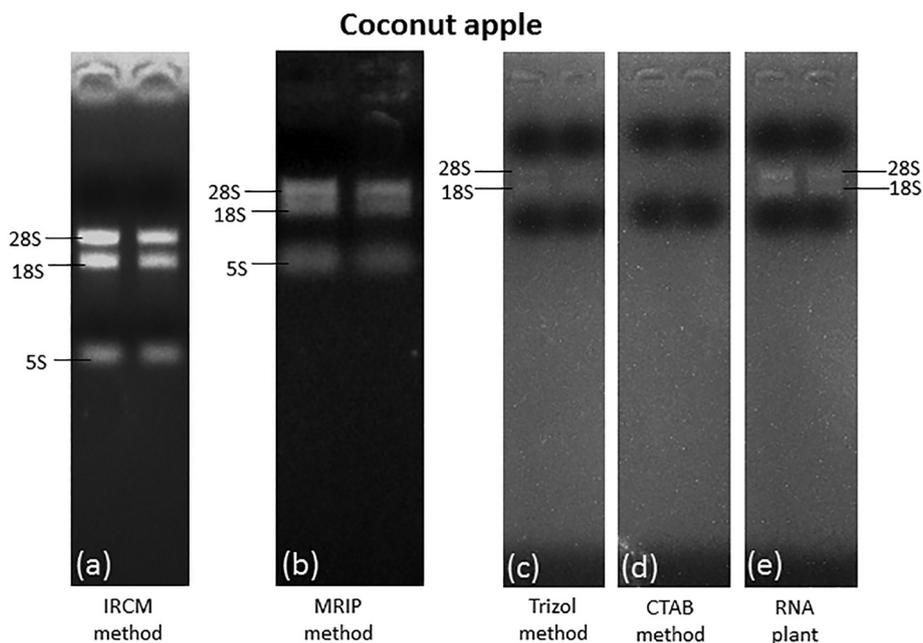
should also be free of any RNase. If these are not ensured, false negative results would be likely.

**2.5.5. Time breakup**

Pulverization of sample: steps 1 to 3 took 30 min for 4 samples.

RNA extraction: steps 5 to 6 took 25 min.

RNA precipitation: steps 7 to 9 took 25 min.



**Fig. 3.** Isolation of RNA from the stored coconut apple by various methods. (a) represents RNA bands that were obtained after ran 3  $\mu$ l of extracted RNA from the coconut apple by IRCM method on the agarose gel for 12 min; (b) represents RNA bands that were obtained after ran 3  $\mu$ l of extracted RNA from the coconut apple by MRIP method; (c) represents RNA bands that were obtained after ran 6  $\mu$ l of extracted RNA from the coconut apple by Trizol method on the agarose gel for 12 min; (d) represents RNA bands that were obtained after ran 6  $\mu$ l of extracted RNA from the coconut apple by CTAB method; (e) represents RNA bands that were obtained after ran 6  $\mu$ l of extracted RNA from the coconut apple by RNA plant kit method; 28S, 18S, and 5S represent the different bands of rRNA. All the gel photographs were cropped from their respective original photographs using Microsoft Office Picture Manager.

## 2.6. Reverse transcription or cDNA synthesis

The first strand cDNA was synthesized with TaKaRa PrimeScript II 1st strand cDNA synthesis kit. According to the manufacturer's protocol, the reaction of the RT was conducted in the following way: About 2 µg of the isolated RNA was mixed with 1 µl oligo dT primer (50 µM) + dNTP mixture (10 mM), and the volume was adjusted to 10 µl with DPEC water in a Microtube. The tube was incubated at 65°C for 5 min and then chilled on ice. Subsequently, 5× PrimeScript II buffer (4 µl), RNase inhibitor (0.5 µl), and PrimeScript II RTase (1 µl) were added to the tube, and the final volume was adjusted to 20 µl with DPEC water. The mixture was incubated at 42°C for 60 min. To inactivate the enzymes, the mixture was incubated for a further 5 min at 95°C, followed by cooling on ice.

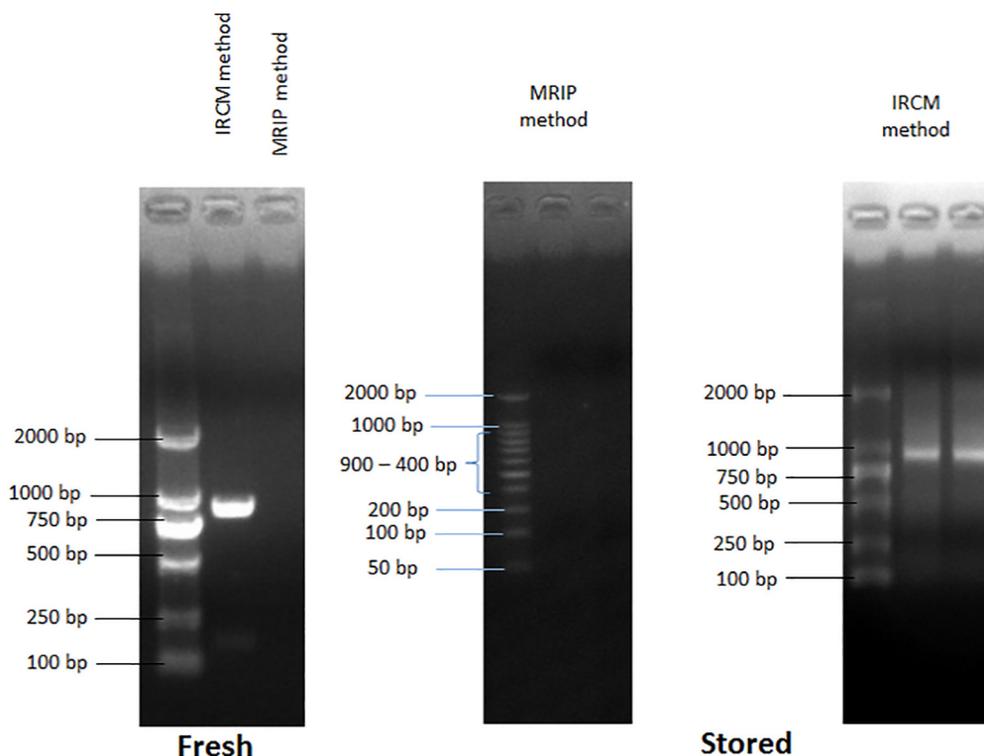
## 2.7. RT-PCR

After the synthesis of first strand cDNA, RT-PCR was done using a kit from the Real Times to amplify the cDNA. An LPAAT-α gene was tested using primers CnLPAAT-For: ATGGATGCTTCAGGGGCAAGTTCG and CnLPAAT-Re: TTATGAATTTGACCTCCGCTAGCATCC for coconut endosperm and coconut apple samples, respectively whereas a housekeeping gene Actin was tested using primers CnACT-For: ATAAAGTATGGCTGATGCTGAGG and CnACT-Re: CAACAATGCTTGGG AACACA for coconut leaf bud. cDNAs were amplified by mixing 2 µl of cDNA, 1 µl of reverse primer (10 µM), 1 µl of forward primer (10 µM), 5 µl 10× TAQ buffer, 1 µl dNTP mix (10 mM), 0.5 µl TAQ (5 U/µl), and 39.5 µl ddH<sub>2</sub>O in a microcentrifuge tube. The tube was then placed in a thermocycler with the following settings: 94°C for 3 min; 35 cycles (94°C for 30 s, 54°C for 30 s, 72°C for 1 min); 72°C for 5 min. A control was carried out with RNA in the absence of reverse transcriptase to check for chromosomal DNA contamination.

## 3. Results

The newly developed IRCM protocol for the extraction of RNA from complex matrices rich in carbohydrates, lipids, proteins, and polyphenols provided us with high quality RNA in appreciable quantities. The A260/280 ratio of RNA extracted by IRCM method ranged from 1.89 to 1.91 for the solid endosperm of coconut, while MRIP (1.53–1.62), CTAB (1.05–1.09), TRIZOL (1.38–1.47), and RNA plant (1.47–1.53) gave far lower values (Table 1). The result also revealed higher concentrations of the extracted RNA from various tissues of coconut by the IRCM method (146.2 to 1052.5 µg/g FW) as compared to MRIP (67.5 to 628.8 µg/g FW), CTAB (7.5 to 18.8 µg/g FW), TRIZOL (130 to 560 µg/g FW), and RNA plant (38.8 to 57.5 µg/80 g FW). Moreover, the electrophoretogram showed that the RNA extracted with IRCM method has sharp bands of 28S, 18S, and 5S rRNA without any contamination. Likewise, the 28S rRNA band was intense than the 18S rRNA band indicated towards the quality of the extracted RNA by the IRCM method, whereas the MRIP, CTAB, and TRIZOL were failed to extract RNA (Fig. 1a, Fig. 2a, Fig. 3a and Fig. 4a, Fig. 4d) from the solid endosperm of coconut (Fig. 1 and Fig. 2) and other tissues (Fig. 3 and Fig. 4). Furthermore, RNA plant (commercial kit) extracted the RNA from coconut apple and coconut bud tissues, but in lower concentrations (Table 1, Fig. 3 and Fig. 4).

RT-PCR confirmed the quality of RNA through the synthesis and amplification of cDNA. The RNA that was extracted from the solid endosperm of coconut (both stored and fresh) by IRCM method successfully produced the amplified RT-PCR product. The MRIP method, on the other hand, failed to produce cDNA from the extracted RNA from the solid endosperm of coconut, which meant that the RNA was not of good quality to be used in downstream experiments (Fig. 5). The quality of the extracted RNA from coconut apple and coconut bud by MRIP method, although not great, is good enough to produce faint



**Fig. 5.** RT-PCR product of isolated RNA from stored and fresh coconut endosperm by IRCM and MRIP method. Lane 1 of this figure with "\*" sign represents the marker (DNA ladder) of different molecular weight, whereas lanes 2 and 3 with "\*" represent the samples. Lane 2 of panel a represents the RT-PCR product of RNA isolated from fresh coconut endosperm by IRCM method, and lane 3 represents the RT-PCR product of RNA isolated from fresh coconut endosperm by MRIP method. Lanes 2 & 3 of panel b represents the RT-PCR product of RNA isolated from stored coconut endosperm by IRCM method. Similarly, lanes 2 & 3 of panel c represent the RT-PCR product of RNA isolated from stored coconut endosperm by MRIP method. Each lane of the agarose gel was loaded with 3 µl of DNA marker or RT-PCR product. The gel was run for 20 min, and the bands were observed in the Gel Doc system. All the gel photographs were cropped from their respective original photographs using Microsoft Office Picture Manager.

bands of RT-PCR product (Fig. 6 and Fig. 7). The gel electrophoresis indicated that the IRCM protocol is suitable for isolating high quality RNA from complex coconut tissues.

#### 4. Discussion

RNA extraction is the initial and most important step for various investigations in the field of molecular biology, including RNA sequencing, cDNA library construction, and profiling the gene expression [15,16,17]. To achieve the goal of high-quality RNA extraction from plant and animal tissues, various methods were adopted over the years. As palmaceae is a complicated family, it is difficult to extract intact RNA with high yields. Being a part of the tropical region of China, Wenchang is famous for its high-quality coconuts with diverse germplasm. To study the various attributes of the palm at the molecular level, one should be certain of the RNA that can be used effectively in downstream experiments. The Biotechnology Laboratory at the Coconut Research Institute is actively involved in the extraction of quality RNA from the complex matrices of the coconut species. Quite recently, the laboratory tested numerous methods, such as CTAB, TRIZOL, and Tiangen RNA plant, for the extraction of RNA from

the coconut leaf, yet all methods have failed [7]. During this research, the above mentioned methods were also used to isolate RNA from the solid endosperm of coconut, coconut apple, and coconut bud, which are rich in carbohydrates, fatty acids, proteins, and polyphenols. However, the tested methods failed to isolate quality RNA in appreciable quantities from the complex tissues of the coconut. Therefore, we developed a new method (IRCM) that is cost effective, easy, and less time-consuming for extracting quality RNA from the matrices that are high in carbohydrates, lipids, proteins, and polyphenols.

As plant species vary in composition of macro- and micro-biomolecules, they require suitable methods for the extraction of quality RNA because the efficiency of an RNA extraction method does not stay the same for different plant species. Further, the presence of polysaccharides, phenols, proteins, and lipids in a plant species not only interferes with the extraction of RNA, but quite likely degrade it as well. Moreover, after comparing the chemical composition and steps of the various methods (Table 2), it was found that a proper combination of chaotropic agent and polyphenol- and polysaccharide-binding agent might be necessary in the lysis buffer. Besides, the lipid-removing agent will aid in the isolation of quality RNA from the oil rich tissues. The presence of high amounts of macromolecules (carbohydrates and lipids) in the coconut tissues might have affected the efficiency of the column used for RNA extraction in RNA plant (a commercial kit). Correspondingly, the buffer composition of CTAB [18], Trizol, and MRIP might have been lacking one or more important component(s) that are vital to extract RNA from the coconut tissues. The buffer recipe of MRIP and Trizol are lacking in PVP and  $\beta$ -mercaptoethanol, and CTAB is lacking in acid-buffered phenol that can remove the polysaccharide, polyphenols, and proteins from the pulverized tissues of the coconut. On the other hand, the IRCM buffer was prepared with all the necessary components (acid buffered phenol, PVP, and  $\beta$ -mercaptoethanol) for the removal of polysaccharide, polyphenols, lipids, and proteins in optimum amounts. The above mentioned buffer components in optimized amounts successfully isolated RNA from the complex tissues of the coconut that are abundant in polysaccharides and lipids, with appreciable quantities of polyphenols and proteins. The combination of buffer components might be the key to recover highly intact RNA from complex tissues of coconut with larger yields in the shortest possible time (2 h) by the IRCM method. The method was validated for the extraction of RNA from coconut tissues, but might be extended to various other tissues from plant and animal origins that are rich in macromolecules and other metabolites.

#### 5. Conclusions

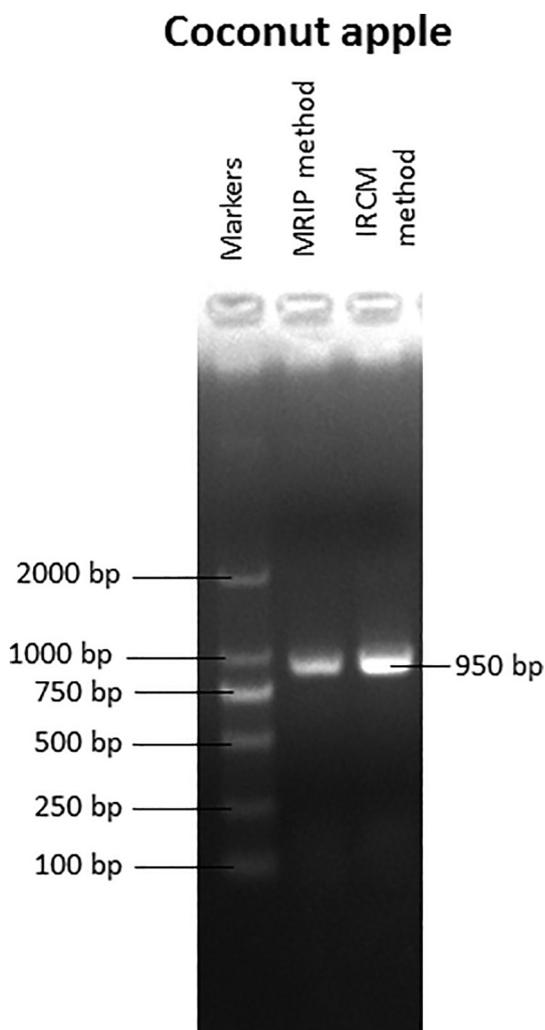
The IRCM buffer was prepared with all necessary components which were required for the successful isolation of RNA from the complex tissues of the coconut (abundant in polysaccharides and lipids with appreciable quantities of polyphenols and proteins). The buffer is the key that allows the IRCM method to provide highly intact RNA in larger amounts within a short time (2 h). The method was validated for the extraction of RNA from the complex coconut tissues. Furthermore, the method might perform well in the isolation of RNA from various tissues of other plant and animal species that are rich in macromolecules and other metabolites.

#### Conflict of interest

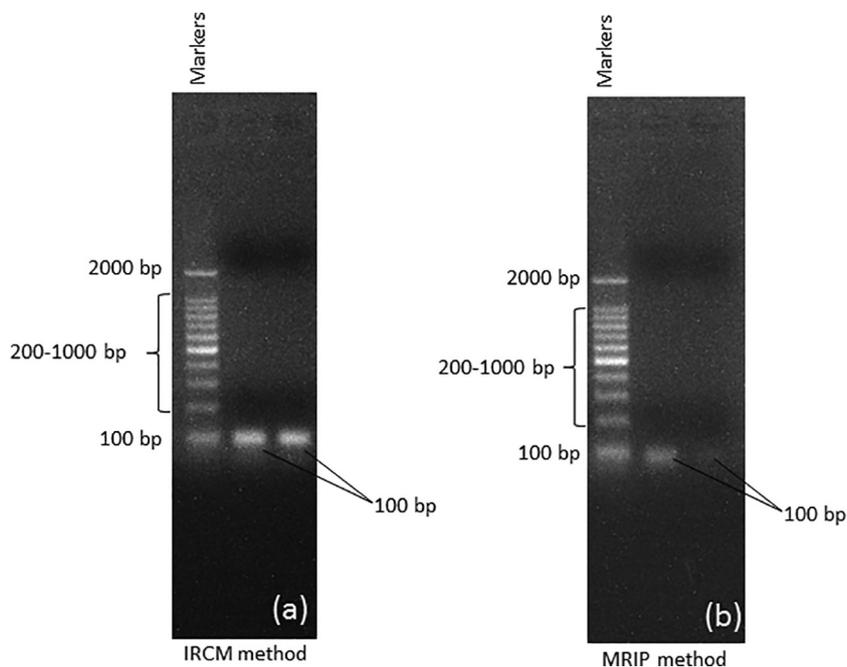
The authors declare that there are no financial and non-financial interests related to this manuscript.

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**Fig. 6.** RT-PCR product of isolated RNA from coconut apple by IRCM and MRIP method. Lane 1 of this figure represents the marker (DNA ladder) of different molecular weight, lane 2 represents the RT-PCR product of RNA isolated from coconut apple by MRIP method, and lane 3 represents the RT-PCR product of RNA isolated from coconut apple by IRCM method. Each lane of the agarose gel in Fig. 5 was loaded with 3  $\mu$ l of DNA marker or RT-PCR product. The gel was run for 20 min, and the bands were observed in the Gel Doc system. All the gel photographs were cropped from their respective original photographs using Microsoft Office Picture Manager.



**Fig. 7.** RT-PCR product of isolated RNA from coconut bud by IRCM and MRIP method. Lane 1 of panel a represents the marker (DNA ladder) of different molecular weight, and lanes 2 & 3 represent the RT-PCR product of RNA isolated from coconut bud by IRCM method. Similarly, lane 1 of panel b represents the marker (DNA ladder) of different molecular weight, and lanes 2 & 3 represent the RT-PCR product of RNA isolated from coconut bud by MRIP method. Each lane of the agarose gel in panels a & b was loaded with 3 µl of DNA marker or RT-PCR product. The gel was run for 20 min, and the bands were observed in the Gel Doc system. The gel pic was cropped from the original photograph using Microsoft Office Picture Manager.

**Table 2**  
Chemical composition of the RNA extraction buffer and steps in various protocols.

IRCM	MRIP	CTAB	TRIZOL	RNA plant kit
Chemical composition of various extraction buffers				
GTC, ATC, glycerol, phenol, βME, PVP, NaOAc	GTC, ATC, glycerol, phenol, NaOAc	Tris-HCl, EDTA, CTAB, PVP, βME spermidine	Trizol	Commercial lysis RB buffer
Differences in the steps of various protocols				
Extraction of the nucleic acids from the pulverized tissues using the IRCM buffer	Extraction of the nucleic acids from the pulverized tissues using the ICRM buffer	Extraction of the nucleic acids from the pulverized tissues using the CTAB buffer (pre-warmed to 65°C)	Extraction of the nucleic acids from the pulverized tissues using the TRIZOL buffer	Extraction of the nucleic acids from the pulverized tissues using the RB buffer
Removal of contaminants with acid-buffered phenol, βME, PVP, and chloroform	Removal of contaminants with acid-buffered phenol, and chloroform	Removal of contaminants with EDTA, Tris-HCl, βME, PVP, and chloroform/isoamyl alcohol (24:1 v/v)	Removal of contaminants with trizol and chloroform	Removal of contaminants with a column and buffers provided with kit
Further cleaning of the supernatant with chloroform/isoamyl alcohol (24:1 v/v)	N/A	Further cleaning of the supernatant with chloroform	Same as IRCM	Further cleaning of the supernatant with the column and buffers provided with the kit
Precipitation of RNA with equal volume of isopropanol	Same as IRCM	Precipitation of RNA with 2x volume of isopropanol	Same as IRCM	Buffer in kit was used
N/A	Wash with 70% ethanol	Same as MRIP	Same as MRIP	Wash with buffer

GTC = guanidinium thiocyanate; ATC = ammonium thiocyanate; βME = beta-mercaptoethanol; PVP = polyvinyl pyrrolidone; NaOAc = sodium acetate.

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