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EVALUATION OF METHODS FOR TOTAL RNA EXTRACTION FROM THE ENDOSPERM OF COCOS NUCIFERA VAR. MAKAPUNO IN VIETNAM FOR MOLECULAR ANALYSIS

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ABSTRACT

Sap coconut (*Cocos nucifera* L. var. *makapuno*) in Vietnam is a mutant coconut variant; coconut water is in the state of lotus glue, and coconut rice is like cream. For high-quality transcriptome, sequencing, quality, purity and concentration of RNA are the key factors. However, coconut endosperm tissue has higher stiffness and fatness than the leaf tissue, which complicates the extraction process. Moreover, RNA is much more difficult to preserve than DNA. In this study, various RNA extraction methods were examined in Vietnamese waxy coconut endosperm tissue samples. Optimum extraction and preservation of the RNA using the simplest possible chemicals was the objective of this study. The modified CTAB method with LiCl and the TRI reagent method were tested and evaluated. The purity, concentration and quality of RNA after storage were improved. The findings indicated that the TRIsure extraction method with the addition of NaCl and β-mercaptoethanol yielded optimum RNA quality. The RNA concentration was 159 ng/μL, with a purity ratio of 1.94 ± 0.04 for A260/A280 and 1.58 ± 0.02 for A260/230. RNA samples remained stable for up to 3 weeks when stored in absolute ethanol at 8°C–10°C, which significantly reduced their degradation during transportation. This study facilitated the use of simple chemicals for high-quality RNA extraction from coconut endosperm and its preservation for applications in high throughput sequencing.

Keywords: *Cocos nucifera var. makapuno*, extraction method, total RNA

INTRODUCTION

Coconut (*Cocos nucifera* L.) is a tree with high economic value, and coconut water is a beverage that is preferred by many people. Coconut is also an important perennial oil crop around the world. In particular, the Sap coconut variant originating from the Philippines, called *Makapuno coconuts*, is considered a specialty, with the coconut sap in a state of gelatinous creamy paste and high in fat content. Interestingly, there are normal fruits with solid endosperm like other coconut variants, and some fruits contain waxy endosperm on the same coconut tree (Arellano *et al.* 2019).

RNA isolated from tissue samples is important for several research projects. Ribosomal RNA sequencing is often used for the identification, classification, or assessment of biodiversity. Furthermore, messenger RNA (mRNA) is used for gene expression research, novel gene discovery, or gene isoform analysis. Short fragments can be sequenced to assess the expression of certain genes, or whole mRNAs can be encoded for comparative transcriptomics. mRNA is used in reverse transcription PCR (polymerase chain reaction) to synthesise cDNA for sequencing, which helps identify gene variants and is also useful in gene expression studies. In addition, mRNA is used in Northern blotting to analyse gene expression over time or under specific conditions based on the size and amount of mRNA. However, for RNA sequencing, reverse transcription PCR and quantitative real-time PCR, an intact RNA with high purity is essential.

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Extraction of RNA from coconut endosperm tissue for mRNA transcriptome sequencing helps to evaluate the differences in gene expression between waxy and normal endosperm tissues, thereby aiding in future fruit characterisation studies. However, obtaining high quality RNA from endosperm tissue is more challenging than obtaining it from leaf tissue. The reason is that coconut endosperm possesses numerous polysaccharides, proteins, polyphenols, and lipids that bind to nucleic acids, thereby posing a challenge in the isolation of RNA (Angeles *et al.* 2005, Zhang *et al.* 2022). Moreover, sequencing results are affected owing to inadequate preservation during the transportation process over an extended period. Hence, obtaining of RNA samples that are highly concentrated and pure is of utmost importance in ensuring the success of RNA sequencing and genetic analysis (Gallego Romero *et al.* 2014, Scholes *et al.* 2020).

Several studies have been performed to enhance the RNA separation techniques to facilitate subsequent molecular investigations. Most of these studies were based on guanidine thiocyanate as the main lysate. In 2012, MRIP (Methods for RNA Isolation from Palms) extraction buffer, which contains a series of different components, i.e. ammonium thiocyanate, guanidine thiocyanate, sodium acetate, phenol and glycerol, was first used for RNA isolation from palm leaves. Higher purity and integrity of the RNA molecules were achieved in comparison with CTAB, TRIzol methods and Tiangen RNA plant kit. The electrophoresis results from MRIP showed clear lines of 28S, 18S and 5S rRNA whereas the other techniques did not. Nonetheless, the concentration of the RNA product from each method was not discussed and the study was performed on the leaf only. Subsequently, Iqbal et al. (2019) provided an improved method, QRREM (Quick and Reliable RNA Extraction Method), which was similar to MRIP in all aspects except for the addition of β-mercaptoethanol (2%) and polyvinylpyrrolidone-40 (3%). Intact RNA was efficiently isolated, and high RNA concentration (16.2 μg/80 mg) was obtained from stored coconut endosperm compared with CTAB (0.1), TRIzol (10.4) and RNA plant kit (0.5). This method had many advantages over other methods in terms of time and cost efficiency (Iqbal *et al.* 2019). Later, in 2020, the research group of Iqbal *et al.* proposed another improved RNA extraction method, IRCM (Isolation of RNA from Complex Matrices), by halving the concentrations of β-mercaptoethanol (1%) and polyvinylpyrrolidone-40 (1.5%) in comparison with QRREM. The protocol was applied for coconut endosperm, coconut mortise and coconut buds, and the results were higher when compared with CTAB, TRIzol and plant RNA extraction kit (Iqbal *et al.* 2020). In all the above methods, the main lysis component was guanidine thiocyanate. Although it was more effective than CTAB and TRIzol, the components were quite complex and acquiring the products from the suppliers was a time consuming process, which sometimes affected the progress of the project. However, TRI reagents, the major component of which is guanidine thiocyanate, are often accessible from vendors, making them more convenient to purchase.

Contrary to guanidine thiocyanate-based methods, Souza-Perera *et al.* (2018) enhanced the CTAB extraction method for RNA from various tissue samples, such as leaves, inflorescences and primary and secondary roots based on the CTAB extraction protocol for DNA. This study examined the characteristics of grade, zygote embryos, and solid endosperm in mature coconut trees. The authors successfully isolated RNA from coconut endosperm tissue with higher integrity compared with the TRIzol method and without any carbohydrate or protein contamination (Souza-Perera *et al.* 2018). Therefore, various RNA isolation methods should be evaluated in coconut endosperm tissue samples and optimised to obtain high quality RNA. This study aimed to develop a protocol for the extraction, preservation, and transportation of total RNA to minimise RNA quality loss.

MATERIALS AND METHODS

Collection of coconut tissue samples

Throughout the study, the waxy endosperm (WE) sample from coconut was the main target for the investigations, i.e. evaluation of RNA extraction efficiency, purity improvement test, lysis temperature test and RNA preservation test during transportation. Particularly, in the

evaluation of RNA extraction efficiency of the two methods CTAB-LiCl and TRIsure, additional samples of non-waxy endosperm (NWE) tissues and leaf (L) tissues from the same coconut cultivar were used as comparison controls. All samples were collected from Tra Vinh province, Vietnam.

The morphology of coconut shells and skulls of waxy and non-waxy fruits indicated no discernible differences in their shape or coloration (Figure 1). The only external difference between the two types of fruits was the characteristic sound upon shaking. The waxy fruit made a rattling sound owingto its thick, viscous, overdeveloped endosperm. However, this sound may also arise during the growing stages of non-waxy fruit. Hence, precise differentiation is possible only by identifying the endosperm tissues. The endosperm of waxy coconut is pliable and contains colloidal water and a disorganised tissue arrangement. In contrast, non-waxy fruit exhibits high water content and a dense and rigid endosperm.

Therefore, for waxy samples, sterile spoons were used to collect viscous endosperm from the non-cut contact area, which was then promptly transferred to labelled sterile bags. The non-waxy endosperm and the leaf tissue were prepared by partially cutting and cleaning them with distilled water and 70% alcohol. After drying, the samples were aseptically transferred to labelled sterile bags. All pretreated samples were promptly utilised or preserved at −80°C.

Extraction of total RNA

Guanidine thiocyanate and CTAB methods have been extensively evaluated but have shown varying results in different studies. Hence, in this study, these two approaches were tested for high-performance RNA extraction from the coconut endosperm tissue. As the composition of lytic compounds is quite complex in IRCM and QRREM and orders for these chemicals are time consuming, the main lysate guanidine thiocyanate via the TRIsure reagent was used with some improvements. The total RNA extraction methods investigated included CTAB-LiCl (Chang *et al.* 1993, Ghangal *et al.* 2009, White *et al.* 2008) and TRIsure (Thermo Fisher 2010).

After assessing the extraction efficiency, several follow-up investigations were conducted to improve the quality of the RNA product. For enhancing the purity of RNA, the addition of NaCl and β-mercaptoethanol to the lysis process was tested and the RNA washing step was repeated thrice. For increasing the efficiency of tissue resolution, different incubation temperatures (room temperature, 50°C, 55°C and 60°C) were used during the lysis process. As RNA is easily degraded, it was stored under conditions similar to transportation conditions to determine its degradation time. Each treatment was performed in triplicate.

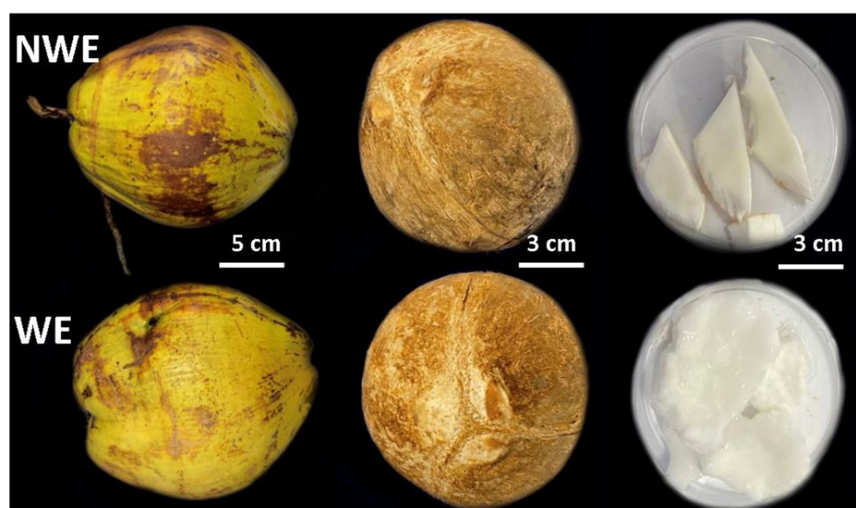

Figure 1 Coconut samples collected in the study
Notes: top row: non-waxy coconut; bottom row: waxy coconut. From left to right: unpeeled fruit; fruit skull, endosperm samples in petri dish.

Investigation of the preservation process under transportation conditions

RNA is inherently unstable and prone to degradation. This experiment aimed to examine the preservation of total RNA post-extraction. Two methods were used to preserve total RNA: (i) Total RNA was stored at −20°C in diethylpyrocarbonate (DEPC)-water after dissolution. (ii) Total RNA was mixed with 0.1× volume of 3M sodium acetate CH_3COONa (pH 5.5) and twice the volume of 100% ethanol after dissolution in DEPC. It is a chemical compound that inactivates RNase, the enzyme that degrades RNA. DEPC is used to preserve RNA integrity in laboratory settings during various experimental procedures. Meanwhile, sodium acetate acts as a co-precipitant in the presence of ethanol. When added to an RNA sample, it neutralises the negative charges on the RNA molecules, facilitating their aggregation and precipitation, which in turn condenses the RNA structure and resists degradation. Both methods are often used for RNA preservation. RNA samples were then stored at −20°C. The examination was performed over a period of 1 month, with weekly testing.

Quality assessment of the extracted RNA

For transcriptome sequencing, the concentration and purity of RNA are of primary concern. RNA purity and total electrophores concentration were checked using the NanoDrop™ - Thermo Fisher Scientific spectrophotometer at wavelengths A260, A280 and A230. For Illumina sequencing, the minimum required weight is 500 ng and concentration should be >10 ng/μL (Hong *et al.* 2020). As for purity, the A260/A280 ratio should be in the range of 1.8–2.0 and A260/A230 should be between 2.0 and 2.2.

Electrophoresis was also used to assess the relative concentration and integrity of RNA bands of different sizes. The extracted RNA samples were electrophoresed on a 2% (w/v) agarose gel together with a DNA scale. However, the size of the mRNA varied greatly depending on the length of the corresponding gene; hence, the number or position of the lanes on the electrophoresis gel was not evaluated. The DNA scale preliminarily assessed the length of the RNA lines that appeared. Brighter the bands, higher the concentration of RNA; clear bands represented intact RNA molecules of certain lengths, whereas smeared bands represented different sizes of RNA, which could be attributed to degradation. To enhance the objectivity while analysing the electrophoresis images, the GelAnalyzer 19.1 software (Lazar Jr. *et al.*) was used in combination.

Statistical analysis

The data obtained from the above experiments were statistically analyzed using the MiniTab 17 software (Minitab Inc. 2020). The data were statistically evaluated using ANOVA with the Turkey algorithm.

RESULTS AND DISCUSSION

Evaluation results of RNA extraction procedures using CTAB-LiCl and TRIsure

The WE sample was used as the main target for investigating the effects of the two extraction methods CTAB-LiCl and TRIsure on the quality of the extracted RNA. In addition, the NWE and L samples from the same plant were included as control samples. In total, three types of tissue samples were used for the two extraction methods. Each treatment was repeated thrice for calculating statistical reliability.

With regard to the total RNA concentration, the results showed the statistical difference between the two methods (P value < 0.05) in all types of tissues. The total RNA concentrations obtained from WE tissue samples were 142.00 ± 2 ng/μL and 157.00 ± 1.7 ng/μL for the CTAB-LiCl and TRIsure methods, respectively (Table 1). In both methods, there was no statistically significant difference between WE and NWE samples. However, both endosperm tissues (statistically classified as B) had significantly lower extraction efficiency than leaf tissues (statistically classified as A), and the difference was significant (P value < 0.05) (Table 1). There was no interaction between the two factors and tissue types [P value > 0.05 (0.422)].

Table 1 Total RNA concentrations extracted using CTAB-LiCl and TRIsure

Extraction method	Tissue type		
	Waxy endosperm sample (WE)	Non-waxy endosperm sample (NWE)	Leaf sample (L)
CTAB-LiCl	142.00 ± 2^{bB}	142.33 ± 1.5^{bB}	155.00 ± 2^{bA}
TRIsure	157.00 ± 1.7^{aB}	156.67 ± 0.5^{aB}	167.67 ± 0.5^{aA}
P value (Extraction method)	0 (<0.05)		
P value (Tissue type)	0 (<0.05)		
P value (two-factor interaction)	0.422 (>0.05)		

Note: Two-way ANOVA analysiswas used to investigate the influenceeffects of different extraction methods (CTAB-LiCl versusvs TRIsure) and different tissue types (waxy endosperm, non-waxy endosperm and leaf tissue) on RNA product concentration. The lowercase letters a and b: represent the differences between the extraction methods; the uppercase letters A and B represent the differences between the tissue types. The differences are significant when the P value is <0.05.

As for the OD assessment to determine sample purity, both CTAB-LiCl and TRIsure methods yielded an A260/A280 OD ratio within the optimal range of 1.8–2.0 (Table 2). In contrast, the OD A260/A230 ratio exhibited a distinct disparity. Samples extracted with CTAB-LiCl had a ratio of 1.8–1.9, whereas those extracted with TRIsure had a significantly lower ratio of 0.99–1.02 (Table 3). The optimal purity range for A260/A230 is 2.0–2.2. Macrogen company does not necessitate high-quality samples for sequencing as the issue can be resolved by purifying the sample before sequencing. The index of samples extracted using CTAB-LiCl was acceptable at 1.8–1.9, despite not reaching 2.0–2.2. On the contrary, the A260/A230 ratio of TRIsure-extracted samples fell very much below the standard threshold and should be improved.

Table 2 A260/A280 ratio of samples extracted using CTAB-LiCl and TRIsure

Extraction method	Tissue type		
	Waxy endosperm sample (WE)	Non-waxy endosperm sample (NWE)	Leaf sample (L)
CTAB-LiCl	1.99 ± 0.03 ^{aA}	1.99 ± 0.01 ^{aA}	2.00 ± 0.05^{aA}
TRIsure	1.99 ± 0.01 ^{aA}	2.00 ± 0.03 ^{aA}	2.01 ± 0.01 ^{aA}
P value (Extraction method)	0.482 (>0.05)		
P value (Tissue type)	0.526 (>0.05)		
P value (two-factor interaction)	0.928 (>0.05)		

Note: Two-way ANOVA was used to investigate the effects of different extraction methods (CTAB-LiCl vs TRIsure) and tissue types (waxy endosperm, non-waxy endosperm and leaf tissue) on the A260/A280 ratio of the RNA product. The lowercase letters a and b represent the difference based on the extraction method; the uppercase letters A and B represent the difference based on the tissue type. The differences are significant when the P value is <0.05.

Table 3 A260/A230 ratio of samples extracted using CTAB-LiCl and TRIsure

Extraction method	Tissue type		
	Waxy endosperm sample (WE)	Non-waxy endosperm sample (NWE)	Leaf sample (L)
CTAB-LiCl	1.87 ± 0.005 ^{aA}	1.89 ± 0.01 ^{aA}	1.87 ± 0.02^{aA}
TRIsure	1.01 ± 0.03 ^{bA}	0.99 ± 0.02 ^{bA}	1.02 ± 0.06 ^{bA}
P value (Extraction method)	0.00 (<0.05)		
P value (Tissue type)	0.936 (>0.05)		
P value (two-factor interaction)	1.87 ± 0.02^{aA}		

Note: Two-way ANOVA was used to investigate the effects of different extraction methods (CTAB-LiCl vs TRIsure) and tissue types (waxy endosperm, non-waxy endosperm and leaf tissue) on the A260/A280 ratio of the RNA product. The lowercase letters a and b represent the difference based on the extraction method; the uppercase letters A and B represent the difference based on the tissue type. The differences are significant when the P value is <0.05.

In electrophoretic separation, RNA samples extracted using CTAB-LiCl exhibited limited bands, including two 23S rRNA subunits at approximately 2900 bp, 18S rRNA subunits at approximately 1900 bp and various other bands at approximately 250–500 bp (Thermo Fisher 2006) (Figure 2). On the contrary, electrophoretic separation of RNA samples extracted using the TRIsure method displayed multiple bands in the range of 250–3000 bp in addition to the 23S and 18S rRNA bands, and these bands were more distinct and intense. To evaluate the results of electrophoresis more objectively, a combination of electrophoretic images and the image analysis software Gelanalyzer was used. A significant difference was observed (Figure 3). According to the analysis results, RNA samples extracted using the TRIsure reagent had clearer peaks and were better in terms of quantity than those extracted using CTAB-LiCl (seven peaks vs two peaks).

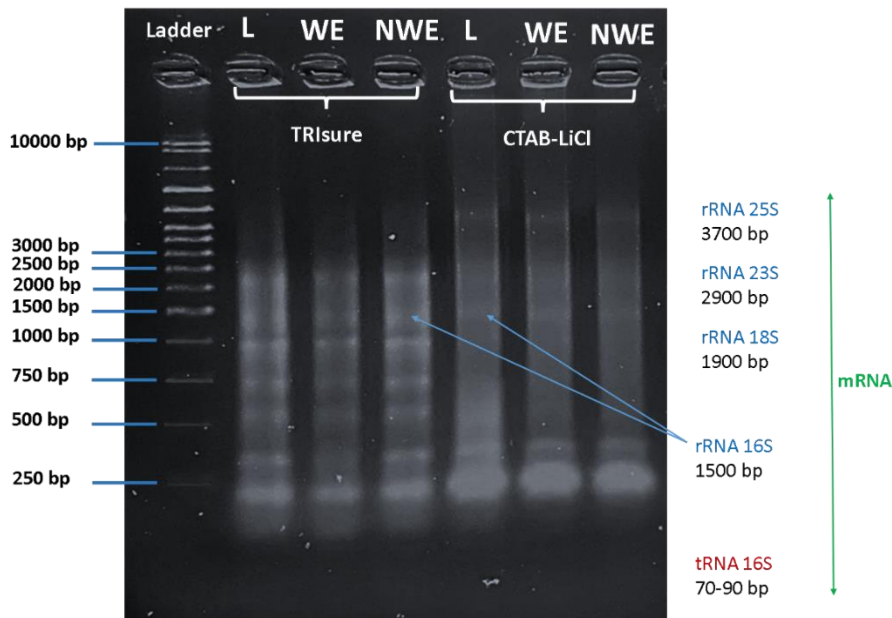

Figure 2 Results of electrophoresis on 2% agarose gel with a scale of 10 kbp; WE: waxy endosperm, NWE: non-waxy endosperm, L: leaf tissue

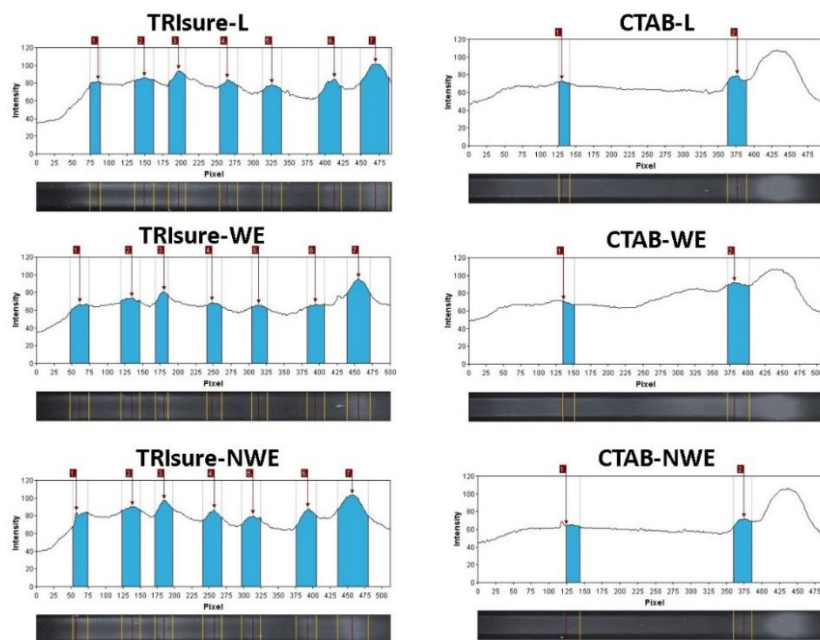

Figure 3 Results of electrophoretic analysis using the GelAnalyzer software

Note: Intensity axis: brightness level based on background; pixel axis: coordinate position on the electrophoresis membrane. The blue area shows the location of the marked RNA bands.

The results indicated that the concentration of total RNA in leaf tissue samples was greater than that in endosperm tissues, specifically in WE and NWE. This finding is consistent with those from previous studies (Iqbal, Yang, Qadri, Wu, Li, Shah, Hamayun & Hussain 2019, Iqbal, Yang, Wu, Li, Hamayun, Hussain & Shah 2020, Souza-Perera *et al.* 2018). Coconut endosperm tissue is hard and has a high lipid content, making it harder to grind than the leaf sample. Furthermore, endosperm is a significant component of seeds and comprises tissues that contain high levels of various polysaccharides (such as starch, cellulose, arabinoxylan, β-glucan, and fructan), proteins, lipids, and secondary metabolites (Angeles, Laurena & Tecson-Mendoza 2005, Ghangal, Raghuvanshi & Chand Sharma 2009, Zhang *et al.* 2019, Liu *et al.* 2018). Most of these components share structural similarities with nucleic acids, including polysaccharides that resemble the ribose sugar of RNA. Consequently, these compounds can bind to RNA and undergo degradation. Low RNA extraction yield was observed because of its encapsulation during centrifugation and its subsequent removal during phase separation using chloroform (White, Venter, Hiten & Burger 2008, Li *et al.* 2005, Wang *et al.* 2012). In addition, small polysaccharide molecules were distributed in the liquid phase during phase separation and co-precipitated with RNA, which reduced the RNA yield and affected subsequent applications (Liu, Han, Yu, Zhang, Xing, Xie & Peng 2018, Li & Trick 2005, Wang *et al.* 2010). Furthermore, a low A260/A230 ratio was detected, which indicated potential guanidine thiocyanate contamination as inferred from the warning messages from the meter. This component was present as a key element in the TRIsure mixture during the extraction procedure (Iqbal, Yang, Wu, Li, Hamayun, Hussain & Shah 2020).

Particularly, for coconut endosperm tissue, RNA extraction yielded mixed results in previous studies. The present study had some interesting findings. The concentration of the product extracted using TRIsure was approximately 157 ng/μL (corresponding to 47.1 μg/g), which was not as high as the QRREM results of Iqbal et al. (2019) (16.2 μg/80 mg corresponding to 202.5 μg/g) or the IRCM results of Iqbal *et al.* (2020) (150 μg/g). Moreover the results were not even comparable to those of the TRIzol method in the aforementioned studies (130 μg/g from Iqbal et al. 2019 and 145 μg/g from Iqbal *et al.* 2020). However, the product from CTAB-LiCL of approximately 142 ng/μL (equivalent to 42.6 μg/g) was significantly higher than the CTAB results of Iqbal *et al.* (2019) (1.25 μg/g) and Iqbal et al. (2020) (7.5 μg/g) and even higher than the results of Souza *et al.* (2018) (28.3 μg/g) in which CTAB was rated as the best reagent. However, in the end, the RNA concentration obtained from TRIsure was still higher than that from CTAB-LiCl in a statistically significant manner [*P* value 0.00 (<0.05)].

The integrity of the RNA fragments is important in assessing the quality of the total RNA obtained. Total RNA differs from DNA as it includes several types of RNA (rRNA, tRNA, mRNA, etc.) that vary in size. The size of rRNA in plants is usually fixed at 25S (3700 bp), 23S (2900 bp), 18S (1900 bp) and 16S (1500 bp) (Thermo Fisher 2006). In contrast, the size of mRNA varies widely because it depends on the length and specificity of the coding gene. Transcriptome sequencing focuses on mRNA molecules, which carry genetic information from the gene and encode functional proteins. mRNA sequencing provides valuable insights into the expressed functional gene and its expression level, which may be significantly lower than that of rRNA. Identifying specific mRNA bands on electrophoresis is challenging owing to variations in size and expression levels. Relative estimates of band quantity and tape compactness may indicate the presence of more intact RNAs and fewer fragmented RNAs (Wang *et al.* 2009, Kukurba *et al.* 2015).

The results demonstrated that the TRIsure method yielded superior electrophoresis and concentration outcomes than the CTAB-LiCl method. Furthermore, the TRIsure procedure required a total extraction time of merely 1 h, which was significantly shorter than the 15 h required for the other methods. This step aimed to determine the relative efficiency of the endosperm tissue compared with the leaf samples, rather than to enhance the extraction efficiency. Therefore, in the next step, the TRIsure method was employed to optimise the extraction of RNA from WE tissue as it offered superior RNA integrity and can potentially overcome the limitations of the current methods.

Improving purity valuethe A260/230 ratio, an indicator of purity, in RNA extraction process with TRIsure

During the extraction of plant tissue samples, A260/230 values are often low owing to the presence of polysaccharides (Wang & Stegemann 2010, Orek 2018). To overcome this problem, nucleotide lysis buffers can be supplemented with NaCl and β-mercaptoethanol to limit the amount of polysaccharides remaining in the sample. NaCl effectively removes polysaccharides during chloroform cleavage (Chang, Puryear & Cairney 1993), and β-mercaptoethanol is a potent reducing reagent that aids in denaturing RNase (Chang, Puryear & Cairney 1993, Mommaerts *et al.* 2015) thereby preventing undesired RNA degradation.

In this study, 0.1 mL of 2M NaCl and 1% β-mercaptoethanol were added simultaneously to the TRIsure buffer, as recommended by Chang *et al.* (1993). The amount of chloroform was increased to a ratio of 1:1 (v/v) for the sample aliquot obtained in the phase separation step. The RNA washing step with 75% alcohol was repeated thrice to eliminate any residual guanidine thiocyanate in the sample. Subsequently, only WE and NWE samples were utilised, with no further inclusion of leaf samples.

The results of statistical analysis using ANOVA signified no difference in the RNA concentration (*P* value 0.067), A260/280 (*P* value 0.124). Furthermore, the A260/230 value was significantly improved when using TRIsure supplemented with NaCl + β-mercaptoethanol (1.58 ± 0.02) compared with the use of TRIsure only (1.01 ± 0.03) [*P* value 0.000 (<0.05)] (Table 4).

RNA quality was measured using the absorption spectrometric ratios of the components present in the sample: pure RNA (Amax = 260 nm), proteins (Amax = 280 nm) and polysaccharides (Amax = 230 nm) (Iandolino *et al.* 2004). The addition of NaCl and the strong reducing agent β-mercaptoethanol to the extraction buffer enhanced the solubility of polysaccharides, decreased their co-precipitation with RNA in later steps and denatured ribonucleases and proteins. Other contaminants were reduced during extraction (Iandolino, Goes Da Silva, Lim, Choi, Williams & Cook 2004, Lodhi *et al.* 1994, Fang *et al.* 1992), thereby improving the purity ratio A260/230.

Testing of the sample incubation temperature atduring the lysis step for improvingto improve RNA concentration

Following the extraction with TRIsure as recommended by the manufacturer of Bioline, the lysate was incubated at room temperature for 5 min. However, coconut endosperm tissues are often hard and contain several lipids. Hence, the grinding process is more difficult than that of the leaf tissue, leading to lower extraction efficiency. In the CTAB method, the sample is usually incubated at a higher temperature (approximately 65°C) to augment the activity of the tissue-degrading enzyme. Therefore, this study tested the effect of increased incubation temperature (50°C, 55°C and 60°C) on the lysis of WE samples for 15 min to determine whether temperature affects the ability to obtain total RNA from coconut endothelial cells in the TRIsure reaction.

The results of ANOVA showed that the WE sample had total RNA concentrations of 157.67 ± 0.57, 156.67 ± 2, 157.00 ± 1 and 156.67 ± 2.5 ng/μL (Table 5), which corresponded to the incubation temperatures of room temperature (approximately 25°C–28°C), 50°C, 55°C and 60°C. However, there was no difference in yield

Table 4 Investigation of the improvement in A260/230 ratio when adding NaCl and β-mercaptoethanol in the lysis step

Buffer	Concentration	A260/280	A260/230
TRIsure	157.33 ± 0.5 [a]	1.99 ± 0.01 [a]	1.01 ± 0.03 [a]
TRIsure + NaCl + β-mercaptoethanol	159.00 ± 1 [a]	1.94 ± 0.04 [a]	1.58 ± 0.02 [b]
P value	0.067 (>0.05)	0.124 (>0.05)	0.000 (<0.05)

Note: One-way ANOVA was used to investigate the influence of the two extraction methods (original TRIsure vs modified TRIsure) on the A260/A230 ratio of the RNA product from the endosperm. The lowercase letters a and b represent the difference in the extraction methods. The differences are significant when the P value is <0.05.

among the investigated temperatures (*P* value 0.875). The OD results were also not different. The A260/A280 ratio was in the range of 1.8–2.0, and the 260/A230 ratio was in the range of 1.58–1.6. The electrophoresis results of the three treatments were also not different (Figure 4). These results revealed that increasing the sample incubation temperature did not increase the amount of total RNA obtained in the TRIsure procedure.

Testing the effectiveness of RNA preservation during transport

In many cases, the sample needs to be sent elsewhere for sequencing, which can take several days to a week, which affects RNA quality. To minimise RNA loss during transport, tests were performed using two preservation methods: DEPC solubilisation and absolute alcohol precipitation. The sample was kept chilled in a Styrofoam container filled with gel ice. The temperature of the container was maintained between 8°C and 10°C, which is similar to the transportation condition. RNA quality during this preservation process was evaluated on a weekly basis using electrophoretic analysis.

After 1 week, in samples that were stored in DEPC and precipitated with absolute alcohol, the electrophoresis bands showed minimal changes. However, from the 2nd week, the electrophoresis band of the RNA product precipitated with DEPC began to become more blurred, and by the 4th week, it was completely blurred and had several streaks (Figure 5). On the contrary, the electrophoresis band of the RNA stored in absolute alcohol did not show many changes compared with the initial time; nonetheless, it began to fade after the 3rd week. The results showed that storage in absolute alcohol is more optimal for RNA preservation than that in DEPC, which could be maintained for up to 3 weeks.

Table 5 Investigation of the possibility of improving the A260/230 ratio when increasing the annealing temperature in the lysis step

Annealing temperature	Concentration	A260/280	A260/230
Room temperature (25°C–28°C)	157.67 ± 0.57 [a]	1.98 ± 0.02 [a]	1.6 ± 0.01 [a]
50°C	156.67 ± 2 [a]	2.00 ± 0.03 [a]	1.58 ± 0.01 [a]
55°C	157.00 ± 1[a]	2.01 ± 0.01 [a]	1.6 ± 0.001 [a]
60°C	156.67 ± 2.5 [a]	1.98 ± 0.02 [a]	1.6 ± 0.02 [a]
P value	0.875 (>0.05)	0.281 (>0.05)	0.205 (>0.05)

Note: One-way ANOVA was used to investigate the influence of four different temperatures in the lysis step on the A260/A230 ratio of the RNA product from the endosperm. The lowercase letter a represents the difference between the screening temperatures. P value >0.05 shows lack of statistical significance.

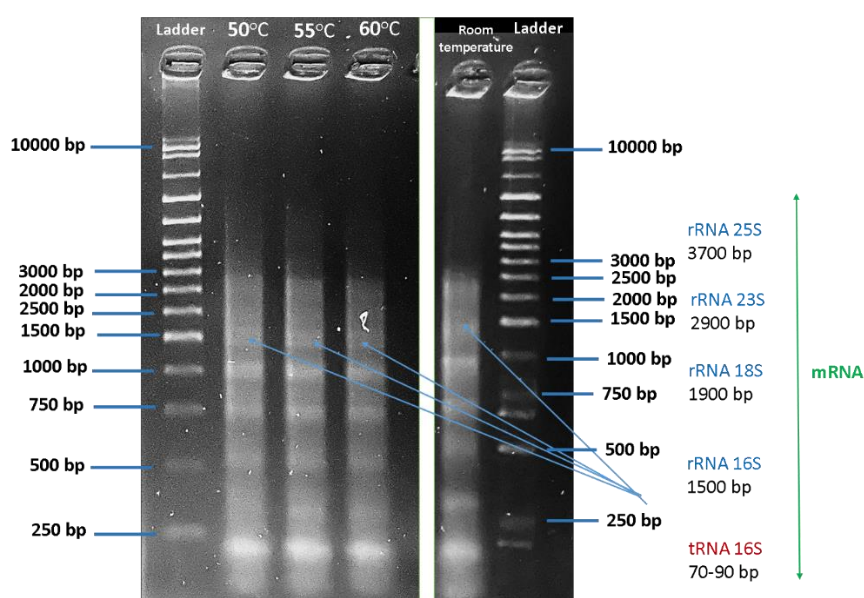

Figure 4 Results of electrophoresis of RNA extracted from WE samples at different temperatures

After RNA extraction, its preservation is crucial. Because RNA is rapidly influenced by temperature and nucleases in the media, it becomes unstable following extraction. This instability could be attributed to the fact that the RNA is often single-stranded and that the hydroxyl group renders the structure unstable, making the nucleotides prone to hydrolysis and destruction (Fordyce *et al.* 2013). Moreover, samples are often sent to sequencing firms located abroad, leading to extended transit times. Unfortunately, this prolonged transportation can immensely affect the quality of the RNA. In such circumstances, precipitation with pure alcohol is a technique that can help prolong the preservation of RNA. Unlike DNA, RNA in its single-stranded structure is inherently more challenging to preserve over an extended period. By precipitating RNA with 99.5% alcohol, the RNA structure condenses, strengthening the bonds and eliminating the enzymes that catalyse hydrolysis reactions. Consequently, RNA becomes significantly more resilient, facilitating its storage, transportation and utilisation for further research.

CONCLUSION

The concentration and purity of RNA, as reflected by the 260/280 ratio, is of utmost concern in next-generation sequencing. Even some sequencing companies now have strict requirements in terms of quality and concentration. This is because prior to sequencing, the extract will be purified so that the product is of the desired purity, but the concentration will be lost. Concentration is also significantly lost during transportation if the time is too long. Therefore, improving the concentration of the extracted RNA is essential. We recommend the use of TRIsure in combination with NaCl and β-mercaptoethanol for efficient RNA extraction instead of the CTAB-LiCl method. Additionally, RNA could withstand temperatures of 8°C–10°C for 3 weeks when stored in a 99.5% ethanol solution, which helps to reduce its degradation when shipping samples over a long distance for an extended period prior to sequencing.

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