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Protocols for the Extraction of High-quality RNA from Pineapple Tiller, Flower, Inflorescence, and Fruits

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ABSTRACT

High-quality RNA is an important genetic study as it has minimal contaminants that can affect gene discovery including degraded RNAs, chemical, and biological residues. Hence, it is a prerequisite for genetic analysis using Next Generation Sequencing (NGS) for accurate and reliable data mining. Despite its importance, extracting high-quality RNA from different samples is often a challenge, as every tissue has a different biochemical composition, thus requiring different protocols. This paper reports protocols for the extraction of high-quality RNA from two type of pineapple tissues, which are thickly lignified hard tissue (tillers, inflorescence, flowers) and watery soft tissue (mature fruit, ripe fruit, and overripe fruit) via modified Kim and Hamada (2005) method. Total RNA was extracted in all six tissues, which showed two distinctive 25S and 18S band on agarose gel. The total RNA in this study was considered high-quality as the minimum concentration was 50 ng/µl, the absorbance ratio (A_{260}:A_{280}) was more than 1.8 and RNA integrity number (RIN) was greater than 7. The obtained results showed that the modified Kim and Hamada (2005) method was effective in extracting high-quality RNA from the challenging MD2 pineapple tissue, which is suitable for subsequent molecular analysis, including the highly sensitive NGS.

Keywords: MD2 pineapple, Next Generation Sequencing, RIN number, RNA extraction

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INTRODUCTION

Next-Generation Sequencing (NGS) is one of the most utilised platforms providing reliable large-scale data within a short time. It facilitates the research of various plant mechanisms and system (Godden et al., 2012; Soltis et al., 2013). Indeed, numerous molecules including DNA, mRNA, small RNA, degraded RNA, long non-coding RNA, exons, and DNA fragments are now being sequenced using various NGS platforms. In the highly sensitive RNA-sequencing or transcriptomic analysis, the quality of the data obtained from these NGS platforms ultimately depends on the quality of the starting materials. However, obtaining high-quality RNA from plants that meets requirement for RNA-sequencing remains a challenge.

Many studies have demonstrated the successful isolation of total RNA from biological samples including human, animal, plant, bacteria, fungus, and viruses (Cox, 1968; Holmes et al., 2014; Kałużna et al., 2016). RNA extraction is the process of isolating purified total RNA from the living source samples with minimal degradation and contamination. However, no standardized protocol can be used due to the different composition of various samples that demand different extraction protocols, especially in high secondary metabolite samples as phenolic compounds readily oxidized to form quinones which bind to nucleic acids, preventing extraction of high-quality RNA (Ghangal et al., 2009). Therefore, the extraction protocol should be modified depending on the sample for the extraction of high-quality RNA (Atshan et al., 2012; D. Li et al., 2009; Liu et al., 2018).

Pineapple (*Ananas comosus*) is an important commercial fruit (Hossain et al., 2015), as well as a model organism for non-climacteric ripening, rich in polysaccharides, polyphenols, water content, lignin, and fibre (Liu et al., 2018). This study reports a protocol suitable for the extraction of high-quality RNA from six pineapple tissues including the tiller, inflorescent, flower, mature fruit, ripe fruit, and overripe fruit. The protocol was modified from Kim and Hamada (2005), which reported DNA and RNA extraction method in sweet potato. The method was equipped to extract total RNA from several tissues of MD2 pineapple which was unsuccessful perhaps due to different tissue composition, resulting in few modifications to obtain high-quality RNA suitable for RNA-based analyses using various platforms utilising NGS technology. Total RNA can be deemed as high-quality if it fits the minimum criteria of NGS requirement which are (a) minimum concentration of 50 ng/µl, (b) absorbance ratio (A_{260}:A_{280}) of more than 1.8, and (c) RNA integrity number (RIN) value greater than 7. In addition, total RNA of high quality can be used for different analytical techniques, including reverse transcription polymerase chain reaction (RT-PCR), real-time fluorescent quantitation polymerase chain reaction (qPCR), and microarray analysis (Ma et al., 2015).

MATERIALS AND METHODS

Plant Materials and Apparatus Treatment

Six MD2 pineapple tissues were used in this study and three of them were hard tissues collected from the tiller, inflorescence, and flower, while the other three were soft tissues collected from mature fruit (fruit at ripening index 1), ripe fruit (fruit at ripening index 4), and overripe fruit (fruit at ripening index 7). The level of maturity of the fruits are measured based on the colour of the skin of the pineapple (Bakar et al., 2013). The samples were collected as per Sabah Department of Agriculture guidance at a farm located in Kampong Poring, Inanam, Sabah, Malaysia, in which the tissues were transported in clean plastic bag. Upon arrival, each tissue was rinsed using sterile distilled water and cut into smaller pieces before placing inside clean sterile plastic. The tissues were then stored at -80°C before extraction. RNAse pre-treatment of apparatus and plastic ware was also done by dipping in diethyl pyrocarbonate (DEPC) solution overnight, before sterilizing via autoclave.

Extraction of Total RNA from Hard and Soft Tissue

Hard Tissues (Tillers, Inflorescence, and Flowers). Total RNA from hard tissues including tillers, inflorescence, and flowers was extracted using a protocol modified from Kim and Hamada (2005). RNA was extracted from approximately 3 g of the frozen sample which was powdered using a chilled mortar and pestle. The samples were transferred into a fresh tube containing 3 mL of lysis buffer consisting of 1.4 M NaCl, 20 mM EDTA, 100 mM Tris (pH 8.0), 2% CTAB, and 2% β-mercaptoethanol, then half volume (v/v) of 6 M sodium chloride (saturated NaCl) was added and incubated at 65°C for 10 minutes. The samples were centrifuged at 16,600 x *g* for 12 minutes at 4°C to collect the supernatants, which were then mixed with an equal volume of phenol: chloroform: isoamyl alcohol (125:24:1; pH~4.5). The samples were centrifuged again at 16,600 x *g* for 12 minutes at 4°C to collect the uppermost aqueous layer, which was transferred into a fresh tube before adding an equal volume of chloroform: isoamyl alcohol (24:1) and centrifuged at 16,600 x *g* for 12 minutes at 4°C. The uppermost aqueous layer was again collected, mixed with an equal volume of absolute ethanol and incubated overnight at -20°C. The RNA precipitate was centrifuged at 16,600 x *g* for 12 minutes at 4°C, the supernatant was discarded, and the RNA pellet obtained was washed twice with 70% ethanol. The RNA pellet was air-dried in a vacuum concentrator before resuspension in 30 µL DEPC-treated water.

Soft Tissues (Mature, Ripe, and Overripe Fruits). For the extraction of total RNA from soft tissues including mature, ripe, and overripe fruits, the same protocol for the extraction of hard tissues was used with minor modifications. First,

the extraction was performed using an extraction buffer containing 400 mM NaCl, 20 mM EDTA, 10 mM Tris-HCl, 1% SDS, and 2% β-mercaptoethanol. Secondly, the soft tissue samples were not incubated at 65°C for 10 minutes.

Modification from Kim and Hamada Protocol

The pineapple tissues were extracted using a protocol modified from Kim and Hamada (2005), from now on referred to as the KH protocol. The five major procedures performed for the extraction of soft and hard tissues of pineapple are as follows:

a. The initial buffer in the KH protocol contained 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris/HCl (pH 9.5), 2% CTAB, and 1% β-mercaptoethanol. This was modified to contain 2% β-mercaptoethanol and Tris/HCl was replaced with Tris (pH 8.0) for the extraction of hard tissues and soft tissues, and 2% CTAB was replaced with 1% SDS for extraction of soft tissues.

b. Instead of adding saturated LiCl (10 M) after the addition of the lysis buffer in the KH protocol, saturated NaCl (6 M) was added for the extraction of hard and soft tissues.

c. The incubation step at 65°C for 10 minutes was omitted for the extraction of soft tissue samples.

d. The KH protocol involved two extractions using a C:I (24:1) mixture, in this study, the first extraction was performed using P:C:I (25:24:1), while C:I (24:1) was used for the second extraction of hard and soft tissues.

e. The RNA samples of the pineapple tissues were precipitated with absolute ethanol rather than LiCl (10 M) in this study.

RNA Concentration, Purity, and Integrity Analyses

Total RNA for each sample was visualised by 1.2% agarose gel electrophoresis with 1X TAE buffer. The total RNA concentration and purity (absorbance A_{260}:A_{280}) were quantified using a NanoDrop™ Spectrophotometer. The RIN was then calculated using an Agilent 2100 Bioanalyzer system and Agilent RNA 6000 Pico Kit.

RESULTS

The six pineapple tissues, namely tillers, inflorescent, flower, mature fruit, ripen fruit, and overripe fruit (Figure 1) were first categorised as hard or soft tissues. Hard tissues (tillers, inflorescence, and flowers) typically are composed of a thick lignified wall (Yahia & Carrillo-López, 2018), while soft tissues (fruit) contain 80% water (Cordenunsi et al., 2010).

The presence of 25S and 18S ribosomal RNA (rRNA) was observed in the RNA extracted from all tissues using the modified protocols (Figure 2) and was consistent for all replicate samples (n = 3). The quality of the extracted RNA was verified using an automated gel electrophoresis system, demonstrating a RIN ranging from 6.3 to 9.5, with a total RNA concentration between

134.3 ng/µl to 1074.6 ng/µl (Figure 3). The gel images also confirmed the RNA integrity. The concentration, purity ratio and RIN for each tissue with respective biological replicate are summarized in Table 1.

Table 1

Concentration, purity ratio and RIN value for each pineapple tissues including biological replicate

Tissue	Biological replicate	Concentration (ng/µl)	A260/280	RIN no.
Tiller	1	442.6	2.01	7.7
	2	308.8	2.12	9.5
	3	338.2	2.13	7.7
Inflorescence	1	678.6	2.05	8.2
	2	528.1	2.08	8.7
	3	673.1	2.08	7.3
Flower	1	1074.6	2.16	7.2
	2	709.3	2.08	7.6
	3	449.8	2.08	7.2
Mature fruit (Fruit at ripening index 1)	1	309.7	2.11	7.4
	2	137.7	1.87	7.2
	3	533.0	2.03	7.8
Ripe fruit (Fruit at ripening index 4)	1	434.5	2.01	7.8
	2	134.3	2.09	7.1
	3	495.1	2.05	9.0
Overripe fruit (Fruit at ripening index 7)	1	243.7	1.96	7.9
	2	200.6	2.12	9.1
	3	205.4	2.13	8.3

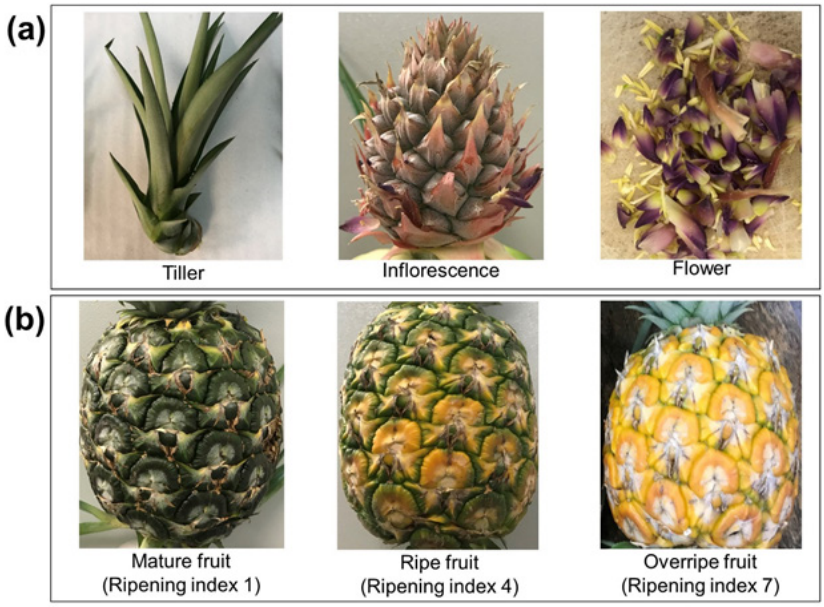

Figure 1. Samples of MD2 pineapple used for RNA extraction. (a) Hard tissue samples of tillers, inflorescence, and flowers; (b) soft tissue samples of mature fruit (at ripening index 1), ripe fruit (at ripening index 4), and overripe fruit (at ripening index 7)

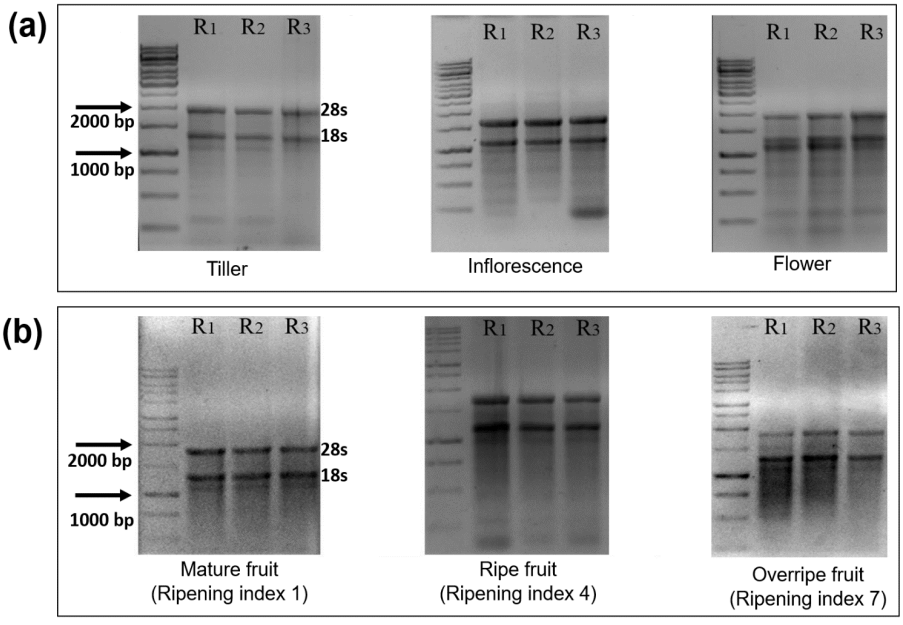

Figure 2. Presence of intact 25S and 18S rRNA in the RNA extracted from pineapple tissues with three biological replicates (R1, R2, and R3), run with '0' GeneRuler™ 1kb DNA ladder. (a) Hard tissue samples of tillers, inflorescence, and flowers; (b) soft tissue samples of mature fruit (at ripening index 1), ripe fruit (at ripening index 4), and overripe fruit (at ripening index 7)

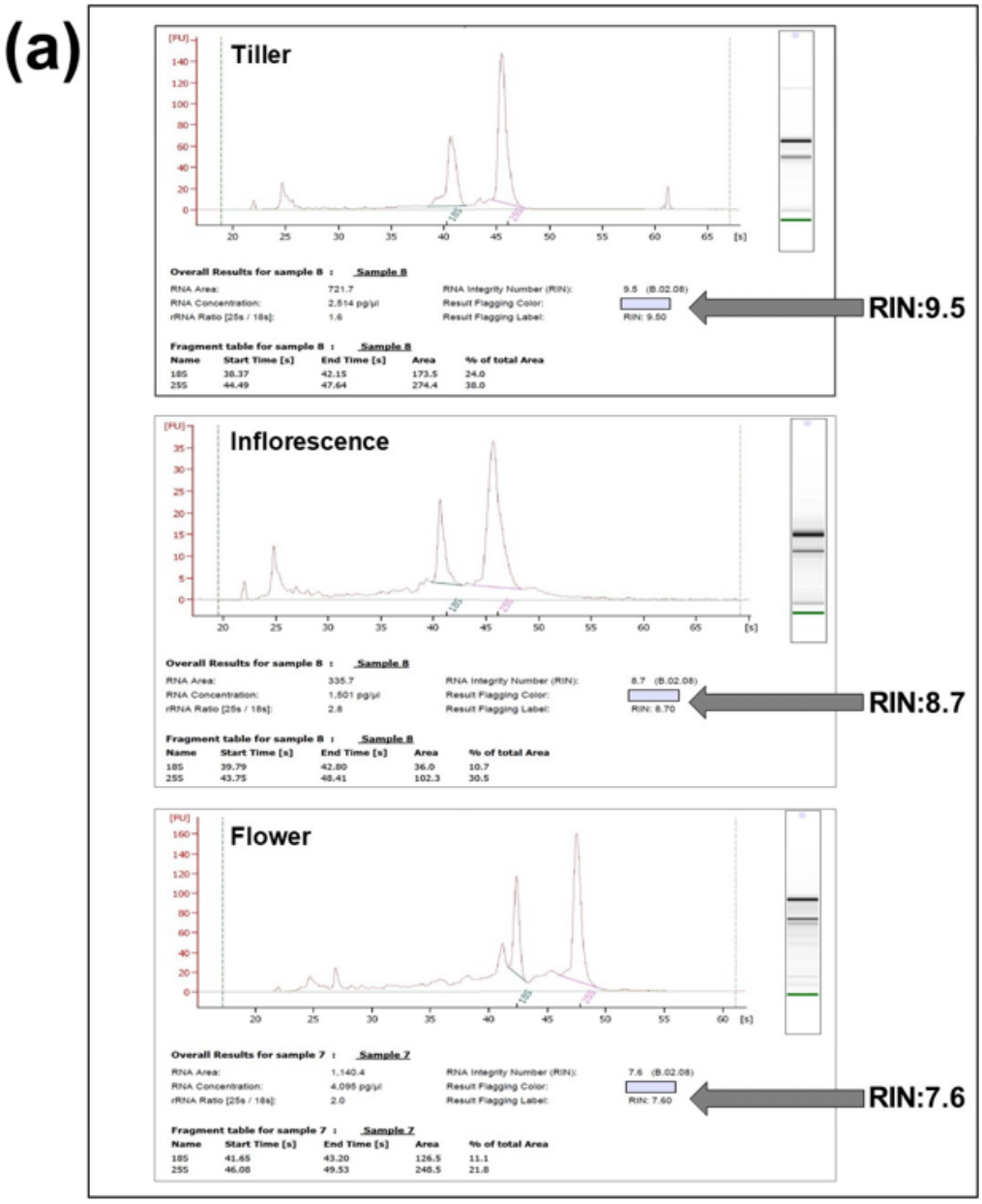

Figure 3. Profile image of intact 25S rRNA, 18S rRNA, and RIN from extracted RNA from (a) Hard tissue samples of tillers, inflorescence, and flowers; (b) soft tissue samples of mature fruit (at ripening index 1), ripe fruit (at ripening index 4), and overripe fruit (at ripening index 7)

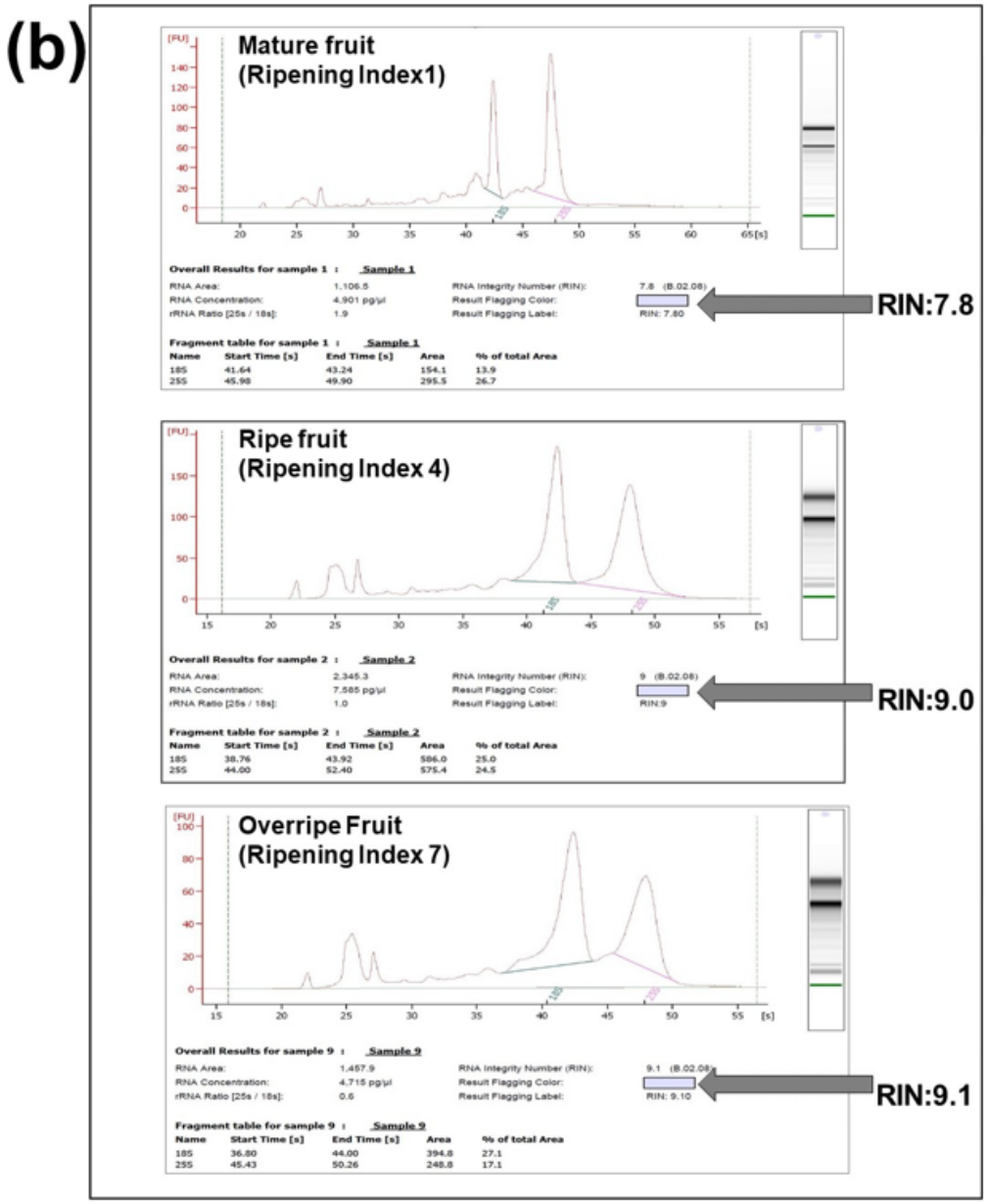

Figure 3. Profile image of intact 25S rRNA, 18S rRNA, and RIN from extracted RNA from (b) soft tissue samples of mature fruit (at ripening index 1), ripe fruit (at ripening index 4), and overripe fruit (at ripening index 7)

DISCUSSION

This study reported protocols for the extraction of total RNA from six pineapple tissues, namely hard tissue samples of the tiller, inflorescence, and flower, as well as soft tissue samples of mature fruit (fruit at ripening index 1), ripe fruit (fruit at ripening index 4), and overripe fruit (fruit at ripening index 7). The KH extraction protocol was first developed to extract RNA from sweet potato (*Ipomoea batatas* (L). Lam.) tissues including the fibrous roots, tuberous roots, flowers, leaves, stems, petioles, thick pigmented roots, and the mature resting tubers (Kim & Hamada, 2005). Hence, it was considered suitable for the extraction of RNA from various pineapple tissues.

The protocol was modified to successfully extract high-quality RNA from both hard and soft tissues of pineapple. β-mercaptoethanol is a strong denaturant commonly used in extraction buffer, with volumes reaching as high as 5% reported (Wang et al., 2010; White et al., 2008). The β-mercaptoethanol concentration in the extraction buffer used in this study was increased from 1% to 2%, thus postulated to increase the rate of RNAse degradation and inhibit its activity, in turn ensuring RNA stability (Azmat et al., 2012; Wang et al., 2010).

Plant tissues contain high levels of polysaccharides, which physiochemically can interact with RNA, thus co-precipitate with RNA, which in turn affects the quality of the RNA. Saturated sodium chloride was used in this study to increase the solubility of polysaccharides, thus reducing the rate of co-precipitation with RNA (Tel-Zur et al., 1999; Wong et al., 2014; X. Li et al., 2011).

The use of CTAB has been reported to be more suitable than SDS for the RNA extraction of samples with leafy-structures including the hard tissues of pineapple (Jordon-Thaden et al., 2015; White et al., 2008; Wong et al., 2014; Yu et al., 2012). As such, CTAB helps to break the thick lignified cell walls of the tissues, separating nucleic acids from polysaccharides (Chaparro-Encinas et al., 2020; Jaakola et al. 2001; Jordon-Thaden et al., 2015). The extraction of high-quality RNA using CTAB from soft pineapple tissues was also done but was found to be unsuitable in this study; hence SDS was used to obtain total RNA. The main factor may be due to the tissue compositions as hard tissues are thickly lignified, whereby the soft pineapple tissues have more water content (85%) and usually are more acidic (pH ~3.5), making the use of SDS more suitable in soft pineapple tissues (Shamsudin et al., 2007). Despite SDS being suitable to extract RNA for soft tissue in this study; however there was no clear correlation between CTAB suitability in hard tissue and SDS in soft tissue as many other protocols have used SDS to extract RNA from hard tissue (Hou et al., 2011; Huded et al., 2018; Ramimoghadam et al., 2012). The incubation at 65°C for extraction was suitable for all hard tissues as it is postulated to increase the efficiency of cell wall degradation.

Phenol was added to the mixture of chloroform and isoamyl alcohol as it provides several advantages in the

process of RNA separation from other contaminants including DNA, proteins, lipids, carbohydrates, and cell debris. Generally, the addition of chloroform alone into a solution that contains RNA forms a biphasic emulsion separating contaminants from total RNA, with the upper layer of the emulsion (the hydrophilic layer) containing the nucleic acids, while the lower layer (the hydrophobic layer) contains other contaminants (Tan & Yiap, 2009). Thus, the use of chloroform alone may not be sufficient to extract RNA without DNA contamination. As such, the acidic conditions developed with the addition of phenol causes the RNA to remain in the upper layer, with most DNA and proteins remaining in the interphase or lower layer (Chomczynski & Sacchi, 2006; Maes & Messens, 1992). Because of this, this study also used acid phenol for separating RNA from contaminants including DNA. On the other hand, the presence of isoamyl alcohol helps to continuously inhibit RNAse activity maintaining RNA stability (Tüzmen et al., 2018). Ethanol is volatile, it is useful in the preparation of samples for NGS purposes, to ensure that the sample is free from chemical residues, as compared to LiCl (Hopkins et al., 2009).

Based on the modification of Kim and Hamada (2005) method, total RNA was successfully extracted from pineapple tissues of different compositions (soft and hard tissues). Total RNA was present in each tissue as seen on Figure 2 with two distinctive band of 25S and 18S representing intact total RNA. Further assays via Nanodrop™ Spectrophotometer (Table 1) and Agilent Bioanalyzer (Figure 3) show total RNA extracted having concentration of more than 50 ng/µl, absorbance ratio (A260:A280) of more than 1.8 and RIN value of more than 7, which are deemed acceptable for RNA sequencing via NGS platform (Kukurba & Montgomery, 2015; Sheng et al., 2017).

CONCLUSION

High-quality RNA was successfully extracted using modified Kim and Hamada (2005) protocol which can be used on tissue with thick lignified cell wall (hard tissues) as well as high water content tissues (soft tissues) of pineapple. The high-quality total RNA reaching minimum concentration, absorbance ratio, and RIN value are applicable for various analytical techniques like reverse transcription polymerase chain reaction (RT-PCR), real-time fluorescent quantitation polymerase chain reaction (qPCR), microarray analysis, and RNA-sequencing via NGS platforms.

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