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Research Article

An Improved Cost-Effective Method of RNA Extraction from Aquilaria malaccensis

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Abstract

Aquilaria malaccensis produces one of the costliest woods known as agarwood in its stem and trunk, which is used in perfume industry and traditional medicines. Agarwood contains abundant polysaccharides and secondary metabolites that make RNA isolation arduous. In this study, we have compared six different protocols for RNA extraction from wood tissues of *A. malaccensis*. The efficiency of each method was evaluated by RNA integrity, yield, and purity. Low yield and poor RNA quality was the key problems faced while using other common procedures which hindered downstream processes like cDNA preparation and quantitative real-time polymerase chain reaction (qRT-PCR). Commercial kits though available and have been used are found to be costly and did not give good quality RNA with agarwood. We have illustrated an abridged and proficient RNA extraction protocol that combines cetyltrimethyl ammonium bromide and Polyvinylpyrrolidone with sequential extraction of chloroform followed by selective salt precipitation, which can persistently isolate good quality RNA from wood tissues of *A. malaccensis*. The extracted RNA was used in downstream applications like cDNA library construction, qRT-PCR, which requires high-quality RNA. Our modified protocol exhibited a prodigious enhancement in purity, yield, and integrity of RNA. This modified protocol renders a new insight, which can be used in different wood tissues with enormously high levels of polysaccharides and polyphenols.

Keywords: Aquilaria malaccensis; Agarwood; Extraction; Isolation; Protocol; RNA

Abbreviations

CITES: Convention on International Trade in Endangered Species of Wild Fauna and Flora; CTAB: Cetyltrimethylammonium Bromide; Ct: Cycle Thresholds; DEPC: Diethylpyrocarbonate; LN_2: Liquid Nitrogen; PVP: Polyvinylpyrrolidone; qRT-PCR: Quantitative Real-Time Polymerase Chain Reaction

Introduction

Agarwood generates in woody tissues of *Aquilaria* and *Gyrinops* plant species in response to either microbial infection or mechanical wounding. Due to high commercial value and high demand, natural *Aquilaria* forests have been rigorously demolished in nearly all the countries where agarwood has been commercially exploited for perfume production, and because of this high demand for the agarwood products all *Aquilaria* spp. being listed in the Appendix II of the CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora). To understand the mechanism of agarwood formation a lot of experiments have been undertaken particularly for deciphering the agarwood induction mechanism and for elucidating the chemical composition of the induced agarwood [1-5]. Because of its emerging significance, Agarwood is a goal forest plant in South East Asia now for gene expression and transcriptome study designed at altering terpenoid biosynthetic pathway and determination of transcripts responsible for imparting characteristic aroma to agarwood. To decrypt these mechanisms significantly pure and high-quality RNA required for processes like molecular cloning, qRT-PCR, cDNA library construction, and RNA interference.

Although there are several RNA extraction methods available nowadays but most of the methods are tissue or species-specific [6] which makes it challenging to get high-quality RNA particularly for woody plant. High-quality RNA extraction is difficult especially from wood and other tissue types of plant. The major problems in RNA extraction from woody plants are the existence of bulk amount of polysaccharides that precipitate with RNA; a number of

phenolic compounds, containing tannins, huge contents of RNases; less nucleic acids concentrations and the presence of lignin which makes it difficult to lyse the cell.

With boom in the fragrance industry, *Aquilaria* species enjoys a privileged position and has attacked importance from research community. Over 150 classes of compound have been known to date [7], largely sesquiterpenoids, phenylethyl chromones, and volatile aromatic groups as well as the regular aldehydes, ketones, phenols and alkaloids groups, in agarwood. *Aquilaria* species even in a non-agarwood producing stem comprises a huge quantity of compounds such as benzaldehydes, alkaloids, terpenoids, phenols, and several other compounds [8]. Presence of these complexed compounds creates a big problem for high-quality RNA extracting from wood tissue.

In recent years, several methods, in conjunction with their alterations, have been utilized throughout the years for nucleic acid (RNA) isolation from *Aquilaria* species. Kumeta and Ito [1] extracted RNA from callus culture of *Aquilaria* spp. Most of the RNA isolation experiments conducted till now have used commercial plant RNA extraction kits (RNeasy plant mini kit Qiagen Germany, Favor PrepTM Plant total RNA purification mini kit Favorgen, USA, Norgen total RNA extraction kit Biocat Germany etc.) obtainable from life science or Biotech companies, these kits are commonly manufactured and optimized for leaf samples. Chomtong., *et al*. [9] used Favor PrepTM Plant total RNA purification mini kit (Favorgen, USA) to extract total RNA from stem tissues of *Aquilaria crassna*. Gao., *et al*. [5] used Norgen total RNA extraction kit (Biocat Germany) to extract RNA from induced callus tissue of *Aquilaria sinensis*. Xu., *et al*. [10] used TRizol kit (Invitrogen, USA) to isolate total RNA from the wounded and Agarwood tissue of *A. sinensis* for transcriptome analysis. Use of total RNA Purification Kit (Aidlab, China) to extract total RNA from callus tissue for molecular cloning was carried out by Xu., *et al*. [11]. Isolation of total RNA from *A. sinensis* calli using a Tiangen RNA extraction kit (RNA prep pure Plant Kit, Tiangen Biotech Beijing Co., Ltd. China) was reported by Liao., *et al* [12]. Extraction of total RNA using extraction kit (Norgen, Biocat Germany) from callus tissue of *A. sinensis* was reported by Wang., *et al* [13]. Extraction of RNA by using the modified guanidinium isothiocyanate-Cetyltrimethylammonium bromide (CTAB) method from induced agarwood was reported by Ye., *et al* [14]. Kenmotsu., *et al*. [15] and Chen., *et al*. [16] used RNeasy Plant Mini Kit (Qiagen, Germany) to extract RNA from induced callus tissue of *Aquilaria agallocha* and *Aquilaria microcarpa* respectively, for draft genome assembly. Earlier, Siah., *et al*. [17] reported use of RNeasy plant mini kit (Qiagen, Germany) with some modifications to be the most suitable method for extracting RNA from *A. malaccensis* wood tissue, but unfortunately this protocol did not work in our experiment, this may be because of the different chemical composition of the samples we used in our experiment. The protocol presented herein is adopted from Lorenz., *et al*. [18], we report here the modifications of Lorenz protocol carried out for RNA extraction from healthy wood and Agarwood (infected wood) of *A. malaccensis*. For comparative purpose, an established protocol CTAB based RNA extraction method of Yang., *et al*. [19], second a protocol reported by Tao., *et al*. [20], third method by Lorenz., *et al*. [18] reported for Loblolly Pine (*Pinus taeda* L.), along with two commercially available extraction kits RNeasy plant mini kit (Qiagen, Germany) and Nucleospin RNA plant protocol (MN, Germany). The protocol describe here uses an extraction buffer comprising polyvinyl pyrrolidone (PVP) and β-mercaptoethanol. PVP inhibits browning effect of polyphenols and eliminates phenolic compounds and secondary metabolites from nucleic acid preparations. B-mercaptoethanol, a reducing agent, irrevocably denatures RNases by reducing disulfide bonds and abolishing the natural conformation, which is essential for the enzyme roles. The objective of this experiment was to establish a simple, low cost, and efficient RNA extraction protocol for *A. malaccensis* with good quality, high purity, integrity, and higher yield by modifying the method established by Lorenz., *et al*. [18] for subsequent applications.

Methods and Materials

Plant materials

Three mature *A. malaccensis* tree were identified for wood tissue collection from Naharani (N 27º 08' 13'', E 94º 49' 12''), Assam, and India. The trees selected are ones with agarwood formation taking place naturally. The presence of darkened agarwood was confirmed by drilling into the trunk and from the odor of agarwood. Two different categories of wood tissue were harvested a) Agarwood (infected wood), b) Healthy wood (non-infected wood) located around the drill site. Small slice (approximately 2 to 4 cm size) of wood was collected in a labeled cryo container by wood chisel and immediately glaciated in liquid nitrogen. The frozen wood tissues were transported to the laboratory and kept in -80°C for further use.

Pretreatment

All the glassware's and mortar pestle used in the RNA extraction procedure were treated overnight with 0.1% (v/v) diethylpyrocarbonate (DEPC) in room temperature (RT) and then oven baked at 200°C for 6 hours. Plastic wares (50 ml polypropylene tube, 15 ml polypropylene tube, 2 ml microfuge tube, and 1.5 ml microfuge tube and micro tips) were dipped in 0.1% (v/v) DEPC

treated water at room temperature for night long and autoclaved at 121ºC for 15 minutes and oven dried at 80ºC for 2 hours. All the solutions except Tris-HCl (pH 8.0) were prepared with 0.1% (v/v) DEPC treated and double autoclaved water. Tris-HCl (pH 8.0) was prepared in Millipore water.

RNA isolation protocols

In this study, six different methods of RNA extraction were tested. In every method tested, the woody tissues were pulverized into a fine residue in a pre-chilled mortar pestle using liquid nitrogen.

A CTAB based method [19] was used as one of the first protocol in our experiment. Two gram wood tissue was grinded and the fine powder was transferred to 18 ml extraction buffer containing 2% (w/v) CTAB, 1.4M NaCl, 0.1M Tris-HCl (pH 8), 20 mM EDTA (pH 8), 2% (w/v) PVP and 2% β mercaptoethanol. Then aqueous phase was extracted using chloroform, followed by phenol: chloroform (1:1) and finally using chloroform: isoamyl alcohol (24:1). Phenol-chloroform extraction was repeated 3 times. One-fourth volume of 10M LiCl was added to the extracted aqueous phase and incubated night long at -20°C to precipitate RNA and pelleted down by centrifugation at 10000g for 30 minutes at 4°C.

Second protocol used in this study is the one reported by Tao., *et al*. [20] with a minor modification. Ground fine samples were first mixed with absolute alcohol and precipitated by centrifugation. To the pellet preheated 1:1 mixer of extraction buffer (100 mM Tris-HCl pH 8.0, 1% SDS, 100 mM LiCl, 20 mM EDTA pH 8.0) and phenol was added, after brief vortexing 5 ml of chloroform: isoamyl alcohol (24:1) was added and extraction of the aqueous phase was done by centrifugation (repeated 4 times). Finally, the aqueous phase was precipitated over night by adding 10M LiCl and pelleted by centrifugation.

In the third method [18], 4g wood tissue was used as starting material for extraction, after grinding, the powder were transferred to 20 ml of extraction buffer (0.4% CTAB, 0.4% PVP-k30, 10 mM Tris-HCl, 1.25 mM EDTA, 0.8M NaCl, 2% β Mercaptoethanol). Aqueous phase was then extracted using chloroform and one-fourth volume of 10M LiCl was added and incubated overnight at 4°C to precipitate RNA and pelleted down by centrifugation at 14450 X g for 30 minutes at 4°C.

In the fourth protocol, RNeasy plant mini kit (Qiagen, Germany) was used following the manufacturer's guidelines with few alterations. The plant material was increase to 1g and subsequently, the whole protocol was modified accordingly. The same RNeasy spin column was used to centrifuge all the sample aliquots. The spin column membrane was washed twice between samples using Buffer RW1. Buffer RLT and RLC was used for the healthy wood and agarwood respectively.

The fifth method used was Nucleospin RNA plant protocol (MN, Germany), following the manufacturers protocol. Unwanted DNA contamination was avoided by adding DNase after binding of the nucleic acid to the silica membrane. Finally, the bound RNA was eluted by centrifugation after adding RNase free water.

The sixth method opted in the study is the one proposed by Lorenz., *et al*. [18] with several major modification. The details of this method are described in the following section.

Solutions and reagents

0.1% (v/v) DEPC-treated and double autoclaved distilled water, Chloroform, 10M LiCl, β-Mercaptoethanol, 70% ethanol, Liquid nitrogen (LN_2), extraction buffer (0.4% CTAB, 0.4% PVP-k30, 10 mM Tris-HCl, 1.25 mM EDTA, 0.8M NaCl) and 2% β Mercaptoethanol-added just prior to the extraction procedure, SSTE buffer (250 mM NaCl, 0.025% SDS, 0.1 mM Tris-HCl, 0.002 mM EDTA).

RNA extraction day 1

The extraction buffer was autoclaved (prepared at least 12 hours earlier). 17 ml of autoclaved extraction buffer was incubated in a water bath at 65°C (until the proper grinding of the sample). 5g of wooden tissue was frozen in liquid Nitrogen (LN_2) and ground initially in a mixer grinder (wiped with RNase away solution and pre-cooled with LN_2) and then in LN_2 using mortar and pestle (pre-cooled in LN_2) until a fine quality powder is obtained. The samples were not allowed to thaw throughout the grinding procedure, 2% (340 µl) of Beta- mercaptoethanol was added into 50 ml Falcon tube containing 17 ml extraction buffer (preheated at 65°C). The grounded tissue was transferred into pre-heated extraction buffer and mixed by gentle shaking. Five gram ground samples were added to the Falcon tube containing 17 ml extraction buffer. The tube was jolted and heated at 65°C for 5 minutes (shake the tube every after 1 minute), to the solution equal volume of chloroform was added. This chloroform phase separation step was repeated until a clear interphase is obtained (repeat 3 times at least). The supernatant from the final phase separation step was transferred into 15 ml falcon tube and one-fourth volume of 10M LiCl was added to precipitate RNA and kept overnight at -20°C.

RNA extraction day 2

The 15 ml polypropylene tube was taken out from -20°C and allowed to thaw in ice for 20 minutes followed by centrifugation at 7000 rpm for 30 minutes at 4°C to precipitate RNA. SSTE buffer was prepared freshly and incubated at 60°C for 10 minutes. The supernatant was decanted carefully and 500 µl of SSTE buffer (pre-

heated at 60°C) was added to it. The RNA pellet was dissolved carefully by pipetting smoothly. The dissolved RNA was shifted into a 2 ml autoclaved microfuge tube and the same volume of chloroform was added and was centrifuged at 12000 rpm for 20 minutes at 4°C. The upper phase was then carefully moved to a new microfuge tube without interfering the chloroform phase. Two volumes of absolute ethanol was added and incubated at -20°C for 2 hours to precipitate RNA followed by centrifugation at 12000 rpm at 4°C for 20 minutes to pellet down the RNA. The supernatant was carefully decanted and the RNA pellet was washed with 500 µl of ice-cold 70% ethanol prepared in 0.1% DEPC treated water. The RNA was finally pelleted down by centrifugation at 12000 rpm for 25 minutes at 4°C. The supernatant was carefully decanted and pellet was air dried in a laminar air flow (LAF) until removal of any residual droplet of ethanol. Finally, the pellet was dissolved in 30 µl of 0.1% DEPC treated water and stored at -80°C for further use. All the protocols were repeated at least 5 times and in different hand to ensure that there was no any handling error during the extraction process.

RNA quantification and purity analysis

The quantity and purity of extracted was evaluated spectrophotometrically using NanoDrop (ND-1000, Thermo Scientific, Delaware, USA) by determining the absorbance ratios of A_{260}/A_{230} and A_{260}/A_{280}, suggestive of impurity by polyphenols/carbohydrates and proteins, respectively. RNA integrity was determined by loading 1 µg of RNA on a standard 1% agarose gel and visualize under Uvitec gel documentation system (Cambridge, USA).

Superscript III 1st strand synthesis kit (Invitrogen, USA) was used to synthesize first strand cDNA, 1 µg total RNA was reverse transcribed as per the manufacturer's instructions. The synthesized cDNA was then diluted to 100 ng/µl with 0.1% DEPC treated water and stored at −20°C for PCR amplification. The 1-deoxy-D-xylulose-5-phosphate synthase (*ADXPS*), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*ADXPR*), and *A. sinensis* farnesyl pyrophosphate synthase (*AFPS*), glyceraldehyde 3- phosphate dehydrogenase (*GAPDH*), α-tubulin (*TUA*) gene were PCR amplified using the cDNA templates. Primer pairs used for some representative genes were as follows: *ADXPS* {5´-GATGCTTCCAGACAGATACA-3´ (F) and 5´- TGCCTCCCTAGAAGAGATAG-3´ (R)}, *ADXPR*{5´-GTTCCACTGGCTCTATCG-3´ (F) and 5´-CCTGGTCAGCAAGAAGAG-3´ (R)}, *AFPS* {5´-CGCTCTAGGATGGTGTATTG-3´ (F)and 5´-CAACCTTGGGCAGTCTAAA-3´(R)}, *GAPDH* {5´-AAGCCAGCATCCTATGATCAGATT-3´(F) and 5´-CGTAACCCAGAATACCCTTGAGTTT-3´ (R)} and *TUA*{(5´-GCCAAGTGACACAAGCGTAGGT-3´(F) and (5´-TCCTTGCCAGAAATAAGTTGCTC-3´ (R)}. Primers were designed by using primer quest and oligoanalyzer tool of Integrated DNA Technologies (IDT). The 25 µl PCR reaction mix consisted of 10X PCR buffer: 2.5 µl, 10 mM d-NTPs: 0.2 mM, Forward Primer: 0.2 µM, Reverse Primer: 0.2 µM, Taq Polymerase 1 Unit, cDNA: 100 ng and 18.4 µl sterile nuclease-free water. The PCR amplification was performed at 94°C for 2 minutes and subsequently 35 cycles of 94°C for 30s, 58°C for 45s and 72°C for 60s, and a final extension period at 72°C for 5 minutes. The amplified product was separated in a 2% (w/v) agarose gel and visualized by gel Uvitec gel documentation system (Cambridge, USA).

Quantitative RT-PCR Analysis

qRT-PCR was performed in a Step One Plus™ Real-Time PCR System (Applied Biosystems, USA) with the Power SYBRR Green PCR master mix (Applied Biosystems, USA) according to the manufacturer's instructions. qRT-PCR was performed thrice with three biological repeats of cDNA. The results were analyzed with SDS1.7 software (Applied Biosystems) and recorded as C_T (threshold cycle) values. The quantification of each transcript was done as compared to that of the *A. malaccensis GAPDH, TUA* gene, using the comparative CT method.

Results

A high-quality RNA extraction from non-model plants, such as *A. malaccensis* is a challenge because of the presence of great amount of polysaccharides, polyphenols, and other organic compounds present in the wood. Prior to the development of an improved protocol, we tried to isolate RNA using 3 different in-house methods which are 1. Yang., *et al*. [19], 2. Tao., *et al*. [20] and 3. Lorenz., *et al*. [18], along with 2 commercial RNA extraction kit based on silica columns which are 1. RNeasy plant mini kit (Qiagen, Germany) 2. Nucleospin RNA plant (MN, Germany) but were unsuccessful in obtaining good quality RNA. The kit-based protocols were carried out as per the manufacturer's instructions; unfortunately, no visible RNA bands were detected in agarose gel (Figure 1). The protocols illustrated in the literature gives smear and low yield of RNA when used as delineated. Application of a few modifications of Lorenz., *et al*. [18] improved results significantly. In the present study, we have found that the modified CTAB method produces best quality RNA with higher yield. A high-quality pure RNA sample produces ratio of A_{260}/A_{280} and A_{260}/A_{230} around 2 [21]. The outcomes of our experiment revealed that to obtain a high-quality RNA was more perplexing in the Agarwood as compared to non-infected wood as a result of oxidation of phenolic compounds in agarwood. Crushing of the wood tissue is a captious point for gaining a high yield in the isolation of RNA. To evade degradation of RNA it is crucial to

crush the tissue as finer as possible and do not allow the samples to thaw throughout the grinding process. The yield of extracted RNA in our study ranged from 0.360 µg to 100.6 µg per gram of wood tissue. The purity of extracted RNA was determined by calculating A_{260}/A_{280} andA_{260}/A_{230} ratios which was found to be from 1.38 to 2.07 and 1.3 to 2.05 respectively. The yield and purity of each RNA sample extracted by six different protocol is listed in table 1.

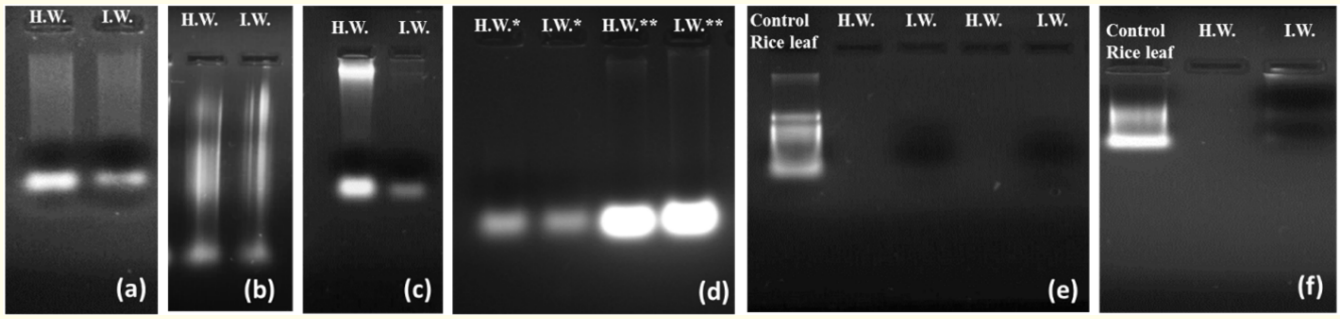

Figure 1: Total RNA resolving through 1% agarose gel. RNA samples were isolated using (a) Yang., et al. (b) Tao., et al. (c) Lorenz., et al. (d) Lorenz., et al. (represented by single*) vs modified CTAB protocol (represented by double**) (e) RNeasy plant mini kit (f) Nucleospin plant Takara. H.W.: Healthy wood, I.W. infected agarwood.

Sl. no	Method	Type of sample	Starting material (g)	Yield (µg/g)	A_{260}/A_{280}	A_{260}/A_{230}
1	CTAB based method (Yang., *et al*. 2008)	Agarwood	1	1.420	1.500	1.600
		Healthy wood	1	0.600	1.510	1.600
2	SDS, EDTA based method (Tao., *et al*. 2004)	Agarwood	1	0.360	1.430	1.300
		Healthy wood	1	0.420	1.410	1.300
3	CTAB based method (Lorenz., *et al*. 2010)	Agarwood	1	0.365	1.500	1.500
		Healthy wood	1	1.620	1.380	1.720
4	RNeasy plant mini kit (Qiagen, Germany)	Agarwood	1	N.A.	N.A.	N.A.
		Healthy wood	1	N.A.	N.A.	N.A.
5	Nucleospin RNA plant protocol (Takara, Germany)	Agarwood	1	N.A.	N.A.	N.A.
		Healthy wood	1	N.A.	N.A.	N.A.
6	Modified CTAB based method	Agarwood	1	100.200	2.040	2.030
		Healthy wood	1	102.600	2.070	2.050

Table 1: Comparative RNA yield and purity in Agarwood and healthy wood using different extraction protocol.

RNA extraction using our modified CTAB method yielded the highest RNA concentration 100.6 µg per gram starting material. The ratio of A_{260}/A_{230} was greater than two for each samples (maximum = 2.05) and the A_{260}/A_{280} ratios ranged from 2.04 to 2.07. The Extracted RNA showed good integrity in 1% agarose gel with no DNA, protein and polysaccharide contamination.

Super-Script III Reverse Transcriptase (Life Technologies, USA) was used to reverse transcribed the extracted RNA into cDNA and which was used to amplify three terpenoid genes and two housekeeping genes. The amplified PCR products were assayed for the occurrence of the objective band on 2% agarose gel (Figure 2). PCR products for detecting the *ADXPS*, *ADXPR*, *AFPS* gene and *GAPDH* and *TUA* housekeeping gene exhibited band size of approximately 100 bp, 250 bp, 250 bp 100 bp, and 100 bp, respectively. According to real-time PCR results obtained in this study, the qPCR cycle thresholds (Ct) for *ADXPS*, *ADXPR*, *AFPS* gene, and *GAPDH*, *TUA*

housekeeping gene was between 18.80 - 29.06 and 18.14 - 27.43 cycles in different positive samples respectively (Table 2). The fluorescence sign appeared after 38.3 cycles in negative control and after 39 cycles in the control samples (no template) which suggests that there was negligible DNA impurities exist in the extracted RNA samples. The melting curve was specific, with a single peak occurring at about 58°C, for *ADXPS*, *ADXPR*, *AFPS*, *GAPDH* and *TUA* gene. In general, all these information validate that the modified RNA isolation method illustrate here is proficient in production of higher yield, high quality, and integrity of total RNA from agarwood and healthy wood. No DNA contamination was detected in the agarose gel (Figure 2) which generally appears as high molecular weight (MW) bands in gel. The RNA extracted by this modified method from different samples e.g. agarwood and healthy wood can be used for cDNA library construction, cDNA-AFLP, Northern blot analysis and Molecular cloning (data not shown) in addition to usual application like semi qPCR and qRT-PCR (shown in this experiment). This modified protocol provide reliably pure and high-quality RNA particularly from *A. malaccensis* and other plant tissue comprising high contents of polysaccharides and polyphenolics compounds.

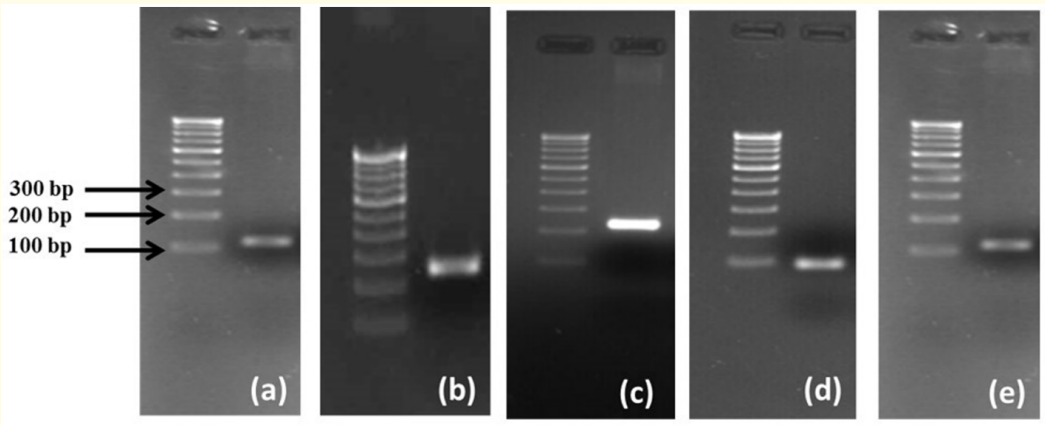

Figure 2: PCR amplified products separated in 2% agarose gel for the primers (a) DXPS, (b) DXPR, (c) FPS and (d) GAPDH (e) TUA gene with cDNA samples.

Sl. no	Gene Name	C_t Values of healthy wood	C_t Values of Agarwood
1	*ADXPS*	24.10 - 25.80	23.00 - 24.10
2	*ADXPR*	26.34 - 29.06	26.16 - 27.43
3	*AFPS*	25.12 - 28.03	24.64 - 26.50
4	*GAPDH*	18.80 - 22.10	18.14 - 19.78
5	*TUA*	26.45 - 26.63	24.64 - 25.55

Table 2: qRT-PCR analysis of total RNA extracted from healthy and agarwood.

Discussion

High-quality RNA extraction is a critical step and can be a confining cause in downstream experiments, for example, microarray, northern blotting assay, RT-PCR and cloning. Although currently many protocol available to extract plant RNA but most of the protocols are either tissue or species specific, therefore it is obligatory to acquire new or enhanced methodology for various plant tissues and species even for similar tissues at various growing phases or identical tissues grown under different environmental condition. The significant changeability in quantity and quality of RNA obtained from any protocol is mainly depends on the composition and content of photochemical [22-24]. This is because diverse plant tissues exhibit differences in their chemical composition [25,26]. One of the critical steps in RNA isolation is to remove polysaccharides and polyphenols [27] caused by oxidation of polyphenols and the resemblance of physicochemical characteristics betwixt polysaccharides and RNA initiate co-precipitation of these molecules along with RNA.

The problems encounter in RNA extraction have been described by various publications [19,28-36]. The RNA extraction methods which uses guanidinium thiocyanate, SDS/Phenol and CTAB indicates that modifications of the protocols are prerequisites for

effective RNA extraction from various *Aquilaria* species and moreover from the same plant grown-up in diverse atmosphere. Escalating the reaction and combining multiple aliquots of reaction into a single RNeasy spin column the commercial RNeasy Plant Mini kit protocol provides maximum yield as well as good integrity of the RNA extracted from healthy wood and agarwood [17]. Surprisingly, the same protocol with the same modification did not work in our experiments; this may be because of different environmental conditions where the plant *A. malaccensis* were grown resulting in presence of inhibitor molecules hindering the RNA extraction process.

The RNA isolated by utilizing the method of Yang., *et al*. [19], had very deprived purity (A_{260}/A_{280} value was 1.5 and A_{260}/A_{230} was 1.6), and poor yield (1.42 µg/g) because of this it is essential to add an extra cleaning step to ameliorate its purity and which is time-consuming and not cost effective. The protocol adopted from Tao., *et al*. [20] yielded poor quality and quantity of RNA, the gel images shown in figure 1b clearly shows the smear when the RNA was separated in 1% agarose gel. RNA extracted by using the method obtained from Lorenz., *et al*. [18] results in poor yield and genomic DNA contamination as shown in figure 1c. Both the commercial kits unable to extract RNA from our samples (agarwood and healthy wood) although it was successful in extracting good quality RNA from control rice plant (Figure 2d and 2f).

RNA extraction using our modified CTAB method, yielded the highest RNA concentration as well as purity (Table 1). The quality of isolated RNA by our modified CTAB method reported in this experiment was tested by many techniques. At first, we separate the extracted RNA in 1% agarose gel which revealed that the RNA has good integrity and brightness, indicates that the RNA extracted by using our modified protocol provides intact and pure RNA without genomic DNA contamination (Figure 1d). We also performed spectroscopic study which exhibit that the A260/A280 ratios of extracted RNA samples were between 2.04 and 2.07 (Table 1), representing that the extracted RNA is free of proteins, polyphenolics, and other reagents used in the extraction protocol. Additionally, A_{260}/A_{230} values were ranged from 2.03 to 2.05 (Table 1), suggesting that the extracted RNA is free from polyphenolics, polysaccharides, other secondary metabolites. An abnormal A_{260}/A_{230} values indicates a problem either with the sample or with the extraction procedure, so it is important to consider both. Although purity ratios are important indicators of RNA quality, the best indicator of RNA quality is functionality in the downstream application of interest. Therefore, the degree of purity of extracted total RNA was further measured by using qRT-PCR analysis. These results indicates that our modified CTAB method is effective in extracting high-quality RNA from healthy wood and agarwood of *A. malaccensis* and which can be used in downstream transcript analysis.

This method is cost effective as compared to the other in-house method or commercial kits. The total cost involved in this method per sample was less than a dollar which is 10 times cost effective as compared to the commercial kits. Approximately 300 ml and 700 ml of liquid nitrogen was needed to grind 4g of healthy wood and 4g of agarwood, respectively. Isolation of intact and high-quality pure RNA is a daunting task. All the protocols verified in our experiment produces low yield and poor purity, as well as the RNA, was either partially or totally degraded. Therefore, we have developed an improved cost-effective method of RNA extraction to overcome these problems.

Conclusion

Present work demonstrates that the modified CTAB based method is the best protocol for extracting RNA from wood and leaf tissues of *A. malaccensis* with maximum yield reported till date and also efficacious downstream application of extracted total RNA. High quality total RNA with a satisfactory degree of purity was isolated consistently from Agarwood and healthy wood with this protocol. Isolated total RNA has been successfully used in downstream processes like qRT-PCR, cDNA-AFLP, and cloning. We have used biological and technical replicates of the samples in different hands to confirm the consistency of the protocol. In addition, this protocol is very cost effective and does not involve any toxic chemicals (e.g. Guanidinium thiocyanate, Guanidinium hydrochloride). This modified protocol is also cost-efficient and require lesser time which can be used as a substitute protocol for extracting total RNA from other fractious plant tissues.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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