

EXTRACTION OF TOTAL RNA FROM MANGROVE PLANTS TO IDENTIFY DIFFERENT GENES INVOLVED IN ITS ADAPTABILITY TO THE VARIETY OF STRESSES

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Nucleic acids extraction from mangroves as woody plants needs lots of practical experience. In the present study, three different methods and the RNeasy plant mini kit were used to extract nucleic acids from mangrove plant. Modified CTAB method provided high integrity and concentration of DNA and RNA, respectively from roots and leaves. This method was successful in extraction of RNA from roots but failed for leaves of mangrove. The CTAB with β -mercaptoethanol increases RNA concentration while it decreases the purity of RNA. High yield of RNA extracted from mangrove leaves was obtained by modifying the SDS method. However, the integrity of RNA decreased. When polyvinylpyrrolidone was used to inhibit polyphenol oxidase activity, the yield and integrity of RNA had improved significantly. Therefore, the objective of this study was to isolate and identify the different genes involved in the adaptation of mangrove plants to different kind of stresses. Two-month old seedlings of the *Rhizophora apiculata* were exposed to 450 mM NaCl for 24 hours under hydroponic culture. Then, the expression of *dihydrolipoamide dehydrogenase* (DLDH) gene was studied using reverse transcriptase-PCR and real-time qRT-PCR.

Keywords: Mangrove, nucleic acids extraction, RNA integrity, RNA extraction, modified methods

INTRODUCTION

Extraction of nucleic acids especially RNA from woody plants containing high polysaccharides and polyphenol is quite challenging task. Since, these components have been released during cell disruption. The mangrove plant is a woody plant (Rubio-Pina and Zapata-Perez, 2011) which contains large amounts of polysaccharides and polyphenols. Mangrove plants comprise of 16 families and 22 genera (Kathiresan and Bingham, 2001) and the *Rhizophora apiculata* is one of the "true mangrove" among 149 species of the Rhizophoraceae family (APG II, 2003) which contributes to make pure sands. The vast humid and sub-tropical marine shorelines are usually covered with mangrove plants. Mangrove plants have adapted to biotic and abiotic stresses which accrued in a wide range of tidal and wetland areas during different cycles of development (Hibino *et al.*, 2001). Due to the increasing population growth in recent years in most parts of the world, the need for a sustainable farming system is being felt more than ever. Stable farming can be achieved either by increasing agricultural products or by producing cereal and higher plants that are resistant to a wide spectrum of biotic or abiotic stresses.

Though different resistant genes have been identified and

isolated from different plants, it is still not sufficient to thoroughly support the need for stable farming products. Hence, continued identification of different plants as the source of tolerance to a wide range of stresses is indispensable and plays a vital role in the attainment of a sustainable farming system. Isolation and identification of different resistance genes from different plants may also play an important role in developmental plant products. Hence, exploration of plants with high levels of adaptation is the first and most important step.

The mangrove plant is a tropical/sub-tropical plant which is subjected to diverse environmental factors, such as salinity (Jayaraman *et al.*, 2008; Ashraf, 2009; Rubio-Pina and Zapata-Perez, 2011), temperature, drought, and moisture, and has adapted to these different stresses to survive (Hibino *et al.*, 2001) through expression of different genes at different ages and parts of the plant. Hence, the mangrove is a valuable source of genes related to resistance and tolerance and must be studied rigorously. Extracting high quality RNA is the most significant factor in constructing cDNA. Moreover, in the absence of intact RNA, the determination of gene expression seems to be impossible.

Phenolic compounds and polysaccharides in mangrove

plants influence the purity of DNA and especially RNA (Fu *et al.*, 2004; Miyama *et al.*, 2006; Nguyen *et al.*, 2006; Zeng *et al.*, 2006; Gonzalez-Mendoza *et al.*, 2008). Phenolic compounds may promote oxidation and degradation of proteins and nucleic acids through covalent bonding with nucleic acids. In this regard, several methods, including the use of insoluble polyvinylpyrrolidone and soluble polyvinylpyrrolidone, have been employed to separate RNA from polysaccharides and phenolic compounds in the precipitate (Rodrigues *et al.*, 2007). Most of the methods could not achieve pure RNA of high quality since phenolic substances are readily degraded and covalently bind with nucleic acids. It has been reported that the use of soluble polyvinylpyrrolidone for RNA extraction without ultracentrifugation is suitable for eliminating proteins during RNA isolation, although polyvinylpyrrolidone interferes with phenol extraction (Woodhead *et al.*, 1997; Salzman *et al.*, 1999).

Several protocols have been used to isolate RNA from different plant tissues and mangrove species (Nguyen *et al.*, 2007; Ganesan *et al.*, 2008; Miyama and Tada, 2008; Yamanaka *et al.*, 2009; Kavitha *et al.*, 2010), but some are time consuming and the purity of extracted RNA is not appropriate for use in RT-PCR or cDNA construction. Extraction of nucleic acids, particularly RNA, from woody plants is not easily achievable due to the presence of polyphenolic compounds and the inhibitory effects over obtaining intact and pure RNA. The most popular method for RNA extraction from woody plants rich in polyphenol and polysaccharides are based on SDS and CTAB extraction procedures (Kiefer *et al.*, 2000; Miyama *et al.*, 2006; Chen *et al.*, 2007; Miyama and Hanagata, 2007; Rodrigues *et al.*, 2007; Gonzalez-Mendoza *et al.*, 2008; Miyama and Tada, 2008; Yang *et al.*, 2008; Rubio-Pina and Zapata-Perez, 2011). Three different manual extraction methods including CTAB, SDS Methods and the RNeasy plant mini kit (Qiagen) were investigated. Following some modifications, two rapid, convenient and reliable protocols, without the need of ultra-centrifugations is introduces to extract high quality DNA, pure RNA suitable for both RT-PCR and construction of cDNA from the leaves and roots of the *Rhizophora apiculata*.

MATERIALS AND METHODS

Plant materials: Seeds of the mangrove plant (*Rhizophora apiculata*) were collected from Kuala Sepetang [04° 50.150'N, 100° 37.620'], Taiping Perak, Malaysia. They were grown in hydroponic-culture for two months and then treated with 450 mM NaCl for 24 hours. Subsequently, the roots and leaves of the plants were collected separately, instantly washed with distilled water and frozen in liquid nitrogen to facilitate the nucleic acids extraction process.

Extraction buffer 1: CTAB (2% w/v = 2 g),

polyvinylpyrrolidone (2% w/v = 2 g), ethylenediaminetetraacetic acid (25mM~ 0.74 g), tris-base (0.1M =1.2 g, pH 8.0), NaCl (1.4M =8.1 g) and 100 mL DEPC treated water.

Extraction buffer 2: SDS (1% w/v =1 g), polyvinylpyrrolidone (4% w/v =4 g), ethylenediaminetetraacetic acid (25M ~0.75 g), tris-base (50mM =0.6 g, pH 8.0), NaCl (0.25M =1.46 g) and addition of 100 mL DEPC treated water.

Chemicals: Liquid nitrogen, 3M sodium acetate (pH 5.2), chloroform :isoamyl alcohol (24:1 v/v), phenol:chloroform (1:1 v/v), phenol : chloroform:isoamyl alcohol (25:24:1 v/v/v), 8 and 10M LiCl (DEPC-treated and autoclaved), cold Isopropanol, β -mercaptoethanol, cold absolute and 70% (v/v) ethanol, 0.1% (v/v) DEPC-treated and autoclaved water. All solutions were prepared with DEPC treated water.

RNA extraction: Method 1: The first method that has been considered in this study is the modified protocol of Kiefer (Kiefer *et al.*, 2000). About 0.1 g of fresh mangrove leaves and roots were frozen and separately ground in liquid N to fine powder using a pre-cooled (overnight at -20°C) mortar and pestle and transferred into a 2 mL eppendorf tube. One thousand μ L pre-warmed (65°C) extraction buffer 1 added and the mixture vortexed vigorously for a short time, followed by addition of 500 μ L chloroform:isoamyl alcohol (CI). The solution was vortexed (high speed) at room temperature (RT) for 10 min and centrifuged at 13000 g for 5 min at 4°C. The supernatant was then transferred to another eppendorf tube, 250 μ L CI added and briefly vortexed and centrifuged at 13000 g for 5 min at 4°C. The supernatant was transferred to a new 2 mL eppendorf tube, cold isopropanol (4°C, 2 volume) added, then incubated on ice (5 min), and centrifuged at 13000 g for 10 min at 4°C, followed by critical modification step: a short spin for 2 minutes at 14.1 g. Then, light brown liquid phase for root and almost white liquid phase for leaf were observed. The supernatant was decanted and the same volume of cold isopropanol (4°C) added as the final step and the mixture centrifuged for 5 min (13000 g, 4°C). The resultant white pellets obtained were washed with 70% cold ethanol, air-dried for 20-30 min and re-suspended in 30 μ L DEPC treated autoclaved water. Note: In order to increase the concentration of RNA, 2% β -mercaptoethanol was added just after addition of the extraction buffer (in the second step followed by the remaining modified steps).

Method 2: The second method used in this study is the modified protocol of Chan (Chan *et al.*, 2007). About 0.15 g fresh mangrove leaves and roots were ground separately to fine smooth powder with liquid nitrogen in a pre-chilled mortar and pestle and transferred into a 2 mL eppendorf tube. Extraction buffer 2 (700 μ L) was mixed with 700 μ L of chloroform: isoamyl alcohol, and vortexed forcefully until the sample was completely re-suspended, followed by the

protocol steps. The extraction process was stopped after incubation at 4°C for 30 min and the sample centrifuged at 12860 g at 4°C for 20 min. The pellet was washed with 70% cold ethanol, air-dried for 15 min, and dissolved in 30 µL of DEPC-treated autoclaved water; or continued the protocol.

Method 3: The third method considers the total RNA extracted by Rubio-pina and Zapata-perez (Rubio-Pina and Zapata-Perez, 2011) procedure. The extraction process was stopped after the third centrifugation steps by dissolving the pellet in 30 µL of DEPC-treated autoclaved water or continued the protocol.

Method 4: The fourth method concerns the total RNA extraction through the RNeasy plant mini kit (QIAGEN, Germany). This method was performed according to the manufacturer's procedures with minor modification including the addition of 600 µL RLC buffer in the first step and then repeating the seventh step twice.

Analysis of nucleic acid integrity: The A_{260}/A_{280} ratio was calculated to determine the amount of contamination caused by proteins and the A_{260}/A_{230} ratio for phenol or carbohydrates contamination, using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). Total RNA was fractionated using electrophoresis on 1.5% ethidium bromide-stained agarose gel.

Semi-quantitative RT-PCR analysis of dihydrolipoamide dehydrogenase: Total extracted RNA was treated with RQ1 RNase Free DNase (Promega, California, USA) and semi-quantitative RT-PCR performed to analyze the *dihydrolipoamide dehydrogenase* gene expression. Based on the manufacturer's protocol for the Super Script™ III reverse transcriptase kit (Invitrogen, Carlsbad, California, USA), 5 µL of total RNA was used to synthesize single-stranded cDNA. The synthesized cDNA was then applied in PCR using the KAPA HiFi™ HotStrat kit to estimate the expression level of *dihydrolipoamide dehydrogenase* genes. The following primers were used: for DLDH (5' GTC ATT GGG GCA GGC TAC AT 3') (5' TCC AGA TGC TGG CTC AAG TG 3') and *actin* as housekeeping gene (5' CAC TAC TAC TGC TAA ACG GG AAA 3') (5' ACA TCT GCT GGA AGG TGC TG 3'). The following PCR program was used: 95°C for 3 min and 35 cycles of 98°C for 30 sec, 60°C for 60 sec, and 72°C for 60 sec. The PCR program was concluded with a final extension of 5 min at 72°C. The PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide.

Real time-PCR: One µL (100 ng) of total DNase treated-RNA was used as a template in real-time PCR, and the master mix for real-time PCR prepared according to the manufacturer's protocol for the KAPA SYBR FAST (Boston, USA). One-Step qRT-PCR kit and the same primers as mentioned in the section above were used for *dihydrolipoamide dehydrogenase* and *actin* as a reference gene with the following PCR program: 42°C for 5 min, 95°C for 3 min and 40 cycles of 95°C for 5 sec, 40 cycles of 60°C

for 30 sec, 40 cycles of 72°C for 10 sec.

RESULTS AND DISCUSSION

The samples showed sharp bands representing 28S and 18S ribosomal subunits of RNA, which confirmed the purity of the RNA (Figs. 1-6). The study revealed that extraction of DNA and RNA from mangrove plants differed between roots and leaves. The integrity and yield of nucleic acids depended on several chemical substances including the use of LiCl, β-mercaptoethanol, or even repetition of some critical steps of the extraction methods, which can vary for different tissues based on the main purpose. The integrity of extracted RNA from the mangrove roots was increased considerably by using the modified CTAB method, which aided in eliminating protein contamination. High yield of RNA was extracted from the leaves of mangrove plant by modifying the SDS method, although the integrity of RNA was decreased due to phenol/chloroform/isoamyl alcohol extraction and easily degradation of phenolic substances through covalent binding with nucleic acids which interfered with the quality of the required RNA for RT-PCR and cDNA library synthesis.



Figure 1. Separation of total RNA and DNA extracted from roots and leaves of mangrove according to the method 1 using 1.5% agarose gel and stained with ethidium bromide. L: Ladder (O'GeneRuler™ DNA Ladder Mix, 100bp, Fermentas)

Evaluation of modified methods for extraction of nucleic acid: The shortest protocol, the RNeasy plant mini kit, which was used in original and modified forms, failed in getting total RNA. Two modified procedures, methods 1 and 2 were examined practically for their effectiveness in

Table 1. The average integrity and yields of extracted RNA from mangrove using different extraction protocols.

| Protocols | Tissue | | Purity | | Concentration of RNA yield |
|--------------------|---------------------------|------|---------------|---------------|----------------------------|
| | Root (Brown & Lateral) | Leaf | $A_{260/280}$ | $A_{260/230}$ | |
| Method 1 | ✓ | | 2.04 | 1.43 | 440.3 |
| Method 1 | | ✓ | 1.80 | 0.68 | 62.3 |
| Method 2 | ✓ | | 1.47 | 0.30 | 947.3 |
| Method 2 | | ✓ | 1.70 | 0.64 | 377.4 |
| Method 3, Buffer 1 | ✓ | | 1.91 | 1.31 | 50.6 |
| Method 3, Buffer 1 | | ✓ | 2.09 | 1.70 | 122.0 |
| Method 3, Buffer 2 | ✓ | | 1.56 | 0.36 | 146.7 |
| Method 3, Buffer 2 | | ✓ | 1.91 | 1.48 | 370.6 |

isolating DNA and RNA from the *Rhizophora apiculata*. Both modified methods were quick and reliable for isolation of intact RNA from the roots and leaves of woody plants for further molecular analysis, such as the construction of a cDNA library and RT-PCR. Contrary to what was said on the original CTAB method, after adding cold isopropanol for the first or second time and centrifuge, no precipitation was formed. Using method 1 the extracted DNA from mangrove leaves showed high yield and purity but failed in extracting RNA from the leaves. The average concentration of extracted RNA from the leaves and roots of mangrove plant were 440.3 and 62.3 ng, respectively using method 1, 377.4 and 947.3 ng using method 2. Using method 1, containing CTAB extraction buffer effective on cell disruption, the integrity of the extracted nucleic acids, especially RNA from the root was significantly increased unlike for the leaf, where the presence of phenol compounds leads to obtain just high quality and adequate amount of DNA (Fig. 1; Table 1). In method 2, SDS increased the concentration of extracted RNA and DNA from the roots and leaves significantly (Fig. 2; Table 1). The average yield of extracted RNA from the leaves and roots of the mangrove plant through modified method 3 and using extraction buffers were 122 and 50.6 ng for buffer 1 and 370.6 and 146.7 ng for buffer 2. Method 3 in combination with buffers 1 and 2, followed by LiCl and sodium acetate can remove the enlacing polysaccharides of nucleic acids for precipitation of RNA, while β -mercaptoethanol helps to prevent oxidation reactions resulting in high integrity and amount of extracted RNA (Fig. 3 and Table 1).

Estimation of quality and quantity of RNA: The average $A_{260/280}$ ratio of the RNA extracted from the roots of the mangrove plant using the two modified methods 1 and 2 were 2.04 and 1.47, respectively, and by using modified method 3, with buffers 1 and 2, 1.91 and 1.56, respectively. The average $A_{260/280}$ ratio of RNA extracted from the mangrove leaves using methods 1 and 2 were 1.80 and 1.70,

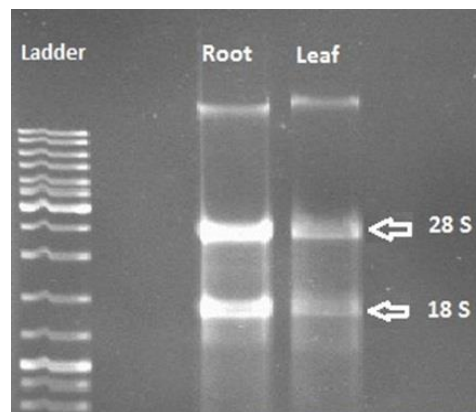


Figure 2. Electrophoretic separation using 1.5% agarose gel of nucleic acids (total RNA, DNA) extracted from roots and leaves of mangrove using method 2 and stained with ethidium bromide. L: Ladder (O'Gene Ruler™ DNA Ladder Mix, 100bp, Fermentas)

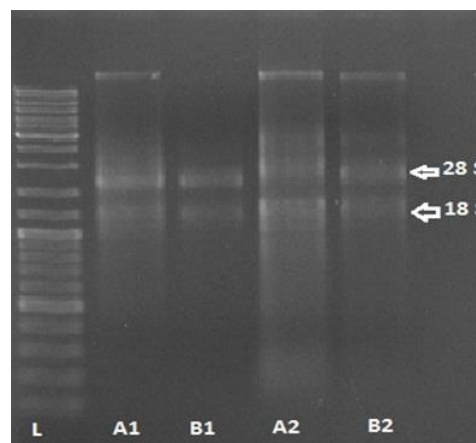


Figure 3. Separation total RNA and DNA from roots and leaves of mangrove according to method 3; A1: Leaf of mangrove using extraction buffer 1. B1: Root of mangrove using extraction buffer 1. A2: Leaf of mangrove using extraction buffer 2. B2: Root of mangrove using extraction buffer 2. L: Ladder (O'GeneRuler™ DNA Ladder Mix, 100 bp, Fermentas)

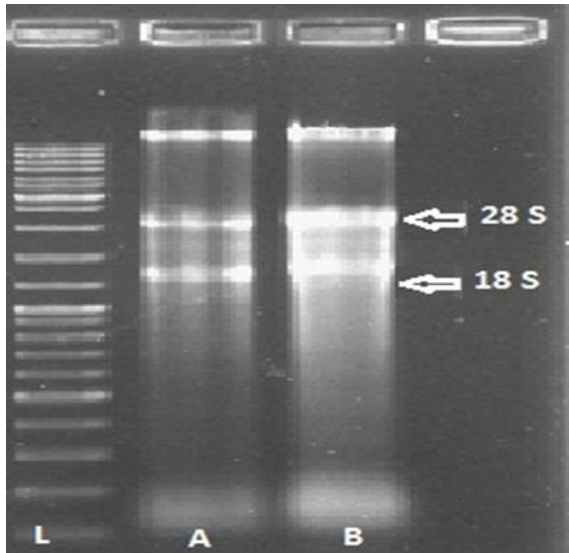


Figure 4. Comparison between concentrations of total extracted RNA using method 1 and 1.5% agarose gel stained with ethidium bromide. A: Extracted nucleic acids from roots without using β -mercaptoethanol; B: Extracted nucleic acids from roots using β -mercaptoethanol, L: Ladder (O'GeneRuler™ DNA Ladder Mix, 100 bp, Fermentas)

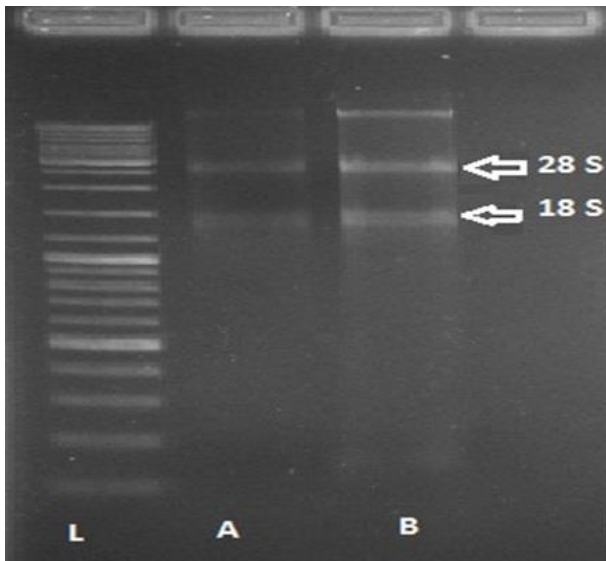


Figure 5. Extracted total RNA from roots of mangrove according to the method 1, with and without LiCl. A: Extracted RNA from roots without using LiCl. B: Extracted RNA from roots using LiCl. L: Ladder (O'GeneRuler™ DNA Ladder Mix, 100 bp, Fermentas)

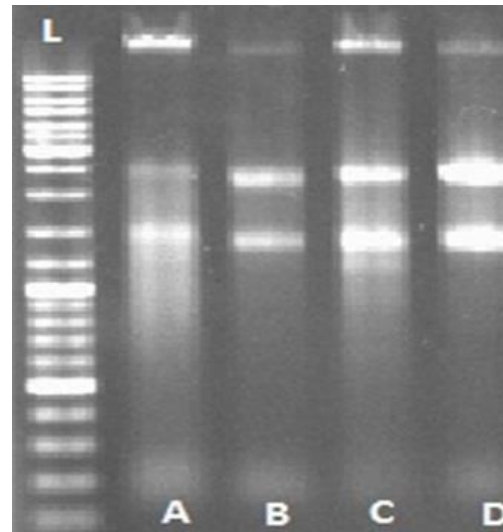


Figure 6. Comparison between concentrations of total extracted RNA, using method 2 and 1.5% agarose gel stained with ethidium bromide. A, B: Extracted nucleic acids without using LiCl from leaves and roots. C, D: Extracted nucleic acids using LiCl from leaves and roots, respectively. L: Ladder (O'GeneRuler™ DNA Ladder Mix, 100 bp, Fermentas)

respectively and using modified method 3, 2.09 (buffer 1) and 1.91 (buffer 2). The extracted RNA using method 1 had high integrity and was successfully used for cDNA synthesis, following the analysis of the differential expression for *actin* and *dihydrolipoamide dehydrogenase* genes from salt-treated and non-treated mangrove plants (Figs. 7-8; Table 2).

Table 2. The integrity and yield of the extracted RNA from mangrove roots by method 1 using with (A) and without using β -mercaptoethanol (B).

| Tissue | Concentration | Purity | |
|----------|-------------------|---------------|---------------|
| | | $A_{260/280}$ | $A_{260/230}$ |
| Root (A) | 440.3ng/ μ L | 2.04 | 1.43 |
| Root (B) | 1199.7ng/ μ L | 1.93 | 0.83 |

The RNA extracted from the mangrove roots using Method 1 was of good quality due to the precise differential expression by *dihydrolipoamide dehydrogenase* and *actin* genes during Real time-PCR (Fig. 9; Table 3). In contrast, method 2 failed to extract RNA from the mangrove roots for cDNA synthesis and for subsequent RT-PCR amplification and real time-PCR (data not shown). The purity and concentration of extracted RNA from the leaves of the mangrove plant was much higher using method 2 as compared to method 1 (Table 1). Hence, if the main purpose is RNA extraction, then method 1 would not be recommended as this approach is only able to extract DNA.

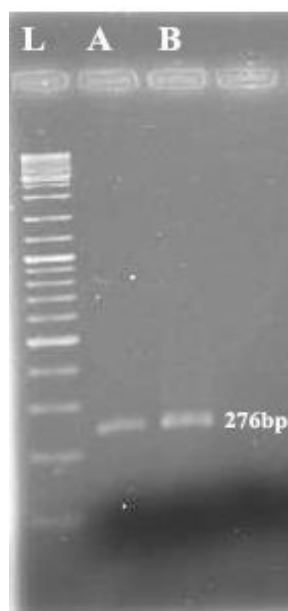


Figure 7. RT-PCR analysis with agarose gel electrophoresis. RT-PCR amplification of *dihydrolipoamide dehydrogenase* gene fragment from roots of *R. apiculata*. A: Non-treated mangrove roots, B: Salt treated mangrove roots. L: Molecular marker (1 kb) in base pairs (bp)

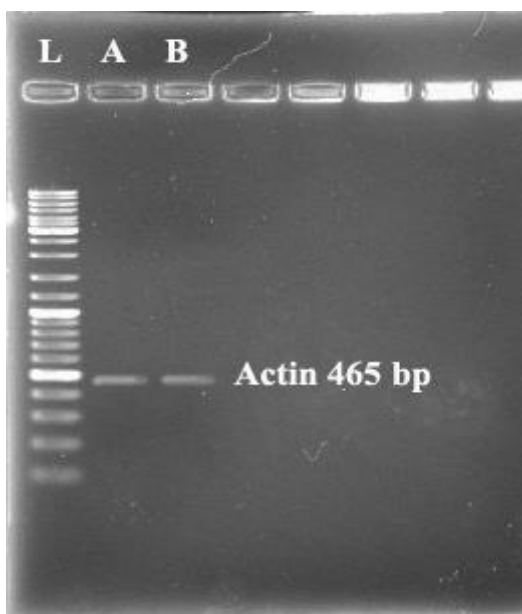


Figure 8. RT-PCR analysis with agarose gel electrophoresis. RT-PCR amplification of *actin* gene fragment from roots of *R. apiculata*. A: Non-treated mangrove roots, B: Salt treated mangrove roots. L: Molecular marker (1 kb) in base pairs (bp)

Table 3. The PCR quantification spreadsheet data for FAM-490 (Extracted RNA from roots of mangrove with using methods 1 and 2).

| Gene | Ct (Method 1) | Ct (Method 2) |
|-----------------------------|---------------|---------------|
| <i>Actin</i> (Non-treated) | 23.1 | N/A |
| <i>Actin</i> (Non-treated) | 22.8 | N/A |
| <i>Actin</i> (Salt-treated) | 22.9 | N/A |
| <i>Actin</i> (Salt-treated) | 22.6 | N/A |
| <i>DLDH</i> (un-treated) | 20.3 | N/A |
| <i>DLDH</i> (un-treated) | 21.2 | N/A |
| <i>DLDH</i> (Salt-treated) | 16.3 | N/A |
| <i>DLDH</i> (Salt-treated) | 17.4 | N/A |

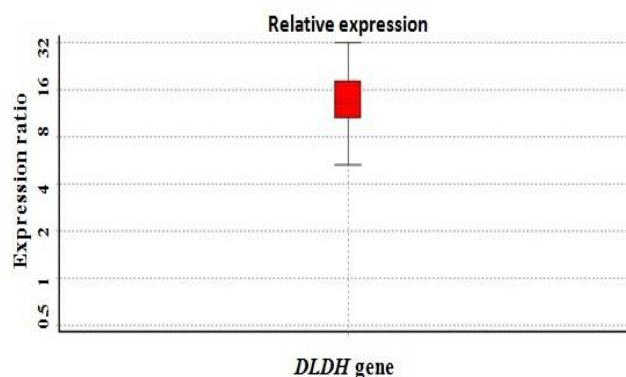


Figure 9. Expression of *dihydrolipoamide dehydrogenase* gene in salt treated mangrove plants

Effects of LiCl on RNA integrity and yield: Given the individual goals of RNA extraction from roots and awareness of the interference of LiCl in the steps involved in molecular studies, the use of LiCl in method 1 was shown to lead to increased concentration of total extracted RNA, from 385 to 780 ng/ μ L and the formation of more sharp bands on the agarose gel (Fig. 5). Further, almost good quality and high yield of RNA was obtained from the leaves and roots of the mangrove plant with the addition of LiCl, whereby the RNA yield increased to 304.2 - 1106.7 (leaves) and 811.2 - 1787.8 (roots). This remarkable increase in yield of RNA with the addition of LiCl did not result in any significant change in terms of RNA integrity (Table 3).

The integrity and amount of extracted RNA obtained using method 3 in combination with buffers 1 and 2 was out of range, which is probably related to the use of LiCl. The use of LiCl increases the amount of polyphenols and polysaccharides during RNA extraction, with subsequent effects on the quality and quantity of the extracted RNA (Chan *et al.*, 2004). In contrast, the use of β -mercaptoethanol helps to avoid oxidation reactions.

Effect of β -mercaptoethanol on purity of total RNA: Based on the results obtained from these nucleic acid extraction protocols, use of β -mercaptoethanol was seen to increase the concentration of RNA considerably while slightly

decreasing its integrity from (2.02-2.04) to (1.89-1.93). Conversely, the use of β -mercaptoethanol in modified method 1 assisted in significantly improving the concentration of the RNA obtained from the mangrove roots, from 440.3 to 1199.7 ng/ μ L (Table 2). Hence, if concentration of total RNA is the top priority, then the use of β -mercaptoethanol is warranted. The improved method 1 presented a unique opportunity to isolate absolutely good quality DNA from the roots and leaves of the mangrove plant and also provide pure and high concentration of RNA from the roots. This method resulted in the least contamination of proteins and polysaccharides from the mangrove roots, especially after using β -mercaptoethanol which constraints RNase activity and avoids samples being oxidised when reacting with phenolic compounds (Tables 1 and 2). Although the purity of the total extracted RNA from the roots of the mangrove was 2% lower when using β -mercaptoethanol in the CTAB extraction buffer (method 1), however, the concentration of the RNA increased significantly. The protocol also showed negligible amounts of degraded RNA. This is confirmed by clear bands related to 18 S, 28 S rRNA without any smear sign. The results of this study confirmed that the best protocol for extraction RNA from the roots of mangrove in terms of quality is method 1; and for leaves, method 3 with use of buffer 1 is the best, although method 3 is more time consuming compared to method 2. Hence, if the concentration of extracting RNA from leaves is not important, method 2 is recommended (Tables 5 and 6).

Table 5. The ANOVA of concentration and purity of extracted total RNA from leaves (A) and roots (B) of mangrove using four different protocols.

| (A) | | | |
|--------|----|-------------------------|----------------------|
| S.O.V. | DF | Concentration | Purity |
| Block | 2 | 79.911 ^{ns} | 0.0002 ^{ns} |
| Treat | 3 | 84715.647 ^{**} | 0.0387 ^{**} |
| Error | 6 | 58.044 | 0.0032 |
| Total | 11 | - | - |
| CV | - | 3.299 | 3.019 |

(B)

| S.O.V. | DF | Concentration | Purity |
|--------|----|--------------------------|----------------------|
| Block | 2 | 2735.853 ^{ns} | 0.0144 ^{ns} |
| Treat | 3 | 516035.694 ^{**} | 0.2616 ^{**} |
| Error | 6 | 2588.833 | 0.0036 |
| Total | 11 | - | - |
| CV | - | 12.052 | 3.454 |

^{**} and ^{ns} are significant at 1% and non-significant, respectively.

Conclusion: Method 3 resulted in total extracted RNA with high integrity as compared to methods 1 and 2. However, this method is time consuming as it requires two-steps sample incubation at -20°C during the extraction process. The modified method 2 is introduced as a straightforward protocol, suitable for isolating intact and high yield RNA from the leaves of the mangrove plant. However, brown sediment was obtained when using the phenol reagent during nucleic acid extraction. The oxidative effect leads to reduced integrity of extracted RNA from the roots of the mangrove. The improved method 1 on the other hand, allowed isolation of high quality DNA from the roots and leaves of the mangrove and also provided pure and high concentration of RNA from the mangrove roots particularly after the addition of β -mercaptoethanol. This study also provides a simple protocol for RNA extraction that helps in indentifying adapted mangrove genes that overcome various stress effects. The present study focuses on *dihydrolipoamide dehydrogenase*, as an adapted mangrove gene to overcome salt stress effects. Furthermore, in this study the modified CTAB method provides DNA that can be easily eliminated through DNase treatment before cDNA construction.

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Table 6. The mean comparison of different protocols in RNA extraction.

| Method | Leaf | | Root | |
|-------------------|--------|---------------|--------|---------------|
| | Purity | Concentration | Purity | Concentration |
| Modified CTAB | 1.86 b | 60.767 c | 2 a | 531.43 b |
| Modified SDS | 1.74 c | 380.133 a | 1.46 c | 960.50 a |
| Method 3,buffer 1 | 2.02 a | 112.333 b | 1.90 b | 50.50 c |
| Method 3,buffer 2 | 1.89 b | 370.333 a | 1.54 c | 146.20 c |
| Mean | 1.88 | 230.892 | 1.73 | 422.16 |
| CV(%) | 3.02 | 3.299 | 3.45 | 12.05 |

Same letter(s) in a column did not differ significantly at $p \leq 0.05$.

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