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# Isolation of total RNA from tissues rich in polyphenols and polysaccharides of mangrove plants

**Jorge A. Rubio-Piña[1] · Omar Zapata-Pérez[1]** ✉

1 Centro de Investigación y de Estudios Avanzados del IPN, Departamento de Recursos del Mar, Unidad Mérida, Mérida, Yucatán, México

✉ Corresponding author: ozapata@mda.cinvestav.mx
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**Abstract** RNA extraction from mangrove tissues can be difficult due to the presence of polyphenolic and polysaccharide compounds upon cell disruption. Besides, a successful RNA isolation from mangrove tissues sometimes can be strain and species specific. Two different methods were used to extract RNA from tissues (stems, leaves and roots) of black mangrove (*Avicennia germinans*) and white mangrove (*Laguncularia racemosa*), collected from the mangrove area at Progreso, Yucatan, Mexico. An optimized and modified total RNA isolation method was developed for these recalcitrant species. The protocol is based on the CTAB method including β-mercaptoethanol and PVPP with sequential Phenol/Chloroform/isoamyl alcohol extractions to remove protein, and polyphenols, followed by two selective purifications with LiCl and sodium acetate to eliminate polysaccharides. Although, the introduced modifications are not new, their addition proved to be decisive for success in RNA isolation. This modified procedure resulted in high quantity and quality RNA. The RNA obtained is suitable for cDNA synthesis, RT-PCR experiments and the phytochelatin synthase 1 gene amplification.

**Keywords:** mangrove, plant tissues, polysaccharides

## INTRODUCTION

Mangroves are woody plants which form the dominant variation in tidal, saline wetlands along tropical and subtropical coasts. The ecological and sociological importance of mangroves is well recognized (Wu et al. 2008). Mangroves are also physiologically interesting as potential models for stress tolerance. It is known that the accumulation of low-molecular-weight organic solutes such as sugars, some amino acids, and quaternary ammonium compounds are involved in the adaptation to abiotic stress (Hibino et al. 2001). Mangrove plants may have acquired specific genes essential for salt tolerance during the course of their evolution. Finding such genes may greatly influence agricultural productivity in the future, because saline stress is an important factor which limits plant growth and productivity throughout the world. Many researchers have analyzed the salt-tolerance mechanisms using either tissue parts or the whole plant (Jayaraman et al. 2008; Ashraf 2009). In the recent years, the isolation of salinity tolerant genes from a few mangrove species through the generation and analyses of expressed sequence tags (ESTs) from *Avicennia marina* (Mehta et al. 2005; Ganesan et al. 2008; Kavitha et al. 2010), *Acanthus ebracteatus* (Nguyen et al. 2006; Nguyen et al. 2007) and *Bruguiera gymnorrhiza* (Miyama et al. 2006; Miyama and Tada, 2008; Yamanaka et al. 2009), differential gene expression studies of Bruguiera spp. by using differential display, cDNA Representational Difference Analyis (cDNA RDA) and suppression subtractive hybridization (SSH) (Nguyen et al. 2007), have been reported. However, the molecular mechanism of salt tolerance remains unclear. On the other hand, molecular cloning and characterization of individual genes have also shed light on the understanding of salinity tolerance of mangrove plants (Yamada et al. 2002; Zeng et al. 2006; Kavitha et al. 2010). These characteristics have motivated us to study and focus on

exploring the physiological mechanism of tolerance to saline stress at molecular level in species: black mangrove (*Avicennia germinans*) and white mangrove (*Laguncularia racemosa*).

A clean and intact RNA is important for functional genomic studies. Unfortunately, the task of extracting intact RNA is difficult due to the susceptibility of RNA molecules to the enzymatic degradation by RNase (Rubio-Piña and Vázquez-Flota, 2008). The problem of isolating RNA from mangrove is magnified by the release of high contents of polyphenolics and metabolites after disruption of the cells, which are embedded in viscous polysaccharides. Furthermore, samples from different species and tissues show differences in their composition (Hibino et al. 2001; Wu et al. 2008). The difficulties of RNA extraction have been reported by numerous publications (Fu et al. 2004; Miyama et al. 2006; Nguyen et al. 2006; Zeng et al. 2006; Gonzalez-Mendoza et al. 2008; Vasanthaiah et al. 2008; Yang et al. 2008). These RNA isolation protocols using acidic guanidinium thiocynate, CTAB and SDS/Phenol, suggests that it requires different methods for successful RNA isolation from different mangrove species and also from the same species, grown under different environment.

At the beginning of this study, we were not successful in isolating RNA from the procedures routinely used in our laboratory (Trizol, Invitrogen and RNeasy Mini Kit, Qiagen) because we were not able to observe the RNA by electrophoresis fractionation. Even using protocols (CTAB - and SDS - based method) developed for total RNA extraction from true mangrove plants (Gonzalez-Mendoza et al. 2008; Yang et al. 2008), the RNA samples were still neither able to be used as template for cDNA synthesis.

Surfactants agents (CTAB and SDS) are widely used in RNA isolation to inhibit RNase activity. However, the efficiency of such methods varies depending on the composition of the treated tissues (Chan et al. 2004; Rodrigues et al. 2007). Organic solvents like phenol and chloroform are used to dissociate RNA from proteins, separating them into two different phases. LiCl is used to precipitate RNA at certain concentrations, while potassium acetate is introduced to remove polysaccharides from the solution. However, standard procedures that use these reagents, rarely result in RNA recovery with enough integrity or purity, when performed on tissues with high polysaccharide and polyphenol content (Rubio-Piña and Vazquez-Flota, 2008). Furthermore, these procedures are frequently modified to suit specific tissues and conditions.

In this work, we compared two RNA extraction methods with modifications using mangrove tissues (*A. geminans* and *L. racemosa*) (Gonzalez-Mendoza et al. 2008; Yang et al. 2008). The objective of this study was to develop a reproducible method for isolation of RNA from different tissues of mangrove plants. The proposed protocol overcomes the problems associated with polyphenol, polysaccharide, and metabolite contamination and it can be easily carried out in any laboratory. The yield and quality of the RNA obtained were consistently high, as confirmed by spectrophotometric analysis, separation on agarose gel electrohphoresis, Reverse Transcription-Polymerase Chain Reaction (RT-PCR), and the phytochelatin synthase 1 (PCS1) gene amplification.

## MATERIALS AND METHODS

### Plant material

Plants of *A. germinans* and *L. racemosa* were collected from wild populations in Progreso (21º16'47''N 89º39'47''O), Yucatan, Mexico and transported to the laboratory. Upon arrival, plants were thoroughly washed with deionized sterile water and dissected leaves, stem and roots separately. The samples were immediately frozen in liquid nitrogen and stored at -80ºC until the RNA to was extracted.

### Solution required

Extraction buffer 1: 2% (w/v) CTAB; 0.1 M Tris-HCl (pH 8); 1.4 M NaCl; 20 mM EDTA (pH 8); 2% (w/v) PVPP. Add β-mercaptoethanol to a final concentration of 10% (v/v) just before use.

Extraction buffer 2: 3% SDS (w/v); 0.5 mM EDTA (pH 8); 0.1 mM Tris-HCl (pH 8); 2% (w/v) PVPP. Add β-mercaptoethanol to a final concentration of 10% (v/v) just before use.

Sodium acetate 3 M (pH 5)

Chloroform: isoamyl alcohol (24:1)

Phenol: chloroform (1:1)

8 M LiCl

100% and 70% ethanol

All solutions were prepared with molecular biology water (MBW; Invitrogen). The mortars and pestles were baked in oven at 200ºC.

**Total RNA extraction**

Method 1, is a modified protocol from Yang et al. (2008).

About 200 mg of each sample tissue were ground to a fine powder using liquid nitrogen and transferred into a 2 ml centrifuge tubes. 900 µl of extraction buffer 1 and 100 µl of β-mercaptoethanol were added. The mixture was shaken for 30 sec and then incubated at 65ºC for 10 min, inverting the tube 3-4 times every now and then during incubation.

Then, 800 µl of chloroform were added. The mixture was shaken for 30 sec. Later the mixture was centrifuged at 10,000 rpm for 10 min at 4ºC. The supernatant was transferred to a new tube. Next, 800 µl of phenol/chloroform (1:1) were added and shaken for 30 sec. Later the mixture was centrifuged at 10,000 rpm for 10 min at 4ºC. The supernatant was transferred to a new tube and an equal volume of chloroform/ isoamyl alcohol (24:1) was added. Samples were shaken for 30 sec and centrifuged at 10,000 rpm for 10 min at 4ºC. The final supernatant was transferred to new tube containing 1/3 volume of LiCl (8 M) and kept at -20ºC for at least 4 hrs.

Next, the samples were centrifuged at 10,000 rpm for 20 min at 4ºC. The pellets were successively washed with 100% and 70% ethanol. After a short drying time at room temperature, the pellets were dissolved in 250 µl of MBW. At this stage, the RNA preparation was treated with RNAse-Free DNAse (Promega). Following standard phenol/chloroform extraction, RNA was precipitated by adding 0.1 volume of sodium acetate (3 M) and 2 volumes of ethanol 100%, then incubated at -20ºC for 2 hrs. The RNA was recovered by centrifugation (13,000 rpm for 15 min at 4ºC), washed with 70% ethanol, air dried and then dissolved in 20-50 µl of MBW.

Method 2, is a modified protocol from Gonzalez-Mendoza et al. (2008).

About 200 mg of each tissue sample were ground to a fine powder using liquid nitrogen and transferred into a 2 ml centrifuge tubes. 900 µl of extraction buffer 2 and 100 µl of β-mercaptoethanol were added. This method was performed following the above procedure.

**Estimation of RNA quality**

The quality and quantity of the RNA extracts were assessed spectrophotometrically by a standard procedure. Contamination due to phenol/carbohydrates and proteins was determined by recording the OD ratios; $A_{260/A230}$ and $A_{260/A280}$, respectively. In order to verify RNA integrity, extracts were fractioned by electrophoresis in a 1.2% agarose gel, stained with ethidium bromide, and visualized under UV light.

**RT-PCR**

Single-stranded cDNA was synthesized from 2.5 µg total RNA using ImProm-II™ Reverse Transcriptase and oligo (dT), following the manufacturer's protocol (Promega). The synthesized cDNA was used in a reaction for PCR in order to estimate the expression level of the actin gene. Actin-specific primers were used Fw (5' CAC TAC TAC TGC TAA ACG GG AAA 3') and Rv (5' ACA TCT

GCT GGA AGG TGC TG 3'). The following PCR program was used: 94ºC for 3 min, 30 cycles of 94ºC for 30 sec, 60ºC for 30 sec, and 72ºC for 30 sec. The program ended with a 10 min extension at 72ºC. The amplified products were separated on a 1.2% agarose gel and visualized after staining with ethidium bromide.

**Semi-quantitative RT-PCR analysis of PCS1**

RT-PCR analysis was performed according to the above protocol; 1 µl of cDNA was used as a PCR template. To evaluate PCS1 gene expression, PCR reactions with equal amounts of cDNA were performed using PCS1 primers Fw (5' AAA TGG AAA GGG CCT TGG AG 3') and Rv (5' CAG CAT TAT ATA GCC ACC AAT AGG 3'), as well as actin primers Fw (5' CAC TAC TAC TGC TAA ACG GG AAA 3') and Rv (5' ACA TCT GCT GGA AGG TGC TG 3'). The actin gene was used as housekeeping and load control. The following PCR program was used: 94ºC for 3 min, 30 cycles of 94ºC for 30 sec, 60ºC for 30 sec, and 72ºC for 30 sec. The program ended with a 10 min extension at 72ºC. The amplified products were separated on a 1.2% agarose gel and visualized after staining with ethidium bromide.

## RESULTS AND DISCUSSION

Our laboratory has routinely used the Trizol protocol to isolate RNA from sample tissues without significant problems. We initially tried to apply this protocol on mangrove plants (*A. germinans* and *L. racemosa*). However, the quality of RNA obtained was not adequate enough to be properly visualized when fractionated by electrophoresis in an agarose gel. We also failed to obtain RNA using several commercially available RNA extraction Kits (RNeasy Mini Kit and RNAspin Mini). The samples obtained had no absorbance at $A_{260/280}$ nm and could not be visualized. This result has been reported taking into account that the osmoprotection might be carried out by small osmolytes like glycine betaine or the oligosaccharide; it is possible that the main problems associated with RNA extraction might be caused by oligosaccharides or even monosaccharides (Fu et al. 2004; Valenzuela-Avendaño et al. 2005).

Though the existence of reports for RNA extraction from mangrove plants (such as guanidine thiocyanate-phenol-chloroform), it was not considered in our plants, since Trizol reagent and RNeasy Mini Kit are based on the same method (Chomczynski and Sacchi, 1987), which previously proved to be ineffective.

On the other hand, two of the most used methods for RNA extraction of mangrove and woody plants are based on CTAB and SDS extractions (Chang et al. 1993; Fu et al. 2004; Miyama et al. 2006; Miyama and Hanagata, 2007; Rodrigues et al. 2007; Miyama and Tada, 2008; Gonzalez-Mendoza et al. 2008; Yang et al. 2008), which are modified for plant tissues rich in polysaccharides and polyphenols. We compared two RNA extraction protocols using CTAB method for the RNA extraction from *Hibiscus tiliaceus* tissues (leaves and roots) (Yang et al. 2008) and SDS method developed for leaves of *Avicennia germinans* in our lab (Gonzalez- Mendoza et al. 2008). The RNA extractions were performed according to the above protocols. These methods did not allow us to obtain good-quality and sufficient amount of RNA for subsequent experiments (Figure 1a and Figure 1b).

Yang et al. (2008) used CTAB as the cell disrupting agent, while β-mercaptoetanol was added to the extraction buffer as a reducing agent to prevent any possible oxidation reactions. They introduced the use of polyvinylpolypyrrolidone (PVPP) in the extraction buffer and LiCl precipitation. The insoluble PVPP has been used for RNA extraction from recalcitrant tissues (Rubio-Piña and Vázquez-Flota, 2008), as it binds and removes phenolic compounds. Fu et al. (2004) reported that extraction buffers containing LiCl could eliminate the entangling polysaccharides from nucleic acids in *Aegiceras corniculatum*; however, this was not suitable for the RNA extraction of *A. germinans* and *L. racemosa* (Figure 1a). In some cases, high LiCl concentrations may result to an increase in the amount of impurities (polysaccharides and polyphenols) in RNA extractions of plant tissues, thus limiting the concentration and quality of RNA (Chan et al. 2004). Gonzalez-Mendoza et al. (2008) used SDS as the cell disrupting agent. Additionally, they applied an extraction with isopropanol, and reported the quality and integrity of the RNA obtained. However, the RNA isolated with this method were partially degraded (Figure 1b), brown in colour and difficult to be dissolved in some tissues. The poor quality of RNA may be attributed to co-precipitation of polysaccharides and oxidation of phenolic compounds that interact

irreversibly with nucleic acids, while the brown colour in the supernatant is developed upon oxidation of the homogenate (Chan et al. 2004; Vasanthaiah et al. 2008).

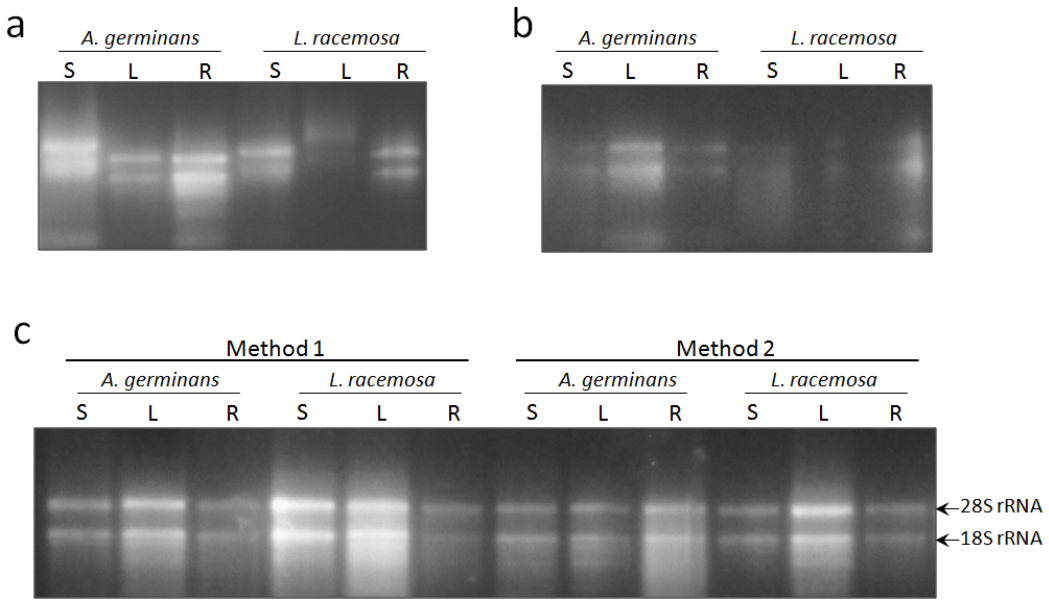


**Fig. 1 Comparison of total RNA isolated from tissues of *A. germinans* and *L. racemosa* by different methods.** The arrows indicate the 28S and 18S units of rRNA. S: stem, L: leaf, R: root. a) RNA extracted by Yang et al. (2008). b) RNA extracted by Gonzalez-Mendoza et al. (2008). c) RNA extracted by modified methods 1 and 2.

Based on these results, these RNA extraction methods were modified in order to develop a protocol which will be able to obtain RNA for recalcitrant tissues in our plants. Method 1 (M1), is a modified protocol from Yang et al. (2008) and Method 2 (M2), is a modified protocol from Gonzalez-Mendoza et al. (2008). These protocols were modified using LiCl/ sodium acetate to remove polysaccharides during the RNA precipitation steps. This approach has been used previously (Chan et al. 2004; Nguyen et al. 2006; Rodrigues et al. 2007; Rubio-Piña and Vázquez-Flota, 2008). The results of the two RNA extraction methods used on the tissue samples are shown in Table 1.

The M1 and M2 methods for RNA extraction were tested to evaluate which protocol would be the most efficient in terms of total yield and purity. The RNA yield using M1 method was higher (55-375 µg per gram of fresh tissue) compared to the yield obtained with M2 (63-261 µg per gram of fresh tissue) (Table 1). The absorbance $A_{260/280}$ ratio ranged from 1.86 to 2.04 from M1. It indicated little or no protein contamination, while in the M2 method the ratio ranged from 1.00 to 2.02, indicating protein contamination in some cases. In all samples, the absorbance $A_{260/230}$ ratio values were higher than 1.90 with the M1, suggesting that the RNA is relatively free from polysaccharides and polyphenols contamination. However, the low $A_{260/230}$ ratio obtained from 1.00 to 2.02 in the M2 method, suggested polysaccharides and polyphenols contamination (Table 1). We evaluated the total RNA integrity on agarose gel electrophoresis. All samples clearly demonstrated a visible intact band, which represents 28S and 18S ribosomal RNA subunits (Figure 1c). Besides, RNA integrity was evaluated by determining the differential expression of phytochelatine synthase gene (PCS1) by RT-PCR. The RNA with high quality of M1 method was successfully used for cDNA synthesis and differential expression analysis of PCS1 for both mangrove plants (Figure 2; Table 1). The M2 method did not provide a RNA capable of being used as a template for cDNA synthesis, neither adequate for RT-PCR amplification (Figure 2; Table 1). The carbohydrate and polyphenol contamination was probably the cause for the RNA inability for subsequent experiments.

M1 method showed a much better RNA quality than M2 method since RNA pellet colour and soluble solution were white; M2 method was yellowish or sometimes brown. The brown colour indicated the

presence of proteins, carbohydrates and phenols compounds mixed with the extracted RNA after M2 method (Table 1). As described in Material and Methods, the basic difference between M1 and M2 methods is the extraction buffer (CTAB and SDS extraction buffer, respectively) and concentration of specific components. In the M1 method, the concentration was higher than M2. The relationship between the extraction buffer and the concentration of organic and inorganic salts can affect the performance of the physical properties and the ability to separate the complexes formed by nucleic acids and surfactants agents (CTAB and SDS) in aqueous solution (Zhang and Teng, 2008). In addition, hyposaline concentrations in these buffers do not allow the precipitation of contaminants (polysaccharides, phenolic compounds, and proteins) from the complex of nucleic acids and can be removed by extracting the aqueous solution with an organic phase (Aiello et al. 2010). On the other hand, brown colour and contamination (phenolic compounds) were reduced by adding of PVPP and high concentrations of β-mercaptoetanol in the extraction buffers. The PVPP produces complexes with polyphenols through hydrogen bonds, which allows to be separated from nucleic acids (Wang et al. 2005). β-mercaptoetanol was used to inhibit RNase activity and prevent sample oxidation as well since phenolic compounds are readily oxidized to form covalently linked quinines and avidly bond nucleic acids which caused irreversible damage to RNA (Gonzalez-Mendoza et al. 2008). An important step in the development of M1 method was the precipitation of the RNA with LiCl followed by the precipitation of sodium acetate. As confirmed in our results, these compounds was very effective in avoiding co-precipitation of polyphenols, polysaccharides, and nucleic acids resulting in the precipitation of RNA free from these contaminants (Table 1). Furthermore, these modifications allowed a significant improvement in both yield and quality of RNA from plant tissues of *A. germinans* and *L. racemosa*. In addition, our method has two major advantages over previous reports. We improved the extraction of RNA quality by increasing the yield of the extracts compared with reports for mangrove tissues (Fu et al. 2004; Yang et al. 2008). Also, this protocol is not complicated and can be completed in a period of seven to eight hours. These assets have an important difference with respect to others reported methods for mangrove plants, since they are more complex and require many hours to be completed (Banzai et al. 2002; Miyama et al. 2006; Miyama and Tada, 2008).

**Table 1. Spectrophotometric quantification of total RNA extracted from different tissues of mangrove plants.**

| Method | Plant species | Tissue | Absorbency ratios | | Total RNA[1] (µg/g FW) |
|---|---|---|---|---|---|
| | | | $A_{260}/A_{280}$ | $A_{260}/A_{230}$ | |
| **1** | *A. germinans* | Stem | 1.96 | 2.39 | 160 (34.8) |
| | | Leaf | 2.02 | 2.41 | 283 (23.3) |
| | | Root | 1.94 | 2.39 | 75 (19.4) |
| **1** | *L. racemosa* | Stem | 2.04 | 1.90 | 217 (49.7) |
| | | Leaf | 1.86 | 2.43 | 375 (75.6) |
| | | Root | 2.00 | 2.04 | 55 (14.1) |
| **2** | *A. germinans* | Stem | 1.97 | 1.20 | 163 (40.3) |
| | | Leaf | 1.94 | 1.35 | 154 (26.2) |
| | | Root | 2.02 | 0.49 | 63 (38.8) |
| **2** | *L. racemosa* | Stem | 1.16 | 0.32 | 121 (2.1) |
| | | Leaf | 1.00 | 0.30 | 261 (86.2) |
| | | Root | 1.65 | 0.71 | 106 (42.4) |

[1]Average of three independent repetitions, with standard deviation between brackets.

In conclusion, the modified method 1 developed in this study allowed the isolation of intact, high yield and quality RNA of leaves, stem and roots from two mangrove plants (*Avicennia germinans* and *Laguncularia racemosa*). The introduction of some modifications, previously described in the literature, to avoid precipitation of RNA, that consisted in of the use of LiCl and sodium acetate proved to be decisive for success in RNA isolation. The results show that this protocol can be applied to some other plant tissues containing high phenolic and polysaccharide compounds for further RT-PCR analysis and molecular studies. This method is effective, simple, and requires non-specialized equipment.

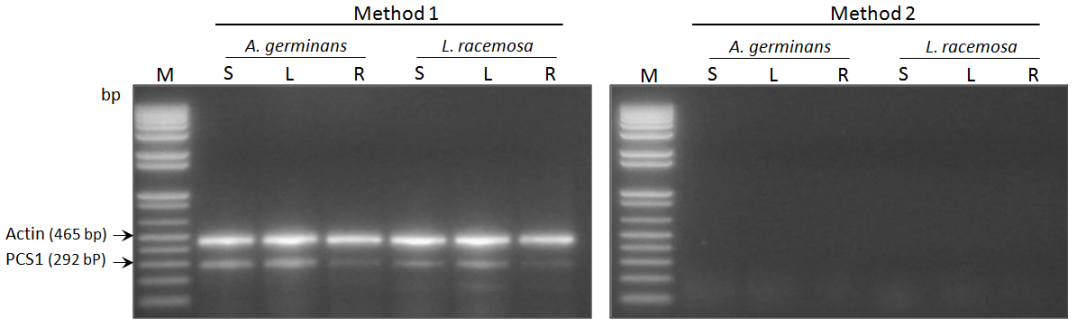


**Fig. 2 Agarose gel electrophoresis of the RT-PCR analysis.** RT-PCR amplification of phytochelatine synthase 1 gene fragment from tissues of *A. germinans* and *L. racemosa*. The arrows indicate the actin and PCS1 genes. S: stem, L: leaf, R: root. M: molecular marker (1 kb) in base pairs (bp).

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