

An Efficient Protocol for Isolation of Functional RNA from Peel Tissue of Different Banana (*Musa* spp.) Cultivars for Gene Expression Studies on Anthracnose Development

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Abstract: Extraction of good quality RNA in larger quantities is a prerequisite for gene expression studies. Existing protocols for RNA extraction from banana pulp tissues were not successful on peel tissues of banana (*Musa* spp.) as they contain higher concentrations of polyphenols, polysaccharides and latex. This study developed a new protocol by modifying the existing protocols. The modifications included combining of pre-warmed Tris-Borate extraction buffer, incorporation of CTAB in the extraction buffer, incubation in extraction buffer at 65°C for one hour, and a three-day long extraction procedure with phenol, phenol:chloroform (1:1) and chloroform:isoamyl alcohol (24:1) together with centrifugation steps at high speeds (i.e. 12,000 –14,000 rpm). Spectrophotometric analysis of the extracted RNA, denaturing agarose gel electrophoresis, cDNA library construction, sequence information of cDNA inserts and RT-PCR confirmed the quality of RNA extracted by the method developed in the present study for gene expression work. Furthermore, it is shown that the developed method is useful to extract good quality RNA from peel tissues of a range of dessert- and cooking-type banana cultivars.

Keywords: cDNA library construction, *Colletotrichum musae*, RNA extraction, RT-PCR

INTRODUCTION

Banana (*Musa* spp.) is grown in more than 120 countries worldwide and has been ranked second in world fruit production (Lassoos *et al.*, 2010), and is a vital source of income, employment, and export revenue for many developing countries in Latin America, West Indies, Southeast Asia and Africa. In Sri Lanka, 29 banana cultivars have been reported and among them 22 cultivars are used as dessert banana (*et al.*, 2002). Anthracnose is one of the most economically significant postharvest diseases of dessert banana in Sri Lanka. As reported by Anthony *et al.* (2004), the postharvest diseases were responsible for 20% of yield losses in Sri Lanka in 1997. *Colletotrichum musae* has been reported as the causal organism of anthracnose (Coomaraswamy *et al.*, 2009). Studies based on natural infections and artificial inoculation of *C. musae* have revealed that the incidence and severity of anthracnose development at the postharvest stage vary among local banana cultivars (De Costa and

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Chandima, 2014). Therefore, studies at gene expression level are essential to elucidate the genetic basis of differential anthracnose development in different banana cultivars.

Isolation of high quality RNA is an important prerequisite for gene expression analyses. However, isolation of high quality RNA from plant tissues rich in polysaccharides and polyphenolic substances is often difficult (Asif *et al.*, 2000). The presence of latex in addition to the above substances affects the quality and the quantity of the RNA extracted. Several methods developed by Doyle and Doyle (1987), Fütterer *et al.* (1995), Asif *et al.* (2000) and Liu *et al.* (1998) have been used to extract total cellular RNA from tissues rich in polysaccharides and polyphenols. Moreover, isolation of RNA for genomic studies has been reported from fruit pulp tissues of banana (Clendennen and May, 1997). However, no method has been reported to isolate RNA for functional studies from banana peel tissues, which are rich in polyphenolic compounds and latex. The amount and specific composition of different polyphenolic compounds in banana peel tissues could vary with the type of banana cultivar (*i.e.* dessert- or cooking-type) and the maturity stage of the fruit (Iandolino *et al.*, 2004). This further complicates the extraction of total cellular RNA for gene expression studies aimed at determining the genetic basis of differential anthracnose development in different banana cultivars. The major problem in RNA extractions is the co-precipitation of polysaccharides and polyphenols together with RNA (Fütterer *et al.*, 1995; Liu *et al.*, 1998). These compounds bind to RNA and render it unsuitable for gene expression analysis (Vasanthaiya *et al.*, 2008). Therefore, the objective of the present study was to develop an efficient protocol for isolation of high quality functional RNA in higher quantities from banana fruit peel tissues obtained from a range of cultivars for subsequent functional studies, namely construction of a cDNA library, identification of EST sequences towards identification of putative gene responsible for possible resistance to anthracnose development in some banana varieties and also for RT-PCR analyses

METHODOLOGY

Plant tissues

Well-grown and fully-matured *i.e.* at Maturity index 1 according to the standard charts by Kader (2005), banana bunches of cultivars *Seenikesel* and *Kolikuttu* (*Musa acuminata*) and cultivar *Alukesel* (*M. balbisiana*), which were free from external blemishes, were collected randomly from three semi-commercial plantations in Kandy, Sri Lanka. In the field, the banana bunches had been covered with plastic sleeves to avoid natural inoculation of *C. musae*. In the case of banana, placing a perforated plastic sleeve around bunches limits the circulation of rainwater and reduces the level of fruit contamination with *C. musae* by more than 82% (de Lapeyre de Bellaire *et al.*, 2000).

Banana fruits (*i.e.* fingers) were separated from banana hands and washed with running tap water. Surface sterilization was done by dipping in 10% commercial bleach solution for 5 min followed by rinsing in sterilized distilled water and subsequent air drying. The surface sterilized fingers were inoculated with an aliquot of 10 μ l (10^5 spores ml^{-1}) of *C. musae* spore suspension. Two spots per finger were inoculated. Twenty one fruits were inoculated and incubated in incubation chambers at room temperature (28°C) and 90% RH. Using three fruits per a given time period, peel tissues were collected from the immediate surroundings of the point of inoculation at one hour before and 1, 24, 48, 96, 144, and 192h after inoculation of *C. musae* to determine the sequence of gene expression along different time intervals, just before and also from the onset of inoculation until symptom development. The

collected peel tissues were ground separately into a fine powder, with an adequate amount of liquid nitrogen in a 0.1% Diethylpyrocarbonate (DEPC)-treated motor and pestle and stored at -80 °C until further use. The extracted RNA samples were pooled using equal concentrations from each time interval to be used for extraction of mRNA. The extracted RNA was used for a cDNA library construction and subsequent identification of EST sequences that allow identification of putative genes responsible for possible resistance to anthracnose development in some banana cultivars and also for RT-PCR analyses.

Extraction method No. 1

The RNA extraction method described by Fütterer *et al.* (1995) was employed as the method No 1 on banana peel tissues of the three cultivars (*i.e.* *Seenikesel*, *Kolikuttu* and *Alukesel*). An aliquot of 0.5 ml of the extraction buffer [1M TrisHCl (pH 9.0), 50 mM EDTA (pH 8.0), 1% SDS (w/v)] and 0.5 ml of Phenol:Chloroform:Isoamyl alcohol (24:24:1) was added into the Eppendorf tube containing 500mg of fine powder of banana peel tissues. Content of the tube was shaken well and placed on ice for 1 hour with shaking at every 10 min. Then the samples were centrifuged for 5 min at 10,000 rpm (24x3g) under 4 °C. The aqueous phase was separated and extracted with 0.5 ml of Phenol: Chloroform: Isoamyl alcohol (24:24:1) as described above. Supernatant was collected and re-extracted with 0.5 ml of Chloroform:Isoamyl alcohol (24:1). The supernatant was collected and 1/10 volume of 4M LiCl and 2 volumes of 100% ethanol were added, mixed well and incubated for 30 min at -70 °C. The samples were then centrifuged for 5 min at 10,000 rpm at 4 °C. Then the pellet that appeared at the bottom of the Eppendorf tube was dissolved in 750 µl DEPC-treated water, 300 µl of 4M LiCl was added, mixed well, incubated overnight at 4 °C. Then the samples were centrifuged at 10,000 rpm for 5 min at 4 °C. Subsequently, the pellet was washed with 100% ethanol and centrifuged at 10,000 rpm for 5 min at 4 °C. Finally, the pellet was dried and dissolved in 10 µl DEPC water and stored at -80 °C.

Extraction method No. 2

The RNA extraction method No. 2 was attempted by combining the methods described by Liu *et al.* (1998) and Asif *et al.* (2000). An aliquot of 600 µl of extraction buffer [100 mM TrisHCl (pH 7.5), 500 mM NaCl, 25 mM EDTA (pH 8.0), 1.5% SDS, 2% PVP, 0.7% β-mercaptoethanol] was mixed with 500mg of fine powder of banana peel tissues of cultivars *Seenikesel*, *Kolikuttu* and *Alukesel*. The content was placed on ice with occasional shaking for 1 hour and then centrifuged at 10,000 rpm (24x3g) for 15 min at 4°C. The supernatant was retained, 1/3 volume of 3 M NaAc (pH 5.2) was added, mixed well and kept on ice for 30 min followed by centrifugation at 10,000 rpm for 15 min at 4°C. The supernatant was transferred to a new tube extract with an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1) and mixed by vigorous vortexing. Afterwards, the phenol and aqueous phase was separated by centrifugation at 10,000 rpm for 10 min. at 4°C. The aqueous phase extracted was mixed with an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged at 10,000 rpm for 10 min at 4 °C. The aqueous supernatant (upper) phase was transferred to a new tube, 2-3 volumes of absolute ethanol were added and stored for 30 min at -80 °C to precipitate RNA. The RNA was pelleted by centrifuging at 10,000 rpm for 15 min at 4 °C and the pellet dissolved in DEPC-treated water was re-extracted with phenol:chloroform:isoamyl alcohol (24:24:1) and chloroform:isoamyl alcohol (24:1) and precipitated with absolute ethanol as given in Method No. 1. Inclusion of additional re-extraction steps with phenol and chloroform helped removing contaminants and recovering a higher quantity of RNA. Inefficient removal of polysaccharides and polyphenolic compounds results in co-precipitation with RNA, which affects its yield and

quality (Logemann *et al.*, 1987). Thereafter, the RNA was pelleted, the pellet was washed twice with 70% ethanol and air dried. Then the pellet was dissolved in 10 µl DEPC treated water and stored at -80 °C.

Extraction method No. 3

This extraction method No 3 attempted to extract functional RNA from peel tissues of different banana cultivars of dessert-(i.e. *Seenikesel* and *Kolikuttu*) and cooking- (*Alukesel*) types. In this method, 0.1% βMercaptoethanol was added to the extraction buffer [100mM Tris-Borate (pH 8.2), 1.4 M NaCl, 20 mM EDTA (pH 8), 2% (w/v) CTAB] prior to pre-warming of the extraction buffer at 65°C for 30 min. An aliquot of 1 ml of pre-warmed extraction buffer was added to 500mg of powdered banana peel tissues, mixed thoroughly by vortexing and incubated at 65°C for one hour. Samples were cooled to room temperature and centrifuged at 12,000 rpm (24x3g) for 15 min at room temperature. The supernatant was collected into new Eppendorfs and extracted twice with chloroform:isoamyl alcohol (24:1). After centrifuging at 12,000 rpm for 15 min at room temperature, the supernatant was separated into new Eppendorfs and mixed with 10 M LiCl to a final concentration of 3M. Contents were incubated overnight at 4°C and RNA was recovered by centrifuging at 14,000 rpm for 20 min at 4°C. The supernatant was decanted carefully and the pellet was dissolved in 500µl of DEPC-treated water and extracted thrice with phenol, phenol:chloroform (1:1) and chloroform:isoamyl alcohol (24:1), respectively. The supernatant was mixed with 1/30 volume of 3M NaAc (pH 5.2) and 0.1 volume of absolute alcohol. The mixture was kept on ice for 30 min and centrifuged at 14,000 rpm for 25 min at 4°C. The supernatant was collected carefully without disturbing the white jelly like precipitate and mixed with 3 M NaAc (pH 5.2) to a final concentration of 0.3 M and 3 volumes of absolute alcohol. The mixture was incubated at -80°C overnight and RNA was recovered by centrifuging at 14,000 rpm for 20 min at 4°C. The RNA was rinsed with 70% ethanol and recovered by centrifuging at 10,000 rpm 5 min at 4°C. Ethanol was removed by air drying the sample and the pellet was dissolved in 10 µl of DEPC-treated water and the RNA was treated with RQ-1 DNase (Promega).

Analyses of Quality and Quantity of the Extracted RNA

Purity and integrity of the extracted RNA

The concentration and purity of the extracted RNA samples were quantified spectrophotometrically (Biospecnano spectrophotometer, Shimadzu, Japan) at A_{230} , A_{260} and A_{280} nm by diluting the RNA sample in DEPC-treated water to detect polysaccharide/polyphenolic contaminations and protein contaminations. The absorption ratios $A_{260}/_{230}$ and $A_{260}/_{280}$ were used to detect polysaccharide/polyphenolic contaminants and protein contaminants, respectively (Iandolino *et al.*, 2004; Zhen *et al.*, 2011). The ratio of $A_{230:260:280}$ of the RNA samples was also quantified as a measure of purity (Iandolino *et al.*, 2004). Integrity of the RNA samples was assessed on a 2% denaturing formaldehyde agarose gels by electrophoresis at 50V (Vasanthaiiah *et al.*, 2008).

Construction of cDNA library

The extracted total cellular RNA from peel tissues of banana collected at different times of inoculation with *C. musae* was purified by magnetic bead- mRNA purification kit (DynaL Biotech ASA, Invitrogen, USA) and cDNA synthesis was done by Superscript first strand synthesis system for RT-PCR (Invitrogen, USA). The cDNA library construction was done using pDONRTM 222 cloning vector by Cloneminer 11 cDNA library construction kit

(Invitrogen, USA). The constructed cDNA library mix was dispensed into several Eppendorf tubes and stored at -80°C until further use. Titer of the cDNA library mix was checked by spreading a 25 µl of cDNA library mix on LB medium supplemented with Kanamycin (50 µgml⁻¹). Size of the cDNA inserts in clones of the cDNA library was estimated by selecting ten random cDNA clones. The cDNA inserts of the selected clones were amplified using M13 specific primers as M13 sequences are flanking the cDNA insert region of the cDNA clones constructed using pDONRTM 222 vector. A few of the amplified cDNA inserts (*i.e.* PCR products) were subjected to DNA sequencing and subsequent homology search with NCBI BLAST and ESTik BLAST databases (www.musagenomics.org/esttik.cirad.fr). Accession numbers of the sequenced PCR products of five selected cDNA clones were obtained from the EMBL (*i.e.* European Molecular Biology Laboratories, UK).

RT-PCR analysis

Total cellular RNA from extraction method No 3 was purified by the magnetic bead-mRNA purification kit and mRNA was obtained. The cDNA was synthesized from 2 µl of total RNA extracted by the extraction method No3 using peel samples of cultivars *Seenikesel*, *Kolikuttu*, *Emban*, *Embul*, and *Alukeselby* Superscript RT First Strand Synthesis kit (Invitrogen, USA). Quality of the extracted mRNA was assured by the construction of cDNA from purified mRNA. Concentration and purity of cDNA were measured using a Biospecnano spectrophotometer (Shimadzu, Kyoto, Japan). Furthermore, the success of cDNA synthesis was confirmed by amplifying the housekeeping genes (*i.e.* Internal control genes for RT-PCR) *RPS-2* gene using the primer pair 5'- TAG GGA TTC CGA CGA TTT GT-3' and 5'- TAG CGT CAT CAT TGG CTG GGAG-3' and *MaEBF-1* gene using the primer pair 5'- CCT CAA TAA GCG ATT ACG GTG-3' and 5'-TCA GAC TCC CAA GAG ATT CC-3' (Chen *et al.*, 2011) with the synthesized cDNA. Thereafter, one of the putative genes deduced based on the sequence information of a cDNA insert of a selected clone was amplified by RT-PCR. The putative gene of the ETS sequence (Accession No. HF567810) given by the selected cDNA clone was homologous to Gama glutamyltransferase gene (GGT) and the following primer pair was designed for the amplification of putative Gama glutamyltransferase gene (*i.e.* 5'-TAT GGA AGA CCC TTG GAT-3' and 5'-CAC TTT CTT CCA TGG CAC CT-3'). The PCR reaction conditions were maintained for 40 cycles at 95 °C for 2 min, denaturation at 95 °C for 30 sec, annealing at 60°C for 30 sec, extension at 72 °C for 2 min and final extension at 72 °C for 5 min for amplification of the *RPS-2* and *MaEBF-1* genes. Amplification of the GGT transcript was done under the same PCR conditions with the exception of the annealing temperature of 58 °C for 30 sec.

RESULTS AND DISCUSSION

Purity and integrity of RNA

Purity and concentration of the RNA, extracted by the three methods and determined spectrophotometrically, are given in Table 1.

Table 1. Yield and quality of total RNA extracted by the three methods from peel tissues of banana fruits of cultivars *Seenikessel*, *Kolikuttu* and *Alukesel*.

Protocols	Cultivar	A 260/230*	A 260/280*	A ₂₃₀ :A ₂₆₀ :A ₂₈₀ *	Yield (µg/g FW)*
Extraction method No. 1	<i>Seenikessel</i>	0.65	1.09	1:1.4:1.8	28.94
	<i>Kolikuttu</i>	0.58	1.15	1:1.4:1.8	29.42
	<i>Alukesel</i>	0.25	0.65	1:1.4:1.8	19.81
Extraction method No. 2	<i>Seenikessel</i>	1.35	1.38	1:0.6:1.7	56.40
	<i>Kolikuttu</i>	1.30	1.32	1:0.6:1.7	58.23
	<i>Alukesel</i>	0.82	1.04	1:0.6:1.7	48.68
Extraction method No. 3	<i>Seenikessel</i>	2.03	2.01	1:2:1	238.10
	<i>Kolikuttu</i>	1.92	2.08	1:2:1	242.62
	<i>Alukesel</i>	1.68	1.65	1:2:1	108.36

*Results are expressed as the mean values of extractions from three different cultivars.

Absorption ratios of A₂₆₀/A₂₃₀, A₂₆₀/A₂₈₀ and A₂₃₀:A₂₆₀:A₂₈₀ are measures to determine RNA quality. A₂₆₀/A₂₃₀ and A₂₆₀/A₂₈₀ ratios near 2.0 were given by the method No. 3, indicating the absence of significant contaminations with polysaccharides and phenolic materials respectively, in the RNA extracted. Normally a 1:2:1 ratio in the A₂₃₀:A₂₆₀:A₂₈₀ is expected in high purity RNA isolates and such a ratio was observed in the extraction method No. 3.

The RNA extracted by methods Nos. 1, 2 and 3 from cultivars *Seenikessel*, *Kolikuttu* and *Alukesel* are shown in Figures 1a, 1b and 1c, respectively, when separated on a 2% denaturing agarose gel. The RNA extracted by the methods 1 and 2 (Figures 1a and 1b) and analyzed on 2% denaturing agarose gel, did not show clear and intense bands representing 18s and 28s rRNA along with a smear showing additional rRNA (between 18s and 5s rRNA) that should be present in a high quality RNA sample (Vasanthaiiah *et al.*, 2008). In comparison to methods No. 1 and 2, the protocol implemented in method No. 3 gave a better separation of 28s rRNA and 18s rRNA bands on 2% denaturing agarose gel. In addition to the intense, resolved ribosomal bands, a smear of RNA could be observed and genomic DNA closer to the wells of the gel is not visible (Figure 1c).

During the extraction of RNA by extraction methods No. 1 and 2, the final RNA precipitate developed a brown colour. This indicated that purification steps of methods No. 1 and 2 are not sufficient to remove all polysaccharides and polyphenols in the samples.

The method No. 3 was able to extract the total cellular RNA from a range of banana cultivars namely, *Seenikessel* (239.85 µg/g), *Emban* (109.57 µg/g), *Kolikuttu* (241.72 µg/g), *Embul* (106.20 µg/g) and *Alukesel* (107.93 µg/g). Denaturing agarose gel electrophoresis indicated that the extracted RNA was of good quality and integrity to be used in downstream applications such as cDNA construction, sequencing and gene expression analysis by RT-PCR (Fig. 2).

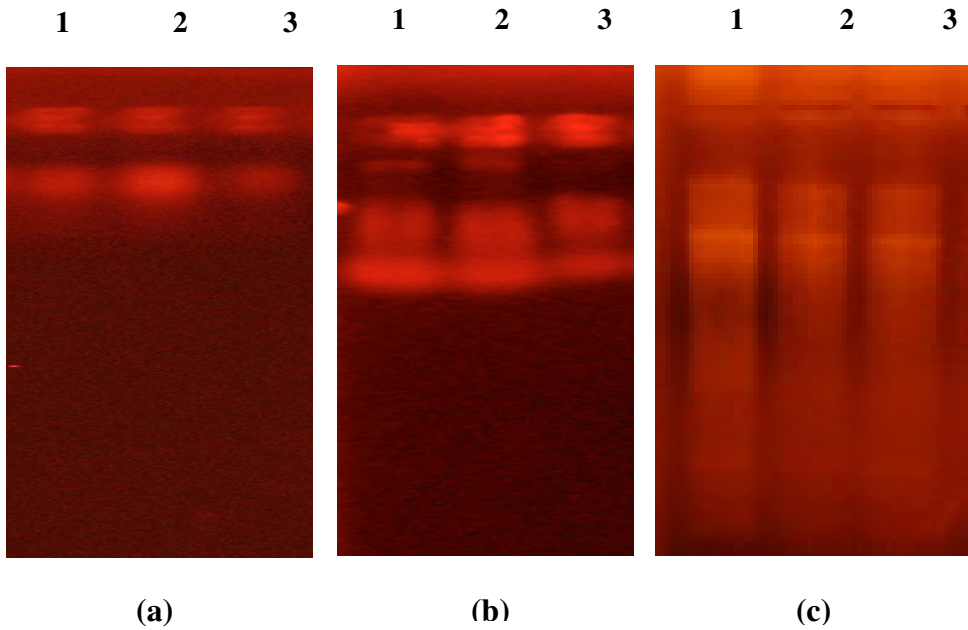


Fig. 1. Total cellular RNA (8 μ l RNA) extracted from the extraction methods No. 1 (a), 2 (b) and 3 (c), and analyzed by 2% denaturing agarose gel electrophoresis. Lanes 1-3 indicate the RNA extracted from banana cultivars *Seenikesel*, *Kolikuttu* and *Alukesel*, respectively.

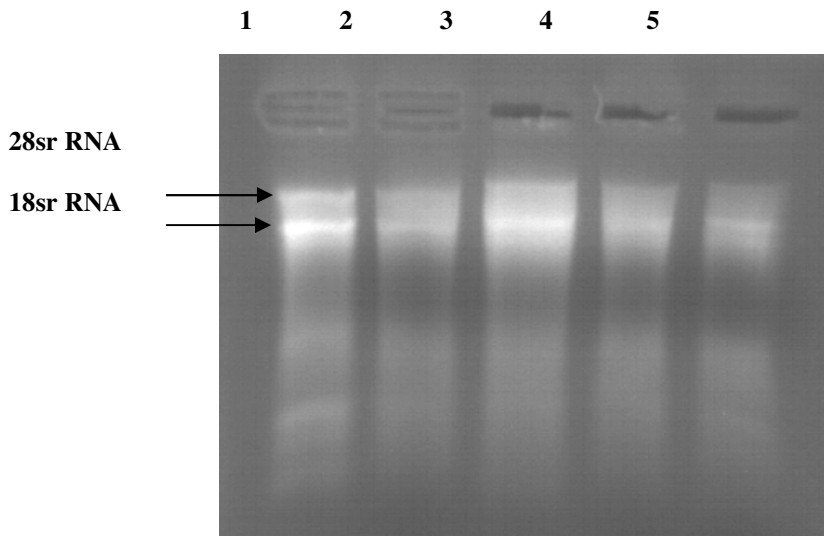


Fig. 2. Total cellular RNA extracted from the developed protocol (*i.e.* extraction method No. 3) analyzed by 2% denaturing agarose gel electrophoresis (8 μ l of total cellular RNA). Lanes 1-5 contain RNA extracted from banana cultivars *Seenikesel*, *Emban*, *Kolikuttu*, *Embuland* and *Alukesel*, respectively.

After verifying the quality of RNA samples, the mRNA were extracted from paramagnetic particle technique using Dynal beads. The concentration of poly (A) + RNA was 0.24µg/µl. Then, 2.4µg of poly(A)+ RNAs was reverse transcribed in corresponding single stranded cDNAs and the doubled stranded cDNA (ds cDNA) that was synthesized and analyzed in 2% agarose gel showed that the size of cDNA fragments ranged from 100 kb to 1500 kb (Fig. 3).

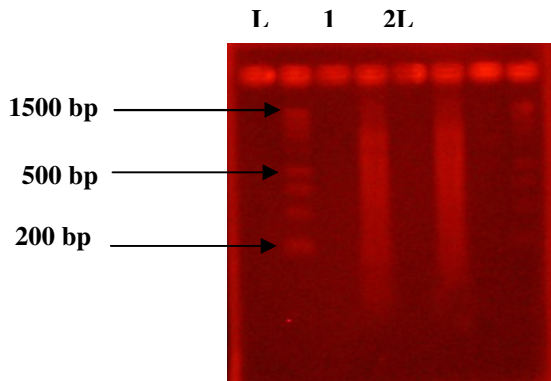


Fig. 3. Range of cDNA fragment size when analyzed on a 2% agarose gel. L- 100 bp marker (Promega), Lanes 1 and 2 – cDNA constructed from RNA samples extracted from cultivar *Seenikesel* by extraction method No. 3.

Quality of cDNA library

The cDNA library was constructed from the generated cDNA from extracted mRNA by using method No. 3 and it had a titer of 6.8×10^6 cfu/ml. Fig. 4 shows the sizes of cDNA inserts of 10 selected cDNA clones. Inserts of the transcripts in cDNA clones ranged from 400 to 1200 bp. Moreover, the sequencing and homology search of PCR products of several transcripts of the selected cDNA clones revealed that they were homologous with transcripts of the *Musa* genome (Table 2). This indicates the successful construction of a cDNA library from the extracted mRNA.

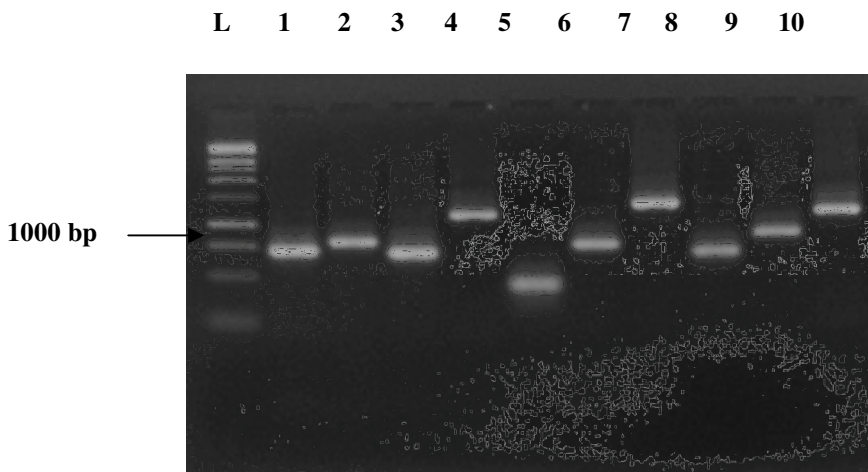


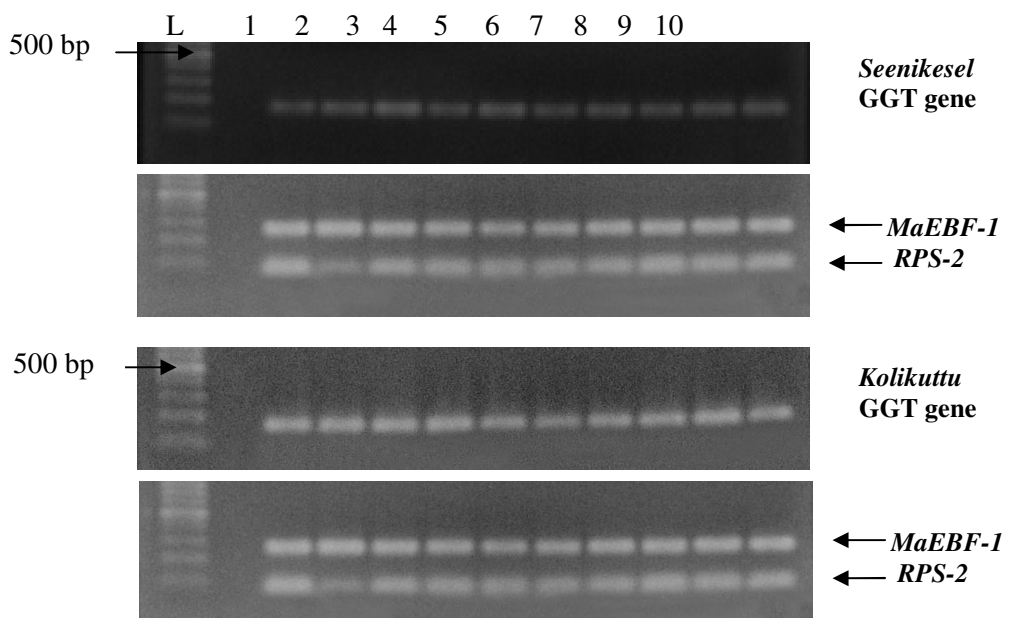
Fig. 4. PCR products amplified with M13 specific primers to determine the size of cDNA insert of ten selected cDNA clones (1-10). L- 1 kb ladder (Promega).

Table 2. Sequencing and homology search of PCR products of several transcripts of the selected cDNA clones that were homologous with transcripts of the *Musa* genome

Clone number	Accession Number	Putative protein/ Homologous host genome	% Similarity
<i>MaSINI</i> 184	HF567809	Beta-1,3 Glucanase / <i>Musa acuminata</i>	98 (2.00E-63)
<i>MaSINI</i> 507	HF567824	Mitochondrial ATP synthase G subunit family protein / <i>Musa balbisiana</i>	92 (5.00E-61)
<i>MaSINI</i> 565	HF567826	Expansin1 / <i>Musa acuminata</i>	71 (2.00E-67)
<i>MaSINI</i> 680	HF567833	Germin-like protein/ <i>Musa acuminata</i>	94 (7e-101)
<i>MaSINI</i> 693	Accession#: HF567835	Endochitinase / <i>Musa</i> <i>acuminata</i>	96 (7.00E-132)

The cDNA constructed from total cellular RNA extracted from the extraction method No. 3 and its mRNA had a A_{260}/A_{230} ratio greater than one, and the ratio of A_{260}/A_{280} ranged from 1.65 to 1.8. As reported by Asif *et al.* (2000), the ratio of A_{260}/A_{230} indicated that the RNA extractions are free from polysaccharides and polyphenols. The OD ratio of A_{260}/A_{280} is also at a satisfactory level. The integrity of the synthesized cDNA assessed by 2% agarose gel is illustrated in Fig. 3.

Fig. 5 shows the PCR amplification of internal control genes *MaEBF-1*, Ribosomal Protein S-2 (*RPS-2*) and gene of interest GGT genes using the synthesized cDNA from cultivars *Seenikesel*, *Kolikuttu*, *Emban*, *Embul*, *Suwadela* and *Alukeselas* the template DNA under inoculated and non-inoculated conditions of *C. musae*. The expected sizes of the PCR products of *MaEBF-1* (280bp), *RPS-2* (84bp) and GGT (171 bp) could be obtained indicating that successful amplification of the genes was possible.



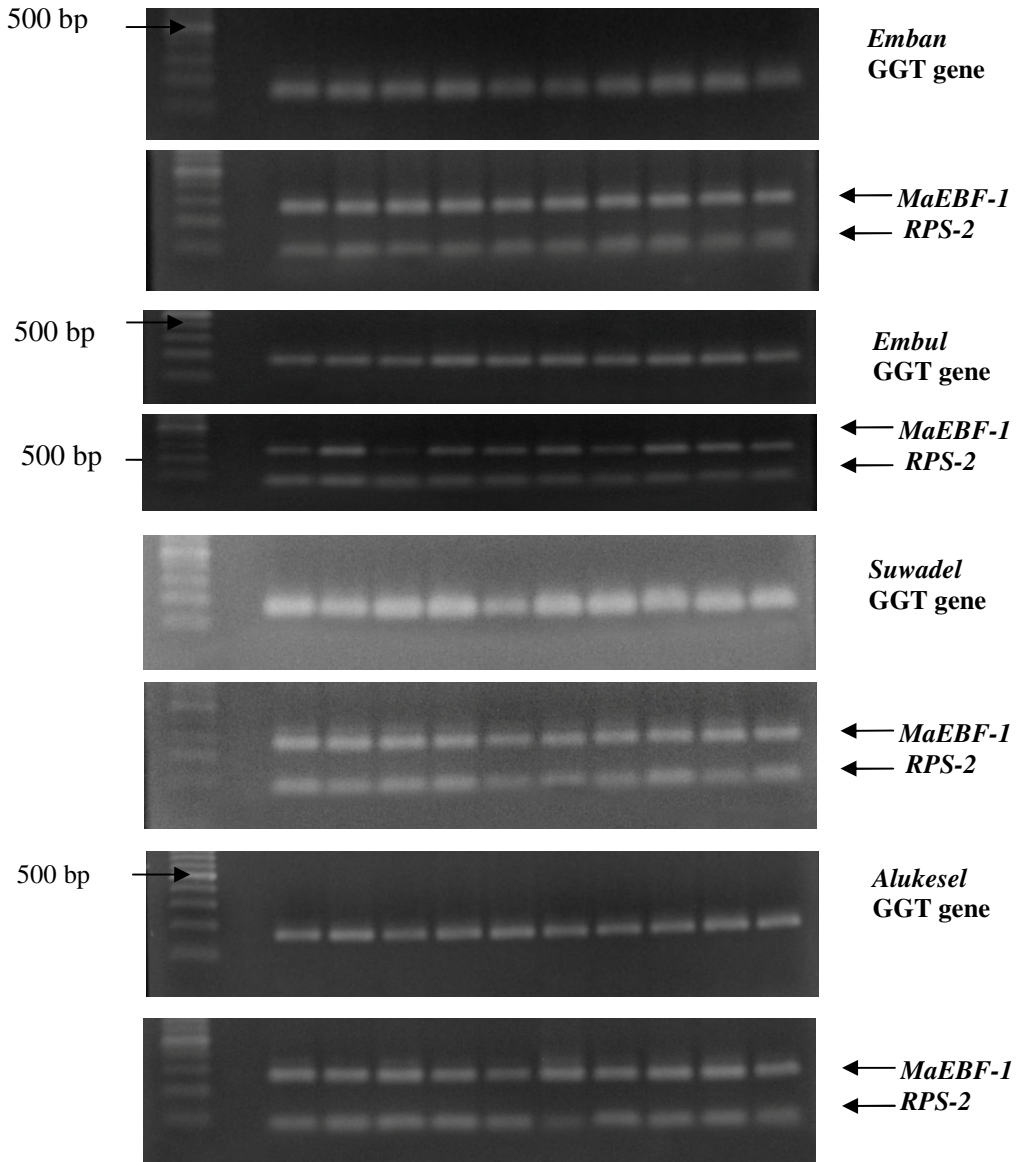


Fig. 5. RT-PCR products of the internal control genes *MaEBF-1*, Ribosomal Protein S 2 (*RPS-2*) and gene of interest Gama glutamyltransferase (GGT) transcript from cDNA constructed from the total RNA isolated from cultivars *Seenikesel*, *Kolikuttu*, *Emban*, *Embul*, *Suwadel* and *Alukesel* by extraction method No. 3 with control and treatment cDNA samples for five time points. Lane L: 100bp DNA ladder (Promega), lane 1-5 control samples: 1HAI, 24HAI, 48HAI, 96HAI, 144HAI respectively, lane 6-10 treatment samples: 1HAI, 24HAI, 48HAI, 96HAI, 144HAI respectively. cDNA was generated from 1µg of template RNA. Total volume of PCR product was separated on 2% agarose gel and electrophoresis was performed for 45 min at 50V.

Different plant species and different parts of the same plant have specific physical and chemical characteristics of their own and contain various endogenous substances (Hughand Robert, 2006). Therefore, extraction methods to obtain good quality RNA should be designed to suit the specific physico-chemical properties of the tissue from which RNA is extracted. In the present study, three different extraction methods were tested for their effectiveness in extracting good quality RNA from banana peel tissue in which RNA extraction by standard established methods is difficult due to the presence of high concentrations of polyphenols, polysaccharides and latex. Our results have confirmed the success of extraction method No.3, which was developed through this study. This protocol is a modification of several RNA isolation protocols from plants as reported by Fütterer *et al.* (1995), Liu *et al.* (1998) and Asif *et al.* (2000).

The extraction method No.3 gave an adequate RNA yield ranging from 100 to 250 $\mu\text{g g}^{-1}$ FW, which is on par with RNA yields of successful extraction methods reported previously (Iandolino *et al.*, 2004; Vasanthaiah *et al.*, 2008). The spectrophotometric analyses and electrophoresis by denaturing gel electrophoresis confirmed the quality of the extracted RNA by method No.3 based on absorbance ratios and separation of ribosomal RNA bands (Vasanthaiah *et al.*, 2008). Furthermore, RNA extracted from method No.3 had A_{260}/A_{230} and A_{260}/A_{280} ratios of 1.87 and 1.92, respectively, which are within the expected range of samples having no contaminations of polysaccharides, polyphenolics and proteins. These levels of quality and integrity of extracted RNA are comparable to those obtained by standard methods by many other workers (Zhen *et al.*, 2011; Gesteira *et al.*, 2003; Vasanthaiah *et al.*, 2008; Meisel *et al.*, 2005).

The method No. 2 employed in this study is a combination of the methods used by Liu *et al.* (1998) and Asif *et al.* (2000) to extract RNA from banana plant tissues. However, in the present study, this method failed to yield good quality RNA from banana peel tissues especially when the peel tissue sample had a combination of mature and ripen stages. The banana peel tissue undergoes significant changes in structural and soluble metabolites such as polyphenols, polysaccharides and specific proteins during the fruit maturing phase (Iandolino *et al.*, 2004). Hence, peel tissues collected from different maturity stages and from different varieties could have significant variations in the above chemical compounds which inhibit or lower the efficiency of RNA extraction.

Several specific features of extraction method No. 3 may have contributed to its ability to extract high quality RNA suitable for functional genomics studies such as cDNA library construction and RT-PCR. Firstly, it used pre-warmed extraction buffer, which removes only the contaminating phenols and proteins without affecting the RNA yield (Vasanthaiah *et al.*, 2008). Moreover, pre-warmed extraction buffer helps to inactivate RNase activity which is high in mature plant tissues (Vasanthaiah *et al.*, 2008). The extraction buffer used in method No. 3 contained boric acid, which together with β -mercaptoethanol, facilitates the recovery of RNA by enhancing the dissolution of cell walls in peel tissues of different maturity stages and by the denaturation of proteins. Further, it inhibits oxidation of the endogenous phenolic compounds and subsequent binding of phenolics to RNA (Wan and Wilkins, 1994). Moreover, polyphenol oxidase activity can be reduced by the highly alkaline pH of the extraction buffer (Gesteira *et al.*, 2003). The extraction buffer used in the method No. 3 contained CTAB, which is a surfactant used as a cell disrupting agent in RNA extraction protocols of recalcitrant plant species and plants with high polyphenolic compounds and polysaccharides (Rubio-Piñ and Zapata-Pérez, 2011). Therefore, CTAB included in the extraction buffer of method No.3 has an advantage over the extraction buffers used in the other two methods. These could have contributed to the higher quantity of extracted RNA

per unit fresh weight of peel tissue and to the absence of a brown colour appearance in the final RNA precipitate of extraction method No.3. The brown colour indicated the presence of proteins, carbohydrates and phenolic compounds mixed with the extracted RNA (Rubio-Piñ and Zapata-Pérez, 2011).

Furthermore, the method No. 3 has an extraction period of up to three days with overnight precipitation, to recover adequate quantity of RNA. According to Vasanthaiah *et al.* (2008), for mature and disease-infected tissues that contain higher amounts of polyphenols, polysaccharides and other interfering compounds, it is necessary to extend the extraction period up to three days. In the extraction method No. 3, more extraction steps with phenol/chloroform/isoamyl alcohol were involved. Also, the addition of sodium acetate used at two steps of the extraction procedure may have contributed positively for elimination of polysaccharides which is the role of sodium acetate in RNA protocols (Rubio-Piñ and Zapata-Pérez, 2011). Moreover, the method No. 3 used higher centrifugation speeds (*i.e.* 12000 – 14000 rpm) in comparison to the other two methods (*i.e.* 10000 rpm). Above modifications of the RNA extraction in method No. 3 could have improved its quality and quantity of RNA extracted in comparison to the RNA obtained from the other two methods.

CONCLUSION

Extraction of good quality RNA from banana fruit peel tissues is a difficult task due to their higher polyphenol and polysaccharide concentrations. The modified protocol (*i.e.* extraction method No. 3) with the inclusion of a high concentration of pre-warmed Tris-Borate extraction buffer containing CTAB and a three-day extraction step yielded adequate quantities of good quality RNA. Intact RNA, $A_{260/230}$ nm ratios greater than one and $A_{260/280}$ ratios of almost two, cDNA profile, sequence data of cDNA inserts, successful RT-PCR of a housekeeping genes and a putative Gama glutamyltransferase (GGT) gene confirmed the quality of RNA. This method is especially useful for extracting RNA from a combination of mature green, ripe and disease-infected peel tissues of a range of banana cultivars including both desert and cooking-types.

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