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# Efficient and simple procedure for isolation of RNA from pulp and peel tissues of ripe banana

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**ABSTRACT :** Sensitive techniques of molecular biology, such as identification of differentially expressed genes, transcriptional profiling etc., require a high quality RNA in suitable quantities. Isolation of good quality RNA from banana pulp and peel tissues is troublesome and challenging owing to rich phenolic compounds and polysaccharides that coprecipitate with nucleic acids. Interaction of phenols with nucleic acids leads to oxidation and degradation of RNA making it unsuitable for downstream processes. We have developed a protocol to isolate good quality RNA from banana fruit pulp and peel tissues. This involves two precipitation steps with sodium acetate with 100 per cent ethanol and reducing the precipitation time which led to the reduction in loss of RNA and risk of degradation. The protocol developed is simple, fast and can extract 81.85 and 40.54 µg/g of pulp and peel tissues, respectively. The absorbance ranged from 1.9-2.0 at the ratio of 260/280 indicating very high quality of RNA suitable for molecular analyses. RNA purity was confirmed through reverse transcription-polymerase chain reaction (RT-PCR) by using β-1,3 glucanase primer pair. The clear banding pattern obtained in RT-PCR analysis revealed that RNA isolated through this protocol could be used for further downstream processes.

**KEY WORDS :** Banana, Ripe fruit, Pulp, Peel, RNA isolation

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Hybridization-based approaches and next generation sequencing have become handy for the recent progress in banana research that is mainly focused on understanding various molecular level mechanisms of diverse biological processes. Most of these approaches require RNA as their starting material and for example, a successful transcriptional profiling largely relies on getting high yield of clean, intact, high-quality RNA molecules suitable for reverse transcription, labelling and hybridization. Cruz *et al.* (1997) reported that despite the availability of lower concentrations of RNA molecules in fruit tissues, there are other challenges in isolating RNAs owing to the presence of higher concentrations of lipids, sugar, phenolic compounds and polysaccharides. The polysaccharides have densities and ion-binding properties similar to those of RNA, and the phenolics bind to macromolecules by hydrogen or covalent bonding when quinones form upon oxidation (Noonberg *et al.*, 1995). Very few protocols are available for isolating RNA form banana fruit and are not highly reproducible. This motivated us to develop a method to isolate RNA molecules that would work well for banana fruit eliminating the coprecipitation of polysaccharides and oxidation of phenolic compounds that interact with the nucleic acids.

## RESEARCH METHODS

Ninety per cent mature green bunch of cultivar Poovan (Mysore-AAB) were collected from National Research Centre for Banana, Trichy farm and allowed to ripe at 24$^0$C. When the fruits attained full yellow stage, samples were collected across the hands and pooled. Then pulp and peel were sliced separately into small pieces, and immediately frozen in liquid nitrogen and stored at -80$^0$C and used independently.

### Total RNA extraction :

Three different RNA extraction protocols namely, root RNA isolation protocol developed at NRCB (method 1)

(Sowmiya, 2008) , green fruit RNA isolation protocol by LiCl precipitation (method 2) (Asif *et al.*, 2000) and modified method of CTAB (method 3) were followed.

**Method 1 :**

One gram each of frozen banana pulp and peel tissues was ground by adding extraction buffer (0.1M Tris HCl, 0.25M sucrose, 0.2M NaCl, 10mM$MgCl_2$) followed by phenol: chlorphorm (1:1), 0.5M potassium EDTA and 20% SDS. For this 150µl of β-mercaptoethanol was added and incubated for 20 minutes at 42$^0$C and centrifuged (14000rpm, 30min, 4$^0$C). The aqueous phase was transferred to fresh tube and equal volume of cholorform: isoamyl alcohol (49:1) was added and centrifuged (15000rpm, 15min, 4$^0$C). Nucleic acid was precipitated by adding 1/10th volume of 3M sodium acetate (pH 5.2) and equal volume of isoproponol by keeping overnight at 40$^0$. Following centrifugation (14200rpm, 30min, 40$^0$), RNA was precipitated and washed with 75% ethanol. The RNA pellet obtained was dissolved in 200µl of RNase free water.

**Method 2 :**

10ml of rewarmed extraction buffer (10mM Tris HCl, 1.4M NaCl, 20mM EDTA, 2% CTAB and 0.1% β mercaptoethanol) was added to 1 g of frozen banana samples. The mixture was incubated at 65$^0$C for 1hr and then extracted twice with equal volume of chloroform : isoamyl alcohol. Then RNA was precipitated overnight at 4$^0$C by adding 10M LiCl. RNA pellet was extracted with phenol:chloroform and again precipitated with 3M sodium acetate followed by 100% ethanol. Finally, RNA pellet was dissolved in 200µl of RNase free water.

**Method 3 :**

Each one g frozen pulp and peel were extracted with prewarmed CTAB buffer as mentioned in method 2. Then aqueous phase was re-extracted twice with equal volumes of phenol: chloroform (14000 rpm, 30min, 4$^0$C); and chloroform : isoamyl alcohol (15000rpm, 15min, 4$^0$C). After that RNA was precipitated with 1/10 volume of 3M sodium acetate + equal volume of isoproponal for four hours and washed with 75% ethanol. The pellet was dissolved in 200µl of deionized formamide. Again RNA was precipitated with 3M sodium acetate and 100% ethanol by keeping it at -70$^0$C for three hours. Finally, after washing with 75% ethanol, RNA pellet was dissolved in 200µl of RNase free water.

**Quantitative and qualitative analysis:**

Quantitative analysis of RNA was carried out using the absorbance in a UV spectrometer by measuring the OD at 260nm and 280nm. Polysaccharide contamination was determined by their maximum absorbance measurement at 230nm. The ratio of measurements at wavelengths 230, 260 and 280 indicated the degree of purity of the RNA. The quality of RNA was confirmed on 1.5% formaldehyde agarose gel.

**Reverse transcription polymerase chain reaction (RT-PCR) analysis:**

Five µg of total RNA extracted from pulp and peel were used for the first strand cDNA synthesis using RevertAid First strand cDNA synthesis kit (Fermentas,Inc, USA) according to the manufacturer's instructions. RT PCR was performed in a 25µl reaction mixture containing 1µl cDNA template, 2µl forward primer (10pmol/µl), 2µl of reverse primer (10pmol/µl), 2.5µl of 10X PCR buffer, 2µl of DNTP (10mmol/L each) and 0.5µl of Taq polymerase (0.25 U). The PCR amplification condition was 94$^0$C for 2min, followed by 40 cycles of 94$^0$ C for 1 min, 60$^0$C for 1 min, 72$^0$C for 1 min and then a final 10 min extension at 72$^0$C. RT-PCR amplification was performed using gene specific primer β-1,3 Glucanase GLUF (5'GGATGAGACTCTACGATCCA3') and GLUR (5'GCCTGATCAAGTTCTGGTTG3') which were designed using Primer 3 software. The PCR products were analysed in a 2.0% agarose gel.

## RESEARCH FINDINGS AND DISCUSSION

Three different protocols were tried to isolate the total RNA from fresh banana pulp and peel tissues. The relative yield of total RNA per gram of fresh pulp and peel tissues ranged from 18.6-81.8µg and 10.5-40.4µg, respectively. The $A_{260}/A_{280}$ ratios for isolated RNA ranged from 1.3-2.0. Previously this protocol was tried with the banana root RNA isolation protocol at NRCB. In this method, RNA yield of pulp was high (30.4µg/g of fresh weight) but exhibited contamination of polysaccrides, pectins and proteins which was confirmed by $A_{260}/A_{280}$ ratio (1.3) while the yield of RNA from the peel was significantly low (10.5µg/g). These results suggested that banana root RNA isolation protocol will not be useful for isolating RNA from both pulp and peel tissues. Lizada *et al*. (1990) reported that drastic biochemical changes will occur during fruit ripening stages which leads to increase in solubale sugars and organic acids, breakdown of chlorophyll and polymerization of phenolic compounds in fully ripe fruits. Hence, we assumed that buffering agent may not be sufficient to isolate RNA from starch as well as phenols of banana fruit pulp and peel tissues. It also indicted that this protocol lacks sufficient steps to remove the high starch and phenols available in the pulp and peel tissues and its inability to apply across tissues. Similar suggestions were also put forth by Stephanie *et al.* (1997) to isolate good quality and quantity of RNA.

In the second method, fruit RNA isolation protocol involved two precipitation steps ie. LiCl and sodium acetate. The results showed that both pulp and peel tissues had 35.3 and 28.6µg per gram of tissue, respectively which was comparatively lower quantity of RNA over method 1 (Table

**Table 1 : Yield and absorbency ratios of total RNA isolated from banana pulp and peel tissues**

| Methods | Tissue | Absorbancy ratios | RNA yield µg/g of fresh tissue | Time required (in hrs) |
|---|---|---|---|---|
| I | Pulp | 1.3±0.22 | 42.4±2.1 | |
| | Peel | 1.4±0.12 | 10.5±0.8 | 25-26 |
| II | Pulp | 1.7±0.08 | 35.3±3.0 | |
| | Peel | 1.6±0.10 | 28.6±1.6 | 25-26 |
| III | Pulp | 2.0±0.05 | 81.8±4.8 | |
| | Peel | 1.9±0.08 | 40.54±2.0 | 10-12 |

1). Although this protocol was proven in 90% mature unripe/ green fruits, it is not suitable for extracting RNA from fully ripe fruits. Liu *et al*. (1998) also found that the use of LiCl after potassium acetate precipitation resulted in a large water insoluble precipitation and loss of RNA. RNA could directly be precipitated with ethanol than overnight precipitation with LiCl (Isabella *et al.*, 2008) and better yield of smallest RNA fragments (Cathala *et al.*, 1983).

Hence in the third method, precipitation was carried out twice with 1/10 volume of 3M sodium acetate (pH 5.2) and 2.5 volume of 100% ethanol which ultimately increase the efficiency of RNA precipitation. This method yielded 81.8µg and 40.4µg/g of pulp and peel tissues, respectively (Table 1). The ratio of $A_{260}/A_{280}$ was 1.9-2.0 for both the tissues which was suggested that the RNA is least contaminated with polyphenols and polysaccharides. This could be attributed to the high buffering and magnesium chelating capacity of the lysis buffer which protected the RNA from degradation. Moreover two precipitations (3M Sodium acetate : Isoproponal/100% ethanlo) involved in the purification phase could have facilitated the removal of residual polysaccharides. Thus among the three methods tried, third method resulted in the good RNA yield with high purity, making it suitable for further downstream analysis.

RNA quality was further assessed by visulazing the major rRNA following their separation on agarose gels. Ribosomal RNA normally represents >90% of the total RNA and any degradation in the RNA preparation can easily be visualized on an EtBr agarose gel. In the lanes, two distinct bands corresponding to 28S and 18S rRNA were distinct with no apparent degradation (Fig. 1). A smeared background was also visible and probably corresponded to mRNA. The contamination of DNA in cDNA was also checked by using banana RPS2 primer. The amplicon size of 200bp alone was observed in the agarose gel which implies the amplification of exon region alone (data not shown). This result revealed that there was no contamination of DNA in cDNA and confirms the purity of total RNA isolated from this modified CTAB protocol.

Apart from the integrity of the ribosomal bands, the intactness of RNA was monitored by studying the expression of β 1,3 glucanse in both pulp and using RT-PCR (Fig. 2). The expression of this gene was represented as a single band at the expected size of 730bp without any smears. This suggested that the RNA obtained from this protocol is free of any DNA contamination and efficient for further downstream analysis like cDNA library construction and

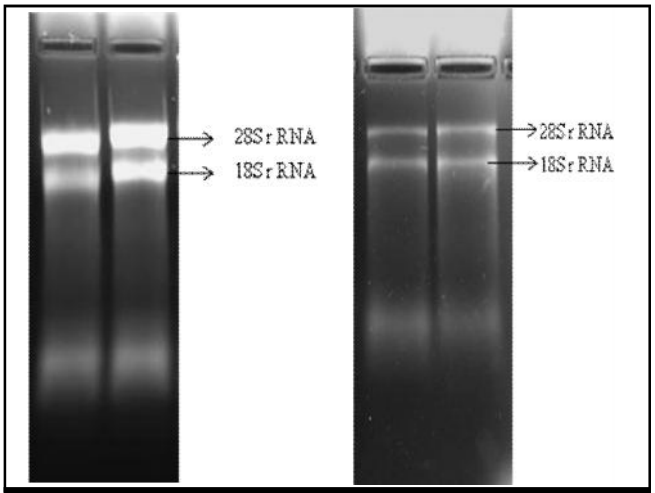


**Fig. 1 : Electrophoretic analysis of banana pulp and peel RNA. Total RNA (5µg) was resolved in nondenaturing conditions on 1% agarose gel. A) RNA from banana pulp b) RNA from banana peel**

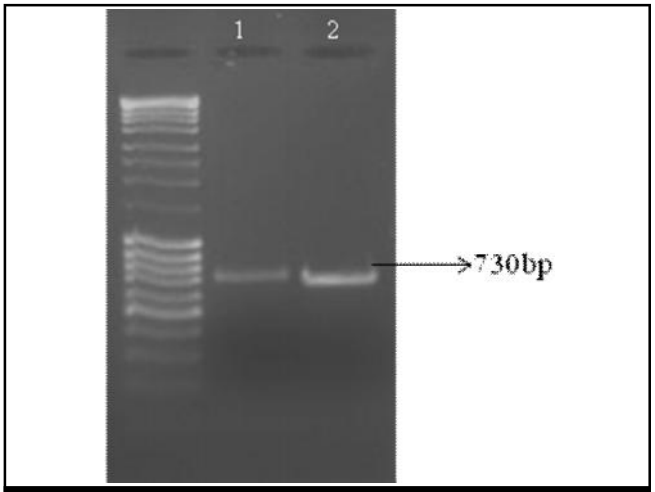


**Fig. 2 : Electrophoretic analysis of RT-PCR amplified cDNA of a pulp and peel tissues of ripe banana fruit. Lane 1 represents DNA marker, lanes 2. And 2 are RT-PCR amplicons of cDNA obtained from total RNA of Pulp and peel, respectively**

Northern blot analysis.

This modified protocol (method 3) has proven advantageous over the other two methods for the following reasons 1) reducing yield (RNA) loss and risk of degradation which resulted in higher RNA yield 2) reduction of DNA contamination which resulted in high quality RNA. Main modifications in this protocol are use of sodium acetate instead of LiCl; shortening the duration for precipitation from 12 hrs to 3 hrs; and reducing time for centrifugation.

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