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An efficient and rapid method for the isolation of RNA from different recalcitrant tissues of mango (*Mangifera indica* L.)

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SUMMARY
The isolation of high quality RNA from different tissues of mango (*Mangifera indica* L.) is relatively challenging due to the presence of interfering substances such as polysaccharides, polyphenols, and proteins. All these compounds render available isolation protocols useless by reducing the quality (purity and integrity) and quantity of the RNA that can be recovered. Several tissue-specific protocols for the isolation of RNA have been developed specifically for mango, however they are cumbersome, expensive and time-consuming. To overcome these drawbacks, we have developed a comprehensive (CTAB-free, guanidine-free, and LiCl-free) RNA isolation protocol using SDS (sodium dodecyl sulphate) plus phenol which works well for most mango tissues such as leaves, flowers, and fruit, at different stages of development or ripening, as well as fruit peel and seed kernels. This rapid protocol allowed us to process large numbers of samples (12 – 15) simultaneously in a single day. Using this method, we obtained good quantity RNA (16 – 80 μg g^{-1} tissue) from various mango tissues at different stages of development. RNA isolated by this method was pure and amenable to various downstream molecular applications such as RT-PCR and the construction of a cDNA library.

Ribonucleic acid (RNA) isolation is a critical step for subsequent molecular experiments involving reverse transcription-polymerase chain reactions (RT-PCR), rapid amplification of cDNA ends (RACE), northern hybridisation, microarray analysis, and transcriptome analysis to decipher mechanisms of gene expression, gene regulation, signal transduction, and post-translational studies (Birtic and Kranner, 2006; Portillo *et al*., 2006; Ghawana *et al*., 2011).

Isolation and purification methods for RNA from plant tissues vary with plant type, as well as the type of tissue in the same plant. The isolation of good quality RNA from cells containing high amounts of polysaccharides, polyphenolic compounds, and/or proteins can be challenging as these molecules interact with nucleic acids and can form insoluble complexes (Bugos *et al*., 1995).

Mango (*Mangifera indica* L.) is a biochemically complex crop from which the isolation of RNA has proved to be difficult (Lopez-Gomez and Gomez-Lim, 1992). The chemical composition of mango varies greatly from tissue-to-tissue. In fruit tissue, significant compositional changes occur during development and ripening, which may include sudden shifts in pH (Germain and Linden, 1981; Kansci *et al*., 2003), as well as alterations in fatty acid, lipid, and protein concentrations and compositions (Bandopadhyay and Gholap, 1973). The conversion of starch to sugars, or protopectins to pectin (Kansci *et al*., 2003), and changes in the pulp, from being terpenoid-rich to alcohol- or aldehyde- ester-rich (Saby *et al*., 1999) occur during fruit-ripening. All these components can contribute to the rapid degradation and low yields of functional mRNA through mechanisms such as polyphenol oxidation and co-precipitation with polysaccharides (Wang *et al*., 2000).

Various protocols have been developed to isolate good quality RNA from mango tissues rich in polysaccharides and secondary metabolites, but most have failed (Logemann *et al*., 1987; Levi *et al*., 1992; Lopez-Gomez and Gomez-Lim, 1992). Conventional methods used for the extraction of RNA from different tissues in biochemically complex crops include the use of guanidinium compounds together with density gradient centrifugation onto caesium chloride (CsCl) cushions (Chirgwin *et al*., 1979), proteinase K (Mozer, 1980), detergents such as sodium dodecyl sulphate (SDS) or cetyltrimethyl ammonium bromide (CTAB; Taylor and Powell, 1982), lithium chloride (LiCl) precipitation (Thompson *et al*., 1983), hot or cold phenol extractions (Galau *et al*., 1981; Morgens *et al*., 1987), and/or combinations of all of the above materials (Logemann *et al*., 1987). Some of these have been successful, to some extent, but most of these methods involve tedious procedural steps and require 1 – 2 d to complete the RNA extraction process. Some of these methods have been limited to the isolation of RNA from specific plant tissues, and not all can necessarily be applied to other plant tissues. Thus, we aimed to develop a new protocol for RNA isolation which was simple, rapid, and economical, as well as being comprehensive and efficient

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for the isolation of good quality RNA from different tissues in mango.

MATERIALS AND METHODS

Plant material

'Amrapali' mango (*Mangifera indica* L.) trees, maintained at the orchard of the Division of Fruits and Horticultural Technology, IARI, New Delhi, India, were used for the collection of different organs and tissues. Fresh tissue from young leaves, flowers, fruit pulp at different stages of maturity and ripening [i.e., S_1, 30 d after pollination (DAP); S_2, 60 DAP; S_3, 90 DAP; S_4, fully-mature unripe pulp 120 DAP; and S_5, mature ripe pulp], fruit peel, and seed kernels were used to extract total RNA. The different tissues or organs were excised, sliced, minced, and aliquoted immediately, then frozen in liquid nitrogen before use for RNA isolation. The different tissues used for RNA isolation were excised from five randomly selected replicate tree samples.

Reagents used

All the chemicals used were of molecular biology (MB) grade and obtained from HiMedia Laboratories Pvt. Ltd., New Delhi, India. The distilled water used was pre-treated with 1% (v/v) diethyl pyrocarbonate (DEPC), following standard procedures (Sambrook *et al*., 1989). All reagent solutions were prepared using DEPC-treated water. Chemicals such as 10 mM ethylenediaminetetraacetic acid (EDTA) made from a 0.5 M stock solution, pH 8.0, sodium acetate (32 mM NaOAc), sodium dodecyl sulphate [0.2% (w/v) SDS], phenol saturated with Tris-HCl pH 7.9, chloroform, 75% (v/v) ethanol, and isopropanol were used and the extraction buffer contained 10 mM EDTA, 32 mM NaOAc, and 0.2% (w/v) SDS.

Pre-treatment

All the equipment and accessories such as plastic-ware, glassware, and mortars and pestles used for RNA isolation were pre-treated with DEPC (Sambrook *et al*., 1989). They were immersed in 0.1% (v/v) DEPC in water and left overnight at room temperature. The next day they were autoclaved at 121ºC and 105 kPa for 15 – 20 min to inactivate RNases by wrapping in sterile aluminium foil.

RNA isolation protocol

The steps used were as follows:

(i) Each frozen tissue sample (0.1 g leaf tissue, 0.5 g flower, peel or kernel, or 1.0 g of fruit pulp) was thoroughly ground to a fine powder in liquid N_2 using a pre-chilled mortar and pestle.

(ii) Two ml of a 1:1 (v/v) pre-heated mixture of extraction buffer (see above) and Tris-HCl-saturated phenol was added to each fine powder which froze immediately and was then ground thoroughly to produce a homogeneous mixture.

(iii) The mixture was allowed to thaw completely with intermittent grinding, then 800 µl of DEPC-treated water was added and mixed thoroughly.

(iv) The resultant mixture was transferred equally to two 2.0 ml micro-centrifuge tubes which were incubated at room temperature (25º – 28ºC) for 5 min.

(v) Then 200 µl of 100% (v/v) chloroform was added to each tube, vortex-mixed thoroughly, and incubated at room temperature for 10 min.

(vi) The tubes were then centrifuged at 11,000 × *g* for 10 min, at room temperature (25º – 28ºC) and the upper aqueous phase was carefully transferred to a fresh, sterile DEPC-treated 1.5 ml Eppendorf tube without disturbing the layer of precipitate between the phases.

(vii) Chilled isopropanol (0.6 volumes) was added to the aqueous phase, vortex-mixed thoroughly, and incubated at room temperature (25º – 28ºC) for 10 min.

(viii) The mixture was centrifuged again at 11,000 × *g* for 10 min to sediment the RNA and the upper aqueous solution was carefully discarded, retaining the pellet at the bottom of the tube.

(ix) If the pellet was too large, it was redissolved in 2 ml of extraction buffer plus Tris-HCl-saturated phenol and the above steps (iii – viii) were repeated.

(x) The final pellet was washed by mixing and resuspending it in 1.0 ml of chilled 75% (v/v) ethanol (MB-grade) and centrifuging it for 10 min at 11,000 × *g* at room temperature (25º – 28ºC).

(xi) The ethanol was discarded and the RNA pellet was air-dried in an incubator at 27ºC.

(xii) Based on the size of the RNA pellet that was recovered, it was dissolved in 15 – 20 µl of microfiltered DEPC-treated water prepared using 0.22 µm MILLEX®-GV Syringe Driven Filter Unit (Millipore Corp., Billerica, MA, USA).

(xiii) The dissolved RNA was kept at 65ºC for 5 min, then cooled immediately by placing the tube in ice flakes for 2 – 3 min.

(xiv) The RNA was finally stored at –80ºC for future applications.

RNA analysis

The purity and concentration of each isolated RNA sample was assessed spectrophotometrically using a Nano-Drop spectrophotometer (Thermo-Fisher Scientific, Wilmington, DE, USA) by determining its absorbance at 230 nm, 260 nm, and 280 nm and by calculating its A_{260}:A_{280} and A_{260}:A_{230} ratios. The integrity of each RNA was evaluated by electrophoresis in 1.2% (w/v) agarose gels followed by staining with 0.1 µl ml^{-1} ethidium bromide (EtBr) and visualisation under UV light using an Alpha Imager 1220 gel documentation system (Syngene Bio-imaging Pvt. Ltd., Gurgaon, Haryana, India).

RT-PCR

Each isolated RNA sample was treated with 100 µl ml^{-1} DNase I (PROMEGA, Madison, WI, USA) and first-strand cDNA was synthesised from 1.0 µg of total RNA using the Verso cDNA Synthesis Kit (Thermo-Scientific, Fermentas, Lithuania) according to the manufacturer's instructions. To assess the quality of each RNA and its suitability for reverse transcription, RT-PCR amplification was performed using primers constructed on the basis of conserved sequences in the house-keeping gene for *actin*. The sequences of the forward and reverse primers were: 5'-AGCGAGTCTTC ATAGGGCGATTGT-3' and 5'-TAGCTCTGGGTTCG AGTGGCATTT-3'.

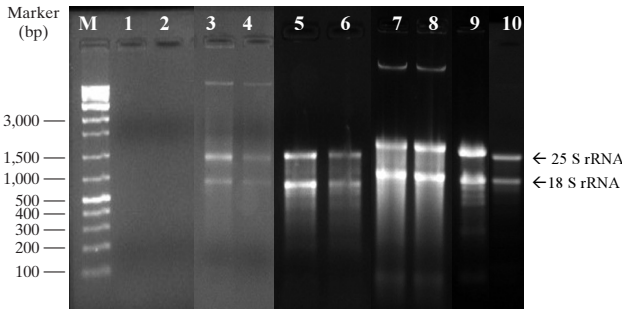

FIG. 1
Comparative 1.2% (w/v) agarose gel electrophoresis of the total RNA extracted from mango leaves and fruit tissue in alternate lanes, using different methods. RNA was visualised by staining with 0.1 µg ml^{-1} ethidium bromide and observed under UV light. Lane M, 1 kb DNA ladder; lanes 1, 2 the Trizol® method; lanes 3, 4 the CTAB-LiCl method; lanes 5, 6 the CTAB-LiCl method with overnight incubation at the precipitation step; lanes 7, 8 a modified CTAB-LiCl method with the addition of 2% (w/v) soluble PVP; lanes 9, 10 SDS/phenol method described in this paper.

Each 25 µl PCR reaction was carried out in Emerald Amp® Max PCR Master Mix (TaKaRa-Bio Inc., Shiga, Japan) and it contained 12.5 µl Master mix, 10 nmoles each of the forward and reverse primers, and 1.0 µl of the cDNA reaction above. An initial denaturation step at 94ºC for 4 min was followed by 35 cycles of denaturation at 94ºC for 45 s, annealing at 60ºC for 45 s, and extension at 72ºC for 1 min. A final extension step was carried out at 72ºC for 10 min.

RESULTS

Prior to the development of this protocol, we tested many other published methods for the isolation of RNA from various mango tissues, especially leaves and fruit. The first attempt at isolating RNA used the most common commercial products TRIzol® (Invitrogen BioServices India Pvt. Ltd., Bangalore, India) and the One-Step RNA reagent (Bio Basic Inc., Ontario, Canada), but both failed to isolate the good quality RNA (Figure 1). Next the CTAB-LiCl protocol for RNA isolation (Pandit *et al*., 2007) was tested. This yielded negligible quantities of RNA, especially from fruit pulp tissue (Table I). Thus, some modifications in the latter protocol were examined.

In the first modification, the incubation time for the LiCl precipitation step was increased to 12 h. In the second modification, 2% (w/v) soluble polyvinylpyrrolidone (PVP) was added to the extraction buffer. These modifications in the CTAB-LiCl method increased RNA yields and quality significantly (Figure 1; Table I), however they were cumbersome, time consuming, and expensive, and limited the scope for simultaneous processing of large numbers of samples. Hence, we attempted to develop a new method using SDS-phenol to extract higher amounts of good quality RNA from various mango tissues in less time.

TABLE II
Quality and yield of RNA isolated from various tissues of mango using the protocol described in this paper

Mango tissue	A_{260}/A_{280}	A_{260}/A_{230}	Yield (µg g^{-1} FW)
Young leaf	1.57	1.39	84.83
Flower	1.59	1.41	40.89
Fruit stage-S_1 (30 DAP)‡	2.00	2.20	41.68
Fruit stage-S_2 (60 DAP)	2.01	2.13	28.66
Fruit stage-S_3 (90 DAP)	2.03	2.14	21.48
Fruit stage-S_4 (mature unripe)	2.05	1.98	18.37
Fruit stage-S_5 (mature ripe)	2.03	1.68	16.57
Fruit peel	1.47	1.48	19.44
Seed kernel	1.78	1.61	52.94

‡DAP, days after pollination.

Using our new standard SDS-phenol method described above, we obtained good quality RNA from all mango tissues such as leaves, flowers, seed, fruit peel and fruit pulp at different stages of development and ripening. RNA yields were highest from leaves [84.83 µg g^{-1} fresh weight (FW)] followed by seed kernels (52.94 µg g^{-1} FW), flowers (40.89 µg g^{-1} FW), and fruit pulp at S_1 (41.68 µg g^{-1} FW). RNA yields were moderate from fruit pulp at the S_2 stage (28.66 µg g^{-1} FW) followed by pulp at S_3 (21.48 µg g^{-1} FW), and were relatively low from fruit peel (19.44 µg g^{-1} FW), and pulp at the S_4 (18.37 µg g^{-1} FW) and S_5 (16.57 µg g^{-1} FW) stages of fruit development (Table II).

Absorbance (A_{260}/A_{280}) ratios were between 1.60 – 2.05 for RNAs from most tissues, indicating high purity and low or no contamination with secondary metabolites such as proteins or polyphenols. Similarly, the A_{260}/A_{230} absorbance ratios for most tissues were between 1.6 – 2.2, indicating low or no contamination with polysaccharides (Table II). The above data indicate that the new protocol described above was efficient at yielding good quantity RNA from various problematic tissues in mango including fruit pulp at various stages of development and ripening

When the isolated RNAs were analysed by agarose gel electrophoresis (Figure 2), two discrete major bands with little degradation were observed corresponding to 25S

TABLE I
Qualities and yields of RNA isolated from young leaf tissue and fruit pulp of mango using different methods

Method‡	Tissue	A_{260}/A_{280}	A_{260}/A_{230}	Yield (µg g^{-1} FW tissue)
TRIzol®	Young leaf	1.04	0.17	1.85
	Fruit pulp	0.97	0.15	1.56
CTAB-LiCl (Pandit *et al*., 2007)	Young leaf	1.93	1.80	3.91
	Fruit pulp	1.58	1.19	1.96
CTAB-LiCl with 12 h incubation	Young leaf	2.11	2.20	13.57
	Fruit pulp	2.04	2.18	9.29
CTAB-LiCl with 2% (w/v) soluble PVP	Young leaf	1.91	2.16	79.14
	Fruit pulp	2.06	2.22	58.95
This protocol (SDS/phenol)	Young leaf	1.57	1.39	84.83
	Fruit pulp	2.00	2.20	41.68

‡TRIzol® (Invitrogen BioServices India Pvt. Ltd., Bangalore, India); CTAB, cetyltrimethyl ammonium bromide; LiCl, lithium chloride; PVP, polyvinylpyrrolidone.

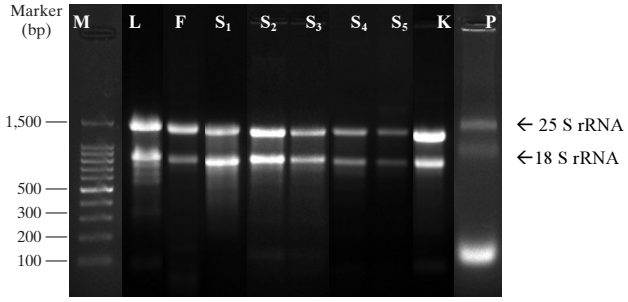

FIG. 2
Comparative 1.2% (w/v) agarose gel electrophoresis of total RNA isolated from different mango tissues. RNA was visualised by staining with 0.1 µl ml^{-1} ethidium bromide and observed under a UV light. Lane M, 1 kb DNA ladder; lane L, leaf; lane F, flower; lane S_1, pulp at 30 DAP; lane S_2, pulp at 60 DAP; lane S_3, pulp at 90 DAP; lane S_4, mature unripe pulp; lane S_5, mature ripe pulp; lane K, seed kernels; lane P, fruit peel.

and 18S plant ribosomal RNA which represent 98 – 99% of the RNA in any cell. In most cases, we also observed a faint background smear or faint bands at the bottom of the gel that corresponded to mRNA or 5S ribosomal RNA, respectively. The absence of high molecular weight bands indicated low or no contamination by genomic DNA (Figure 2).

The functionality of the isolated RNA was tested by constructing a cDNA library and performing RT-PCR analysis. Reverse transcriptase is highly sensitive to contaminants such as polyphenols, proteins, or polysaccharides (Li *et al*., 2006; Ghangal *et al*., 2009). RT-PCR amplification was observed for the house-keeping gene, *actin* (Figure 3). The presence of a single discrete band, without any smearing, following RT-PCR analysis confirmed that the RNAs isolated from the different mango tissues were of high integrity and purity. The new standard RNA isolation protocol could be completed within 1 – 2 h, allowing simultaneous processing of large numbers of samples.

DISCUSSION

Several protocols are currently available to isolate RNA from different plants. However, no single method is effective at isolating RNA from all samples because plant tissues vary in their composition and concentrations of inhibitory components. This has necessitated the development of plant- and tissue-specific protocols for RNA isolation (Sharma *et al*., 2003; Wang *et al*., 2007).

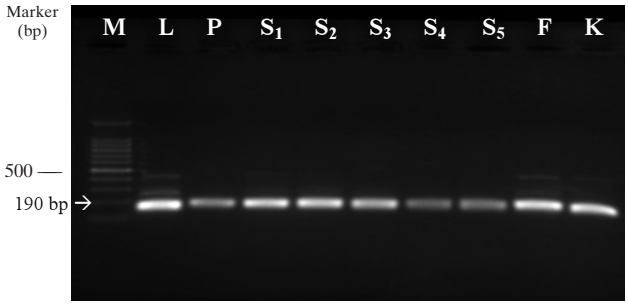

FIG. 3
RT-PCR amplification of a 190 bp amplicon from transcripts of the *actin* gene isolated from various mango tissues using the RNA extraction protocol described in this paper. Lane M, 100 bp molecular marker; lane L, leaf; lane F, flower; lanes S_1–S_5, fruit stages S_1–30 DAP; S_2–60 DAP; S_3–90 DAP; S_4 mature unripe; S_5 mature ripe; lane K, seed kernels; lane P, fruit peel.

Mango is a problematic perennial, woody fruit crop which contains abundant quantities of polysaccharides, proteins, and secondary metabolites such as polyphenols in all its recalcitrant tissues. As a result, RNA isolation procedures using commercial reagents such as TRIzol® are challenging compared to other herbaceous plant tissues (Liu *et al*., 2005).

Polyphenols and polysaccharides are the most difficult molecules to remove during RNA isolation procedures (Pandit *et al*., 2007). During RNA isolation, polyphenols are oxidised to quinones which are irreversibly linked to nucleic acids and impede the utility of the RNA for downstream applications such as RT-PCR or cDNA library construction. Furthermore, contamination with polysaccharides results in their co-precipitation with RNA, as both have similar physico-chemical properties (Wang *et al*., 2000).

In some traditional methods based on a CTAB lysis buffer, the aqueous RNA phase is separated from proteins, polysaccharides, and genomic DNA using one or two extractions with phenol-chloroform-isoamyl alcohol (PCI; Chang *et al*., 1993; Kiefer *et al*., 2000; Meisel *et al*., 2005). The RNA is then precipitated using LiCl and anhydrous alcohol. Such methods produce inferior quality RNA due to high losses of total RNA during repeated extractions with PCI when 20 – 25% of the supernatant is discarded, leading to the inefficient removal of proteins and polysaccharides (Sambrook and Russell, 2001). However, in the protocol describd here, we have removed the PCI extraction step by adding phenol together with the extraction buffer, then the chloroform was added later. This modification of conventional RNA extraction protocols not only reduced RNA losses, but also reduced the chemicals used and the overall time required for RNA isolation to approx. 2 h.

In our protocol, we used SDS which is a strong ionic detergent that removes anions from proteins, destroys their conformation and tertiary structure (Rio *et al*., 2010). Moreover, proteins and lipids in cell membranes are damaged and solubilised, leading to the breakdown of membranes and the nuclear envelope to expose nucleic acids. In addition to removing membrane barriers, SDS also releases ribosomal RNA, disrupts protein-nucleic acid interactions, and inactivates ribonucleases (Rio *et al*., 2010).

The other chemicals used in our RNA protocol included phenol, chloroform, sodium acetate, and EDTA. The major role of phenol was to separate the cell lysate during centrifugation into two phases (i.e., an upper aqueous phase containing the RNA and a lower organic phase containing DNA and proteins; Rio *et al*., 2010; Song *et al*., 2011). Chloroform denatures lipids and proteins and renders them soluble in the organic (phenol) phase or at the interface, while nucleic acids remain in the aqueous phase. Sodium acetate was added to the extraction buffer to maintain the pH of the denatured cell lysate during extraction and to provide the Na^+ ions necessary for RNA precipitation. EDTA was present in the lysis buffer to chelate divalent cations which can catalyse RNA degradation (Morante-Carriel

et al., 2014). The role of the isopropanol was to precipitate the RNA by binding water molecules and preventing the RNA from being dissolved. The final ethanol step acts as a purification agent. Ethanol washing allows the sample to be centrifuged to collect a 'clean' RNA pellet, after discarding the supernatant that contained any contaminating salts or proteins. During the isolation and purification of RNA, 75% (v/v) ethanol is used as a wash solution because RNA is precipitated at this ethanol concentration, while most proteins and salts remain soluble. At lower concentrations of ethanol, both RNA and proteins are soluble. In contrast, at higher concentrations of ethanol, both RNA and salts remain in the solid (pellet) form, rendering them inseparable (Lopez-Gomez and Gomez-Lim, 1992; Gonzalez-Mendoza, 2008).

In conclusion, the protocol described in this paper proved to be efficient for the isolation of good quality RNA (i.e., high purity and integrity) and yielded higher quantities of RNA from various problematic tissues of mango with reduced chemical use and of lower toxicity (i.e., CTAB-free, guanidine-free, and LiCl-free) within only 1 – 2 h. Due to the reduced number of steps and shorter time of extraction, this method can be used for high-throughput sampling. The RNA isolated using this protocol was suitable for downstream applications such as the construction of a cDNA library and RT-PCR.

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