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Modified TRIzol Method for extraction of Good quality RNA from Spikes of Black Pepper (*Piper nigrum* L.)

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ABSTRACT: Obtaining high quality RNA is a basic requirement for plant molecular biology, genetics, and physiological studies. High-quality RNA isolation from spikes of *Piper nigrum* L. is crucial for inflorescence specific gene studies for crop improvement. The present study includes comparison of reported CTAB, SDS, and TRIzol methods for extraction of RNA from spikes of black pepper. The quality and yield of RNA was comparatively poor in all these methods. Black pepper spikes, in particular, are rich in polyphenols and secondary metabolites, which comprise a diverse group of naturally occurring compounds contributing to the unique flavour and aroma of the plant. However, the isolation of RNA from black pepper spikes is challenging due to the presence of these compounds, which interfere with the isolation, purification, and resuspension of precipitated RNA, as well as contaminate the RNA pellets through co-precipitation, thus inhibiting downstream applications. Therefore, we have modified original Trizol protocol by inclusion of 2% PVP, 4% β Mercaptoethanol and 1% SDS to reduce the polyphenols at the initial stage of isolation itself. We also increased the incubation time and followed this with repeated chloroform extractions and ethanol washes to remove the remaining contamination. We kept the precipitation at 4ºC to improve the quality and yield of the RNA. The RNA extracted using the modified TRIzol method in the present study was intact, clear, non dispersive with good integrity and high yield (530ng/µl). There was no evidence of DNA or other contaminants in the isolated RNA (A_{260}/A_{280} – 1.99; A_{260}/A_{230} – 2.01). The quality was reconfirmed with gel electrophoresis and RT-PCR. Extracted RNA was successfully converted into cDNA and validated by amplification with primer for housekeeping gene β-actin. The present modified TRIzol-based protocol provides valuable insight to isolate high quality RNA from crops with high content of polyphenols, polysaccharides and other metabolites for further downstream techniques.

Keywords: Polyphenols, Secondary metabolites, CTAB, SDS, TRIzol method.

INTRODUCTION

Black pepper, *Piper nigrum* L., belonging to the *Piperaceae* family is the highest traded spice crop worldwide. It is a perennial, climbing woody vine, indigenous to humid, tropical evergreen forests of the Western Ghats in India, which is also the richest hub of its diversity. Currently, more than thirty countries cultivate black pepper for economic purposes with majority of the cultivation concentrated in India, Indonesia, Brazil, Vietnam, Malaysia, China, Thailand, Madagascar, Sri Lanka, Cambodia, Ethiopia and Mexico (Newerli-Guz and Smiechowska 2022). In addition to being used in the human diet as a spice and flavour, black pepper has a number of additional uses as well, including fragrance, traditional medicine, preservative, and insecticidal use (Srinivasan, 2007). The therapeutic potential of secondary metabolites of *P. nigrum* L. exhibit their role in gastrointestinal diseases, hepatoprotection, antithyroid, anti-inflammation, antidepressant, immunomodulation, antispasmodic, cardiovascular protection, antiobesity antioxidant properties and also promote blood circulation (Gulcin, 2005; Ahmad *et al.*, 2012; Gorgani *et al.*, 2017; Lailiyah *et al.*, 2021; Wang *et al.*, 2021). Piperine is a bioactive compound of black pepper, used as a natural bioenhancer in formulation development of medicines for various disorders (Pingale *et al*., 2023).

Isolation of good quality RNA is a prerequisite for molecular biology research involving gene expression

profiling by reverse transcription polymerase chain reaction, quantitative real time polymerase chain reaction, cDNA preparation, northern blot analysis, molecular cloning, etc. involved in crop improvement studies of black pepper (Sah *et al.,* 2014; Liu *et al.,* 2018; Vennapusa *et al..,* 2020). RNA isolation is difficult in black pepper, as it contains high amounts of polyphenols, polysaccharides, flavonoids, and other metabolites. Based on their physical and chemical properties, contaminants such as polysaccharides and polyphenols irreversibly bind to RNA and proteins and co-precipitate during the purification and precipitation steps resulting in poor quality and yield of the isolated RNA hindering their use for further molecular studies (Rezadoost *et al.,* 2016; Liu *et al.,* 2018; Liao *et al*., 2020; Gaafar *et al*., 2021). The rapid oxidation of the polyphenol causes browning of the final RNA pellet, which in turn inhibits the activity of different enzymes like reverse transcriptase, Taq polymerase, DNase, etc. used in the different molecular biology techniques. Furthermore, the polysaccharides that get co-precipitated with the RNA make the pellet viscous and subsequently make the loading also difficult during gel electrophoresis. Successful isolation of good quality RNA from spike samples of *Piper nigrum* L. should ensure efficient removal of polyphenols and polysaccharides. Therefore, in the present study, isolation of the total RNA from spikes of black pepper was compared using the reported protocols of CTAB (White *et al*., 2008), SDS (Valderrama-Chairez *et al.,* 2002) and TRIzol methods (Chomczynski and Sacchi 2006) and further modifications were incorporated in the TRIzol method to develop a standard protocol for the isolation of good quality RNA from spike tissues of black pepper.

MATERIALS AND METHODS

Collection of plant tissue. The spikes of black pepper at immature stage1 (1-2 cm, 12-15 days after bud emergence) were collected from the field of College of Agriculture, Vellayani, Kerala, cleaned with 70 percent ethanol followed by double distilled water and immediately snap frozen in liquid nitrogen and stored at -80 °C in a freezer until use.

Preparation for RNA extraction. Preparations for total RNA isolation needs more care compared to preparations for DNA isolation because the risk of RNase (catalyzes RNA degradation) contamination during RNA isolation is more as RNases do not require any cofactor such as Mg^{2+} ion for activity, unlike DNases. 0.1% (v/v) diethyl pyrocarbonate (DEPC; Gbiosciences) was used for the preparation of all solutions, buffers and for dissolving the RNA pellet. Glassware, plastic ware, mortar-pestle etc. were washed and treated with 0.1% DEPC water, overnight followed by double autoclaving at 121ºC for 40 min and then kept in hot air oven at 80ºC for drying. The Working platform was cleaned with 3% H_2O_2 followed by RNase OUT (Gbiosciences). The mortar and pestle used for the grinding of tissue samples were also cooled in a refrigerator and cleaned with RNase OUT (Gbiosciences) for the removal of traces of RNAase if any.

Methods for RNA isolation. CTAB method. 100mg of frozen sample tissue was ground into fine powder in prechilled mortar and pestle using liquid nitrogen and immediately transferred into a prechilled 2mL RNase free micro centrifuge tube. 1mL pre warmed (at 65ºC) CTAB extraction buffer [2% CTAB; 2% soluble polyvinyl pyrrolidone (PVP); 25 mM ethylenediamine tetra acetic acid (EDTA); 100 mM Tris-HCl (pH 8.0); 2 M NaCl; 0.5 g/L spermidine and 3% β-mercaptoethanol] was added and mixed by vortexing for 1 min. After that samples were incubated in water bath at 65˚ C for 30 min and inversed after every 5 min to ensure proper mixing. The sample was cooled down at room temperature and centrifuged at16,000 g for 10 min at 4ºC. The supernatant was collected into a fresh 2mL micro centrifuge tube and an equal volume of chloroform: isoamyl alcohol (C:I, 24:1) was added, mixed and centrifuged at 16,000 g for 15 min at 4ºC. The aqueous phase was transferred into a fresh 2mL microfuge tube and the C: I wash was repeated twice. The clear supernatant was collected and to that one-third volume of 8M Lithium Chloride (LiCl) was added, mixed and kept at 4ºC overnight. Centrifugation was done at 16,000 g for 60 min at 4ºC, supernatant was removed and the pellet was washed with 70% EtOH. The RNA pellet was air dried for 10 min and dissolved in 30-50µl ddH_2O and stored at -80ºC.

SDS method. Collected spike samples (100 mg) were placed in a cooled mortar and crushed to make a fine powder using liquid nitrogen. 1mL extraction buffer [2% (w/v) SDS; 150 Mm Tris base; 100 mM EDTA and 30 µL β-ME (1%, v/v)] was added to fine powder, mixed using a cut tip. The homogenate (approx. 750 µl) was transferred to cool RNase free 2 mL micro-centrifuge tube and precipitated using 5 mM potassium acetate (66 µL) and absolute ethanol (150 µL).850 µL of chloroform-isoamyl alcohol (49:1, v/v) was added and briefly vortexed before centrifuging at 16,000 g for 20 min at room temperature. The supernatant was taken into fresh 2mL micro centrifuge tubes, an equal volume (850 µL) of phenol, chloroform, and isoamyl alcohol (25:24:1) was added, vortexed for 10 s and centrifuged at 16,000 g for 15 min at room temperature. The supernatant was collected into fresh tubes and 850 µL of chloroform-isoamyl alcohol (49:1, v/v) was added to it. Tube was quickly vortexed for 10 s and centrifuged at 16,000 g for 15 min at 4ºC. 3M LiCl was added to the clear supernatant that was collected and mixed by inversion and kept at -20ºC overnight. Tube was centrifuged at 16,000 g for 20 min at 4ºC, supernatant was decanted and RNA pellet was washed twice with 70 percent ethanol (500µL). It was air dried at room temperature and dissolved in 30-50µl DEPC treated double autoclaved water, and stored at -80ºC.

TRIzol method. Spike samples (100mg) were ground in prechilled mortar and pestle using liquid nitrogen to make into fine powder. One mL TRIzol was added to finely crushed tissue, mixed gently to homogenize the mixture and incubated at room temperature for 5 min. The homogenate was transferred to 2mL prechilled

RNase free micro centrifuge tubes. 200µL chloroform was added to it, mixed by vigorous shaking for 15 sec and incubated at room temperature for 5 min and centrifuged at 12,000 g for 15 min at 4ºC. The clear aqueous phase was carefully transferred to a fresh 1.5 mL micro centrifuge tube without disturbing the middle protein layer. 0.5 ml of chilled absolute isopropanol was added to the supernatant that was collected, mixed slowly by inversion and incubated at room temperature for 10 min. The tube was centrifuged at 12,000 g for 10 min at 4ºC. The supernatant was gently decanted and the pellet was washed with 1mL of 75% ethanol followed by centrifugation at 7,500 g for 5 min at 4ºC. RNA pellet was air dried for 10 min and dissolved in 30-50µl RNase free water, incubated at 55º-60ºC for 10 min and stored at -80 ºC.

Modified TRIzol method. Stored frozen spikes samples were weighed (100 mg) and crushed in a prechilled mortar and pestle using liquid nitrogen to make a fine powder. Crushed tissue along with liquid nitrogen was immediately transferred into 15 mL prechilled RNase free centrifuge tubes. The liquid nitrogen was allowed to evaporate by keeping the cap of the tubes loose. After that 1mL TRIzol, 2%PVP, 4% β mercaptoethanol and 1% SDS were added, mixed by hand inversion, and kept for incubation at room temperature for 8-10 min. The content was centrifuged at 12,000 g for 15 min at 4ºC. The supernatant was transferred into 2mL prechilled RNase free microcentrifuge tube and 200µL chloroform was added to it. The content was mixed by hand inversion at least 20 to 30 times and incubated at room temperature for 8-10 min. The tube was centrifuged at 12,000 g for 10 min at 4ºC and the supernatant was collected in a fresh 2mL microcentrifuge tube. Again, 200µL chloroform was added to it and centrifuged as in the earlier step. The clear supernatant was transferred carefully into fresh 1.5 mL tube and equal volume of chilled isopropanol was added, mixed slowly by inversion and kept at 4ºC for 10-15 min. Centrifugation was done at 12,000 g for 15 min at 4ºC, supernatant was discarded and RNA pellet was washed with 75% ethanol (prepared in DEPC treated water) twice followed by centrifugation at 7,500 g for 7 min at 4ºC. The pellet was air dried under sterile laminar airflow for 10-15 min and resuspended into appropriate volume of DEPC treated double autoclaved water. The resuspended RNA pellet tube was initially stored at -20ºC for 5-6 hrs and then stored at -80ºC in a freezer which helped to dissolve the RNA pellet completely.

Analysis of RNA quality and quantity. The quality and quantity of total RNA isolated by the different methods were checked using nanodrop spectrophotometer (Thermo Fisher Scientific) and absorbance ratio of 260/280 and 260/230 were noted. Good RNA quality is indicated by A_{260}/A_{280} ratios between 1.8 and 2. The integrity of extracted RNA was evaluated on 1.2% agarose gel electrophoresis in 1X TBE buffer and visualized under gel documentation system (Bio-Rad).

Synthesis of cDNA. cDNA was synthesised from extracted RNA by modified TRIzol method using Verso cDNA synthesis kit (Thermo Scientific, AB-1453/A), as per the manufacturer's protocol. Total 20µL of the reaction mixture was prepared by using 5X cDNA assay buffer, 2µL dNTPs (10mM), oligo dT primer 0.5µL, random hexamer 1µL, RT enhancer 1µL, Verso enzyme mix 1µL (U/µL), RNA 1µg and ultimate volume was filled with nuclease-free water. Further, the reaction mixture was mixed properly and incubated for 30 min at 42°C. The tubes were then rapidly placed on ice and briefly spun after being incubated at 92ºC for 2 min (for denaturation of RNA-cDNA hybrids) and cDNA samples were stored at -20°C for further use.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) analysis. To check cDNA conversion from the isolated RNA, RT-PCR was done using *Piper nigrum* L. specific house keeping gene β actin (Forward-5'-ACATCCGCTGGAAGGTGC-3' and Reverse-5'-TCTGTATGGTAACATTGTGCTC-3').

The standard 20µL reaction mix prepared included 2µL of PCR buffer, 0.6µL of dNTPs, 1µL of each forward and reverse primer (10pmol), 1µL of cDNA template (50ng), 1U of Taq polymerase and molecular grade water to make up the reaction volume, and cDNA was amplified in Thermal cycler (T100TM Bio-Rad). The PCR thermal condition was set to an initial denaturation at 94ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 30 s, annealing at 58.4ºC for 45 s, extension at 72ºC for 1 min and final extension was set to 72ºC for 10 min. The PCR product was stored at 4ºC till further use. The PCR products were separated on a 1.5% agarose gel using 1xTBE buffer at 75 V for 40-50 min and the gel image was observed using a gel documentation system (Bio-Rad).

RESULTS AND DISCUSSION

The understanding of molecular aspects of different cellular, metabolic and signaling pathways and developmental processes for crop improvement of black pepper requires isolation of total RNA of good quality, yield and integrity. Three reported methods of isolation were compared with a new protocol with modifications in the TRIzol method for isolation of RNA from spike samples of black pepper, a spice crop rich in polyphenols, polysaccharides and other secondary metabolites. The modifications in the TRIzol method followed in the present study included addition of 2% PVP, 1% SDS, 4% β mercaptoethanol, a longer incubation time followed by repeated chloroform extractions, ethanol washes, and precipitation at 4ºC RNA. The better quality and yield of RNA in the modified protocol was evaluated using gel electrophoresis, quantitative nucleic acid detection, and RT-PCR analysis.

Agarose gel electrophoresis is a common method followed for checking the quality of the isolated RNA (Ghawana *et al.*, 2011; Ma *et al.*, 2015; Betts *et al.*, 2017). In 1.2% agarose, the gel profile of 28S and 18S rRNA were observed to be intact, clear, bright, crisp and distinct with no prominent degradation and shearing, in the modified TRIzol method followed in the present study (Fig. 1D). On the other hand, the gel profile of RNA extracted following the already reported

protocols of CTAB, SDS and TRIzol methods showed shearing and degradation (Fig. 1-A-C). While following the CTAB, SDS and TRIzol protocols, the supernatant was observed to be highly viscous and dense compared to the modified TRIzol protocol developed in the present study.

The OD value is also routinely used to determine the purity and yield of total RNA. Under neutral pH RNA shows maximum absorbance at 260nm and the yield can be determined based on the absorbance value. A_{260}/A_{280} ratio depicts the purity of RNA samples. A_{260}/A_{230} ratio is used to analyse the presence of impurities such as polysaccharides and other associated metabolites in the isolated RNA. Using the modified TRIzol method, the A_{260}/A_{280} value of RNA isolated from spike samples of black pepper was approximately 1.99, A_{260}/A_{230} was 2.01, and the concentration was 530 ng/µL. On the other hand, the A_{260}/A_{280} and A_{260}/A_{230} values were observed to be comparatively poor in SDS (1.45; 0.32), CTAB (1.52; 0.43) and TRIzol (1.65; 0.81) methods. RNA concentration was comparable in all methods except the modified TRIzol method which showed a significantly higher yield (Table 1). Genomic DNA contamination was also observed in all the reported protocols and was negligible in the modified TRIzol method developed in the present work.

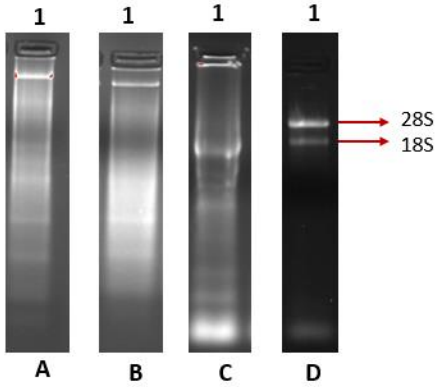

Fig. 1. Gel profile of total RNA isolated from black pepper spikes. (A) CTAB method (B) SDS method(C) original TRIzol method (D) Modified TRIzol method.

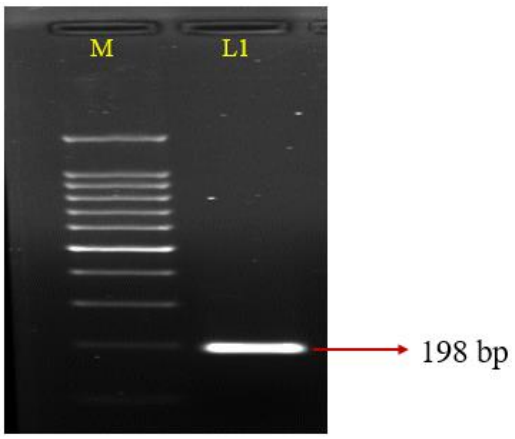

Fig. 2. Quality assessment of cDNA using RT-PCR with β actin gene primer. Lane M-100bp ladder, Lane 1- β actin gene.

Table 1: Purity and yield of total RNA isolated from spikes of black pepper using different methods.

Method used	$A_{260}/_{230}$	$A_{260}/_{280}$	Concentration of RNA (ng/µl)
CTAB	0.43	1.52	165
SDS	0.32	1.45	112
TRIzol	0.81	1.65	213
Modified TRIzol	2.01	1.99	530

The quality of total RNA extracted using the modified TRIzol method was determined using reverse transcriptase polymerase chain reaction. Verso cDNA synthesis kit Thermo scientific (AB-1453/A) was used to reverse transcribe RNA samples into cDNA, and the resulting cDNA was amplified using β actin a housekeeping gene. RT-PCR product on agarose gel electrophoresis showed a prominent band of expected size (198 bp) (Fig. 2). These results indicate that total RNA extracted from spikes of *Piper nigrum* L. using the modified protocol of TRIzol method is suitable for further molecular biology techniques. When compared to the original TRIzol method, the modified TRIzol method performed significantly better in terms of concentration and purity compared to the reported protocols. Furthermore, CTAB and SDS methods were more time consuming and laborious.

In RNA extraction, degradation of RNA is the major problem caused by ribonuclease (RNase) enzyme. Ribonuclease can be denatured by heat, but when the temperature is reduced, the enzyme spontaneously refolds into its native active configuration. Since, TRIzol (Chomczynski and Sacchi 2006) is a monophasic reagent containing, phenol, guanidine thiocyanate, etc. during phase separation, precipitation and purification of RNA using the original Trizol method the resultant isolated RNA was found to be contaminated with impurities and also low yield. This is in accordance with the earlier reports (MacRae, 2007; Kansal *et al.,* 2008). Conventional protocol using TRIzol reagent is not often successful for different types of cells, tissues and crops in samples with high polyphenol and polysaccharide content. The original TRIzol method is reported to have less success in many crops, including potato tubers, soybean leaves, wheat seeds, and medicinal plants (Bilgin *et al*., 2009; Ghawana *et al*., 2011; Behnam *et al*., 2019; Vennapusa *et al*., 2020). In the present study, the original TRIzol method was modified by incorporating 2% PVP, 4% β mercaptoethanol and 1% SDS. PVP helps to tackle the phenol interference and in liquid can also interact with polysaccharides and assist in their elimination. Moreover, it unfolds the RNase at a much faster rate than RNA hydrolysis. Furthermore, β-mercaptoethanol commonly used as a strong reducing agent or antioxidant helps to destroy disulfide linkages between proteins thereby causing the RNase enzyme to be irreversibly denatured (Ma *et al.,* 2015; Rashid *et al.,* 2016). Thus, PVP and β-mercaptoethanol work independently to block the oxidation of polyphenols and secondary metabolites in the plant samples thereby enhancing the yield and quality of the isolated RNA.

SDS, a strong anionic detergent, has no interactions with nucleic acids (negatively charged) but can play an important role in the dissociation of nucleoprotein complex and separation of proteins from RNA, thereby leading to an improvement in the efficiency of RNA purification (Tan and Yiap 2009; Chang *et al.,* 2016; Barbier *et al.,* 2019, Ekatpure *et al.,* 2019). The addition of the small quantity of TRIzol to the ground sample in the mortar and pestle often causes a time lapse required for evaporation of the liquid nitrogen and often leads to browning and poor yield and quality of the isolated RNA. In the current protocol, this lapse was reduced by transferring the sample to the tubes along with liquid nitrogen followed by the addition of the required TRIzol reagent directly into the tubes.

In the modified TRIzol method incubation time was doubled compared to the original TRIzol method to assure the complete lysis of cells. This incubation process efficiently degraded carbohydrates, proteins, suspended lipids, and other celluloid components. Repetition of organic extraction using chloroform and pellet washing with ethanol was found to be helpful in removing contaminants (phenol, carbohydrates, lipids, cell debris, protein, etc.) and other chemicals that were used for the extraction procedure such as phenol, salts, TRIzol, guanidium thiocyanate etc (Farrell, 2017). This in turn improved RNA recovery. Also, precipitation of RNA at 4ºC compared to room temperature was found to significantly increase the RNA quality. Thus, the modified TRIzol method used in the present study is simple, rapid, and ideal for the extraction of RNA from black pepper spikes and can also be tried for the improvement of efficiency of RNA extraction in other crops with high polyphenol and polysaccharide content.

CONCLUSION AND FUTURE SCOPE

To conclude, total RNA extraction from spikes of black pepper using the modified TRIzol method resulted in higher yield and better quality compared to the already reported protocols using CTAB, SDS and TRIzol. The modifications helped in the efficient removal of polyphenols and polysaccharides. The good quality and quantity of the RNA extracted using the present modified method were validated using gel electrophoresis, quantitative nucleic acid detection, and RT-PCR. The modified protocol has the potential for extraction of good quality RNA from samples with high polyphenol and polysaccharide content.

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Conflict of Interest. None.

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