Bioscience Method 2023, Vol.14, No.1, 1-8
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Research Article Open Access

# A Modified Method of Total RNA Isolation for Mango Leaf Tissues

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Bioscience Method, 2023, Vol.14, No.1 doi: 10.5376/bm.2023.14.0001
Received: 14 Feb., 2022
Accepted: 20 Oct., 2022
Published: 17 Mar., 2023



**Preferred citation for this article**:
Huang Z.B., Ren H., Dong L., Cai Z.Y., Fang W.K., Wang X.M., Su W.Q., Liu Y.Q., and Qiu W.W., 2023, A modified method of total RNA isolation for mango leaf tissues, Bioscience Method, 14(1): 1-8 (doi: 10.5376/bm.2023.14.0001)

**Abstract** Complex metabolic components in mango leaves lead to high difficulty in RNA extraction. In order to improve the quality of total RNA extraction, on the basis of the existing methods reported in the literature, an RNA extraction method combining acetone washing liquid nitrogen abrasive material with 0.3 mol/L lithium chloride (LiCl) solution and 2 times the volume of anhydrous ethanol as the second precipitator was developed. The experimental results showed that the improved method significantly reduced the amount of impurity precipitation in the RNA extraction process. The electrophoretic bands of total RNA extracted were complete. The absorbance ratio of $OD_{260/280}$ was about 1.9, and the average was 329.8 g/g FW±11.2 g/g FW. Further validation experiments of RNA reverse transcription and the polymorphic amplification of double-stranded cDNA related sequences (RAP-PCR) obtained clear polymorphic bands, showing that the total RNA from mango leaves had high quality and was suitable for molecular biology experiments.

**Keywords** Mango; RNA abstraction method; Polysaccharides; Polyphenols

The complex metabolic components of plant tissue have a strong interference effect on RNA extraction, which is one of the difficulties in extracting high-quality RNA from plant tissue. There are great differences in secondary metabolites in different plant tissues, so it is necessary to select and improve the existing extraction methods for RNA from many plant tissues. The commonly used extraction methods of polyphenol-rich plant RNA mainly include guanidine isothiocyanate method, Trizol method, SDS-phenol method and CTAB-LiCl method (Liu and Guan, 2015). However, the existing methods can not be used to extract RNA from different plant tissues, so they often need to be improved according to different tissues. Lithium chloride (LiCl) can selectively precipitate RNA molecules when used as a precipitator, and potassium acetate (KAc) can remove polysaccharides. These two reagents are often used individually or in combination to improve the quality of RNA extraction. CTAB-LiCl method can be used to extract total RNA from *Dioscorea opposita* Tbunb tissues rich in polysaccharides (Qin et al., 2009). Compared with the existing Trizol and CTAB methods, the improved method combining potassium acetate (KAc) and SDS phenol method could better extract total RNA from the leaves of *Platycladus orientalis* with rich content of polysaccharide and polyphenols (Wang et al., 2012). Combined with the method of guanidine isothiocyanate, total RNA of melon tissues rich in polysaccharides was successfully extracted by combining potassium acid (KAc) and lithium chloride (LiCl) with the method of Guanidine isothiocyanate (Hao and Hasi, 2010). These studies indicate that the selection or optimization of RNA extraction methods according to the characteristics of different methods and steps plays an important role in the extraction effect of RNA.

*Mangifera indica* L. is a large evergreen tree in the family of Anacardiaceae, which is native to India. Its popular name is mango. It is a tropical and subtropical fruit with expanding industrial scale. To carry out basic research on fine gene mining, growth and development mechanism, disease and insect resistance mechanism and stress resistance mechanism related to mango will help promote the healthy development of the industry. These basic studies often involve reverse transcription experiments to understand how genes are expressed under specific conditions, and therefore need to extract high-quality RNA. It is difficult to extract RNA from various tissues of mango fruits. An SDS two-step precipitation method for extracting RNA from mature mango peel was reported

earlier (Lopez and Lim, 1991). Based on SDS method, selective precipitation of RNA in LiCl solution was used. Combined with certain concentration of potassium acetate (KAc) and ethanol solution to remove impurities, and through two steps of RNA precipitation, RNA was successfully extracted from the tissue. This method was improved several times later. The grinding sample was washed with acetone and SDS two-step precipitation method was combined to obtain an improved method that could successfully extract RNA from mango cotyledon tissue (Xiao et al., 2003). SDS two-step precipitation method has also been improved to extract total RNA from young mango leaves, peel and flesh, but the extraction quality of leaf tissue RNA is easily affected by leaf age and other factors (Du et al., 2005). Another CTAB method was reported to extract RNA from fruits, flowers and leaves of mango, and total RNA from different periods of mango tissues could be obtained from 100~250 μg/g (Pandit et al., 2007). However, the electrophoresis bands of RNA extracted from leaves of mango by this method were not as clear as those of other tissues. Leaf tissue may be more difficult to extract than other tissues. In addition, the extraction of this method requires 10 mL extraction buffer per gram of sample, so it needs to use a large centrifugal tube for extraction, which has higher requirements for equipment and operation, and higher extraction cost. The improved Tirs-Boric acid method could also extract high-quality total RNA from mango pulp tissue, and the RNA yield was 143.2 μg/g. This method also required more than 10 mL centrifuge tube for extraction (Zhang et al., 2009).

The above studies showed that SDS two-step precipitation method was a classical RNA extraction method, and after many improvements, the RNA extraction ability of mango skins, flesh and leaves was improved. However, the current methods of extracting total RNA from Sijimi mango (*Mangifera indica* L.) leaves are still easy to produce a large amount of impurities precipitation, and it is difficult to extract RNA successfully. Therefore, this study took Sijimi mango as experimental materials and improved the existing methods to further improve the ability of the improved SDS two-step precipitation method to extract high-quality total RNA from plant tissues rich in complex metabolic components based on the existing methods, so as to provide support for subsequent basic research.

## 1 Results and Analysis

### 1.1 Lithium chloride (LiCl) precipitator and acetone washing treatment reduce the generation of impurities precipitate and obtain clear RNA bands

In order to verify the effect of the improved steps on reducing the amount of impurities produced, six groups of comparative experiments were carried out. The results showed that lithium chloride (LiCl) solution instead of potassium acetate (KAc) solution as the secondary precipitant could significantly reduce the generation of impurity precipitates during RNA extraction, while potassium acetate (KAc) was used as the precipitant in the two experimental groups "C, D" and "I, J", a large amount of visible precipitates were generated. The amount of precipitation in other groups without potassium acetate (KAc) precipitator was significantly less; Acetone washing treatment can also effectively prevent the formation of impurities precipitate. The amount of precipitate generated in groups "C" and "D" is significantly less than that in groups "I" and "J" without acetone washing treatment (Figure 1). Pure RNA is colorless and transparent, and adhesion to the tube wall generally requires a careful backlight inspection to identify. These visible large amounts of precipitation are impurities that are difficult to dissolve in water.

Gel electrophoresis showed that the RNA extracted from "C, D" and "I, J" groups using potassium acetate (KAc) as precipitator could not obtain clear RNA bands. The RNA extracted in the "A, B" and "G, H" groups using 0.3 mol/L lithium chloride and 2 times the volume of anhydrous ethanol as precipitant had clearer RNA bands than that in the "E, F" and "K, L" groups using only 2 times the volume of anhydrous ethanol as precipitant. The results showed that the composition of precipitant had an important effect on RNA extraction (Figure 2).

The comparative experimental results showed that the use of acetone to wash the material after grinding with liquid nitrogen and the replacement of 0.3 mol/L lithium chloride (LiCl) solution with potassium acetate (KAc) solution as the precipitant for the second precipitation can reduce impurities precipitation and obtain clear electrophoretic bands. Therefore, the "A, B" experimental group is the best improvement method.

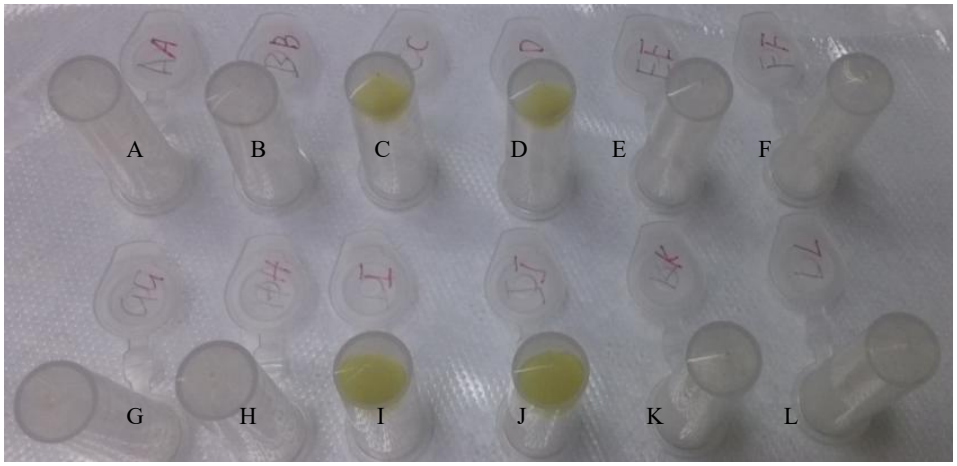


Figure 1 The amount of impurity precipitation in A~L experimental group

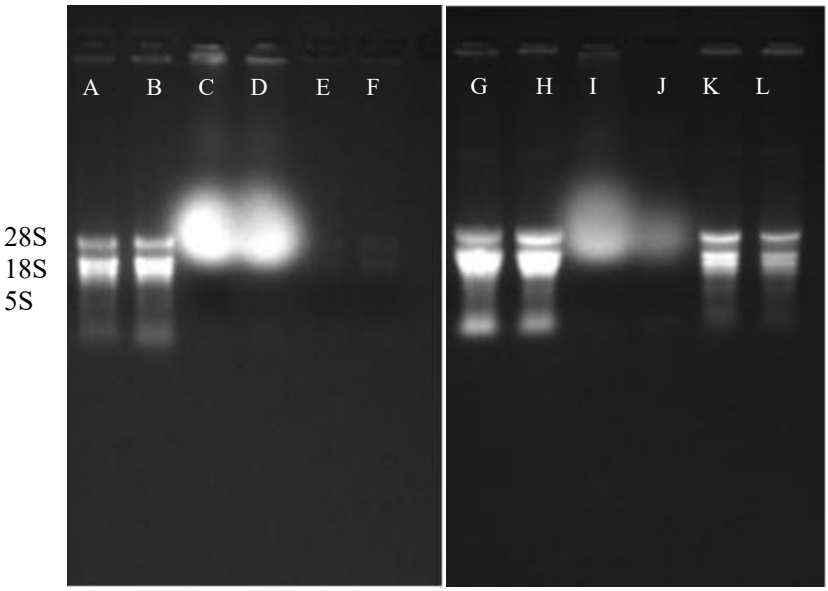


Figure 2 Agarose gel eletrophoresis of total RNA extracted in A~L experimental group

### 1.2 The integrity, yield and purity of total RNA extracted from mango leaves by modified method were better

The total RNA extracted from mango leaves by modified method was directly detected by "IMPLEN P330 NANOPHOTOMETER". $OD_{260/280}$ is used to indicate whether the extracted RNA products have residues of protein or other organic pollutants. The ratio of high purity RNA is in the range of 1.8~2.2. The RNA $OD_{260/280}$ extracted from mango leaves by modified method was about 1.9, with an average yield of (329.8±11.2) μg/g FW, indicating that it had high purity and yield (Table 1). Clear bands of 28S, 18S and 5S were obtained by gel electrophoresis, indicating good integrity of the total RNA (Figure 3).

Table 1 Purity and yield of RNA extracted by modified method

| RNA sample No. | Purity ($OD_{260/280}$) | Yield (μg/g FW) | Average yield (μg/g FW) |
|---|---|---|---|
| 1 | 1.905 | 324.1 | 329.8±11.2 |
| 2 | 1.932 | 345.5 | - |
| 3 | 1.936 | 319.9 | - |

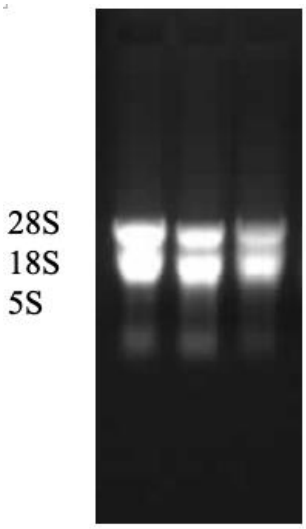


Figure 3 Agarose gel eletrophoresis detection of total RNA extracted by modified method from mango leaf tissue

### 1.3 The results of reverse transcription and related sequence polymorphism amplification (SRAP-PCR) showed that the quality of RNA extracted by modified method was higher

In order to further test the quality of RNA extraction, the total RNA extracted from mango leaves by modified method was prepared into double-stranded cDNA using Takara reverse transcription kit. The gel electrophoresis results obtained 0.3~2 kb dispersive bands, indicating good quality (Figure 4). Four primers ME2-EM2, ME4-EM4, ME44-EM44 and ME44-EM2 were used to amplify the related sequence polymorphism (SRAP-PCR) of the double stranded cDNA made from 1, 2, 3 and 4 mango leaf samples. The polymorphic bands were detected by agar-electrophoresis and gel-imaging system. The results showed that the RNA extracted by modified method could meet the requirements of molecular biology experiments (Figure 5).

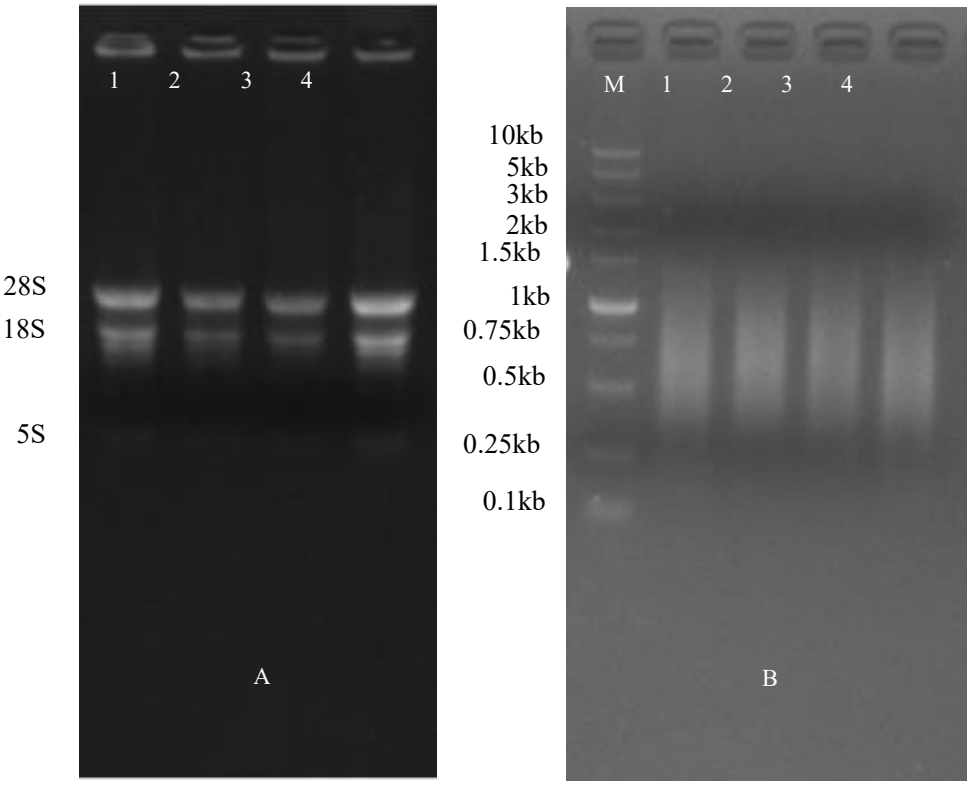


Figure 4 Reverse transcription of total RNA abstracted by modified method

Note: A: 1~4: Total RNA of 4 mango leaf samples prepared for RT-PCR; B: 1~4: Double strand cDNAs obtained by RT-PCR

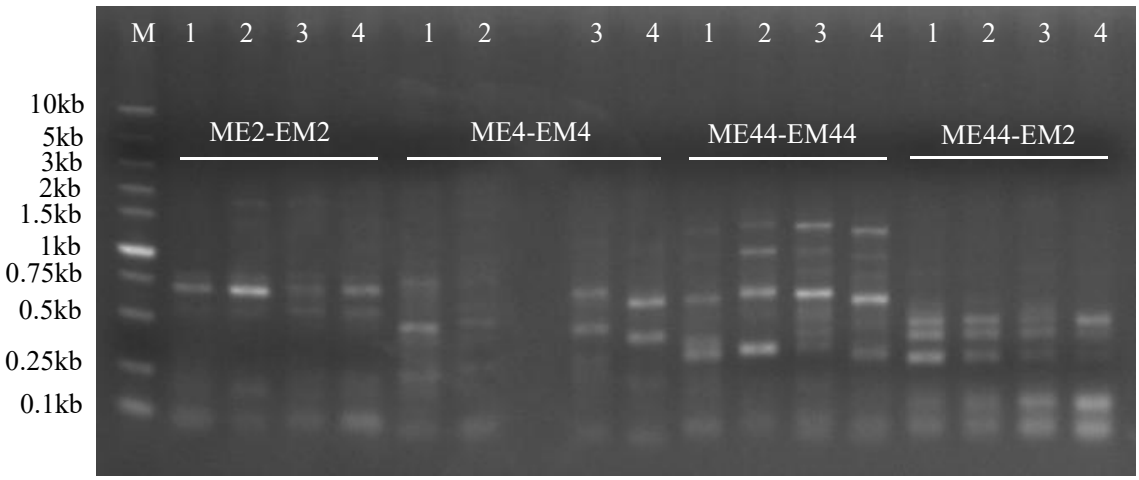


Figure 5 SRAP-PCR amplified polymorphic bands of agarose gel electrophoresis using cDNA as template
Note: M: CW2583 Super DNA Marker; ME2-EM2, ME4-EM4, ME44-EM44, ME44-EM2: 4 primers; 1~4: Double strand cDNAs of 4 mango leaf samples

## 2 Discussion

The difficulty of plant RNA extraction is to avoid the degradation of RNA in the extraction process, and to separate and remove interfering substances from RNA. Polysaccharides, polyphenols, proteins and various secondary metabolites are considered as interfering substances in the process of RNA extraction from plant tissues, and the effective removal of these components has become the objective of optimization of many RNA extraction methods. The extraction of RNA from leaves of woody plants is prone to produce brown precipitates that are insoluble in water, and almost insoluble RNA-phenol-polysaccharide complexes are produced when extracted by SDS/ phenol method (Zeng and Yang, 2002). The abundant secondary metabolites in the leaves of *Reaumuria songarica* also lead to the formation of sticky colloidal complexes during RNA extraction (Wang et al., 2011). Light yellow floc insoluble precipitation was also easy to occur when RNA was extracted from mango leaves. This phenomenon was especially obvious when the extracted materials were Sijimi mango, which might be related to the complex metabolic components in leaves. Studies on metabolites in mango leaves show that mango leaves contain complex components such as glycosides, esters and phenolic acids (Guo et al., 2012; Gu et al., 2013; Zhang et al., 2014).

A certain amount of polyvinyl pyrrolidone (PVP) was added to the improved method to facilitate RNA extraction, as 1% PVP effectively removed phenolic complexes (Salzman et al., 1999). Reductant method, Tris-boric acid method, acetone method and chelation method were used to prevent the oxidation of phenolic substances during RNA extraction. $Li^+$ and $Ca^+$ could precipitate RNA and remove unoxidized phenols (Li et al., 1999). The content of phenolic substances in the cell wall varies greatly in different tissues and at different growth stages, and the effect of phenolic substances on RNA extraction can be effectively removed by treating the grinding sample with acetone (Schneiderbauer et al., 1991). Acetone washing was also used to extract apple RNA (Asif et al., 2006). The preservation of samples with acetone can maintain the stability of proteins, DNA and RNA for a long time (Fukatsu, 1999), so the washing treatment of samples with acetone will not adversely affect the stability of RNA. However, this study found that the extraction quality of RNA would be affected if the sample was in a wet state due to high acetone residue. Therefore, after the grinding sample is washed with acetone, the acetone should be sucked clean as far as possible, and the sample in the centrifugal tube is gently pressed along the tube wall to disperse and adhere to the inner wall of the tube, so as to facilitate the volatilization of residual acetone and sample drying.

When the existing two-step precipitation method was used to extract RNA from the leaves of Sjimi mango, it was easy to produce a large number of insoluble floc-like precipitation when potassium acetate (KAc) was used as precipitator in the supernatant in the second step of precipitation, leading to the failure of RNA extraction. This phenomenon may be related to the large number of complex metabolites in leaf tissues of mango leaves. It was

found that when 0.3 mol/L potassium acetate (KAc) solution was replaced with lithium chloride (LiCl) solution of the same concentration as the precipitant in the second precipitation step, the selective precipitation of RNA could be retained and the formation of impurity precipitation could be effectively avoided. In extracting RNA from mango leaves, acetone is usually used to wash the material after grinding liquid nitrogen with acetone and 0.3 mol/L lithium chloride (LiCl) solution instead of potassium acetate (KAc) solution is used as the precipitate for the second precipitation. This may occur in the extraction of *Mangifera indica* leaves or *Mangifera indica* leaves, but KAc free precipitators must be used. This indicates that the metabolic components in leaf tissues of different varieties and leaf ages of mango may also be different, and affect the difficulty of RNA extraction. The use of anhydrous ethanol alone as a precipitator in the second precipitation step will affect the precipitation of RNA and is not conducive to RNA extraction. In the improved method, the RNA extraction step required the use of acidic phenol: chloroform: isopentyl alcohol (25:24:1). The corresponding alkaline reagent could not be successfully extracted, so the pH of the reagent also had a key influence on RNA extraction.

After synthesis of double-stranded cDNA, agar-electrophoresis detection showed that it was generally dispersed and concentrated between 300 and 2 000 bp, which indicated that the cDNA obtained by reverse transcription was relatively complete and the quality of extracted RNA could meet the requirements of reverse transcription (Xu et al., 2008; Hu et al., 2013; Sun et al., 2015). Genetic diversity analysis of mango germplasm resources based on genomic DNA using SRAP molecular markers (Liu et al., 2019), single-strand cDNA can also be amplified to study the differential genes in the development process of *Chimonanthus praecox* blossom (Zhao et al., 2012), indicating that SRAP molecular marker technology can be used to obtain polymorphic bands from double and single chain DNA molecules. In this study, SRAP molecular markers were used to amplify double-stranded cDNA from mango fruits prepared by reverse transcription. The size distribution of polymorphic bands obtained was consistent with that reported in literature, which further verified the quality of extracted RNA and reverse transcription cDNA.

## 3 Materials and Methods

### 3.1 Experimental material

Leaf materials of Sijimi mango were collected from the Mango Plantation of Guangxi Academy of Agricultural Sciences (N22º50.6464', E108º14.7136'), cut and quickly put into liquid nitrogen, and then stored in the refrigerator at -40℃ for use. DEPC treated water was sterilized and used to configure extraction reagents and dissolve RNA products.

### 3.2 A modified extraction method

Take 0.5 g mango leaves into a mortar, add 0.05 g polyvinyl pyrrolidone, grind mango leaves into powder with liquid nitrogen, quickly transfer to 2 mL centrifuge tube, wash with pre-cooled acetone solution for 2-3 times, centrifuge at 12 000 r/min at 4℃ for 5 min after each washing, discard acetone. Place the centrifuge tube to collect precipitation in an ice opening for an appropriate time until the sample is dry.

Add 600 μL extraction buffer (2% SDS, 1% 2-Hydroxy-1-ethanethiol, 50 mmol/L EDTA, 150 mmol/L Tris (adjust pH to 7.5 with 1 mol/L boric acid)), shake and mix at room temperature.

150 μL anhydrous ethanol, 66 μL 5 mol/L potassium acetate, 600-800 μL acidic phenol: chloroform: isopentyl alcohol (25:24:1), centrifugation at 12 000 r/min for 10 min; The recovered supernatant was repeatedly extracted once.

Carefully absorb the supernatant, add LiCl solution to the final concentration of 3 mol/L, and precipitate overnight in the refrigerator at -20℃.

RNA was collected by centrifugation at 12 000 r/min at 4℃ for 10 min, and washed twice with 3 mol/L LiCL. After each washing, RNA was collected by centrifugation at 12 000 r/min at 4℃ for 10 min.

DEPC treated water was used to re-dissolve RNA precipitation, and LiCL solution was added to the final concentration of 0.3 mol/L. Anhydrous ethanol was added to the solution, and precipitation was carried out

overnight in the refrigerator at -20℃. The precipitation was collected by centrifugation at 12 000 r/min at 4℃ for 15 min. The precipitation was washed and precipitated with 75% and 100% ethanol, respectively. After each washing, the RNA precipitation was collected by centrifugation at 12 000 r/min at 4℃ for 10 min.

The opening of the centrifuge tube containing RNA products was inverted and properly dried, and the RNA solution was dissolved with appropriate DEPC treatment water.

### 3.3 Effectiveness comparison and verification of improvement steps

In order to verify whether the improved procedure can effectively reduce the production of RNA precipitation, six groups of comparative experiments were designed according to different material treatment methods and different components of the second precipitant (Table 2).

Table 2 Comparative experimental design for the effect of different material treatments and precipitant composition on the amount of impurities precipitated during RNA extraction

| Sample No. | Material treatment | Agent composition in the second precipitation |
|---|---|---|
| A, B | Liquid nitrogen grinding, Acetone washing | 0.3 mol/L LiCl, double volume of ethanol |
| C, D | Liquid nitrogen grinding, Acetone washing | 0.3 mol/L KAc, double volume of ethanol |
| E, F | Liquid nitrogen grinding, Acetone washing | Double volume of ethanol |
| G, H | Liquid nitrogen grinding | 0.3 mol/L LiCl, double volume of ethanol |
| I, J | Liquid nitrogen grinding | 0.3 mol/L KAc, double volume of ethanol |
| K, L | Liquid nitrogen grinding | Double volume of ethanol |

### 3.4 Quality detection of RNA extraction

The concentration and purity of RNA were tested using an "Implen P330 Nanophotometer" (purchased from Implen, Germany). Electrophoresis test conditions: 1.2% agarose gel, 1×TAE buffer.

## Authors' contributions

HZB is the experimental designer and executor of this study, and has written the first draft of this paper. DL, CZY, FWK, WXM participated in the test results and data analysis; SWQ, LYQ and QWW participated in the revision of the paper; RH is the designer and principal of the project, directing experimental design, data analysis, paper writing and revision. All authors read and approved the final manuscript.

## Acknowledgement

This study was supported by Guangxi Key Research and Development Program (Guike AB18294031, AB16380068), Basic Scientific Research Business Special Project of Guangxi Academy of Agricultural Sciences (Guinongke 2020YM28; 2015JZ82) and Team Project of Special Advantage Disciplines for Basic Research Business of Guangxi Academy of Agricultural Sciences (Guinongke 2015YT47).

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