RESEARCH ARTICLE

RAPID AND RELIABLE METHOD FOR HIGH QUALITY RNA ISOLATION FROM MAJOR SEED SPICES

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Abstract: In plant molecular biology, isolation of high quantity with best quality of RNA is a crucial technique. The quality of RNA determines the reliability of downstream process like real time PCR. Isolation of RNA from seed spice crops is in itself a major challenge as there is an abundance of polysaccharides and oleoresin in the plant material. Three major seed spices Fenugreek, Coriander and Fennel were considered for this study. For all seed spices, where conventional isolation procedures gave poor results the present study describes a modified and more consistent method, which yields a greater quantity of RNA compared to the use of conventional protocols for seed spices. The protocol, in the present paper yielded 1.2-2.7 µg of RNA per 100 mg of fresh tissue and took only 3 hrs to complete. In-spite of the quantity, RNA obtained was of high quality and proved suitable to RT-PCR.

Keywords: Seed spices, Coriander, Fennel, Fenugreek, RNA, RT-PCR, Nano-drop

INTRODUCTION

Spices have a profound influence on the course of human civilization. They permeate our lives from birth to death (Anwer *et al*., 2011). Coriander (*Coriandrum sativum*), Fennel (*Foeniculum vulgare*) and Fenugreek (*Trigonella foenum-graecum*) are the seed spices which are identified as annual herbs. In everyday life, spices succour us, cure us and relax us and are widely used as seed or leaf in food, beverages and medicines as a spice or an added ingredient. They are very rich source of antioxidants and used in many medicines to cure diseases (Bagdassarian *et al.*, 2013). Seed spices are the cash crops and play a significant role in Indian economy and yet very little information is available about genome of these crops. Now a days, use of advanced biotechnological tools is becoming a very important part of breeding programmes. Conventional breeding processes are very tedious and time consuming but molecular marker assisted breeding is time saving process (Kalinowska *et al.*, 2012). Molecular markers are very essential tools for advance breeding programmes but unfortunately, the present scenario reveal that very few molecular markers are available for seed spices. So, to enhance the molecular studies for these crops, it has become a necessity to devise rapid and effective method of DNA and RNA isolation.

Decrypting the entire mechanisms of gene expression, gene regulation, signal transduction and transcriptome analysis requires an unabridged range of techniques such as reverse transcription, southern hybridization, northern hybridization, polymerase chain reaction (RT-PCR), and construction of cDNA libraries. Substantially pure and un-degraded DNA and RNA are fundamental necessities for all these techniques. A large number of protocols have been developed or extensively modified, and commercial kits are also available for isolation of RNA from plant tissues. Most of these methods, including kits, were found to be unsuitable for isolation of RNA from the seed and leaves of seed spices. Some renounced protocols which are available for RNA isolation in Jute (Khan *et al.*, 2004), *Arabidopsis thaliana* (Suzuki *et al.*, 2004), *Fallopia multiflora* (Chen *et al.*, 2012), sweet potato (Lam *et al.*, 2009) and tea (Muoki *et al.*, 2012) are found to be unsuitable in case of seed spices as these crops have a high concentration of flavonoids and phenolic contents. These antioxidants can directly affect the quality as well as the quantity of nucleic acids isolated, as the phenolic substances interact irreversibly with nucleic acids and proteins (Loomis, 1974), leading to their oxidation and degradation (Dabo, 1993) and often interfere with downstream applications thereby making the nucleic acid unusable. A single nucleic acid isolation method is not likely to be suitable for all plants as different plants contain different amounts of nucleic acids (Loomis, 1974). Therefore, it can be inferred that the single isolation protocol is unlikely to be equally effective for all species (Weishing *et al.*, 1995).

Looking to the need to characterize the genome and gene expression in coriander, fennel and fenugreek, in the present study a suitable and highly efficient RNA isolation protocol is devised. Special attention was paid to extraction efficiency and recovery of RNA from seeds of different seed spices to be used in RT-PCR and RNA sequencing. It is a modified protocol that was derived from the TRIzol and EZNA RNA extraction kit, using this protocol we have isolated high quality DNA and RNA from coriander, fennel and fenugreek that is time saving and cost effective.

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MATERIAL METHOD

Plant material

Coriander (*Coriandrum sativum*L.), Fennel (*Foeniculum vulgare* L.) and Fenugreek (*Trigonella foenum-graecum* L.) fresh leaf tissue were used for RNA isolation. The seed were collected either from the local market. The seeds were placed on a moist filter paper in a Petri dish, subjected to no prior treatment,10 seeds/plate at 25ºC for overnight and next day transplanted in pots. The seed were allowed to grow for eight days. Leaves of these eight days old plants were taken for RNA isolation.

Solutions and reagents

1. TRIzol
2. RNA wash buffer
3. DNase Buffer
4. Isoamyl alcohol
5. Sodium Acetate
6. Isopropenol
7. 70% ethanol
8. Liquid nitrogen and dry ice
9. Chloroform
10. Isopropanol (2-propanol)
11. Diethyl pyrocarbonate (DEPC)
12. 2X RNA loading dye
13. High Range RNA ladder
14. Agarose

Equipments

DEPC water treated and autoclaved pestle and mortar
High speed centrifuge (Backman Coulter)
Nano-Drop (Thermo NanoDrop2000)
Agarose gel electrophoresis equipment (Thermo Easycast B1)
Power supply (Thermo EC3000 XL)
Vortex mixer (VWR vortexer mini)
Q-PCR (Bio-Rad)

An Improved method for RNA Isolation: Two replications of 100mg fresh leaves were homogenized in liquid nitrogen and 1 ml of Trizol was added to a fine powder using DEPC water treated mortar and pestle. (Kept for thawing for 5 min)

1. The samples were transferred to 2ml micro centrifuge tubes and mixed well with 200µl chloroform by inversion, vortexed and incubated at room temperature for 5 min.
2. The samples were centrifuged at 12,000rpm for 15 min at 4ºC. 250-500 µl aqueous phase was transferred to a fresh 2ml micro centrifuge tubes.
3. 500µl ethanol was added to aqueous phase and vortexed for 2min, a precipitate was formed which was applied to HiBind^R RNA 2ml mini columns.
4. Centrifuged at 13,000 rpm for 1min at room temperature. Filtrate was discarded and collection tubes were reused in next step.
5. 250 µl RNA wash buffer was added directly onto the HiBind^R RNA mini column and centrifuged at 13,000rpm for 1min. Filtrate was discarded and tubes were collected.
6. Freshly prepared 75µl DNase 1 buffer was pipetted directly onto the surface of the HiBind^R matrix in each column and incubated at room temperature for 15 min.
7. 500 µl RNA wash buffer1 was added and column was placed on bench top for two minutes then centrifuge at 13000rpm for 1min, filtrate was discarded and collection tubes were reused.
8. 500 µl RNA wash buffer 1 was added and centrifuge at 13000rpm for 1min, filtrate was discarded and collection tubes were reused. (Step repeated x 1).
9. The HiBind^R RNA mini column were centrifuged with empty collection tubes for 2min at 13000rpm to completely dry the HiBind^R RNA mini columns.
10. Place the column in a clean 1.5 ml micro-centrifuge tube (not supplied) and add 40-70 µl of DEPC water make sure to add water directly onto the centre of the column matrix. Let it sit for 1 min and then centrifuge for 2 minutes at maximum speed to elute the RNA. A second elution may be necessary if the expected yield of RNA>30 µg.
11. Alternatively, RNA may be eluted with a greater volume of water. While the additional elution increase the total RNA yield. The concentration will be lower since more than 80% of RNA has been recovered in the first solution.

RNA Quality and Quantification

Gel analysis

The RNA was size fractionated on a 1% formaldehyde agarose gel (Sambrook and Russell, 2001) and visualized using UV illumination.

NanoDrop Analysis

Purity and concentration of RNA was assessed by determining the absorbance of the sample at 260 and 280 nm using a NanoDrop. (Specord 200, Analytica Jena, AG, Germany).

RT-PCR analysis

Reverse Transcription-PCR was carried out using the RT-PCR System in a single tube reaction. The reverse transcription was carried out at 48 °C for 50 min, followed by 94 °C for 2 min. The PCR reaction was carried out for 30 cycles. The conditions for each cycle were: denaturation at 94 °C for 30 sec, annealing at 42 °C for 1 min and extension at 70 °C for 2 min.

RESULT AND DISCUSSION

Till date a range of methods are reported for the isolation of RNA from plants, but failed to yield high quality ribonucleic acids from seed spices. The presence of polysaccharides, oleoresins and other metabolites can hamper isolation procedures and reactions such as amplification and cloning. The major obstacles, such as low yield degradation and

poor PCR amplification were easily overcome by using the above devised protocol specifically for major seed spices, rich in polysaccharide, oleoresins and other metabolites.

The distinctive steps, which make this protocol different from others are chloroform extractions placed at critical steps as not to lower the yield. Also, an alternative method of growing the plants was devised. In conventional methods the seeds were sown in soil with fertilizer and the plants were harvested at various stages of growth. It was observed that younger plants have, lower polysaccharide and metabolite content. However, even the youngest plants harvested had very high polysaccharides which made nucleic acid isolation difficult. The ideal growing method was devised by allowing the seeds to germinate on moist filter paper in Petri dishes at a controlled temperature of 25^{o} to 28^{o}C for 8-9 days. This method proved to be advantageous in overcoming the effect of oleoresins and other polysaccharide as well as soil contamination by allowing the plants to use only the materials stored in the endosperm and sterile soil for growth and development.

The quantity and purity of RNA preparations were found to be superior when using this simple modification as compared to other protocols. RNA concentration was very much improved. Electrophoresis on Ethidium Bromide Agarose Gel resolved RNA bands with no DNA contamination and degradation in all samples. The RNA yield measured was about 720 µg per gram of tissue.

The $A_{260/280}$ and $A_{260/230}$ ratios of RNA extracts indicated there was no protein, phenol or other contaminants that absorb strongly at or near 280 nm (Table 1). RNA extracts obtained with our modification were suitable for RT-PCR. RT-PCR with various primers (Fig. 1), was executed based on the intactness and quality of RNA bands paving the way to clone genes for diverse significant morphological as well as genetic characters.

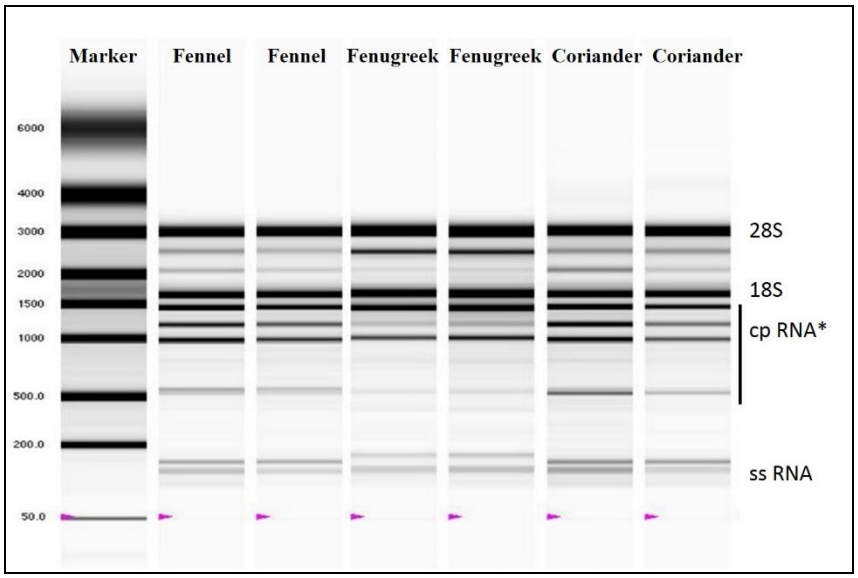

Figure 1: RT-PCR amplified RNA products on bioanalyser with specific primers for Fennel, Fenugreek and Coriander. Image of typical high quality RNA showing the clear cytosolic and plastidic (Cp,asterisks) ribosomal bands. RNA species of low molecular weight are also apparent. gDNA contamination is effectively removed by DNase treatment.

CONCLUSION

The distinguishing feature of the protocol was its success, with seed spices tissues wherein the commonly used protocols failed as they contain different polysaccharides, oleoresins and metabolites. The protocol is simple, does not require any specialized material, chemical, instrument and procedure such as ultracentrifugation step through Cesium chloride gradient or lithium chloride precipitation, thereby greatly reducing the complications and the time required. The results of this study also indicate that, apart from the choice of chemical reagents etc., the method of growing seedlings and tissue homogenization is a critical step.

Table 1. $A_{260/280}$ and $A_{260/230}$ ratios of RNA extracts and yield in ng/ µl

Crop/ Accession	R1			R2		
	Purity (nm)		Yield	Purity (nm)		Yield
	$A_{260/280}$	$A_{260/230}$	Con. (ng/µl)	$A_{260/280}$	$A_{260/230}$	Con.(ng/µl)
Cor1	2.15	2.36	1338.3	2.15	2.19	2230.3

Cor2	2.16	2.39	1207.2	2.16	2.09	1743.0
Fel1	2.17	2.36	1602.5	2.14	2.33	2046.0
Fel2	2.16	2.32	2079.6	2.14	2.42	1240.1
Fng1	2.16	2.35	2102.7	2.12	2.40	2082.1
Fng2	2.19	2.43	2566.4	2.16	2.35	2746.5

Cor=Coriander, Fel=Fennel, Fng=Fenugreek, 1=replication1, 2=replication2.

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