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High Quality RNA Isolation from Leaf, Shell, Root Tissues and Callus of Hazelnut (*Corylus avellana* L.)

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Abstract

Extraction of high quality RNA is a critical step in molecular genetics studies. Hazelnut is one of the most important nuts plants in the world. The presence of the taxol and other taxanes in hazelnut plant necessitates explaining their biosynthesis pathway and identifying the candidate genes. Therefore, an easy and practical method is necessary for RNA extraction from hazelnuts. Hazelnut has high levels of phenolic compounds. High amounts of polyphenolic and polysaccharide compounds in plants could be causing problems in RNA extraction procedures. To avoid these problems, a simple and efficient method can be used based on cetyltrimethylammonium bromide (CTAB) extraction buffer and lithium chloride for extraction of high quality RNA from different parts of hazelnut plant. Using this method, a high-quality RNA sample (light absorbed in the A260/A280 was 2.04<A260nm/A280nm<1.94 and yield of RNA was 50-110 µg RNA/g fresh weight for different parts) was obtained from the leaf, shell, root tissues and callus of hazelnut.

Keywords: CTAB extraction, Hazelnut, RNA extraction, Taxol.

Introduction

Hazelnut (*Corylus avellana* L.) belongs to the dicot group of angiosperms and the betulaceae family. It is reported that this plant contains important taxanes that are involved in cancer treatment (Hoffman *et al*., 1998). Further studies confirmed the presence of taxanes in the leaf, green shell, brown shell and suspension cell culture of hazelnut (Ottaggio *et al*., 2008; Hoffman and Shahidi, 2009). According to these results, it can be predicted that there should be a metabolic pathway for biosynthesis of taxol in hazelnut tissues. The presence of these valuable compounds in the plant hazelnuts has made this plant very important. Therefore, the determination of the key genes that are involved in this pathway is crucial to molecular studies. High quality RNA isolation for gene expression studies, such as RT-PCR, real-time PCR, RACE and functional genomic studies, such as the generation of expressed sequence tags, next generation sequencing, and cDNA library plays a key role in molecular studies. On the other hand, the presence of secondary metabolites such as phenolic compounds prevents the extraction of high quality RNA.

Many protocols have been reported for RNA extraction from various plants. However, a few of them were suitable for plants containing secondary metab-

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olites, phenolic or polysaccharide compounds (Table1). Diversity and differences among different plant species and their biochemical properties make it difficult to use the same RNA extraction protocol for all of the species (Weishing *et al*., 1995). Therefore, it is difficult to introduce a general RNA extraction protocol suitable to all kind of plants. Also, most of these protocols have been recommended for specific plant species or specific organs that may limit them to certain conditions.

Table 1. RNA isolated from some plants with high contains phenolic, secondary metabolites or polysaccharide compounds.

Plant /scientific name/species	Reference
Pine Trees	Chang *et al*., 1993 Claros and Cánovas, 1998
Sorrel (*Rumex acetosa*)	Ainsworth., 1994
Grapevine	Geuna *et al*., 1998
Bilberry *(Vaccinium myrtillus L.)*	Jaakola *et al*., 2001
Eucalyptus	Suzuki *et al*., 2003
rheum (*Rheum australe*) and arnebia (*Arnebia euchroma*)	Sanjay *et al*., 2011
Myrciaria dubia "CAMU CAMU"	Gómez *et al*., 2013
Hylocereus sp	Li-Min *et al*., 2014

Hazelnut shell and leaves contain high levels of phenolic compounds (Amaral *et al*., 2005; Ciarmiello *et al*., 2014), which make it difficult to isolate RNA from the Hazelnut tissues. Phenolic compounds are a group of secondary metabolites (Scalbert and Williamson, 2000). These compounds have negative effects on RNA extraction procedures. Plant tissues are oxidized and converted to quinone. Quinones bind to the nucleic acids through covalent bonds and deposition with RNA (Loomis, 1974). Therefore, elimination of this sort of compounds is crucial. Presence of these compounds in cell extract makes RNA extraction time-consuming and tedious. Phenolic compounds often reduce the RNA quality and quantity of the extract.

In this research, some protocols for plants with high phenolic compounds were studied (Table 1) and with some modifications, an easy, practical and efficient method for RNA extraction from hazelnut shell, leaves, root and callous is introduced.

Materials and Methods

Plant material

Shells and leaves were collected from Gilan Eshkevarat and immediately frozen into liquid nitrogen and stored at –80ºC until needed. Roots obtained from seed that cultivated in pot and callus was obtained from seeds (after sterilization of seeds and separate the woody seed coat, seed placed in the Callus-inducing medium).

Solutions

All solutions were prepared with distilled water and autoclaved, Diethylpyrocarbonate (DEPC) was not used.

Extraction buffer

(The modified CTAB method (Chang *et al*., 1993; Jaakola *et al*., 2001) was followed with some modification): 2% CTAB (hexadecyltrimethylammonium bromide), 3% PVP (polyvinylpyrrolidone, molwt 25,000), 150mM Tris- HCl (pH 8.0),

25mM EDTA, 2.0M NaCl, 3.5mM spermidine. These solutions are mixed together and autoclaved.

2-mercaptoethanol as much as 2.5% and 20mM ascorbic acid are added just prior to use (Ascorbic acid and 2-mercaptoethanol is not stability at high temperatures, so these materials are not autoclavable and must added just prior to use (Sambrook *et al*., 1989; Leland *et al*., 2011)). 10M lithium chloride, Chloroform: isoamyl alcohol at a ratio of 24:1

Phenol (Sigma; phenol phase equilibrated to pH 4.3).

Methods (Wear gloves and protective eyewear at all times)

1. Grind 300-400 mg of fresh (frozen) tissue in a mortar and pestle (autoclaved twice and pre-cooled with liquid nitrogen) using liquid nitrogen to a fine powder. To obtain best conceivable quality yield of RNA the liberation of cellular components, perfect tissue disruption is necessary. After grinding, leaf tissue can be stored at 80° until ready for use.

2. Transfer (approximately 80-100 mg) of powder into three sterile 2 mL tubes (pre-cooled with liquid nitrogen)

3. Add 850 µL extraction buffers (Per-warmed to 65°C in Bain-marie) to each tube.

4. Add 22 µL 2-mercaptoethanol and 20mM ascorbic acid (final concentration) in each tubes and mix thoroughly in a vortex.

5. Incubate the tubes at 65°C for 10-15 minutes, during the incubation time, vortex the tube 3–5 times.

6. Add the 850 µL of ice-cold Chloroform: isoamyl alcohol (IAA) (24:1) and mix gently by inversion.

7. Centrifuge samples at 12,000 rpm for 10 min at room temperature.

8. Collect upper aqueous phase (the samples are including of three phases) and carefully transfer to a fresh tube. (Fig. 1A). It is very important that upper aqueous phase not admix with other phases so, should be performed carefully and take only the top phase and not cell wall debris, denatured protein, and polysaccharides complexed to CTAB at the interface.

9. Add equal volume of phenol: chloroform: IAA (25:24:1) to the sample and invert several times Incubate on ice for five minutes.

10. Add an equal volume of ice-cold Chloroform: isoamyl alcohol (IAA) (24:1) then invert gently several times and centrifuge at 12,000 rpm for 10 minutes at room temperature.

11. Collect upper aqueous phase carefully (To escape of genomic contamination and get high purity RNA should be avoiding the white interface between the aqueous and organic layers and it is better that ignore some of the aqueous layer (Fig. 1B).

12. Repeat the above step twice.

13. Combine the samples into one (or two) sterile 2 mL (1.5 mL) tubes.

14. Add approximately 30% volume10M lithium chloride and mix lightly (final concentration of Lithium Chloride 2.8M)

15. Incubation 60-90 minutes at -80 °C or 90-120 minutes -20 °C for RNA precipitation.

16. Centrifuge the tubes at 13,400 rpm for 30 min at room temperature.

17. Wash the RNA pellet with ice cold ethanol %70 twice.

18. Dry pellet and add 25-40 µL double distilled water.

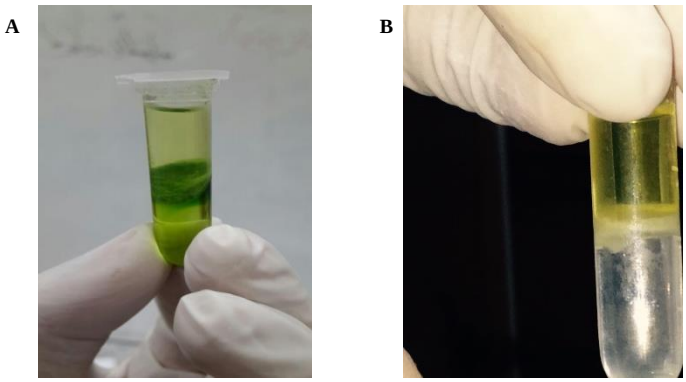

Fig. 1. Step 8 and formed three separate phases **A)** In this step cell wall debris, denatured protein, and polysaccharides complexed to CTAB while retaining the nucleic acids in solution will be removed. **B)** In this step after centrifugation, a white interface, i.e., the CTAB-protein/polysaccharide complexes to be eliminated should be visible. (Ralph, 2000)

Results

Determine the quantity and quality of RNA

After RNA extraction, the quality and quantity were measured using a spectrophotometer (Table 2) and 1% agarose gel (Fig. 2).

Table 2. The amount of light absorbed in the A260 / A280 and A260 / A230 in different parts of hazelnuts

Samples	A260/A280	A260/A230	RNA µg /g
Leaf	2.01± .34	2.25± .3	50-90
Shell	1.94±.37	2.53± .1	40-80
Root	2.04±.22	2.37± .08	70-100
Callus	1.98±.21	2.33±.06	70-110

Fig. 2. RNA isolation Gel.1- Callous RNA 2-Root RNA 3-Shell RNA 4- Leaf RNA.

Discussion

We have proposed a modified method for isolating RNA from four parts of hazelnut. The isolation of RNA inactivation of RNase is essential. This method is a relatively quick procedure and RNA is converted to cDNA (Fig. 3) to reduce RNase activity materials. The equipment was autoclaved twice and chemical agents were not used for inactivation of RNase. For long term storage of RNA, we recommend using the RNase inactivation agent such as DEPC.

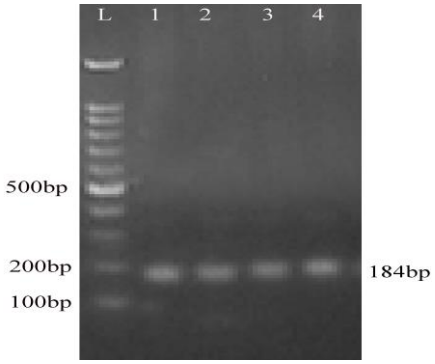

Fig. 3. cDNA synthesized from total RNA extracted from different parts of hazelnut with reference gene
L: 100bp ladder; lane 1: callous, lane 2: root, lane 3: shell, lane 4: leaf

In our proposed method, the ratio of absorptions at 260 nm and 280 nm (A260nm/ A280nm) means were 2.04< A260nm/ A280nm<1.94 demonstrated that total RNA obtained was not contaminated by proteins. The A260nm/ A230nm means were 2.33<A260nm/A230nm<2.53, indicating that the total isolated RNA was not contaminated by polysaccharides and polyphenols. The presence of phenolic compounds, polysaccharides and complex secondary products cause loss of ability to acquire successful RNA isolation (Shultz *et al*., 1994). To remove phenolic compounds, we used 3% PVP 2% PVP has been used in some studies (Zeng and Yang, 2002; Jaakola *et al*., 2001). PVP connects with phenolic compounds and forms a complex with them (Loomis, 1974) and therefore, these compounds separate from RNA.

The chemical agent 2-Mercaptoethanol has an important role and was used as much as 2.5% in this protocol. 2-Mercaptoethanol cleaves disulfide bonds in ribonuclease (Claros and Canovas, 1999) and causes disruption of RNases. Moreover, 2-Mercaptoethanol prevents the oxidation of samples, (Claros and Ca´novas, 1998) and it can reduce quinone (phenol oxidation products) formation due to the oxidation of phenolic compounds. Quinones with the formation of radicals can break phosphodiester bonds and connections to nucleic acids and can compromise the integrity of the nucleic acid samples (Farrell, 2010). To sequester the contaminated DNA, we used 10M LiCl (final concentration of LiCl was 2.8M) precipitation and incubated it for 60-90 minutes at -80°C (or 90-120 minutes -20°C). Jaakola *et al.*, (2001) and Chang *et al*., (1993) extracted RNA from pine trees and bilberry. They used 25% the volume of the previous stage (the same step of our protocol) of 10M LiCl. LiCl selectively precipitated the RNA (Farrell 2010) and compared with other monovalent cations, is more efficient because there is less DNA and protein precipitation, which improves RNA isolation and cDNA synthesis efficiency (Barlow *et al*., 1963; Cathala *et al*., 1983). Also, acid phenol (pH~4.3) in phenol–chloroform extraction step has been used to remove DNA. 20mM ascorbic acid was used in this protocol. 10Mm ascorbic acid have been recommended for RNA isolation from bark and fruit (Farrell, 2010). Ascorbic acid is an antioxidant that could be useful to isolate nucleic acids in plants that contain a large amount of polyphenols (Seiob and Tolbert, 1982; Borse *et al*., 2011). Ascorbic acid prevents oxidation of polyphenols, changes the pH of extraction buffer as well as prevents the activity of polyphenol oxidase and oxidation (Borse *et al*., 2011). The presence

of spermidine in extraction buffer helps to remove the DNA by forming a low-solubility complex with genomic DNA (Farrell, 2010).

In this protocol, we optimized RNA extraction from the hazelnut plant that has a high phenolic compound with suitable quality (Table 2). This protocol can be useful for RNA isolation in other plants with high phenolic compounds.

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