Electronic Journal of Biotechnology ISSN: 0717-3458
Vol.11 No.3, Issue of July 15, 2008

Received April 15, 2007 / Accepted January 23, 2008
DOI: 10.2225/vol11-issue3-fulltext-5
RESEARCH ARTICLE

Efficient protocol for isolation of functional RNA from different grape tissue rich in polyphenols and polysaccharides for gene expression studies

Hemanth K.N. Vasanthaiah*
Division of Plant Biotechnology
Center for Viticulture and Small Fruit Research
Florida A & M University
6505 Mahan Drive, Tallahassee
Florida - 32317, USA
Tel: 1 850 412 5191
Fax: 1 850 561 2617
E-mail: hemanth.vasanthaiah@gmail.com

Ramesh Katam
Division of Plant Biotechnology
Center for Viticulture and Small Fruit Research
Florida A & M University
6505 Mahan Drive, Tallahassee
Florida - 32317, USA
Tel: 1 850 412 5190
Fax: 1 850 561 2617
E-mail:katamr@yahoo.com

Mehboob B. Sheikh
Division of Plant Biotechnology
Center for Viticulture and Small Fruit Research
Florida A & M University
6505 Mahan Drive, Tallahassee
Florida - 32317, USA
Tel: 1 850 412 5189
Fax: 1 850 561 2617
E-mail: mehboob.sheikh@famu.edu

Website: http://www.famu.edu/oldsite/acad/colleges/cesta/vit-faculty.htm

Financial support: The research was supported by Florida Grape Growers Association.

Keywords: differential display RT-PCR, grape, RNA isolation, RT-PCR, subtractive hybridization, *Vitis*.

Abbreviations: CTAB: Cetyl Trimethyl Ammonium Bromide
DEPC: Diethyl pyrocarbonate
GT: Guanadine thiocyanite
PVP: Polyvinylpyrrolidone
RT-PCR: Reverse transcription polymerase chain reaction
SDS: Sodium dodecyl sulfate

Extraction of RNA from plant tissue containing high levels of polyphenols and polysaccharides is tedious and difficult in grapes. Although several protocols have been published for plant RNA isolation, most have failed to yield high quality RNA in sufficient quantity from mature and diseased grape tissue. We describe a protocol for isolating intact and high quality RNA from various grape tissues as evident by high A_{260}/A_{280} absorbance ratio (1.8 to 1.9) and electrophoretic profile on denaturing formaldehyde agarose gel. On an average, 205 µg RNA per g of fresh tissue were obtained using this modified protocol. RNA quality was further assessed through RT-PCR, differential display RT-PCR and subtractive hybridization, and found to be suitable for molecular studies.

Isolation of high quality RNA is an important first step in gene expression studies. However, presence of high levels of polyphenols and polysaccharides in certain plant tissue makes RNA extraction often difficult and requires extensive modification of protocols. Thus, RNA recovery differs from tissue to tissue based on the levels of polyphenols and polysaccharides (Malnoy et al. 2001). This is particularly true for grape as it contains high levels of

*Corresponding author

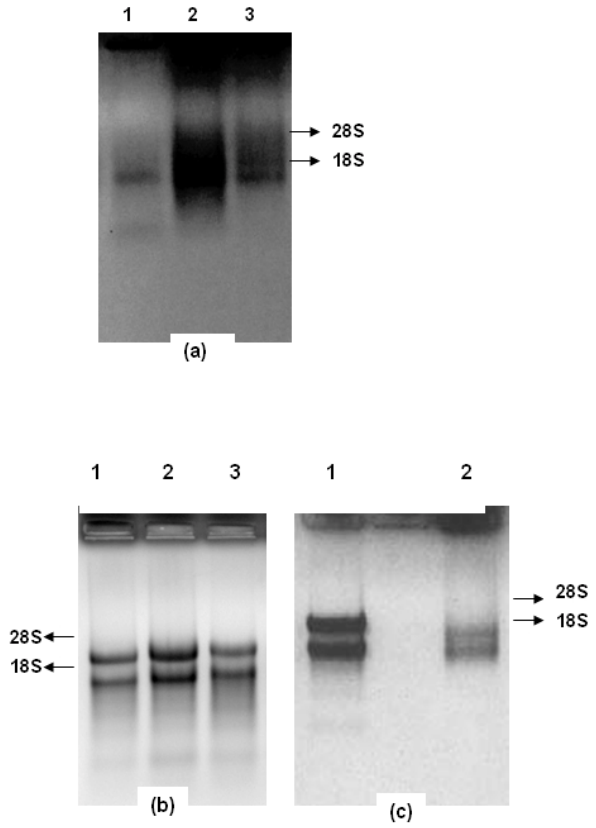

Figure 1. Total RNA isolated from different mature and diseased grape tissue.
(a) Salzman method: Zinfandel (Pierce's disease infected), Lane 1: Xylem, Lane 2: Phloem and Lane 3: Petiole.
(b) Modified method: Zinfandel (Pierce's disease infected), Lane 1: Xylem, Lane 2: Phloem and Lane 3: Petiole.
(c) Lake Emerald, Lane 1: Healthy leaf and Lane 2: Anthracnose infected leaf.

extractable phenols and polysaccharides (Claros and Canovas, 1998; Geuna et al. 1998; Thomas and Schiefelbein, 2002; Tattersall et al. 2005), and especially for mature and infected leaf tissue (Shellie et al. 1997).

Several CTAB, SDS and GT methods (Levi et al. 1992; Lopez-Gomez and Gomez-Lim, 1992; Chang et al. 1993; Liu et al. 1998; Salzman et al. 1999; Kiefer et al. 2000; Iandolino et al. 2004; Thomas and Schiefelbein, 2002; Tattersall et al. 2005; Vasanthaiah et al. 2006) have been developed to isolate total RNA from tissue containing high levels of polyphenols and polysaccharides. In addition to these there are several commercial kits and Trizol (Sigma Co.) available for RNA isolation. However, isolation of RNA from grape tissue using above methods failed to yield adequate amount of high quality RNA, especially in mature and infected tissues necessary for subtractive hybridization, differential display RT-PCR (DDRT-PCR) and RT-PCR. The major problem appears to be co-precipitation of polyphenols and polysaccharides (Levi et al. 1992; Lopez-Gomez and Gomez-Lim, 1992; Malnoy et al. 2001) along with the RNA. These compounds bind to RNA and render it unsuitable for gene expression studies. Our aim has been to study differential expression of genes during the course of fungal and bacterial infection, in particular anthracnose and Pierce's disease of grape, respectively. Although Salzman et al. (1999) method yielded RNA from mature and infected grape tissue, it was found to be inadequate and degraded. DDRT-PCR using this RNA resulted in amplification of fewer cDNA transcripts while subtractive hybridization yielded lower number of partial cDNA clones, which are not adequate for gene expression comparative studies. Hence, we modified the RNA extraction method of Salzman et al. (1999) to provide high quality and quantity RNA suitable for gene expression studies from various grape tissue viz. grape leaf (healthy and infected), xylem, phloem and petiole. This is a modification of the RNA extraction method described by Salzman et al. (1999). Our method is a three step precipitation protocol and includes high concentration of insoluble PVP and pre-warmed extraction buffer, which removes only the contaminating phenols and polysaccharides but does not affect the RNA yield, and also excludes Lithium chloride precipitation.

MATERIALS AND METHODS

Plant material

Young, mature healthy and infected leaf, xylem, phloem and petiole tissue of California bunch (*Vitis vinifera* cv.

Table 1. PCR primers and its sequence used in DDRT-PCR analysis.

Sl. No	Primers	Sequence
1	H-T_{11} G	AAGCTTTTTTTTTTG
2	H-T_{11}A	AAGCTTTTTTTTTTA
3	H-T_{11}C	AAGCTTTTTTTTTTC
4	H-AP1	AAGCTTGATTGCC
5	H-AP2	AAGCTTCGACTGT
6	H-AP3	AAGCTTTGGTCAG
7	H-AP4	AAGCTTCTCAACG
8	H-AP5	AAGCTTAGTAGGC
9	H-AP6	AAGCTTGCACCAT
10	H-AP7	AAGCTTAACGAGG
11	H-AP8	AAGCTTTTACCGC

Source: GenHunter Corporation, TN.

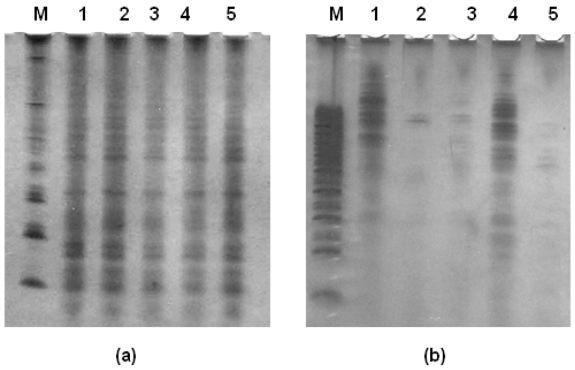

Figure 2. Gel profile representing cDNA transcripts expressed in different grape tissue of Zinfandel (Bunch type) using our modified method and Salzamn method. (a) Modified method and (b) Salzman method. Lane M: 100 bp Molecular ruler (BioRad), Lane 1: Xylem, Lane 2: Phloem, Lane 3: Petiole, Lane 4: Healthy leaf and Lane 5: Infected leaf (PD).

Zinfandel, Merlot and Pinot Noir), Florida hybrid bunch (*Vitis* spp cv. Blue Lake, Lake Emerald, Blanc du Bois and Suwannee) and muscadine (*Vitis rotundifolia* cv. Carlos and Fry) grape genotypes were collected from the plants growing at the Center for Viticulture and Small Fruit Research, Florida A & M University, Tallahassee, Florida. The tissue were immediately frozen in liquid nitrogen and stored at -80ºC until further use. Xylem and phloem were separated by stripping off the bark (Biddulph et al. 1958; Sheldrake, 1971) using a sharp blade. Equal amount of xylem and phloem were processed and used for RNA isolation.

Reagents used

Extraction buffer: 4 M guanidine thiocyanate, 100 mM Tris-HCl, pH 8.0, 25 mM sodium citrate pH 8.0, 0.5% N-Lauryl sarcosine.

PVP, insoluble.

5 M NaCl and β-Mercaptoethanol.

Chloroform: isoamyl alcohol (24:1).

Tris-saturated phenol (pH 8.0): chloroform: isoamyl alcohol (25:24:1) (prepared fresh).

Phenol: Chloroform (1:1).

Pre-cooled, isopropanol / absolute ethanol.

75% Ethanol prepared from DEPC treated water.

Protocol

All the solutions were prepared using DEPC treated water. Pestle and mortar, glassware and centrifuge tubes were soaked in 0.1% DEPC water overnight and autoclaved next day at 15 lb/in^2 for 20 min. The following procedure was used:

(Note: In between each extraction steps after precipitation, it is necessary to keep pellet on ice till it dissolves).

Sample preparation.

1. 1.5 g of frozen tissue was ground to a fine powder with liquid nitrogen using mortar and pestle in presence of 2% (W:V, 200 mg tissue per 10 ml extraction buffer) insoluble polyvinylpyrrolidone.

2. Powdered sample was transferred to a 50 ml sterile Oakridge polypropylene centrifuge tube, to which 20 ml pre-warmed extraction buffer at 65ºC was added. (Note: Add 0.1 volume of 5 M NaCl and 1% β-Mercaptoethanol to extraction buffer fresh prior to heating).

Extraction I.

3. Vortex the sample for 1 min and incubate on ice for 5 min. Add equal volume of Chloroform: isoamylalcohol (24:1) and hand shake the mixture vigorously for 5 to 10 min.

4. Centrifuge the sample at 14000 g for 15 min at 4ºC.

5. Transfer the supernatant to a fresh centrifuge tube and repeat the above step one more time.

6. Transfer the supernatant to a fresh 50 ml centrifuge tube and add 0.1 volumes of 5 M NaCl to the supernatant and mix gently, and then add either cold isopropanol (1 Volume) or cold absolute ethanol (2 Volumes). Precipitate overnight at -20ºC.

7. Pellet the RNA by centrifuging at 14000 g for 20 min at 4ºC. Wash the pellet with 75% ethanol and air dry for 10 min. Resuspend the pellet in 5 ml DEPC treated sterile water.

Extraction II.

8. Add equal volume of Phenol:chloroform:isoamylalcohol (25:24:1) to the sample suspension and hand shake vigorously for 10 min.

9. Centrifuge at 14000 g for 15 min at 4ºC. Collect the upper supernatant and repeat the above step (step 8) twice. Avoid pipetting any whitish interphase.

10. Collect the supernatant in a fresh centrifuge tube and add 0.1 volumes of 5 M NaCl, mix gently and then add either cold isopropanol (1 Volumes) or

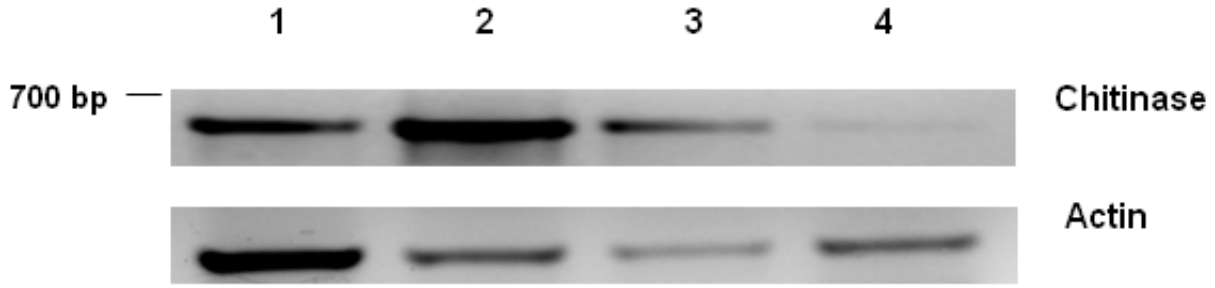

Figure 3. **RT-PCR profile of Muscadine grape cv. Carlos using synthetic oligonucleotide chitinase and actin specific primers using RNA as template from both modified protocol (Lane 1 and 2) and Salzman method (Lane 3 and 4).** Lane 1 and 3: Healthy leaf tissue and Lane 2 and 4: Infected leaf tissue (Anthracnose).

cold absolute ethanol (2 Volumes). Precipitate overnight at -20ºC or at least for 1½ hr at -80ºC.

11. Pellet the RNA by centrifuging at 14000 g for 20 min at 4ºC. Wash the pellet with 75% ethanol and air dry for 10 min.

12. Resuspend the pellet in 500 µl DEPC treated sterile water.

Extraction III.

13. Transfer the precipitate in a sterile 1.5 ml Eppendorf tube and add equal volume of chloroform. Hand shake vigorously for 2 to 3 min.

14. Centrifuge at 12000 g for 15 min at 4ºC and collect the supernatant.

15. Add equal volume of phenol: chloroform (1:1). Hand shake vigorously for 5 min.

16. Centrifuge at 12000 g for 15 min at 4ºC and collect the supernatant. Repeat the above step till there is no whitish interphase.

17. Add equal volume of chloroform, hand shake vigorously for 2 to 3 min and centrifuge at 12000 g for 15 min at 4ºC.

18. Transfer the final supernatant to a fresh 1.5 ml sterile Eppendorf tube and add 0.1 V 5 M NaCl and mix gently and then either add cold isopropanol (1 Volume) or cold absolute ethanol (2 Volumes). Precipitate overnight at -20ºC or at least 1½ hr at -80ºC.

19. Pellet the RNA by centrifuging at 12000 g for 20 min at 4ºC. Wash the pellet with 75% ethanol and air dry for 10 min. Resuspend the pellet in sterile water.

20. Store the RNA sample at -20ºC.

RNA analysis

RNA was quantified by measuring absorbance at 230, 260 and 280 nm in water. The integrity of the RNA sample was analyzed on 1.2% denaturing formaldehyde agarose gel electrophoresis (Sambrook et al. 2000).

Differential display RT-PCR

DDRT-PCR was carried out for total RNA extracted from both modified and Salzman methods using RNAimage kit as for the manufacturer's instructions (GenHunter, TN USA) to compare differences in gene expression between RNA populations. Purified total RNA (100 ng each) were reverse transcribed (RT) using T_{11} MN anchored primer (where M stands for dA, dC, dG and N stands for dA, dC, dG, dT as described) in presence of MMLV reverse transcriptase. The reaction was carried out in a thermocycler as follows: 65ºC for 5 min, 37ºC for 60 min and 75ºC for 5 min. MMLV reverse transcriptase was added after the tubes have been at 37ºC for 10 min.

This first strand cDNA (2 µl, according to the manufacturer's protocol) was used as template in the PCR reaction containing the same T_{11} MN anchored primer in combination with an H-AP 1 to 8, 13 mer primers (Table 1); 1 x PCR buffer, and 1 unit of *Taq* DNA polymerase. The PCR conditions used were as follows: 94ºC for 30 sec, 40ºC for 2 min and 72ºC for 30 sec repeated for 40 cycles, followed by 72ºC for 5 min. The PCR product was resolved by gel electrophoresis on 12% polyacrylamide gel at a constant voltage (60 V) and stained with silver (Sambrook et al. 2000).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

One step RT-PCR kit from Promega was used according to manufacturer's instructions. RT-PCR amplification was performed by primers, which were constructed based on the conserved sequences for chitinase and actin. The sequences of the chitinase and actin primers are as follows:

Table 2. Yield and quality* of total RNA obtained from different grapevine tissue and species.

Species and cultivar	Grapevine Tissue		Absorbance ratios		Yield (µg/g FW)
			A_{260}/A_{280}	A_{260}/A_{230}	
Vitis vinifera cv. Zinfandel (Bunch type)	Leaf	Young	1.90	2.12	540
		Mature healthy	1.67	2.22	384
		Infected	1.87	2.14	272
	Xylem	Young	1.87	2.36	520
		Mature healthy	1.70	2.11	347
		Infected	1.54	2.24	284
	Phloem	Young	1.84	2.10	287
		Mature healthy	1.76	2.32	246
		Infected	1.52	2.22	132
	Petiole	Young	1.64	2.16	460
		Mature healthy	1.55	2.24	244
		Infected	1.54	2.36	178
Vitis spp cv.Lake Emerald (Florida Hybrid)	Leaf	Young	1.87	2.34	276
		Mature healthy	1.72	2.24	214
		Infected	1.64	2.11	110
Vitis rotundifolia cv. Carlos (Muscadine)	Leaf	Young	1.90	2.28	532
		Mature healthy	1.86	2.14	480
		Infected	1.68	2.10	275
	Xylem	Young	1.88	2.26	209
		Mature healthy	1.62	2.13	178
		Infected	1.54	2.11	112
	Phloem	Young	1.72	2.31	184
		Mature healthy	1.66	2.29	096
		Infected	1.61	2.10	054
	Petiole	Young	1.82	2.27	140
		Mature healthy	1.75	2.18	084
		Infected	1.72	2.14	032

*Results are expressed as the mean of 3 samples.

Chitinase-forward (5’ – GGCATCAAGGTTGGTTCAGT-3’)

Chitinase-reverse (5’ – GGCTACAACATAGGCCTCCA-3’)

Actin-forward (5’ – ACTGCTGAACGGGAAATTGT- 3’)

Actin-reverse (5’-ACGGAATCTCTCAGCTCCAA- 3’)

Equal amount of RNA (100 ng) from both healthy and PD infected leaf tissues of muscadine cultivar Carlos was used. The reaction condition for chitinase primers was as follows: First strand cDNA synthesis was carried out at 45ºC for 45 min, followed by 45 cycles of denaturization at 94ºC for 30 sec, annealing at 55ºC for 30 sec, and extension at 72ºC for 45 sec. For actin, first strand cDNA synthesis was carried out at 45ºC for 45 min, followed by 35 cycles under the following conditions: 30 sec at 94ºC, 30 sec at 50ºC, and 1 min at 72ºC. For both the genes, the final extension was carried out at 72ºC for 10 min. The obtained PCR product was separated on 1.2% agarose gel stained with Ethidium Bromide and visualized under UV transilluminator.

RESULTS AND DISCUSSION

RNA extraction methods of Lopez-Gomez and Gomez-Lim (1992), Salzman et al. (1999), Hu et al. (2002), Iandolino et al. (2004), Thomas and Schiefelbein (2002), Tattersall et al. (2005), Vasanthaiah et al. (2006), and also Commercial kits

and Trizol method did not yield sufficient amount of good quality RNA from different grape tissues. Only Salzman et al. (1999) method yielded RNA but it was found to be inadequate and degraded. This suggests that RNA might have been lost by binding to polysaccharides, polyphenols or other components during extraction. For successful DDRT-PCR and subtractive hybridization it is necessary to use high quality RNA to not to miss any low copy expressed genes. Our modified protocol yielded adequate amount of RNA and was free from contaminants. The A_{260}/A_{280} nm ratio of 1.52 to 1.87 from RNA of mature and diseased tissue indicated presence of only trace levels of polyphenols and polysaccharides while in the young tissue, the ratio was 1.8 to 1.9 indicating that the RNA from young tissue is relatively free from polyphenols and polysaccharides contamination. The A_{260}/A_{230} nm ratio for all the samples was higher than 2.0 indicating that the RNA is of high purity and without polyphenols and polysaccharides contamination. The RNA yield ranged between 32 to 540 µg per g of fresh sample (Table 2). The integrity of the RNA is evident on 1.2% denaturing agarose gel where 28s and 18s bands are clearly visible along with a smear showing additional rRNAs (between 18s and 5s rRNA) (Figure 1). No apparent degradation of RNA was observed indicating that RNA resulting from our protocol is relatively free of RNases.

This protocol involves addition of 2% insoluble polyvinylpyrrolidone which facilitated removal of most of the polyphenols and polysaccharides while pre-warmed extraction buffer helped in inactivating RNase which is high in mature and diseased tissue. PVP concentrations below or above 2% did not increase RNA recovery. Earlier studies indicated that PVP in extraction buffer is incompatible with phenol extraction and binds to nucleic acids (Asif et al. 2000). In our experience, the insoluble PVP is compatible with the buffer and helps in removing most secondary metabolites. Inclusion of PVP during grinding the tissue helped in recovering higher quantity of RNA (20%) compared to adding PVP in the extraction buffer. PVP helps in dissociation of complexes of polysaccharides, phenols and other compounds (Ainsworth, 1994), which can be removed later by phenol: chloroform extraction. The RNA yield obtained using pre-warmed extraction buffer was higher (32 to 540 µg per g of fresh sample) compared to the yield obtained with buffer at room temperature (15 to 140 µg per g of fresh sample). Since mature and diseased tissue have less RNA synthesis and more secondary metabolites, it is necessary to use pre-warmed extraction buffer to recover more RNA. About 1.5 g of tissue is sufficient to obtain adequate amount (on an average 305 µg) of RNA, which should be sufficient for most studies. Inclusion of an additional re-extraction step with phenol and chloroform helped remove contaminants and recover high quantity of RNA. Inefficient removal of polysaccharides and polyphenolic compounds results in co-precipitation with RNA, which affects the yield and quality (Logemann et al. 1987).

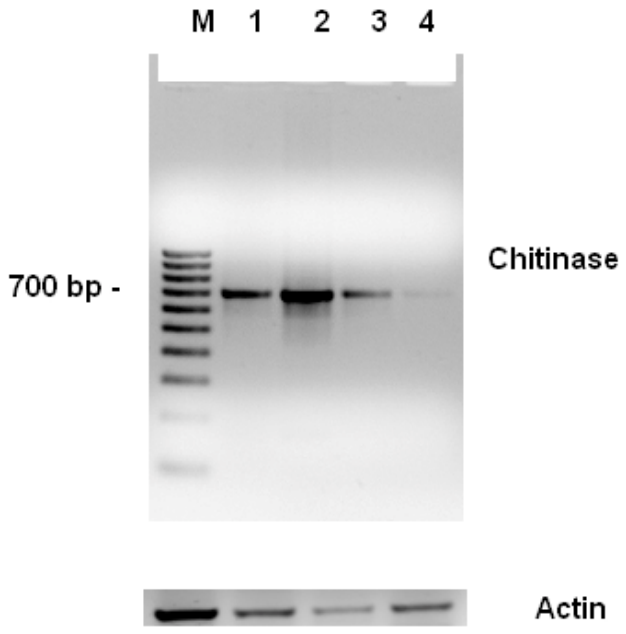

Figure 4. RT-PCR profile of Muscadine grape cv. Carlos using synthetic oligonucleotide chitinase and actin specific primers using RNA as template from both modified protocol (Lane 1 and 2) and Salzman method (Lane 3 and 4). Lane 1 and 3: Healthy leaf tissue and Lane 2 and 4: Infected leaf tissue (Anthracnose). Lane M: Molecular ruler (100 bp).

For mature and diseased tissue which contains higher amounts of polyphenols, polysaccharides and other interfering compounds it is necessary to extend the extraction period to three days with overnight precipitation to recover adequate RNA. For tissue containing low to moderate levels of polyphenols (young tissue), the protocol can be shortened by precipitating the RNA for only 1½ hr instead of overnight in extraction steps II and III. RNA was directly precipitated using cold absolute ethanol/isopropanol instead of LiCl to avoid any water insoluble precipitate and loss of RNA (Liu et al. 1998). There was no significant yield difference when either cold absolute ethanol or isopropanol was used for RNA precipitation. RNA yield from the leaves at advanced stages of infection decreased which is believed to be due to apoptosis (programmed death) of the cells near the infection site (Ciccarelli and Bork, 2005) that leads to less RNA synthesis. Salzman et al. (1999) protocol failed to yield sufficient amounts of pure RNA from mature and diseased tissue even after lithium chloride precipitation, and the resulting RNA was found to be degraded and highly contaminated.

This RNA was successfully used in gene expression studies as evident by DDRT-PCR analysis (Figure 2) and RT-PCR (Figure 3). From the consistently obtained profiles the differentially expressed cDNAs were successfully identified, eluted and re-amplified. DDRT-PCR and subtractive technique has been used frequently to compare the expression pattern of genes between two RNA

Table 3. A comparison of mRNA recovered and number of recombinant colonies obtained from Xylem tissue of Zinfandel (PD-Susceptible) and Blanc du Bois (PD-Tolerant) infected with Pierces Disease (PD) between original and modified protocol through subtractive hybridization.

Protocol	Xylem Tissue			
	Zinfandel (Bunch type)		Blanc du Bois (Florida hybrid)	
	mRNA (µg/100 µg of total RNA)	No. of recombinant colonies obtained	mRNA (µg/100 µg of total RNA)	No. of recombinant colonies obtained
Modified method	3.6	180	3.2	140
Salzman method	1.4	40	1.0	35

populations. DDRT-PCR using RNA prepared from the Salzman et al. (1999) method resulted in amplification of fewer cDNA transcripts compared to our modified protocol, which are not adequate for gene expression comparative studies. Subtractive hybridization using mRNA recovered from RNA extracted from our protocol yielded several partial cDNAs. On an average 3.4 µg of mRNA was recovered (3.4%) from 100 µg of total RNA prepared using our protocol, when compared to 1.2 µg of mRNA (1.2%) from the total RNA extracted using Salzman method. Qiagen mRNA isolation kit was used to recovery mRNA from the total RNA according to the manufacturers instruction. Subtractive hybridization was carried out between Pierce's disease tolerant (Blanc du Bois) and susceptible (Zinfandel) grape genotypes challenged with *Xylella fastidiosa*,which causes Pierce's disease. These cDNAs were cloned into pGEM T Easy vector (Promega), transferred into JM109 competent cells (Promega), plated on Luria-Bertani (Fisher ChemAlert) agar plates and incubated overnight at 37ºC. Next day, colony counting was done to determine the number of clones obtained. Around 300 subtracted cDNA clones (data unpublished) were recorded from subtractive hybridization using mRNA from our protocol in both bunch and Florida hybrid bunch grapes compared to 75 clones from Salzman method (Table 3). This indicates that the RNA resulting from our protocol is intact, PCR amplifiable and can be used in DDRT- PCR and subtractive hybridization based gene expression studies. Higher percentage of total RNA and mRNA recovery from this procedure indicates that the RNA obtained can also be used in constructing cDNA libraries, which requires adequate quantity of high quality mRNA.

The quality of RNA was further confirmed throughRT-PCR using degenerated synthetic oligonucleotide primers which were designed based on the conserved domain of chitinase and actin gene. The RT-PCR yielded the expected cDNA fragment (700 bp) as a single band, indicating that the RNA is PCR amplifiable and is free of any inhibitors (Figure 3). The expression of chitinase was found to be high in *Elsinoe ampelina* (anthracnose) infected leaf tissue compared to healthy leaf tissue, revealing the effect of infection on the metabolism of the plant system. Similarly, RT-PCR using RNA derived from Salzman method resulted in lower expression of Chitinase gene in both healthy and infected leaf tissue. Further, actin an abundant gene in the plant system was also used in RT-PCR reaction to determine the quality of the extracted total RNA. The expression of actin was found to be higher with RNA extracted using our modified method compared to Salzman method (Figure 3). Using our refined procedure, we were able to isolate RNA from different tissue of muscadine, bunch and Florida hybrid grape genotypes (data not shown). This protocol can be successfully used in other plants, where higher polyphenols and polysaccharides problem exists, especially in mature and diseased tissue.

CONCLUDING REMARKS

Grape is a woody perennial and contains large amounts of polyphenols and polysaccharides. Because of these compounds isolation of good quality RNA from grapevine tissue is difficult. Most of the published protocols failed to yield sufficient quantity of high quality RNA from various grape tissue suitable for gene expression studies. Our refined protocol with the inclusion of high concentration of PVP, pre-warmed extraction buffer and three extraction steps yielded good quality and quantity RNA, especially from mature and diseased tissue containing high levels of polyphenols and polysaccharides. Intact RNA, high A_{260}/A_{280} ratio (1.52 to 1.90), high A_{260}/A_{230} ratio (2.10 to 2.36), higher amount of mRNA recovery (3.4%), consistent cDNA profile through Differential Display RT-PCR, amplification of higher number of subtracted cDNA transcripts through subtractive hybridization and RT-PCR using gene specific primer confirmed the quality of RNA. Hence, this protocol will be useful for isolating high quality RNA suitable for gene expression studies and also for isolating RNA from plants containing high concentration of polyphenols and polysaccharides.

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