A COMPARISON OF SIX TOTAL RNA ISOLATION METHODS FOR DIAGNOSIS OF GYSVD-1 (GRAPEVINE YELLOW SPECKLE VIROID-1) ON *VITIS VINIFERA* L. LEAVES

İSMAIL CAN PAYLAN[1]*, AYŞE ÇANDAR[1], MUSTAFA GÜMÜŞ[1], SERCAN PAZARLAR[1] AND SERKAN ÖNDER[2]

[1]*Department of Plant Protection, Ege University, 35100 Izmir, Turkey*
[2]*Manisa Viticulture Research Station, 12 Manisa, Turkey*
Corresponding author e-mail: ismail.paylan@ege.edu.tr, +902323111010-2669

Abstract

Isolation of high quality RNA from plant tissues is one of the most critical steps for the successful application of diagnostic tests such as reverse transcription polymerase chain reaction (RT-PCR), northern blotting, microarray hybridization. The presence of inhibitors such as secondary metabolites, phenolic compounds and RNAses can cause inaccurate and undesirable results. Grapevine is rich in a wide range of metabolites which interfere with RNA isolation. From this point of view, we researched six different total RNA extraction methods on leaves of *Vitisvinifera* L. to find the best one that contribute the purity and high quality. The methods tested are silica-capture, modified silica-capture, commercial kit, the new combined, lithium chloride and citric buffer. RNA quality was analyzedspectrophotometrically by nanodrop, agarose gel electrophoresis and RT-PCR. As a result of all, it is clear that the most suitable TNA isolation protocol is the new combined method which experienced and named firstly by us, in terms of RNA purity, concentration, less time consuming of isolation step and achievement on detection of GYSVd-1.

Keywords: Grapevine, GYSVd-1, RNA isolation, TNA extraction methods, Viroid diagnosis

Introduction

Diseases and pests have major importance among factors limited the production in vineyards of World, particularly China, Spain, France, Italy, Turkey which constitute the most productive areas (Emmett *et al.,* 1992). Among these, viruses, viroids and virus-like agents cause serious yield and quality losses. After determination of a viroid-like RNA in vineyards first time at 1984 (Sano *et al.*, 1985), the presence of six grapevine viroids were exhibited in Japan, Australia, USA and Europe (Flores *et al.*, 1985). These six viroids in question are *Citrus exocortis viroid-grapevine* (CEVd-g) (Garcia-Arenal *et al.*, 1987), *Hop stunt viroid-grapevine* (Sano *et al.*, 1985), *Grapevine yellow speckle viroid*-1 (GYSVd-1), *Grapevine yellow speckle viroid*-2 (GYSVd-2) (Koltunow & Rezaian 1988), *Grapevine viroid-cucumber* (GVd-c) and *Australian grapevine viroid* (AGVd).

Grapevine yellow speckle viroid 1 (GYVSd-1) is a plant pathogenic viroid in the family *Pospiviroidae*. GYSVd-1 is found in the important production areas, is very easily spread mechanically by contaminated cutting tools (Szychowski *et al.*, 1988), infected graft (Szychowski *et al.*, 1988; Staub *et al.*, 1995), propagation materials (Kultonow *et al.*, 1988) and causes severe damage to quality and quantity of grapevines.

The reliable diagnosis of viroids is essential in quarantine and certification programs that were developed in order to obtain viroid-free propagation material and make the usage of these materials widespread. GYSVd-1 is not determined by biological indexing via mechanical inoculation to herbaceous test plants because of that it doesn't have any herbaceous hosts, just as not detected by serological methods owing to the fact that viroids don't have coat protein (Reisner & Gross, 1985; Garnsey & Jones, 1967). Nowadays, the most frequently used techniques for detection of this agent are molecular methods. In addition, the most preferred method among molecular techniques is reverse transcriptase PCR (Flores *et al.*, 1985, Garnsey & Jones, 1967; Szychowski *et al.*, 1988).

RNA isolation is one of the most critical step forqualified diagnostic and gene expression studies such as reverse transcription polymerase chain reaction (RT-PCR), northern blotting, microarray hybridization. Although there are lots of factors that prevent to obtain the good quality nucleic acid, inhibitors such as tannins, latex, gums and phenolic compounds are the most important factors among them (Martelli, 1994; Sipahioğlu *et al.*, 2007). Grapevine leaves have the high concentration of secondary metabolites polysaccharides and polyphenols, struggling with total RNA isolation (Tattersall *et al.*, 2005, Reid *et al.*, 2006). TNA extraction process should be made suitable for each viroid and plant species in order to reduce and even annihilate effects of inhibitors. From this point of view, we researched into effects of different and modified TNA extraction methods on detection of GYSVd-1 in this study.

Material and Methods

Plant material and viroid source: 57 leaf samples which were obtained from grapevine variety collection in "Manisa Viticulture Research Station, Turkey" during surveys in 2012 were tested by RT-PCR from the point of presence of GYSVd-1. 5 leaf samples from 'Isa' variety which were found positive for GYSVd-1 and viroid isolates from these leaf samples constituted the material of research.

Total nucleic acid (TNA) extraction: TNA extraction was carried out according to six different RNA extraction methods. These are silica-capture method, modified silica-capture method, GeneJET Plant RNA Purification Mini Kit procedure (Fermentas, USA)(named commercial kit protocol in the article), new combined method, lithium chloride method and citric buffer method. Also, six different procedures were applied under same ambient conditions and with same samples. Thus, effects of different and modified TNA extraction methods on detection of GYSVd-1 were researched.

After TNAs were obtained according to six different protocols, they were separated on 1.5% agarose gel with electrophoresis and visualized by ethidium bromide (Sambrook *et al.*, 1989).

I. Silica-capture method: 100 mg fresh leaf samples were grinded and homogenized with 2 ml grinding buffer (4.0 M Guanidine thiocyanate, 0.2 M NaOAc, 25 mM EDTA, 1.0 KOAc, 2.5% (w/v) PVP-40, 1% 2-mercaptoethanol). Later on, five hundred μl of extract was transferred to new eppendorf tubes and mixed with 100 μl of 10% sodium lauryl sarcocyl in these tubes. After tubes containing extract and sodium lauryl sarcocyl mix, were respectively incubated at 70°C for 10 min., and in ice for 5 min, centrifuged at 14000 rpm for 10 min., 300 μl of supernatant which obtained by centrifugation, was transferred to new eppendorf tubes containing 300 μl of 6M sodium iodide, 25 μl silica and 150 μl of 96% ethanol. Then, the mixture was incubated at room temperature for approximately 10 min on intermittent shaker. After tubes were centrifuged at 6000 rpm for 1 min., the supernatants were removed and pellets in the tubes were washed two times with washing buffer (10 mMTris-HCl, pH 7.5; 0.5 mM EDTA; 50 mMNaCl; 50% ethanol). 150 μl of RNase-free water was added to the obtained pellets after the washing, they were mixed. The mixture was centrifuged at 14000 rpm for 3 min., the supernatant was transferred to new tubes (Foissac *et al.*, 2000). Lastly, tubes containing TNA were kept at -20°C until used.

II. Modified silica-capture method: Although this method was also carried out with silica-capture method according to Foissac *et al.*, (2000), the unique distinction from Method I is that we used 500 mg fresh leaf sample instead of 100 mg sample.

III. Commercial kit protocol: TNA was recovered from infected leaf samples using "GeneJET Plant RNA Purification Mini Kit" (Fermentas, USA) with slight modifications on the protocol.

According to this, 500 mg grapevine leaf sample was homogenized with 500 μl Plant RNA Lysis Solution containing 10 μl of 2M Dithiothreitol (DTT). Then, whole mixture was transferred to clean eppendorf tubes and tubes were centrifuged at 14000 rpm for 5 min after they were incubated at 56°C for 3 min. After the supernatant (approximately 450-550 μl) was transferred to new micro centrifuge tubes, 250 μl of 96% ethanol was added to the supernatant and mixed by pipetting. The prepared mixture was transferred to purification columns inserted a collection tube. Later on, columns were centrifuged at

11000 rpm for 1 min and after the flow through liquid was discarded, columns and purification tubes were reassembled. 700 μl of Wash Buffer 1 (WB 1) containing 96% ethanol was added to purification column and purification tubes inserted the collection tubes were centrifuged at 11000 rpm for one min. Then, both the flow through solution and collection tube were discarded and purification columns were placed into a set of clean 2 ml collection tubes. 500 μl of Wash Buffer 2 (WB 2) containing 96% ethanol was added to the purification column, tubes were centrifuged at 11000 rpm for 1 min and the flow through liquid was discarded. After column and collection tube was reassembled, the previous step (washing step using WB 2) was repeated. At the end of this step, the empty purification column inserted the collection tube was spinned at 14000 rpm for 1 min and the collection tube containing the flow through solution discarded. 50 μl of nuclease-free water was added to the purification column which was transferred to a RNase-free 1.5 ml collection tube and collection tube containing purification column was centrifuged at 11000 rpm for 1 min. Finally, purification column was discarded and the TNA in 1.5 ml collection tube was kept at -20°C until used.

IV. New combined method (Silica-capture + Commercial kit protocol): This method is a new technique which was experienced by us for the first time, as the combination of Method II and Method III. Later on, it was termed by us as new combined method.

According to this method, 500 mg leaf sample was homogenized with 2 ml of grinding buffer containing 4.0 M Guanidine thiocyanate, 0.2 M NaOAc, 25 mM EDTA, 1.0 KOAc, 2.5% (w/v) PVP-40, and 1% 2-mercaptoethanol (Foissac *et al.*, 2000). Then, 500 μl of homogenized mixture was transferred to new eppendorf tubes and tubes were centrifuged at 14000 rpm for 5 min after they were incubated at 56°C for 3 min. After the supernatant (approximately 450-550 μl) was transferred to new microcentrifuge tubes, 250 μl of 96% ethanol was added to the supernatant and mixed by pipetting. Following, the method was continued such in Method III (Fermentas, USA).

V. Lithium chloride method: TNA extraction via lithium chloride method was carried out according to Hughes & Glau (1988) and Sipahioğlu *et al.*, (2007) with minor alterations. For that purpose, 500 mg fresh sample was homogenized with 1 ml of extraction buffer (200 mMTris-HCl pH 8.5, 1.5% sodium dodecylsulphate, 300 mM Lithium chloride, 10 mM EDTA, 1% sodium deoxycholate, 1% NP-40) involving 0.5% 2-mercaptoethanol. Later on, 500 μl of homogenized mixture was transferred to 1.5 ml micro centrifuge tubes. After tubes were incubated at 65°C for 15 min., 500 μl of potassium acetate (pH 6.5) was added to tubes and they were incubated on ice for 10 min. Tubes were centrifuged at 14000 rpm for 15 min., and then, 600 μl of the obtained supernatant by centrifugation was transferred to new sterile tubes to which 600 μl isopropanol was added. The mixture was incubated at -20°C overnight. The following day, the mixture was centrifuged at 14000 rpm for 15 min and the obtained pellet was washed with 70% ethanol. Finally, TNA was kept at -20°C until used.

VI. Citric buffer method: This method was carried out according to Wetzel *et al*., (1992) with minor modifications. Approximately 500 mg leaf sample was homogenized with 1 ml of citric buffer (50 mM sodium citrate, 2% PVP, 20 mM DIECA) containing some quartz sand. The extract was centrifuged at 8000 rpm for 3 min. 50 µl supernatant was collected and 450 µl of citric buffer was added. TNA was stored at -20°C for further studies.

Total RNA analyses: The total nucleic acid of per sample was analyzed using a spectrophotometer (NanoDrop 2000c UV-Vis Spectrophotometer, Thermoscientific, USA) by UV light absorbance at 260 and 280 nm. The ratio A_{260}/A_{280} was calculated in order to assess the purity and contamination level of isolated TNA. Also, RNA concentration were calculated using the value of A_{260} (Sambrook *et al*., 1989; Reid *et al*., 2006, Yang *et al*., 2011; Yu *et al*., 2012).

cDNA synthesis by reverse transcription: The TNA obtained from these six methods, were used for complementary DNA synthesis via reverse transcription according to the First Strand cDNA Synthesis Kit Protocol. For this, after 2 µl of TNA, 1 µl of oligo $(dT)_{18}$ primer (0.5 µg/µl) and 9 µl of DEPC- treated water were mixed in a eppendorf tube, the mixture was incubated at 70°C for 5 min., and then, 4 µl of 5x reaction buffer, 1µl of Ribolock[TM]Ribonuclease inhibitor (20u/µl) and 2 µl of 10mM dNTP mix were added to the tube. They were mixed and incubated at 37°C for 5 min. Finally, 1 µl of Revert Aid[TM] M-MuLV Reverse Transcriptase (200u/µl) was added to the mixture and the mixture was incubated at 42°C for 60 min., and 70°C for 10 min., respectively (Fermantas, USA). The synthesized cDNA, was stored at -20°C until used.

Amplification of cDNA by polymerase chain reaction (PCR): After 1 µl of cDNA was mixed with 24 µl amplification mixture involving 2.5 µl of 10x reaction buffer (200 mMTris-HCl pH 8.4, 500 mMKCl), 1.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 0.5 µl of each primer (100 pmol µl^{-1}), 0.2 µl of *Taq* DNA polymerase and 18.3 µl of RNase-free sterile water, cDNA mixture was amplified in thermo cycler (Gene Amp. PCR System 9700) under appropriate PCR conditions. For this, the mixture was heated at 95°C for 5 min., and then, subjected to 35 cycles 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1.5 min., with an additional 10 min extension at 72°C.

Primers which were used to detect GYSVd-1, were P-sense 5'-TTG AGG CCT GGC GTA ACG C-3'and P-anti- sense 5'-GGA CGC GAA CGT GAA TAG G-3' (Koltunow and Rezaian 1988). Also, 10 µl amplified cDNA (PCR product) were separated on 1.5% agarose gel by electrophoresis and visualized by ethidium bromide (Sambrook *et al*.,1989).

Results and Discussion

Results were compared and evaluated according to TNA and PCR gel pictures and spectrophotometric RNA analyses. Firstly, we observed TNA images in new combined, modified silica-capture, silica-capture, commercial kit and lithium chloride methods with assessments on TNA gel pictures. However, no TNA image was observed in citric buffer method. The adequate results were obtained from new combined, lithium chloride, and modified silica-capture methods. The quality of RNAs extracted with commercial kit protocol and silica-capture methods were significantly lower than lithium chloride, modified silica-capture and new combined methods. In case we assessed the quality of RNAs extracted with lithium chloride, new combined and modified silica capture methods, they had put in order from most to least qualified as lithium chloride, new combined and modified silica-capture method, respectively (Fig. 1).

In this study, we also amplified TNA by thermal cycler and assessed the effects of six different total RNA extraction methods on determination of GYSVd-1. As a result of electrophoresis of PCR products obtained from TNAs extracted with new combined, lithium chloride, modified silica-capture, silica-capture methods and commercial kit protocol, bands were observed on gel. The most qualified band was showed in new combined method. On the other hand, lithium chloride, modified silica capture, commercial kit and silica-capture methods were succeed on detection GYSVd-1, but they had lower band quality than new combined method. Especially, silica-capture and commercial kit methods had the least band quality. Citric buffer method was failed on diagnosis GYSVd-1 because of that they did not isolated any TNA (Fig. 2).

Finally, we assessed TNA purity and concentration spectrophotometrically. For this, A_{260} and A_{280} absorbance values of TNA extracted with the four most successful methods were determined. And then, Table 1 was created. In lithium chloride and commercial kit methods, A_{260}/A_{280} had values between 0.61 and 1.65 that was too low. For this reason, these two methods were failed for TNA purity. Because the pure total RNA should have values in between 1.8 and 2.1 (Wilfinger *et al*., 1997). While the modified silica-capture method resulted in higher values, ranging from 1.69 to 1.84, the best values were observed in the new combined method. The purity of TNAs extracted with in question method, ranged from 1.87 to 2.10 (Table 1). Also, in order to understand why we didn't observe any or low quality bands in some methods, we considered the TNA concentration. According to First Strand cDNA Synthesis Kit Protocol, 0.1-5 µg of total RNA requires for cDNA synthesis (Fermentas, USA). For this reason, modified silica-capture method and commercial kit protocol didn't have sufficient total RNA concentration because of their concentrations ranged from 0.016 to 0.055 µg µl^{-1}. On the other hand, lithium chloride (0.18-0.71 µg µl^{-1}) and new combined (0.22-0.50 µg µl^{-1}) methods had adequate values (Table 1). Similar results for lithium chloride obtained by Tattersall *et al*., (2005) and Gambino *et al*., (2008).Although lithium chloride method had the best values for RNA concentration; the new combined method gave the best band quality since it had higher RNA purity than the lithium chloride method.
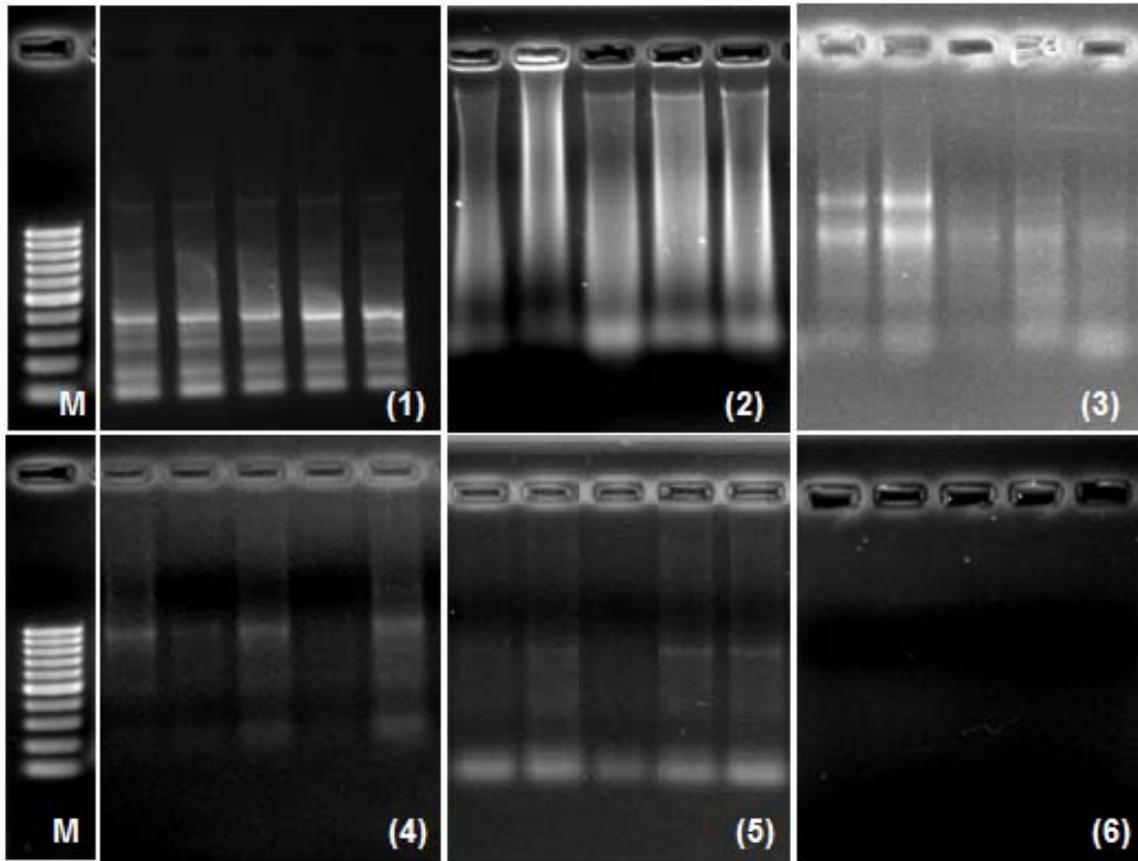
Fig. 1. Agarose gel electrophoresis of isolated TNA samples with six different extraction methods. (1) New combined method (Silica-capture+ commercial kit), (2) Lithium chloride method, (3) Modified silica-capture method, (4) Commercial Kit Protocol, (5) Silica-capture method, (6) Citric buffer method, M: Molecular size marker.
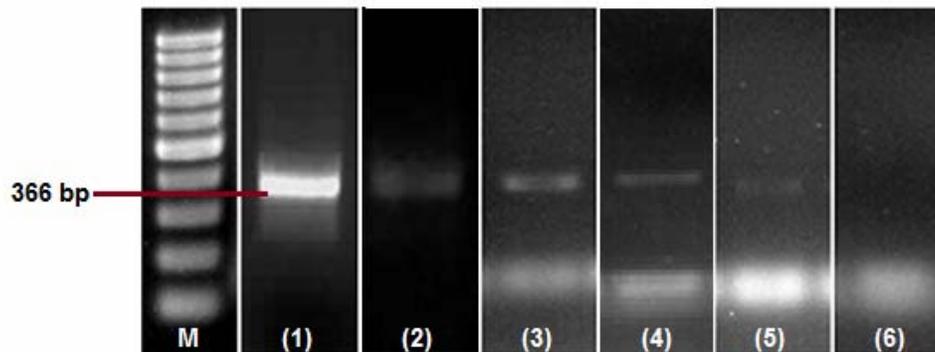
Fig. 2. Agorose gel electrophoresis of amplified PCR products, obtained from six different TNA extraction methods. (1) New combined method (Silica-capture+ commercial kit), (2) Lithium chloride method, (3) Modified silica-capture method, (4) Commercial Kit Protocol, (5) Silica-capture method, (6) Citric buffer method, M: Molecular size marker.

Table 1. A_{260}/A_{280} values and concentrations of total RNA extracted with the four most successful methods.

Sample No.	Modified silica-capture		New combined method		Lithium chlorid method		Commercial kit protocol	
	A260/A280	Concentration ($\mu g\ \mu l^{-1}$)	A260/A280	Concentration ($\mu g\ \mu l^{-1}$)	A260/A280	Concentration ($\mu g\ \mu l^{-1}$)	A260/A280	Concentration ($\mu g\ \mu l^{-1}$)
1.	1,78	0,053	1,99	0,22	1,37	0,71	1,03	0,029
2.	1,69	0,055	1,93	0,35	0,61	0,19	1,04	0,028
3.	1,74	0,054	2,10	0,38	1,65	0,35	1,05	0,016
4.	1,84	0,053	1,92	0,50	1,29	0,43	1,03	0,027
5.	1,75	0,054	1,87	0,40	1,52	0,18	1,03	0,021

Conclusion

Total RNA isolation is an essential step for RT-PCR applications. Especially, quality and quantity of isolated RNA has vital issues in order to obtain high quality bands on gel and so accuracy of RT-PCR. Nevertheless, there are still some troubles associated with total RNA isolation steps. Therefore, different TNA extraction methods were experienced in this study with intent to obtain high quality TNA. Consequently, the best method is the new combined method, although modified silica-capture, lithium chloride, new combined, commercial kit and silica-capture methods may be used to isolate total RNA for detection of GYSVd-1. Because, total RNA extracted with the new combined method had the best RNA purity and sufficient concentration. So, its PCR products showed the best band quality. Besides, the new combined method has several advantages, except having the best research results. For instance, total RNA isolation step takes less time than lithium chloride and silica-capture methods. It is clear that the most suitable TNA isolation protocol is the new combined method in terms of RNA purity, concentration, less time consuming of isolation step and achievement on detection of GYSVd-1.

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