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Comparison of Three Conventional Extraction Methods for the Detection of Plant Virus/Viroid RNAs from Heat Dried High-phenolic Host Leaves

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Abstract: The presence of virus/viroid infections can go unnoticed since symptoms appear only if additional viruses are present. Detection of *Plum bark necrosis stem pitting associated virus* (PBNSPaV), *Apricot latent virus* (ApLV), *Apple scar skin viroid* (ASSVd), *Prunus necrotic ringspot virus* (PNRSV) and *Potato virus Y* (PVY) by reverse transcriptase polymerase chain reaction (RT-PCR) or nested-RT-PCR is possible; however, these assays could be unreliable if the tissue contains interfering compounds. This study reports on use of three extraction procedures in recently developed heat-dried virus/viroid preserved plant tissues. The methods tested were lithium chloride, silica-capture and citric buffer. The results showed that (1) the silica-capture RNA extraction method appears to be superior for total RNA extraction; (2) the increase in volume of silica improves the efficiency of RNA extraction from dried infected leaves and (3) the use of silica method minimizes the fragmentation of PCR products and improves the PCR detection of tested pathogens. The results of study indicate that the use of appropriate RNA extraction method is crucial for a successful PCR and an appreciable yield of PCR product from heat-dried infected leaves.

Key words: Heat-dried infected leaves, RNA extraction methods, virus/viroid preservation, RT-PCR, nested-RT-PCR

INTRODUCTION

The molecular biological study of plant virus and viroids from fresh plant tissues is relatively straightforward, since good quality RNA/DNA can be easily extracted. Recently, Sipahioglu *et al*. (2006) have described the preservation of virus/viroid isolates by heat drying method for the long term storage and subsequent detection by PCR methods. By this method it was shown that the RNA for use as template material for cDNA synthesis could be extracted, not only from fresh tissue, but also from dried leaf preparations of infected plants. Drying the infected plant leaves has several advantages over the frozen tissues for the detection of pathogens. It can give an opportunity to researcher for the arrangement of best testing period of surveyed crops, it is an alternative method for the transportation of the virus/viroid isolates among the research laboratories where analyses performed and starting sample volume is relatively lower (100 versus 1-10 mg).

Polymerase Chain Reaction (PCR) has been widely used due to its high specificity, sensitivity and turn around time in diagnostic and research laboratories. In order to amplify nucleic acids (DNA or RNA), these molecules need to be extracted and purified from other components of microorganisms or cellular materials which may inhibit amplification reactions (Wiedbrauk *et al*., 1995; Schepetiuk *et al*., 1997; Aygan, 2006). In a plant matrix many components are known to inhibit the PCR reaction, such as tannins, latex, gums, phenolic compounds (Martelli, 1994). The chief limiting factor in the application of the PCR technique in routine diagnosis lies in the preparation of good quality nucleic acid, free of PCR inhibitors. This is especially true in the case of woody species such as tree fruit varieties of *Malus* and *Prunus* origin (Korschineck *et al*., 1991). Most standard nucleic acid extraction procedures do not remove contaminating plant polysaccharides or polyphenolic compounds, which can have direct inhibitory effects on subsequent PCR amplification (Demeke and Adams, 1992; Henson and French, 1993). Sipahioglu *et al*. (2006) has suggested that drying method for extracting total RNA could be effective at reducing inhibitors in all plant species they tested.

Many methods of extracting viral RNA from the crushed extraction buffer, including rapid methods and commercial kits, have been compared and evaluated mainly from fresh plant tissues (Faggioli *et al*., 1998;

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Zhang *et al*., 1998; Roberts *et al*., 2000). However, no studies have been focused on RNA extraction from heat-dried infected plant tissues and there is no standardized RNA extraction method available for heat dried tissue samples to date. Thus the aim of our work was to define the best protocol to extract viral/viroid RNA from heat-dried infected host leaves by comparing three commonly used extraction methods. With this, we intended to discuss some critical points on extraction of virus/viroid RNAs as heat-dried conserved preparations.

MATERIALS AND METHODS

Virus/viroid source and preparation of samples: Leaf samples of ApLV, PBNSPaV, PNRSV, PVY and ASSVd infected hosts were dried at 65°C for 48 h and grounded into dry powder and stored at 4°C in air proof plastic bags until use. Dried and powdered leaf samples were prevented exposure to moisture, by sealing with plastic bag and transported safely at room temperature as long as the seal is maintained (Sipahioglu *et al*., 2006). An apple (*Malus sylvestris* Mill.) isolate of ASSVd, apricot (*Prunus armeniaca* L.) isolates of ApLV and PNRSV, a potato (*Solanum tuberosum* L.) isolate of PVY and a cherry (*Prunus avium* L.) isolate of PBNSPaV were used as reference isolates. Dried leaves of systemically infected apple, apricot, cherry and potato plants were used in RT-PCR and nested-RT-PCR analyses.

RNA extraction methods and reverse transcription: The following extraction methods: silica-capture, citric buffer and lithium chloride were used to prepare viral RNA from dried-infected leaf tissues belonging to different plant species. Extractions were carried out under the same conditions, regardless of the species of plant sample. Volumes in which the pellet was resuspended for each technique were adjusted to provide the optimal conditions for each protocol and ranged 40-100 μL depending on the method used. Negative controls were included in the course of each extraction. Purified RNA was used as template to generate the single stranded cDNA for ApLV, PNRSV, PVY, ASSVd and PBNSPaV. Reverse transcription was carried out according to Usta *et al*. (2005). The sequences of reverse complementary primers were used for all isolates in cDNA synthesis (Table 1).

Silica-capture method (SC): The silica-capture extraction procedure was done according to Foissac *et al*. (2000) with minor modifications. Approximately 10 mg of heat-dried infected leaf powder was placed in a sterile mortar and homogenized with 2 mL of 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-mercapto ethanol). Aliquots of 500 μL of extracts were mixed with 100 μL of 10% sodium lauryl sarcosyl solution in a new set of sterile eppendorf. Tubes were incubated at 70°C with intermittent shaking for 10 min and incubated in ice for 5 min. After centrifugation at 14,000 rpm for 10 min, 300 μL of the supernatant was transferred to a new eppendorf set containing 150 μL of ethanol, 25 μL of resuspended silica and 300 μL of 6 M sodium iodide. The mixture was then incubated at room temperature for 10 min with intermittent shaking. After centrifugation at 6,000 rpm for 1 min, supernatant discarded and the pellet washed twice with washing buffer. The pellet then was resuspanded with 150 μL of RNase-free water and incubated for 4 min at 70°C followed by a centrifugation at 14,000 rpm for 3 min. Finally the supernatant was transferred to a new eppendorf set and stored at -20°C until use.

Citric buffer method (CB): The citric buffer method was used according to the study made by Wetzel *et al*. (1992) with slight modifications. Ten mg of dried plant material were ground with quartz sand in the presence of 1 mL of citric buffer pH: 8.3 (sodium citrate 50 mM, PVP 2%, DIECA 20 mM). The extract was then centrifuged at 8.000 rpm for 3 min. Fifty microliter of the supernatant was collected and 450 μL of citric buffer were added. The TNAs were kept at -20°C for further manipulation.

Table 1: The primer pairs used for various viruses and viroid in RT-PCR and nested-RT-PCR

Virus/ viroid	Primers	Amplified fragment	Reference	PCR cycling conditions
PVY	PVY[1] 5'-ACGTCCAAAATGAGAATGCC-3' PVY[2] 5'-TGGTGTTCGTGATGTGACCT-3'*	480 bp	Nie and Singh,(2001)	36 X (92°C/1 min, 57°C/1 min, 72°C/1 min), 72°C/10 min
PNRSV	A1: 5'GAGCTCTGGTCCCACTCAGG-3' A2: 5'-TCACTCTAGATCTCAAGCAG-3'*	616 bp	Spiegel *et al*. (1999)	94°C/3 min, 41 X (92°C/30 sec, 54°C/30 sec, 72°C/1 min), 72°C/5 min
ASSVd	AS1: 5'-CCGGCCTTCGTCGACGACGA-3' AS3: 5'-TGAGAAAGGAGCTGCCAGCAC-3'*	330 bp	Hashimoto and Koganezawa,	36 X (95°C/1 min, 55°C/2 min, 72°C/3 min)
ApLV	ApLV[1] 5'-GGAATAGAGCCCCAAGAAG-3' ApLV[2] 5'-AGCAAGGTAAACGCCAAC-3'*	200 bp	(1987) Nemchinov and Hadidi, (1998)	36 X (94°C/30 sec, 58°C/30 sec, 72°C/45 sec), 72°C/7 min
PBNSPaV	ASP1 5'-CGGTAGGGCTGTGACTACCG-3' ASP2 5'-GTAGTCCGCTGGTACGCTACAAG-3'*	290 bp	Abou Ghanem *et al*. (2001)	94°C/1,5 min, 36 X (94°C/20 sec, 58°C/30 sec, 72°C/45 sec), 72°C/5 min
Nested primer	ASPn1 5'-ACGAATCCGAGTTTCGTCGC-3' ASPn2 5'-AGGCACTACTGACCTGTAGG-3'	190 bp	Amenduni *et al*., (2004)	94°C/1,5 min, 36 X (92°C/20 sec, 58°C/30 sec, 72°C/45 sec), 72°C/5 min

* = Reverse complementary primer

Lithium chloride method (LC): The lithium chloride based extraction procedure was done according to Hughes and Galau (1988) with minor modifications. Ten mg of dried plant tissue sample (ca. 10 mg) were homogenized in a sterile mortar with 1 mL of extraction buffer (200 mM Tris-HCl, pH 8.5, containing 1.5% sodium dodecylsulphate, 300 mM lithium chloride, 10 mM EDTA, 1% sodium deoxycholate, %1 NP-40 and 0.5% 2-mercaptoethanol). Five hundred microliter extract was collected into 1.5 mL micro centrifuge tube. Tubes were incubated at 65°C for 15 min and equal volume of 5M potassium acetate (pH 6.5) was added and the mixture was incubated in ice for 10 min and centrifuged at 14.000 rpm for 15 min. Six hundred microliter of the supernatant was collected in a new sterile tube to which 600 μL isopropanol was added. The mixture was incubated overnight at -20°C. Next day, the pellet was obtained by centrifugation at 14.000 rpm for 15 min and washed with 70% ethanol and conserved at -20°C until use.

Polymerase Chain Reaction (PCR): The PCR method varied depending on the intended virus/viroid to be detected and the number of primers involved. For the detection of ApLV, PNRSV, PVY and ASSVd RT-PCR method was used. A final volume of 50 μL contained: 2 μL of cDNA, 5 μL of 10 X reaction buffer (200 mM Tris-HCL pH: 8.4, 500 mM KCl), 3 μL of $mgCl_2$ (25 mM), 1 μL of dNTPs (10 mM each), 1 μL of each primer (100 pmol/μL), 0.4 μL of Taq DNA polymerase and 36.6 μL of RNase free sterile water. However, for the detection of PBNSPaV, nested-RT-PCR method was used. A final volume of 50 μL contained: 5 μL of cDNA, 5 μL of 10 X reaction buffer (Fermentas), 2 μL of $mgCl_2$ (25 mM), 1 μL of dNTPs (10 mM each), 1 μL of each primer ASP1 and ASP2 (10 μM each), 0.25 μL of Taq DNA polymerase (Fermentas) and 34.75 μL of sterile water for the first step. One microliter of RT-PCR amplicons, 5 μL of 10 X PCR buffer, 2 μL of 25 mM $mgCl_2$, 1 μL of 10 mM dNTPs, 1 μL of specific primer ASPn1 and ASPn2 (10 μM each), 0.25 μL of Taq DNA polymerase (Fermentas) and 38.75 μL of RNase free water were mixed at the second (nested) step. Samples were amplified in a ThermoHybaid PX2 thermo cycler. The cycling conditions are given in Table 1.

Aliquots of 10 μL PCR products were separated either on 5% polyacrylamide gel (PAGE) in 1 X TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.0) or on 2% agarose gel in TAE buffer (40 mM Tris pH 7.8, 20 mM acetic acid, 2 mM EDTA). In both methods, the DNA was visualized by ethidium bromide staining (Sambrook *et al*., 1989).

RESULTS AND DISCUSSION

The results of this study show that, RNA extraction with silica-capture method yielded the highest concentration and best purity of total RNA, with significant difference among these three methods tested, in the detection of final RT-PCR products. The quality of RNA extracted with lithium chloride and citric buffer was significantly lower than that with silica-capture (Fig. 1 and 2).

Although the lithium chloride method appears to be more affective in extracting viral RNA from infected dried leaves than citric buffer method, its major disadvantages include being time consuming, labor intensive and requiring more laboratory disposables. Lithium chloride extraction method takes nearly 4 h spread over two days (because of precipitation step with isopropanol) whereas extraction using the silica capture and citric buffer method take only 2 h and 15 min, respectively (based on 6 samples on per run). Despite its labor intensity the lithium chloride method was found more effective compared to citric buffer method.

One of the rapid methods for the extraction of viral/viroid RNA from infected tissue is to citric buffer method, which takes only 15 min. Citric buffer method seemed to be the least useful method in all detection studies carried out. Except ASSVd, the method yielded sufficient RNA for subsequent detection of APLV, PBNSPaV, PNRSV and PVY with limited success. In detection of ASSVd, two of three protocols (LC and CB) have failed for RNA isolation from dried infected leaves mainly because of RNA degradation. These two methods resulted negative when the assay was repeated (Fig. 2).

It was shown that, in detection of PVY the use of lithium chloride method was resulted fragmentation in PCR products; typical example is shown in Fig. 1 (PVY LC lane). The phenomenon was determined as presumably due to annealing of primers to the non-specific sites on the template. Arezi *et al*. (2003) has indicated that Taq DNA enzyme formulations can also cause fragmentation and smears.

Present results showed that, in silica capture method, 4M-guanidine thiocyanate is sufficient to break rehydrated dried tissue fragments and release RNA from the tissue. Of three evaluated procedures were assayed in 5 purposely-selected plant virus and viroid isolates conserved as dried leaf tissue preparations and tested as a method of removing the remaining inhibitors and improving the PCR detection. To date, there are no published data comparing different methods of RNA extraction from heat-dried tissues. The sensitivity of drying method had been previously tested in our laboratory by performing assays with silica-capture method. It was demonstrated that RNA isolated from heat-dried leaf tissue was suitable for downstream applications (cDNA, RT-PCR and nested-RT-PCR). In the course of study it was found that for RNA extraction, 280 day stored ApLV, 31 day stored ASSVd, 20 day stored PVY, 233 day stored PBNSPaV and 35 day stored PNRSV

Table 2: The results of PCR analysis and the source of heat-dried leaf samples used in PCR reactions

Pathogen tested	Source of tissue	Age of the sample at the time processed (days)	Test used	Band intensity		
				LC	SC	CB
PNRSV	Apricot leaf	35	RT-PCR	+	+++	+
PBNSPaV	Cherry leaf	233	Nested-RT-PCR	++	+++	+
PVY	Potato leaf	20	RT-PCR	+	+++	+
ApLV	Apricot leaf	280	RT-PCR	++	++	+
ASSVd	Apple leaf	31	RT-PCR	-	+	-

LC: Lithium Chloride, SC: Silica Capture, CB: Citric Buffer, +: Week positive, ++: Good positive, +++: Strong positive, -: Negative

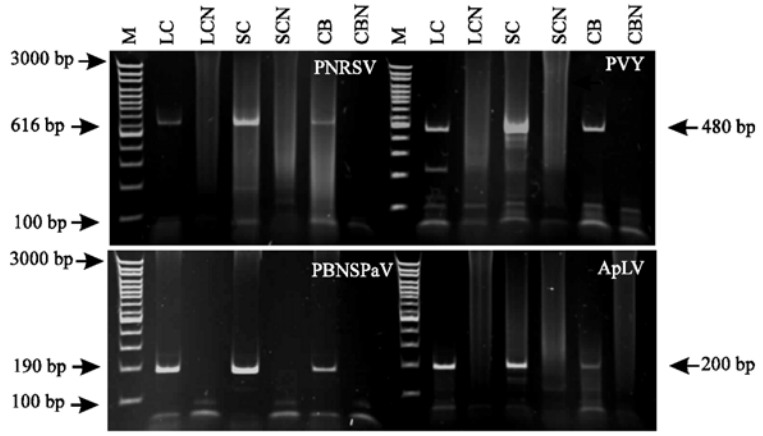

Fig. 1: Comparison of genomic RNA derived PCR products of PNRSV, PVY, PBNSPaV and ALV from heat-dried infected host leaves on ethidium bromide stained 5% PAGE gel. Positive (LC, SC and CB) and negative (LCN, SCN and CBN) samples extracted by Lithium Chloride (LC), Silica-Capture (SC) and Citric Buffer (CB) methods, respectively, M: Molecular size marker

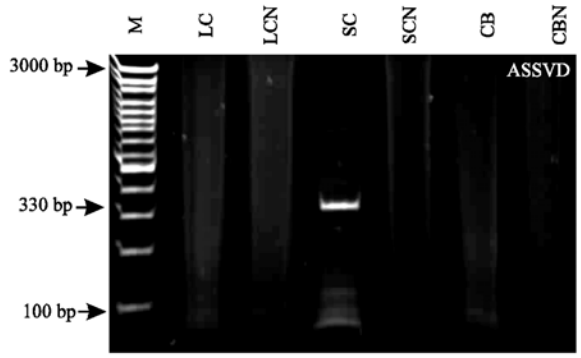

Fig. 2: Comparison of genomic RNA derived PCR products of ASSVd from heat-dried infected host leaf on ethidium bromide stained 5% PAGE gel. Positive (LC, SC and CB) and negative (LCN, SCN and CBN) samples extracted by Lithium Chloride (LC), Silica-Capture (SC) and Citric Buffer (CB) methods, respectively, M: Molecular size marker

infected dried leaf samples produces significant amounts of RNA by silica-capture method (Table 2). Jaiprakash *et al*. (2003) demonstrated that RNA isolated from freeze dried tea leaves was suitable for all molecular biology methods examined, including Northern blotting, reverse transcription and microarray analysis.

To detect viruses using PCR, it is important that the samples of nucleic acids from the original specimens be as pure as possible. The purification stage is particularly important when the virus concerned is an RNA virus, for which a Reverse Transcription (RT) stage is necessary. Indeed, the high susceptibility of reverse transcriptase to interfering or inhibitory substances is a major limiting factor in amplification reactions (Wilde *et al*., 1990). Due to the sensitivity of RNA to ribonuclease degradation, which is present ubiquitously, the guanidine hydrochloride or guanidine thiocyanate is widely used for extraction of RNA from tissues and cells (Gough, 1988). The results obtained after RT-PCR, nested-RT-PCR and PAGE showed that the technique using silica (Sigma S 5631) and guanidine thiocyanate (Appli Chem A 1107) was the most effective and sensitive as compared to lithium chloride and citric buffer methods. The silica technique described in materials and method has several advantages. First, it is very efficient and can produce relatively large amounts of RNA from as little as a few dried tissue sections (less than 10 mg). Second, the use of this method minimizes the fragmentation of PCR products. Third, the RNA isolated by silica-capture method improves the PCR detection and the extracts can be stored for up to several months. Thus, this extraction method

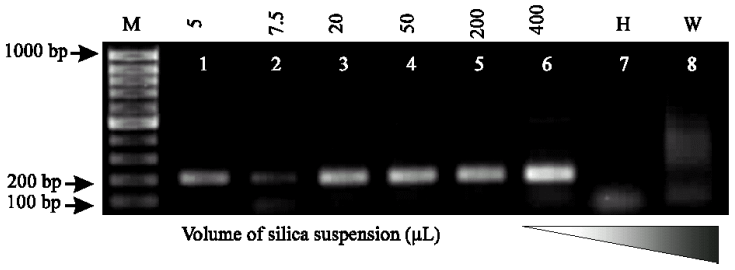

Fig. 3: The effect of silica suspension volume on RNA extraction from heat dried tissue in detection of ApLV by RT-PCR, W: water control, H. Healthy control, M: Molecular size marker.

could be safely used to produce RNA preparations on which many analyses could be performed over a long period of time.

Because of superior results of silica capture method over lithium chloride and citric buffer methods; an additional research was carried out to determine the effect of silica volume on RNA extraction and subsequent PCR yield. An apricot isolate of ApLV was chosen for this purpose. It was shown that, the use of gradually increased volume of silica suspension greatly improves the yield and quality of RNA that can be extracted. Higher volume of silica suspension (i.e., 200 and 400 µL) did not produce any deleterious effect on diagnosis of infected leaves. The use of increased quantity of silica suspension dramatically improved the RT-PCR signal in detection of ApLV (Fig. 3).

The silica method applied is an efficient, simple and reproducible for the isolation of RNA from heat-dried leaf tissues of infected hosts. The isolated RNA is of high quality and could serve as a robust template for reverse transcription. Concerning the impact of freezing, a preliminary study demonstrated, without explanation, that the freezing of infected material could reduce the nucleic acid content of extract (Hajduk, 1999). There is still a lack of information concerning DNA and RNA changes in frozen and heat-dried infected tissues (Giacomazzi *et al*., 2005; Sipahioglu *et al*., 2006). Moreover, there are no studies focusing on impact of these physical treatments on viral RNA.

The choice of an extraction technique that can be used for routine testing of a large number of samples must also take into account simplicity of use (with the possibility of automatization) and rapidity of execution (Arnal *et al*., 1999). Citric buffer presents a short extraction method for RNA extraction, which combines only extraction and centrifugation steps. It is quickest method for obtaining total RNA among the three RNA extraction methods tested. It is more suitable for large-scale survey activities employing PCR methods. But in situations where maximum sensitivity is needed, the moderately labor intensive silica capture method is recommended. With increased volume of silica suspension (200-400 µL), the silica-capture method can be applied safely to detect both viruses and viroid RNA from heat-dried tissue sections and is most suitable for routine diagnostic use, in view of its rapidity and sensitivity.

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