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Improvement of RT-PCR Sensitivity for Fruit Tree Viruses by Small-scale dsRNA Extraction and Sodium Sulfite

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Woody plant tissues contain great amounts of phenolic compounds and polysaccharides. These substances inhibit the activation of reverse transcriptase and/or *Taq* polymerase in RT-PCR. The commonly used multiple-step protocols using several additives to diminish polyphenolic compounds during nucleic acid extraction are time consuming and laborious. In this study, sodium sulfite was evaluated as an additive for nucleic acid extraction from woody plants and the efficiency of RT-PCR assay of commercial nucleic acid extraction kits and small-scale dsRNA extraction was compared. Sodium sulfite was used as an inhibitor against polyphenolic oxidases and its effects were compared in RNA extraction by commercial extraction kit and small-scale double-stranded RNA (dsRNA) extraction method for RT-PCR. During nucleic acid extraction, addition of 0.5%-1.5%(w/v) of sodium sulfite to lysis buffer or STE buffer resulted in lighter browning by oxidation than extracts without sodium sulfite and improved the RT-PCR detection. When commercial RNA extraction kit was used, optimal concentrations of sodium sulfite were variable according to the tested plant. However, with dsRNA as RT-PCR template, sodium sulfite 1.5% in STE buffer improved the detection efficiency of *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem grooving virus* (ASGV) in fruit trees, and reduced the unspecific amplifications significantly. Furthermore, when viruses existed at low titers in host plant, small-scale dsRNA extractions were very reliable.

Keywords : *Apple chlorotic leaf spot virus, Apple stem grooving virus*, detection, dsRNA, RT-PCR

Apples and pears are the most widely grown fruits in Korea. Area devoted to these fruits occupies about 30% of the 167,000 ha allotted for fruit trees. Out of the many viruses infecting apples and pears, the more economically important and common ones among commercial cultivars are apple chlorotic leaf spot virus (ACLSV, *Trichovirus*), apple stem pitting virus (ASPV, *Foveavirus*), apple mosaic virus (ApMV, *Ilarvirus*), and apple stem grooving virus (ASGV, *Capillovirus*) (Campbell, 1963; Posnette et al., 1963; Desvignes, 1999).

However, ASGV and ACLSV are considered to be the major viruses infecting apples and pears in Korea (unpublished).

To detect ASGV and ACLSV, researchers have widely used the woody indicators or the enzyme-linked immunosorbent assay (ELISA). However, ELISA frequently lacks the sensitivity that can effectively detect these viruses, particularly under low viral concentrations in their woody hosts.

In addition, there are no existing internal controls that can prevent false or negative ELISA results. Indexing by using woody indicators is time consuming, usually spanning over a period of up to three years. It is also expensive and sometimes, symptoms are difficult to interpret.

For routine diagnosis, the procedures should be essentially reliable, rapid, simple, and inexpensive; however, plant extracts have not always been found reliable for use in RT-PCR. Tissues from woody plants, especially when field-grown, contain higher amounts of phenolic compounds and polysaccharides which are thought to have inhibitory activity (Newbury and Possingham, 1977; Rezaian and Krake, 1987; Demeke and Adams, 1992; John, 1992; Kim et al., 2001).

To overcome the problem of unreliability, researchers have come up with longer or more complicated nucleic acid extraction procedures. However, these procedures have been found to contaminate and degrade RNA during the extraction process.

In an effort to reduce the levels of inhibitors, researchers have used IPVP-40, which has complex polyphenols, and/or mercaptoethanol acting as an antioxidant, into the extraction procedure (Monette and James, 1990; Henson and French, 1993; Rowhani et al., 1993; Mackenzie et al., 1997). However, this procedure has also some weaknesses.

Therefore, researchers are now looking at nucleic acid-based diagnostic techniques, such as reverse transcription and polymerase chain reaction (RT-PCR), to provide them with a possible reliable alternative.

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Recently, sodium sulfite is known to inhibit polyphenolic oxidases (Wu et al., 1999) and viral double-strand RNAs (dsRNA) reduced unspecific amplification when used as RT-PCR template (Okuda and Hanada, 2001).

Materials and Methods

Virus and plant materials. The virus sources used in this study were ACLSV from leaf of 'Hwahong' apple and 'Kurakatawase' peach, and ASGV from leaf of 'Niitaka' pear and 'Hongro' apple. Young leaf samples and bark tissues were collected from potted plants in mid June (2002). The samples were stored in plastic bags at -70°C until extraction of total nucleic acids. Samples were taken from the experimental glasshouse of the National Horticultural Research Institute, Suwon, Korea. The trees infected with ACLSV and ASGV were selected through prior experiment using RT-PCR.

RNA extraction with sodium sulfite. Sodium sulfite from 0.0% to 1.5% (w/v) with 0.5% increment in the lysis buffer was used in total RNA extraction using the Plant RNeasy kit from Qiagen according to the manufacturer's instructions. The RNA was eluted by applying 50 μL RNase free H_2O, and then left for 1 min at room temperature, and centrifuged for 60 sec at 8000 g. Total RNA extracts were used in one-tube RT-PCR or stored in small aliquots at -70°C.

Viral dsRNA isolation. Cellulose column chromatography was used to isolate a variety of plant viral dsRNA (Morris and Dodds, 1979). In this study, more easy and simple procedure was used (Zhang et al., 1998). All steps were performed at room temperature. A total of 100 mg samples were ground in mortar and pestle with 600 μL of 1 × STE (0.1 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 6.8), 80 μL of 10% SDS, 800 μL of water-saturated phenol and sodium sulfite at the concentrations 0.0%, 0.5%, 1.0%, 1.5% respectively.

Mixture treatment. The mixture was spinned in a vortex motion, and was centrifuged for 5 min at maximum speed. The upper aqueous phase was transferred to a clean micro centrifuge tube and ethanol was added to a final concentration of 16.5% and approximately 10 mg cellulose (Whatman CF-11).

The mixture was vortexed thoroughly and centrifuged for 30 sec at 14,000 rpm to pellet the CF-11, and the supernatant was discarded. The CF-11 cellulose was washed three times with 1 mL of 1 × STE in 16.5% EtOH and collected through centrifugation at 14,000 rpm for 30 sec.

The dsRNA was eluted from the CF-11 by resuspending it with 200 μL of 1 × STE and by centrifuging it for 5 min at 14,000 rpm. The supernatant was transferred to a clean tube and was eluted repeatedly. Approximately 400 μL of eluted dsRNA was precipitated by adding 40 μL of 3 M NaOAC and 1 mL of 100% ethanol.

Incubation. The mixture was incubated at -70°C for 20 min, centrifuged for 10 min at 14,000 rpm, and rinsed with 80% ethanol. The pellets were air-dried and resuspended in 15 of nuclease-free water. The dsRNA extracts were used for RT-PCR. The entire extraction from fresh leaf or bark chip tissue was completed within 1-1.5 hr.

Detection of ACLSV and ASGV. One-step RT-PCR, based on the procedure of Mackenzie (1997), was taken to detect ACLSV and ASGV with primers (Table 1; Baek, 2002). Prior to RT-PCR, each 5 μL of total RNAs were serially diluted dsRNAs (1:5, 10, 50, 100, 1000), mixed with 1 μL of 10 pmol reverse primers, and incubated at 95°C for 8 min. The tubes were quickly chilled in ice, and 5 μL of reactions was used for one-step RT-PCR template RNA.

RT-PCR mixture. The RT-PCR mixture (final volume of 50 μL) contained 5 μL template RNA, 10 μL of 5 × reaction buffer (Promega, USA), 0.2 mM dNTP mixture. The mixture also contained 1 mM $MgSO_4$, 0.1 U AMV reverse transcriptase, 0.1 U *Tfl* DNA polymerase, 0.6 pmol each with reverse and forward primers. Amplification was carried out in a thermocycler (MJ Research PTC-220, USA).

Incubation. The mixture was incubated once at 50°C for 30 min (reverse transcription) and at 94°C for 2 min, followed by 40 cycles at 94°C for 1 min, 54°C for 1 min, and 68°C for 2 min. It was incubated again at 68°C for 7 min. Amplified products were stored at 4°C until analysis, and 5 μL of the RT-PCR products underwent electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and photographed under UV light.

Use of Plant RNeasy kit. RNAs were extracted by using Plant RNeasy kit with lysis buffer for viruses from young leaf and bark chip tissues of 'Hongro' apples which were not detected by ELISA. The buffer contained 1.0% sodium sulfite and small-scale dsRNA extraction with 1 × STE containing 1.5% sodium sulfite.

DMSO. The dsRNA was denatured with an equal volume of dimethyl sulfoxide (DMSO) at 95°C for 5 min, and the mixture was directly used as a RT-PCR template. Duplex RT-PCR was conducted by using the same thermo-profile and concentration reagent with two primer pairs (ACLSV-CPf, CPr and ASGV-Pf, Pr). The RT-PCR product 5 μL underwent electrophoresis in 1.5% agarose gels, and the products were stained for photograph.

Table 1. Primer sequences and expected size of RT-PCR product for each primer pair

Primer name	Primer sequence from 5'-3' orientation	Primer position	Product size
ASGV-Pr (reverse)	TGGAGGAAAAGAACTTTGGG	531-551	404bp
ASGV-Pf (forward)	GTGACCAATCGCTTCTTTTCT	148-168	
ACLSV-CPr (reverse)	GCAAATTCAGTCTGTAAAAG	7288-7307	566bp
ACLSV-CPf (forward)	GAGAGTTTCAGTTTGCTAGACA	6742-6763	

Table 2. RNA extraction from leaf of fruit trees, pear, apple and peach through commercial RNA extraction kit

Sample	Sodium sulfite concentration (%)	Purity of total RNA (A_{260}/A_{280})	Concentration of total RNA (μg/mL)	Yield (μg)
Pear leaf	0.0	0.59	10.4	0.52
	0.5	1.07	89.6	4.48
	1.0	1.05	172.0	8.60
	1.5	1.07	126.4	6.32
Apple leaf	0.0	1.29	7.2	0.36
	0.5	1.65	26.4	1.32
	1.0	1.59	49.6	2.48
	1.5	1.46	79.2	3.96
Peach leaf	0.0	1.85	59.2	2.96
	0.5	1.82	48.0	2.40
	1.0	1.47	37.6	1.88
	1.5	1.35	40.0	2.00

Results and Discussion

Woody plants and their RNA extracts contain high amounts of polysaccharides or phenolic compounds that inhibit the effects on reverse transcriptase and/or *Taq* polymerase (Demeke and Adams, 1992; Staub et al., 1995; Pandey et al., 1996). Sodium sulfite has a potent inhibitor of polyphenol oxidases and peroxidases (Wu et al., 1999) and is used widely to inhibit enzymatic and non-enzymatic browning in fruits and vegetables (Molnar-Perl and Friedman, 1990).

Effect of sodium sulfite. To determine an optimal concentration of sodium sulfite for nucleic acid extraction, 0.5 to 1.5%(w/v) sodium sulfite with increments of 0.5% was tested. The color of extracts became lighter brown than that of extracts without sodium sulfite in all leaf samples of apples, peaches, and pears. The concentration of total RNA was determined by using a spectrophotometer (Table 2).

In RT-PCR assay, product band intensity of sodium sulfite treated lanes was stronger than that without sodium sulfite treatment. When commercial RNA extraction kit was used, optimal concentrations of sodium sulfite varied according to the host plants in agarose gel electrophoresis. Sodium sulfite at 0.5% was most effective in pear leaf samples infected with ASGV. Sodium sulfite at 1.0-1.5% o was optimal in apple leaf samples infected with ACLSV, and at 0.0-0.5%, it was also optimal in peach leaf samples infected with ACLSV (Fig. 1).

RT-PCR sensitivity. Polyphenol oxidases and peroxidases are more highly concentrated in apples than pears and peaches. Total RNA yield and RT-PCR sensitivity were reduced when using commercial RNA extraction kit with excessive sodium sulfite. The specific fragment was amplified in 10-fold-dilution of RNA, while many fragments were not in undiluted RNA (Fig. 1C, D).

Generally, 90% of polysaccharides in rigid cells inhibit enzyme catalysis (Nakahara et al., 1999). In extracting polysaccharides from pear leaf, the elution of RNA from gelatinous precipitate was the major problem. Therefore, it was thought that the purity of total RNA from pear leaf was lower than that from apple and peach (Table 2), and it led to the RT-PCR sensitivity reduction and non-specific fragment

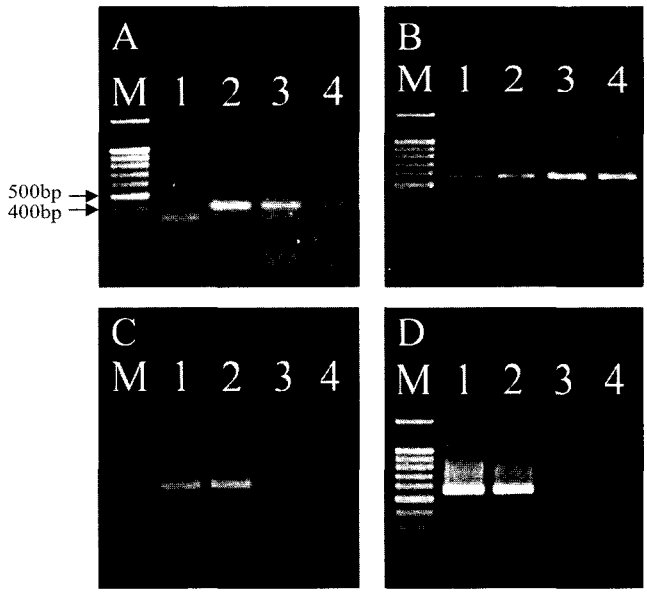

Fig. 1. Agarose gel electrophoresis of RT-PCR products by commercial RNA extraction kit with differential concentration of sodium sulfite in lysis buffer from ASGV infected pear (A), ACLSV infected apple (B) peach (C), and RT-PCR products of 10 fold diluted RNAs that extracted from peach (D). M, size marker (100bp ladder); Lane 1, 0.0% sodium sulfite; Lane 2, 0.5% sodium sulfite; Lane 3, 1.0% sodium sulfite; Lane 4, 1.5% sodium sulfite.

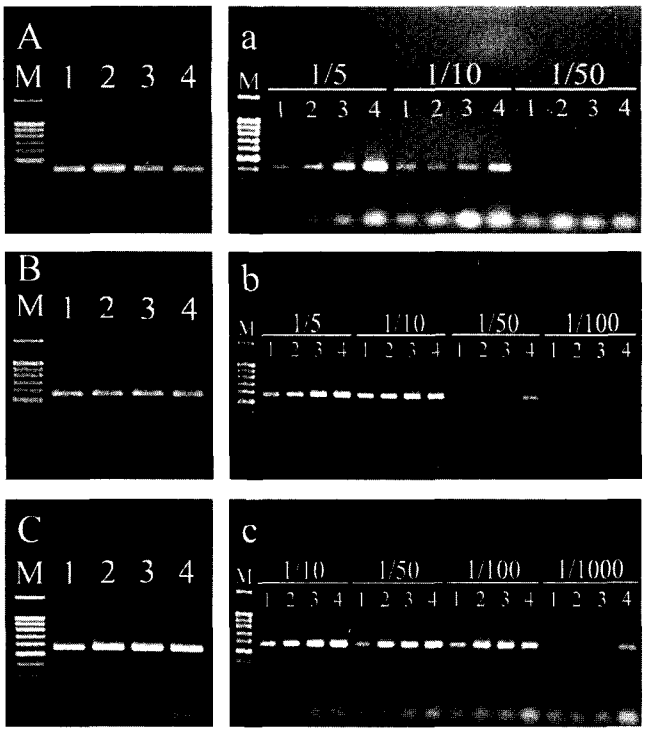

Fig. 2. Agarose gel electrophoresis of RT-PCR products by small-scale dsRNA extraction with differential concentration of sodium sulfite in 1 × STE buffer from ASGV infected pear (A), ACLSV infected apple (B) peach (C), and RT-PCR products of serial diluted dsRNAs (a, b, c). M, size marker (100bp ladder); Lane 1, 0.0% sodium sulfite; Lane 2, 0.5% sodium sulfite; Lane 3, 1.0% sodium sulfite; Lane 4, 1.5% sodium sulfite.

amplification (Fig. 1A).

Sometimes, non DNase-treated RNA extracts are contaminated by DNA (Nassuth et al., 2000). So, the same size will be amplified for RNA and DNA or the other fragments will be amplified (Fig. 3 lane 1). To eliminate these problems, researchers treat extracts with Dnase.

Each additional extraction step consumed more time, raised costs, and increased the risk of contamination. However, unspecific amplifications were drastically reduced when RNA was purified with STE containing less than 16.5% ethanol (Okuda and Hanada, 2001). Furthermore, viral dsRNA, which was more stable than viral genomic RNA, was used for identification of virus and cloning template (Zhang and Rowhani, 2000).

In dsRNA based RT-PCR, product band intensity was two to three times higher than that of total RNA based RT-PCR. In all cases, 1.5% of sodium sulfite in STE buffer very effectively detected viruses in pears, apples, and peaches (Fig. 2A, B, C). The dilution end-point of the dsRNA template for RT-PCR and agarose gel electrophoresis was 1:50 in ASGV-infected pears, 1:100 in ACLSV-infected apples, and 1:1000 in ACLSV-infected peaches (Fig. 2a, b, c).

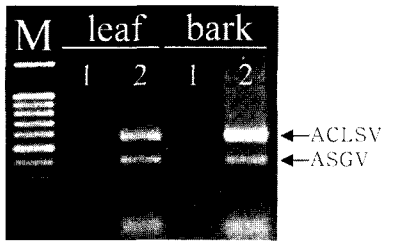

Fig. 3. Agarose gel electrophoresis of duplex RT-PCR products from ACLSV and ASGV doubly infected leaf and bark chip tissue in apple that couldn't detect any viruses by ELISA. M, size marker (100bp ladder); Lane 1, commercial RNA extraction kit with lysis buffer containing 1.0% sodium sulfite; Lane 2, small-scale dsRNA extraction with 1 × STE buffer containing 1.5% sodium sulfite.

Viruses from leaf and bark chip tissues from 'Hongro' apples that were not detected by ELISA were detected through duplex RT-PCR for ACLSV and ASGV. The modified commercial RNA extraction kit and small-scale dsRNA extraction were used. Both viruses were detected in small-scale dsRNA extraction lane (Fig. 3).

Therefore, small-scale dsRNA extraction will be very reliable in detecting viruses at low titers in host plants. It was found very useful in detecting phloem-limited viruses such as *Grapevine leafroll-associated* 1 and *Grapevine leafroll-associated* 3 *Ampelovirus* in grapevines. Sodium sulfite inhibited polyphenolic compounds in nucleic acid extraction. When combined with small-scale dsRNA extraction, the sensitivity to detect viruses increased. This report will be highly useful in detecting other major woody plant viruses and selection of virus-free plants.

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