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Full Length Research Paper

Simultaneous detection of Apple mosaic virus in cultivated hazelnuts by one-tube RT-PCR

Birol Akbas* and Kemal Degirmenci

Plant Protection Central Research Institute, Ankara, Turkey.

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The most economically damaging ilarvirus affecting hazelnut on a worldwide scale is the related apple mosaic virus (ApMV). Attempts were made to isolate the virus RNA from hazelnut tissues using different extraction methods. The most suitable extraction method that could detect the virus occurring naturally in hazelnut by reverse-transcription polymerase chain reaction (RT-PCR) methodology was selected. RT-PCR was applied successfully using flower, husk and leaf tissues. The most suitable extraction method and hazelnut tissues determined were sensitive, simple, rapid and reliable for simultaneous detection of ApMV in hazelnut tissues. To our knowledge, this is the first report of the simultaneous detection of the virus by RT-PCR, an alternative detection of ApMV in hazelnut hosts.

Key words: RT-PCR, hazelnut, apple mosaic virus.

INTRODUCTION

Apple mosaic virus (ApMV), a member of the genus *Ilarvirus* (Fulton, 1972), occurs worldwide and infects a number of woody plants of over 65 species in 19 families including rose, hop, birch and raspberry (Brunt et al., 1996; Gotlieb and Berbee, 1973; Postman and Cameron, 1987; Sweet and Barbara, 1979; Wong and Horst, 1993) and induces bright yellow patterns on leaves (Nemeth, 1986). ApMV, the main and sole known virus affecting hazelnut (*Coryllus avenea*) trees is also one of the major pathogens infecting them. The virus occurs frequently in all hazelnut production areas in the world and cause significant yield reduction (Postman and Cameron, 1987; Aramburu and Rovira, 1995; Rovira and Aramburu, 1998; Postman, 2002).

The only effective way to prevent virus spread in woody crops is through the use of healthy material. Control of viral diseases in woody plants is best accomplished by establishing new virus-free plantings from virus tested plants. Fast and sensitive screening methods are essential for detection of viruses in certificated nursery stocks. Bioassays such as grafting method with sensitive herbaceous and woody indicator species as well as enzyme-linked immunosorbent assay (ELISA) are the routine methods used for detecting viruses. However, these techniques are not sufficient to detect low concentrations of virus in plant tissues, and sometimes variations occur in the responsiveness of indicator host plants to various isolates of virus. Meanwhile, ELISA fails occasionally due to low virus titre, or inhibitory effects of polysaccharides or phenolic compounds in tissue extracts of woody plants (Nemchinov et al., 1995; Kinard et al., 1996; MacKenzie et al., 1997; Menzel et al., 2002). For hazelnut trees, so far, only herbaceous indexing and ELISA techniques have been valid (Postman and Cameron, 1987; Aramburu and Rovira, 2000; Akbas et al., 2004; Kobyłko et al., 2005). Indexing to herbaceous plants is time consuming, usually spanning over a period of a minimum of several weeks to a few months. It is also not specific and sometimes symptoms are difficult to interpret. Serological detection by ELISA using commercially available antisera represents the first alternative for biological indexing, but its use is limited to certain time period in the growing season and appears inappropriate with dormant woody tissues (Postman and Cameron, 1987; Aramburu and Rovira, 2000; Kobylko et al., 2005). Indeed, this method, even

*Corresponding author. E-mail: birol_akbas@zmmae.gov.tr. Tel: + 90 312 344 59 94/ extension 103. Fax: + 90 312 315 15 31.

Abbreviations: ApMV, Apple mosaic virus; **RNA,** ribonucleic acid; **ELISA,** enzyme-linked immunosorbent assay; **RT-PCR,** reverse transcription polymerase chain reaction; **SDS,** sodium dodecyl sulfate; **DEPC,** diethylpyrocarbonate; **dNTP,** deoxynucleotide triphosphate; **AMV,** avian myeloblastosis virus.

though reliable, sometimes gives background with leaf tissues as shown from our previous studies and personal communication with other researchers and the results are often difficult to interpret. Furthermore, there are no existing internal controls that can prevent false or negative ELISA results. So, rapid, reliable and simultaneous virus detection method is required for virus-free germplasm of hazelnut, propagated vegetatively.

Great efforts have been made during recent years to improve the sensitivity and speed of diagnostic methods. Therefore, molecular hybridization based techniques represent additional alternative for biological and serological assays. Polymerase chain reaction (PCR) has contributed to increasing the detection sensitivity for low virus titre or for the presence of inhibitors (Bariana et al., 1995; Rowhani et al., 1995; Kummert et al., 2001). Reverse transcription-PCR (RT-PCR) has the potential to be an extremely sensitive method in some woody plants even during seasons of low titre of virus (Kinard et al., 1996; Spiegel et al., 1996; Sanchez-Navarro et al., 1998). It has allowed the simultaneous detection of viruses, saving time, reagents and reducing costs. ApMV detection methods based on RT-PCR have been reported by Choi and Ryu (2003), Crowle et al. (2003) and Petrzik (2005). Recently Saade et al. (2000), Menzel et al. (2003) and Sanchez-Navarro et al. (2005) reported multiplex RT-PCR detection of ApMV with some viruses from apple and some other host tissues. One step multiplex RT-PCR systems have been developed, allowing 3 or more viruses detection to be conducted in the same test without any additional steps, thus reducing the test number and giving an opportunity for the detection of mixture infection.

A critical step for routine use of PCR technology is template isolation. The standard sample extraction procedure for RT-PCR detection of ApMV is based on nucleic acid isolation (total RNA) from different host, but not from hazelnut (MacKenzie et al., 1997; Choi and Ryu, 2003; Crowle et al., 2003; Petrzik, 2005). Potentially improved sample processing procedures for plant virus detection by PCR have been reported (Choi and Ryu, 2003; Foissac et al. (2001). In this study, various RNA extraction methods and different part of plant tissues were used and the ability of RT-PCR to detect the virus clarified crude extracts prepared from hazelnut tissues was examined. Amplification of the virus directly from hazelnut trees was realized. Thus, reliable and simultaneous detection of ApMV by RT-PCR in hazelnut tissues was provided. Using flower, husk and leaf tissues, the test can be carried out almost half of the year. To our knowledge, this is the first report detecting ApMV simultaneously through a RT-PCR from hazelnut tissues.

MATERIALS AND METHODS

Plant materials

Plant tissues were obtained from known ApMV sources in previous studies in hazelnut (*C. avenea*) trees from different orchards in Bartin, Duzce and Zonguldak provinces of the West Black Sea region in Turkey. Flowers were collected in winter season (January - February). Husk and young leaves were collected at the beginning of the vegetation period (April - May). In total, 150 samples were collected for RT-PCR tests and 24 samples among them were selected for comparison tests with ELISA.

Nucleic acid extraction

Five extraction methods were tried and evaluated for total RNA purification. These methods are Verwoerd et al. (1989), Parakh et al. (1995), Spiegel et al. (1996), the RNeasy plant mini kit (QIAGEN, GmbH, Hilden, Germany) and Foissac et al. (2001) methods. In all extraction methods, 100 mg plant tissues were powdered in liquid nitrogen in the presence and absence of charcoal (2.5 mg/ml) (Luis et al., 2005). Then the other procedures were followed as described below:

(I) In Verwoerd et al. (1989), extraction buffer (0.1 M LiCl, 100 mM, Tris-HCl pH = 8.0, 10 mM EDTA, 1% SDS: Phenol (1:1)) heated at 80°C was added to powdered plant tissues and vortexed. Supernatant was removed after chloroform: isoamylalcohol (24:1) treatment and RNA was precipitated with 4 M LiCl by overnight incubation at 4°C and then centrifuged at 13.000 g. The pellet was dissolved in sterile distilled water and precipitated once more with two volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2). Final preparation was dissolved in 30 µl of diethylpyrocarbonate (DEPC)-treated water and stored at - 70°C until use.

(II) In Parakh et al. (1995), powdered plant tissues were extracted with 1 ml of guanidine thiocyanate, 20 mM Tris-HCl (pH 7.8) containing 100 mM NaCl, 10 mM EDTA and 1% SDS. After centrifugation at 9000×g for 10 min, the pellet was discarded and 500 µl of supernatant was mixed with 5 µl of protinase K (20 mg/ml) and incubated at 37°C for 1 h. Then 500 µl of phenol/ chloroform was added, the mixture vortexed, and centrifuged at 9.000×g for 10 min. This step was repeated, the aqueous phase removed and mixed with 2.5 volume of cold absolute ethanol to precipitate the nucleic acid. The pellet was resuspended after centrifugation in 30 µl of DEPC-treated water.

(III) In Spiegel et al. (1996), briefly, crushed plant material was extracted with 500 µl of 1 M Tris-HCl (pH 8) containing 4 M LiCl, 0.5 M EDTA, 10% SDS and 0.2% sodium deoxycholic acid. 500 µl of 5 M potassium-acetate was added, the mixture vortexed and centrifuged at 15 000 rpm for 15 min, the pellet was discarded and supernatant was mixed with equal volume of isopropanal and incubated at - 20°C for 1 h. Then RNA was precipitated with centrifugation at 15 000 rpm for 15 min. The aqueous phase was removed and washed with 1 ml 70% cold ethyl alcohol. The pellet was dried and resuspended in 30 µl of DEPC-treated water.

(IV) In Qiagen protocol, the RNeasy plant mini kit was applied using manufacturer's instructions with slight modifications. Plant tissues (100 mg) were crushed in liquid nitrogen, 450 µl of RLT buffer was added with further grinding, then another (450 µl) RLT buffer was added and mixed thoroughly. This was followed by adding 600 µl of lysate and this mixture was then applied to a QIA shredder spin column. The flow-through phase was captured in a microfuge tube. Subsequently, absolute ethanol (0.5 vol.) was added to the lysate and the mixture was then applied to a RNeasy column. After washing with RW1 buffer as well as RPE buffer, RNA was eluted in two subsequent steps with 30 µl of RNase-free water.

(V) In Foissac et al. (2001) method, powdered plant tissues were

extracted using 500 µl extraction buffer (6 M guanidine thiocyanate containing 0.2 M sodium acetate, 25 mM EDTA, 1 M potassium acetate, 2.5% PVP-40 and 1% mercaptoethanol) and then mixed with 100 µl of 10% sodium lauryl sarcosyl solution in a new set of sterile tube. 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 was discarded and the pellet was washed twice with washing buffer (10 mM Tris-HCl containing 0.05 mM EDTA, 50 mM NaCl and 50% ethanol). The pellet was then resuspended with 150 µl of RNase-free water and incubated for 4 min at 70°C followed by centrifugation at 14.000 rpm for 3 min. Finally the supernatant was transferred to a new eppendorf set and stored at - 20°C.

RT-PCR mixture and procedures

The RT-PCR mixture (final volume of 50 µl) contained 5 µl template RNA, 2.5 µl of 5x reaction buffer (Sigma), 1 mM dNTP mixture. The mixture also contained 3 mM $MgCl_2$, 2.5 U avian myeloblastosis virus (AMV) reverse transcriptase, 2.5 U *Taq* DNA polymerase, 2 pmol each with reverse and forward primers: 5′-3′ ATCCGAGT GAACAGTCTATCCTCTAA (forward), 5′-3′ GTAACTCACTCGTTA TCACGTACAA (reverse) were used as previously described by Menzel et al. (2002) to amplify viral sequences; specifically a 262 bp product.

In the cycling condition, reverse transcription step was carried out at 37°C for 50 min, activation of the Taq polymerase at 94°C for 2 min, followed by 35 cycles of 94°C at 30 s, 52°C at 30 s, 72°C at 1 min, and a final extension step at 72°C for 7 min. Amplifications were carried out in a eppendorf mastercycler gradient thermal cycler. PCR products were separated by electrophoresis in 1.5% agarose gels in TAE buffer, stained with ethidium bromide and visualized under UV light.

Validation of RT-PCR

Validation assays were carried out by testing selected 24 samples among collected 150 samples from hazelnut trees of known infections with ApMV in previous studies. The flowers, husks and leaves were collected in the vegetation period from January to mid of May. Collections were tested by DAS-ELISA using manufacturer's (Agdia, USA) protocol to ApMV antisera for comparisons of RT-PCR.

RESULTS

Nucleic acid extraction

The five extraction procedures were tried using samples from naturally infected hazelnut trees. The phenol/ chloroform (Verwoerd et al., 1989), $LiCl_2$ (Spiegel et al., 1996) and guanidine thiocyanate, Tris-HCl (Parakh et al., 1995) extraction procedures were not effective with all part hazelnut tissues for RNA isolation. The RNeasy plant mini kit (Qiagen) was rarely found to be effective when using active charcoal at the grinding step, especially for flower samples, but generally gave erratic results in particular with leaf samples. It sometimes yielded faint bands in husk preparations. On the other hand, total RNA extracted using silica gel method (Foissac et al., 2001) gave reliable, robust results, even for leaf samples. However, this method was also found not effective without the use of active charcoal. Considering the results obtained, the silica gel procedure was adopted and flowers were selected as the most suitable tissues for RNA isolation. Our results clearly show that addition of active charcoal in extraction step improved the integrity and yield of isolated RNA, especially in the cases of guanidinium thiocyanate buffer and Qiagen RLT extraction buffer. Without active charcoal, the isolation leads to either no RNA or mostly degraded RNA. Using the two commercial Qiagen-Kit buffers RLT and RLC with HMW-PEG, only the RLT buffer results gave detectable quantity of RNA, while the RLC buffer gave no RNA at all. RNA prepared with these buffer systems and active charcoal served as a robust template for reverse transcription as indicated by PCR amplification from cDNA for hazelnut isolates of ApMV.

Validation of RT-PCR and its comparison with ELISA

The total RNA extracts obtained from hazelnut tissues infected with ApMV were analyzed by RT-PCR. PCR products of the expected size (262 bp) were obtained (Figure 1). To further evaluate the efficiency and robustness of the optimized one tube RT-PCR for the detection of ApMV, 24 infected hazelnut trees known to be infected with ApMV in previous studies were tested. All samples tested by one tube RT-PCR was shown to contain ApMV. In further tests of the same trees by ELISA and RT-PCR, leaf preparations produced comparable results. In these tests, the six ApMV were false-negative by ELISA and yielded faint bands in leaf preparations. That is, RT-PCR is a more usable and reliable test than the serological test during most of the season.

DISCUSSION

ApMV infecting hazelnut trees occur commonly in hazelnut plantation in the west Black Sea region. As rooted suckers are vulnerable to ApMV infections, the need for clean-stock is apparent. When infected suckers are used, this can result in reduced production. The fast and simple alternative detection method with one tube RT-PCR can help minimize the time and labour required for the diagnosis of ApMV in hazelnut. The current work demonstrates the success obtained in extracted ApMV RNA and in directly detecting ApMV in different part of hazelnut tissues by RT-PCR. Silica gel method allowed for the isolation of RNA from hazelnut trees in a wide range, in which other methods yielded either no RNA or poor-quality RNA unsuitable for reverse transcription. When total nucleic acid extracts from hazelnut tissues were analyzed by RT-PCR, it was observed that the

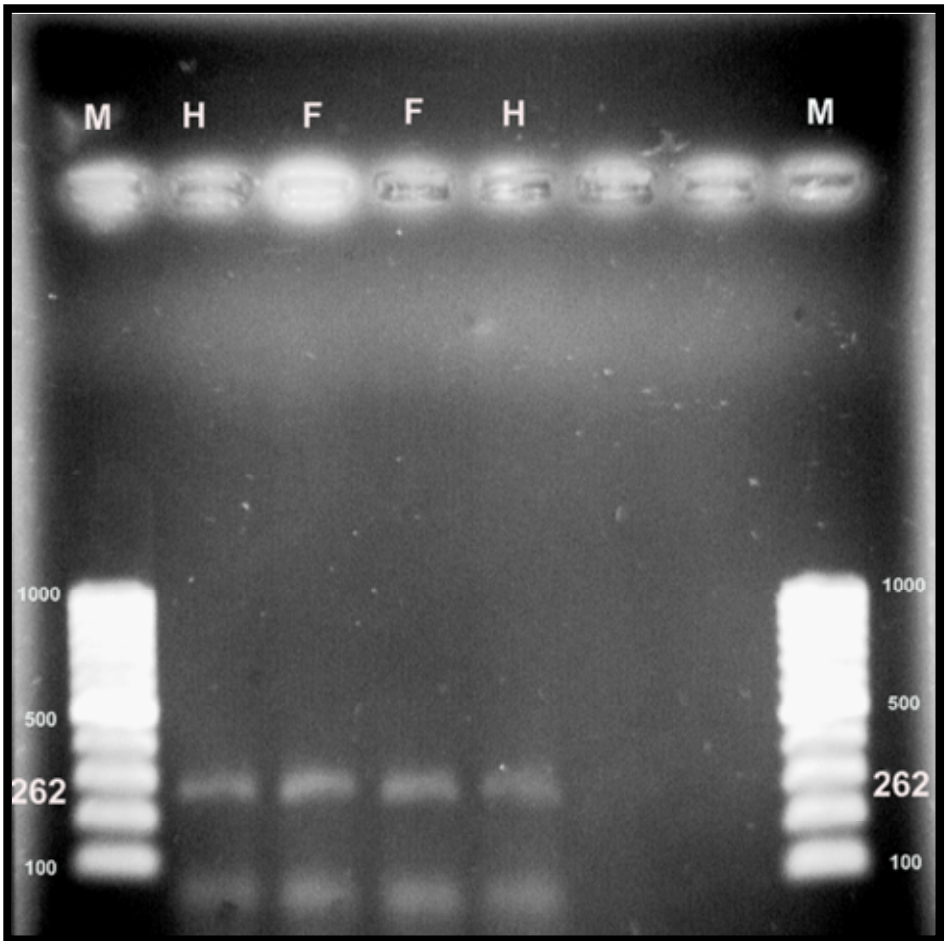

Figure 1. Agarasoe gel electrophoresis of RT-PCR products of ApMV with specific primers 262 bp. Lane M: Molecular marker 100 bp (MBI fermantas); Lane H: husk tissues; Lane F: flower tissues.

sensitivity limits were higher than those obtained in serological analysis. This result was consistent with previous reports using RT-PCR which revealed more infected trees than were discovered by ELISA, and this agrees with the findings of Choi and Ryu (2003) and Hassan et al. (2006). PCR-based technology is several-fold more sensitive than serological assays (Henson and French, 1993; Pallas et al., 1998). ApMV were amplified in extracts of flowers and husks suggesting good reliability of this molecular assay.

The use of RT-PCR is an alternative to the classical detection of ApMV directly from hazelnut tissues. Utilization of RT-PCR system allowed specific detection of ApMV from clarified crude plant extracts from flowers, husk and leaf tissues of hazelnut trees. Crude plant extracts can be readily prepared, and the usual inhibitory effects of plant polysaccharides or the other components of crude plant extracts on PCR amplification (Luis et al., 2005) were avoided by appropriate additives. RT-PCR protocol overcame a significant limitation of ELISA on extracts from hazelnut trees. Flowers and husk tissues were shown to be good templates for reliable amplification of ApMV in one step RT-PCR. In the same conditions, total RNA prepared from leaf tissues and clarified crude extracts gave weak detectable product. There are sufficient reports on the detection of ApMV directly from its other hosts and indicator plants by different kind of PCR methods (Candresse et al., 1998; Saade et al., 2000; Menzel et al., 2002; Petrzik and Lenz, 2002; Choi and Ryu, 2003; Crowle et al., 2003; Hassan et al., 2006). However, there is none directly from hazelnut tissues.

Control of virus diseases in woody plants depends on the detection and elimination of the agents through certification and clean-stock programs. Our experiments have shown that crude extracts from hazelnut tree tissues are good templates for reliable ApMV detection by RT-PCR if combined with using silica gel extraction methods. The ability to detect ApMV using hazelnut tissues will provide a valuable tool for certification programs. This procedure can also be incorporated into testing protocols during post-entry quarantines for rapid initial screening of imported budwood, or in virus eradication programs.

Consequently, this method can be useful for early stage screening of genetic resources of hazelnut plants.

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