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Distribution and molecular detection of apple mosaic virus in apple and hazelnut in Turkey

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Apple mosaic virus (ApMV) is one of the most important diseases limiting the production of hazelnut and apple in Turkey and the objectives of this research were to determine the convenient and reliable method for RNA isolation and also to determine primer pair for real time polymerase chain reaction (RT-PCR) detection of coat protein gene for Turkish ApMV isolates. Apple mosaic virus isolates were collected in 2007 to 2010 and the presence of the pathogen was detected by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and RT-PCR tests. Six different RNA extraction protocols and three primer pairs were applied in RT-PCR amplifications and 44 hazelnut and 15 apple ApMV isolates were obtained. All of the amplicons were subjected to enzymatic digestion with restriction endonuclease enzymes and phylogenetic analysis were performed according to the digestion profiles.

Key words: Apple mosaic virus, coat protein gene, hazelnut.

INTRODUCTİON

Apple (*Malus domestica* L.) is a pome fruit widely distributed and produced in Turkey. Turkey is the fourth largest producer world-wide (Koksal et al., 2010), supplying about 3.5% of the world's production. The major cultivated varieties are Starking Delicious, Golden Delicious, Granny Smith, Fuji and some local varieties such Amasya, Tokat etc. Hazelnut (*Corylus avellana*) is native to Turkey and Turkey is the world leader in hazelnut production (Koksal et al., 2010). One of the most important factor that limits apple and hazelnut production in Turkey is *Apple mosaic virus* (ApMV, family Bromoviridae, genus *Ilarvirus*). The virus is primarily pruning (Brunt et al., 1996). Major hosts of the virus are transmitted by vegetative propagated material and hops, horse chesnut, hazelnut, raspberry, birch, rose, some herbaceous plants (Rybicki, 1995) and strawberry (Tzanetakis and Martin, 2005). The virus causes symptoms such as severe systemic mosaic in apple, mottle, oak leaf patterns and ringspots on the hazelnut trees. The symptoms are evident early in the growing season but they may become partly masked with time and as temperatures increase on hazelnut trees (Kobylko and Nowak, 2006). The virus is also transmitted by root bridges between trees. The virus also causes great reduction in the size and the yield of hazelnut (Gentit et al., 2009). Control of graft-transmissible viruses includes

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Table 1. Primers used for RT-PCR amplification of ApMV coat protein gene targets.

Primer	Reference	PCR product (bp)	Ann. temp. (°C)
Sense: 5'-TCA ACA TGG TCT GCA AGT AC-3' Antisense: 5'-CTA ATC GCT CCA TCA TAA TT-3'	Lee et al. (1998)	680	54
Sense: 5'-ATC CGA GTG AAC AGT CTA TCC TCT AA-3' Antisense: 5'-GTA ACT CAC TCG TTA TCA CGT ACA A-3'	Menzel et al. (2002)	262	62°C
Sense: 5'- GGC CAT TAG CGA CGA TTA GTC- 3' Antisense: 5'- ATG CTT TAG TTT CCT CTC GG-3'	Petrzik and Lenz (2002)	820	50°C

sanitation, quarantine and certification programs but the success of those programs depends on specific and sensitive methods for pathogen detection. The most effective way of controlling viral infections in woody plants is establishing virus-free plantations with virus-tested planting material.

This research was conducted to investigate the suitable RNA extraction method and the primer pairs work with both RNAs in RT-PCR analysis of capsid protein (CP) gene of ApMV and also to determine the genetic variability on the basis of restriction analysis of apple and hazelnut CP genes since those genes are frequently used in genetic variability (Petrzik and Lenz, 2002).

MATERIALS AND METHODS

Virus source

Hazelnut samples were collected from different varieties from the Eastern and Western Black Sea Region of Turkey (Duzce, Sakarya, Samsun, Ordu, Giresun and Trabzon provinces) in 2007 to 2010. Each plant sample was taken per 1,000 km^2 area of plantation. Apple samples were çollected from a number of varieties in the major apple production areas (Ankara, Antalya-Korkuteli, Tokat, Nevşehir, Konya, Nigde, Isparta provinces) with the same procedure. Young leaves, bark tissues and fruits were collected from symptomatic and asymptomatic plants and kept in deep-freezer at -20°C until they were processed.

ELISA procedure

Standard DAS-ELISA procedure was applied to all of the apple and hazelnut specimen collected with commercial antiserum (Agdia), according to manufacturer's instructions. Young leaves were subjected to the test.

RNA extraction

Six different RNA extraction procedures were applied to both ApMV infected plant tissues. Young bark scraps and leaf tissues of hazelnut and apple leaves were used for RNA extraction. Six different RNA extraction methods were applied to both specimens: 1) Spiegel et al. (1996) method, 2) Menzel et al. (2002) method, 3) Rott and Jelkman (2001) method, 4) Zhang et al. (1998) method, 5) Verwoerd et al. (1989) method and 6) QIAGEN RNeasy Plant mini kit. Total plant RNAs were isolated by using silica in the procedures of Menzel et al. (2002); Rott and Jelkman (2001), ds-RNA's were isolated in the procedure of Zhang et al. (1998) and then subjected to RT-PCR. After total RNAs were extracted, they were quantified by Nanodrop 2000 (Thermo Scientific).

Primers and PCR mix for RT-PCR

One step RT-PCR was performed using the primers of the coat protein gene of ApMV shown in Table 1. PCR mix was organised and used throughout the RT-PCR analysis. It was as follows: virus specific primers (up and down stream) (0.2 µM each), 1.5 mM dNTP mix (Fermentas), 2.5 µl 10 X Taq buffer, 8 u MMLV-RT (Fermentas), 1.2 u RNase inhibitor (Promega), 0.6 µM $MgCl_2$, 1 U Taq DNA Polymerase (Fermentas), 2 µl RNA template. Total reaction volume was 25 µl.

RT- PCR amplification

PCR protocols were applied according to the thermocycling conditions of each technique and for Menzel et al. (2002), it was as: 30 min at 42°C, 15 min at 95°C and 34 cycles of 30 s at 94°C, 30 s at 62°C and 1 min at 72°C, amplification was finalised by 7 min at 72°C. Amplified fragments of ApMV CP genes were all subjected to electrophoresis in 1% agarose slab gels at 100 V for 1 h and then stained with 0.5 µgml $^{-1}$ of ethidium bromide.

Phylogenetic analysis

Restriction enzymes used for digestion of RT-PCR amplified products were Hinf*I* (Genemark), *BamHI* (Genemark), Ava*I* (Genemark), *EcoRI* (Sigma), Ssp*I* (Fermentas), Rsa*I* (Fermentas), *HindIII* (Genemark), and Hinc*II* (Genemark). PCR products (8 µl), 1 µl enzyme buffer and 0.5 µl of specific restriction enzyme were incubated overnight at 37°C and then subjected to gel electrophoresis in 6% polyacrylamide at 100 V for 2 h. Gels were stained with ethidium bromide and visualised by Bio-imaging system (Gene Genius) (Ulubas et al., 2009). Phylogenetic analysis has been performed according to the restriction profiles, by using Jacord's similarity index programme of Numerical Taxonomy and Multivariate Analysis System, Version 2 programme (Rohlf, 1998).

RESULTS

Virus source

During the surveys performed between 2007 to 2010, a

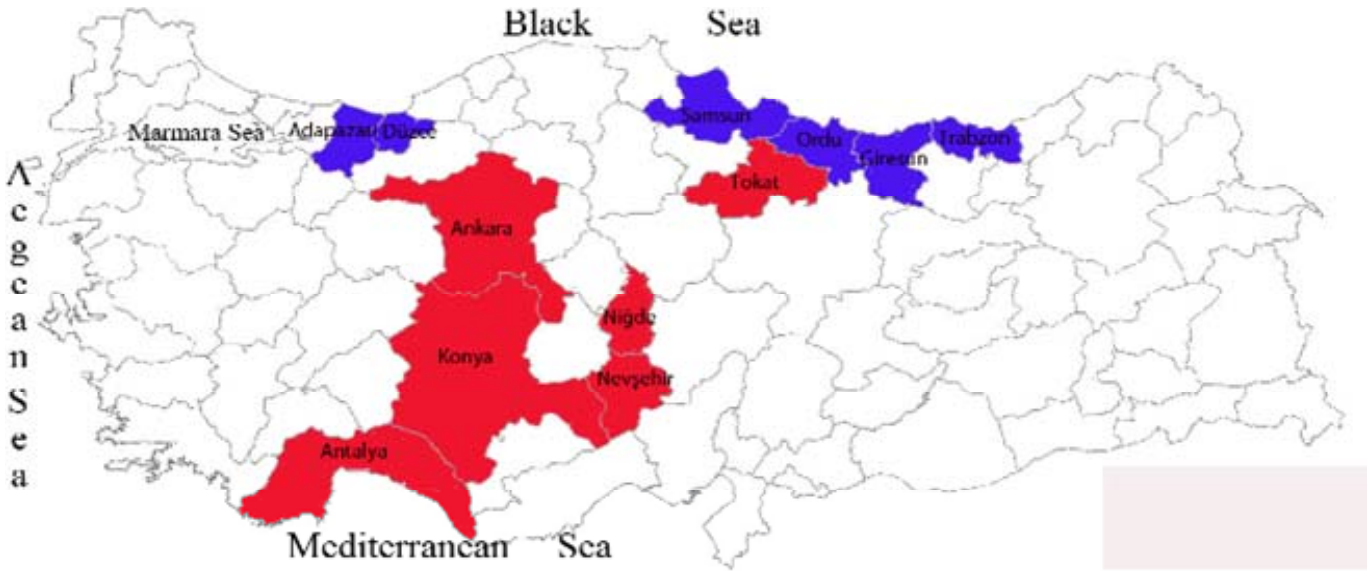

Figure 1. The survey area (areas shown in blue are the provinces surveyed for hazelnut and areas shown in red are the provinces surveyed for apple).

Figure 2. The systemic mosaic symptoms of ApMV on hazelnut (top) and apple (bottom) trees.

total of 194 hazelnut and 47 apple specimens were collected from the survey area as shown in Figure 1. Since the infection can be masked in arid climates, both symptomatic and asymptomatic specimens were collected from the research area. Major symptoms of virus infection in hazelnuts were chlorotic oak leaf pattern mosaic, vein clearing and diffuse ringspots whereas the symptoms observed on apples were severe systemic mosaic (Figure 2). The symptomatic plants were commonly present at the side rows of hazelnut whereas symtomatic plants were usually in the middle of the apple orchards. The infection was present only on Granny Smith variety of apples but in the case of hazelnut, it was common on local varieties; such as, Karafindik, Tombul, Giresun yaglisi, Sivri, Foca, Mincane, Palaz, Cakildak and Badem. The virus was present along all the Black Sea Coast.

DAS-ELISA

According to DAS-ELISA results, ApMV infections were detected in 44 out of the 194 of hazelnut specimens (22%) and in 15 out of 47 apple specimens (31.9%). All of the ApMV positive isolates were symptomatic and showed symptoms typically associated with infection by ApMV as shown on Figure 2. The ApMV infection was present in all of the provinces surveyed for apple. Crop loss was greater in hazelnut production than in apple and the infection caused almost no yield. Although, ELISA is routinely used for detecting the plant viruses, this technique is not sufficient to detect the low concentrations of the virus that occur in some tissues or

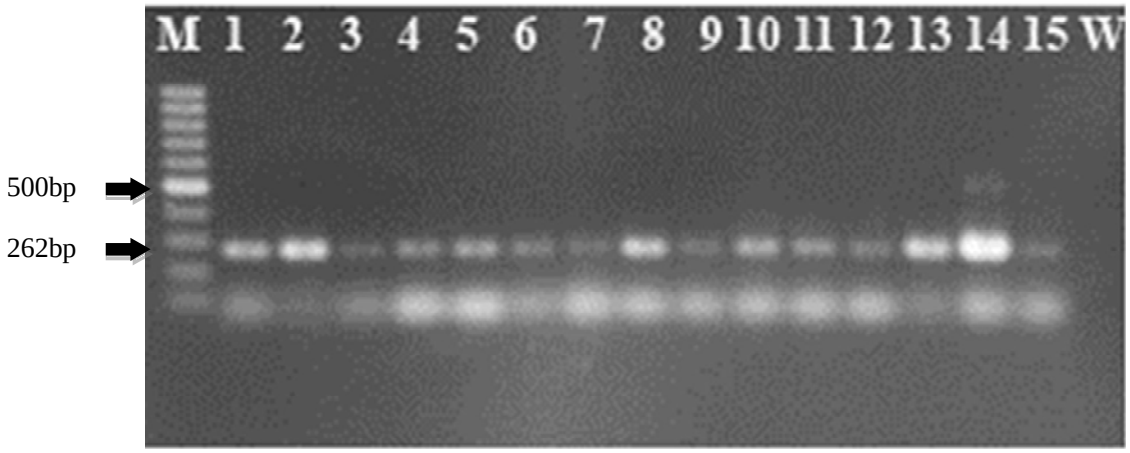

Figure 3. RT-PCR amplification of Turkish ApMV apple isolates. M, GeneRuler 100bp DNA ladder(Fermentas); 1, Ankara; 2, Antalya-Korkuteli; 3-5, Isparta-Eğirdir; 6, Isparta-Eğirdir BKAE (fruit); 7-8, Isparta-Gelendost; 9, Isparta-Gelendost (fruit); 10 Isparta-Gelendost; 11-12, Tokat, 13-15, Nevşehir isolates.

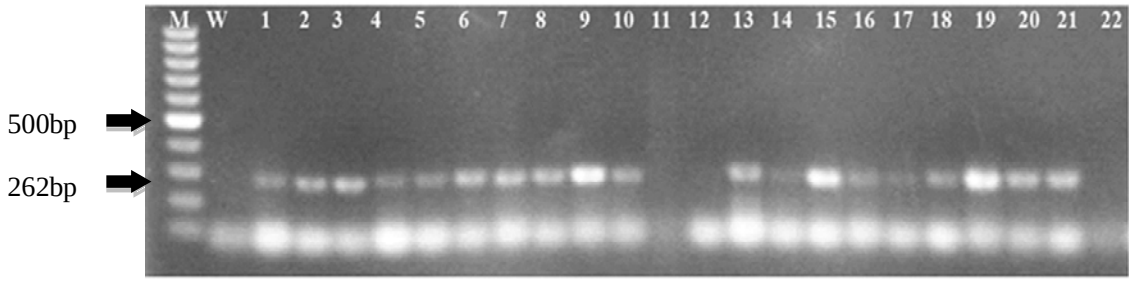

Figure 4. RT-PCR amplification of Turkish ApMV hazelnut isolates. M, GeneRuler 100bp DNA ladder, Fermentas; 1-3, Düzce; 4-11; Adapazarı; 12-17, Giresun; 18-19, Ordu; 20-21, Giresun isolates; 22, water control.

in some hosts. ELISA may fail because of the inhibitory effects of polysaccharides or phenolic compounds in tissue extracts of woody plants, thus ELISA seems inappropriate with dormant woody tissues (Menzel et al., 2002; Kobylko and Nowak, 2006; Akbas and Değirmenci, 2010). Some of the hazelnut isolates showing the typical symptoms of infection were detected as negatives by ELISA but were then detected as positives in RT-PCR amplification. Therefore, ELISA alone is not sufficient for a reliable detection of ApMV from the infected hazelnut tissues. The infection was not present in asymptomatic apple and hazelnut trees.

RNA extraction

Extracted RNAs were amplified only with the Menzel et al. (2002)'s primers for coat protein gene of ApMV (the same primer pair was used to amplify both apple and hazelnut RNAs) and the amplified products of RT-PCR were 262 bp long fragments (Figures 3 and 4). PCR mix used in this research gave better results with hazelnut and apple extracted RNAs in RT-PCR amplification compared to the original PCR mix of Menzel et al. (2002). RNA isolation from infected hazelnut specimens is known to be problematic because of the high tannin content and uneven distribution of the pathogen in the infected tissues (Kobylko and Nowak, 2006) so this problem was overcomed by using the RNA extraction procedure of Rott and Jelkman (2001) for hazelnut specimens. The yield and purity of hazelnut RNA extracts obtained using Rott and Jelkman's procedure was quite high compared to the other procedures tested, 3.5 to 4 µg per 100 mg fresh tissue and 260/280 ratios were between 1.7 to 1.9 and quality and amount of the RNA extracts obtained from bark scraps were better comparing the leaf tissues.

RT- PCR amplification

The extracted RNAs were amplified only with Menzel et al. (2002)'s primer pair and no amplification was obtained with the other primer sets tested. Some RNAs obtained from apple fruits were amplified and showed clear bands

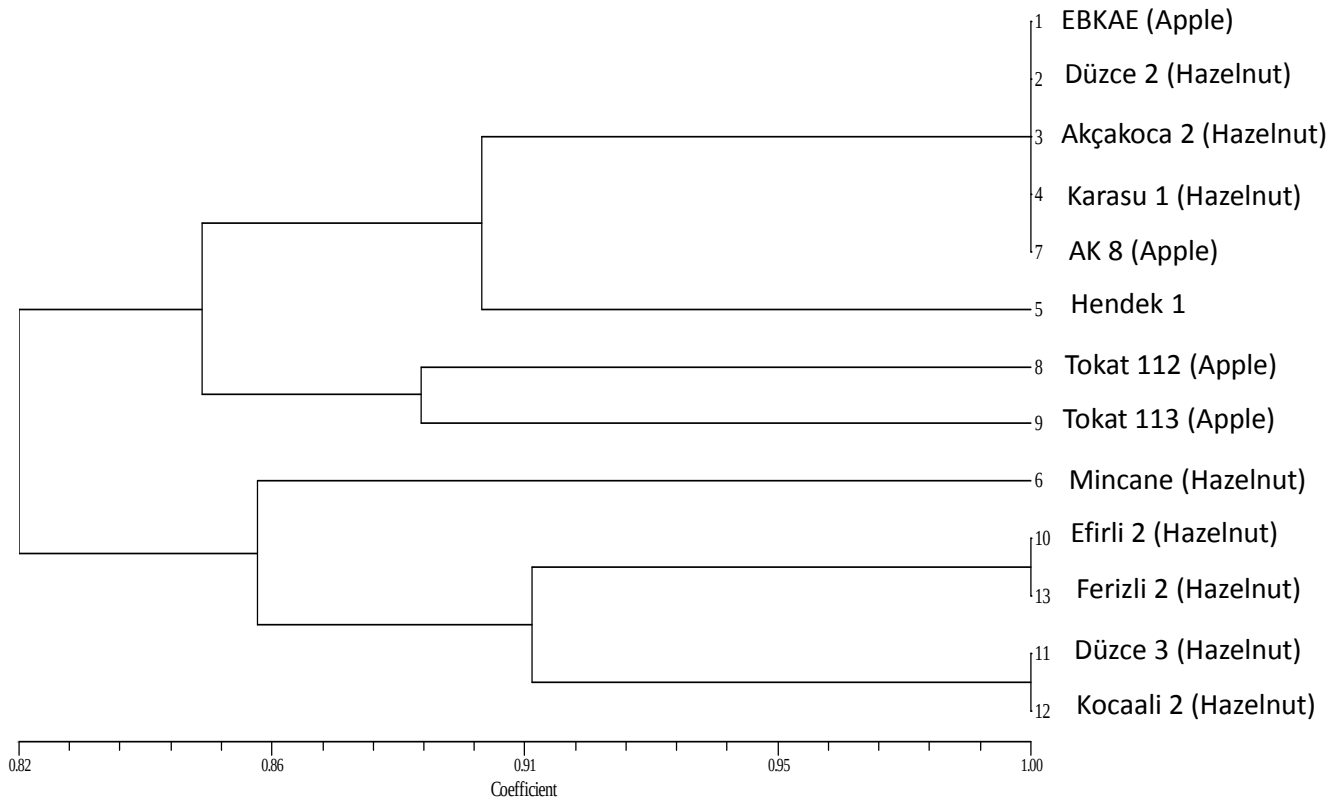

Figure 5. Dendogram of Turkish *Apple mosaic virus* isolates.

on gels whereas hazelnut fruits were generally empty or small and very weak amplification bands were obtained from those samples. Therefore, leaves and fruits of apple and bark of young branches of hazelnut were preferred for total RNA extraction. Among the primers tested, only amplifications were obtained with Menzel et al. (2002)'s primer set. RNA extracts of hazelnut flowers were also amplified with the same primer pair (Akbas and Degirmenci, 2010). Our research demonstrated that optimization of the PCR protocol by suitable additives to the PCR mix, reduced the adverse affects of the plant polysaccharides and phenolics. Hazelnut bark tissues and apple leaf tissues were shown to be good templates for reliable detection and amplification of ApMV in one step RT-PCR. As a result of RT-PCR procedure, 44 hazelnut and 15 apple isolates were amplified and produced very clear and sharp bands as shown in Figures 3 and 4.

Phylogenetic analysis

In order to detect genetic polymorphism between the capsid protein genes of isolates of ApMV from hazelnut and apple, all of the RT-PCR products were subjected to enzymatic digestion with restriction endonuclease enzymes of Ava*I,* Bam H*I*, Hinf*I*, Ssp*I* EcoR*I*, Hinc*II*, Hind*III* and *Rsa*I. Ava*I*, Bam H*I*, Hinf*I* and Ssp*I* enzymes digested the RT-PCR products whereas the other enzymes (EcoR*I*, Hinc*II*, Hind*III* and Rsa*I*) had no effect on the amplified product and failed to digest them. The present results demonstrate that genotypic variation of Turkish ApMV isolates were quite high, at 85% similarity; four clusters were obtained in phylogenetic analysis (Figure 5). In the first cluster, two apple and three hazelnut isolate collected from Western Black Sea Region were similar and settled in the same cluster. The second cluster consisted only 2 apple isolates collected from the same province (Tokat). Third cluster is consisting of hazelnut isolates collected from Eastern Black Sea Region and the fourth cluster is consisting of hazelnut isolates of Western Black Sea Region.

DISCUSSION

The results of the present study indicates that ApMV can be detected by one step RT-PCR and it is possible to extract total RNAs from leaf tissues of apple and also dormant tissues of hazelnut although the tissues contain high amount of phenolic compounds and tannins. In Turkey, ApMV is a widespread pathogen and has been detected previously in hazelnut (Akbas et al., 2004), apple (Ulubas and Ertunc, 2003) cherry (Gumus et al.,

2008), and rose tissues (Yardimci and Culal, 2009) by ELISA. In Poland, ApMV was detected only in Negret and Gustav Zellemus cultivars among the 49 cultivars tested by ELISA (Piskornik et al., 2002; Kobylko and Nowak, 2006). Furthermore, it was not possible to obtain reliable RNA extraction from hazelnut tissues because of the high tannin and phenolic compounds. Investigators have tried to overcome this problem by inoculation to an herbaceous host such as *Phaseolus vulgaris* (Sokmen, 2003). Recently, Akbas and Degirmenci (2010) have isolated total RNA only from flower tissues of hazelnut and Ertunc et al. (2011) analysed Turkish and Ukranian ApMV apple isolates and compared them for coat protein composition. We succeeded to detect ApMV from the leaves and dormant tissues of hazelnut and apple trees within this work by using the same primer pair. The ability to detect ApMV in hazelnut and apple tissues will provide a valuable tool for certification programs and this protocol can easily be incorporated into the testing protocols of quarantine for rapid screening of imported mother plants of apple and screening of national collections of hazelnut in Turkey.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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