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Detection of Four Apple Viruses by ELISA and RT-PCR Assays in Turkey

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Abstract: Plant samples were collected from the main apple growing provinces of Turkey in order to evaluate the incidence of 4 important apple virus diseases during spring 2004. Collected leaves and shoots were tested using enzyme linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR) for *Apple mosaic virus* (ApMV), *Apple stem grooving virus* (ASGV) and *Apple chlorotic leaf spot virus* (ACLSV). Since no commercial antiserum is available, *Apple stem pitting virus* (ASPV) was tested only using RT-PCR and the results were compared. A total of 174 apple samples were collected from varietal collections belonging to governmental and university institutions and also from commercial orchards. Out of 174 plants, 126 were infected by at least one virus disease. The incidence of the 4 viruses in varietal collections was 70.21%, while it was 75.00% in commercial orchards. The results obtained from the comparison of ELISA and RT-PCR in this study showed that with the RT-PCR technique 8.6% more samples were positive for ACLSV, ASGV and ASPV. Mixed infections were also very common in both varietal and commercial orchards. Among the mixed infections, the most common one was ASPV + ACLSV (84.21%), followed by ASPV + ASGV (36.84%), ACLSV + ASGV (26.32%) and ASPV + ApMV (5.26%). The incidence of the ASPV + ASGV + ACLSV combination was 26.32%. The number of plants infected with any viruses was higher when tested using RT-PCR comparing to ELISA. This study showed that apple virus diseases, especially on symptomless trees, were very common in different provinces of Turkey and RT-PCR can be successfully applied in certification programs of pome fruit trees.

Key Words: Apple, virus, RT-PCR, ELISA, Turkey

Türkiye'de Dört Elma Virüsünün ELISA ve RT-PCR Yöntemleriyle Saptanması

Özet: Türkiye'de elma yetiştiriciliğinin yoğun olarak yapıldığı bölgelerde elmalarda önemli dört virüs hastalığının yaygınlık durumlarını değerlendirmek amacıyla 2004 yılının ilkbahar döneminde bitki örnekleri toplanmıştır. Toplanan bu örnekler Elma mozaik virüsü (*Apple mosaic virus* = ApMV), Elma gövde yivlenme virüsü (*Apple stem grooving virus* = ASGV) ve Elma klorotik yaprak leke virüslerine (*Apple Chlorotic Leaf Spot Virus* = ACLSV) karşı hem ELISA hem de RT-PCR yöntemleriyle testlenmiştir. Elma gövde çukurlaşma virüsüne (*Apple stem pitting virus* = ASPV) karşı ise henüz ticari olarak üretilmiş antiserum bulunmadığı için sadece RT-PCR yöntemi kullanılmış ve sonuçlar kıyaslanmıştır. Toplam 174 örnek bakanlık ve üniversitelere ait olan çeşit koleksiyon bahçelerinden ve ticari bahçelerden toplanmıştır. Bu bitkilerden 126 tanesi en az bir virüs ile enfekteli olarak saptanmıştır. Hastalığın yaygınlık oranı ticari bahçelerde % 70.21 olarak saptanırken çoğunluğu çeşit bahçelerinden alınan gözlerle üretilen fidanlarla kurulmuş olan ticari bahçelerde ise enfeksiyon oranı daha yüksek olup % 75.00 olarak bulunmuştur. ACLSV, ASGV ve ASPV'nin tanılanmasında ELISA ve RT-PCR tekniklerinin kıyaslanması sonucu elde edilen bulgular, RT-PCR yöntemi ile % 8.6 oranında daha fazla sayıda bitkinin pozitif olarak saptandığını göstermiştir. Gerek çeşit gerekse ticari bahçelerde karışık enfeksiyon oldukça yaygın bulunmuştur. Karışık enfeksiyonlar içinde en yaygın olanlar sırasıyla ASPV + ACLSV (% 84.21), ASPV + ASGV (% 36.84), ACLSV + ASGV (% 26.32) ve ASPV + ApMV (% 5.26)'dir. ASPV + ASGV + ACLSV üçlü enfeksiyon oranı ise % 26.32 olarak saptanmıştır. Virüslerin herhangi biri ile enfekteli bitki örneklerinin sayısı RT-PCR ile test edildiğinde ELISA yöntemine göre daha fazla olmuştur. Bu çalışma elma virüs hastalıklarının Türkiye'nin farklı bölgelerinde özellikle de simptom göstermeyen ağaçlarda çok yaygın olduğunu ve RT-PCR yönteminin yumuşak çekirdekli meyve ağaçlarının sertifikasyon çalışmalarında başarıyla kullanılabileceğini ortaya koymuştur.

Anahtar Sözcükler: Elma, virus, RT-PCR, ELISA, Türkiye

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Introduction

Turkey has suitable ecological conditions for most fruit species and is considered an important germplasm source for them. More than 30 different fruit species from temperate to citrus and other subtropical plants have been cultivated for centuries. Nine million tons of fruit crops were produced in 2000, representing approximately 4.5% of annual total fruit production worldwide. The most important fruit crop in tonnage terms is apple, with production of 2.5 million tons (SIS, 2003). Although apple production is very important in Turkey, the sanitary status of apple trees is still unknown and no certification program has been established. The studies on apple viruses in Turkey are very limited and only some symptomatical observations and woody indexing have been reported. The first survey was conducted in Central Anatolia during 1966-1969, which recorded the symptoms of Green crinkle, Star crack and Dapple apple virus diseases, recently regarded as virus-like diseases (EPPO, 1999), and apple proliferation phytoplasma disease in Central Anatolia (Özkan and Kurçman, 1976). Some viroid and phytoplasma-like symptoms were also reported in this study. Çalı (1992) observed small fruit symptoms resembling phytoplasma symptoms on different apple cultivars in Isparta. He found no graft transmissible pathogen responsible for those symptoms according to biological indexing and electron microscope observations. Another study was carried out during 1992-1997 in the mother block foundation of Atatürk Horticulture Research Institute by woody indexing of apple and pear rootstocks. When 19 apple cultivars were grafted on to different indicator plants, small fruits and depressions were observed similar to those caused by apple chat fruit disease and apple green crinkle disease, respectively (Nogay et al., 2001).

Although there are more than 40 virus and virus-like diseases in pome fruit trees (Nemeth, 1986), the economically important virus diseases of apple trees are *Apple chlorotic leaf spot virus* (ACLSV, *Trichovirus*), *Apple mosaic virus* (ApMV, *Ilarvirus*), *Apple stem pitting virus* (ASPV, *Foveavirus*) and *Apple stem grooving virus* (ASGV, *Capillovirus*) (Mink, 1989; Desvignes, 1999). Except ApMV, these viruses are symptomless in most commercial apple varieties and occur frequently in combination. For the certification of plant material, apple plants have to be tested along with other pathogens for these 4 distinct virus diseases (EPPO, 1999). Because many apple viruses are symptomless on apple plants, the testing of mother plants with reliable methods is very important in order to prevent virus spread. Since woody indexing and ELISA have some disadvantages for apple viruses, reverse transcription polymerase chain reaction (RT-PCR) has been adapted to detect ApMV, ACLSV, ASGV and ASPV in woody tissues (Jelkmann, 1994; MacKenzie et al., 1997). Even recently, multiplex assays were developed for testing different viruses (Nie and Singh, 2000; Sharman et al., 2000) as well as apple viruses (Menzel et al., 2002) in order to decrease the test cost and the number of samples.

This paper describes the use of ELISA and RT-PCR assays to determine ApMV, ACLSV, ASGV and ASPV incidence in different apple cultivars growing in varietal collections and commercial orchards in Turkey.

Materials and Methods

Sample Collection

The samples were collected from germplasm sources, varietal collections, and commercial orchards located in the main pome fruit production provinces during the spring of 2004. The provinces from which samples were collected and the number of samples taken are shown in Figure 1. Many of the visited orchards were over 15 years old but some of the samples were collected from young commercial orchards, which were mainly established using imported varieties. The samples were collected randomly for ACLSV, ASGV and ASPV because they are symptomless in most apple cultivars, whereas they had mosaic symptoms for ApMV. The most common apple cultivars and their numbers among the collected samples were as follows: different Golden varieties (33), Starkrimson (27), Amasya (14), Vista Bella (13) and Anna (12). The rest of the samples were unknown cultivars.

Source of control plant materials

Healthy and infected apple tissues for the 4 viruses were obtained from the virus collection at the Federal Biological Research Center for Agriculture and Forestry (BBA) in Dossenheim, Germany. Other tested plants were collected directly from different orchards in Turkey.

Enzyme-linked immunosorbent assay (DAS-ELISA)

All samples were tested using DAS-ELISA for the detection of ACLSV, ASGV and ApMV (Clark and Adams,

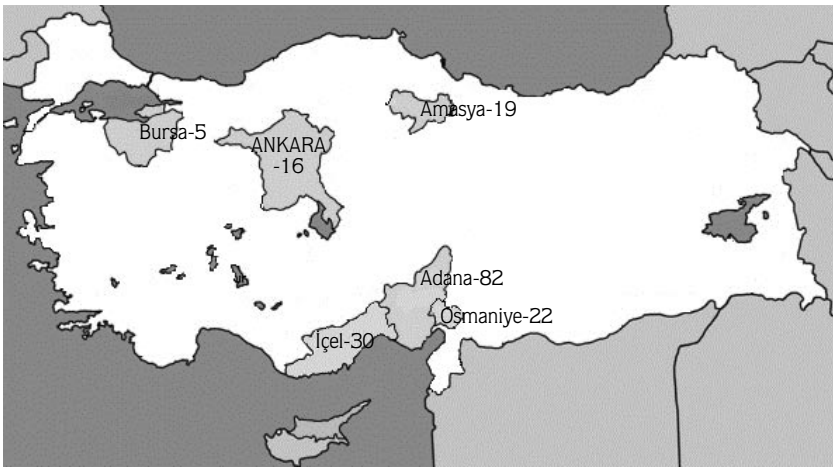

Figure 1. Surveyed provinces for apple viruses in Turkey and the number of the tested samples.

1977). Since no commercial antiserum is available, it was not possible to test ASPV using ELISA. Serological reagents were commercial kits and were supplied by Loewe (Germany).

Extraction of total nucleic acids

Total nucleic acids were isolated from apple tissues using the silica-capture method described originally by Boom et al. (1990), and adapted for the detection of plant viruses from woody plants by Rott and Jelkmann (2001).

Virus specific primers and RT-PCR

The primers used in the research for the 4 viruses were kindly supplied by Dr. Jelkmann, BBA Institute, Dossenheim, Germany, and additional sequence information was given in the literature (*Apple chlorotic leaf spot virus*: Jelkmann, unpublished data; *Apple stem pitting virus*: Jelkmann & Keim-Konrad, 1997; *Apple stem grooving virus*: MacKenzie et al., 1997; *Apple mosaic virus*: Menzel et al., 2002).

Complementary DNA synthesis was carried out in a 1.5 ml microcentrifuge tube containing 5 µl of extracted nucleic acid, 2 µl of random hexamer primer (100 µg μl^{-1}), 2 µl of oligo$(dT)_{18}$ (70 µg μl^{-1}), and 4.5 µl of RNase-free water. This mixture was incubated for 10 min at 70 ºC. Then 4 µl of 5 X reaction buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 µl of dNTP mix (10 mM), and 0.5 µl of MMLV-Reverse transcriptase (200 units) were added. Reverse transcription was carried out at 37 °C for 10 min and followed by 42 °C for 1 h and finally at 70 °C for 10 min. PCR mix containing 1 µl of the cDNA, 0.6 µl of $MgCl_2$ (50 mM), 1.6 µl of dNTPs (2.5 mM), 1 µl of sense and antisense primer mix (10 µM each), 2 µl of 10 X PCR reaction buffer (Invitrogen GmbH, Germany), 0.2 µl of Taq-polymerase (Goldstar 5 U μl^{-1}), and 13.6 µl of water were added. The cycling parameters were as follows: initial denaturation of 2 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 1 min at 46 °C, and 1 min at 72 °C for ACLSV and ASGV. The procedure was the same for ASPV except that the annealing temperature was 57 °C instead of 46 °C. The reaction was extended to 5 min at 72 °C. The samples were tested for ApMV using RT-PCR (in one tube). The difference from the PCR reaction mixture described above was that reverse transcriptase enzyme was added to the PCR reaction mix. The cycling parameters were 45 min at 42 °C for reverse transcription, 2 min at 94 °C and followed by 34 cycles 30 s at 94 ºC, 30 s at 62 ºC, 1 min at 72 ºC and the final extension was 7 min at 72 ºC. The amplification products were analyzed using electrophoresis through 1.5% agarose gels.

Results

In the course of field surveys to collect plant samples more than 1000 trees were individually inspected in 20 commercial or governmental orchards. No obvious symptoms were observed on trees except for some mosaics on the leaves and poor growth (Figure 2).

Figure 2. Early spring symptoms of *Apple mosaic virus* (ApMV) disease on Granny Smith apple variety in Ankara.

As a result of surveys, 174 apple tree samples were collected from varietal collections (94 samples) and commercial orchards (80 samples). According to ELISA tests, the incidences of the 3 viruses provided from varietal collections were 13.83% for ACLSV, 21.28% for ASGV and 2.13% for ApMV. The incidence of ACLSV in the commercial orchards was higher than the rates in the varietal collections, with an incidence of 32.5%. ASGV infection was much lower (1.25%) and no trees infected by ApMV were found in commercial orchards. RT-PCR results showed that the same or greater amounts of plants were infected in both varietal and commercial orchards, as expected, since PCR is more sensitive and accurate method to test the viruses. ELISA-based detection of ApMV, ASGV and ACLSV in all tested samples showed a 35.63% infection level, whereas it was 44.25% when the same samples were tested by RT-PCR (Table 1).

The PCR fragments of ACLSV (258 bp), ASGV (523 bp), ASPV (243 bp) and ApMV (262 bp) were amplified in all tested cultivars on expected levels except in the water control (no fragment) (Figure 3). The most infected cultivars were different Golden varieties (13/33), Starkrimson (15/27), Vista Bella (3/13) and Anna (4/12). Cv. Amasya, which is one of the most important local cultivars, was also infected by more than one virus (6/14).

Mixed infections were also very common, with an incidence of 10.92%. Among the mixed infections the most common one was ASPV + ACLSV (84.21%), followed by ASPV + ASGV (36.84%), ACLSV + ASGV (26.32%) and ASPV + ApMV (5.26%). The incidence of the ASPV + ASGV + ACLSV combination was 26.32%.

Discussion

Although apple virus and virus-like diseases have been studied in Turkey since 1966, there are only a few reports on these diseases based on symptomatology, biological indexing and electron microscopy (Özkan and Kurçman, 1976; Çalı, 1992; Nogay et al., 2001). In the present study, 4 of the most important apple viruses, which are on the quarantine list of EPPO, were detected using ELISA and RT-PCR for the first time in Turkey. The results indicate a high incidence of latent apple viruses like ACLSV, ASGV and ASPV in the selected orchards. High infection rates of these 3 viruses in varietal collections revealed that they might spread very quickly and widely through those infected budwoods to commercial apple orchards. As a result of this research, many commercial orchards, mainly established using budwoods provided

Table 1. The incidence of *Apple stem grooving virus* (ASGV), *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV) and *Apple mosaic virus* (ApMV) in tested plants growing in both commercial and varietal apple orchards by ELISA and RT-PCR (%).

	Varietal collections (No. of tested plants: 94)				Commercial orchards (No. of tested plants: 80)			
	No. of infected plants		Incidence (%)		No. of infected plants		Incidence (%)	
Viruses	ELISA	RT-PCR	ELISA	RT-PCR	ELISA	RT-PCR	ELISA	RT-PCR
ACLSV	13	15	13.83	15.96	26	26	32.50	32.50
ASGV	20	20	21.28	21.28	1	2	1.25	2.50
ApMV	2	6	2.13	6.38	0	8	0.00	10.00
ASPV	–	25	–	26.60	–	24	–	30.00

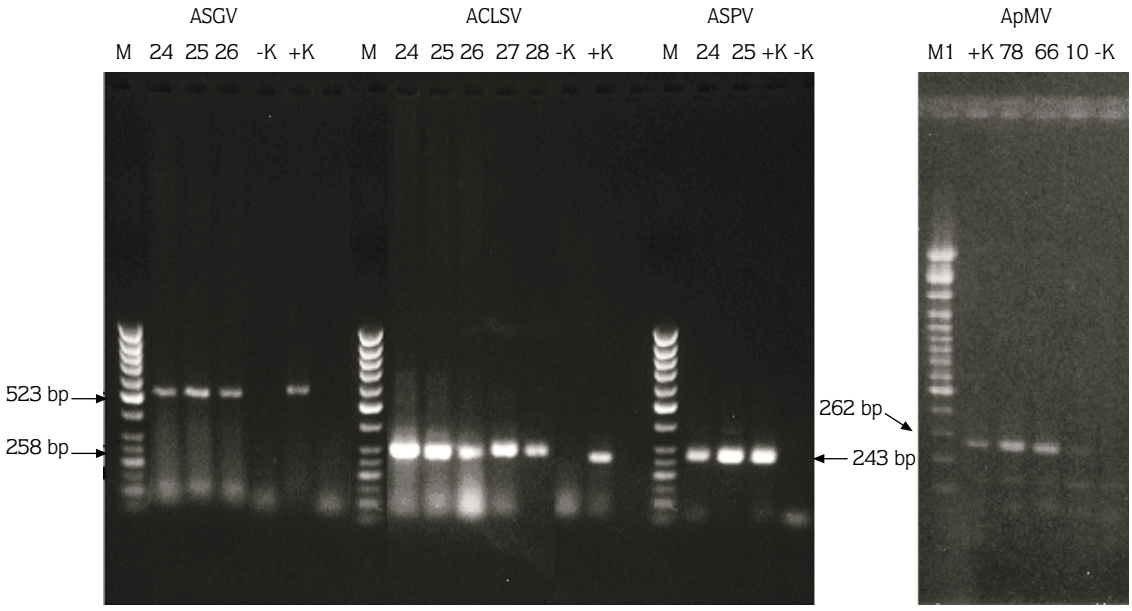

Figure 3. Representative results for the detection of *Apple stem grooving virus* (ASGV), *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV) and *Apple mosaic virus* (ApMV) by RT-PCR. M: 50 bp DNA ladder; M1: 100 bp DNA ladder Plus (Fermentas GmbH Germany); Lanes 24, 25, 26, 27, 28: Apple tree samples from Pozantı-Adana; 78: Granny Smith from Ankara; 10, 66: Golden from İçel; +K: Positive control sample for ASGV, ACLSV, ASPV and ApMV; - K: Water control.

from those varietal collections, were found to be highly contaminated, particularly by ACLSV and ASPV. Since ApMV causes obvious symptoms on most apple cultivars and its infection rate is fairly low compared to other 3 latent virus diseases, it may not be a big danger in Turkey. Our results correspond to those presented by Myrta et al. (2003), who described a high incidence of ASGV, ASPV and ACLSV but a very low incidence of ApMV in Albania.

Since no natural vectors for spreading these viruses have been described, the production of virus-free reproductive and planting material and its usage for the establishment of new plantings are key for effective virus control. Sensitive, reliable and rapid methods for virus detection are a major prerequisite for the production of healthy plant material. The RT-PCR assay offers a very effective and reliable detection method for the determination of ApMV, ACLSV, ASGV and ASPV incidence. The results obtained from the comparison of ELISA and RT-PCR in this study showed that with the RT-PCR technique 8.6% more samples were detected as positive, emphasizing the possibility of using this technique for routine diagnostic purposes. Kinard et al. (1996) obtained similar results and reported that ELISA-based techniques often fail because of low virus titer and the inhibitory effect of compounds in the sap of woody plants. Since no commercial antibody for ASPV detection is available (Nemchinov and Hadidi, 1998), a comparison of ELISA and RT-PCR for this virus detection was not carried out in this study. The obtained results clearly proved that ASPV can be detected using RT-PCR at a high frequency, which was 26.60% and 30.00% for varietal and commercial orchards, respectively (Table 1). ASPV detection by means of RT-PCR has previously been reported (Foissac et al., 2001; Salmon et al., 2002).

Mixed infections with 2 or 3 viruses were very common and recorded in all tested cultivars in different provinces. The mixed infection of 3 main pome fruit viruses including ACLSV, ASGV and ASPV has previously been reported (Leone et al., 1998) and yield losses up to 60% were pointed out, especially for frequent mixed infections (Campbell, 1963; Schmidt, 1972; Zahn, 1996).

The present results indicate that a sensitive assay like RT-PCR for the detection of 4 important apple viruses can be used easily in the certification program. However,

a final conclusion on the incidence of these viruses can only be made after detailed screening of a large numbers of orchards. The eradication of infected mother plants is a significant matter for the certification system of nursery material for apple growing in Turkey.

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