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Update on *Plum pox virus* distribution in Turkey

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Summary. Extensive surveys to determine the occurrence of *Plum pox virus* (PPV) in Turkey were carried out between 2007 and 2010 in commercial stone fruit orchards and nurseries, in non commercial stone fruit trees at other locations, and in rural and urban residential properties located in 56 of Turkey's 81 provinces. A total of 5,762 samples were collected from almond, apricot, mahaleb, nectarine, plum, peach, sweet cherry and sour cherry and tested by biological indexing, DAS-ELISA and RT-PCR. Two hundred and twenty two samples from 4 regions (the Aegean region, the Central Anatolia region, the Marmara region and the Mediterranean region) were found to be infected with PPV. This virus has occurred in Turkey since 1968. This is the first record of PPV occurrence in Aksaray, Çanakkale, İzmir, Kayseri, and Konya provinces.

Key words: stone fruits, sharka, ELISA, RT-PCR, virus detection.

Introduction

Plum pox potyvirus (PPV), the causal agent of sharka, is one of the most serious diseases of stone fruits, including peaches, apricots, plums, nectarines and almonds. Sharka causes considerable loss in yield and is of great economic importance (Dunez and Sutic, 1988; Németh, 1994). The disease significantly limits stone fruit production in those areas where it is established. It produces blemished, misshapen fruit, can cause fruit to drop prematurely from the tree or, in some cases, completely prevents fruit development. Both wild and ornamental *Prunus* species are susceptible to PPV, and are considered potential reservoirs of the virus. PPV was first reported in Bulgaria in 1918, and was discovered to be a viral disease in 1932. Since then, it has gradually spread to a large part of Europe, around the Mediterranean basin and the Middle East. It has also been reported in restricted areas of South and North America, and in Asia (EPPO, 2006).

To date, seven PPV strains have been described on the basis of their serological and molecular properties: PPV-D (Dideron); PPV-M (Marcus); PPV-EA (El Amar); PPV-C (Cherry); PPV-W (Winona); PPV-Rec (Recombinant) and PPV-T (Turkish) (Kerlan and Dunez, 1979; Wetzel *et al.*, 1991; Cambra *et al.*, 1994; Nemchinov and Hadidi, 1996; Crescenzi *et al.*, 1997; James and Varga, 2005; Glasa *et al.*, 2005; Candresse and Cambra, 2006; Ulubaş Serçe *et al.*, 2009). Most PPV isolates belong to the D and M strains.

PPV occurred in Turkey for over 40 years. It is one of the most studied virus diseases of stone fruit trees and other plant crops in Turkey (Şahtiyancı,

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1969; Kurçman, 1973; Yürektürk, 1984; Elibüyük, 2004; Koç and Baloğlu, 2006; Candresse *et al.*, 2007; Gümüş *et al.*, 2007; Ulubaş Serçe *et al.*, 2009). These studies have been conducted in different areas of Turkey; PPV was first reported in Edirne province (in the Thrace region, the European part of Turkey) in 1968 (Şahtiyancı, 1969), it was later found in Ankara (the Central Anatolia region) in 1973 (Kurçman, 1973). PPV was also reported in the Marmara Region (Yürektürk, 1984). More recently, it has been identified in two provinces of the Mediterranean Region (Koç and Baloğlu, 2006), and in one location between Central Anatolia and the Mediterranean regions (Candresse *et al.*, 2007) and in the Aegean region (Gümüş *et al.*, 2007). Hotspots of PPV infection were recorded in some residential areas of Ankara province. Though PPV has occurred here since 1973, it has remained limited to only a few sites. For the last 34 years PPV has only been reported from Isparta province once; this province is located in the Mediterranean region about 300 km far from Ankara. The Isparta finding remained an exception as there have been no further reports of PPV in this region.

Since PPV epidemics are a major threat to fruit cultivation, national surveys of *Prunus* orchards and nurseries were initiated by the Turkish Ministry of Agricultural and Rural Affairs (MARA) in 2007 to determine the areas were the infection was occurring and to establish quarantine zones to control the further spread of the disease. Besides commercial orchards and nurseries, rural and urban residential properties and gardens were also included in the survey. Commercial stone fruit orchards are common in some provinces of the Aegean, Mediterranean, Marmara and Eastern Anatolia regions. In spite of the relatively limited areas for stone fruit cultivation, some provinces within these regions have a significant number of stone fruit trees. Moreover, private owners of gardens and small orchards often cultivate stone fruit trees such as pome fruits, walnut, grapevine (up to twenty trees in some gardens and orchards). Peach and nectarine orchards are predominant in the Marmara region and are important for the local economy. Apricot is grown in Eastern and South-East Anatolia. Plum, apricot, sweet and sour cherry production are grown in the Mediterranean and Aegean provinces, fruit orchards are smaller in the Black Sea and the Central Anatolia regions than elsewhere in Turkey. Commercial stone fruit orchards are less widespread in provinces of Central Anatolia and Black Sea; however, their number is increasing. It is a common practice for growers to plant a variety of *Prunus* species instead of one species in their orchards. Peach and nectarine trees, sweet and sour cherry trees are usually grown together. Cherry orchards, therefore also include significant numbers of sour cherry trees, and peach orchards, especially those recently established, likewise contain significant numbers of nectarine trees.

Before this study, PPV in Turkey was known to occur in some locations in the Marmara region, in Ankara province in the Central Anatolia region, in a few orchards of three provinces of the Mediterranean region and in the Aegean region (Şahtiyancı, 1969; Kurçman, 1973; Yürektürk, 1984; Koç and Baloğlu, 2006; Candresse *et al.*, 2007; Gümüş *et al.*, 2007) (Figure 1).

This study reports on extensive surveys carried out within all regions of Turkey to identify the

Figure 1. Surveyed areas infected with *Plum pox virus*.

distribution of PPV in stone fruit species in commercial orchards, in nurseries, and in rural and urban residential properties. The surveys were structured and carried out in cooperation with the MARA to provide a complete picture of the extent of PPV infection, with the ultimate aim to implement measures to eradicate the virus.

Materials and methods

Surveys and sampling

The surveys were conducted annually between March and July 2007–2010 in 56 provinces covering all regions of Turkey. Commercial nurseries and orchards, noncommercial stone fruit trees in other sites, and in rural and urban residential properties were surveyed. All *Prunus* fruit trees were included in the survey. *Prunus* spp. used for landscaping in each nursery and orchards were also included. Residential sites (properties and private gardens) were randomly selected for the surveys. At each site, domestically grown *Prunus* fruit trees and landscaping plants were surveyed. In most cases, *Prunus* fruit trees were sampled individually, 6 to 12 leaves (approximately 10 g of leaf tissue) being harvested from each tree. In some cases, if the virus was not known to occur in a given area and there were no obvious sharka symptoms, samples from gardens and orchards were bulked from six trees. Leaf and flower samples from apricot, plum, peach, sweet cherry, sour cherry, mahaleb and almond trees were collected from the entire tree canopy. Samples were stored on ice prior to processing and tested by DAS-ELISA. For each whenever an ELISA-negative sample fell within 10% of the detection threshold, or whenever an ELISA sample was positive the trees producing the sample were resampled and retested by ELISA and by the polymerase chain reaction (PCR). If a similar result was obtained in a bulked sample, all the trees were re-tested individually in the same way. Field inspections were carried out in 879 locations (153 residential sites, 16 nurseries, 569 orchards and 141 other fruit orchards and fields) (Table 1). In total, 5,762 samples were collected from 2,387 apricot, 1,031 plum, 862 peach, 150 nectarine, 864 cherry, 103 sour cherry, 62 mahaleb and 303 almond trees (Table 2).

Testing for PPV

Samples from symptomatic apricot and plum trees were grafted by chip-budding to peach GF 305 and Myrobalan plum in controlled conditions. *Nicotiana clevelandii* and *Chenopodium foetidum* indicator plants were grown in an insect-proof greenhouse at 20–25°C until they were 4 to 5 weeks old, then mechanically inoculated. At least five replicates were used per plant. PPV transmission was monitored by symptoms development and by double sandwich (DAS)-ELISA. All 5762 samples collected were tested serologically for PPV

Table 1. Sites inspected in the surveyed areas.

Region	No. of provinces	No. of residential sites	No. of nurseries	No. of orchards	No. of other orchards	Total inspected sites	No. of samples
Aegean	8	31	-	98	35	164	942
Black Sea	9	36	1	30	20	87	733
Central Anatolia	13	63	2	74	68	207	1,720
Eastern Anatolia	2	-	3	86	-	89	852
Marmara	12	7	3	27	6	43	291
Mediterranean	8	16	5	203	12	236	784
South-East Anatolia	4	-	2	51	-	53	440
Total	56	153	16	569	141	879	5,762

Table 2. *Prunus* species inspected in surveyed areas.

Region	Apricot	Plum	Peach	Nectarine	Cherry	Sour cherry	Mahaleb	Almond	Total
Aegean	278	231	271	-	116	18	4	24	942
Black Sea	126	215	170	7	124	33	45	13	733
Central Anatolia	1,023	256	183	12	141	33	13	59	1,720
Eastern Anatolia	632	40	28	-	135	12	-	5	852
Mediterranean	276	118	149	72	120	4	-	45	784
Marmara	15	159	51	59	-	-	-	7	291
South-East Anatolia	37	12	10	-	228	3	-	150	440
Total	2,387	1,031	862	150	864	103	62	303	5,762

within 7 days using the Agdia Inc. (Elkhart, IN, USA) antisera kit for DAS-ELISA. This antibody detects strains C, M, D, EA and W of PPV. Positive and negative samples obtained from Agdia were used. DAS-ELISA was carried out following manufacturer's instructions (Agdia Inc). A sample was taken to be positive if both wells had an OD_{405} value greater than three times the negative control. Any sample that was suspect or positive for PPV was resampled from the field within 2 weeks and retested with ELISA and RT-PCR.

In the molecular tests, nucleic acids were extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA was purified according to manufacturer's instructions with slight modifications (QIAGEN). The RNA was eluted in two steps with 50 μL of RNase-free water. For general PPV detection, universal primers (P1, 5'–3' ACC GAG ACC ACT ACA CTC CC; and P2, 5'–3' CAG ACT ACA GCC TCG CCA GA) were used according to Wetzel *et al*. (1991) to amplify specifically a 243 bp product. PCR was done as in Olmos *et al*. (2002) with modifications; the reaction mixture consisted of a 25 μL volume containing 1 μL of RNA and 25 pmol mL^{-1} of each primer, and the ingredients for the cocktail reaction were prepared as follows: H_2O 16.45 μL; 10× *Taq* polymerase buffer 2.5 μL; 25 mM $MgCl_2$ 1.5 μL; 5 mM dNTPs 1.25 μL; 25 μM primer P1 1 μL; 25 μM primer P2 1 μL; Rnasin inhibitor 0.1 μL; 10 U μL^{-1} MMV 0.1 μL; 5 U μL *Taq* polymerase 0.1, to a total volume of 25 μL. The RT-PCR was performed under the following thermocycling conditions: reverse transcription 55 min at 37°C, activation of the *Taq* polymerase at 92°C for 2 min, followed by 40 cycles of 92°C for 30 s, 60°C for 30 s and 72°C for 1 min, and a final extension step at 72°C for 10 min; and hold at 4°C. Amplifications were realized using an Eppendorf thermal cycler (Mastercycler, Hamburg, Germany). PCR products were separated by electrophoresis in 2% agarose gels in TAE buffer, stained with ethidium bromide, visualized under UV light, and photographed using a Polaroid gel camera system.

Results

Surveys

No symptoms associated with a viral infection were found in any plants in the field nurseries. The majority of these nurseries were far from PPV-infected areas (more than 25 km), a few were 5–25 km from a PPV-infected area. Symptoms associated with a viral infection were seen in some apricot and plum trees in some residential sites of Kayseri and Konya provinces, on a plum tree in a peach orchard, surrounded by other fruit trees and some apricot trees in Ortaköy district of Aksaray province in Central Anatolia, on almond, apricot, plum, peach and nectarine trees of Bilecik, Bursa, Çanakkale, Kırklareli, Kocaeli, Sakarya and

Table 3. ELISA test results of PPV (*Plum pox virus*) detection in Turkey (except Ankara province since this was too heavily infected).

Region	Province	District	No. of PPV infected *Prunus* species	No. of infected trees
Aegean	İzmir*	Menemen	2 Apricot 1 Plum	3
Central Anatolia	Aksaray*	Ortaköy	5 Apricot	5
		Center	3 Plum	3
	Kayseri*	Kocasinan	7 Apricot 3 Plum	10
	Konya*	Center	3 Apricot 1 Plum	4
Marmara	Bilecik	Osmaneli	3 Plum	3
	Bursa	Gürsu	7 Plum 3 Peach	10
		İnegöl	16 Plum 5 Peach 2 Nectarine	23
		Kestel	32 Plum	32
		Osmangazi	2 Plum	2
	Çanakkale*	Gelibolu	3 Apricot 1 Nectarine	4
	Edirne	Center	3 Plum 2 Apricot	5
	İstanbul	Center	2 Apricot	2
	Kırklareli*	Center	2 Plum	2
	Kocaeli	Gölcük	9 Plum	9
		Karamürsel	2 Plum	2
	Sakarya	Geyve	3 Plum	3
	Tekirdağ	Center	4 Almond	4
	Yalova	Altınova	27 Nectarine	27
Mediterranean	Adana		16 Peach 12 Nectarine	28
	İçel	Mezitli	22 Apricot 11 Peach 12 Nectarine	45
Total				221

*, Newly PPV-infected areas.

Tekirdağ provinces in the Marmara region, and on some apricot and plum trees in a mixed orchard of İzmir province in the Aegean region. One was repeated in Tekirdağ province, Thrace, where according to first inspection, a few almond trees were suspected to be infected with PPV (Table 3).

Detection of PPV

Biological indexing

Isolates of PPV transmitted from apricot and plum caused the same symptoms on the infected woody and herbaceous plants. Infected GF 305 peach developed distortion of the leaves, tissue

clearing along the veins and chlorotic blotching. *P. mahaleb* showed moderate green mottling. As to the herbaceous plants, the PPV isolates caused local chlorotic and necrotic spots on *Chenopodium foetidum* and *Nicotiana clevelandii*. PPV was detected in symptomatic plants with the ELISA test.

DAS-ELISA

Of the 5,762 trees individually tested by ELISA, PPV was found in samples from 221 trees in sites from regions other than Ankara. In the residential sites of Ankara, almost all plum and apricot trees were infected with PPV. For that reason, stone fruits of residential sites from central Ankara province were not taken into account in this study to determine the PPV situation. However, PPV infection was detected in two rural areas (Karapürçek and Sincan) close to Ankara, but not in suspected samples from the Ayaş and Beypazarı districts also in Ankara province.

Trees infected with PPV were found in Aksaray, Kayseri and Konya provinces. A number of stonefruit trees were found positive for PPV: (i) a plum tree in an orchard containing peach, apricot and nectarine, (ii) a few apricot trees in the Ortaköy district of Aksaray province, some plum and peach trees in the Konya province, and (iii) 15 apricot and 5 plum trees from 6 residential sites in two different locations in the Kayseri province. PPV was not detected in the other *Prunus* fruit trees in orchards or gardens. The samples collected from almond, apricot, nectarin peach, and plum orchards in Bilecik, Bursa, Çanakkale, Kırklareli, Kocaeli, Sakarya, Tekirdağ and Yalova provinces (the Marmara region) and from apricot and plum orchards in İzmir province (the Aegean region) were positive for PPV. PPV infection was most common and most widespread in the Marmara region with 161 infected trees. ELISA failed to detect PPV in any of the samples from nurseries. However, entrance of a PPV infected apricot seedling that was being imported into the directorate nursery of Malatya, province belonging to the Eastern Anatolia region, was intercepted, and immediately eradicated (Table 3).

Molecular tests

The conventional RT-PCR assay discriminated between virus-free and virus infected trees.

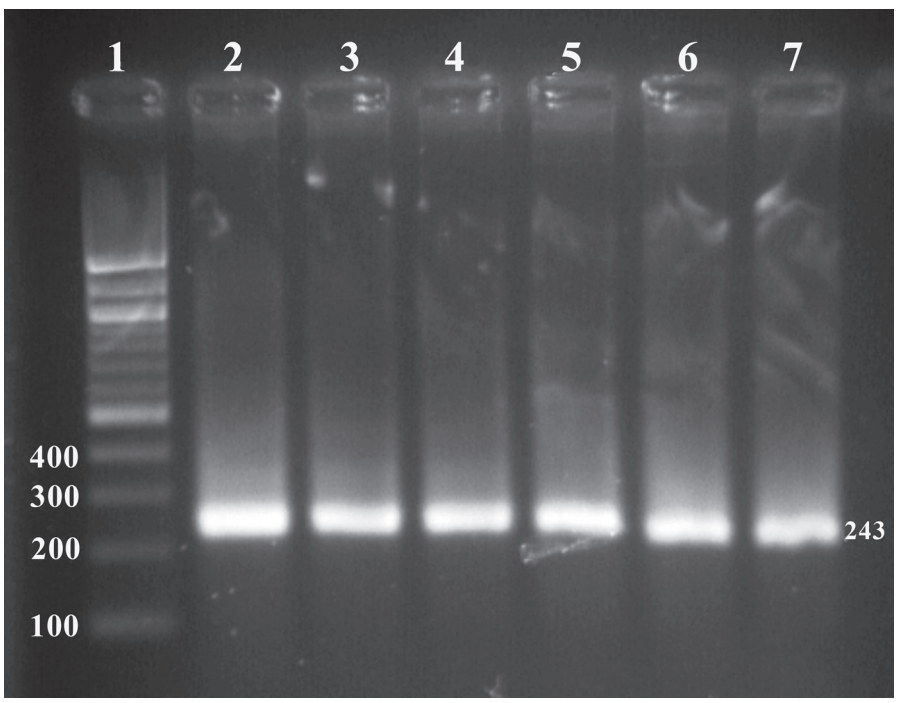

Figure 2. Gel electrophoresis of RT-PCR products of *Plum pox virus* (PPV) with specific primers. Lane 1, molecular marker 100 bp (MBI); lane 2–5, different PPV apricot isolates (2, Ankara; 3, Konya; 4 and 5, Kayseri); lane 6–7, PPV plum isolates (both from Ankara).

Results obtained with total nucleic acid preparations from a set of PPV isolates from leaf and flower samples of apricot and plum trees are shown in Figure 2. Sixty two samples were used in the molecular tests, some of these samples came from symptomless trees (Table 4). PPV was not detected in symptomless leaves of stone fruit trees. Samples that were positive in the PCR test were confirmed by DAS-ELISA. RT-PCR amplification of PPV isolates gave a product of approximately 243 bp with the PPV primer set described above. The nucleotide sequences of the two apricot isolates from Ankara were deposited in the GenBank database under accession numbers HM245754 and HM245757.

Discussion

Sites known to be infected with PPV were sampled more intensively than other sites (residential, gardens, orchards) known to be free of sharka. Any spread of PPV was carefully monitored in districts within PPV infected areas. Sharka symptoms from PPV were less frequent in districts of Ankara province (found only in a few apricot trees); however, elsewhere the infection rates (approximately 90% of apricot trees and approximately 60% of plum trees) increased in residential areas of Ankara city center, in spite of the existing eradication program. Sharka symptoms were also seen in locations Karapürçek and Sincan, two neighboring rural sites. Surprisingly, virus symptoms were not seen in any stone fruit trees in Isparta province, nor in any samples from this province, which is known to be infected with PPV (Candresse *et al*., 2007). While this does not prove that PPV does not occur here, it does indicate that either infected tree(s) was (were) eradicated or that PPV was not detected in the region during the 2007–2010 survey. On the other hand, Candresse *et al*. (2007) detected recombinant strain of PPV in Isparta, and the antiserum we used was not specifically geared to detect recombinant strains. However, since a set of closely related isolates is characterized by a unique ancestral recombination event between PPV-M and PPV-D with a breakpoint in the NIb polymerase gene, according to Glasa *et al*. (2005), then if any antiserum detects either the M or the D strain, it should detect any recombinant strain from M and D as well. In the other parts of the Turkish Mediterranean region, PPV-infected areas were not found to expand. In the Marmara region, there were a few general reports of PPV in some locations of the region, but these reports were depended on symptomatology and biological tests, and not confirmed by conventional tests (Şahtiyancı, 1969; Yürektürk 1984). In addition, it was indicated that limited PPV infections occurred in the region in these reports. PPV symptoms occurred in almost all provinces surveyed of the region.

Table 4. Samples used for the RT-PCR tests.

Original host	Location	No. of tested samples	No. of infected samples
Apricot	Central Anatolia Region (Aksaray)	5	4
Plum	Central Anatolia Region (Aksaray)	4	2
Apricot	Central Anatolia Region (Ankara)	16	16
Plum	Central Anatolia Region (Ankara)	9	6
Cherry	Central Anatolia Region (Ankara)	3	0
Apricot	Central Anatolia Region (Kayseri)	5	4
Apricot	Central Anatolia Region (Konya)	5	2
Plum	Central Anatolia Region (Konya)	4	1
Peach	Marmara Region (Bilecik)	4	2
Plum	Marmara Region (Bursa)	3	1
Nectarine	Marmara Region (Yalova)	4	3
Total		62	41

Trees displaying sharka were believed to have been propagated from a single plant and to have been spread further by various aphids in the Kayseri area over the past five years, or propagated from a single plant in the Konya and Aksaray area over the past three years. The orginal infected parent tree did no longer exist. In the Aegean area, PPV has been reported before this study. However, in that report location was not indicated (Gümüş *et al.*, 2007). During the survey, a mixed apricot-plum orchard was found to have trees with sharka in the İzmir province. PPV was believed to have been introduced here by infected propagation materials in the last decade. PPV was thought to have been disseminated in newly infected areas of the Marmara region by infected propagation materials, and also by aphid vectors after initial reports back to 1968 and 1984. In the Black Sea, South-East Anatolia and Eastern Anatolia regions, differences with previous surveys were very small (Elibüyük and Erdiller, 1998; Sipahioglu *et al.*, 1999). These regions are still PPV-free. In general, PPV management was excellent, with most if not all home and orchard owners agreeing to have their properties surveyed. Apart from the PPV-infected graft that was intercepted as it was about to enter Malatya province, all the infected trees found in Aksaray and Konya provinces during the current survey were eradicated within 3 to 4 weeks after detection. Any other trees in the Aegean, the Marmara and the Mediterranean regions that become infected are eradicated by the local authorities.

Surveys of stone fruit of the *Prunus* species from commercial nurseries in the other 56 provinces did not detect any PPV. Most nursery stock consisted of second-year material or mother plants and had undergone at least one cycle of dormancy required for symptom or virus expression in some species. While this does not prove that these species are susceptible to PPV, it does indicate that no PPV was detected in any nursery plantings of these provinces in 2007–2010. Since the distribution of PPV in trees is often uneven, and since virus levels fluctuate over the year (Olmos *et al.*, 1997; Cambra, 1999), it may be necessary to monitor the trees regularly, as well as to carry out field surveys. The reliability of PPV detection depends largely on the concentration of the virus, the host species, the time of collection, and the location where the sample was collected (Gruntzig *et al.*, 1986). Further, it is usual to observe a delay of several years between the introduction of PPV and the systemic expression of the virus on many hosts (Dicenta *et al.*, 1999).

While this survey examined many parts of the country, some errors in diagnostics due to these factors are inevitable. Errors in sampling due to variation in the virus distribution within trees were reduced by repeated monitoring of PPV-infected apricot and plum trees throughout the survey demonstrated that the ELISA continued to detect the virus. When resampling was necessary it was done as much as possible from the original trees to minimise sampling error. Ongoing studies in our laboratory are examining aphid vectors and other native plant species by enabling aphids to infect them naturally under high inoculum pressure in containment facilities. While these trials are carried out and supplemented, eradication program of infected trees is being implemented.

Plum pox virus was not detected in tested mahaleb, or in sweet or sour cherry trees in Turkey, not even in the Ankara area. This is consistent with other studies in the Ankara area, where PPV was not found to occur naturally in cherry or sour cherry trees (Elibüyük, 2004). However, the only almond trees without PPV came from Tekirdağ province and the Thrace region. Therefore, cherry, sour cherry and mahaleb do not appear to be a significant reservoir of PPV and therefore do not have a prominent role in the etiology of sharka. Extensive sampling of trees done in areas of high inoculum pressure and where aphid species occurred failed to detect any natural infection over the 3 years of the survey.

The results strongly suggest that the incidence and distribution of PPV will increase in Turkey if growers do not become aware of the risk. Because of the existence of sources of PPV, the lack of control measures, especially measures to prevent the dissemination of propagating materials over long distances, poses a great threat. Although moderate numbers of various aphid species were found in the orchards when the samples were collected, it was noticed that especially nursery and orchard owners currently already take measures to control aphids. Meanwhile, in residential sites, it has been agreed to prioritize aphid control.

Residential sites in Kayseri province (other than Ankara) were found to have cases of PPV in susceptible *Prunus* stone fruit trees, grown for

landscaping or other purposes. On the other hand, residential sites landscaping *Prunus* trees did not have a high incidence of PPV on rural residential properties. Such sites do not appear to represent a significant reservoir of PPV, and hence do not present an obstacle to the current eradication and containment program.

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