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Description and Detection of Almond Virus Diseases in The Trakya Region of Turkey

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ABSTRACT

Almond *Prunus dulcis* is a well known fruit grown for its unripe fruits and delicious seeds in Turkey. Agro ecological conditions in Trakya are suitable for almond production and promise great potential for establishment of commercial almond orchards. Some prevailing virus infections on almond trees however have reduced yield and quality in the region. In order to describe diseases and identify viruses 158 flower and 260 leaf samples were collected from symptomatic trees in 10 districts of region in 2010. By employing enzyme linked immunosorbent assay (DAS–ELISA), TAS-ELISA) and reverse transcriptase polymerase chain reaction (RT–PCR), *Prunus necrotic ring spot virus* (PNRSV), *Prune dwarf virus* (PDV) and *Plum pox virus* (PPV) viruses were identified. As a result of detection studies 31.15 % samples was found infected with PNRSV, 4.23 % of them had PDV, 1.92 % of them were infected with PPV individually and 1.54 % of them however infected with the mixture of PNRSV+PDV and PNRSV+PPV. So 38.85% of almond leaf samples were found infected with viruses in Trakya region of Turkey.

Key words: *Prunus dulcis*, PNRSV, PDV, PPV, RT-PCR

INTRODUCTION

Almond species, *Prunus dulcis* Mill and *Prunus fragilis* C. Schneid are belong to *Rosaceae* family and *Prunoidea* subfamily in the Botanical Taxonomy. Almond is produced for its nutritious and delicious seeds as well as for unripe fresh fruits in Turkey. Beside two species *Prunus amara* C. Schneid containing cyanuric acid is also produced as a raw material of industry. Almond production is possible everywhere in Turkey except North Eastern part of the country and high mountains in Eastern Anatolia. However, the most of the almond production takes place in western part of Turkey including Trakya region. Trakya region of Turkey is located 28˚, 1' and 29˚,1' East longitude and 40˚,5'- 42˚,2' North latitude having hilly and plain topography. 65 % of land is arable land, 15 % is pasture and meadow and 16% of land is covered with forests. With an average 45 000 tones of annual almond seed production Turkey is ranking 8 in the world (Anonymous, 2008). But this amount of production does not meet the demand of consumption. Because of this, Turkey imports more than 1000 tones of almond seed every year. Almond is a marketable nut fruit which can adapt extreme soil and climatic conditions. That is why almond trees take place almost everywhere in the flora of Turkey. Like every fruit species, almond trees are also susceptible to some virus infections. At the first time Waterworth and Fulton (1964) separated both *Prunus necrotic ringspot virus* (PNRSV) and *Prune dwarf virus* (PDV) by using biological and serological methods. Fulton (1970a) indicated almond as a host of PNRSV as described PDV as another important virus for almond (Fulton, 1970b). Smith (1972) reported

three types of virus infection caused by PNRSV strains. By employing reverse transcription polymerase chain reaction (RT-PCR) Rosner et al. (1999) identified PNRSV on peach and almond trees in Israel. Beside these investigations in almond orchards, Kölber et al. (2001) identified *Plum pox virus* (PPV) as another important virus on almond orchards in Czech Republic, Hungary, Russia and Bulgaria. In Turkey PPV was the first virus identified on local plum varieties in the Trakya region by Sahtiyancı (1969). By employing DAS-ELISA serological tests Azeri and Çiçek (1997) identified PNRSV, PDV, *Raspberry ring spot virus* (RpRSV), *Peach rosette mosaic virus* (PRMV) and *Tomato ringspot virus* (ToRSV) on almond trees in Izmir, Balıkesir and Aydın provinces in Turkey. Sipahioğlu et al. (1999) searched the presence of 11 viruses and 2 viroids including PNRSV, PDV, PPV, *Apple mosaic virus* (ApMV), RpRSV, *Peach rosette mosaic virus* (PRMV) and ToRSV on almond and the other stone fruits in Eastern Anatolia. They revealed the presence of PNRSV as 21 % and PDV was 33% as the other viruses and viroids did not occurred. Sertkaya et al. (2004) identified *Apple chlorotic leaf spot virus* (ACLSV) on an almond tree as Ilbağı et al. (2008) determined *Prunus spinosa* as the wild host of ApMV, ACLSV and PPV. Establishment of almond and walnut orchards in Trakya Region have been supported by government since 2002. Hence, to reduce risk of epidemic virus infections in the new almond plantations and orchards detection of viruses is necessary. In order to determine the important virus diseases on almond and identify the viruses in the Trakya region of Turkey, this study was performed.

MATERIALS AND METHODS

Survey and Collection of Samples

Almond virus disease survey was conducted by visiting 10 districts of Trakya region of Turkey as indicated in Figure 1. Samples were obtained from naturally infected symptomatic almond (*Prunus dulcis)* trees in commercial orchards established with the cultivars of Dokuzoğuz and Texas most of the samples however were collected from individual almond trees located field borders and home gardens belonging local varieties exhibiting chlorotic and necrotic leaf spots, leaf deformations and mosaic as observed in 2009. The flower samples were collected in February and March 2010 as suggested by Silva et al. (2003) and the leaf samples were collected in June 2010. Distribution of almond samples was exhibited in Table 1. So, 158 flower and 260 leaf samples were obtained from 418 almond trees.

Figure 1. Almond virus disease survey conducted by visiting 10 districts of Trakya region of Turkey

Table 1. Number of almond samples collected from 10 Districts of Trakya Region of Turkey in 2010

Province name	District name	Number of flower samples	Number of leaf samples
Edirne	Merkez	12	44
	Keşan	35	45
	Süloğlu	10	6
Kırklareli	Merkez	12	14
	Pınarhisar	13	4
	Babaeski	4	52
Tekirdağ	Merkez	36	30
	Şarköy	25	40
	Çorlu	4	16
	Çerkezköy	7	9
Total	10	158	260

Serological Tests: All the almond samples were tested for presence of *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV) and *Plum pox virus* (PPV). For this purpose double antibody sandwich enzyme linked immune sorbent assay (DAS-ELISA) and Triple antibody sandwich enzyme linked immune sorbent assay (TAS-ELISA) tests were employed as suggested by Clark and Adams (1977), formulated to PNRSV, PDV and PPV by Bertozzi et al. (2002). Necessary kits, compounds, positive and negative samples, polycolonal antibodies against PNRSV, PDV and PPV were obtained from Sediag Firm (Longvic-France). Both DAS-ELISA and TAS-ELISA tests were performed according to the protocol of Sediag Firm. Absorbance values were evaluated visually and reading by THERMO-Multiskan FC (Thermo Fisher Scientific Instruments Co. Ltd., U.S.A.). Samples were considered to be positive when reading of the average of two wells gave on optical density (OD_{405}) of at least two times OD_{405} of the healthy control. All the other samples revealed negative results subjected to reverse transcriptase polymerase chain reaction (RT-PCR) tests.

Molecular Test Studies

Total Nucleic Acid Extraction

Total nucleic acid extractions of PNRSV, PDV and PPV from flower and leaf samples of almond were made by employing nucleic acid extraction methods of Foissac et al. (2001). Nucleic acids were isolated from 100 mg portions taken from flowers and leaf samples. Each portion of material was homogenized in 1ml extraction buffer at pH:5-6. 500 µl portions of sap put into sterile Eppendorf tubes and 100 µl solution of 10% sodium lauryl sarcosyl was added and tubes were shaken time to time and incubated for 10 minutes at 70˚C. Tubes were cooled for 5 minutes in ice and were centrifuged at 14.000 rpm for 10 minutes. After centrifugation 300 µl amounts of supernatant were transferred to the series of Eppendorf tubes, 150 µl ethanol, 25 µl silica suspension at pH:2.0 and 300 µl 6M sodium iodide solution were added to each tubes. Mixture in the tubes were incubated and shaken on shaker platform at room temperature for 10 minutes. Tubes were centrifuged for 1 minute at 6000 rpm once again supernatants were discarded and pellets were dissolved with 500 µl washing buffer. Those pellets were incubated 4 minutes at 70˚C. All tubes were centrifuged for 3 minutes at 14 000 rpm and supernatants were transferred to new series of Eppendorf tubes and pellets were discarded, obtained total nucleic acids were kept -20˚C until they were used for cDNA.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Test

Synthesis of complementary DNA (cDNA) was performed according to protocol of MBI Fermentas (GmbH,St. Leon-Rot, Germany) where kits were obtained. For this purpose 2 µl total RNA, 1 µl Reverse complementary primer and 9µl RNAse free water were mixed into nuclease free microcentrifuge tube. The mixture was vortexed for 5 minutes at 65˚C and cooled and incubated in ice granules. The mixture of 4 µl 5 first stand buffer, 1 µl ribonuclease inhibitor, 2 µl dNTPs (10mµ) and 1µl reverse transcriptase enzyme were added into tube

and mixed by pipetting. The final mixture was incubated 1 hour at 42˚C and kept in 5 minutes at 70˚C. Obtained cDNA was kept in the -20˚C until it is used for polymerase chain reaction (PCR) test. The primer pairs were used to perform RT-PCR to detect to PNRSV by reported Spiegel et al. (1999), to PDV by reported Mekuria et al. (2003) and to PPV by reported Hadidi and Levy (1994). Determination of those three viruses, PCR tubes were added with 25 µl of the mixture of 2.5 µl 10 X PCR buffer (200 m MTris-HCL pH:8.5, 500 m M KCL), 1.5 µl $MgCl_2$ (25mµ), 0.5 µl dNTP (10 mµ), 0.5 µl Primer 1, 0.5 µl Primer 2 (MBI Fermentas, GmbH,St. Leon-Rot, Germany), 0.2 µl Taq DNA polymerase enzyme (MBI Fermentas), 1µl cDNA and 18,3 µl RNAse free water for PNRSV. Another mixture for PPV and PDV are 2.5 µl 10xPCR buffer, 3.75 µl $MgCl_2$ (25mµ) 1µl dNTP (10mµ), 1.5 µl Primers1, 1.5 µlPrimers2, 0.2µl Taq DNA polymerase enzyme (MBI Fermentas), 2 µl cDNA and 12.55µl RNAse free water. PCR amplification consisted of an initial denaturation step of 5 min at 94 ˚C followed by 35 cycles of 94 °C for 1 min, 50°C for 1 min, 72°C for 1 min and the final extension at 72°C for 10 min in Techne thermal cycler (Staffordshire ST15 0SA, UK). The amplification protocol for PPV as follows: initial denaturation was at 95 °C for 5 min, followed by 30 cycles at 94 °C for 30 sec, 62°C for 30 sec, 72 °C for 45 sec and the final extension step at 72°C for 10 min in Techne thermal cycler. PCR products were analyzed by electrophoresis in 2 % agarose gel, stained with ethidium bromide (EtBr) and viewed under UV illumination in a gel documentation system (VilberLourmat, MarnelaValleeCedex 1 France).

RESULTS and DISCUSSION

Survey studies during the year of 2010 revealed that the most severe characteristic symptoms occurred on old almond trees as chlorosis, chlorotic and necrotic ring spots, leaf deformations, mosaic, bud failure and the die back on shoot and the death of branches. Those infected old almond trees served as the sources of viruses for new orchards which are transmitted by infected propagation materials pollen, seed and vectors. Serological ELISA test and RT-PCR test results revealed the presence of PNRSV, PDV and PPV viruses on almond leaf samples as exhibited in Table 2. Only 1 out of 158 flower sample revealed the presence of PPV. Rest of the other flower samples exhibited any virus at all. On the other hand almond leaf samples revealed the presence of viruses with 81 out of 260 PNRSV as the rate of 31.15 %, with 11 out of 260 PDV as the rate of 4.23 %, with 5 out of 260 PPV as the rate of 1.92 % individually. Three out of 260 leaf samples had the mixture of PNRSV+PDV as only 1 out of 260 leaf sample had the mixture of PNRSV+PPV having totally 1.54 % mixed infections. As a result of DAS-ELISA and TAS-ELISA serological tests and the RT-PCR test, 101 out of 260 leaf samples of almond trees revealed the presence an infection of viruses with the disease rate of 38.84%.

Table 2. The number of almond leaf samples found infected with viruses as a result of ELISA and RT-PCR tests

Name of Provinces	Name of Districts	Number of Infected samples by ELISA and RT-PCR					Infected number of samples	Rate of Diseases %
		PNRSV	PDV	PPV	PNRSV+PDV	PNRSV+PPV PPV		
Edirne	Merkez	4+1*	-	-	-	-	5	11.36
	Keşan	24+2*	4	1	-	-	31	68.88
	Süloğlu	1	-	-	-	-	1	16.6
Kırklareli	Merkez	12+1*	-	-	-	-	13	92.86
	Babaeski	15	-	1	-	-	16	30.77
	Pınarhisar	-	-	-	-	-	-	-
Tekirdağ	Merkez	14	-	-	3	1	18	60.0
	Şarköy	4	4	-	-	-	8	20.0
	Çorlu	2+1*	3	3	-	-	9	56.25
	Çerkezköy	-	-	-	-	-	-	-
TOPLAM	10	81	11	5	3	1	101	38.84

*Number of samples exhibit positive by RT-PCR as they were found negative with ELISA tests

Samples of almond trees revealed the presence an infection of viruses with the disease rate of 38.84%. Molecular RT-PCR tests revealed only PNRSV were detected on 5 more leaf samples which were undetected by ELISA tests. Rest of the 105 RT-PCR tests just confirmed the results of ELISA tests. As a result of both serological ELISA and RT-PCR tests only one almond flower sample revealed the presence of PPV as indicated in Figure 2.

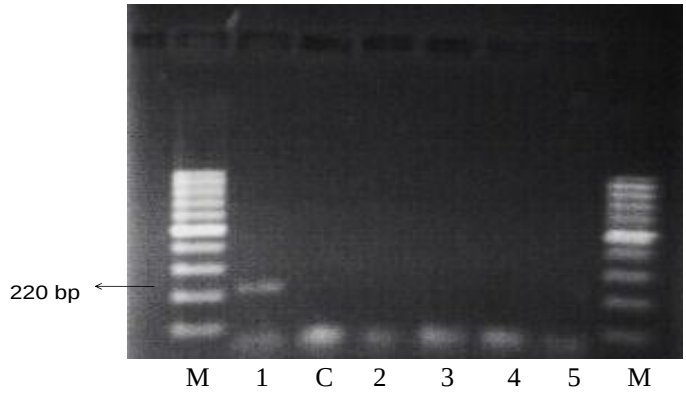

Figure 2. Molecular identification of PPV with DNA fragment on agarose gel band from infected almond flower sample (Lane M: DNA size marker (100 bp), Lanes1-5 flower samples C: Negative Control)

Presence of PPV was detected both serological and RT-PCR molecular tests in leaf samples of almond as indicated Figure3.

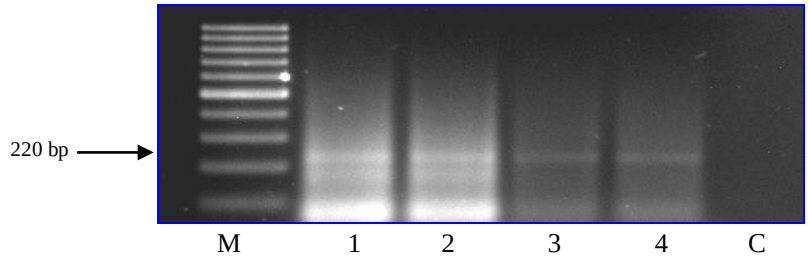

Figure 3. Molecular identification of PPV with DNA fragment on agarose gel bands from infected almond samples (Lane M: DNA size marker (100 bp), Lanes 1-4 leaf samples C: Negative Control)

In addition to ELISA tests 20 leaf samples subjected to RT-PCR test results verified the revealed results of ELISA for PDV as indicated Figure 4.

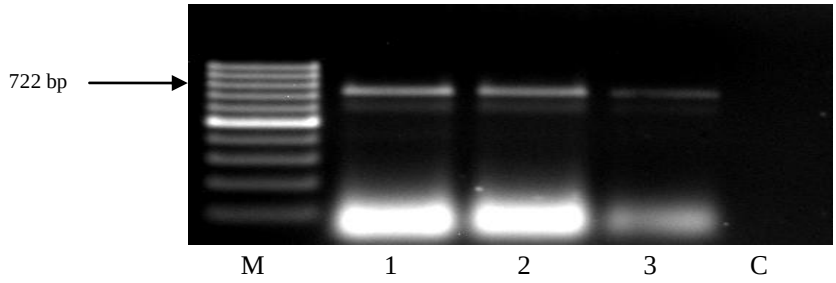

Figure 4. PDV infected leaf samples revealing 722 bp PCR products (Lane M: DNA size marker, Lane 1-3infected leaf samples C: Negative Control)

Beside 76 positive results of PNRSV by serological tests, RT-PCR tests were implemented to 71 leaf samples which revealed negative results. The results of RT-PCR revealed that 5 more leaf samples had PNRSV. Therefore, the number of PNRSV infected leaf samples became 76 + 5 = 81.

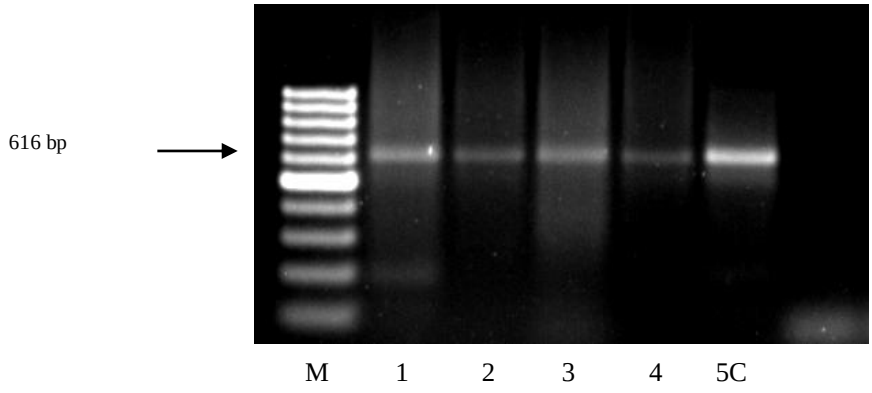

Figure 5. PNRSV infected leaf samples revealing 616 bp PCR products (Lane M: DNA size marker (100 bp), Lanes 1-5 infected leaf samples C: Negative control)

Wild or cultivated almond species are parts of flora of Turkey. Our observations in 2009 revealed sporadic old almond trees have been present everywhere in Trakya region. Almond orchards established with standard varieties and types in Trakya region were rare in the region similar to Western and Mediterranean region of Turkey (Küden et al.,1994) and South Eastern part of Turkey (Kaşka et al., 1994). Those old almond trees were exhibited characteristic disease sypmptoms similar to symptoms described by Smith (1972) and Sutic et al. (1999) except calico symptoms on leaves. All kinds of symptoms were systemic on infected trees reduced the yield and quality. Appearance of those symptoms implied that almond trees have infected with virus diseases. As a matter of fact Smith (1972) reported one, Fulton (1983) reported two and Horst (2008) reported six virus diseases on almond in the World. Some of those virus infections on almond trees were also reported in the orchards and nurseries by Azeri and Çiçek (1997), in Western Provinces of Turkey. Our observations confirmed those literature findings. Our ELISA and RT-PCR tests results revealed the presence of PNRSV and PDV as shown in table 1 confirmed findings of Azeri and Çiçek (1997), Rosner et al. (1999) in Israel and Sertkaya et al. (2004) East Mediterranean region of Turkey. Our detection of PPV on almond flower and leaf samples is the first finding in the Trakya region of Turkey. So our results confirmed the findings of Kölber et al. (2001) who determined the PPV one of the important virus on almond in Central and Eastern European countries including Bulgaria and the findings about *Prunus spinosa* as being the important stone fruit virus sources Ilbagi et al. (2008). In contrary to the reports of Bertozzi et al. (2002) and Silva et al.(2003), samples of almond flowers were not suitable as sources of viruses. In spite of both ELISA tests, which we found them fast and reliable for the detection of PNRSV, PDV and PPV on almond leaf samples, merely RT-PCR was much reliable by revealing the presence of PNRSV in the five leaf samples detected as free of virus by ELISA tests. Hence, important almond viruses PNRSV, PDV and PPV were detected certainly by using RT-PCR. The PPV was detected in the one flower sample as indicated in Figure 2. PCR products of 220 bp for 4 PPV infected leaf samples were exhibited as bands in the Figure 3. The bands of the PCR products of 722 bp of three PDV infected leaf samples were exhibited in the Figure 4. The bands of the PCR fragments of 616 bp for PNRSV infected five almond leaf samples were exhibited in Figure 5. Commercial almond orchards by evaluating obtained results can be established in Trakya region by eradicating all old almond trees, improving and planting new varieties as implemented by Miguel et al. (1997) in Portugal.

ÖZET

TÜRKİYE'NİN TRAKYA BÖLGESİ'NDE BADEM VİRÜS HASTALIKLARININ SAPTANMASI VE VİRÜSLERİN TANILANMASI

Türkiye'de badem *Prunus dulcis* çağlası ve lezzetli tohumları için üretilen kabuklu bir meyve türüdür. Yıllık ortalama 45 000 ton üretimi ile Türkiye, dünyanın önde gelen badem üreticisi ülkelerden birisidir. Türkiye'nin Trakya Bölgesi, sahip olduğu tarımsal ekolojik koşullar nedeniyle verimli badem bahçelerinin kuruluşu için büyük bir potansiyel taşımaktadır. Ancak badem ağaçlarında görülen yaygın virüs hastalıkları bölgedeki badem verimini ve kalitesini düşürmektedir. Söz konusu badem virüs hastalıklarının saptanması ve virüslerin tanılanması için 2010 yılında Trakya Bölgesi'nin 10 ilçesindeki 158 ağaçtan çiçek ve 260 ağaçtan yaprak örnekleri toplanmıştır. Örneklere Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) ve Triple antibody sandwich enzyme linked immunosorbent assay (TAS-ELISA) ile Reverse transcriptase polymerase chain reaction (RT-PCR) testleri uygulanarak *Prunus necrotic ring spot virus* (PNRSV), *Prune dwarf virus* (PDV) ve *Plum pox virus* (PPV) virüsleri tanılanmıştır. Sonuçta bölgedeki badem ağaçlarında bireysel olarak %31.15 oranında PNRSV, %4.23 oranında PDV, % 1.92 oranında PPV saptanmıştır. Ağaçların %1.54 oranında ise PNRSV+PDV ve PNRSV+PPV virüslerinin karışık enfeksiyonlarına rastlanmıştır. Bu durumda Türkiye'nin Trakya Bölgesi'ndeki badem ağaçlarında % 38.85 oranında virüs enfeksiyonları saptanmıştır.

AnahtarKelime: *Prunus dulcis*, PNRSV, PDV, PPV, RT-PCR

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