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Molecular Determination of Some Important Viruses Causing Infection in Potato Fields in Turkey

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Abstract: Potato is one of the most important agricultural crops worldwide and known to be susceptible to more than 40 viruses in nature. In this research,298 leaf samples collected in 2020 from potato fields in Afyon, Nevşehir and Bolu provinces were used to determine viruses affecting potato production in the region. The leaves of potato plants showing virus symptoms (mosaic, deformation, yellowing, curling) were subjected to RT-PCR using virus-specific primers, in order to detect the presence of Potato leafroll virus (PLRV), Potato virus A (PVA), Potato virus X (PVX) and Potato virus M (PVM). As a result of the study, one or more viruses were detected in 46 (15.43%) of the 298 leaf samples tested. A total of 43 samples were infected with PLRV (14.42%) and 3 samples were infected with PVX (1.%). It was determined that the most common virus in plant samples collected from Afyon, Nevşehir, and Bolu Central regions was PLRV followed by PVX. PVA and PVM were not detected in the samples. The sequences of some positive isolates of PLRV were obtained and used along with the isolates registered in the GenBank to reveal phylogenetic relationships among them. PLRV isolates from Turkey were classified in Group 2 along with isolates from Serbia, Canada, and Pakistan based on phylogenetic analyses. As a result, only 5 PLRV isolates were studied in this study. Further studies are planned with more isolates and other protein regions. RT-PCR products obtained with PVX did not give good results in RT-PCR, and it is planned to work with different primers in the future.

Keywords: PVA, PVM, PVX, PLRV, RT-PCR

Türkiye'de Patates Alanlarında Enfeksiyon Oluşturan Bazı Önemli Viral Etmenlerin Moleküler Olarak Belirlenmesi ve Filogenetik Analizi

Öz: Patates, dünyada en önemli tarımsal ürünler arasında yer alır ve doğal koşullarda 40'dan fazla virüs türüne hasssas bir bitki türüdür. Bu araştırmada, Afyon, Nevşehir ve Bolu illerindeki patates tarlalarından 2020 yılında toplanan 298 yaprak örneği, bölgede patates üretimini etkileyen virüslerin tespiti için kullanılmıştır. Virüs simptomu (mozaik, şekil bozukluğu, sararma, yapraklarda kıvrılma) gösteren patates bitkilerinin yaprakları, Potato leafroll virus (PLRV), Potato virus A (PVA), Potato virus X (PVX) ve Potato virus M (PVM)'nin varlığını tespit etmek amacıyla, virüs-spesifik primerler kullanılarak RT-PCR testine tabi tutulmuştur. Test edilen 298 tane yaprak örneğinin 46'sında (%15.43), bir veya birden fazla virüs tespit edilmiştir. Çalışma sonucunda, test edilen örneklerin 43'si PLRV (%14.42), 3'ü PVX (%1) ile enfekteli bulunmuştur. Afyon, Nevşehir ve Bolu illerinden toplanan bitki örneklerinde en yaygın görülen virüsün PLRV, ardından PVX olduğu belirlendi. Örneklerde PVA ve PVM tespit edilmedi. PLRV ile enfekteli olan bazı pozitif izolatların elde edilen sekans verilerinin GenBank'a kayıtlı izolatlarla karşılaştırılması yapılarak filogenetik ilişkileri belirlenmiştir. Filogenetik analize göre, Türkiye PLRV izolatları Sırbistan, Kanada ve Pakistan izolatları ile birlikte Grup 2'de kümelenmiştir. Sonuç olarak, bu çalışmada sadece 5 PLRV izolatı ile çalışılmıştır. İleryen çalışmalarda daha fazla sayıda izolat ile ve diğer protein bölgeleri ile çalışmalar yapılması planlanmaktadır. PVX ile elde edilen RT-PCR ürünleri RT-PCR'da iyi sonuç vermemiş olup, ileride farklı primerler ile çalışılması planlanmaktadır.

Anahtar Kelimeler: PVA, PVM, PVX, PLRV, RT-PCR

1. Introduction

The second most popular food source in people's plant-based diets, after grains, is the potato (*Solanum tuberosum L.*). Today, potato is farmed across Turkey (Çalışkan et al., 2020). In production, imported varieties from nations including the Netherlands, Germany, France, USA, and England are employed in place of native varieties in Turkey, one of the world's major producers of potatoes (Çalışkan et al., 2010). As a result, Turkey has a current account deficit and is dependent on imported seeds. Turkey imports the 500,000 tons of seed potatoes needed for cultivating potatoes from other nations (Onaran, 2014). Turkey is ranked 16th globally in terms of production in 2020 (FAOStat, 2020). Turkey produced 45 50000 tons of potatoes in 2018, and 5200000 tons of potatoes in 2020 (FAOStat, 2020). More than 60% of the nation's production of potatoes, which play a significant role in both food and industry in Turkey, is concentrated in the provinces of Niğde, Nevşehir, İzmir, Bolu, and Afyon

(Yılmaz et al., 2006).

One of the earliest viral diseases examined on potato plants is Potato leafroll virus (PLRV), the culprit that causes Leafroll virus disease in potato fields. PLRV is a member of the *Luteoviridae family* and the *Polerovirus genus*. It has isometric particles with a diameter of 24 nm and single-strand positive RNA (ssRNA) within (Peters, 1970; Hooker, 1986).In nations where potatoes are grown, the potato virus X (PVX) is a relatively prevalent virus. It is transferred and propagated via contact with ill and healthy plants in outdoor settings, as well as by people, animals, or tools and equipment. It has filamentous particles.

The Potato virus M (PVM), which causes crop losses of up to 40% in several European nations, also contains filamentous particles. *Myzus persicae* and *Aphis nasturtii* are significant vectors of the virus, which is *non-persistently* spread by aphids in field circumstances. According to a survey on Potato virus X (PVX), Potato virus S(PVS), PVA, Potato virus Y(PVY), and PLRV that was undertaken in 23 places throughout Turkey, the incidence of PVS was 93.13%, followed by PVX (81.12), PVY+PVA (39.59%), and 32.08% PLRV (Özbayram and Yorgancı, 1989). A survey investigation that was carried out in the provinces of Afyon and Bolu in 2003–2004 employed DAS–ELISA and PCR techniques, and the outcomes were verified by experiments using mechanical inoculation on test plants.

According to Güner and Yorgancı (2009), the most prevalent viruses are PVY and PVS, which are present at rates of 20.46% in Afyon and 13.06% in Bolu. This study examined the prevalence and incidence of PLRV, PVX, and PVM in potato-growing regions in the Turkish provinces of Afyon, Bolu, and Nevşehir.

2. Materials and Methods

2.1 Materials

The surveys conducted to potato growing areas in Afyon, Nevşehir and Bolu provinces in 2020 within the scope of the 1002 TÜBİTAK project. During the surveys, 298 plants showing signs of the virus such as mosaic, deformation, yellowing, curling were collected (Figure 1). Leaf samples stored in the freezer at -20 °C were used in molecular studies.

The primers used for PLRV, PVA, PVM, and PVX are given in Table 3.1.

Figure 1: Infected plants collected during surveys (potato leafroll virus)
Resim 1: Surveyler sırasında toplanan enfekteli bitkiler(patates yaprak kıvırcıklık virüsü)

Table 1. Primers, their polarities and the expected fragment sizes (Peker, 2007; Meena et al. 2017).
Tablo 1. *Primerler, polariteleri ve beklenen ürün büyüklüğü*

Virus	Primer Sequences	Target Gene Region	Polarite	Expected Fragment Size	Annealing Temperature
PVA	5’-GACACTACCAATGCTCAAAG-3’ 5’-CTCTTCTGAAGGTGTGACTAT-3’	Coat protein	Sense Antisense	560 bp	62 ºC
PVM	5'-ATCTGAAATAGTGAGTATGGG-3' 5'-GCCACCTTGGTTACGTGCTT-3'	Coat protein	Sense Antisense	408 bp	56 °C
PVX	5'-TAGCACAACACAGGCCACAG-3' 5'-GGCAGCATTCATTTCAGCTTC-3'	Coat protein	Sense Antisense	562 bp	62°C
PLRV	5'- CGCGCTAACAGAGTTCAGCC-3' 5'-GCAATGGGGGTCCAACTCAT-3'	Coat protein	Sense Antisense	336 bp	62°C

2.2 Method

2.2.1. Isolation of total RNA

In the study, RNA isolation process were carried out using the leaf samples collected from potato production areas of Afyon, Bolu and Nevşehir provinces. RNA isolation was performed according to the method of Astruc et al. (1996) given as follows.

- ❖ Samples were diluted in 1:2(w/) with extraction buffer solution [(100 mM Tris-HCl (pH.8.0), 50 mM EDTA (pH. 7.0), 5 mM NaCl, 10 mM 2-mercapto-ethanol (1/1000)] v) was diluted and crushed.
- ❖ After taking 1 ml of the plant sap into an eppendorf tube, the samples were centrifuged at 4,000 rpm for 3 minutes, then 50 µl of Sodium Dodecyl Sulfate (SDS) (20%) was added to the pellet and mixed by vortex.
- ❖ The tubes were then incubated in heating blocks at 65ºC for 30 minutes.
- ❖ 250 µl of potassium acetate (5M) was added to the tubes, kept in ice for 20 minutes, and then centrifuged at 13,000 rpm for 15 minutes.
- ❖ The supernatant was divided into two parts, 500 µl of it was placed in newly prepared microcentrifuge tubes and stored at -70ºC. The remaining 500 µl of supernatant was placed in the newly prepared microcentrifuge tubes, 500 µl of 96% Ethanol was added, and it was mixed with vortex.
- ❖ Then, 50 µl of sodium acetate (3M) was added to the tubes and the samples were mixed again and kept at -70 ºC overnight.
- ❖ The next day, the samples were centrifuged at 14,000 rpm for 15 minutes, and the liquid part was removed.
- ❖ Microcentrifuge tubes were inverted and dried on filter paper for 5 minutes and washed by adding of 1 ml of ethanol (70%) to the pellet.
- ❖ In order to precipitate RNAs, the tubes were centrifuged at 13,000 rpm for 5 minutes, the ethanol in the tube was discarded, and the microcentrifuge tubes were left to dry at room temperature.
- ❖ Total RNAs obtained from the samples were reconstituted with 50 µl of distilled water and stored at -20ºC until use.

2.2.2. Complementary DNA (cDNA) synthesis

Utilizing RNAs acquired from the total RNA isolation technique, complementary DNA (cDNA) synthesis was done. In microcentrifuge tubes for cDNA synthesis, 2 µl total RNA, 1 µl a random hexamer primer (5'-d (NNNNNN)-3'N = G, A, T, or C) (10 mol), and 7µl of distilled water were mixed. The mixture was put on ice for three minutes after 5 minutes at 65 °C of incubation.

To each tube was filled to a capacity of 20 by adding 5X MMLV buffer (5X), dNTP (25 mM), 0.5 random hexamer primer (10 mol), 0.25 RNAse inhibitor (10 U/l), 0.25 reverse transcriptase (Thermo Scientific), and distilled water. The tubes were then incubated at 25 °C for 5 minutes, 42 °C for 60 minutes, and 72 °C for 10 minutes to produce cDNA.

2.2.3. Polymerase Chain Reaction (PCR)

Using the cDNA templates obtained in the first stage and virus-specific primers, PCR procedures were conducted. At this point, 2µlcDNA, 5µl of 5X Green GoTaq® Flexi Buffer from Promega, 0.2 µl dNTP, 0.5 µl forward primer and 0.5µl of reverse primer each with 100 pmol, 1.5 µl MgCl2 (25 mM), 0.25µlTaq polymerase enzyme from Promega, and 0.25 µl Dimethyl sulfoxside (DMSO). The PCR themocycler was filled with the tubes.

The reaction took place at 94 degrees Celsius for 2 minutes for initial denaturation, 1 minute for denaturation during the cycle, and 30 seconds at 56 degrees Celsius (for PVM) for primer annealing. (62°C for other viruses), 2 minutes at 72°C for synthesis completion (primary extension) and 35 cycles of amplification were used. Finally, the reaction was completed by incubating the samples at 72°C for 10 minutes (final extension).

2.2.4. Agarose gel electrophoresis

Tris-borate-EDTA (TBE) buffer was used to prepare a 1.2% agarose gel for the electrophoresis technique examination of the PCR products that were obtained. Then, 7µlethidium bromide stock solution (10 mg/ml) was added to it. For one hour, the samples were electrophoresed at 100 V. The PCR products were evaluated in the gel imaging device after electrophoresis, and pictures of the products in the gel were taken.

2.2.5. Phylogenetic analysis of Potato leaf roll virus isolates

Five PLRV isolates' RT-PCR products that tested positive at the end were sent for sequencing analysis using the Sanger method in order to conduct phylogenetic investigations. The MEGA10 program was used to evaluate the raw data that was received from the sequencing (Kumar et al., 2018) Following that, BLAST was used to match the sequencing data to those of the reference isolates listed in the National Center for Biotechnology Information (NCBI) (Basic

Local Alignment Search Tool). By using the neighbor joining method, a phylogenetic tree was constructed, and the degree of relatedness was compared.

3. Results

3.1 Results of RT-PCR

Total RNAs were extracted from the 298 leaf samples that had previously been collected in order to identify the existence of viral agents in the potato production regions of the provinces of Afyon, Bolu, and Nevşehir. The extracted RNA samples were used to create cDNA using a hexamer primer in order to detect PLRV, PVM, and PVX. Then, using virus-specific primers, the RT-PCR technique was used.

As a result of RT-PCR performed with primers specific to PLRV, a band of the expected size (336 bp) was obtained in 43 leaf samples (Figure 2).

As a result of RT-PCR performed with PVX-specific primers, a band of expected size (562 bp) was obtained in 3 leaf samples (Figure 3).

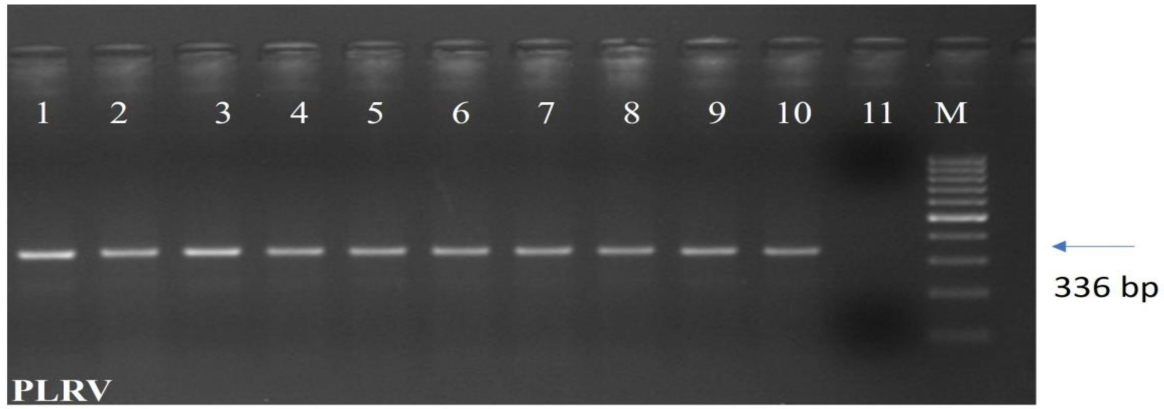

Figure 2. The RT-PCR results of some isolates were obtained with PLRV specific primers. M: 100 bp DNA Ladder (Fermentas), 1: Ş7-1, 2: Ş7-2, 3:Ş7-3, 4: Ş74, 5:Ş7-5, 6: Sa11-7, 7: Ş7-9, 8: Ş7-16, 9: NP22, 10: NP68, 11: Negative control.

***Şekil 2**. PLRV spesifik primerler ile elde edilen bazı izolatlara ait RT-PCR sonuçları M: 100 bp DNA markör (Fermentas), 1: Ş7-1, 2: Ş7-2, 3:Ş7-3, 4: Ş74, 5:Ş7-5, 6: Sa11-7, 7: Ş7-9, 8: Ş7-16, 9: NP22, 10: NP68, 11: Negatif kontrol.*

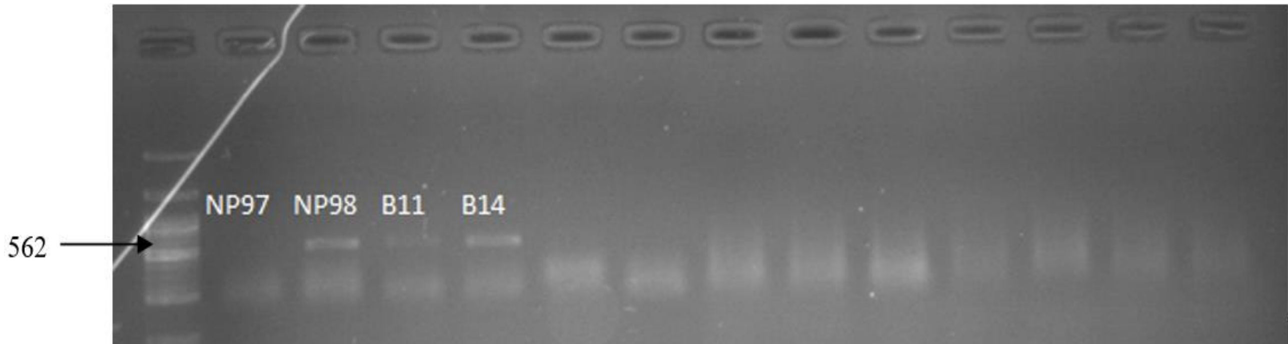

Figure 3. The RT-PCR results of some isolates obtained with PVX-specific primer.M: 100 bp DNA Ladder (Fermentas)

***Şekil 3.** PVX'e özgü primer ile elde edilen bazı izolatların RT-PCR sonuçları.M: 100 bp DNA markör (Fermentas)*

Table 2. The presences of PVM, PVX and PLRV in leaf samples taken from potato production areas of Bolu, Nevşehir and Afyon in the survey studies carried out in 2020.

***Table 2.** 2020 yılında yapılan sürvey çalışmalarında Bolu, Nevşehir ve Afyon patates üretim alanlarından alınan yaprak örneklerinde PVM, PVX ve PLRV varlığı.*

			Number of infected samples (Infection rate, %)			
Province	Number of samples tested	Number of samples infected	PLRV	PVX	PVM	PVA
Bolu	89	14	14 (15.73%)	-	-	-
Nevşehir	98	12	11 (11.22%)	1 (1.02%)	-	-
Afyon	111	20	18 (16.21%)	2 (1.80%)	-	-
Total	298	46	43 (14.42%)	3 (2.82%)	-	-

3.2. Phylogenetic relationships among Potato leaf roll virus isolates

The sequences of the partial coat protein region of five PLRV isolates (two from Nevsehir and three from Afyon) consisting of 337 nucleotides were obtained. The isolates obtained in this study were registered in the NCBI with the following accession numbers: Sa11-7: OP824688, S7-9: OP824689, S7-16: OP824690, NP22: OP824691, NP68: OP824692. BLAST analysis of the sequences of PLRV isolates showed 99-100% nucleotide and 100% amino acide identities with the reference isolates. The table of BLAST analysis of Turkish PLRV isolates is shown in table 4.5. Phylogenetic tree was constructed using the UPGMA method implemented in MEGA X (Kumar et al., 2018) based on the comparison of nucleotide sequences (Figure 4).

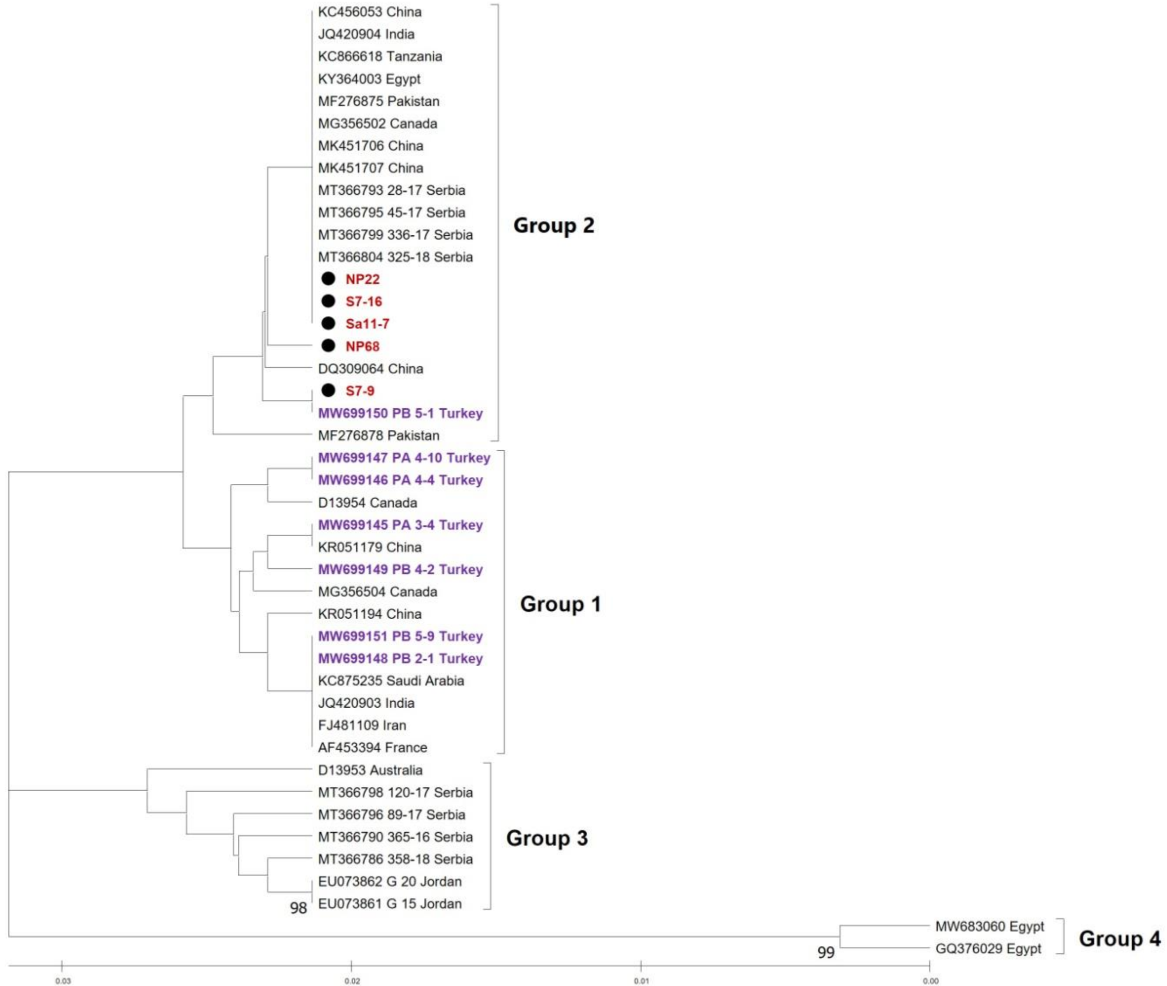

Figure 4. Phylogenetic tree constructed with PLRV isolates using UPGMA method. The evolutionary history was inferred using the UPGMA method The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Kumar et. Al. 2018). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. There were a total of 325 positions in the final dataset.

***Şekil 4**. UPGMA yöntemi kullanılarak PLRV izolatları ile oluşturulan filogenetik ağaç. Evrimsel tarih, UPGMA yöntemi kullanılarak çıkarılmıştır. En uygun ağaç gösterilmiştir. Önyükleme testinde (1000 kopya) ilişkili taksonların bir arada kümelendiği kopya ağaçların yüzdesi, dalların yanında gösterilir (Kumar et. Al. 2018). Ağaç, filogenetik ağacı anlamak için kullanılan evrimsel mesafelerle aynı birimlerde dal uzunlukları ile ölçeğe göre çizilmiştir. Nihai veri setinde toplam 325 pozisyon vardır.*

4. Discussion and Conclusion

The viruses that reduce productivity in many cultivated plants around the world cannot be directly controlled, just as other infections. The majority of plant virus management and control techniques focus more on prevention than on therapy. As a result, in viral infections, the accurate implementation of a disease control strategy depends on the early and

precise diagnosis of the pathogen and the application of sensitive procedures. Serological and molecular methods are employed to produce more accurate results in diagnosis (Erkan et al., 2011).

A variety of symptoms, including yellowing, a reduction in leaf size, stunting, curling and rolling of the top leaves, mosaic, vein clearing/banding, necrotic lesions, deformities and cracks in tubers, and crescent-shaped cavities in the tuber eyes, are all signs of potato viruses. Similar symptoms were observed in plants during surveys done in the provinces of Bolu, Nevşehir, and Afyon in 2021. By using the RT-PCR method, potato leaf samples from the provinces of Bolu, Nevşehir, and Afyon were tested for the presence of several viruses in this study.

The possibility that viruses that spread from year to year through tubers were present in the potato tuber seeds used for planting in these locations is one explanation for this. According to Çıtır and Özbayram (1982), an average of 95% of the seed tubers used by farmers are contaminated with at least one virus. Viruses also enter Turkey via imported seed tubers. Furthermore, Bostan and Haliloğlu (2004) discovered the identical circumstance for the seed tubers that the producers had obtained from the businesses in the areas where potato farming is widespread.

The absence of effective, reliable, and affordable virus testing methods in tubers before they are imported or utilized for seed purposes may be the primary cause of this issue. The major source of virus infection is the infected tuber, and the virus is subsequently transmitted throughout the year via vectors or mechanical mechanisms (Bostan et al., 2006). By using the RT-PCR technology, the presence of PLRV, PVA, PVM, and PVX viral agents was examined in this study's potato production regions of the Central Districts of Bolu, Nevşehir, and Afyon provinces. As a consequence, 180 out of 300 leaf samples collected from potato growing locations had one or more virus species found in them.

The most prevalent virus in plant leaf samples from the provinces of Bolu, Nevşehir, and Afyon was identified as PLRV with single infections. PLRV, PVM, and PVX were found to be the most prevalent viruses in Bolu, Nevşehir, and Afyon, respectively. Two samples from Afyon and one from Nevşehir each had one PVX-infected sample.

The most prevalent potato viruses, according to a study carried out in Erzurum by Yardımcı and Bostan (1999), were PLRV (42.2%), PVX (38.3%), and PVY (7%). Infections of PVX+PLRV (6.29%), PVX+PVY (2.96%), and PLRV+PVY were also found to be mixed. In samples lacking virus symptoms, the DAS ELISA approach was used to identify PVY, PLRV, and PVY+PVS infections. In contrast to our study, this one in Erzurum found that PVY was the virus with the lowest prevalence, whereas PLRV had the highest prevalence.

In this investigation, it was also possible to find combination infections of the aforementioned viral agents. In contrast to our research, it was found that PLRV (14.42%) was the most prevalent virus to infect potatoes, whereas PVM (5.61%) and PVX (1.02%) were found to be present at low levels. In one investigation, DAS-ELISA was used to check samples from field studies for the presence of PVX, PVS, PVY, PVA, PLRV, and PVM.

PVX, PVY, PVS, and PLRV were found in the samples to cause single infections, while PVY+PVS, PVY+PLRV, PVY+PVS+PLRV, PVS+PVA, and PVY+PVA were shown to induce mixed infections. In addition to positive results, the test also produced some negative outcomes. In contrast to our findings, viruses were identified to produce both combined infections (PVA+PVM) and single infections (PVA, PLRV, PVM, and PVX) in the samples (Güner, 2007).

In the phylogenetic tree, the isolates formed 4 different groups. Similarly, Ristic et al. (2021) reported that PLRV isolates were divided into 4 groups in the phylogenetic tree they made with CP sequence data. Within the scope of the study, newly obtained PLRV isolates from Turkey (Purple colored) were included in Group 2 together with isolates from Serbia, Canada, and Pakistan. On the other hand, PLRV Tokat isolates (Red colored) obtained in the master thesis made by Engür (2020), were included in group 1 together with isolates from China, Canada, India, Iran, and France. In the phylogenetic tree, while the isolates NP22, S7-16 and Sa11-7 clustered with isolates from Serbia, Canada, and Pakistan, the isolate NP68 clustered with China isolate in different branches. the isolate S7-9 clustered with the previously reported Tokat isolate (MW699150: PB5-1) (Figure 4.7).

Among the positive PCR products obtained as a result of RT-PCR studies, five samples for PLRV (S7-9, NP68, NP22, S7-16, Sa11- 7) were selected and sent to the sequence for analysis. As a result of the sequence analysis, the results were obtained from the 5 PLRV isolates and continued to be studied with these isolates. RT-PCR products obtained with PVX did not give good results in the RT-PCR, and it is planned to

work with different primers in the future.

Potatoes are an important food source in Turkey as well as in the whole world. These viruses, which are seen in potato production areas, cause significant yield loss in tubers, along with green parts infections, and continue their continuity with seeds to be used in the future. As with other disease agents and harmful insects, viruses cannot be combated with chemical means. For this reason, special attention should be paid to the fact that the tubers to be used as seeds are certified and free from viral factors. The farmers who produce potatoes in Turkey should be educated on this issue and their awareness should be raised. For the control of virus agents such as PLRV that can be transmitted persistently by aphids, aphids and other vector insects carrying these diseases should be combated. In addition, in order to take precautions against PVY, PVS, and PVX transported by mechanical means, attention should be paid to the cleanliness of the tools and equipment used during the harvest and care should be taken not to injure the plants during the green period.

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