Bitki Koruma Bülteni / Plant Protection Bulletin

http://dergipark.gov.tr/bitkorb

Original article

Serological and molecular detection of *Leek yellow stripe virus* infecting onion (*Allium cepa* L.) and leek (*Allium ampeloprasum* L.) in Ankara, Turkey

Ankara'da soğan (*Allium cepa* L.) ve pırasayı (*Allium ampeloprasum* L.) enfekte eden Pırasa sarı çizgi virüsü (*Leek yellow stripe potyvirus*)'nün serolojik ve moleküler olarak tanılanması

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ARTICLE INFO

Article history:
DOI: 10.16955/bitkorb.622150
Received : 19.09.2019
Accepted : 16.12.2019

Keywords:
onion, leek, LYSV, Ankara

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ABSTRACT

Onion (*Allium cepa* L.) has a high economic value in Turkey and it is widely used as main ingredient in Turkish menus. During 2018, 25% of 1.930.695 tons onion production in Turkey was produced in Ankara province. Recently, virus-like symptoms had been observed on onion fields but so far there was no study on onion viral diseases in Ankara province. *Leek yellow stripe virus* (LYSV) mainly infects leek (*Allium ampeloprasum* L.) but also infects and potentially causes yield reduction on onion. In August 2018, 45 onion samples show typical viral infection symptoms such as leaf stripes, leaf malformation, yellowing, and dwarfing and six leek samples showing severe yellow leaf stripe were collected from fields in Ankara province. 6 onion and 2 leek samples were positive to LYSV infection in DAS-ELISA. Total nucleic acid was then extracted from samples to be used in RT-PCR using a pair of specific primer to amplify a fragment of LYSV CP region. 6 onion and 2 leek samples were also positive in the RT-PCR by forming specific bands of 1020 bp on agarose gel. The remaining samples did not produce any band on agarose gel, thus were evaluated as negative for LYSV infection. Phylogenetic analysis showed that LYSV Ankara isolates were most similar to isolates from Serbia, Argentina and Germany. This study confirmed LYSV infection in onion and leek for the first time in Ankara province, Turkey.

INTRODUCTION

Onion (*Allium cepa* L.) and leek (*Allium ampeloprasum* L.) are among the most economically important vegetables cultivated in many regions in the world, including in Turkey, where these *Allium* species are inseparable main ingredient used in many Turkish menus. During 2018, 510.414 tons of onions were produced in Ankara province, which accounted for about 25% of the total onion produced in Turkey. Three hundred sixty-two tons of leeks were also produced in

Ankara province during the same time (TUIK 2019).

Two Potyviruses; *Onion yellow dwarf virus* (OYDV) and *Leek yellow stripe virus* (LYSV) are commonly found infecting *Allium* spp. around the world and potentially causes yield loss on onion (Bos 1983, Elnagar et al. 2011, Lot et al. 1998, Ward et al. 2009). Although rarely detected on *Allium* spp., the wide host range *Cucumber mosaic virus* (CMV) was identified on garlic crops planted in East Mediterranean of Turkey and possibly infecting *Allium* spp. in other regions of Turkey (Fidan 2010). ELISA and RT-PCR have been routinely used as rapid and accurate methods in the detection of onion viruses (Fajardo et al. 2001, Tuzlalı et al. 2016).

LYSV is transmitted mainly by several aphid species, such as *Aphis fabae* and *Myzus persicae*, as non-persistent manner, but can also be transmitted mechanically (Brunt et al. 1996, Lunello et al. 2002). The only open reading frame (ORF) in LYSV genome potentially encodes a polyprotein which includes NIa-Pro (nuclear inclusion a – proteinase), Nib (nuclear inclusion b), VPg (viral protein genome linked) and CP (coat protein) (Adams et al. 2005). CP gene of LYSV was observed to be around 864 bp and encodes a protein with around 288 amino acids (Gupta et al. 2017). In Turkey, LYSV was identified for the first time from infected leek samples that were collected in Marmara region (Korkmaz and Cevik 2009). Since then, it has been reported to infect onion, leek and other *Allium* spp. cultivated in East Mediterranean, Amasya province, and South Marmara region of Turkey (Fidan 2010, Fidan and Baloğlu 2009, Sevik and Akcura 2013, Tuzlalı 2018). Although Ankara is one of main onion producing area, there was no study about virus diseases on onion and leek conducted in this province so far. Therefore, viral symptomatic onion and leek samples were collected from fields in Ankara province and then tested by serological and molecular methods to detect virus infection on them and determine their infection rate.

MATERIALS AND METHODS

Samples collection

In August 2018, 34 samples were taken from Polatlı and 11 samples were taken from Haymana districts of Ankara province for a total of 45 onion samples. In addition, six leek samples were also taken from Polatlı district. The onion samples showed typical viral infection symptoms such as leaf stripes, leaf malformation, yellowing, and dwarfing; and six leek samples showing yellow leaf stripes. The presence of any aphid species in onion fields was also searched during field trips.

DAS-ELISA

Serological test using DAS-ELISA kit specific for each LYSV, OYDV and CMV detection was performed according to the manufacturer instructions (Bioreba, Switzerland). Absorbance values were measured at 405nm (A_{405}) using ELx808TM microplate reader (BioTek Instruments, Inc., USA). A sample was considered positive if A_{405} of the sample at least two times that of negative control. All samples were then tested by RT-PCR only against viruses that gave positive results in DAS-ELISA for confirmation and to obtain some of their nucleotide sequences to be used in phylogenetic analyses.

Total nucleic acid extraction and RT-PCR

Total nucleic acid was extracted from samples following procedure based on tris-EDTA buffer (Presting et al. 1995). 100-200 mg sample was ground in 1.5 ml extraction buffer (100 mM tris, pH 8.0, 50 mM EDTA, 500 mM NaCl, 10 mM 2-mercaptoethanol) then was placed into 2 ml tube. Plant extract was then centrifuged at 4.000 rpm, 3 min. 600 ml of plant suspension was then transferred into new 1.5 ml tube, 70 µl of 10% SDS was added then put in 65 °C for 10 min. After that, 200 µl 5 M potassium acetate was added then tube was put in ice for 30 min. The tube then centrifuged at 10.000 rpm, 10 min, 600 ml of suspension was then removed into new 1.5 ml tube. 300 µl cool isopropanol was added into the suspension then placed in ice for 25-30 min. The tube then centrifuged at 10.000 rpm, 10 min, and then the suspension was thrown away. 500 µl cool 70% ethyl alcohol was pipetted to the pellet then centrifuged twice at 10.000 rpm, for 2 and 10 min respectively. Ethyl alcohol was thrown away; tube was then dried on paper tissue for 10 min. 30 µl nuclease free water then added to the pellet. Total RNA was then measured using spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, USA). Extracted nucleic acids were then used in RT-PCR using a pair of specific primer, F-5′ TCACTGCATATGCGCACCAT 3′ and R-5′ GCACCATACAGTGAATTGAG 3′ to amplify a 1020 bp fragment of LYSV coat protein region (Fajardo et al. 2001).

All of samples were tested using two-step RT-PCR. The procedure to perform RT was done according to Thermo Fisher Scientific (USA) protocols. RT was done in a total volume of 20 µl containing 500-600 ng/µl total RNA, 1 µl (100 pmol/µl) random hexamer primer (Sentegen, Turkey), 2 µl dNTPs (10 mM) (GeneAll, South Korea), 4 µl RT buffer (250 mM tris-HCl (pH 8.3 at 25 °C), 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT) (Thermo Fisher Scientific, USA), 0.25 µl (40 U/µl) RNase Inhibitor (GeneAll, South Korea), 0.5 µl (200 U/µl) Reverse transcriptase (Thermo Fisher Scientific, USA) and Nuclease-free water. PCR was performed in a reaction volume of 25 µl which contains 2 µl cDNA, 1 µl $MgCl_2$

(25 mM), 0.6 µl of each primer (100 pmol/µl) (Oligomer Biyoteknoloji, Turkey), 0.8 µl dNTPs (10mM) (GeneAll, South Korea), 2.5 µl PCR buffer (GeneDirex, Taiwan), 0.3 µl Taq DNA polymerase (5 U/µl) (GeneDirex, Taiwan) and 17.2 µl Nuclease-free water. PCR cycles were carried out using thermocycler (Biometra, Germany), with cycles as follows: initial denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 60 s, 50 °C for 60 s and 72 °C for 60 s, and a final extension of 72 °C for 7 min (Fajardo et al. 2001). Products were visualized on a 1% (w/v) tris-acetate agarose gel stained with ethidium bromide. One of LYSV isolated from onions (LYSV-12.6Po) and 2 isolated from leek samples (LYSV-Ankara-Leek1 and LYSV-Ankara-Leek2) were sequenced then obtained nucleotide sequences were published in NCBI GenBank.

Host range characterization

LYSV isolated from 2 leek (LYSV-Ankara-Leek1 and LYSV-Ankara-Leek2) and one of onion samples (LYSV-12.6Po) were mechanically inoculated to two onion, leek and garlic plants to find out their ability to infect those species. Each gram of leaf samples were ground using mortar and pestle in 5 ml of 0.01 M Potassium phosphate buffer (pH 7) to create plant sap. Leaves of onion, leek and garlic were dusted with abrasive-celite then rubbed with plant sap using forefinger. The inoculated plants were then rinsed with distilled water and kept in the greenhouse at 25-27 °C (Hill 1984). Symptoms expression were examined during a period of four to five weeks after inoculation. The infection on the inoculated plants were confirmed by RT-PCR.

Phylogenetic analyses of LYSV Ankara isolates

The nucleotide sequence of partial coat protein (CP) gene of one LYSV isolate infecting onion (accession no. MN070127) and two LYSV isolates infecting leek (accession no. MN070132 and MN070133) in Ankara were compared with other isolates from around the world that were obtained from NCBI GenBank. Homologus genes were searched using BLAST program which is available in NCBI website. All sequences were aligned together by ClustalW version 1.6 with default parameters. Similarity percentage among isolates (identity matrix) was calculated using Sequence Demarcation Tool (SDT) v1.2 software (Muhire et al. 2014). A phylogenetic tree was built using maximum-likelihood algorithm on MEGA7 software (megasoftware.net) (Kumar et al. 2016). Tamura-Nei parameter with 1000 bootstrap replicates was used to test the significance of isolate clusters statistically (Tamura and Nei 1993).

MEGA7 software was also employed in the evolutionary analyses of genetic distance and diversity among isolates compared in this study. Tamura-Nei parameter was applied as statistical model in the analysis. Standard error (S.E.) of the analysis was determined the mean evolutionary distances using 1000 bootstrap replicates (Tamura and Nei 1993). The values of within group, between groups and overall, as well as values of mean evolutionary diversity within subpopulations, interpopulation, entire population, and also of evolutionary differentiation coefficient were calculated by the number of base substitutions per site using the nucleotide sequences. The evolutionary distances and diversity analyses included both transitional and transversional substitutions for each sequence pair, with gaps/missing data were completely removed (Kumar et al. 2016).

RESULTS

Six onions and two of leek samples were positive to LYSV infection in DAS-ELISA test. All samples were negative to OYDV and CMV. The six onions and two leek samples positive to LYSV in DAS-ELISA were also gave positive results in RT-PCR by formed specific bands of 1020 bp on agarose gel following electrophoresis (Figure 1). The remaining onion samples did not form any band on agarose gel, thus concluded as negative to LYSV infection.

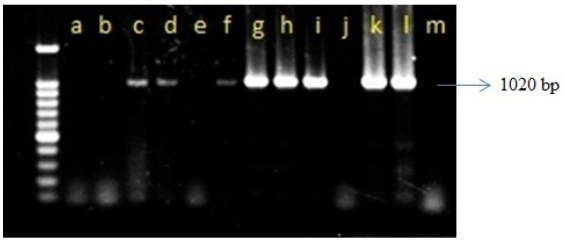

Figure 1. RT-PCR amplification result of 1020 bp fragment of LYSV coat protein region. Onion isolates no. LYSV-4.3Po, LYSV-12.3Po, LYSV-12.6Po, LYSV-12.8Po, LYSV-20.2Po, LYSV-20.6Po (c, d, f, g, h, i), and leek isolates no. LYSV-Ankara-Leek1 and LYSV-Ankara-Leek2 (k, l) were positive to LYSV infection. Onion samples no. 1.3Hy, 2.2Hy, 2.7Po, 4.5Po (a, b, e, j) were included as samples negative to LYSV infection. Negative control (m). 100 bp marker (GeneDirex, Taiwan)

The name LYSV-4.3Po, LYSV-12.3Po, LYSV-12.6Po, LYSV-12.8Po, LYSV-20.2Po and LYSV-20.6Po were assigned to each LYSV isolated from onion, whereas LYSV-Ankara-Leek1 and LYSV-Ankara-Leek2 were assigned to each LYSV isolated from leek. The naming was based on the field number where sample were collected and the sample number. Po stands for "Polatlı" since they were taken from this district. NCBI GenBank accession no. MN070127, MN070132 and MN070133 were obtained for isolates no. LYSV-12.6Po,

LYSV-Ankara-Leek1 and LYSV-Ankara-Leek2 respectively. LYSV infected leeks showing typical yellow stripes symptom while infected onions showed different symptoms such as leaf malformation, leaf stripes and leaf yellowing (Figure 2).

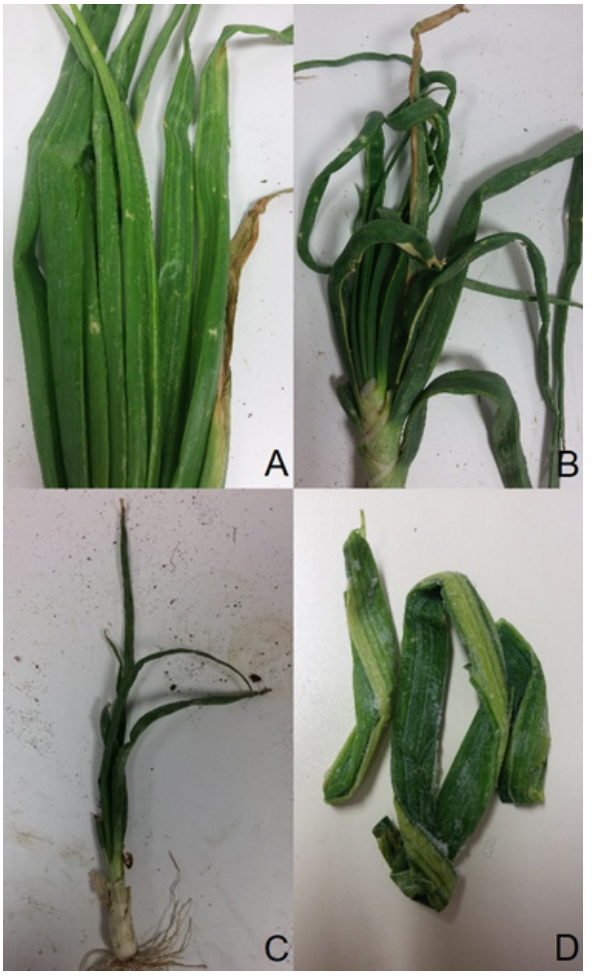

Figure 2. Symptoms on onion and leek infected with LYSV. A) Leaf stripes on onion, B) leaf malformation and yellowing on onion, C) severe leaf malformation on onion, D) yellow leaf stripes on leek

Under greenhouse condition, inoculation of LYSV-12.6Po produced leaf malformation and yellowing symptoms on onion, mild yellow stripes and yellowing on leek, and mild mosaic on garlic. Whereas, inoculation of LYSV-Ankara-Leek1 and LYSV-Ankara-Leek2 produced yellowing on onion, yellow stripes on leek, and mosaic symptoms on garlic. Infections on all inoculated plants were confirmed by RT-PCR (Table 1). These results concluded that isolates no. LYSV-12.6Po, LYSV-Ankara-Leek1 and LYSV-Ankara-Leek2 were able to infect onion, leek and garlic. Calculation by SDT v1.2 software showed that the three LYSV Ankara isolates shared 95.1-99.1% similarities among themselves and 78.2-96.8% similarities to isolates from other countries. A Serbian isolate (KR075504) had the highest similarities (94.8-96.8%) and an isolate from China (AJ409307) had the lowest similarities (78.2-78.7%) to Ankara isolates based on partial CP gene sequences comparison. The constructed phylogenetic tree revealed that isolates that were analysed in this study were divided into two groups: 1 and 2. The three LYSV Ankara isolates together with isolates from Serbia, Germany, the Netherlands, Czech, Italy, Japan and Argentina belong to group 1. Group 2 consisted of isolates belong to Asia Pacific countries such as China, India, Australia, New Zealand, and Mexico (Figure 3).

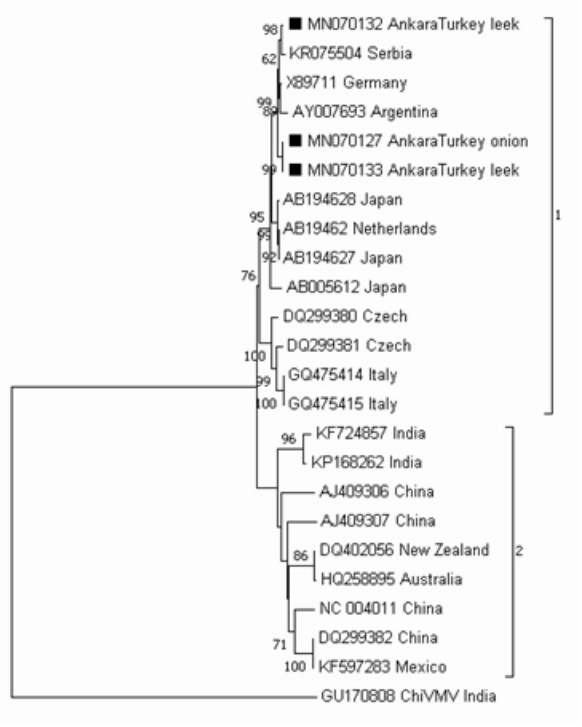

Figure 3. Maximum-likelihood tree based on analysis of partial nucleotide sequences of coat protein (CP) coding region of three LYSV ankara isolates (MN070127, MN070132, and MN070133) and 20 isolates from different countries. The compared isolates were clustered into two groups (1 and 2). An Indian isolate of *Chilli veinal mottle virus* (ChiVMV) was used as outer group

Analyses of evolutionary distances revealed that the mean evolutionary distance value of group 1 and 2 were 0.140±0.012 and 0.249±0.017 respectively, with an overall value of 0.239±0.016 for all tested isolates. Mean evolutionary distance between group 1 and 2 was 0.307±0.022. Mean evolutionary diversity within subpopulations value (0.194±0.012) was higher than that of interpopulation (0.044±0.006) in the genetic evolutionary analysis. Whereas,

Table 1. Isolates of LYSV infecting onion and leek in Ankara that were identified in this study

Sample no.	District	Symptoms	ELISA	RT-PCR
1.10Po	Polatlı	Leaf malformation, yellowing	+	+
2.1Po	Polatlı	Leaf malformation, yellowing	+	+
2.3Po	Polatlı	Leaf malformation	+	+
2.8Po	Polatlı	Leaf malformation	+	+
3.6Po	Polatlı	Yellow leaf stripes	+	+
4.4Po	Polatlı	Yellowing, leaf necrosis	+	+
1. Leek	Polatlı	Yellow leaf stripes	+	+
2. Leek	Polatlı	Yellow leaf stripes	+	+

+ = Positive to LYSV infection

the mean evolutionary diversity value of all population was 0.239±0.015 and the evolutionary differentiation coefficient was determined to be 0.185±0.021.

DISCUSSION

Leek production in Ankara province was negligible compared to onion (TUIK 2019). Therefore, in this study we focused mainly on the detection of viruses on onion crops. However, we also took six symptomatic leek samples that we came across during our field trips as additional data for our study since leek is LYSV main host. OYDV and CMV were not detected in any samples during screening by DAS-ELISA. So, RT-PCR was only performed on all samples against LYSV. Previous studies identified OYDV and CMV on *Allium* species cultivated in other regions of Turkey (Fidan 2010, Sevik and Akcura 2013, Tuzlalı et al. 2016).

With a low infection rate of around 13.3%, it can be suggested that LYSV was not widespread among onions cultivated in Ankara. There were no aphid vectors found in fields during our survey. Onion aphid (*Neotoxoptera formosana*) has not been reported in Turkey (CABI 2001). Besides that, onion fields in Ankara, in general, are routinely sprayed with insecticides since it is considered a high value crop. LYSV is not known to be a seedborne virus (Bos et al. 1978). Therefore, the low LYSV infection rate could most likely be attributed to the absence of aphid vectors on fields that limit the spread of LYSV. Low LYSV infection rates on onions were also reported on surveys in other regions of Turkey and other countries (Dovas and Vovlas 2003, Fidan 2010, Sevik and Akcura 2013, Vučurović et al. 2017). However, infection rate of 60.1% was observed in Tehran region of Iran (Shahraeen et al. 2008).

All of positive samples were collected from Polatlı district. Besides that, both of positive leek samples were also taken from Polatlı. These results gave impression that LYSV is spread more in Polatlı than Haymana. However, this data was probably normal since 65% of onion in Ankara was actually cultivated in Polatlı and most of our samples were taken from this district. Some of onion cultivars planted by farmers in Haymana might have some resistance or tolerance to LYSV since there was different onion types planted in both districts. Onion planted in Haymana were mostly 'Red bulbs' type, while in Polatlı were 'White bulbs' type. Resistant onion cultivars could also be one of reasons for low LYSV infection rate in this study. If so, the use of resistant or tolerant cultivars can be applied in the management of the virus.

Different garlic (*Allium sativum* L.) cultivars had been known to produce different symptoms upon infection by LYSV (Lot et al. 1998). So, the diverse symptoms on infected onion probably were also due to response of different cultivars to the virus. Viral like symptoms on negative samples probably were caused by other pathogen infection such as Aster yellows phytoplasma (Khadhair et al. 2002). Nutritional deficiency also sometimes produced viral-like symptoms on onions (Thangasamy et al. 2018).

Identity matrix and the maximum-likelihood tree analyses of partial LYSV CP gene revealed that LYSV Ankara isolates are more closely related to LYSV isolates from Argentina, Japan, and European countries (Serbia, Germany, the Netherlands, Czech and Italy) than those from Asia Pacific countries such as China, India, Australia and New Zealand. These results showed that there was more genetic interchange among LYSV Ankara isolates and isolates from European countries

which located closer to Turkey than Asia Pacific countries. Japanese isolates used in this study probably were originated from European regions instead of the much closer Asia regions since they are more similar to European isolates. Whereas, one isolate from Mexico are more similar to Asia Pacific isolates and probably was introduced from these regions (Figure 3).

Among all compared isolates, LYSV isolates from Serbia (KR075504), Argentina (AY007693) and Germany (X89711) had the highest homology (93.2-96.8%) to LYSV Ankara isolates. The Serbian isolate (277-13), Argentines isolate (LYSV-L-Arg) and Dutch isolate (no isolate name) were all isolated from leek and had also been reported to have high nucleotide identity to each other by previous analyses (Lunello et al. 2002, Vučurović et al. 2016). LYSV isolates and strains were shown to have different capability to infect *Allium* species (Lunello et al. 2002, Van Dijk 1993). A LYSV isolated from garlic (LYSV-G) was examined to be very difficult to infect leek, while LYSV-L-Arg had been observed to have some difficulty to infect onion but easily infect leek and garlic (Lunello et al. 2002). All three Ankara isolates infected onion, leek and garlic by mechanical inoculation based on our study result. This finding indicated that LYSV-12.6Po, LYSV-Ankara-Leek1 and LYSV-Ankara-Leek2 were not only similar in nucleotide identity but also biologically similar to LYSV-L-Arg (AY007693). LYSV infecting onions and garlics were also reported from other regions of Turkey (Fidan 2010, Sevik and Akcura 2013). This could be an indication that some LYSV isolates in Turkey might have similar biological properties to Ankara isolates and LYSV-L-Arg which capable to infect leek, onion and garlic rather easily. Unfortunately, there is no information regarding nucleotide sequences of LYSV isolates from other regions of Turkey available in NCBI GenBank, thus their homology analysis against Ankara isolates is still not possible to be done up to now.

Analyses of evolutionary distances results suggested that LYSV isolates in group 1 (including Ankara isolates) were highly similar to each other; whilst isolates belong to group 2 had lower similarity among them. Isolates clustered in group 1 had relatively low similarity to isolates in group 2. LYSV isolates examined in these phylogenetic analyses came from various countries in the world and they have rather high diversity in the CP gene sequence and rapid evolutionary differentiation among them based on analyses of evolutionary diversity. These findings were supported by identity matrix study that found a high (18%) diversity in partial CP gene sequences of all LYSV isolates analyzed, which means that they are relatively low conserved. However, since they were taken from nearby areas, the partial CP gene sequences of LYSV Ankara isolates were shown to be much more conserved, with only 4% diversity.

The results of this study confirmed for the first time LYSV infection on onion and leek in Ankara province, Turkey. LYSV Ankara isolates are most closely related to isolates from Serbia, Argentina and Germany based on analysis of partial CP gene. Onion cultivars that commonly planted in Turkey are necessary to be tested for their resistance against LYSV.

ACKNOWLEDGEMENTS

Authors are grateful to Scientific Research Projects Coordinator of Ankara University for the funding of this research (Project No. 18H0447001). This research was part of a Doctoral dissertation at Plant Protection Department, Ankara University. The abstract was published in the 1. International Molecular Plant Protection Congress (10-13 April 2019, Adana, Turkey).

ÖZET

Soğan (*Allium cepa* L.) Türkiye'de yüksek derecede ekonomik öneme sahip olup, Türk menülerinde yaygın olarak kullanılmaktadır. 2018 yılında 1.930.695 tonluk Türkiye üretiminin %25'i Ankara'da yapılmıştır. Son yıllarda soğan tarlalarında virus benzeri belirtiler gözlenmiş ancak Ankara bölgesinde soğan virus hastalıkları üzerine bir çalışma yapılmamıştır. *Leek yellow stripe virus* (LYSV) esas olarak pırasayı (*Allium ampeloprasum* L.) enfekte etmekle birlikte soğanı da hastalandırmakta ve verim düşüklüğüne de neden olmaktadır. 2018 yılı Ağustos ayında, tipik virüs belirtisi, örneğin yaprakta şeritler, yaprak deformasyonu, sararma ve cüceleşme belirtileri gösteren 45 soğan örneği ve yapraklarda şiddetli sarı çizgi belirtisi tasıyan 6 pırasa örneği tarladan toplanmıştır. 45 soğan örneğinin 6 tanesi, 6 pırasa örneğinin 2 tanesi DAS-ELISA çalışmalarında LYSV'ye karşı pozitif sonuç vermiştir. Örneklerden elde edilen toplam nükleik asit RT-PCR'da LYSV'ye karşı geliştirilen örtü proteinine spesifik primerlerle amplifiye edilmiştir. 6 soğan ve 2 pırasa örneği RT-PCR çalışmalarında da pozitif sonuç vermiş ve agarose jelde 1020 bp büyüklüğünde bir bant oluşturmuşlardır. Diğer örnekler agarose jelde bir bant oluşturmamış ve bunlar LYSV enfeksiyonu açısından negatif olarak değerlendirilmişlerdir. Yapılan filogenetik analiz sonucunda LYSV Ankara izolatlarının Sırbistan, Arjantin ve Almanya izolatları ile benzerlik gösterdiği görülmüştür. Bu araştırma ile LYSV enfeksiyonu Ankara ilinde ilk olarak kanıtlanmış bulunmaktadır.

Anahtar kelimeler: soğan, pırasa, LYSV, Ankara

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Cite this article: Santosa, A, Ertunç, F. (2020). Serological and molecular detection of Leek yellow stripe virus infecting onion (Allium cepa L.) and leek (Allium ampeloprasum L.) in Ankara, Turkey. Plant Protection Bulletin, 60-1. DOI: 10.16955/bitkorb.622150

Atıf için: Santosa, A, Ertunç, F. (2020). Ankara'da soğan (Allium cepa L.) ve pırasayı (Allium ampeloprasum L.) enfekte eden Pırasa sarı çizgi virüsü'nün (Leek yellow stripe potyvirus) serolojik ve moleküler olarak tanılanması. Bitki Koruma Bülteni, 60-1. DOI: 10.16955/bitkorb.622150