

Bitki Koruma Bülteni / Plant Protection Bulletin

<http://dergipark.gov.tr/bitkorb>

Original article

Prevalence and molecular characterization of Tomato spotted wilt virus in pepper fields in Tokat province

Tokat ilinde biber alanlarında Tomato spotted wilt virus'un yaygınlığı ve moleküler karakterizasyonu

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ARTICLE INFO

Article history:

DOI: [10.16955/bitkorb.898371](https://doi.org/10.16955/bitkorb.898371)

Received : 17-03-2021

Accepted : 03-06-2021

Keywords:

pepper, RT-PCR, TSWV, Tokat

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ABSTRACT

The study was conducted in Tokat Center, Niksar, Erbaa and Pazar districts where peppers were grown in the summer of 2016, and leaf samples were collected from plants suspected of the virus. During the surveys, a total of 324 plant samples were collected and the infected pepper samples were subjected to DAS-ELISA test with (Tomato spotted wilt virus) TSWV-specific antiserum, and RT-PCR was performed with virus-specific primers. In DAS-ELISA studies, 324 plants were tested and 13% of the samples were found to be TSWV infected. Samples that were positive in ELISA test were subjected to RT-PCR with nucleocapsid gene specific primers in the S segment and three samples were sent for sequence analysis. According to results, Turkey TSWV isolates Np gene region have shown 98-99% nucleotide identity with the isolates from France and South Korea and grouped with them same group.

INTRODUCTION

In Turkey, pepper cultivation is widely done in greenhouse during the winter and open fields during summer months. Tokat province is one of the important vegetable production which has a microclimate in Turkey. Peppers where cultivated in open fields is more susceptible to virus infections (Bogatzevska et al. 2007). To date more than 100 viral pathogens have been detected that infect pepper crops and 43 of them have been reported to have natural infections (Edwardson and Christie 1986). *Cucumber mosaic virus* (CMV) (Chen et al. 2011, Garcia-Arenal et al. 2000, Nakazono-Nagaoka et al. 2005, Oreshkovikj et al. 2018), *Potato virus Y* (PVY) (Fereser et al. 1993, Singh et al. 2008), *Tomato spotted wilt virus* (TSWV) (Roggero et al. 2002),

Tomato mosaic virus (ToMV) (Gilardi et al. 2004), *Tobacco mosaic virus* (TMV) (Boukema 1982), *Potato virus X* (PXV) (Palloix et al. 1994), *Beet curly top virus* (BCTV) (Chen et al. 2011), *Pepper yellow leaf curl virus* (PYLCV) (Dombrovsky et al. 2010), *Pepper mild mottle virus* (PMMoV) (Genda et al. 2007, Guldur and Caglar 2006.), and *Pepper veinal mottle virus* (PeVeMoV) (Fajinmi 2013) are the most important and common viruses infecting pepper plants.

Since TSWV first report in Australia in 1915 (Brittlebank 1919), it has been reported in over 60 countries worldwide (Karavina and Kubba 2017). In Turkey, TSWV was firstly detected in lettuce by Tekinel et al. (1969), then were determined in tobacco plants in Çanakkale region with the rate of infection

of 80-100% (Azeri 1981). Then it was also reported on tomato, pepper, eggplants, lettuce and squash from different parts of Turkey (Arli-Sokmen et al. 2005, Azeri 1994, Guldur et al. 1995, Kamberoglu and Alan 2011, Kamberoglu et al. 2009, Turhan and Korkmaz 2006, Yardimci and Kilic Cular 2009).

The TSWV is the type member of *Orthotospovirus* genus in the family Bunyaviridae (Adams et al. 2017, Milne and Francki 1984) and transmitted by thrips (Thysanoptera, Thripidae) especially *Frankliniella occidentalis* in a persistent and circulative manner (Mandal et al. 2007, Mound 2001, Todd et al. 1995). The genome of TSWV consists of three single-stranded RNAs: the large (L) negative-sense RNA and the middle (M) and small (S) ambisense RNAs (Adkins 2000, Peiro et al. 2014). The *Sw5* gene in tomato and *Tsw* resistance in pepper provide resistance against to the TSWV. However, in different studies, researchers have reported resistance-breaking isolates in both peppers (Deligoz et al. 2014, Gabor et al. 2012, Hobbs et al. 1994, Margaria et al. 2004, Roggero et al. 2002, Sharman and Persley 2006) and tomatoes (Aramburu and Marti 2003, Debreczeni et al. 2014, Fidan 2016, Lian et al. 2013, Lopez et al. 2011, Margaria et al. 2004, Peiro et al. 2014).

In Tokat, presence of TSWV in tomato has been reported by Sin (2015), however there is no available information in pepper plants yet. In recent years, the incidence and spread of TSWV have reached high levels at pepper-producing areas in Tokat. There are many complaints about TSWV from many farmers in Tokat province and there are very few studies on the molecular studies of the virus. This study was conducted to determine occurrence and incidence of TSWV in pepper-growing areas in Tokat and to investigate whether there are isolates that breaking resistance. For this reason, this study involves field surveys combined with DAS-ELISA and RT-PCR method to determine TSWV in pepper in Tokat.

MATERIALS AND METHODS

Survey

Field surveys were carried out to detect viral agents causing disease in the pepper production fields of Tokat province. For this purpose, surveys were carried out on pepper growing fields in the Tokat Center, Turhal, Pazar, Erbaa and Niksar districts of Tokat province. A total of 324 plant samples was collected from the leaves of pepper plants showing symptoms of viruses in the survey areas.

Serological assay

Collected plant samples were subjected to DAS-ELISA test with TSWV specific antiserum.

DAS-ELISA studies were carried out according to the method reported by Clark and Adams (1977), and taking into account the rates specified by the commercial company (BIOREBA AG) from which ELISA kits were supplied.

Molecular assay

Total RNA extraction was performed from plants that gave positive reaction as a result of DAS-ELISA test and RT-PCR studies were carried out with nucleocapsid gene (Np) specific primers in the S segment of TSWV virome. RNA isolation was done according to protocol described by Astruc et al. (1996). Complementary DNA (cDNA) synthesis was performed using extracted RNA with hexamer primers. The cDNA synthesis was carried out in a total reaction mixture of 20 µl including 2.5 µl RNA, 1.0 µl hexamer primers, 0.2 µl of 25 mM dNTPs, 0.5 µl RNase inhibitor, 2.0 µl of 10x RT buffer, 1.0 µl reverse transcriptase enzyme (Thermo Scientific, USA) and sterile ultra-pure water. The reaction mixture was incubated at 25 °C for 5 min and 42 °C for 60 min, followed by incubation at 85 °C for 5 min. cDNA was used as template for reverse transcriptase-polymerase chain reaction (RT-PCR). PCR was carried out in a 25 µl mixture containing 2.5 µl of cDNA, 2 µl of 10 mM dNTPs, 2 µl of 25 mM MgCl₂, 5 µL of 5X Green GoTaq Reaction buffer and 0.5 µl of 10 µM of TSWV Np specific primers (F 5' AAC CTG CAG CTG CTT TCA AGC AAG TTC 3' and R 5' ACA ACT TTT AGG ATC CTC ATG TCT AAG GTT 3') (Maiss et al. 1991), 0.25 µl of 5 units µl⁻¹ Taq DNA polymerase (Promega, USA), and distilled water. PCRs were performed in the thermocycler (Techne Prime Thermal Cycler) using the conditions described below. The PCR conditions were as follows: initial denaturation at 94 °C for 2 min; 35 cycles of denaturation at 94 °C for 30 sec., annealing at 52 °C for 30 sec., and elongation at 72 °C for 2 min. The final cycle was followed by extension at 72 °C for 10 min. Then, PCR products were analyzed by on 1.2% agarose gel containing ethidium bromide and visualized with a UV transilluminator.

Phylogenetic assay

For phylogenetic analysis, after RT-PCR analysis, three PCR products were sent for sequence analysis in both directions (forward and reverse) to Sanger technology. The obtained data were cleaned from beginning and end by using Chromas computer program (Chromas 2.6.6 version) and saved as a consensus file. The obtained nucleotide sequences were analyzed by Molecular Evolutionary Genetics Analysis using the Neighbour-joining tree model with 1,000 replications (software MEGAX, Kumar et al. 2018). The Bootstrap analysis was performed with 1000 replications.

RESULTS

Survey

Within the scope of the study, surveys were carried out on pepper growing areas in the districts of Tokat Central (117), Pazar (46), Erbaa (95) and Niksar (66) of Tokat province in

July-August and a total of 324 leaf samples were collected. Virus-specific symptoms like severe mosaic, chlorosis, leaf deformation, ringspots, mottling, vein clearing were observed on leaves collected during the surveys (Figure 1).

DAS-ELISA results

As a result of DAS-ELISA test, macroscopically samples showing yellow color change and samples with a value of twice the absorbance of the negative sample in the ELISA reader and more were evaluated as positive.

TSWV presence was detected in the samples tested. Out of 324 tested plants 13% of them were detected as infected with TSWV. Considering the presence of the virus by district, the infection rates were detected as 13.7% (n=16), 6.32% (n=6), 12.1% (n=8), and 26.1% (n=12), Tokat Center, Erbaa, Niksar and Pazar, respectively in peppers.

Molecular and phylogenetic results

The presence of TSWV in pepper plants was confirmed by RT-PCR using TSWV specific primers. The expected bands (approximately 800 bp) were observed on 1.2% agarose gel (Figure 2). The sequences analysis was obtained belong to ,

129 s. gene of three TSWV isolates in this study. Three Turkish TSWV isolates were sequenced and submitted to GenBank with the accession numbers: MW751975 (B1), MW751976 (B2), MW751977 (B3). Comparison of sequences of TSWV isolates showed that Turkish TSWV isolates shared 98-99% nucleotide identity with references isolates from Genbank. The nucleotide sequences of TSWV isolates were compared with reference isolates and phylogenetic analysis were performed. Based on the results, the isolates were divided into two major groups (resistance breaking (RB) and non-breaking (NRB) isolates) Turkish TSWV isolates grouped with French and South Korean NRB isolates. These isolates were not grouped with previous reported RB isolates KM379141, KM379142 and MH367502.

DISCUSSION AND CONCLUSION

Pepper ranks second after tomato in terms of vegetable cultivation and vegetable production is an important source of income for farmers. Previously, pepper leaf samples were collected from pepper growing areas Tokat Central, Turhal, Pazar, Erbaa and Niksar districts of Tokat in July-August 2016. During the surveys, typical TSWV symptoms



Figure 1. Symptoms on infected plants collected during surveys

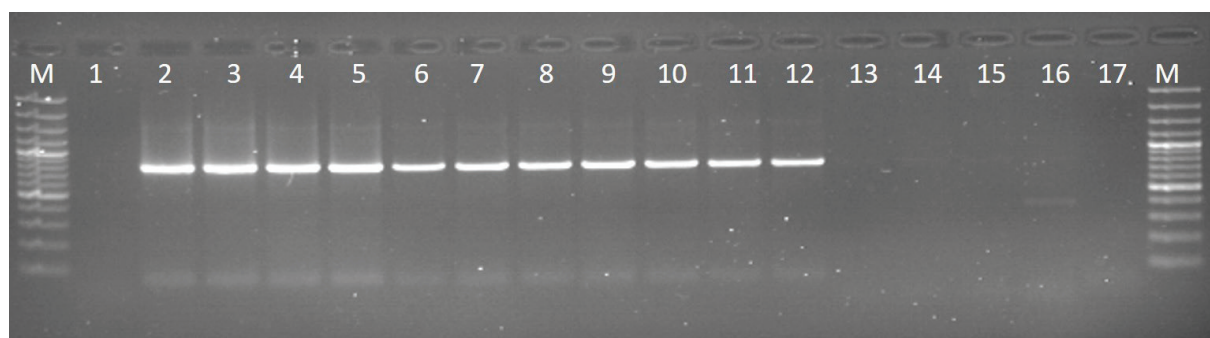


Figure 1. Agarose gel images of RT-PCR results. M: 100 bp Ladder (Fermantas), 1: Negative control, 2-12: TSWV positive samples, 13-17: TSWV negative samples

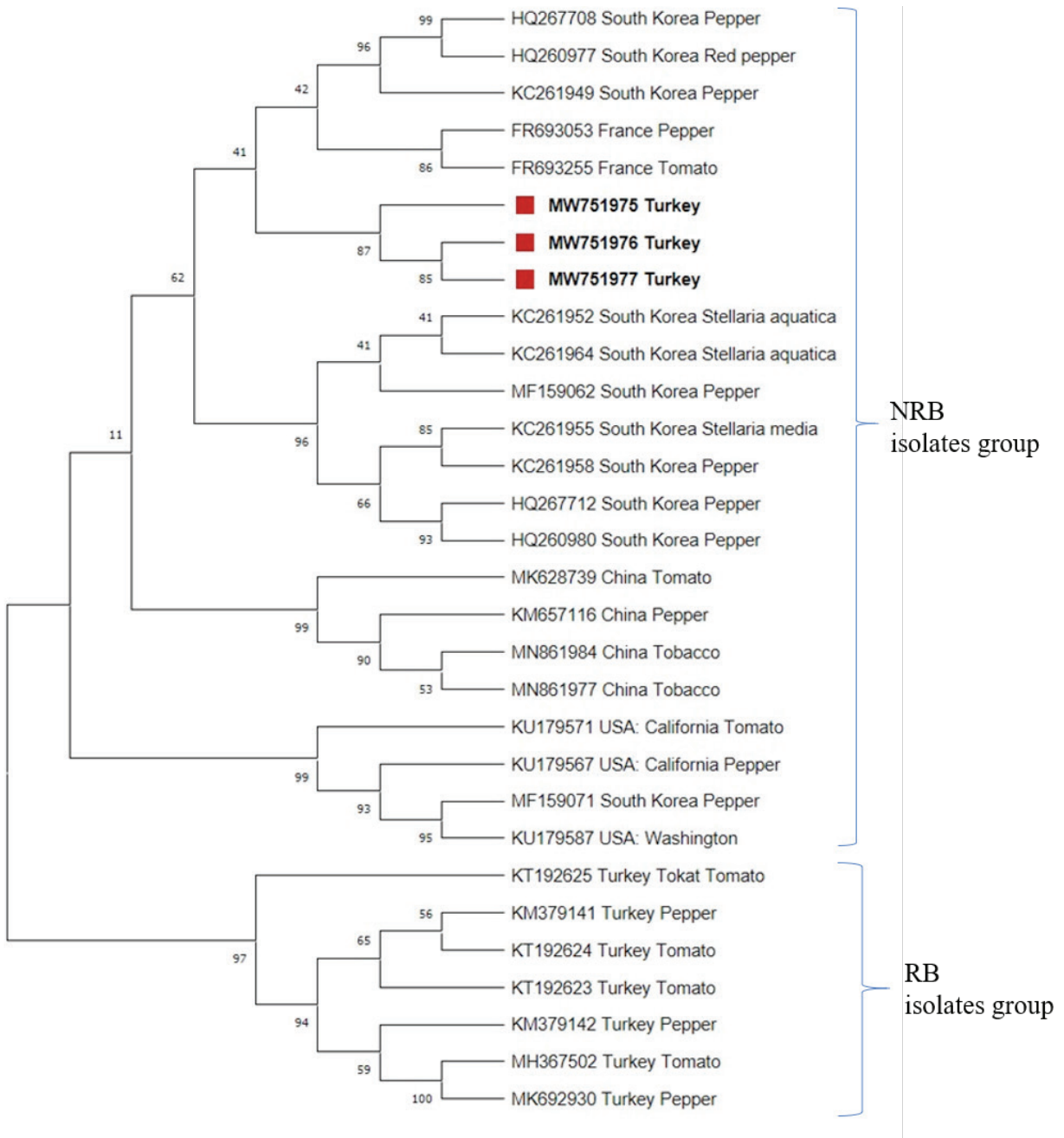


Figure 3. Pylogenetic tree of three Turkish isolates and references isolates. Sequences obtained in this study are shown in bold colour and red mark.

such deformations on the leaves of the plants, yellowing, discoloration of the veins, mosaic, excessive whitening, deformations in the fruits, and ring spot symptoms were observed surveyed area. The results of survey observations were similar to the reported studies of Deligoz et al. (2014), Buzkan et al. (2013), and Bozdogan and Kamberoglu (2015).

The presence of TSWV and other important viruses such as CMV, PVY, PMMoV, and AMV were detected by DAS-ELISA in the samples collected during the surveys. According to DAS-ELISA test results, the rates of viruses

were 43%, 25%, 13%, 15.3%, 11.9%, 9.4% CMV, TSWV, AMV, PMMoV, PVY respectively (not published). Similarly, the presence of these viruses in peppers has been reported by different researches (Arli-Sokmen et al. 2005, Buzkan et al. 2013, Ozdag and Sertkaya 2017). Ozdag and Sertkaya (2017) reported that PVY, CMV, *Beet western yellows virus* (BWYV), *Potato leaf roll virus* (PLRV), *Potato X virus* (PVX), *Tomato mosaic virus* (ToMV) and TSWV were identified in pepper growing areas of Iskenderun and Samandağ districts in Hatay. TSWV also infect other vegetables. Bozdogan

and Kamberoglu (2017) reported that TSWV was detected in 526 samples (88%) from 156 tomato (81%), 316 pepper (92%), 54 lettuce (93%) plants, which are collected from greenhouses in Antalya province with DAS-ELISA tests.

TSWV was firstly observed in tomatoes in the region, and typical symptoms were also observed in peppers over time. The presence of the TSWV was serologically and molecularly determined in this study. The TSWV isolates did not group based on geographical origin, as previously reported by Zindovic et al. (2014) and Karavina and Gubba (2017), but was grouped as resistance breaking (RB) and non-breaking isolates (NRB). In Turkey, RB isolates reported in Samsun and Antalya provinces in pepper plants. Molecular analysis of the TSWV nucleocapsid gene showed that Turkish TSWV isolates from this study were very similar (98-99%, 99.02%) to each other than non-breaking isolates at nucleotide and amino acid sequence levels. These isolates were showed relativity with the previously reported RB isolates KM379141, KM379142 and MK692930. In pylogenetic tree, the isolates were divided into two major groups as RB and NRB isolates. RB isolates reported by Deligoz et al. (2014) from Samsun province (KM379141, KM379142) and Fidan and Sarı (2019) (MK692930, MH367502) from Antalya province showed different clustering in the pylogenetic tree. Three Turkish TSWV isolates grouped with NRB isolates.

Pepper production constitutes an important source of income for Tokat province. In recent years, farmer have given up pepper farming due to yield losses caused by diseases and pests especially viral diseases that do not have chemical control. Due to the easy transmission of viruses [mechanically and by insects (*Frankliniella occidentalis*)] and open field cultivation, yield losses cannot be avoided. While cultural precautions are the most important method to be taken for the management of viral diseases, it is necessary to know their molecular structures well for the correct diagnosis and detection of them. By the improvements on molecular techniques, the diagnosis of viral agents can be done in a shorter time and more accurately in all production areas. In this study, the important viruses were determined both serologically and molecularly in Tokat. Previously, TSWV infections in tomato plants were reported by Sin (2015). This is the first molecular assay that had been employed in characterizing TSWV in pepper in Tokat province. Only partial nucleocapsid gene of S segment of TSWV were studied in this study. It is reported that the TSWV RB isolates related to NSm protein, which has a point mutation on the cell to cell movement gene, caused resistance breaking on TSWV resistant tomato (Fidan and Sarı 2019, Lopez et al. 2011). However, this study also showed that Np gene region of TSWV isolate can be us for discriminate of TSWV isolate as RB and NRB. And, more isolates and further studies are needed for evolutionary analysis of TSWV isolates.

This information will shed light on future studies.

ACKNOWLEDGEMENTS

This study is a part of the project supported by Tokat Gaziosmanpasa University Scientific Research Project with number of 2016/25. Serological studies of this paper were present as abstract with title "Serologic identification of Tomato spotted *wilt virus* in pepper plantations in Tokat province" at "3rd International UNIDOKAP Black Sea Symposium "Sustainable Agriculture and Environment" 21-23 June 2019".

ÖZET

Çalışma kapsamında 2016 yılı yaz döneminde biber yetiştirilen Tokat ili Merkez, Niksar, Erbaa ve Pazar ilçelerine sürveyler düzenlenmiş ve virüs şüphesi gösteren biber bitkilerinden yaprak örneği toplanmıştır. Sürvey çalışmaları sonucunda toplanan 324 adet bitki yaprak örneği *Tomato spotted wilt virus* (TSWV)'e spesifik antiserum ile DAS-ELISA testine tabii tutulduktan sonra virüse spesifik primerlerle RT-PCR işlemi gerçekleştirilmiştir. Testlenen örneklerin %13'nün TSWV ile enfekteli olduğu tespit edilmiştir. ELISA testinde pozitif çıkan örnekler S segmentinde yer alan Nucleocapsid proteine (Np) spesifik primerlerle RT-PCR işlemine tabi tutulmuş ve pozitif bant elde edilen örneklerden 3'ü sekans analizine gönderilmiştir. Analiz sonuçlarına göre Türkiye TSWV izolatlarının Np gen bölgesi Fransa ve Güney Kore izolatları ile %98-99 nükleotid benzerliği göstermiştir ve filogenetik ağaçta aynı grupta kümelenmiştir. Anahtar kelimeler: biber, RT-PCR, Tomato spotted wilt virus, Tokat

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Cite this article: Topkaya Ş. (2021). Prevalence and molecular characterization of Tomato spotted wilt virus in pepper fields in Tokat province. *Plant Protection Bulletin*, 61-3. DOI: 10.16955/bitkorb.898371

Atıf için: Topkaya Ş. (2021). Tokat ilinde biber alanlarında Tomato spotted wilt virus'ın yaygınlığı ve moleküler karakterizasyonu. *Bitki Koruma Bülteni*, 61-3. DOI: 10.16955/bitkorb.898371