

Sensitive Detection of Tomato Spotted Wilt Virus from Pepper Plants by DAS-ELISA, RT-PCR and IC-RT-PCR

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Abstract

This study was conducted to determine the prevalence of Tomato spotted wilt virus (TSWV) in pepper growing areas in Burdur, Isparta and Denizli provinces of Turkey in 2012- 2013. During surveys TSWV suspected leaf samples were collected. Total of 448 leaf samples were collected from different locations in the region. Presence of TSWV was investigated using Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) method. ELISA test results showed that 61 samples were infected with TSWV (13.61%). ELISA positive samples for TSWV were used in the mechanical inoculation studies. Typical symptoms of TSWV were observed on the indicator plants. Molecular studies were carried out on 25 ELISA negative samples and 61 ELISA positive samples (Totally 86 samples). 63 samples were detected positive with RT-PCR (reverse transcriptase polymerase chain reaction) method while 70 samples were found to be infected with TSWV using IC-RT-PCR (immunocapture reverse transcriptase polymerase chain reaction) method. Expected size of band with 276 bp was observed on agarose gel electrophoresis. IC-RT-PCR method was found to be more suitable and sensitive than RT-PCR.

Keywords: Pepper, Tomato spotted wilt virus, DAS-ELISA, mechanical inoculation, RT-PCR, IC-RT-PCR.

1. Introduction

Pepper (*Capsicum annum* L.), a member of the Solanaceae family, is an important warm and temperate climate plant. It has been reported that the homeland of pepper is tropical South America, especially Brazil (ANONYMOUS [1]). Pepper was spread from Spain to England in the 1500s and then all over Europe [1]. Turkey was the third highest pepper producing country in the world in 2013 with 1.975.269 tons (ANONYMOUS, FAO [2]). In Turkey, 1.975.269 tons of pepper was produced totally (2013) and of this was 879.846 tons of long green pepper, 364.930 tons was sweet bell pepper, 730,493 tons was sauceboat pepper (ANONYMOUS [3]). Isparta, Burdur and Denizli provinces are important for pepper production due to their soil and climatic properties and rich irrigation facilities. In the production of pepper, improper or inadequate agricultural practices and biotic and abiotic disease factors cause significant yield losses. There is a wide variety of fungal, bacterial and viral diseases limiting production. Viral diseases have an important place among these factors due to their chemical and physical structures, size, infection types, symptoms, transportation and a lack of effective management strategies (AGRIOS [4]). In order to minimize the damage caused by viral diseases and to improve the control methods, first it is necessary to identify the viruses found in culture plants. Symptomatology studies based on the identification of the viruses should be supported by

serological and molecular tests. In the recent years, PCR techniques which allow precise and accurate diagnosis of pepper viruses from plant tissues have been developed (JACOBI& al. [5]; VINAYARANI& al. [6]). *Tomato spotted wilt virus* was first identified in 1915. The virus belongs to the *Tospovirus* genus of the Bunyaviridae family. TSWV is carried by nine species of thrips belonging to the genera *Thrips*, *Frankliniella* and *Scirtothrips*. Among these species, the most important vectors of TSWV are *Frankliniella occidentalis* Pergande and *Thripstabaci* Lindeman (MAU and MARTIN [7]). The virus can also be transported from plant to plant mechanically. It has been reported that the virus is not transported through root cohesion, seeds or pollens (SHERWOOD& al. [8]). PAPPU [9] reported that the virus causes much damage in plants especially in the early stages of infection and that infected plants are severely stunted and may die. It was reported that TSWV causes mosaic, tanning, leaf curl, wilt, fruit deformation, ring spots on the fruit in pepper (SHERWOOD & al. [8]). In studies on viral diseases in pepper conducted in Turkey, TMV, ToMV, CMV, PMMoV, TSWV, PVY and AMV have all been detected (ŞEVİK [10]; ÖZASLAN & al. [11]). In this study, DAS-ELISA, mechanical inoculation and IC-RT-PCR and RT-PCR methods were utilized for the identification of TSWV in the pepper production areas in Burdur, Isparta and Denizli provinces in Turkey. Additionally, the success rates of the molecular methods used for the identification of TSWV were evaluated.

2. Materials and Methods

Plant Materials

The main material of this study consisted of 448 leaf samples totally (239 samples from Isparta + 94 samples from Burdur + 115 samples from Denizli) showing signs of the disease and thus suspected to be infected with the virus. The samples were collected from pepper production areas in June-September of 2012-2013. During the field surveys, virus-like symptoms including yellowing, mosaic pattern of light and dark green, yellow spotting, malformation symptoms on the leaves, ring-spots or line patterns on leaves or fruit were observed and symptoms are photographed. The collected samples were labeled in polyethylene bags, brought to the laboratory in ice boxes and kept in a freezer (-20°C) until the necessary tests were made.

Serological Test Method (DAS-ELISA)

TSWV DAS-ELISA (BIOREB AG, Switzerland) kit was used in the study. The application was performed according to the method prepared by the manufacturer. Accordingly, 200 µl IgG 1:1000 diluted in coating plate was added to each well of the ELISA plate and kept at 4° C overnight. Then, ELISA plates were washed with washing buffer. Washing was repeated three times. Each well was filled with 200 µL plant extracts obtained by 1/10 dilution with extraction buffer and kept at 4° C overnight. The washing was repeated the next day. Following the washing process, conjugated antibodies were diluted in conjugated buffer in a 1:1000 ratio and 200 µl dilution was added to each well and kept at 37° C for 4 hours. Following the washing process, 200 µL of substrate which was prepared as 1 mg/mL in substrate solution was added and kept at room temperature. The samples with values at least twice the value of the negative control value according to the absorbance at 405 nm were accepted as positive (ÖZASLAN & al. [11]).

Mechanical inoculation studies

Mechanical inoculation was performed on 3-4 leaves by using the samples that were determined as positive as a result of ELISA tests performed on the leaf samples collected during the field trips in the study regions and suspected of TSWV infection. Accordingly, plant tissues taken from the plants infected with TSWV were prepared in 0.01 M phosphate buffer (pH 7.2)

containing 0.01% 2-mercaptoethanol at 1:1 (W/V) and inoculated to the leaves of the test plants. *Catharanthus roseus*, *Pelargonium zonale*, *Chenopodium amaranticolor*, *Nicotiana glutinosa*, *Nicotiana tabacum* L. “Xanthii”, *Nicotiana tabacum* “White Burley” and *Capsicum annum* L. were used as test plants. For symptom emergence, the test plants were placed in plant growth cabins at 18-20°C.

IC-RT-PCR

For the immunocapture-RT-PCR method, PCR tubes were coated with TSWV-specific antibodies during ELISA studies and left to incubate at 4° C overnight. Following the incubation, the tubes were washed three times with washing buffer (PBS-tween buffer) and kept in the freezer until the molecular studies. For molecular studies, 1 g sample was squashed in 10 mL general extraction buffer solution and 200 µL of solution was added to PCR tubes coated with virus specific antibodies. The samples were left to incubate at 4°C overnight. Following the incubation, the tubes were washed three times with washing buffer (PBS-tween buffer) and then washed with pure water. Then, RT-PCR mixture was added to these PCR tubes.

Total RNA Isolation

The isolation of total RNA was performed according to RNA extraction kit (Qiagen, RNeasy Mini Kit GmbH, Germany) protocol suggested by the manufacturer.

RT-PCR studies

RT-PCR studies were conducted in a single step according to the Primescript One step RT-PCR kit (Takara Bio Inc, Japan) protocol in 50 µL volume. Each reaction mixture was prepared with 1 µL virus-specific primer pair (20 µM each), 25 µL 2x1 step buffer (reaction buffer and dNTP mix), 2 µl 1 step enzyme mix (reverse transcriptase, taq polymerase, RNAase inhibitor) and sterile H₂O. In this stage of the study, 25 samples which gave negative results in DAS-ELISA tests and 61 samples which gave positive results in DAS-ELISA tests were used in RT-PCR and IC-RT-PCR studies. In RT-PCR and IC-RT-PCR studies, a primer pair specific to TSWV was used to amplify a 276 bp fragment of the RdRp gene (Forward: 5'-AATTGCCTTGCAACCAATTC-3'; Reverse: 5'-ATCAGTCGAAATGGTCGGCA-3' (SHOUSHTARI & al. [12])). For the amplification, the PCR thermocycling program consisted of 1 cycle at 50 °C for 30 minutes, 94 °C for 2 minutes and 30 cycles at 94 °C for 30 seconds, 54 °C for 30 seconds, and 72 °C for 30 seconds, and finally 72 °C for 1 minute. In molecular studies, pepper plant leaves which were found to be infected with TSWV in DAS-ELISA studies were used as a positive control and the leaves of a healthy pepper plant was used as the negative control. The amplified RT-PCR products were electrophoresed in 1% agarose gel (Bio-Rad, France) and stained with ethidium bromide. Doc-It (UVP, UK) was used for imaging. 100 bp DNA ladder (GeneRuler, ThermoScientific) was used as marker.

3. Results and Conclusions

In survey studies conducted between 2012 and 2013, leaf samples were collected from Burdur, Isparta and Denizli provinces. Leaf deformation, curliness, withdrawal in the vessels, discoloration of the leaves, necrosis, mosaic formation, and plant stunting, ring-spot symptoms were common in surveys. Photographs of these plants are presented in Figure 1. Mechanical inoculation studies were performed with leaf tissues collected from the field that were determined to be infected with TSWV as a result of ELISA tests. In the mechanical inoculation studies, systemic mosaic and leaf deformations were observed in *Catharanthus roseus* and severe stunting and deaths were observed in *Pelargonium zonale* ten days after the inoculation. In *Nicotianaglutinosa* and *Nicotianata bacum* “White Burley”, systemic mosaic symptoms were seen 12 days after the inoculation (Figure 2). In pepper, leaf deformations were observed 15 days after the inoculation. No symptoms were observed in *Nicotiana tabacum* L. “Xanthii” or *Chenopodium amaranticolor*.



Figure 1. Mosaic patterns, interveinal yellowing and deformations on pepper leaves.

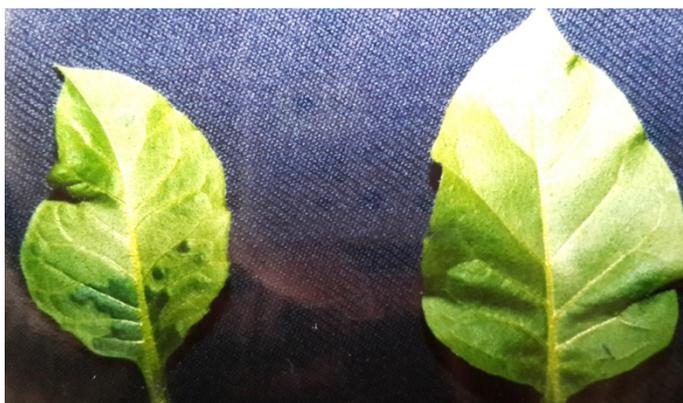


Figure 2. Systemic mosaic symptoms were observed on inoculated *Nicotiana tabacum* "White Burley" leaves.

The symptoms were similar to those obtained in previous studies and support the suggestion that the virus may be TSWV (ADKINS, [13]; ŞEVİK [14]). However, it is a fact that symptoms might not always be observed in mechanical inoculation method studies due to various reasons. Therefore, as the exclusive use of this method may cause errors, identification studies should be supported with more sensitive methods. Initially, ELISA tests were performed in order to determine the presence of TSWV in the leaf samples taken from 448 plants with virus infection symptoms. Among the tested samples, 61 samples gave positive TSWV reactions. TSWV infections have also been detected in studies carried out in other regions in Turkey (GÜLDÜR and ÇAĞLAR[15]; YILMAZ and DAVIS [16]; KAMBEROĞLU and ALAN [17]). The positive reactions for the targeted virus obtained in serological tests performed for the identification of the virus indicated the presence of TSWV in the plant. ELISA tests have been widely used by researchers for the determination of viruses in pepper (CHOI & al.[18]; AROGUNDADE& al. [19]). This method is preferred due to its speed, sensitivity, affordability and reliability (JACOBI & al. [5]). RT-PCR and IC-RT-PCR are molecular methods that are widely used in studies carried out for the identification of viral diseases in plants (FINETTI-SIALER & al. [20]; TSOMPANA& al. [21]). As a result of the RT-PCR studies on plant samples, 63 samples yielded band results of the expected TSWV-specific size (276 bp) and in IC-RT-PCR studies, 70 samples gave expected TSWV-specific bands. No band formation was observed in the negative control (Figure 3). The results obtained in this study are compatible with previous studies conducted by using the same TSWV primer pair (SHOUSHTARI& al.[12]; PEREZ & al. [22]). These researchers also obtained 276 bp bands in their molecular studies. 61 of 448 samples gave positive DAS-ELISA results, while 70 samples were shown to be infected with TSWV via the PCR methods, demonstrating that these PCR-based methods were more sensitive and accurate than DAS-ELISA. Virus-specific bands were obtained in both of the PCR methods applied. The study results indicate that RT-PCR and IC-RT-PCR methods can be successfully used for the identification of TSWV. Positive results obtained in IC-RT-PCR in samples which gave negative results in RT-PCR showed that IC-RT-PCR was more sensitive. Similarly, ANFOKA& al. [23] emphasized that some challenges were eliminated during isolation by directly using plant extracts without nucleic acid isolation in IC-RT-PCR.

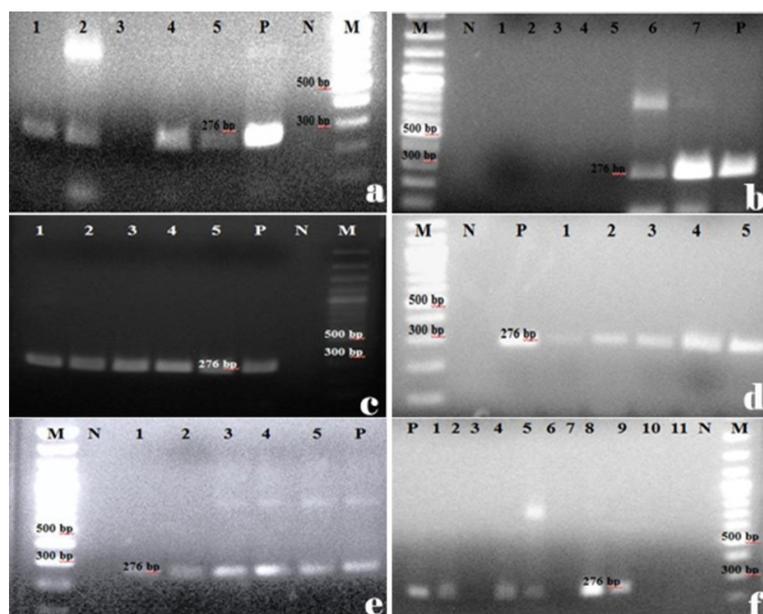


Figure 3.a.b. Agarose gel electrophoretic stained by ethidium bromide analysis of RT-PCR amplification of TSWV RdRp gene from infected field samples of Pepper.c.d.f. Agarose gel electrophoretic stained by ethidium bromide analysis of IC-RT-PCR amplification of TSWV RdRp gene from infected field samples of Pepper. M: Markır 100 bp DNA ladder, P: Positive control, N: Negative control, Lanes: infected field sample.

In the light of these results, IC-RT-PCR tests were found to be the most reliable molecular method for the identification of viral factors. DAS-ELISA remains useful for identification given its simultaneous application to a large number of samples and rapid results. For the identification of TSWV, biological, serological and different molecular methods were implemented. As a result of these studies, the presence of TSWV was demonstrated. All the applied methods supported each other. Also, the usability and accuracy of different PCR methods for the identification of TSWV were demonstrated. RT-PCR and IC-RT-PCR provide possible alternatives to ELISA, which fails because of low virus titre.

4. Acknowledgements

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References

1. ANONYMOUS. Pepper Production, 2013, <http://megep.meb.gov.tr>. (accessed 5 October 2013).
2. ANONYMOUS, FAO, 2012, <http://faostat.fao.org/site/567/Desktop>. (accessed 20 May 2013).
3. ANONYMOUS. *Plant Production*, 2013, <http://tuikrapor.tuik.gov.tr/reports> (accessed 12 April 2014).
4. G. N. AGRIOS. *Plant Pathology*. Academic Press Inc. New York, USA, 1997.
5. V. JACOBI, G.D. BACHAND, R.C. HAMELIN, J.D. CASTELLO. Development of multiplex Immunocapture RT-PCR assay for detection and differentiation of tomato and tobacco mosaic viruses. *J. Virol. Methods*. 74: 167-178 (1998).
6. G. VINAYARANI, K.N. MADHUSUDHAN, S.A. DEEPAK, S.R. NIRANJAND, H.S. PRAKASH. Detection of mixed Infection of Tobamoviruses in tomato and bell pepper by using RT-PCR and Duplex RTR-PCR. *Int. J. Plant Pathol.* 2(2): 89-95 (2011).
7. R.F.L. MAU, J.L. MARTIN. *Frankliniella occidentalis (Pergande) Western Flower Trips*, 2002, www.extentohawaii.edu/kbase/crop/type/f-occid.html (2002). (accessed 10 April 2014).
8. J.L. SHERWOOD, T.L. GERMAN, J.W. MOYER, D.E. ULLMAN. *Tomato spotted wilt. The Plant Health Instructor*, 2003, doi:10.1094/PHI-I-2003-0613-02.
9. H.R. PAPPU. *Tomato spotted wilt virus (Bunyaviridae)*. In: M. BRIAN, V. MARC, eds., *Encyclopedia of Virology*, 3rd edition, Elsevier Ltd, Oxford, UK, 2008, pp. 133-138.
10. M.A. ŞEVİK. Occurrence of *Pepper mild mottle virus* in greenhouse grown pepper (*Capsicum annuum* L.) in the West Mediterranean region of Turkey. *African J. Biotech.* 10 (25):4976-4979 (2011).
11. M. ÖZASLAN, B. BAŞ, T. AYTEKİN, Z. SİĞİRCİ. Identification of Pepper Viruses by DAS-ELISA Assay in Gaziantep-Turkey. *Plant Pathol. J.* 5(1): 11-14 (2006).

12. S. SHOUSHARI, B. JAFARPOUR, M. FALAHATY RASTEGAR. Serological and Molecular Detection of Tomato spotted wilt virus (TSWV) in Khorasan Razavi Province. *J. Plant Protect.* Vol. 26(4): 43 (2013).
13. S. ADKINS, Tomato spotted wilt virus positive steps towards negative success. *Mol. PlantPathol.* 1(3): 151-157 (2000).
14. M.A. ŞEVİK. Determination of some characteristic features of Tomato mosaic virus (TSWV) AND Its spread in tomato fields in Samsun Province. Ondokuzmayıs University, Agriculture Faculty, Plant Protection Department. Doctoral Thesis, 128p (2007).
15. M.E. GÜLDÜR, B.K. ÇAĞLAR. Outbreaks of Pepper mild mottle virus in greenhouses in Şanlıurfa, Turkey. *J. Plant Pathol.* 88(3): 341 (2006).
16. M.A. YILMAZ, R.F. DAVIS. Identification of viruses infecting vegetable crops along the Mediterranean Sea coast in Turkey. *J Turkish Phytopathol.* 14: 1-18 (1985).
17. M.A. KAMBEROĞLU, B. ALAN. Occurrence of Tomato spotted wilt virus in lettuce in cukurova region of Turkey. *Int J Agric. Biol.* 13: 431-434 (2011).
18. G.S. CHOI, J.H. KIM, K.H. RYU, J.S. CHOI, B.N. CHUNG, H.R. KIM, Y.M. CHOI. First report of tobacco mild green mosaic virus infecting pepper in Korea. *Plant Pathol. J.* 18: 323-327 (2002).
19. O. AROGUNDADE, O.S. BALOGUN, K.T. KAREEM. Occurrence and distribution of pepper veinmottle virus and cucumber mosaic virus in pepper in Ibadan, Nigeria. *Virologia J.* 19: 79 (2012).
20. M.M. FINETTI-SIALER, C. LANAVE, M. PADULA, C. VOVLAS, D. GALLITELLI. Occurrence of two distinct *Tomato spotted wilt virus* subgroups in southern Italy. *J. Plant Pathol.* 84: 145-152 (2002).
21. M. TSOMPANA, J. ABAD, M. PURUGGANAN, J.W. MOYER. The molecular population genetics of the *Tomato spotted wilt virus* (TSWV) genome. *Mol. Ecol.* 14: 53-66 (2005).
22. Y.V. PEREZ, A. MEJIAS, E. RODRIGUEZ-ROMAN, D. AVILAN, K.A. ZAMBRANO, J.C. GOMEZ, J.E. OLACHEA, E.E. MARYS. Identification of Tomato spotted wilt virus associated with fruit damage during a recent virus outbreak in pepper in Venezuela. *Plant Diseases.* (2014). <http://dx.doi.org/10.1094/PDIS-11-14-1202-PDN>.
23. G.H. ANFOKA, M. ABHARY, M.R. STEVENS. Occurrence of *Tomato spotted wilt virus* (TSWV) in Jordan. *Bull OEPP/EPPO Bull.* 36: 517-522 (2006)