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Investigation of viruses and phytoplasma infections in tomato plantations in Bilecik province, Türkiye

Filiz Randa-Zelyüt[1,2] **Ali Karanfil**[3] **Adyatma Irawan Santosa**[4]

[1]Department of Plant Protection, Faculty of Agriculture and Natural Sciences, Bilecik Seyh Edebali University,Bilecik, Türkiye
[2]Biotechnology Application and Research Centre, Bilecik Şeyh Edebali University, Bilecik, Türkiye
[3]Department of Plant Protection, Faculty of Agriculture, Canakkale Onsekiz Mart University, Canakkale,Türkiye
[4]Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada, Sleman, Yogyakarta, Indonesia

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Corresponding Author
Filiz Randa Zelyüt
filizrandazelyut@yahoo.com

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Abstract

In this study, the status of infections caused by viruses and phytoplasmas in tomato production areas of Bilecik province was determined using conventional molecular methods. During the 2022 tomato production season, 93 plants exhibiting symptoms such as mosaic, leaf and fruit deformations, flower anomalies, and necrotic spots were collected. Viral agents such as tomato spotted wilt virus (TSWV), cucumber mosaic virus (CMV), southern tomato virus (STV), tobamoviruses, and potyviruses were screened by RT-PCR, while phytoplasmas were detected by nested-PCR. Single, double, and triple infections were detected in 50 of the 93 plants. 17, 5, and 21 plants were infected by a single pathogen for TSWV, STV, and phytoplasmas, respectively. 1, 3, and 2 plants were infected by two pathogens for STV+TSWV, STV+phytoplasma, and TSWV+phytoplasma, respectively. Only one plant detected a triple infection caused by STV, TSWV, and phytoplasmas. The phytoplasma genetic group was determined as 16Sr XII-A by PCR RFLP *in-silico* and *in-vitro* methods. Sequencing studies revealed that TSWV had high nucleotide sequence similarity with other Türkiye isolates for the NSs partial gene and STV entire CP gene region. For phytoplasmas, sequencing studies showed that the obtained tomato strains overlapped one-to-one with stolbur strains. Phylogenetic analyses applied with global isolates for TSWV NSs and STV CP gene regions showed the existence of 2 main groups (Clade I and Clade II). TSWV and STV isolates obtained from this study clustered in large main branches (Clade I).

Keywords: Bilecik, Tomato, Virus, Phytoplasma, PCR, Sequencing

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INTRODUCTION

Tomato (*Solanum lycopersicum* L.), a member of the Solanaceae family, is one of the most widely grown and consumed vegetables. It has a very high climate tolerance and is produced in different systems such as open field, hydroponic, polytunnel, and greenhouse. Currently, tomato production amounts is 180 million tons globally. Türkiye, which has a wide climatic zone, is one of the leading countries with an average annual yield of 13 million tons. In Bilecik Province, located in the South Marmara region and the transitional climate zone, it is recorded that an average of 107.000 tons of fresh and paste tomatoes are produced in an area of approximately 16,000 da. In the region, tomato production is quite common due to the flavour of local tomato varieties and the efficient use of agricultural areas.

It is well known that many phytopathogens cause significant yield and quality losses in tomato cultivation. In particular, viral infections can lead to decreased fruit quality and yield, reduced nutrient content and shorter shelf life, making tomatoes unmarketable (Hanson et al., 2016). Over 130 viruses and virus-like agents have been reported to cause infections in tomato plants. More specifically, viruses of different species have been reported as significant agents in tomato production areas from many countries in the Mediterranean basin, causing substantial

yield and quality loss. These viruses are tomato spotted wilt virus (TSWV), tomato yellow leaf curl virus (TYLCV), tomato infectious chlorosis virus (TICV), cucumber mosaic virus (CMV), tomato chlorosis virus (ToCV), alfalfa mosaic virus (AMV), and pepino mosaic virus (PepMV) have been recorded as agents that cause established and chronic infections (Panno et al., 2021; Roselló et al., 1996; Navas-Castillo et al., 2011). Recently, there have been reports of the emergence of viruses that cause serious restrictions in tomato production, including torradoviruses and tomato brown rugose fruit virus (ToBRFV) (Van der Vlugt et al., 2015; Oladokun et al., 2019). The presence of viral agents such as AMV, TSWV, CMV, southern tomato virus (STV, Amalgavirus), ToBRFV, ToCV, and TYLCV has been reported in tomato production areas located in different geographical regions of Türkiye (ustaeşilyurt and Çevik, 2019; Fidan et al., 2019; Güller and Usta, 2020; Karanfil, 2021; Akdura and Çulal Kılıç, 2022; Randa-Zelyüt et al., 2023; Usta et al., 2023; Güller et al., 2023).

Another pathogen that causes significant infections in tomato is phytoplasma of the phylum Firmicutes (class Mollicutes), a group of cell wall-less microorganisms genetically related to a Gram-positive ancestor (Weisburg et al. 1989). It is well known that various genetic groups of phytoplasmas, which are limited to the phloem tissue in plants and transmitted by insect vectors, cause different or similar symptoms in many agricultural products grown in many parts of the world (Kumari et al. 2019; Hogenhout et al.,2 008). Specifically, '*Candidatus* Phytoplasma solani' (CaPsol) (subgroup 16SrXII-A), which is highly prevalent in Euro-Mediterranean grapevine agroecosystems and a potential threat to viticulture worldwide, has also been reported to cause significant infections in Solanaceae and Apiaceae members (Navrátil et al. 2009; Quaglino et al. 2019; Ember et al., 2016). In Türkiye, it has been reported that CaPsol causes infections in various perennial and annual agricultural products, especially tomatoes (Güller and Usta, 2020; Çağlayan, 2023; Randa-Zelyüt et al., 2022; Randa-Zelyüt, 2023).

This study aimed to determine the possible viral and phytoplasma infections that cause yield and quality loss in products and cannot be controlled by direct chemical control methods in the regions of Bilecik province of Marmara region where tomato production is intensive. The presence of pathogens was determined by conventional molecular methods and molecular characterization of genes of viruses and conserved 16Sr RNA gene regions of phytoplasmas were genetically identified.

MATERIALS AND METHODS

Field studies

Between June and September of 2022, field surveys were done in open fields and plastic tunnels where tomato cultivation was carried out in the province of Bilecik, Türkiye. A total of 93 tomato plants with symptoms caused by viruses and virus-like agents were collected from İnhisar, Osmaneli, Söğüt, Gölpazarı, and Merkez Districts. The samples were brought to the laboratory, labelled and stored at -20 ℃ for molecular studies. A symptomatic description of the samples was recorded

Total Nucleic Acid (TNA) Isolation and cDNA synthesis

CTAB-based Total Nucleic Acid (TNA) isolation method with minor modifications (Li et al. 2008) was applied to ± 150 – 160 mg epidermal and vascular tissues of tomato leaves since the molecular study used both RNA and DNA molecules as PCR templates. cDNA libraries were constructed to detect RNA viruses considered within the scope of this study. In the first step, 3 µL RNA was denatured with 0.2 µg/µL Random Hexamer primer (5'-NNNNNNNN-3') (Thermo Sci., USA) and DEPC-water (Thermo Sci., USA) to a volume of 12.5 µL at 95 °C for 3 minutes and the tubes were denatured at 95 °C for 3 minutes and they were kept on ice for 5 minutes. In the second step, 10 µL of enzyme-containing 100 units of reverse transcriptase enzyme (RevertAid Reverse Transcriptase 200 U/µL, Thermo Sci., USA), 20 units of Rnasin (RiboLock RNase Inhibitor 40 U/µL, Thermo Sci., USA), 2 µL (10 mM) dNTP, 1X RT-buffer (250 mM Tris-HCl (pH 8. 3 at 25°C), 250 mM Tris-HCl (pH 8.3 at 25°C), 250 mM KCl, 20 mM MgCl2, 50 mM DTT) and enzyme mixture containing 1.25 DEPC-water were prepared and added to the tubes on ice. The tubes were incubated at 25°C for 15 min (random primer binding), 42°C for 60 min (enzyme forming the templates) and 4°C for 5 min. The cDNA libraries obtained were stored at -20 °C for use in amplification studies.

Amplification Assays

The presence of CMV, TSWV, tobamoviruses, potyviruses, and STV in tomato plants was investigated in cDNAs obtained. Therefore, molecular detection studies were applied for the detection of specific gene regions of the aforementioned viruses, both species- and genus-specific. For amplifications, 2 µL cDNA, 1X buffer, 2 µL (25 mM) $MgCl_2$, 0.5 µL (forward and reverse; 10 µM) primers, 0.5 µL dNTP (10 µM), and 1.25 units of Taq enzyme (Thermo Sci., USA) were used in a total reaction volume of 20 µL. Details of the primer sequences used for virus detection are given in Table 1.

TNAs obtained in the molecular detection of phytoplasmas were diluted 1:30 with nuclease-free water- NFW (Invitrogen, USA) and then used in nested-PCR studies as 1 µL gDNA template in a total volume of 20 µL. For the first step reaction, 1 µL gDNA, 1X buffer, 2 µL (25 mM) MgCl2, 0.5 µL dNTP (10 µM), 1.25 units of taq enzyme, and 0.5 µL (10 µM) each primer were used in a total volume of 20 µL. For the second step of PCR studies, the first amplification products were diluted 1:30 and reacted with R16F2n and R16R2 universal phytoplasma

primers under the same reaction conditions stated above. Details of the primer sequences used for phytoplasma detection are given in Table 1.

Table 1. Primer sequences used in the detection of viruses and phytoplasmas by PCR methods

The causal agent	Primer name	5'-3' sequence	Size and gene region	Reference
TSVW	NSS2-F	GCTTCAGTCTGGGGATCAACT	724 bp Partial non-structural (NSs)	Abadkhah et al. 2018
	NSS2-R	TTGGAACTCTTAGCCAGAGGC		
Tobamovirus	TobamodF	TKGAYGGNGTBCCNGGNTGYGG	880 bp Partial RdRp	Li et al., 2018
	TobamodR	ACNGAVTBNABCTGTAATTGCT AT		
CMV	CMV_CPF	ATGGACAAATCTGAATCAACC	638 bp Partial CP	Karanfil and Korkmaz, 2017
	CMV_CPR	GATGTGGGAATGCGTTGGTGC		
Potyvirus	Potyvirus-F	GTITGYGTIGAYGAYTTYAAYAA	350 bp Partial Nib	Zheng et al. 2008
	Potyvirus-R	TCIACIACIGTIGAIGGYTGNCC		
STV	STV_ CPF	CTCGTCGTTGCTTCCGTT	1134 bp Complete CP	Randa-Zelyüt et al. 2023
	STV_ CPR	ACCACCACCCCTGTACTT		
Phytoplasma	R16mF2	CATGCAAGTCGAACGGA	1.8 kb Partial 16Sr RNA	Gundersen and Lee, 1996
	R16mR2	CTTAACCCCAATCATCGA		
	R16F2n	GAAACGACTGCTAAGACTGG	1.2 kb Partial 16Sr RNA	Lee et al. 1993
	R16R2	TGACGGGCGGTGTGTACAAACC CCG		

Determination of 16s rRNA Genetic Groups of Phytoplasmas by *in-vitro* and *in-silico* PCR-RFLP Methods

To determine the genetic group of phytoplasmas according to the 16s rRNA region, digestion profiles were prepared using both in-vitro and in-silico methods. For this purpose, PCR-RFLP (Restriction Fragment Length Polymorphism) profiling of the 1.2 kb PCR products obtained with R16F2n/R16R2 primers was performed under in vitro conditions using the AluI endonuclease enzyme. A standard reaction protocol was used for polymorphic profiling. For AluI enzyme (Eurx, Estonia), 5 µL PCR product (≈ 0.1-2 µg), 2.5 µL of 10 X reaction buffer, 0.25 µL of 100 X BSA (Bovine serum albumin) (Eurx, Estonia), 6 units of enzyme and the remaining portion was completed with nuclease-free water in 25 µL volume. The mixture was incubated at 37 0C overnight for at least 16 hours to allow the reaction to take place. The products were then run on a 1.8% agarose gel in 1X TAE buffer solution at 80 V for 90 min. UV device (Syngene, UK) was used for visualisation. After sequence analyses of some R16F2n/R16R2-amplified products selected according to the profiles were completed, computer-simulated in silico PCR-RFLP cutting was performed using iPhyClassifier (Zhao et al. 2013) software.

Sequencing and Phylogenetic Inferences

For sequencing, selections were made based on the regions where infection was detected from the fragments obtained as a result of amplification studies. To extract the nucleotide sequences of these fragments, sequencing was performed by outsourcing services (BM Labosis, Ankara). After the raw nucleotide data of the isolates obtained from viruses and the strains obtained from phytoplasmas were edited in Bioedit software, the studies were continued in MEGA X (Kumar et al., 2018) software. Sequences of relevant global isolates were extracted from GenBank and data sets were prepared. Alignment of relevant gene regions was performed using the ClustalW algorithm. Phylogenetic analyses were applied using the Tamura-3 (T-92) parameter and the Neigbour-Joining method. Additionally, iTOL software was used to visualize the trees. In addition, the similarity ratios of the obtained nucleotide and amino acid sequences were calculated in SDT software.

RESULTS AND DISCUSSION

Symptoms such as chlorosis, necrosis, mosaic, yellowing, embrittlement, epidermal tissue with narrowed vein angles, weak leaf formations, deformations, reddening of veins and proliferation were observed on the leaves of tomato samples during field studies (Figure 1a and 1b). Dead and unpollinated flower structures were observed in the plant's flowers (Figure 1c and 1d). In the fruit, symptoms such as severe deformation, necrosis, round spots, fungal and cracked tissues, and small weak fruit formations were observed (Figure 2a-d).

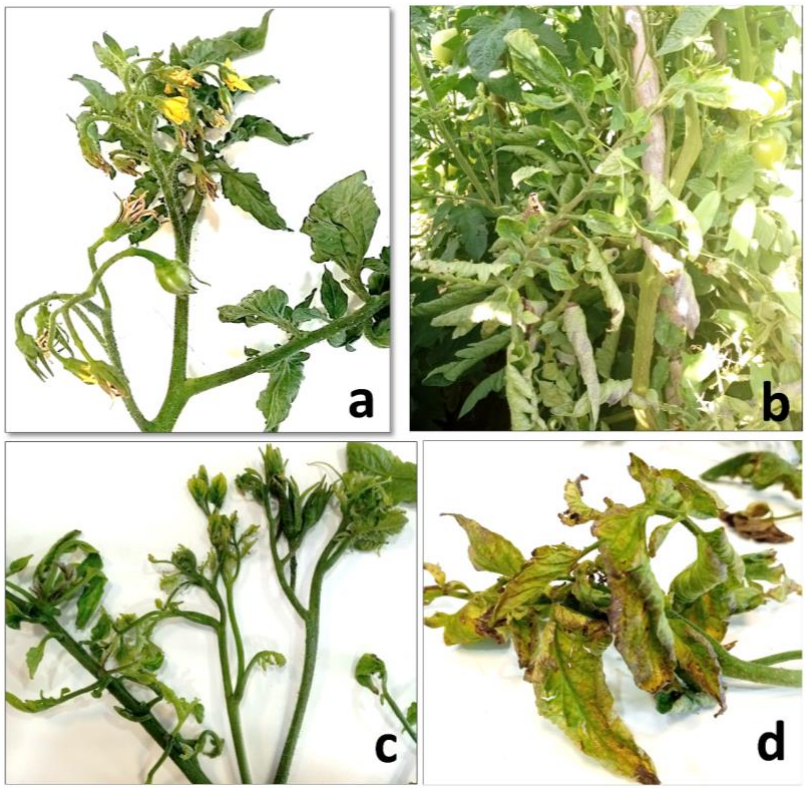

Figure 1. Malformation and death of tomato flowers (a-c). Curling and necrotic spots on leaves (b-d).

Figure 2. Deformation in tomato fruits, yellow ring spots, brown and necrotic sunken areas (a-d).

PCR study revealed fragments of the expected size for STV in 10 samples and TSWV in 21 samples. CMV, potyvirus and tobamovirus agents were not detected in all 5 districts by the molecular method. Fragments of 1134 bp, corresponding to the fully encoded CP gene region for STV, and 774 bp, corresponding to the complete encoded NSs gene region for TSWV, were obtained. For phytoplasmas, 1.25 kb fragments corresponding to a part of the 16Sr gene region were obtained with universal primer sets in 27 tomato samples. these infections were

distributed as single, double and triple in the samples. However, virus and phytoplasma infections were not detected in the Merkez and Gölpazarı districts. Single infection was detected in 17, 5, and 21 samples for TSWV, STV, and phytoplasmas, respectively. STV and TSWV together caused double infections in 1 sample, STV and phytoplasma in 3 samples, and TSWV and phytoplasma in 2 samples. Finally, a triple infection caused by STV, TSWV, and phytoplasmas was detected in 1 sample. Infection rates are detailed in Table 2.

Table 2. Regional distribution and rates of infections

Region	Number of samples	TSWV	STV	16Sr	STV + TSWV	STV + 16Sr	TSWV + 16Sr	STV + TSWV + 16Sr
İnhisar	22	11 (11.8%)	3 (3.2%)	4 (4.3%)	1 (1.1%)	2 (2.2%)	2 (2.2%)	-
Osmaneli	34	-	2 (2.2%)	13 (13.9%)	-	1 (1.1%)	-	-
Söğüt	29	6 (6.5%)	-	4 (4.3%)	-	-	-	1 (1.1%)
Merkez	3	-	-	-	-	-	-	-
Gölpazarı	5	-	-	-	-	-	-	-
Total	93	17 (18.3%)	5 (5.4%)	21 (22.3%)	1 (1.1%)	3 (3.2%)	2 (2.2%)	1 (1.1%)

Virus and phytoplasma diseases are major obstacles in the cultivation of horticultural products worldwide that may lead to significant yield losses. The findings of this study based on molecular detection methods showed that TSWV, STV and CaPsol agents caused damage in tomato plants in the districts of Bilecik province.

The molecular test applied in this study confirmed the presence of TSWV and STV in Söğüt and İnhisar districts as well as phytoplasma in Söğüt, İnhisar, and Osmaneli districts of Bilecik province. TSWV infection rate was especially high in İnhisar where 14 of 22 (64%) of the samples tested positive for the virus. However, infections caused by TSWV have been reported from different geographical regions of Türkiye and a wide variety of hosts (Bozdoğan and Kamberoglu 2016; Morca et al. 2022; Güneş et al. 2022; Usta et al., 2023; Sajid and Elçi, 2024). Severe symptoms (fruit deformations) were observed in the fruits of TSWV-infected plants during field observations, and similar symptoms were also reported by Morca et al. (2022). Furthermore, our study revealed that TSWV-infected plants exhibited leaf symptoms like severe yellowing, necrosis, and brown spots, which have been documented in various parts of Türkiye (Sajid and Elçi, 2024). Vector control may need to be upped in Bilecik. It is also worth noting that infection rates of phytoplasma were much higher than viruses in Osmaneli which may be related to the practice of using varieties resistant to the viruses there. Only a handful of samples were collected from Merkez and Gölpazarı which are not tomato cultivation regions, and the samples were all free from the tested causal agents. Furthermore, infections caused by CaPsol in both tomatoes and other agricultural products have been reported from many different geographical regions of Türkiye (Usta et al. 2018; Güller and Usta, 2020; Usta et al. 2021; Çağlar and Şimşek, 2022; Randa-Zelyüt et al. 2022). Especially in tomatoes, symptoms such as leaf curling, floral distortion and growth retardation have been observed (Usta et al. 2021; Çağlar and Şimşek, 2022; Zelyüt, 2023). Virus and phytoplasma vectors might not widely disperse and thus easier to manage in the two districts than Söğüt, İnhisar, and Osmaneli districts.

Tomato samples infected with TSWV, STV, and phytoplasma were selected to represent each region to perform their molecular characterization. Thus, 7 TSWV, 5 STV isolates and 6 phytoplasma strains were selected and their nucleotide sequences were obtained for the NSs, CP and 16Sr RNA gene regions, respectively. After the obtained nt sequences were edited in MEGAX software, BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) analyzes were performed. The partial NSs gene region of 7 TSWV isolates obtained from Bilecik province showed more than 99.8% nt identity with another isolate (MK922155) obtained from Türkiye. The CP gene region of 5 STV isolates showed more than 99.9% nt identity with other isolates obtained from Türkiye. The 16Sr RNA gene region of 6 phytoplasma strains showed 99-100% nt similarity with other '*Candidatus* Phytoplasma solani' strains. Nucleotide sequence information of these isolates and strains were deposited in GenBank and accession numbers were obtained (Table 3).

Table 3. GenBank accession numbers of virus isolates and phytoplasma strains

TSWV		STV		16Sr XII-A	
Accession no	Isolate	Accession no	Isolate	Accession no	Strain
PP842680	Sogut1	PP836129	Bilecik 1	PP837564	Sogut1
PP842681	Sogut2	PP836130	Bilecik 2	PP837565	Sogut2
PP842682	Sogut3	PP836131	Bilecik 3	PP837566	Osmaneli1
PP842683	Sogut4	PP836132	Bilecik 4	PP837567	Osmaneli2
PP842684	Inhisar1	PP836133	Bilecik 5	PP837568	Inhisar1
PP842685	Inhisar2	-	-	PP837569	Inhisar2
PP842686	Inhisar3	-	-	-	-

The partial NSs gene region of the TSWV agent isolates obtained from this study (N = 7) and in the GenBank (N = 92) was used for phylogenetic analyses. A total of 99 isolates were clustered into 2 major branches Clade I and Clade II (Figure 3). The isolates obtained in this study were grouped in the largest main branch Clade I only with isolates obtained in Türkiye.

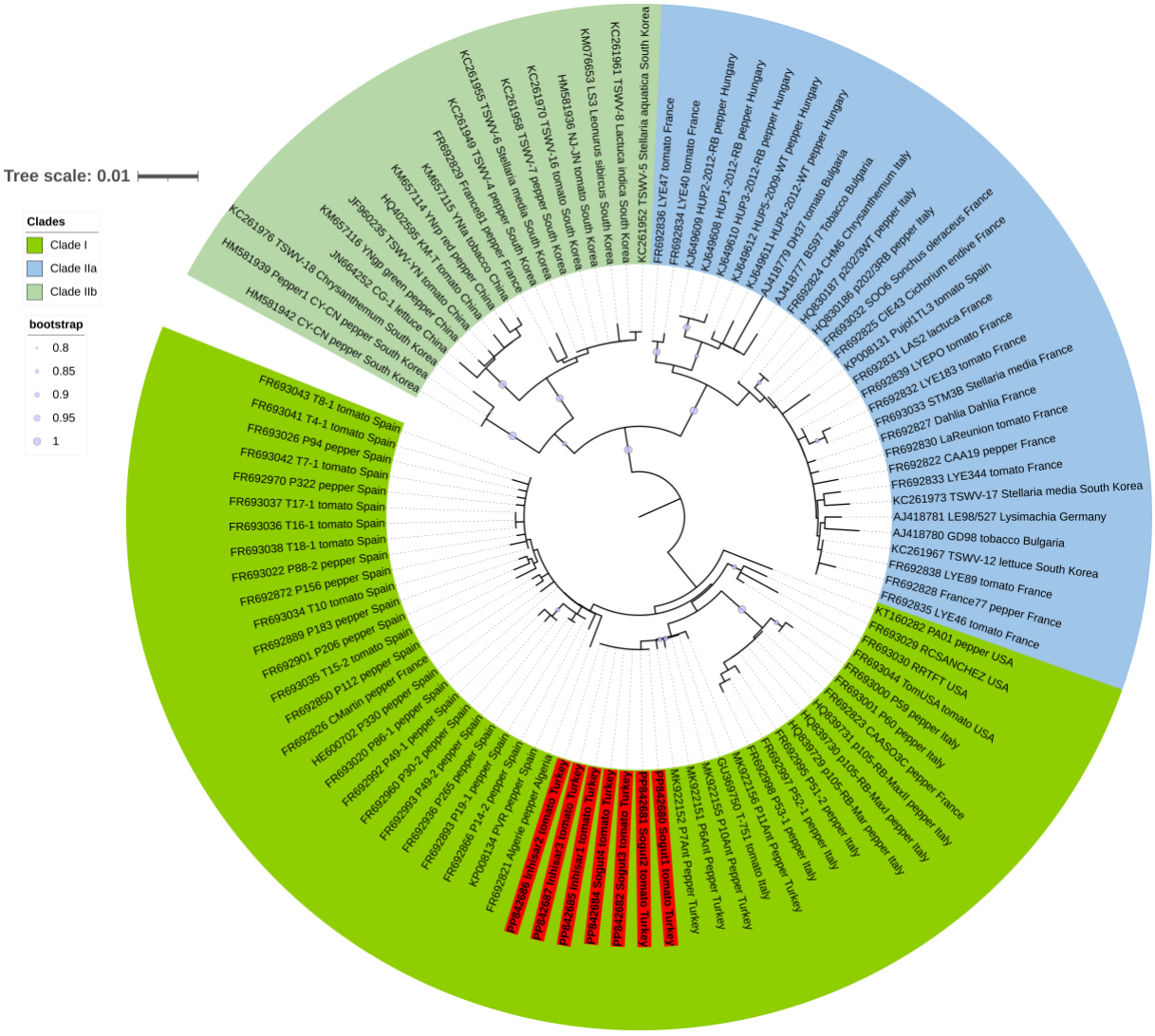

Figure 3. Dendrogram obtained as a result of the phylogenetic analysis applied for the partial TSWV NSs gene. Clusters of isolates from this study are marked in blue. Tamura-3 (T-92) algorithm was created with the Neighbor Joining (NJ) method and no outgroup was used.

For the STV CP gene region, global isolates (N=107) and isolates obtained in this study (N=5) were divided into two main branches Clade I and Clade II, and Turkish isolates were collected in Clade I (Figure 4). Complete CP region of STV isolates from regions of Türkiye had been sequenced, and they are positioned in distant subclusters within Clade 1 together with isolates from other countries in the constructed phylogenetic tree. PP836130 and PP836131 genetically were shown to have the closest relationship with isolates from Bursa while PP836132 and PP836133 with isolates collected in Balıkesir and Çanakkale Provinces which all are geographically proximate to Bilecik. Interestingly, PP836129 were related closely to Slovenia and Spain isolates. The results indicated that STV populations in Bilecik as well as the whole of Türkiye are highly stable as reported in previous studies (Randa-Zelyüt et al. 2023).

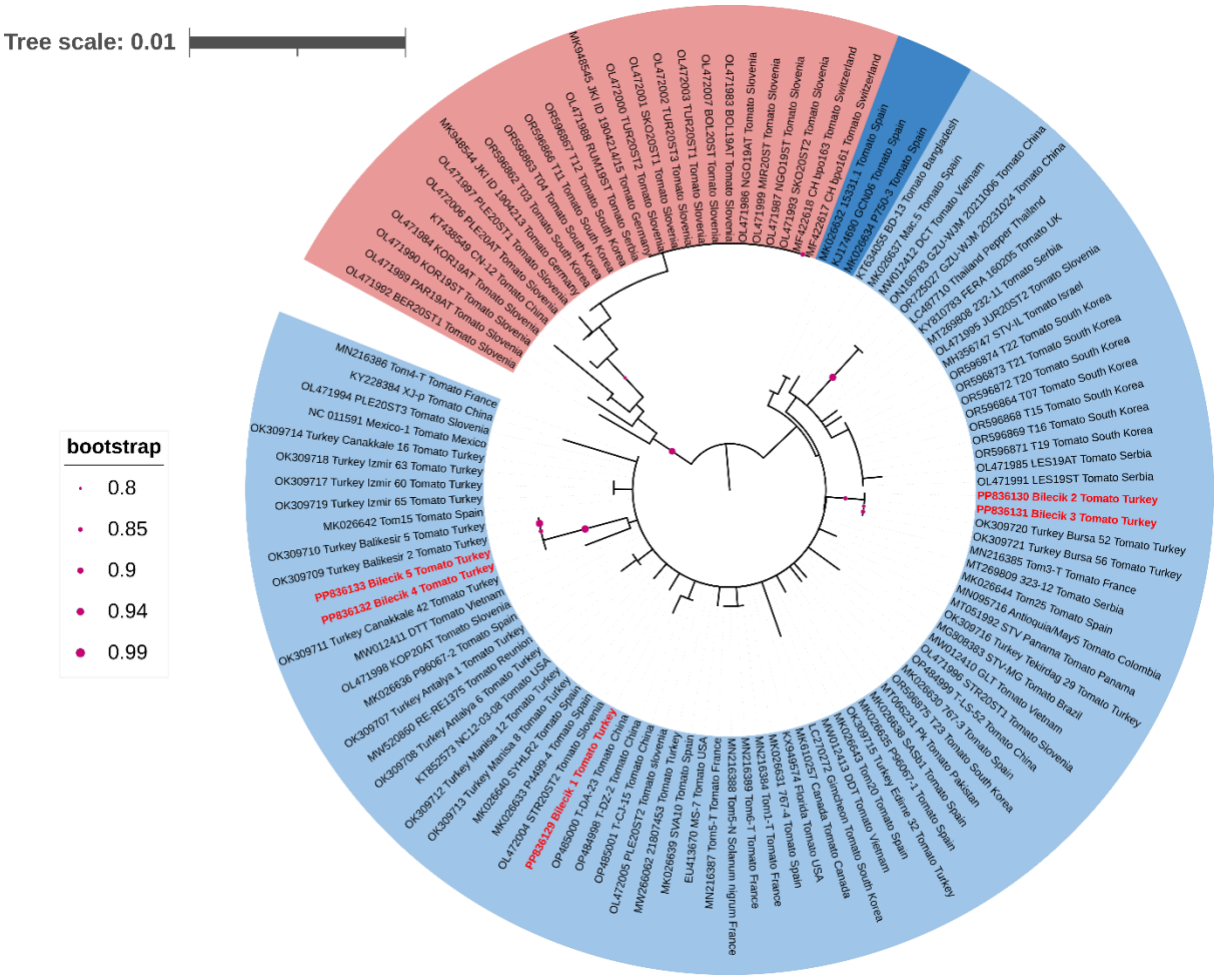

Figure 4. Tree created according to the fully encoded CP gene region of the STV. Clusters of isolates from this study are marked in light blue and the isolates were shown in red. Tamura-3 (T-92) algorithm was created with the Neighbor Joining (NJ) method and no outgroup was used. The blue pattern indicates Clade I and the other color indicates Clade II.

Amplicons obtained from 27 phytoplasma-infected tomato plants, 1.2 kb in size, as a result of nested-PCR studies, were digested with *Alu*I endonuclease under *in-vitro* conditions. The digest products were run on agarose gel with reference isolates and general profiles were obtained (Figure 5). To better understand the profiles, the '*Ca*. P. asteris' related strain Cabbage chloranthy (CHLL) (16Sr I-B) (France, INRAE) strain was used as a reference for comparisons. The digesting profiles obtained with *Alu*I exhibited the same profiles as the 16SrI-B '*Ca*. P. asteris' reference.

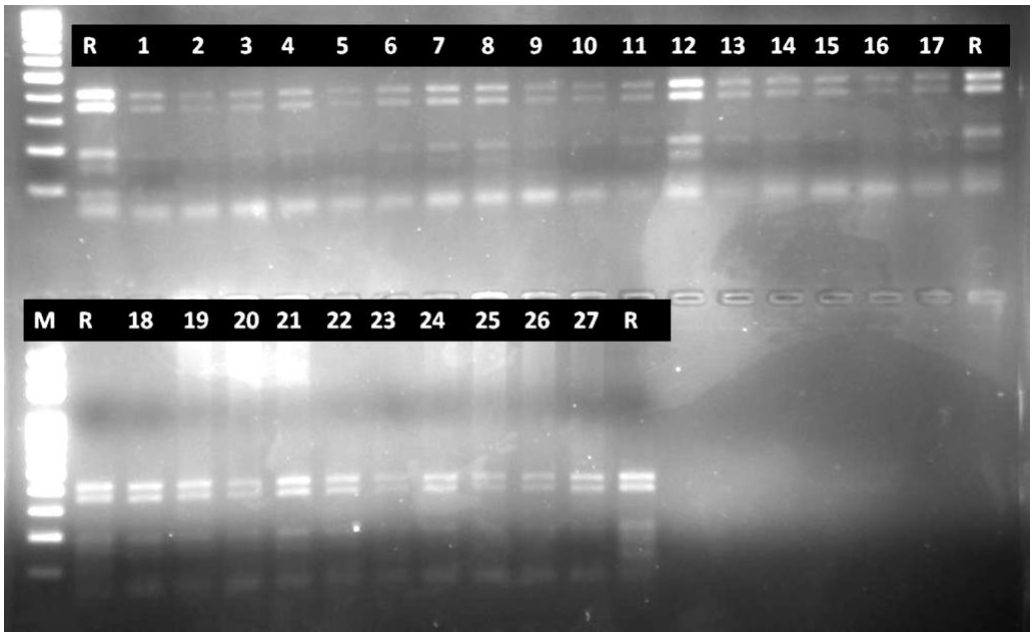

Figure 5. *Alu*I enzyme digesting profiles of fragments obtained with R16F2n / R16R2 primers of phytoplasma infected samples (R: Reference, CHLL Aster yellows 1-27 tomato samples from Bilecik province).

The nt sequences of 6 Bilecik tomato strains selected from these profiles were obtained. These sequences were analyzed using the *iPhyclassifier* software and subjected to in-silico digesting, and each strain was checked for group and subgroup verification in the same software (Figure 6). The software revealed that tomato phytoplasma strains obtained from Bilecik province shared more than 99% sequence identity with the '*Ca*. P. solani' 16Sr XII-A genetic subgroup.

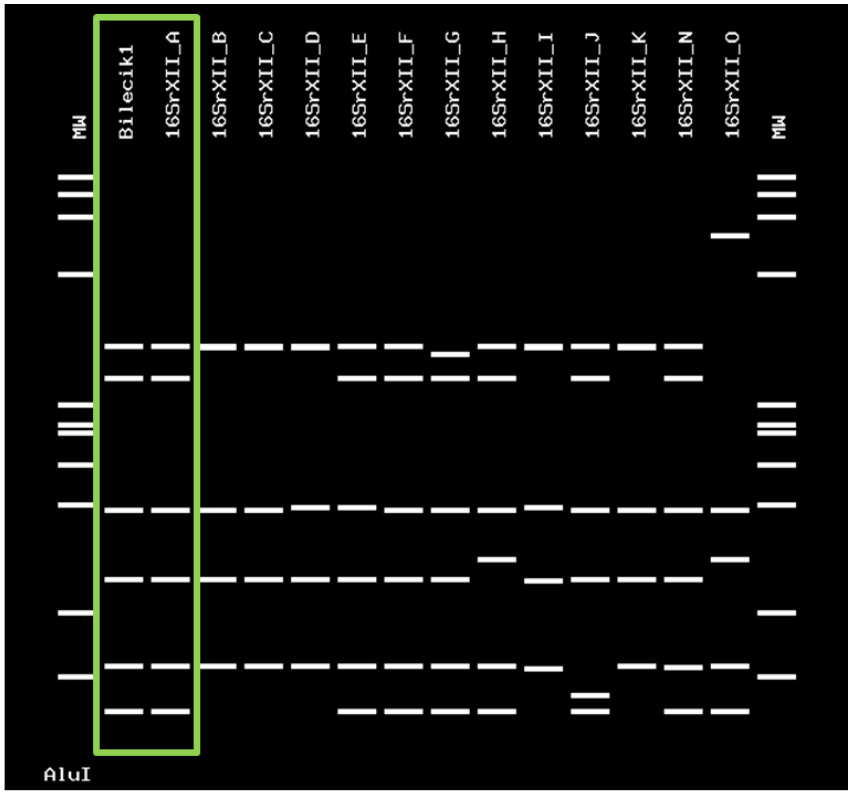

Figure 6. Comparison of 16Sr XII subgroups and *Alu*I endonuclease digesting profiles of 16Sr XII-A reference tomato sample from Bilecik province. The green rectangle shows the pattern of 16Sr XII-A and Bilecik province strain (Sogut1).

Phytoplasma infecting tomato in Bilecik was identified to belong to '*Ca*. P. asteris' 16Sr I-B subgroup due to band patterns after digestion of PCR amplicons using *Alu*I endonuclease *in vitro* analysis. However, *in silico* analysis using iPhyClassifier software suggested that the strains were '*Ca*. P. solani' 16Sr XII-A genetic subgroup. The study confirmed that phytoplasma is increasingly understood as a major pathogen of tomato in Türkiye, and sufficient control, probably through its vector management, needs to be implemented to avoid further damage.

CONCLUSION

The most important method for controlling viral agents is the breeding of resistant varieties. Breeding programs aimed at TSWV in tomato plants in particular are ongoing. However, varieties developed according to the host resistance genes are exposed to the molecular mechanisms of the virus that break the resistance over time. More efforts should be made to understand these mechanisms and genetic characterization studies should be carried out on different gene regions of the virus. However, the global seed trade impacts the spread of viral agents and affects the prevalence of STV. In addition, in controlling CaPsol phytoplasma, which has a very wide host range, it is important to ensure control of vector insects and weeds in cultivation areas.

Compliance with Ethical Standards

Peer-review

Externally peer-reviewed.

Declaration of Interests

The author has no conflict of interest to declare.

Author contribution

Conceptualization: F.R.Z, Methodology: A.K., A.I.S. and F.R-Z., Investigation: A.K. and F.R-Z Formal analysis: A.I.S., A.K., and F.R-Z., Writing—original draft preparation: A.K., F.R.Z., and A.I.S., Writing—review and editing:F.R-Z., Project administration: F.R.Z. All authors have read and agreed to the published version of the manuscript.

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