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Research Article
(Araştırma Makalesi)

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Presence of '*Candidatus* phytoplasma trifolii' in pepper plants showing yellowing and bushy appearance from Iğdır province of Turkey

Türkiye'nin Iğdır İli'nde sararma ve çalımsı görünüm gösteren biber bitkilerinde '*Candidatus* phytoplasma trifolii'nin varlığı

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ABSTRACT

Objective: The objective of this study was to investigate the group/subgroup of phytoplasma agent in peppers showing phytoplasma symptoms.

Material and Methods: In this study, plants collected from Iğdır province in 2020 were analyzed using direct and nested PCR tests, and BLASTn, iphyClassifier, Mega 7, and pDRAW32 programs were used.

Results: In the tests performed, approximately 1.2 kb of DNA fragments specific to phytoplasma were obtained. The 16S rRNA nucleotide sequence (1254 bp in length) (OM663745) revealed that it was showed more than 99.44% nucleotide similarity to other '*Ca*. P. trifolii' members. The tentative RFLP and phylogenetic analyzes performed proved the '*Ca*. P. trifolii' the infection from the Clover proliferation group (16SrVI) group and subgroup A in symptomatic pepper plants.

Conclusion: The presence of '*Ca*. P. trifolii' in naturally infected peppers in Iğdır province of Turkey was detected using PCR-RFLP and cladistic analysis.

ÖZ

Amaç: Bu çalışmanın amacı fitoplazma belirtisi gösteren biberlerde fitoplazma etmeninin grup/alt grubunu araştırmaktır.

Materyal ve Yöntem: Bu çalışmada, 2020 yılında Iğdır ilinden toplanan bitkiler direct ve nested PCR testleri kullanılarak analiz edilmiş ve BLASTn, iphyClassifier, Mega 7 ve pDRAW32 programları kullanılmıştır.

Araştırma Bulguları: Testlerde, fitoplazmaya spesifik yaklaşık 1.2 kb DNA fragmentleri elde edilmiştir. Belirlenen 16S rRNA nükleotit dizisi (1254 bp) (OM663745) %99.44'ten fazla diğer '*Ca*. P. trifolii' üyelerine nükleotit benzerliği göstermiştir. Gerçekleştirilen Sanal RFLP ve filogenetik analizler simptom gösteren biber bitkilerinde Clover proliferation group (16SrVI) grubu ve A alt grubundaki '*Ca*. P. trifolii' etmeninin infeksiyonunu kanıtlamıştır.

Sonuç: Türkiye'nin Iğdır ilinde doğal olarak enfekte olmuş biberlerde '*Ca*. P. trifolii' varlığı PCR-RFLP ve kladistik analiz kullanılarak tespit edilmiştir.

INTRODUCTION

Pepper (*Capsicum annuum* L.) is one of the important solanaceous crops from the Solanaceae family cultivated in numerous countries, accounting for a total share of around 40.5 million tons of production value in about 4 million ha (FAOSTAT, 2017). Turkey serves as an important producer of pepper for the global economy, accounting for around 2.554.974 tons, equivalent to 6.9% of the global pepper production. It is grown in an area of 919.730 ha in Turkey, with a yield of 2.777 kg/da (FAO, 2020). In Turkey, the main pepper-growing regions are Çanakkale, Antalya, and Bursa province. With a total pepper production area of 428 decare, Iğdır province has 878 tons of pepper production, equivalent to 19.14% of the national pepper production (TUIK, 2020). The sanitary of pepper plants is affected by many phytopathogens, such as bacteria, phytoplasma, viruses, fungi, and abiotic factors (Erdoğan, 2009; Julien et al., 2017; Beşkeçili vd., 2021). In particular, viral and phytoplasma diseases of pepper plants cause a confusion because they exhibit similar symptoms such as yellowing, witches' broom, flower infertility, resetting, and abnormal leaf tissue (Özdağ & Sertkaya, 2017; Yılmaz et al., 2019).

Phytoplasmas belong to the class *Mollicutes*, which are polymorphic obligate plant parasites that cannot grow in culture and have no cell wall (Lee et al., 2000). The agent known as the virus was named mycoplasma-like organisms after 1967 and phytoplasma in 1994 and was included in the genus '*Candidatus* Phytoplasma' in 2004 (Lee & Davis, 1992; IRPCM, 2004). The causative agent can be transmitted by vector insects such as leafhoppers and psyllids, by grafting, by natural root fusion, by parasitic plants such as dodder, and by weeds as alternative hosts (Weintraub & Beanland, 2006; Bertaccini, 2007; Fialová et al., 2009; Hogenhout & Music, 2010; Bayram et al., 2014; Flower et al., 2018; Hemmati et al., 2018).

Phytoplasmas are currently categorized into 33 ribosomal groups based on the 16S rRNA gene region, each with subgroups (Bertaccini & Lee, 2018), and are destructive to forest trees, vineyards, orchards, ornamental plants, weeds, and many plant species, including vegetables such as potatoes, tomatoes, peppers, and eggplants throughout the world. Especially in some cultivated plants, yield loss reaches 40% in eggplant, 60% in tomato, 30-80% in potato and 80% in cucumber (Bogoutdinov et al., 2008; Navratil et al., 2009; Rao & Kumar, 2017). In Turkey, various phytoplasma groups were detected in cultivated crops and ornamental plants (Alp et al., 2016; Usta et al., 2017), in orchards (Sertkaya et al., 2008; Usta et al., 2021), in maize from field crops (Güller, 2021), and grapevine (Ertunc et al., 2015). Infections of the phytoplasma-infected plants were typically recognized using 16S rRNA gene segment by molecular tools, including PCR, restriction fragment evaluation, and sequencing (Gundersen et al., 1996; Hodgetts et al., 2007).

Hence, a study was conducted and the objective of this study was to reveal the molecular presence and phylogeny of a possible infectious agent by employing PCR-based techniques on phytoplasma-suspected pepper plants in Iğdır Province of Turkey.

MATERIALS and METHODS

Source of phytoplasma isolate and genome isolation

A survey was conducted in August 2021 in the main vegetable crops cultivating localities of Iğdır Province of Turkey. A total of eight pepper samples exhibiting suspicious phytoplasma symptoms were sampled and stored in deep freeze conditions. Total genome isolation was accomplished from 0.5 g of leaf vein tissue using the GeneJET Genomic DNA Purification Kit (Thermo Fisher, USA) according to kit instructions. The extracted nucleic acids were kept at -80°C until further use.

Phytoplasma detection using universal/specific primers

To amplify the 16S rRNA gene fragment, phytoplasma-specific primers designated R16mF2/R16mR1 and R16F2n/R16R2 were synthesized by Sentebiolab (Ankara/Turkey) as described by Lee et al. (1993) and Gundersen & Lee (1996). The extracted DNAs were subjected to direct PCR for first amplification using R16mF2/R16mR1 primer pairs. The resulting amplified yields were diluted at a 1: 40 ratio using distilled RNase-free water for nested PCR amplification. Termocycling conditions were regulated: 2 min at 94°C for the first denaturation, followed by 35 repetitions of 1 min at 94°C, 2 min at 55°C (60°C for nested PCR), and 3 min at 72°C, finally 10 min at 72°C. The 50 µl of both PCR reactions mixes consisted of 4 µl of DNA as a template, 6 µl of 10x PCR buffer, 3 µl of MgCl2 (25 mM), 1 µl of dNTP (10 mM), 1 µl of reverse and forward primer (20 pmol), 0.4 µl of Taq DNA polymerase (0.5 U) (Thermo Scientific, USA), and 33.6 µl of distilled RNase free water. For stability of amplification assays, reaction mixtures, devoid of DNA, and phytoplasma isolates formerly detected were utilized as negative and positive controls, respectively. Nested PCR-amplified products were separated electrophoretically (at 90V for 50 min) in an agarose gel with EtBr and monitored with a UV light device (Synoptic, Cambridge, UK).

Cloning, sequencing, and nucleotide BLAST

A nested PCR positive isolate was purified from the gel (GeneJET Gel Extraction Kit, Thermo Fisher), promptly cloned into an appropriate bacterial plasmid, and transferred to competent cell *E. coli* bacteria. The 16S rRNA gene sequence of phytoplasma isolate in the pepper plant was revealed by sequencing the recombinant plasmids recovered from the transformed bacteria. The sequencing process was carried out by the relevant company using Next Generation Sequencing (NGS) (Sentebiolab, Turkey). The nucleotide identity matrix of the obtained sequence was checked for sequence consensus using the FASTA and BLASTN analysis tools in the online program NCBI GenBank. The related sequence was called and recorded in the GenBank database.

Similarity index and tentative analysis of Iğdır phytoplasma isolate

The similarity index of 16S rRNA segment was estimated by the *i*Phyclassifier program used for the assignment of phytoplasma species and the similarity coefficient (Zhao et al., 2009). A further analysis was executed by employing tentative Restriction Fragment Length Polymorphism (T-RFLP) analysis through pDRAW32 scientific software. In a tentative analysis, seventeen endonuclease enzymes were used to compare the restriction profile patterns (Lee et al*.,* 1998) with this of the reference isolate (AY390261) reported for 16Sr VI-A (Clover proliferation group) from *Trifolium hybridum* in Canada (Hiruki & Wang, 2004).

Phylogenetic dendrogram

To determine the phylogenetic relationship of the Iğdır isolate revealed in this study, a phylogenetic tree was created using various phytoplasma isolates available in the NCBI database. For this, sequences from chosen distinct geographic origina together with Iğdır sequence were aligned and trimmed to ensure sequence homogeneity of 16S rRNA nucleotide fragments. For the phylogenetic dendrogram, a neighbor-joining method was used with 1000 bootstrap scores (Saitou & Nei, 1987).

RESULTS and DISCUSSION

Symptomatology

In the surveyed region of Iğdır province, suspicious phytoplasma disease symptoms in pepper plants i.e. such as dwarfing, witches' broom, curling leaves, and diminished leaf sizes (Figure 1) were observed. Our field observations of pepper phytoplasma have also been extensively reported by various plant pathologists in Lebanon (Choueiri et al., 2007), Turkey (Sertkaya et al., 2007), Mexico (Santos-Cervantes et al., 2008), and Indonesia (Harling et al., 2009). Similar phytoplasma signs of pepper plants were reported by Oksal et al. (2017) who identified '*Ca*. P. trifolii', a member of the 16S VI-A group, in Turkey and by Khan & Raj (2006) who detected '*Ca*. P. asteris' from 16SrI Aster Yellows group in India using PCR-based approaches.

Figure 1. Pepper plants showing phytoplasma symptoms such as yellowing, shoot proliferation, dwarfing, leaf curling, and small leaves directed upwards in Iğdır Province of Turkey.

Şekil 1. *Türkiye'nin Iğdır ilinde sararma, sürgün çoğalması, bodurlaşma, yaprak kıvrılması ve yukarıya doğru küçük yaprak gibi fitoplazma belirtileri gösteren biber bitkileri.*

A total of eight samples with 3 symptomless and 5 symptomatic were tested against phytoplasma bacteria using 16S rRNA-specific primers. Nested and direct PCR tests yielded 1.2 kb and 1.8kb expected size DNA amplicons in one sample and the positive control, respectively, but not from other samples and the negative control (data not shown). In the current study, 4 out of 5 symptomatic samples in nested PCR tests gave a negative reaction to phytoplasma. This situation is probably because phytoplasmas and pepper viruses express similar symptoms. Many studies showed that pepper-associated viral signs resemble those of phytoplasma worldwide. Pepper-associated viral symptoms such as yellowing and severe dwarfing were reported in pepper plants using DAS-ELISA in Turkey (Hatay) (Özdağ & Sertkaya, 2017). In Mexico, similar symptoms on chile peppers were tested against *beet mild curly top virus* (BMCTV). It has been suggested that the causative agent is a new strain of virus named *Pepper yellow dwarf virus* (PeYDV), although closely related to BMCTV (Lam et al., 2009). In Saudi Arabia, symptoms such as yellowing and curling leaves associated with four viral pathogens in bell pepper specimens were affirmed using PCR tests by Kamran et al. (2018). With an infection incidence of 47.14%, the presence of *pepper mild mottle virus* (PMMoV) was reported by using DAS-ELISA on pepper plants showing stunting, leaf malformation, and chlorosis from Turkey (Antalya) (Şevik, 2011).

Nucleotide BLAST and tentative analysis

The nested PCR-positive amplicon detected was successfully cloned, sequenced, and revealed the nucleotide sequence. Iğdır phytoplasma sequence of 1254 bp was termed as Igdır I3 and recorded with accession code OM663745 to GenBank database. According to BLASTN analysis, 16S rRNA sequences displayed nucleotide identity between 100% and 99.44% with alfalfa isolate from Russia (KP864671) and *Datura* sp. isolate from India (MW261864), respectively, compared to consensus genes of '*Candidatus* Phytoplasma trifolii' isolates submitted to the GenBank site.

Based on the *i*PhyClassifier program, 16S ribosomal group/subgroup assignment of the Igdır I3 isolate was identified as a member of Clover Proliferation Group (16SrVI-A), with 1.00 the similarity index. The agent was closely related to the reference isolate '*Ca*. P. trifolii' under GenBank accession code AY390261. Clover Proliferation Group (16SrVI) consists of three main subgroups: VI-A ['*Ca*. P. trifolii', clover proliferation (CP)] (Hiruki & Wang, 2004), VI-B [Strawberry multiplier disease (SMPD)] (Jomantiene et al., 1998), and VI-C [Illionis elm yellows (ILEY)] (Jacobs et al., 2003). To fortify the subgroup of the Iğdır sequence in the 16SrVI group, the tentative restriction was performed using the 17 restriction

enzymes (*Alu*I, *Bam*HI, *Bfa*I, *Bst*UI (*Tha*I), *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hin*fI, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI (*Mbo*I), *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I) by the pDRAW32 program. Isolate Iğdır I3 showed an identical 16S RNA RFLP model with the reference isolate of '*Ca*. P. trifolii' (AY390261) (data not provied).

Cladistic analysis of phytoplasma Igdır i3 isolate

The Iğdır phytoplasma sequence detected was analyzed phylogenetically using 26 different phytoplasma groups and 16S rRNA gene sequences selected from different plants submitted to the GenBank. The phylogenetic tree revealed that the studied sequence (OM616883) clustered with the '*Ca*. P. trifolii' from different hosts and the reference sequence from Clover Proliferation Group VI, sub-group A (AY390261). Probably due to the close ecogeographic origin, the '*Ca*. P. trifolii' phytoplasma sequence in pepper exhibited closer sequence homology to other plant materials, including tomato (MF564268), cucumber (KR080212), and pear isolates (MH709141, MH730561) from Van province, tomato isolate (MT344968) from Iğdır province, and maize (MK372596), and tomato isolates (MT279852) from Bingöl province of Turkey (Fig. 2).

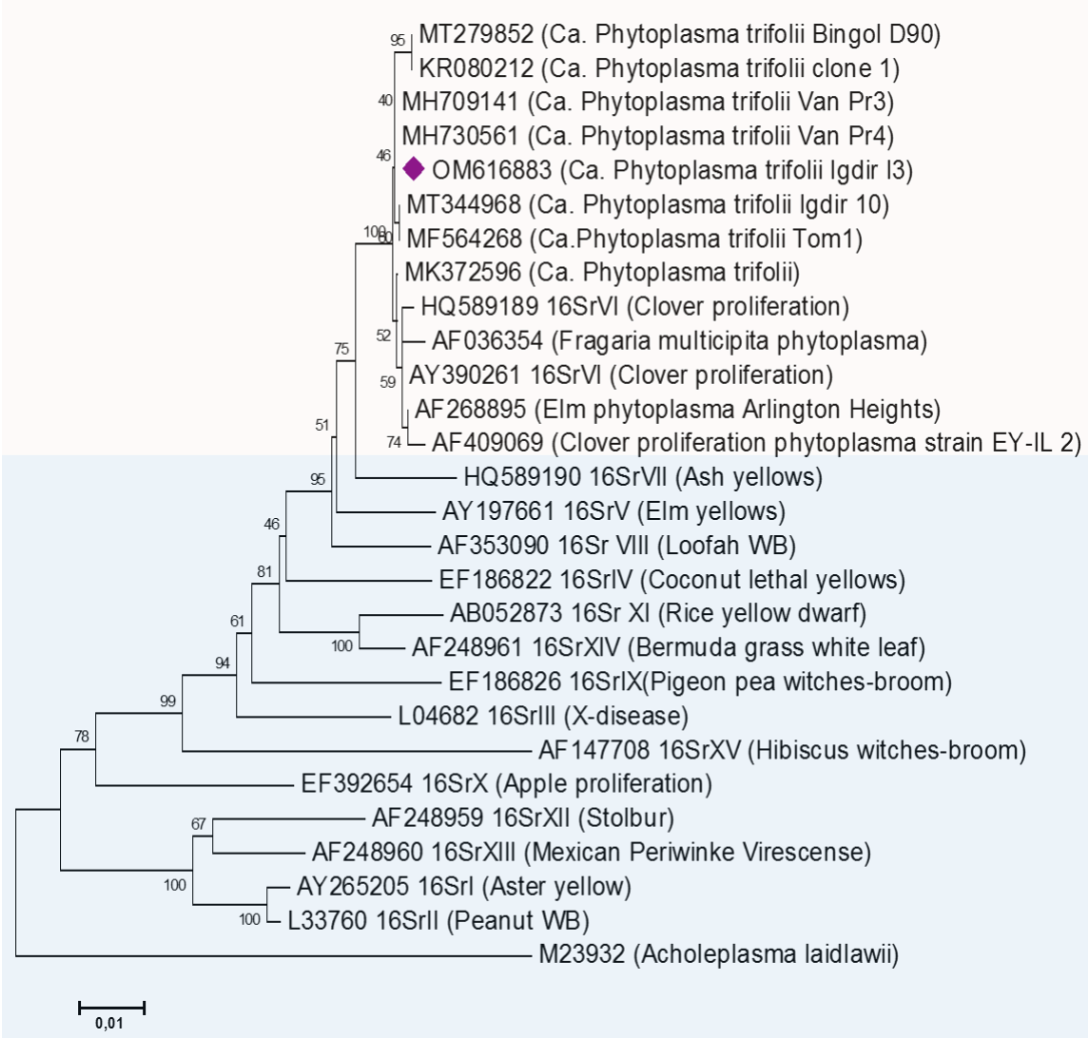

Figure 2. Neighbour-joining molecular phylogeny based on the 16S rRNA nucleotide sequence of '*Ca*. P. phytoplasma' isolate (Igdir I3, OM616883) from Iğdır province. *Acholeplasma laidlawii* is assigned as an outsource.

Şekil 2. *Iğdır ilinden 'Ca. P. phytoplasma' izolatının (Igdir I3, OM616883) 16S rRNA nükleotid dizisine dayalı Neighbour-joining metoduyla oluşturulan moleküler filogeni. Acholeplasma laidlawii dışgrup olarak atanmıştır.*

Pepper plant species have been reported to be an important host of phytoplasmas from different 16Sr groups. Literature screening revealed that phytoplasmas from five different 16Sr groups were infectious for pepper plants. Khan & Raj (2006) identified the '*Ca*. P. asteris' from 16Sr I-B ribosomal subgroups using phytoplasma-specific primer sets in pepper plants expressing symptoms of dwarfed and reduced leaves. Similarly, Zheng-Nan et al. (2013) reported the same phytopathogen in pepper plants showing witches' broom symptoms. Sertkaya et al. (2007) determined 16SrXII-A ('*Ca*. P. solani') in pepper with big bud symptoms. In addition, Harling et al. (2009) and Faghihi et al. (2016) reported the'*Ca*. P. aurantiolia' (16SrII group) in pepper plants exhibiting symptoms such as small-leaved, phyllody (virescence), big bud, and rosetting from Indonesia and Iran. In Bolivia, Arocha et al. (2010) identified the phytoplasma of X-disease (16SrIII group) in pepper plants with reduced leaf size, yellowing, shoot proliferation, shortened internodes, and a little fruits. The 16SrVI: The clover proliferation group was also ascertained in phytoplasma symptomatic pepper plants. In this study, we determined the presence of '*Ca*. P. trifolii' pathogen, a member of 16SrVI-A subgroup: Clover proliferation group, in pepper plant in Iğdır province based on nested PCR sequence-RFLP and phylogenetic analysis outputs. '*Ca*. P. trifolii'**,** which has a wide host range in many plant families, was first described in the 60s, and was included in the Clover proliferation group after being characterized in 2004, with the access code AY390261 (Chiykowski, 1965; Hiruki & Wang, 2004). Apart from pepper, the phytopathogen '*Ca*. P. trifolii' is also infectious to other solanaceous, cucurbits, leguminous, and graminae, fruit trees, weeds, some insects, ornamental plants, and weeds (Alp et al., 2016; Usta et al., 2017; Girsova et al., 2017; Salas-Muñoz et al., 2018; Oksal et al., 2017, 2020; Güller, 2021; Usta et al., 2021, 2022).

In the Igdır province of Turkey, the causative agent of pepper plants was first characterized in tomato plants expressing symptoms of curling and deformed leaves, infertility, and bushy upper tissues (Usta & Güller, 2020). In summary, the agent is present in the field conditions of this region, and if appropriate control strategies for phytoplasma are not implemented, the agent will continue to parasitize the other crops such as solanaceous, cucurbits, and fruit trees grown in this region. The use of appropriate molecular detection methods for phytoplasma infections will provide clues for the region's resident community in terms of disease management and agricultural production. Considering the continuity of phytoplasmas overwintering in insects and weeds to the next season, it is recommended to inform the producers about the primary control methods.

CONCLUSION

The association between phytoplasma and the appearance of the pepper plant was revealed using symptomatology, molecular tests, and computer-supported bioinformatic analyzes. Phytoplasma-infected pepper plants exhibited phytoplasma-specific signs such as yellowing, dwarfing, and bushy features in field conditions. The tests and analyzes proved the occurrence of '*Ca*. P. trifolii' from the 16SrVI-A subgroup. Performing phytoplasma screenings on vector and weed transmission of phytoplasmas will help minimizing phytoplasma infections under field conditions in this region.

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