https://doi.org/10.17221/163/2020-PPS

First report of '*Candidatus* Phytoplasma trifolii' associated with leaf reddening and upright growth in pears (*Pyrus communis* L.)

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Citation: Usta M., Güller A., Sipahioğlu H.M. (2021): First report of '*Candidatus* Phytoplasma trifolii' associated with leaf reddening and upright growth in pears (*Pyrus communis* L.). Plant Protect. Sci., 57: 188–195.

Abstract: The natural occurrence of '*Candidatus* Phytoplasma trifolii' in pear trees (*Pyrus communis* Linnaeus) is reported here for the first time. In 2017, a total of thirty-five pear trees, two of them exhibiting leaf rolling along the midvein, reddening, bushy appearance, and upright growth symptoms were sampled in different locations in Van province, Turkey. The total deoxyribonucleic acid was extracted from symptomatic and asymptomatic plants. The purified DNA served as a template in nested polymerase chain reaction (nested-PCR) assays, performed to amplify 16S rRNA sequences using universal primer pairs (R16mF2/R16mR1 and R16F2n/R16R2). The resulting PCR products were then cloned into a pGEM T-Easy vector and sequenced bidirectionally. The phytoplasma strain, group, and subgroup identity were determined using the *in silico* restriction fragment length polymorphism (RFLP) analysis of the 16S ribosomal RNA-encoding gene sequences profiling with seventeen distinct restriction enzymes. Of the thirty-five pear samples, only two yielded 1 256 bp and 1 258 bp DNA fragments and were designated as Van-Pr3 (Acc. No. MH709141) and Van-Pr4 (Acc. No. MH730561), respectively. Based on the *in silico* virtual RFLP pattern analysis of the 16S rRNA sequences, we confirmed the presence of '*Ca*. P. trifolii' belonging to the clover proliferation group and both identified phytoplasmas were identical with the similarity coefficient of 1.00 to the reference pattern of 16Sr group VI, subgroup A (Acc. No. AY390261). Here we report that the pear tree is an alternate host of the '*Ca*. P. trifolii'.

Keywords: clover proliferation group; detection; new host; pear; nested-PCR; virtual RFLP; Turkey

Phytoplasmas that evolved from low-GC gram-positive bacteria such as *Bacillus*, *Clostridium*, and *Streptococcus* spp. were first discovered in the 1960s and classified as mycoplasma-like organisms (MLOs) and later included within the Mollicutes class as a monophyletic group with the '*Candidatus* Phytoplasma' taxonomic unit because they could not be cultivated *in vitro* (Davis et al. 1997; Seemüller & Schneider 2004; Bai et al. 2006). They colonise in phloem bundles and insects' glands or other organs, transmitted from plant to plant, mostly by leafhoppers, planthoppers, and psyllids in the Hemiptera order, infecting more than a thousand plant species, including pome fruit trees (Seemüller et al. 2002; Weintraub & Beanland 2006). Phytoplasmas are specialised plant parasites characterised by having no cell walls, but have a standard phospholipid membrane, no flagella, and the smallest genome among all the bacteria (530–1 350 bp) (Seemüller 1989; Hogenhout et al. 2009).

Since the first discovery of phytoplasmas, they have been identified using Transmission electron

https://doi.org/10.17221/163/2020-PPS

microscopy (TEM) imaging, disease symptomatology, relationships between the host-pathogen, sensitive indicator plants and 4',6-diamidino-2-phenylindole (DAPI) staining (Nejat & Vadamalai 2013). Then, serological methods were developed thanks to the development of phytoplasma-specific antigens, because TEM imaging and DAPI staining could not distinguish different types of phytoplasmas (Brzin et al. 2003). Currently, the reliable diagnosis of phytoplasma infections and their group/subgroup discrimination have been carried out by restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) tests performed targeting the 16SrRNA region of the pathogen (Bertaccini & Duduk 2009). In addition to the 16S rRNA gene, recently, various conserved genetic markers, such as rp, secY, secA, groEL, tuf, and vmp1, can also be used as a supplemental tool for the taxonomy of phytoplasmas (Sugio et al. 2010; Wu & Baldwin 2010; Rashid et al. 2018).

The pear (*Pyrus communis* Linnaeus) is in the Pomoideae subfamily of the Rosaceae family and is a commercial crop grown in almost every region of the world after apples (Westwood & Bjornstad 1971; Bell & Hough 1986). The pear is an important host of the phytoplasma agents that cause growth reductions, redness of leaves, leaf curl, early leaf fall, swelling and death of shoots, tree decline and death of the whole plant (Németh 1986; Seemüller 1992; Gazel et al. 2007).

Apple proliferation phytoplasma ('*Ca*. Phytoplasma mali', 16SrX-A) and Pear decline phytoplasma (PDP) ('*Ca*. Phytoplasma pyri', 16SrX-C) infections, reported in most countries of the world, are considered to be the two main diseases of pears, PDP is especially on the European and Mediterranean Plant Protection Organization (EPPO) A2 quarantine list (EPPO 2020). Turkey is an important pear producer in the world with its 350 000 tonnes of annual production (FAO 2009). To date, in conducted surveys in pome fruit trees in Turkey, no phytoplasma diseases have been reported except '*Ca*. Phytoplasma pyri' and '*Ca*. Phytoplasma mali' (Sertkaya et al. 2005; Ulubaş Serçe et al. 2006; Canik & Ertunç 2007; Gazel et al. 2007; Sertkaya et al. 2008a, b; Kaya et al. 2016; Canik Orel et al. 2019).

In the present study, we investigated the presence of a possible causal agent responsible for pear leaf reddening and swollen twigs observed in pear orchards in Van province (Turkey). This paper reports the findings of these observations and the characterisation of '*Ca*. P. trifolii' detected in pear trees based on the analysis of the 16S rRNA sequences.

MATERIAL AND METHODS

Symptom observation and collecting plant material. Surveys were carried out from August to September 2017 in four pear orchards in Van province located in eastern Turkey. Typical leaf reddening, leaf rolling, and upright growth symptoms were observed in the collected local pear varieties. Leaf samples were collected from thirty-three non-symptomatic and two symptomatic plants.

DNA isolation and Nested-PCR assay. The total nucleic acid was extracted from 1 g of the leaf sample using a DNA extraction kit (Spin column based genomic DNA preparation kit, Jena Bioscience, Germany) following the instructions of the manufacturer. The purified DNAs were stored at –20 °C until further use. Direct and nested polymerase chain reactions were performed to amplify the 16S rRNA gene sequences of the phytoplasmas. The first-round PCR was primed by R16mF2/R16mR1 resulting in 1 800 bp DNA fragments and the second-round PCR was primed by R16F2n/R16R2 resulting in an approximately 1 250 bp DNA product. Both reactions were catalysed by the *Taq* DNA polymerase enzyme (Thermo Scientific, Lithuania) under the conditions previously described (Lee et al. 1994; Gunderson & Lee 1996). The first-round PCR products were diluted 30-fold in sterile distilled water and used as a template for the second round.

The isolated pear sample DNA that had previously tested negative for phytoplasma infection was used as the negative control. The total DNA from a previously characterised '*Ca*. P. trifolii' infecting pepper plants belonging to the clover proliferation phytoplasma group (subgroup 16SrVI-A, Acc. No. MF564266), was used as the positive control. Each PCR reaction was performed in a total volume of 50 µL, containing 1 µL of the sample DNA, 5.0 µL of a 10 × PCR reaction buffer, 1.5 mM of $MgCl_2$, 1.0 µL of a dNTP mixture (each at 2.5 mM), 0.25 µL of each primer (50 pmol), and 0.5 U of the Taq DNA polymerase enzyme (Thermo Scientific, Lithuania). The thermal cycling parameters used were one cycle at 94 °C for 1 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min and extension at 72 °C for 3 min and an additional cycle at 72 °C for 5 min (Lee et al. 1993). Twelve microliters of the PCR products were separated by electrophoresis on 2% agarose gel, stained by ethidium bromide, and visualised by gel imaging and analysis system (Syngene™ UV Transilluminator 2020LM).

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Cloning of PCR products and sequencing. Nested PCR-amplified phytoplasma fragments of the positive field samples were purified by a PCR product clean-up kit (Thermo Scientific GeneJET Gel Extraction Kit, Lithuania) according to the manufacturer's instructions, they were ligated into a pGEM-T Easy vector, and transformed into *Escherichia coli* JM109 (Promega, USA), following the manufacturer's instructions. Colonies were screened for the presence of cloned rRNA gene sequences using a colony PCR primed by R16F2n/R16R2 and the positive plasmids were sequenced by Sanger sequencing commercially (Sentebiolab, Turkey). The sequences were deposited in the GenBank database under Acc. Nos: MH709141 and MH730561.

Analysis of cloned DNA sequences and computer-simulated virtual restriction fragment length polymorphism. The 16S rRNA gene nucleotide sequence identity of the Van-pear phytoplasma isolates was analysed using the BLAST program in the National Center for Biotechnology Information (NCBI) GenBank. To study the relationships of the new phytoplasma isolates to the known phytoplasma isolates in the clover proliferation group (16SrVI), the nucleotide sequences of the 16S rRNA gene of '*Ca*. P. trifolii' isolates identified in the present study were used for the phylogenetic analyses. The sequence similarity, multiple alignment and the construction of the phylogenetic tree were performed using the Mega 7 software package with 1 000 bootstrap replicates. For the molecular evolutionary analysis, the nucleotide sequences from this study were aligned with the other thirty other phytoplasma accessions from the GenBank using the same software.

Identification of the phytoplasmas is further supported by the virtual RFLP analyses of the 16S rRNA F2nR2 fragments with a set of two key endonucleases (AluI and MseI) using the virtual gel plotting program pDRAW32 software (version PDRAW32 1.1.130). The similarity coefficients of the obtained sequences were estimated with the iPhyClassifier software, an online phytoplasma classification tool (Wei et al. 2007; Zhao et al. 2009). The restriction patterns of the sequences, obtained with seventeen distinct restriction enzymes, were compared to identify the group to which the studied phytoplasma belongs.

RESULTS

Symptoms of diseased plants. During our field survey conducted in pear orchards, we found two pear trees suffering from early reddening of leaves, defoliation of the shoots, reduction of the leaf size, and swollen twig tips (Figure 1). Further molecular analyses confirmed the presence of the phytoplasma '*Ca*. P. trifolii'.

Detection of phytoplasma. The nested PCR assays of all the collected samples tested using R16F2n/R16R2 generated fragments of 1 250 bp typical to phytoplasmas from two symptomatic pears and the positive control. No fragment was obtained from the other thirty-three samples from non-symptomatic trees collected in different

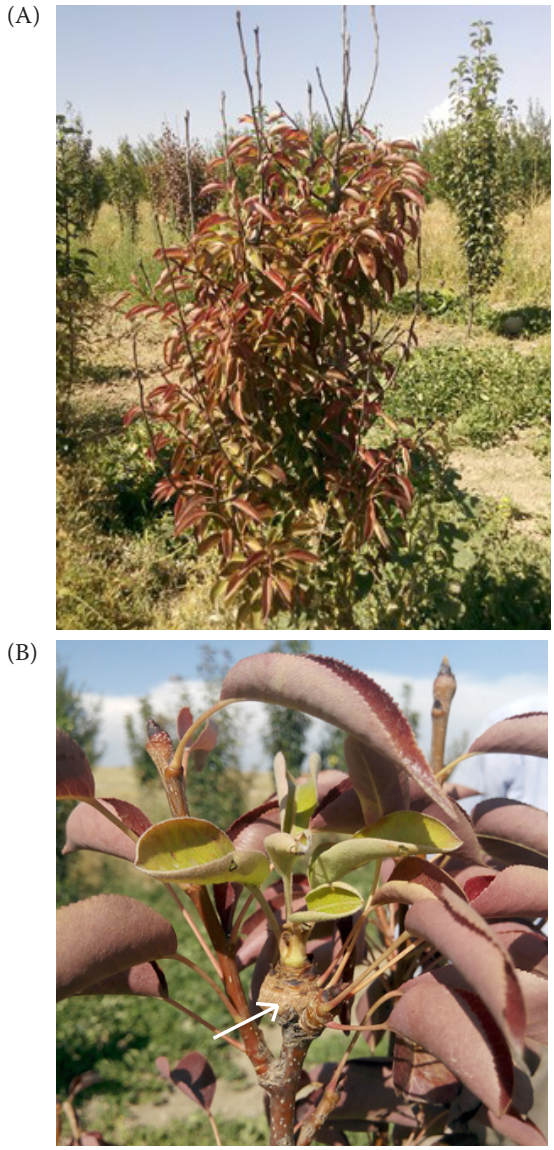

Figure 1. Severe phytoplasma symptoms observed on a pear tree in Van province; (A) redness of leaves and premature leaf defoliation and (B) swelling at the shoot tips, and the leaf curl along the midrib
arrow indicates swelling point

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pear orchards. The DNA from a healthy tree, used as negative control, generated no amplicon. The DNA sequencing homology of two isolates was conducted using the BLAST program. This search ascertained the presence of '*Ca.* P. trifolii' (16Sr VI-A group) isolates in pears and designated them as Van-Pr3 and Van-Pr4.

Sequencing and phylogenetic analysis. The 16S rRNA sequences of the Van-Pr3 (1 256 nt) and Pr4 (1 258 nt) isolates were deposited in the GenBank under Acc. Nos MH709141 and MH730561, respectively. The BLASTn analysis of the obtained 16S rRNA sequences showed that the sequence of two '*Ca*. P. trifolii' isolates (Van-Pr3 and 4) from the pear samples share 100% sequence identity to each other and 98.57% identity with the '*Ca*. P. trifolii' reference strain (Acc. No. AY390261). Similarly, pear isolates obtained from Van province revealed 98.96% TUR_Bas_634_HC (MT994432) and 98.97% (MT994432) nucleotide sequence identity with other '*Ca.* P. trifolii' isolates worldwide. The phylogenetic tree, created by a neighbour-joining algorithm, indicated that the '*Ca*. P. trifolii' isolates from Van province clustered with the reference strain (AY390261) and other isolates of the same phytoplasma species (Figure 2). Among the '*Ca*. P. trifolii' isolates from the GenBank, the isolates we detected (Van Pr3 and Van Pr4) were in a close relationship with the Turkish isolate of '*Ca*. P. trifolii' (KY321932) identified in peppers (Oksal et al. 2017).

***In silico* RFLP analysis**. The *in silico* analyses of the 16S rRNA sequences of the two '*Ca.* P. trifolii' isolates (GenBank Acc. Nos MH709141 and MH730561) and reference strain (AY390261) with two restriction endonucleases revealed a high-level

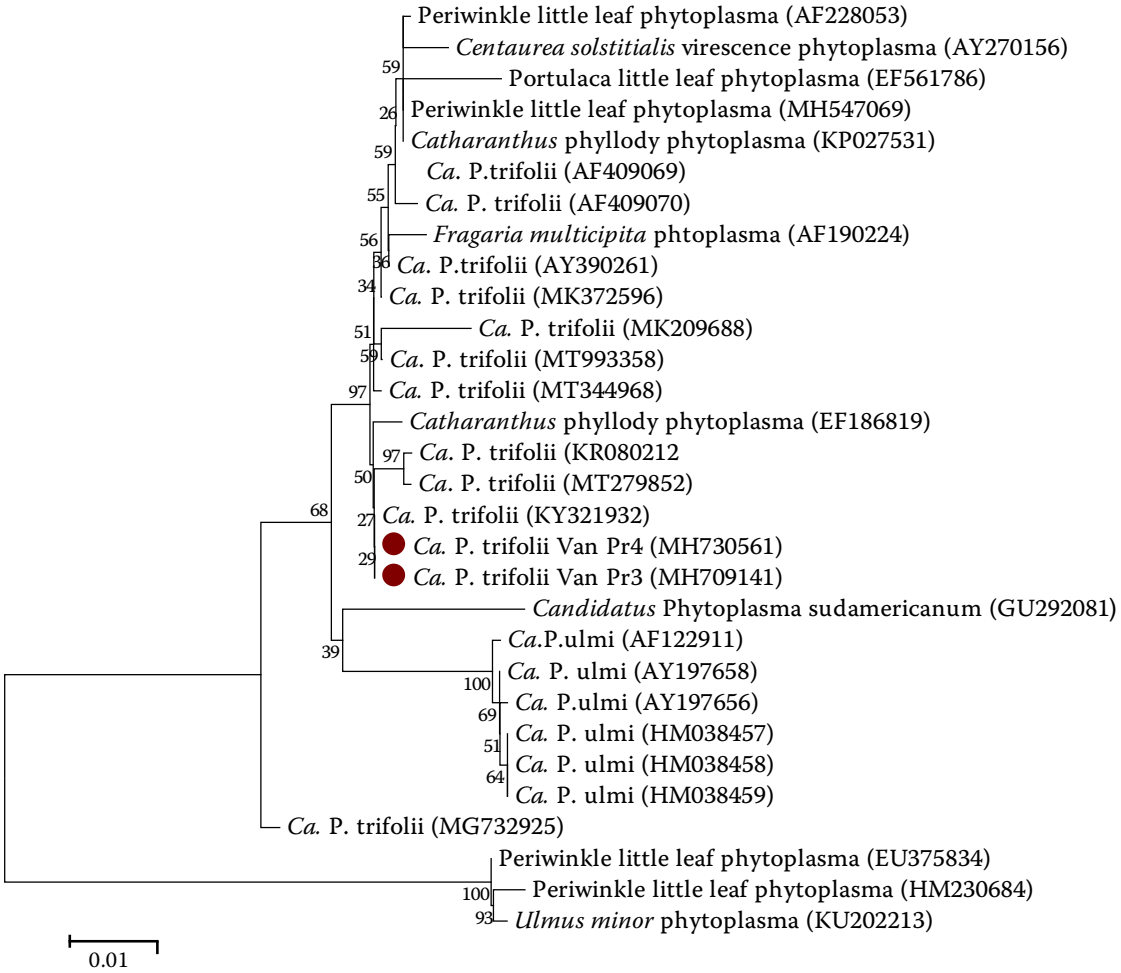

Figure 2. Dendrogram created by the neighbour-joining method of the 16S rRNA gene sequences of the '*Ca*. P. trifolii' isolates; Van-Pr3 (MH709141) and Van-Pr4 (MH730561)

Bootstrap values, consisting of 1 000 replicates, are located at the branch roots of the phylogenetic tree; the GenBank accession numbers of the '*Ca*. Phytoplasma' species are given in parentheses; bar – 0.01 nucleotide substitutions per site

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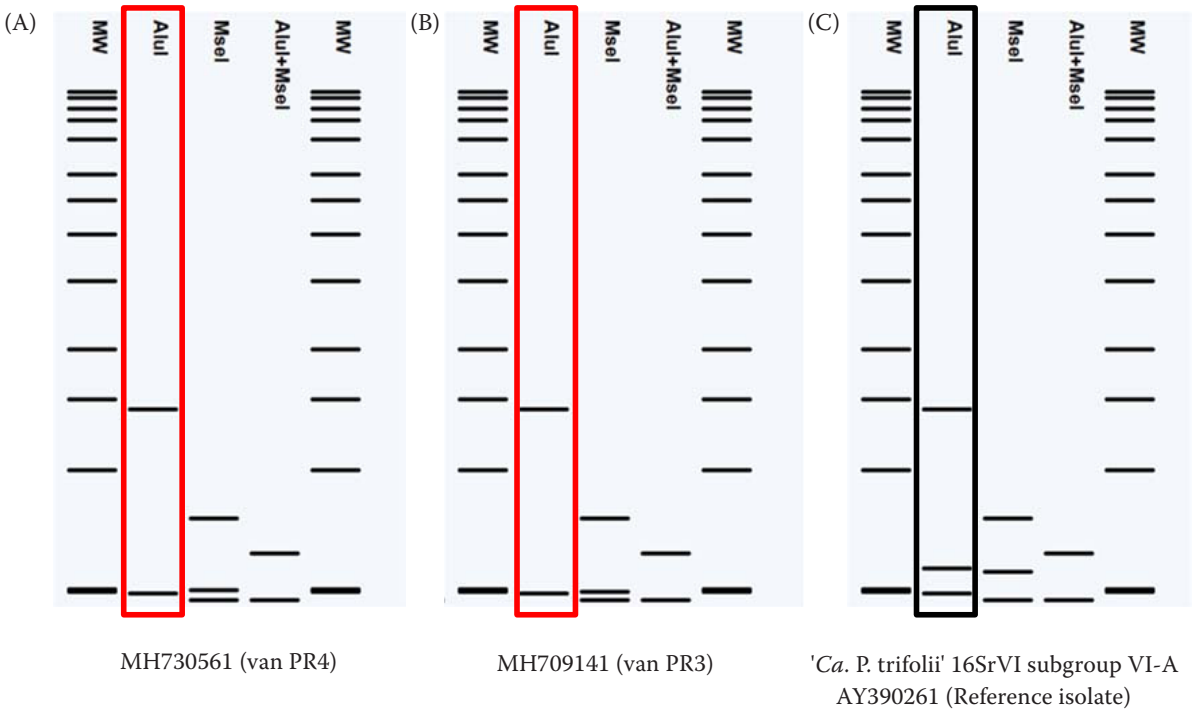

Figure 3. Virtual RFLP of the reference strain of '*Ca*. P. trifolii' belonging to Clover proliferation group and pear isolates of '*Ca*. P. trifolii': (A) Van-Pr4 isolate (MH730561), (B) Van- Pr3 isolate (MH709141) and (C) 16SrVI (AY390261)

The pear isolates exhibited unique RFLP patterns with the restriction endonucleases *Alu*I (red boxes) that clearly differentiated from reference pattern of reference strain (Acc. No. AY390261) (balck box); MW – DNA ladder size range 250–10 000 bp (Promega)

of homology between the Van isolates and the reference strain (Figure 3). Both isolates (Van-Pr3 and Pr4) exhibited highly similar restriction patterns (with a similarity coefficient of 1.00) to the reference pattern of 16Sr group VI, subgroup A. The R16F2n-R16R2 fragment of the 16S rRNA gene sequences of two '*Ca*. P. trifolii' phytoplasmas we studied and the reference strain were each digested in silico with two restriction endonuclease enzymes. The virtual RFLP analysis indicated that these two isolates were identical and slightly diverse from that of the reference phytoplasma isolate (Clover proliferation group, 16SrVI-A) (Figure 3). Both isolates exhibited unique RFLP patterns with the restriction endonucleases *Alu*I that clearly differentiated them from the reference pattern of 16Sr group VI, subgroup A (Acc. No. AY390261). However, the profiles performed with both digestion enzymes were identical for both our isolates and the reference isolate.

DISCUSSION

The molecular tests and *in silico* analysis of the 16S rRNA sequences were identified in association with the 16SrVI-A subgroup phytoplasma with the pears in Van province. In recent years, numerous phytoplasma survey studies have been completed in economically important crops and on their surrounding weeds in different geographical regions of Turkey (Alp et al. 2016; Oksal et al. 2017; Kaçar et al. 2019; Gazel et al. 2020; Oksal 2020). These surveys revealed the presence of phytoplasma agents belonging to different phytoplasma groups including 16Sr I (Aster yellows), II (Peanut witches' broom), V (Elm yellows), VI (Clover proliferation), IX (Pigeon pea witches' broom), X (Apple proliferation), XII (Stolbur), and XIV (Bermudagrass white leaf) (Canik & Ertunç 2007; Ertunc et al. 2015; Özdemir & Çağırgan 2015; Usta et al. 2017; Çağlar et al. 2019).

In the world, phytoplasmas in the 16 SrVI ribosomal group commonly affect plants grown in different geographic regions (Jamshidi 2014; Ayanur et al. 2018). This group, called Clover Proliferation, consists of 8 subgroups (A, B, C, D, E, F, H, I), including '*Ca*. P. trifolii' (Bertaccini et al. 2014). '*Ca*. P. trifolii' from the members of the 16SrVI-A ribosomal subgroup have been reported commonly in vegetables and weed species. It has been identified in potatoes

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(Rao et al. 2018), tomatoes, cucumbers, and peppers (Usta et al. 2017, 2018), soybeans (Ghayeb Zamharir & Aldaghi 2018), eggplants (Yadav et al. 2015), weeds, ornamental plants, woody plants, and fruit trees (Gupta et al. 2010; Zirak et al. 2010; Priya et al. 2016; Flower et al. 2018). In Turkey, '*Ca*. P. trifolii' has been reported in the sesame, periwinkle, pepper, eggplant, *Orosius orientalis* (Sertkaya et al. 2007; Oksal et al. 2017; Özdemir 2017), tomato and cucumber (Usta et al. 2017, 2018), *Brassica oleracea* (Ulubaş Serçe & Yılmaz 2020), and *Amaranthus retroflexus* (Oksal 2020). To our knowledge, this is the first report of '*Ca*. P. trifolii' belonging to the 16SrVI-A group infecting pears in Turkey and first record for the world.

This work shows that pear trees could be a host for the '*Ca*. P. trifolii' pathogen. Phytoplasmas and certain insect species are closely related. More than 100 weeds and insects are an important intermediate host for phytoplasmas (Singh & Upadhyaya 2013). Insects feeding on infected plants belonging to the family Cicadellidae (leafhoppers), Fulgoridae (planthoppers), and Psyllidae (psyllids) including *Circulifer haematoceps*, *Hyalesthes obsoletus*, *Thylocyba quercus*, *Macrosteles laevis*, *Pentastridius leporinus* are the main vectors for the spread of the phytoplasmas (Weintraub & Beanland 2006; Şahin et al. 2007; Weintraub & Jones 2009; Salehi et al. 2017). Recently, adults of *Empoasca decipiens* and *Zyginidia pullula* were reported on symptomatic pepper plants in the region (Oksal et al., 2017). However, the significance of the epidemiological role played by such pests is not known yet. In cases where the phytoplasma is present in this area and no precautions are taken for such vectors, it seems possible to spread more and may form epidemics.

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Received: November 9, 2020
Accepted: May 3, 2021
Published online: June 3, 2021