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Detection, *in silico* analysis and molecular diversity of phytoplasmas from solanaceous crops in Turkey

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Abstract: Phytoplasma-like symptoms of leaf yellowing and calyx malformation were observed in eggplant (*Solanum melongena* L.), upward leaves and fruit malformation in pepper (*Capsicum annuum* L.), and aerial tuber formation in potato (*S. tuberosum* L.) during the survey performed in the late season (August to September) of 2015 and 2016 in Van province (Turkey). A total of 100 samples were tested by nested-PCR using universal primer pairs to assess the sanitary status of the solanaceous crops and to characterise the phytoplasma isolates. Among them, seven samples resulted in a 1.25 kb DNA fragment, and five (two eggplants, two peppers, and one potato) were molecularly characterised (Accession No.: KY579357, KT595210, MF564267, MF564266, and MH683601). BLAST and the virtual restriction fragment length polymorphism (RFLP) analysis of 16S rRNA genes revealed the presence of two distinct phytoplasma infections in solanaceous crops: '*Candidatus* Phytoplasma trifolii' a member of the clover proliferation group (16SrVI) and subgroup A and '*Candidatus* P. solani' a member of the stolbur group (16SrXII) and subgroup A. The virtual RFLP analysis and calculated coefficients of RFLP pattern similarities further revealed a remarkable genetic diversity among the '*Candidatus* P. solani' isolates infecting pepper (similarity coefficient of 0.90) and eggplant (similarity coefficients of 0.98 and 1.00) at the same geographical area. This is the first report of the natural occurrence of '*Candidadtus* P. trifolii' in potato from the Eastern Anatolia region, Turkey.

Keywords: '*Candidatus* Phytoplasma solani'; '*Candidatus* Phytoplasma trifolii'; nested-PCR; virtual RFLP; 16S rRNA; pepper; eggplant; potato

Phytoplasmas are prokaryotic phytopathogens of agricultural crops belonging to *Acholeplasmataceae* family, Mollicutes class, and '*Candidatus* Phytoplasma' genus adopted by International Organization of Mycoplasma (IRPCM 2004). They have been first explored by Doi et al. (1967) since then, many distinct new species have been described in nature (Acosta-Pérez et al. 2017; Kra et al. 2017; Naderali et al. 2017). They are gram-positive, noncell walled, having a pleomorphic structure in different morphological forms, phloem restricted bacteria with a low quantity of guanine and cytosine bases in their genetic material (Bertaccini & Duduk 2010; Sugio et al. 2011).

Phytoplasmas cause destructive damage to many plant species including ornamental plants, vegetables, grapevines, and fruit trees around the world and are considered an economically restricting factor in many cultivated plants of high agricultural value (Bertaccini et al. 2014; Maejima et al. 2014; Usta et al. 2018). Phytoplasmas characteristically cause witches' broom, proliferation in shooting and rooting, flower defects, and increase in the host's metabolic activities by altering phytohor-

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mones activity (Duduk & Bertaccini 2011). Phytoplasmas have a broad host range and have been reported in many plant species throughout the world (Usta et al. 2018).

Solanaceae is a large plant family, encompassing 90 genera and more than 2 000 species, including potato (*Solanum tuberosum* L.), tomato (*Solanum lycopersicum* L.), pepper (*Capsicum annuum* L.), and eggplant (*Solanum melongena* L.), which are significant crops grown almost everywhere in the world (Shah et al. 2013). These crops are important hosts of phytoplasma associated diseases in Turkey and the world (Pracros et al. 2006; Choueiri et al. 2007; Sertkaya et al. 2007; Çağlar et al. 2010).

In recent years, the PCR amplification of the 16S rRNA gene and its sequence analysis has become the method of choice for detection and identification of phytoplasmas in their plant and insect hosts (Nejat & Vadamalai 2013; Bertaccini et al. 2014; Marcone 2014). By amplifying this specific region by PCR assay with appropriate primer sets and the use of *in silico* virtual restriction fragment length polymorphism (RFLP) analysis, the infectious agent can be diagnosed effectively (Zibadoost et al. 2016). The combination of both techniques has been proved to be useful for the identification and taxonomic grouping of mixed and single infections (Lee et al. 1998). By extending our previous survey study conducted in Van province, the current survey was carried out to discover and characterise the undetected phytoplasmas present in Van province with particular reference to solanaceous crops.

MATERIAL AND METHODS

Sampling and DNA isolation. Of 100 collected plants, sampled in 2015 and 2016 from solanaceous crops, 45 were peppers, 35 were potatoes and 20 were eggplants. The sampling was carried out from the plants with and without symptoms from Tuşba, Erciş, Edremit, and Gevaş locations of Van province, Turkey. Sampling was done to diagnose phytoplasmic diseases, especially in regions where pepper and potato production is intensive. Total genomic DNA was prepared using 0.5 g of leaf tissue containing midrib by a commercial kit (Genomic DNA Purification Kits; Jena Bioscience, Germany) according to the manufacturer's instructions. The isolated DNA was suspended in 50 µl of elution buffer and kept at –20 °C until use.

Detection of phytoplasmas by nested-PCR. Nested-PCR was used to detect phytoplasmas by amplification of a 16S rRNA gene fragments of about 1 250 bp length using two universal phytoplasma nested primer sets for the first step (R16F1 5'-AAGACGAGGATAACAGTTGG-3'; R16R0 5'-GGATACCTTGTTACGACTTAACCCC-3') and second step R16F2n 5'-ACGACTGCTAAGACTGG-3'; R16R2 5'-TGACGGGCGGTGTGTACAAACCCCG-3') (Lee et al. 1994; Gundersen & Lee 1996). The amplicons of the first step PCR obtained with R16F2/R2 primers were diluted 1 : 30 with nuclease-free water and used as a template for the nested step. Each 50 µL PCR reaction mix contained 5 µL of purified DNA, 28.75 µL of RNAase free H_2O, 3 µL of $MgCl_2$ (25 mM), 1 µL of dNTPs (10 mM), 10 µL of 5X Green buffer (Promega, USA), 0.25 µL of Go Taq DNA Polymerase (10 IU/µL) (Promega, USA), and 1 µL of sense and antisense primer (100 µM each). For the reliability of nested-PCR, '*Candidatus* P. solani' isolate previously obtained from tomato (*Solanum lycopersicum* L.) was used as a phytoplasma-positive control (Usta et al. 2018). Asymptomatic leaves from the plants belonging to the same family grown in a cool chamber served as the negative control. Thirty-five cycles of first and second step PCR reactions were performed under the following conditions: after initial denaturation for 2 min at 94 °C, denaturation at 94 °C for 1 min, annealing for 1 min at 60 °C (55 °C for the nested step PCR), and primer extension for 3 min at 72 °C (10 min in the final elongation cycle). 10 µL of PCR products were electrophoresed at 120 V for 45 min by loading 1% agarose gel, stained with ethidium bromide along with the 3 000 bp DNA ladder (Thermo Fisher Scientific, Lithuania) and separated based on fragment size of the PCR products.

Cloning, sequencing and the phylogenetic analysis of 16S rDNA sequences. The amplified 16S rRNA genes obtained from the symptomatic plants were cloned into the pGEM®-T Easy prokaryotic cloning vector and transformed into *E. coli* JM109 (Promega, USA). Purified recombinant, plasmids bearing the cloned phytoplasmic genome, were sequenced in an automated new generation sequencing system (Sentebiolab, Turkey). The nucleotide sequences were aligned with other '*Candidatus* P.' isolates in NCBI data utilizing CLC Main Workbench version 6.2 software. Based on 16S rRNA gene fragments, a phylogenetic tree was created with 1 000 bootstrap values using the neighbour-joining algorithm. For better separation of the phylogenetic

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tree, *Acholeplasma laidlawii* (M23932) was selected as the out-group.

Computer-aided virtual RFLP analysis. Virtual restriction fragment length polymorphism (V-RFLP) was performed by pDRAW32 program based on 16S rRNA gene sequences with 17 various restriction endonuclease enzyme as described by Lee et al. (1998) for determination of structural diversity. 16S rRNA group/subgroup classification concerning similarity coefficient and affiliation of detected '*Candidatus* P.' species was carried out via the current mode of *i*Phy Classifier computer-assisted tool (Zhao et al. 2009).

RESULTS

Survey and phytoplasma symptoms. During the entire survey period, a severe susceptibility of infected solanaceous crops to phytoplasma diseases was recorded. In both surveyed years (2015 and 2016), typical symptoms were appeared during the late summer, mainly in August and September. All cultivated solanaceous crops (eggplant, pepper and potato) seemed equally susceptible to phytoplasma diseases. Some plants showed small leaves and dwarfing with short internodes, others exhibited proliferation of several lateral individual branches (Figure 1) mostly with an upright growth which is summarized in Table 1.

On potatoes, the most characteristic symptoms were aerial tuber formation, a decrease in the number of leaves, and upward growth. The main symptoms observed on the infected eggplants were fruit deformation, hypertrophy in calyxes, and yellowing. On diseased peppers, as described by Oksal et al. (2017), the main symptoms observed were the presence of small leaves rolled upward, dwarfing,

Figure 1. Symptoms associated with phytoplasma disease in eggplant, pepper, and potato
(A) Leaf yellowing and fruit malformation in eggplant, (B) upward leaf curling, decreased leaf lamina, yellowing, and deformation in the fruit in pepper, (C) phytoplasma symptoms on the potato. Aerial tubers and upward rolling of the top leaves in '*Candidatus* Phytoplasma trifolii'-infected potato plant

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Table 1. Solanaceous host, number of characterised samples, symptoms observed on the host plant, detected phytoplasma species and the name of isolates, accession numbers and collection site of phytoplasmas from Van province, Turkey

Infected host	No. of sequenced samples	Symptoms observed	Detected phytoplasma species	Similarity coefficient	Isolate name	Accession No.	Collection site
Eggplant	2	leaf yellowing, calyx malformation, flower infertility	'*Candidatus* Phytoplasma solani'	0.98	VE1	KY579357	Alakoy/Tuşba
			'*Candidatus* Phytoplasma solani'	1.00	VE2	KT595210	
Pepper	2	apical upward leaves, fruit malformation, yellowing, bushy and stunting plant	'*Candidatus* Phytoplasma solani'	0.90	VB1	MF564267	Alakoy/Tuşba
			'*Candidatus* Phytoplasma trifolii'	1.00	VB2	MF564266	
Potato	1	aerial tuber formation, leaf curling, swollen nods, dwarfing	'*Candidatus* Phytoplasma trifolii'	1.00	VP	MH683601	Alakoy/Tuşba

yellowing, flower sterility, and hardened severe fruit malformation. The late-season new fruits produced by the symptomatic pepper, and eggplant were abnormal, generally small and malformed, almost all unmarketable (Figure 1). Only the symptomatic ones tested positive in nested PCR reactions.

Nested-PCR results. The R16F2n/R16R2 primers yielded DNA amplicons in nested-PCR of the expected size (approx. 1 250 bp) from all seven symptomatic solanaceous plants and the infected tomato (*Solanum lycopersicum* L.) (positive control) (Usta et al. 2018). Amplification products were obtained only from the vegetables collected from the symptomatic plants. According to the nested-PCR test results using gene-specific 16Sr RNA primers, seven out of 100 samples resulted in a 1.25 kb DNA fragment, with the same size as the positive control, among them two eggplants, two peppers, and one potato isolates were molecularly characterised (Figure 2). Particular '*Candidatus* P. solani' was identified from two eggplants and one pepper while '*Candidatus* P. trifolii' was identified from one pepper and two potato samples with the primers R16F2n/R16R2 (Figure 2). Molecular tests implementing amplification of the phytoplasma 16S rRNA gene from symptomatic potato, pepper, and eggplant revealed that the phytoplasma diseases occur in only Alakoy/Tuşba region (Table 1). The phytoplasma was detected in 8.8% of pepper, 2.9% of potato and 10% of eggplant samples collected in Van province. All symptomless plants of solanaceous species were tested negative.

Virtual RFLP analysis and phylogenetic tree. Results of nucleotide sequence analysis using iPhyClassifier software (Wei et al. 2007), and virtual RFLP analysis of 16S rDNA amplicons allowed

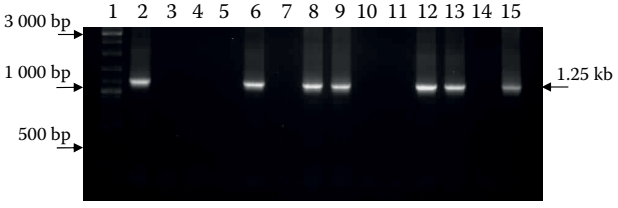

Figure 2. Agarose gel image showing '*Candidatus* Phytoplasma solani' and '*Candidatus* P. trifolii' by nested-PCR test from suspected potato, eggplant and pepper plants
Column 1 – marker (100–3 000 bp); Columns 2 and 6 – DNA bands of tested eggplant; Columns 8 and 9 – DNA bands of tested pepper; Columns 12 and 13 – DNA bands of tested potato; Column 14 – healthy plant; Column 15 – positive control ('*Candidatus* P. solani' isolate)

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identifying '*Candidatus* P. solani' and '*Candidatus* P. trifolii' in surveyed solanaceous crops. The virtual RFLP patterns, resulting from the digestion of PCR products of 16S rDNA fragment, were obtained *in silico* using 17 distinct restriction enzymes (Figure 3). Based on computer-simulated RFLP analyses, the isolates VB2 and VP showed profiles identical to the control reference isolate (AY390261) from subgroup 16SrXII-A with the similarity coefficient of 1.00. The isolates VE1 and VE2 showed profiles identical to the control reference isolate (AF248959) from the stolbur group (16SrXII) and subgroup A, with the similarity coefficient of 0.98 and 1.00, respectively. Comparison of virtual gel RFLP of sequences presented in this study showed that at least four isolates (VE1, VE2, VB2, VP) identified a high level of similarity with the representative RFLP pattern (Figure 3). However, the isolate VB1 from the stolbur subgroup (16SrXII-A) (similarity coefficient of 0.90) showed diverse profiles after virtual RFLP analyses with 17 restriction enzymes (Figure 3).

Nucleotide and phylogenetic analysis of the 16S rRNA gene sequences of the phytoplasma isolates was in agreement with the existence of two distinct phytoplasma species belonging to clover proliferation subgroup (16SrVI-A) and the stolbur subgroup (16S rRNA XII-A). Neighbour-joining analysis of 16S rRNA gene sequences confirmed the existence of two phylogenetic clusters corresponding to '*Candidatus* P. solani', and '*Candidatus* P. trifolii' (Figure 4). All phytoplasma isolates of a given species were clustered on the identical branch fortified by bootstrap values of a thousand. '*Candidatus* P. solani' pepper-1 (VB1) genotype was discriminated from the others, differing by multi nucleotide polymorphisms when compared with the other world isolates and native '*Candidatus* P. solani' genotype. The number of mutations between genotypes of the identical species was more abundant in '*Candidatus* P. solani' than in '*Candidatus* P. trifolii'. The amplicons obtained from R16F2n/R16R2 primers of pepper-2 (VB2) and eggplant-1 (VP) isolates, showed an identity of 99% with clover proliferation (16SrVI-A subgroup) in agreement with the phylogenetic tree (Figure 4).

DISCUSSION

Phytoplasma DNA belonging to clover proliferation and stolbur group were detected in solanaceous crops in Van province. In eggplants, the only '*Candidatus* P. solani', in potato, the only '*Candidatus* P. trifolii' was detected. However, based on PCR testing, in peppers, both phytoplasmas were identified. These findings indicate that '*Candidatus* P. solani' and '*Candidatus* P. trifolii' are the most important phytoplasmas associated with solanaceous crops in the region. Both are important pathogens of solanaceous crops in the world, which have been reported in the same region in other crops such as cucumber, tomato, pear, and marigold (*Tagetes erecta* L.) (Alp et al. 2016; Usta et al. 2017; 2021). Phytoplasmas are extremely virulent and destroy numerous plants such as potato, tomato, pepper,

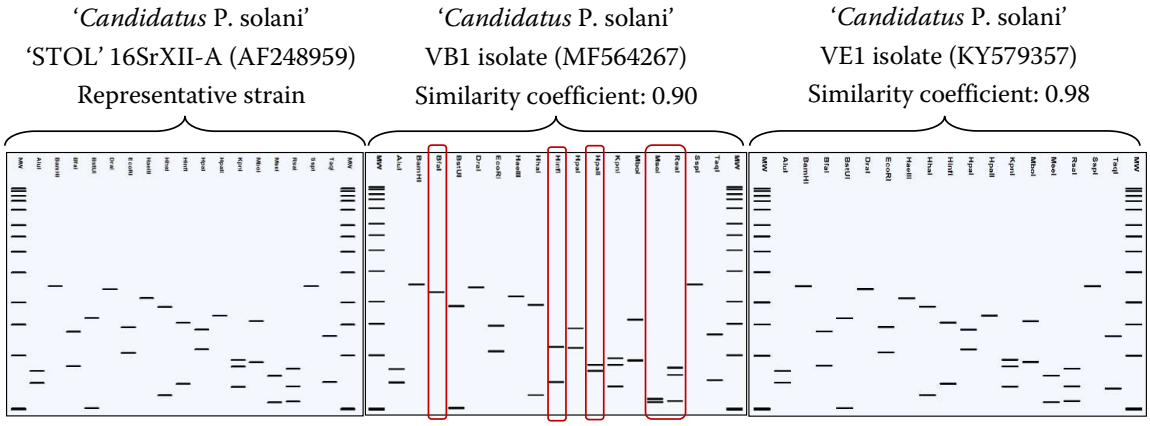

Figure 3. Virtual restriction fragment length polymorphism (RFLP) pattern with 17 different restriction enzymes of 16S rRNA genes of phytoplasma isolates infecting pepper, potato and eggplant

Virtual RFLP pattern was created from reference strain 16SrXII-A (AF248959), VB1 (MF564267), and VE1 (KY579357) isolates of '*Candidatus* Phytoplasma solani'

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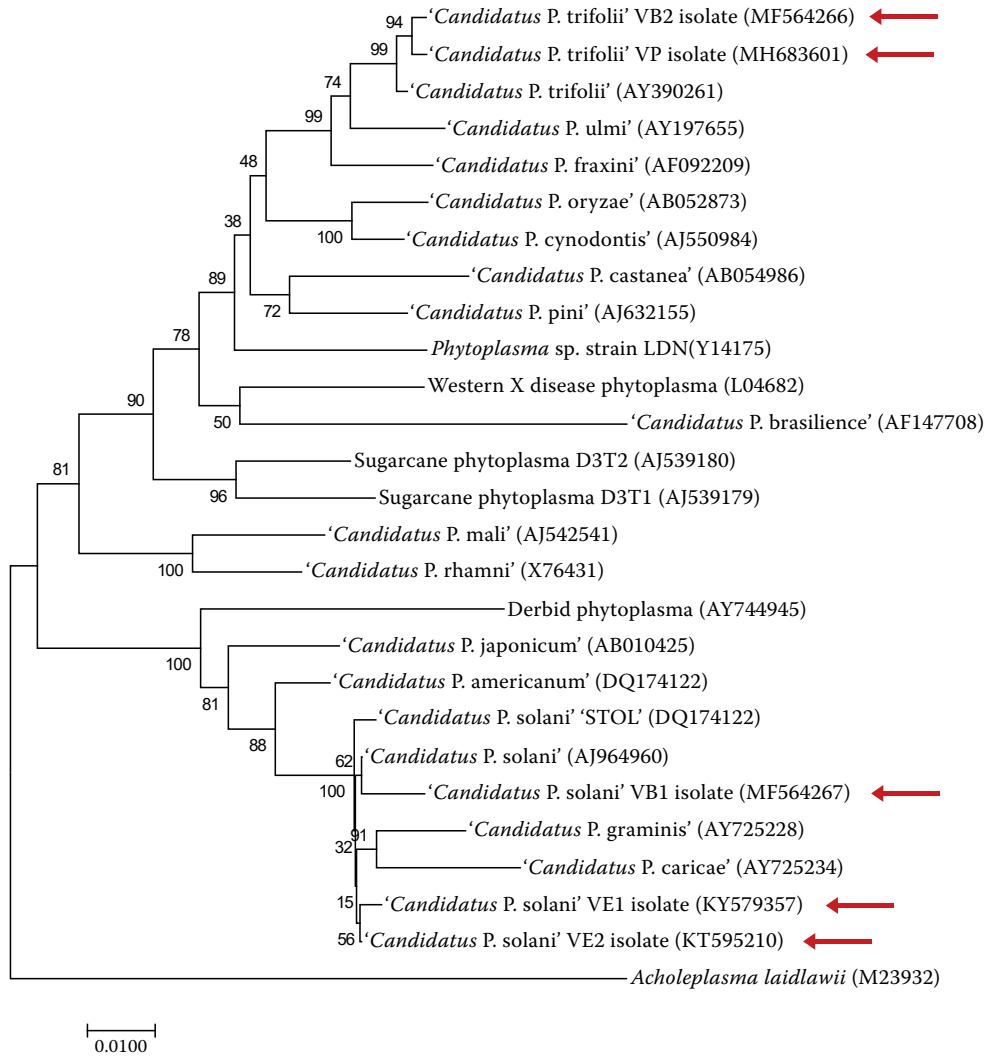

Figure 4. Phylogenetic relationships among 16S rDNA sequences of '*Candidatus* Phytoplasma solani', '*Candidatus* P. trifolii' and selected phytoplasmas, retrieved from NCBI Genbank, constructed by the neighbour-joining algorithm *Acholeplasma laidlawii* was selected as an outgroup to root the tree. The numbers of each branch indicate bootstrap values

and eggplant belonging to the *Solanaceae* family and result in substantial damage to both financial and environmental perspectives in crop plantations. '*Candidatus* P. solani' and '*Candidatus* P. trifolii' have been reported in various solanaceous crops in Turkey (Oksal et al. 2017; Usta et al. 2018) and in the world such as in pepper (Çağlar et al. 2010; Delić et al. 2016), in eggplant (Ember et al. 2011; Venkataravanappa et al. 2018) and potato (Holeva et al. 2014; Mitrović et al. 2015; Rao et al. 2018).

Analysis of the 16S rRNA genes of the 16SrVI-A and 16SrXII-A phytoplasma strains exhibited genetic variation. The isolates belonging to the clover proliferation group (16SrVI) shared high 16S rRNA gene sequence similarity with reference strains. However, the isolates of the stolbur group (16SrXII) showed different degrees of genetic variability through this gene. A high degree of genetic variability in similarity coefficient of '*Candidatus* P. solani' strains from pepper and eggplant and reference '*Candidatus* P. solani' strain (AF248959) indicates that the isolate from the pepper is not the same as the isolate from eggplant and reference strain of the same pathogen from GenBank (AF248959).

The R16F2n-R16R2 fragment of the 16S rRNA gene sequences of three '*Candidatus* P. solani' we studied, and the reference strain were each digested *in silico* with 17 restriction enzymes. The virtual 16S rRNA gene RFLP pattern of '*Candidatus* P. solani' VB1 isolate was highly diverse from that of stolbur phytoplasma (16SrXII-A, AF248959). Vir-

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tual RFLP analysis generated distinct RFLP patterns were generated by digestion of five key restriction enzymes, including *Bfa*I, *Hinf*I, *Hpa*II, *Mse*I, and *Rse*I. In contrast, the RFLP pattern of the VE1 isolate was identical to that of stolbur phytoplasma (16SrXII-A, AF248959), although the similarity coefficient was 0.98. This can be explained by the presence of nucleotide sequence variation in the 16S rRNA gene outside of the cleavage site of compared endonuclease enzymes. Both '*Candidatus* P. trifolii' (MF564266, MH683601) isolates clustered together with the reference strain of the clover proliferation group to form a 16SrVI clade. However, two isolates of '*Candidatus* P. solani' of closest similarity coefficient (0.98 and 1.00) to reference strain (AF248959) were formed an individual subclade separately. However, the distinct isolate '*Candidatus* P. solani' VB1 (similarity coefficient 0.90) was clustered with the reference strain to form a 16SrXII clade. These genetic diversities can probably be due to point mutations occurring in the 16S rRNA gene region, which indicates the development of new strains due to continuing evolvement (Arocha-Rosete et al. 2011).

The main principle in combatting plant pathogenic phytoplasmas is based on the prevention of the transmission and spread of the disease to new areas rather than the treatment of infectious plants (Alma et al. 2015). Numerous wild plants (approx. 100 weed species) and leafhoppers belonging to *Cicadellidea*, *Fulgoridae*, and *Psyllidae* family such as *Hyalesthes obsoletus*, and *Reptalus panzeri* are natural hosts of phytoplasmas and act as phytoplasma infection sources and thereby assist its propagation (Weintraub & Beanland 2006; Johannesen et al. 2012). Especially wild plants such as *Urtica dioica* (nettle), *Convolvulus arvensis* (bindweed), *Cuscuta* spp. (dodder) play incomparably the role of phytoplasma epidemics (Langer et al. 2003; Cvrković et al. 2013; Přibylová & Spak 2013). For this reason, in the case of phytoplasma disease, it is important to fight against weeds and vector insect populations in the affected areas where solanaceous crops are grown. Both are ensured to inhibit the spread of phytoplasma and diminish their economic influence (Mori et al. 2008). The present survey revealed the presence of two plant phytoplasma diseases ('*Candidatus* P. solani' and '*Candidatus* P. trifolii') on eggplant, pepper, and potato fields of Van province. Although the presence of potato phytoplasma diseases is common in Turkey, the present results reveal the first detection of stolbur phytoplasma associated with big bud disease and the first report of '*Candidatus* P. trifolii' associated with pepper proliferation in Turkey. Further studies are needed for the detection of other possible hosts of these phytoplasmas and vectors.

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