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Full Length Article

Molecular Analysis of '*Candidatus Phytoplasma trifolii*' and '*Candidatus Phytoplasma solani*' Associated with Phytoplasma Diseases of Tomato (PDT) in Turkey

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Abstract

Tomato plants displaying severe fruit deformation, flower sterility, aerial rooting, purplish leaves and leaf rolling were observed in tomato fields at Van province (Turkey). Samples were collected, and total DNA was extracted from symptomatic and asymptomatic plants. Nested polymerase chain reaction (nested-PCR) assays were performed to amplify 16S rDNA sequences for molecular detection using universal primer pairs. Out of 100 tested tomato samples, 11% of tomato samples yielded a DNA fragment of 1.25 kb. Amplified PCR products were then cloned into pGEM T-Easy vector and sequenced using new generation DNA sequencing (NGS) system. The virtual restriction fragment length polymorphism (RFLP) analysis of 16S rDNA sequences and molecular detections were allowed to characterize possible phytoplasmas associated with diseased plants. Our results revealed the presence of two *Phytoplasma* species belonging to two different ribosomal groups; '*Candidatus Phytoplasma trifolii*' (16Sr VI-A group) (Acces no. MF564268, MG732925) and '*Candidatus Phytoplasma solani*' (16SrXII-A group) (Acces no. KY579358, MF576263). Despite a high variation in their similarity coefficient of '*Ca. P. solani*' VTS2 (0.91) and '*Ca. P. trifolii*' VTT1 (0.88) isolates, the infected tomato plants generally displayed similar disease symptoms during field observations. Due to its commercial interest, co-existing of these phytoplasmas in tomato fields is of great phytosanitary significance not only for tomato plants but also for other crops such as vegetables, ornamentals and field crops. With this study, '*Ca. P. trifolii*' associated with phytoplasma diseases of tomato (PDT) has been reported for the first time in tomato in Turkey. © 2018 Friends Science Publishers

Keywords: '*Candidatus Phytoplasma trifolii*'; '*Candidatus Phytoplasma solani*'; Molecular analysis; *Solanum lycopersicum*

Introduction

Phytoplasmas are plant-pathogenic and phloem-limited agents that are responsible for hundreds of diseases in several hundred plant species world-wide (McCoy *et al*., 1989). Many species of plants are susceptible to phytoplasma diseases, including, fruit and vegetables crops, ornamentals and weeds.

Tomato (*Solanum lycopersicum* L.) is one of the economically most important vegetable crops in Turkey and known as susceptible against phytoplasma infections (Shaw and Kirkpatrick, 1993; Sertkaya *et al*., 2007). In many production areas of the world, phytoplasma diseases of tomato (PDT), have been described under different names including 'big bud' (Xu *et al.,* 2013), 'stolbur' (Ploaie, 1981), 'tomato yellows or yellows', (Tapia-Tussell *et al.,* 2012), hoja de perejil' (Arocha *et al*., 2007), and mal azul (EPPO/CABI, 1996). It has been reported that the causal agents of PDT are genetically diverse pathogens belonging to different ribosomal groups of phytoplasmas (Santos-Cervantes *et al.,* 2008). To date, six distinct ribosomal groups of phytoplasmas have been recognized that are responsible from PDT. These include: stolbur (16SrXII) subgroup A, clover proliferation (16SrVI), elm yellows (16SrV), Western-X (16SrIII), peanut witches' broom (16srII) subgroups A and D, aster yellows (16SrI) subgroups A and B (Marcone *et al.,* 1997; Okuda *et al.,* 1997; Lee *et al.,* 1998; Del Serrone *et al.,* 2001; Anfoka *et al.,* 2003, Amaral-Mello *et al.,* 2006; Arocha *et al.,* 2007; Sertkaya *et al*., 2007; Vellious and Lioliopoulou, 2007; Omar and Foissac, 2012; Singh *et al.,* 2012; Tapia-Tussell *et al.,* 2012; Du *et al.,* 2013; Xu *et al.,* 2013).

In Turkey and the world, tomatoes have been reported showing typical symptoms of phytoplasmas. The infections of tomato have been recorded in several countries including Italy, Jordan, the United States, Australia, India, Israel and Turkey (Granett and Provvidenti, 1974; Dale and Smith, 1975; Zimmerman-Gries and Klein, 1978; Varma, 1979; Shaw and Kirkpatrick, 1993; Serrone *et al*., 2001; Anfoka *et al*., 2003; Sertkaya *et al*., 2007; Ozdemir *et al*., 2009; Çağlar *et al*., 2010; Ozdemir and Saygili, 2012).

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Here, we report detection and molecular characterization of '*Ca. P. solani*' and '*Ca. P. trifolii*' belonging to two different ribosomal groups of phytoplasmas associated with tomato big bud and heavy fruit deformation of tomato at Van province. We also report for the first time, natural infections of '*Ca. P. trifolii*' occurring in tomato, in Turkey.

Materials and Methods

Tomato Samples, DNA Extraction and Nested Polymerase Chain Reaction

Over the late growing season of 2017, a total of 100 tomato plants showing typical phytoplasma symptoms and without symptoms were sampled from commercial tomato fields of Van province in Turkey. DNA samples were extracted from approximately 100 mg of fresh leaf tissue (Lee *et al*., 1993). Nested-PCR was carried out in a final volume of 50 μL of reaction mixture using universal primer pairs (R16mF2/R16mR1 and R16F2n/R16R2) (Lee *et al*., 1993; Gundersen and Lee, 1996). Total genomic DNA of '*Ca. P. solani*' from a previously characterized isolates infecting marigold (*Tagetes erecta*) was used as positive control (Alp *et al*., 2016). DNA extracted from asymptomatic tomato plant was used as negative control. The temperature regime of thermocycler was conducted with one cycle of 1 min at 94°C 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 of 5 min at 72°C (Lee *et al.*, 1993). The amplified DNA was analysed by electrophoresis in a 1% agarose gel visualised by staining with ethidium bromide under ultraviolet trans-illuminator.

Molecular Cloning, Sequencing and Phylogenetic Analysis of 16SrDNA Sequences

Purified two PCR products from each '*Ca. P. trifolii*' and two from '*Ca. P. solani*' were ligated onto pGEM T Easy Vector (Promega, USA) and transformed to *Escherichia coli* JM 109 by electroporation using micropulser following the manufacturer's instructions. Plasmid DNA from cultures containing insert DNA was purified using miniprep kit (Fermentas, Vilnius, Lithuania). Cloned DNA fragments were sequenced by new generation sequencing (NGS) system. The 16S rDNA sequences from the '*Ca. P. solani*' (MF576263 and KY579358) and '*Ca. P. trifolii*' (MG732925 and MF564268) have been assigned the Gen Bank. The assigned sequences were aligned and compared with the sequences of world isolates of 16S rDNA retrieved online from NCBI nucleotide sequence database. Phylogenetic relationships among phytoplasmas from both world isolates and the isolates sampled from Van province were assessed using neighbour-joining method and maximum parsimony methods of MEGA 4.0 (Tamura *et al*., 2007) and the robustness of the tree was assessed after 1000 bootstrapping replicates.

In silico Restriction Fragment Length Polymorphism (RFLP) Analysis

The 16S rDNA sequences corresponding to the R16F2/R16R2 fragment were evaluated *in silico* RFLP analysis after endonuclease digestion patterns were generated with pDRAW32 program (http://www.acaclone.com). The virtual gel patterns of 16S rDNA belonging to '*Ca. P. trifolii*' and '*Ca. P. solani*' Van isolates were compared with reference strains of '*Ca. P. solani*' (AF248959) and '*Ca. P. trifolii*' (AY390261), respectively. DNA fragments amplified in nested PCR were digested with various endonucleases by pDRAW32 software using 17 distinct restriction endonucleases that been routinely used *in silico* phytoplasma analyses (Lee *et al*., 1998) and plotted in a virtual 1% agarose gel. Phytoplasma 'species' assignment and 16Sr group/subgroup classification of tomato isolates were determined automatically by comparing with each reference strain. The incidence of similarity of obtained pattern from this study were generated using *i*PhyClassifier (http://www.ba.ars.usda.gov/data/mppl/) (Zhao *et al.*, 2009).

Results

Field Survey, Symptomatology and DNA Amplification

A total of 100 tomato leaf samples were screened against possible phytoplasma diseases from commercial tomato fields of Van province in 2017. Phytoplasmas were detected by nested-PCR in 11 out of 100 tomato samples associated with hardened tomato fruit, big bud, aerial roots on tomato trunk, and heavy fruit deformation symptom of tomato plants. As shown in Fig. 1, approx. 1.25 kb typical DNA bands were visualized with a UV light transilluminator in agarose gel, specific to phytoplasma from tomato samples. No amplicon was observed from asymptomatic tomato plant. Based on sequencing data of four randomly selected tomato isolates, two were determined as '*Ca. P. trifolii*' (16Sr VI-A group) and other two were ascertained as '*Ca. P. solani*' (16SrXII-A group).

In tomato fields of Van province, an infection rate of up to 11% was recorded. The main disease symptoms of PDT included hardened, discoloured and rectangular shaped fruits, aerial roots along the stem, phyllody and virescence, floral sterility and abnormalities, whitish fruit flesh and necrosis on seeds, fruit malformation and failure to fruit, rolled and purplish leaves and big bud (Fig. 2). The positively reacted samples in PCR, all were apparently symptomatic. The remaining plant samples neither exhibited PCR bands nor any of PDT symptoms.

Cladistic Analysis and Sequence Similarity

The presence of '*Ca. P. trifolii*' and '*Ca. P. solani*' genomes in tomato samples, exhibiting same symptomatology, were revealed by PCR amplification and RFLP analysis of phytoplasmal 16S rDNA fragments.

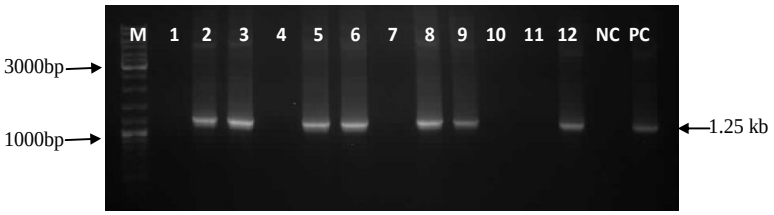

Fig. 1: Gel electrophoresis of PCR products for detection of '*Ca*. *Phytoplasma*' isolates of tomato. Lanes 1 to12 tomato samples, lanes 2, 3, 5, 6, 8, 9 and 12 positively reacted samples, lane NC: negative control, lane PC: positive control, Lane M, 3 kb DNA ladder

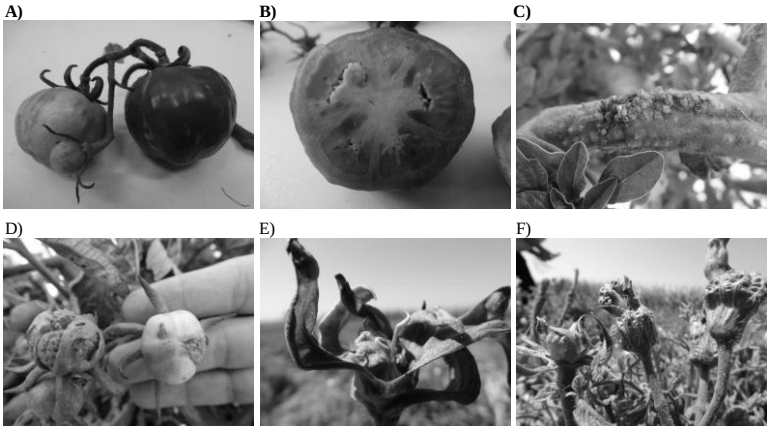

Fig. 2: Heavly phytoplasma infected *tomato* fruits that are often *unusable.* Hardened tomato fruits at different growing stages (A), whitish fruit flesh and necrosis on seeds (B), aerial roots on tomato trunk (C), heavy fruit deformation (D), giant *calyx* (E), and big bud (F) symptoms due to '*Ca*. *P. solani*' or '*Ca*. *P. trifolii*' infections

Out of 11 phytoplasma isolates detected, the only four were randomly selected for sequencing and cloning due to a limited supply of reagents. The sequences were deposited in GenBank and the isolates were designated as '*Ca*. *P. trifolii*' Van-tomato trifolii1 (VTT1) (MG732925), '*Ca*. *P. trifolii*' Van-tomato trifolii2 (VTT2) (MF564268), '*Ca*. *P. solani*' Van-tomato solani1 (VTS1) (MF576263) and '*Ca*. *P. solani*' Van-tomato solani2 (VTS2) (KY579358).

Using the neighbour joining and maximum parsimony methods, DNA sequences representing the '*Ca*. *P. trifolii*' (MG732925 and MF564268) and '*Ca*. *P. solani*' (MF576263 and KY579358) were compared with 20 other phytoplasmas from GenBank and a phylogenetic tree was constructed. The topology of phylogenetic tree was basically similar to that of 16S rDNA sequences. The phylogenetic tree (Fig. 3) revealed that the both agents belong to phytoplasma clade.

Similarity Coefficients and Virtual RFLP Analysis

Using the 16S rDNA gene sequences of '*Ca*. *P. trifolii*' and '*Ca*. *P. solani*' VT isolates and other reference strains of the 16SrVI and 16SrXII,the similarity coefficient were calculated and analysed by RFLP pattern comparison by iPhyClassifier software (Zhao *et al*., 2009). Results showed that the pattern similarity coefficient value of '*Ca*. *P. trifolii*' VTT1 and '*Ca*. *P. solani*' VTS2 isolates were lower than 0.97 and higher than 0.85 with the representative phytoplasmas classified previously in 16SrVI and 16SrXII groups, respectively. The VTT1 and VTS2 isolates were taxonomically grouped with the reference strains of '*Ca*. *P. solani*' and '*Ca*. *P. trifolii*' with the percentage of 98–99%. The virtual RFLP patterns of the 16S rDNA sequence of VTS1 and VTT2 isolates were closely related the members of 16SrVI and 16SrXII group, with the similarity coefficient value of 1.0 for both. However, the virtual RFLP patterns of VTT1 and VTS2 isolates were clearly distinct from the closely related members of the 16SrVI and 16SrXII group, with the similarity coefficient value of 0.88 and 0.91, respectively (Fig. 4).

The virtual RFLP profile of the 16SrRNA gene of VTT1 and VTS2 were similar with majority of the restriction enzyme. However, the genomic restriction pattern of the phytoplasma sequence of VTT1 digested with *Alu*I, *Bfa*I and *Mse*I endonucleases were exhibited significant differences with the close related reference strain (Fig. 4, panel A). Likewise, the genomic restriction pattern of the phytoplasma sequence of VTS2 digested with, *Kpn*I and *Rsa*I endonucleases exhibited significant differences with the closely related reference strains (Fig. 4, panel B).

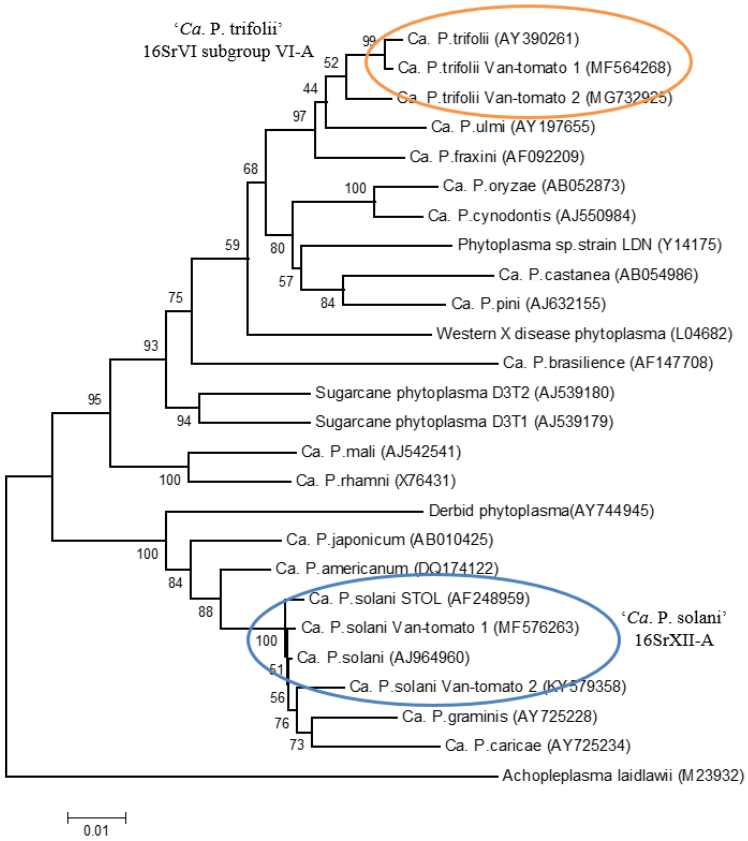

Fig. 3: Phylogenetic relationships among 16SrDNA sequences of '*Ca. P. solani*', '*Ca. P. trifolii*' and selected phytoplasmas, retrieved from NCBI Genbank, constructed by the neighbor-joining algorithm. *Acholeplasma laidlawii* was selected as the outgroup to root the tree. The numbers of each branch indicate bootstrap values

Discussion

Here, we analysed the genetic structure of tomato-associated '*Ca. P. trifolii*' VTT1 (MG732925) VTT2 (MF564268) and '*Ca. P. solani*' VTS1 (MF576263) and VTS2 (KY579358) isolates which are responsible for the PDT disease on tomato. One isolate of each species, identified by nested-PCR and RFLP analysis of 16SrDNA sequences, were exhibited distinct restriction patterns. For example, '*Ca. P. trifolii*' VTT1 (subgroup 16SrVI-A) and '*Ca. P. solani*' VTS2 isolates, (subgroup 16SrXII-A) sharing more than 98% 16SrRNA gene sequence similarity with members of subgroup 16SrVI-A and 16SrXII-A, respectively, were demonstrated that the variants of the same pathogen can cause same disease in the same individuals of a given species.

The isolate '*Ca. P. trifolii*' VTT1 showed three DNA fragments when digested by *Bfa*I and two fragments when digested by *Mse*I, which were two and three in the reference strain of '*Ca. P. trifolii*' 16SrVI subgroup VI-A (AY390261), respectively. This indicates the presence of one more *Bfa*I site and one missing *Mse*I site in '*Ca. P. trifolii*' VTT1 isolate, comparing with reference strain (AY390261). The isolate '*Ca. P. solani*' VTS2 showed two DNA fragments when digested by *Kpn*I and *Rsa*I restriction enzymes which were there in the reference strain '*Ca. P. solani*' "STOL" 16SrXII-A (AF248959), when digested by both enzymes. This indicates the absence of one *Kpn*I and *Rsa*I sites in '*Ca. P. solani*' VTS2 isolate, comparing with reference strain (AF248959). However, the isolates of VTT2 and VTS1 were exhibited similar restriction RFLP pattern with the reference strains in all the restriction enzymes used *in silico* RFLP analysis. The phylogenetic tree was consistent with the results of virtual restriction patterns constructed with the 16S rDNA gene sequences. Despite the low number of isolates on which these differences are based, they indicate the presence of a high level of heterogeneity among the individuals of same phytoplasma species in the same ecology.

Although the RFLP profiles of VTT1 and VTS2 isolates were more similar to those of 16SrVI-A (AY390261) and 16SrXII-A (AF248959), they showed a similarity coefficient of 0.88 and 0.91, suggesting that the VTT1 and VTS2 may represent a new subgroup within the 16SrVI and 16SrXII phytoplasma group, respectively. However, further genetic studies are needed particularly on VTT1 and VTS2 isolates to propose that these phytoplasmas should be given *Candidatus* status.

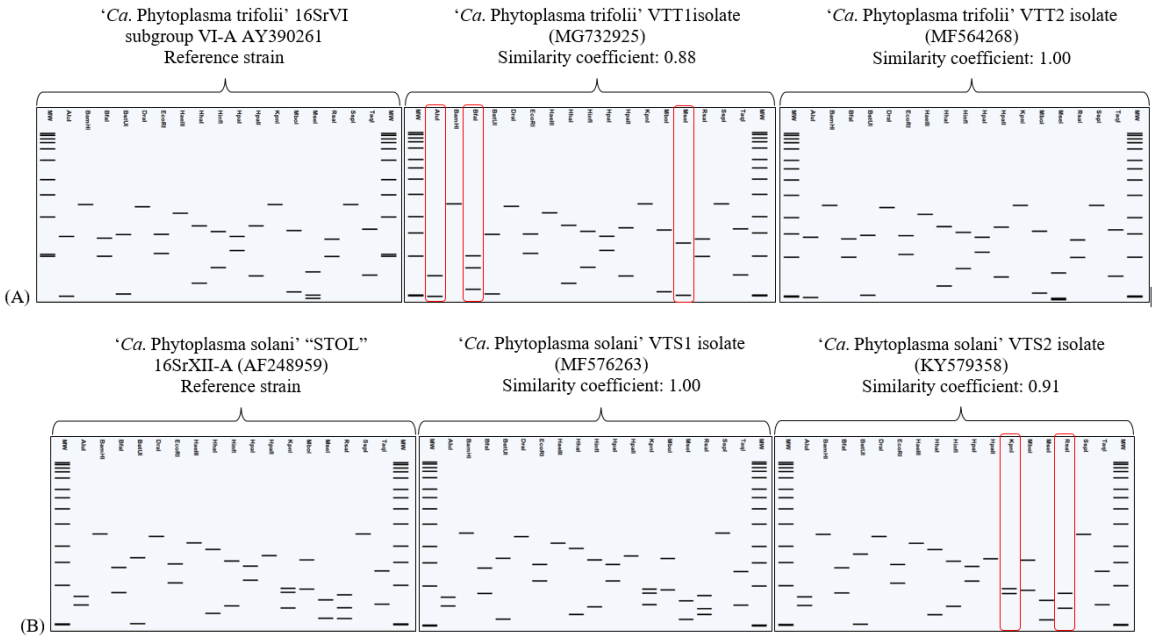

Fig. 4: Virtual restriction endonuclease digestion patterns of tomato-associated '*Ca. P. trifolii*' VTT1 (MG732925) and VTT2 (MF564268) isolates and '*Ca. P. solani*' VTS1 (MF576263) and VTS2 (KY579358) isolates andrepresentative strains of '*Ca. P. trifolii*' 16SrVI subgroup VI-A (AY390261) and '*Ca. P. solani*' "STOL"16SrXII-A (AF248959). In the simulated digestions for the recognition sites, the following 17 restriction enzymes were used: *Alu*I, *Bam*HI, *Bfa*I, *Tha*I (*Bst*UI), *Dra*I, *EcoR*I, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Mbo*I, *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I. Boxes indicate the differences in restriction patterns of the isolates. MW: 1 kb DNA ladder

Recently, we reported the presence of natural infections of '*Ca. P. solani*' and '*Ca. P. trifolii*' infecting cucumbers with similar symptomatology at the same survey area (Usta *et al*., 2017). In another province (Malatya) close to Van Province, Oksal *et al*. (2017) reported the presence of genetically diverse '*Ca. P. trifolii*' isolates at the same ecological niche. Vellious and Lioliopouou (2007) reported mix-infection of aster yellows (16SrI) and stolbur (16SrXII-A) in tomato, which were in correlation with the symptoms they showed. The phytoplasma characteristic 1.25 kb DNA bands amplified by nested-PCR demonstrated the presence of phytoplasma infections in tomato samples exhibiting big bud symptoms. Positive control DNA was exhibited the same size fragments and no DNA product was observed in healthy tomato plants.

Field surveys were performed in tomato growing areas of Van province at the late season (mid. September) of 2017. Our results indicated that phytoplasmas were present in 11% of overall tomato leaf samples tested by molecular methods. At least few symptomatic plants having symptoms similar to those caused by phytoplasma were noticed from each surveyed location. Similar symptoms associated in tomato plants were described by Dale and Smith (1975), Serrone *et al*. (2001), Anfoka *et al*. (2003) reported from other countries.

Phylogenetic analysis is in agreement with the results of RFLP analysis. Our study confirmed the existence of two distinct isolates belonging to 16SrXII-A Stolbur and 16SrVI-A clover proliferation groups. We report the presence of "variant clones" (VTT1 and VTS2) for each detected phytoplasma group. BLAST analysis of phytoplasma tomato isolates, detected in Van, showed 98–99% 16S rDNA sequence similarity with both phytoplasma groups.

Conclusion

The different isolates from same geographic region associated with diseases of tomato plant were examined. Despite a high genetic heterogeneity in field populations of two pathogens, both species induced similar symptoms in tomato suggesting the need for further studies with high number of isolates. The presence of new phytoplasma "variant clones", isolated from severely affected tomato plants, might represent a new threat to vegetable health. The agents play crucial role in the development of phytoplasma diseases of tomato (PDT).

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