Characterization of ‘Candidatus Phytoplasma solani’ associated with a maize leaf reddening disease in Turkey

Multigene characterization of '*Candidatus* Phytoplasma solani' associated with a maize leaf reddening disease in Turkey

Behçet Kemal Çağlar[1], Eray Şimşek[2], Murat Dikilitas[2], Assunta Bertaccini[3]

[1]Faculty of Agriculture, Department of Plant Protection, Çukurova University, Adana, Turkey

[2]Faculty of Agriculture, Department of Plant Protection, Harran University, Şanlıurfa, Turkey

[3]Department of Agricultural and Food Sciences, Alma Mater Studiorum–University of Bologna, Bologna, Italy

Correnspondence: Behçet Kemal Çağlar, Çukurova University, Faculty of Agriculture, Department of Plant Protection, Adana, Turkey, E-mail: kecaglar@cu.edu.tr

Abstract

In recent years, empty or poor grain bearing corn plants with leaf reddening symptoms have been observed in some commercial maize production areas in Adana province of Turkey. The disease has an ongoing increasing trend and cause economic losses. To verify the possible presence of phytoplasmas, leaf samples were tested with P1/Tint and R16F2n/R2 phytoplasma specific primer pairs after DNA extraction. The 16S ribosomal gene sequence analysis followed by sequence *in silico* enzyme digestions and phylogenetic analyses, allowed the identification of '*Candidatus* Phytoplasma solani', subgroup of 16SrXII-A related-strain in all symptomatic samples. The positive samples were subjected to multigene analyses for the molecular characterization of *tuf* and *vmp1* genes. *In silico* restriction fragment length polymorphism and sequence analyses allowed the inclusion of all the positive samples into tuf-type b and V15 molecular types. The sequence analysis indicated the presence of a single genotype in all the tested samples (n=12). This is the first molecular characterization of a '*Ca*. P. solani' strain infecting maize plants in Turkey. The identified pathogen is listed in the EPPO A2 list.

Key words: *Zea mays* L., phytoplasma, 16SrXII-A subgroup, PCR, *tuf, vmp1*

1 | INTRODUCTION

Maize (*Zea mays* L.) is a widely cultivated annual species in the *Poaceae* family followed by wheat and rice throughout the world. Maize is generally cultivated in humid regions in most parts of the world where 90% of its production is used for human and livestock feeding. It is grown in almost all regions of Turkey and has a great economic value to the national economy with a production of 6,500,000 tons. Adana province significantly contributes to the Turkish economy with the 12.6% of national maize production (819.978 tons) (Anonymous, 2020). Diseases associated with phytoplasmas in maize have been reported in some European countries including Serbia, Romania, Bulgaria, and more recently north Italy, Hungary, Bosnia and Herzegovina, Russia and India (Jović *et al*., 2009; Calari *et al*., 2010; Acs *et al*., 2011; Kovačević *et al*., 2014; Grigoryevna, 2015; Rao *et al*., 2017). Maize redness, also known as corn reddening, is a severe disease associated with the presence of '*Ca*. P. solani' or '*Ca*.P. asteris' that belongs to the 16SrXII-A and 16SrI-B ribosomal subgroups, respectively (Quaglino *et al*., 2013; Rao *et al*., 2017). Maize redness disease is transmitted in Serbia by the cixiid planthoppers, *Reptalus panzeri* (Löw) and *Hyalesthes obsoletus* Signoret (Hemiptera: Cixiidae) (Jović *et al*., 2007; 2009; Mori *et al*., 2013). Duduk and Bertaccini (2006) reported the first presence of phytoplasmas in corn with reddening symptoms in Serbia, then, maize redness disease associated with the same phytoplasma was reported in Hungary and Bulgaria (Acs *et al*., 2011; Genov *et al*., 2014). '*Ca*. P. solani' has a wide range of diverse host plants and many of them are of economic importance. Several studies have focused on its molecular identification and multilocus typing, moreover its genetic diversity was evaluated by different

protein coding-genes and molecular approaches (Cimerman *et al*., 2009; Fialová *et al*., 2009; Quaglino *et al*., 2019). Based on *tuf* gene, '*Ca*. P. solani' consists of two main strains named tuf-type a and b; while the tuf-type a strain is having an epidemic cycle that involves the stinging nettle (*Urtica dioica*), the tuf-type b is primarily associated with field bindweed (*Convolvulus arvensis*) (Langer and Maixner, 2004). The gene encoding variable membrane protein 1 (*vmp1*) has been employed for molecular characterization of immunodominant proteins linked to phytoplasma-host interactions (Cimerman *et al*., 2009); it is more variable and has shown its usefulness for '*Ca*. P. solani' epidemiological studies (Murolo *et al*., 2010; Aryan *et al*., 2014; Conigliaro *et al*., 2020).

In Turkey, phytoplasma symptoms in maize plants have recently been detected and studied due to their increasing incidence in fields (Çağlar *et al*., 2016, 2019). In the last two years, symptoms such as redness on leaf veins, empty and poor seed set in corn cobs were commonly observed in maize fields between April and May. This study was, therefore, designed to determine if these symptoms on maize plants are associated with '*Ca*. P. solani' presence, and to verify the possible presence of genetic diversity based on *tuf* and *vmp1* genes in the detected phytoplasma strain(s).

2 | MATERIALS AND METHODS

2.1 | Sample collection

Leaf samples were collected from commercial maize fields expressing reddening symptoms in Adana province, Turkey and tested to verify possible phytoplasma presence and identity. Samples were collected in an area of two hectares, during surveys between April-May 2020. A total of 12 plants exhibiting the symptoms of reddening disease in the midrib of leaf and stem carrying empty corn cob with poor seed set were sampled (three per field) from four

neighboring fields (Figure 1). One asymptomatic plant sample from each field was also collected as a negative control.

2.2 | Nucleic acid extraction

DNA was extracted to determine the possible phytoplasma occurrence from midribs of fresh leaves following the method of Ahrens and Seemüller (1992) with some minor modifications. In brief, 1 g midrib was homogenized in 4 ml of CTAB buffer pH 8.0 (2% w/v cetyl trimethyl ammonium bromide, 1.4 mol L^{-1} NaCl, 20 mmol L^{-1} EDTA, 100 mmol L^{-1} Tris HCl, 0.2% 2-β-mercaptoethanol, 2% polyvinylpyrrolidone-40) followed by an incubation at 65°C for 30 min. After elimination of plant tissue residues by centrifugation at 13,000 *g* for 10 min, the nucleic acids in suspension were clarified through chloroform–isoamyl alcohol (24:1), and precipitated with isopropanol at −20°C overnight. The precipitated nucleic acids were then collected by centrifugation at 13,000 *g* for 15 min, washed with 70% ethanol, dried at room temperature and finally dissolved in 50 µL of sterile double distilled water (SDDW). Before using in polymerase chain reaction (PCR) assays, nucleic acids were quantified and diluted with SDDW to a final concentration of 20 ng/µL.

2.3 | Detection and molecular characterization of phytoplasma DNA

The phytoplasma detection was carried out through nested PCR assays using the primer pair P1 (Deng and Hiruki, 1991) / Tint (Smart *et al*., 1996) amplifying a fragment of 1,600 bp from the 16S rRNA gene and the beginning of the 16S-23S spacer region, followed by R16F2n/R16R2 primers (Gundersen and Lee, 1996), which amplify fragments of 1,250 bp in the 16S rRNA gene. All the samples amplified with R16F2n/R16R2 primer pair were used for amplification of *vmp1* and *tuf* genes using fTuf1/rTuf1 and StolH10F1/R1 primer pairs

followed by nested-PCR with fTufAY/rTufAY and TYPH10F/R primer pairs, respectively (Schneider *et al*., 1997; Cimerman *et al*., 2009; Fialová *et al*., 2009). A DNA concentration of 40 ng was used as template in the final volume of 50 µL containing 1 µL dNTPs (10 mmol L^{-1} mM), 1 µL of each primer (10 pmol), 5 µL of 10X Dream Taq green buffer, 0.25 µL Dream Taq DNA polymerase (5 U/µL) (Thermo Scientific, USA) and 40.75 µL SDDW. PCRs using P1/Tint, fTuf1/rTuf1 and H10F1/R1 were carried out after initial denaturation for 3 min at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 60°C (for P1/Tint) and 55°C (for fTuf1/rTuf1 and H10F1/R1) and 2 min at 72°C followed by an extension step of 10 min at 72°C in Mini Amp Plus Thermal Cycler (Applied Biosystems by Thermo Fisher Scientific). Nested-PCR assays using R16F2n/R16R2, fTufAY/rTufAY and TYPH10F1/R1 were performed using 1 µL of a 1: 50 dilution in SDDW of the PCR product. All the parameters were identical in direct and nested PCR except for the annealing temperatures, which were 55-, 50- and 53°C for the primers R16F2n/R16R2, fTufAY/rTufAY and TYPH10F1/R1, respectively. Samples without DNA template were added as negative controls in all PCR reactions. Total nucleic acid of the BGWL-Tu-M1 ('*Ca.* P. cynodontis', GenBank accession number HE599389) strain (Çağlar *et al*., 2013) was employed as positive control. A strain of '*Ca*. P. solani' (Güldür *et al*., 2018) was used as positive control in the PCR studies with *tuf* and *vmp1* genes. Seven microlitres of amplicons were electrophoresed in 1% of 1X TAE agarose gel, stained with ethidium bromide and photographed under a UV transilluminator.

The nested-PCR products obtained using R16F2n/R16R2 and fTufAY/rTufAY primers from symptomatic maize plants were analyzed by RFLP. Enzymatic digestions were carried out in a final volume of 30 µL including 2 µL Fast Digest Green Buffer, 10 µL amplicons and 1 U restriction enzyme *Hpa*II (Thermo Scientific, Lithuania), according to the manufacturer's instruction at 37°C for 20 min. The digested products were separated in 1.5% of 1X TAE agarose gel, stained with ethidium bromide and photographed under a UV transilluminator.

2.4 | Sequencing and phylogenetic relationships

The R16F2n/R2 and TYPH10F1/R1 amplicons from four fields were purified using ExoSAP-IT® for PCR product clean-up (Thermo Fischer) according to the manufacturer's instructions. The purified products were quantified in a nanodrop spectrophotometer and sequenced in both directions with the same primers used for amplification in an automated sequencing (Macrogen, The Netherlands and Medsantek, Turkey). Sequence editing and assembly were carried out using MEGA 7 (Kumar *et al*., 2016). The sequences obtained were compared with those of phytoplasmas using the Basic Local Alignment Search Tool (BLASTn) system of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). Partial sequences from the 16S rRNA gene corresponding to the R16F2n/R2 fragments were subjected to *in silico* RFLP analyses with the *i*PhyClassifier tool *(*Zhao *et al*., 2009). In addition to 16S rDNA, the RFLP patterns of *vmp1* gene amplicons was studied to verify the phytoplasma identification accuracy . The nucleotide sequences were virtually digested with *Rsa*I endonuclease via pDRAW32 software version 1.1.146 (AcaClone software, http://www.acaclone.com).

Phylogenetic trees were generated using MEGA 7 with the neighbor-joining method and 1,000 replicates for the bootstrap analysis to estimate the stability of the inferred subclades. The 16S rRNA gene sequence from *Acholeplasma laidlawii* (GenBank accession number M23932) was used as an outgroup for the 16S rDNA tree root. Twenty-one nucleotide sequences shown in Table 1 were downloaded from the GenBank database and used together with the nucleotide sequences generated in this study to reconstruct the phylogenetic relationships according to *vmp1* gene.

3 | RESULTS

149 The symptomatic maize plants, mainly located at the edge of the surveyed fields,
150 exhibited severe reddening symptoms in leaves and midribs, and showed atrophied
151 inflorescence and empty corn cob with poor quality of seeds (Figure 1). The use of P1/Tint
152 primer pair enabled to amplify DNA bands (about 1.6 kb) from the positive control and from
153 all symptomatic leaf tissues. However, no bands were observed from the asymptomatic plants
154 negative control (data not shown). The 16S rDNA partial sequences of samples Tr-AcM 1, -2,
155 -3 and -4 showed 100% identity with each other and 99.75% with that of '*Ca.* P. solani'
156 (GenBank accession number AF248959) and were submitted to GenBank under the accession
157 numbers MW344059, MW344060, MW344061, MW344062, respectively. The identification
158 was made with *i*PhyClassifier based on *in silico* RFLP patterns (similarity coefficient 1.00) and
159 this confirmed that the detected phytoplasma belong to the 16SrXII-A subgroup (Figure 2).
160 This identification was further supported by the clustering analysis with other '*Candidatus*
161 Phytoplasma' species and '*Ca*. P. solani' strains deposited to GenBank from Turkey and
162 neighboring countries (Figure 3).
163 For the *tuf* and *vmp1* genes, amplification of approximately 1.4 kb and 0.9 kb, was
164 obtained from the nested-PCR assays with TYPH10F1/R1 and fTufAY/rTufAY primer pairs,
165 respectively (data not shown). Restriction patterns of PCR products from the *tuf* gene with
166 *Hpa*II were identical to each other and confirmed that all maize strains belong to tuf-type-b
167 (Figure 4). The primer pair TYPH10F1/R1 also amplified the expected length fragment in all
168 the maize samples and positive control but not from the negative controls. The *vmp1* amplicons
169 of Tr-AcM 1, -2, -3 and -4 samples were sequenced in both directions, and the sequences were
170 deposited in GenBank under the accession numbers MW984610, MW984611, MW984612,
171 MW984613, respectively. The unrooted phylogenetic tree (Figure 5) constructed using the
172 *vmp1* sequences listed in Table 1 and those obtained from corn from Turkey showed that the
173 nucleotide sequences of maize strains clustered according to the typology of the *Rsa*I/RFLP

(Figure 6). *Vmp1* gene amplicons of the four studied strains were virtually digested using the pDraw32 software that showed only one *Rsa*I profile, identical to the one of the '*Ca*. P. solani' strains enclosed in the lineage V15 (Figure 6).

4 | DISCUSSION

In this study, the presence of '*Ca.* P. solani' in commercial maize production areas in Southern Anatolia including Adana province, was reported for the first time in Turkey. While the phytoplasmas in the 16SrXII group are divided in numerous subgroups distributed in agricultural crops worldwide, only subgroup 16SrXII-A was detected, indicating the presence of a homogeneous phytoplasma population. A prerequisite to manage newly occurring diseases is the detection and proper identification of the associated pathogen. The identification of this phytoplasma strain in corn is, therefore, a first step for the management of the reddening of maize disease via cultural, chemical and breeding approaches.

The molecular characterization of '*Ca*. P. solani' in maize by using the *tuf* and *vmp1* genes confirmed the finding and the phytoplasma identity and represents a new epidemiological information about '*Ca*. P. solani' in Turkey. Based on the *tuf* gene characterization, all the symptomatic maize samples showed the presence of only tuf-type b which agrees with reports from Bosnia and Herzegovina (Kovačević *et al*. 2014; Delić *et al*., 2016). Tuf-type b is primarily associated with field bindweed (*Convolvulus arvensis* L.) (Langer and Maixner, 2004) that is its dominant reservoir plant species throughout most of the Europe and Turkey (Ember *et al*., 2011; Ozturk *et al*., 2017). With the abundance of these plants, '*Ca*. P. solani' has a strong potential to spread out to maize and other crop plants when the insect vector(s) are present. Typing *vmp1* gene with *Rsa*I digestion and sequence analysis indicated that all maize strains in the present study belonged to the same molecular type named V15. It was previously identified in the 22% of 56 *Hyalestes obsoletus* in Spanish vineyards (Sabaté *et al*., 2014), in

diseased grapevine and *Convolvulus arvensis* (Murolo *et al*., 2010*;* Sabaté *et al*., 2014; Quaglino *et al*., 2016; Jamshidi *et al*., 2019), and in tomato (Contaldo *et al*., 2021) in other Mediterranean Countries. In the present study, all symptomatic maize samples resulted infected by this phytoplasma strain. Identification of the same genetic variant of '*Ca*. P. solani' from different fields in the present study indicated that the pathogen population is strongly homogeneous, and the pathogens have a probable common origin. A similar case with severe corn reddening symptoms associated with a similar strain of phytoplasma was reported in Bulgaria (Genov *et al*., 2014) considering that close geographical areas could create potential danger for insect vector mobility. Limited studies have reported the presence of *H. obsoletus* and *Dictyophara europaea*, insect vectors of 16SrXII-A phytoplasmas, in vineyards of Turkey. However, the phytoplasma presence in those insect species were negatively reported previously (Bayram *et al*., 2014). Moreover, *Laodelphax striatellus* (Fallen, 1826), a possible vector of 16SrI, 16SrXII-A and 16SrIII group phytoplasmas (Prota *et al*., 2007), was previously detected in Turkish maize fields (Sertkaya *et al.*, 2010; Bayram *et al.*, 2014). Çukurova region is a large, flat and fertile area suitable for agricultural crop production where almost all agricultural products except tea and hazelnuts could be grown. Since this region is suitable for polycultivation due to the suitable climatic conditions and long vegetation period, two or more harvests can be possible for different crops in one season. As a result of this, any occurrence of new disease or emerging pathogen has great potential in terms of spread mainly due to insect vectors . It is known that phytoplasmas are spread primarily by insects families such as *Cicadellidae* (leafhoppers), *Fulgoridae* (planthoppers), and *Psyllidae* (jumping plant lice), which feed in the phloem of plants, and, in case of infected plants, they may transmit them to the next plant on which they feed. Thus, the host range of phytoplasmas strongly depends on the insect vector (Weintraub and Beanland, 2006). Inthe reported case, it is suggested that insect vectors might have played a significant role for the spread of phytoplasmas to maize plants.

Therefore, the cultivation of maize varieties resistant to the diseases associated with phytoplasmas and the control of insect vectors may be of great significance to manage this emerging disease situation. Further studies are, however, necessary to identify the potential insect vectors, the origin of the disease and its geographical distribution to develop a sustainable disease management. With the global increase in temperature, xerothermic insect vectors might migrate towards northern areas, and the cultivation of various host plant species in the same area would probably increase the possibility of phytoplasma dissemination (Maixner and Langer, 2006; Krishnareddy, 2013; Mamay and Şimşek, 2017). The phytoplasma-associated disease emergence in new geographic areas might create epidemics where susceptible host plant species are cultivated. Therefore, the main strategy for the control of the phytoplasma diseases is to monitor their presence and consider the biological activities of the insect vectors.

CONFLICT OF INTEREST

All the authors declare that there is no conflict of interest in this study.

AUTHOR CONTRIBUTION

BKÇ was responsible for establishing and designing the surveys and experiments. BKÇ conducted the Nested-PCR experiments based on 16S rDNA, BKÇ, EŞ and MD conducted the experiments based on *Tuf* and *VMP1*. AB conducted phylogenetic studies. BKÇ, EŞ, AB and MD wrote the manuscript and analysed the data. All authors have read and approved the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

Acs, Z., Jović, J., Ember, I., Cvrković, T., Nagy, Z., ... & Kölber, M. (2011). First report of maize redness disease in Hungary. *Bulletin of Insectology*, 64(Supplement), S229–S230.

253 Ahrens, U., & Seemüller, E. (1992). Detection of DNA of plant pathogenic mycoplasmalike
254 organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA
255 Gene. *Phytopathology,* 82(8), 828–832.
256 Anonymous. (2020). Crop production statistics. https://turkstatweb.tuik.gov.tr/PreHaber
257 Bultenleri.do?id=30685# (verified: May 04, 2021).
258 Aryan, A., Brader, G., Mortel, J., Pastar, M., & Riedle-Bauer, M. 2014. An abundant
259 '*Candidatus* Phytoplasma solani' tuf b strain is associated with grapevine, stinging nettle
260 and *Hyalesthes obsoletus*. *European Journal of Plant Pathology*, 140, 213-227.
261 Bayram, S., Zeybekoglu, G., Soylemezoglu, U., Canik, D., Karavin, M., Cakir, A. & Ertunc, F.
262 (2014). Presence of putative ınsect vectors of grapevine yellows phytoplasmas in Turkey.
263 *Phytopathogenic Mollicutes*, 41(1), 22–26.
264 Calari, A., Contaldo, N., Ardizzi, S., Bertaccini, A. (2010). Phytoplasma detection in corn with
265 reddening in Italy. In: Bertaccini, A., Laviña, A., Torres, E.. Sitges, (Eds.) *COST Action*
266 *Current status and perspectives of phytoplasma disease research and management*, (p.5).
267 Cimerman, A., Pacifico, D., Salar, P., Marzachì, C., & Foissac, X. (2009). Striking diversity of
268 *vmp1*, a variable gene encoding a putative membrane protein of the "stolbur" phytoplasma.
269 *Applied and Environmental Microbiology*, 75(9), 2951-2957.
270 Conigliaro, G., Jamshidi, E., Lo Verde, G., Bella, P., Mondello, V., Giambra, S., …&
271 Romanazzi, G. (2020). Epidemiological ınvestigations and molecular characterization of
272 '*Candidatus* Phytoplasma solani' in grapevines, weeds, vectors and putative vectors in
273 western Sicily (Southern Italy). *Pathogens*, 9(11), 918.
274 Contaldo, N., Stepanović, J., Pacini, F., Bertaccini, A., & Duduk, B. (2021). Molecular
275 variability and host distribution of '*Candidatus* Phytoplasma solani' strains from different
276 geographic origins. *Annals of Applied Biology*, (submitted).
277 Çağlar, B. K., Satar, S., Bertaccini, A., & Elbeaino, T. (2019). Detection and seed transmission
278 of Bermudagrass phytoplasma in maize in Turkey. *Journal of Phytopathology*, 167(4),
279 248–255.
280 Çağlar, B. K., Satar, S., Güneş, A., & Bozdoğan, O. (2016). Detection and characterization of
281 an agent causing yellowing, shortening of internodes and dwarfing on maize. In: Boyraz
282 N., Baştaş K.K. Eds. 6th Plant Protection Congress, 5-8 September, Konya, Turkey, 796.
283 Çağlar, B. K., Satar, S., & Elbeaino, T. (2013). Detection and molecular characterization of
284 Bermudagrass (*Cynodon dactylon*) white leaf phytoplasma from Turkey. *International*
285 *Journal of Agriculture and Biology*, 15(1), 90-94.

Delić, D., Contaldo, N., Lolić, B., Moravčević, Đ., Milošević, D., & Bertaccini, A. (2016). Multigene characterization of *'Candidatus* Phytoplasma solani' strains infecting pepper, celery and maize in Bosnia and Herzegovina. *Mitteilungen Klosterneuburg*, 66 (Suppl.), 55-59.

Deng, S., & Hiruki, C. (1991). Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes. *Journal of Microbiological Methods*, 14(1), 53–61.

Duduk, B., & Bertaccini, A. (2006). Corn with symptoms of reddening: new host of "stolbur" phytoplasma. *Plant Disease*, 90(10), 1313–1319.

Ember, I., Acs, Z., Munyaneza, J. E., Crosslin, J. M., & Kolber, M. (2011). Survey and molecular detection of phytoplasmas associated with potato in Romania and southern Russia. *European Journal of Plant Pathology*, 130(3), 367-377.

Fialová, R., Válová, P., Balakishiyeva, G., Danet, J. L., Šafárová, D., Foissac, X., & Navrátil, M. (2009). Genetic variability of "stolbur" phytoplasma in annual crop and wild plant species in south Moravia. *Journal of Plant Pathology*, 91(2), 411-416.

Genov, N., Mitrović, J., Genov, M., & Duduk, B. (2014). First report of corn reddening caused by '*Candidatus* Phytoplasma solani' in Bulgaria. *Plant Disease*, 98(7), 991.

Grigoryevna, V. (2015). Phytoplasmatic disease of maize under the conditions of Crimea. *Izvestiya Orenburgskogo Gosudarstvennogo Agrarnogo Universiteta*, 5, 14-17.

Güldür, M. E., Simsek, E., Caglar, B. K., Dikilitas, M., Gumus, H., & Ayvaci, H. (2018). First report of a '*Candidatus* Phytoplasma solani'-related strain (16SrXII-A subgroup) associated with pistachio leaf scorch disease in Turkey. *New Disease Reports*, 38(1), 4.

Gundersen, D. E., & Lee, I-M. (1996). Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. *Phytopathologia Mediterranea*, 35, 144–151.

Jamshidi, E., Murolo, S., Ravari, S. B., Salehi, M., & Romanazzi, G. (2019). Molecular typing of '*Candidatus* Phytoplasma solani'in Iranian vineyards. *Plant Disease*, 103(9), 2412-2416.

Jović, J., Cvrković, T., Mitrović, M., Krnjajić, S., Redinbaugh, M.G., Pratt, R. C., ...& Toševski, I. (2007). Roles of "stolbur" phytoplasma and *Reptalus panzeri* (Cixiinae, Auchenorrhyncha) in the epidemiology of maize redness in Serbia. *European Journal of Plant Pathology,* 118, 85–89.

Jović, J., Cvrković, T., Mitrović, M., Krnjajić, S., Petrović, A., Redinbaugh, M. G., ... & Toševski, I. (2009). "Stolbur" phytoplasma transmission to maize by *Reptalus panzeri* and the disease cycle of maize redness in Serbia. *Phytopathology*, 99(9), 1053-1061.

Kovačević, M., Đurić, Z., Jović, J., Perković, G., Lolić, B., Hrnčić, S., ... & Delić, D. (2014). First report of "stolbur" phytoplasma associated with maize redness disease of maize in Bosnia and Herzegovina. *Plant Disease*, 98(3), 418.

Krishnareddy, M. (2013). Impact of Climate Change on Insect Vectors and Vector-Borne Plant Viruses and Phytoplasma. In Singh H., Rao N., Shivashankar K. (Eds.) *Climate-Resilient Horticulture: Adaptation and Mitigation Strategies* (pp.255-277). Springer.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33(7), 1870–1874.

Langer, M., & Maixner, M. (2004). Molecular characterisation of grapevine yellows associated phytoplasmas of the "stolbur"-group based on RFLP- analysis of non-ribosomal DNA. *Vitis,* 43, 191-199.

Maixner, M., & Langer, M. (2006). Prediction of the flight of Hyalesthes obsoletus, vector of stolbur phytoplasma, using temperature sums. *IOBC wprs Bulletin*, 29(11), 161-166.

Mamay, M., & Şimşek, E. (2017). The Expected Impact of Global Warming and Climate Change on Insect Biodiversity. In: Semiz, G., and Akyildiz, G.K. (Eds.) *The 3rd International Symposium on EuroAsian Biodiversity, 05-08 July 2017, Minsk – BELARUS*, (173).

Mori, N., Mitrović, J., Smiljković, M., Duduk, N., Paltrinieri, S., Bertaccini, A., & Duduk, B. (2013). *Hyalesthes obsoletus* in Serbia and its role in the epidemiology of corn reddening. *Bulletin of Insectology*, 66(2), 245-250.

Murolo, S., Marcone, C., Prota, V., Garau, R., Foissac, X., & Romanazzi, G. (2010). Genetic variability of the "stolbur" phytoplasma *vmp1* gene in grapevines, bindweeds and vegetables. *Journal of Applied Microbiology*, 109(6), 2049-2059.

Ozturk, L., Sivri, N., & Sin, B. (2017). An update of weed flora of vıneyards ın Northwestern Turkey. *International Journal of Environment, Agriculture and Biotechnology*, 2(6), 3050-3055.

Prota, V. A., Garau, R., Paltrinieri, S., Botti, S., Nahdi, S., Calari, A., ... & Bertaccini, A. (2007). Molecular identification of phytoplasmas infecting myrtle plantations in Sardinia (Italy). *Bulletin of Insectology*, 60(2), 383-384.

Quaglino, F., Sanna, F., Moussa, A., Faccincani, M., Passera, A., Casati, P., ... & Mori, N. (2019). Identification and ecology of alternative insect vectors of '*Candidatus* Phytoplasma solani' to grapevine. *Scientific Reports*, 9(1), 1-11.

Quaglino, F., Maghradze, D., Casati, P., Chkhaidze, N., Lobjanidze, M., Ravasio, A., ... & Bianco, P. A. (2016). Identification and characterization of new '*Candidatus* Phytoplasma solani' strains associated with "bois noir" disease in *Vitis vinifera* L. cultivars showing a range of symptom severity in Georgia, the Caucasus region. *Plant Disease*, 100(5), 904-915.

Quaglino, F., Zhao, Y., Casati, P., Bulgari, D., Bianco, P. A., Wei, W., & Davis, R. E. (2013). '*Candidatus* Phytoplasma solani', a novel taxon associated with "stolbur"- and "bois noir"-related diseases of plants. *International Journal of Systematic and Evolutionary Microbiology*, 63, 2879-2894.

Rao, G. P., Kumar, M., & Singh, A. K. (2017). First report of '*Candidatus* Phytoplasma asteris'(16SrI-B subgroup) associated with a maize leaf redness disease in India. *Phytopathogenic Mollicutes*, 7(1), 52-56.

Sabaté, J., Laviña, A., & Batlle, A. (2014). Incidence of "bois noir" phytoplasma in different viticulture regions of Spain and "stolbur" isolates distribution in plants and vectors. *European Journal of Plant Pathology*, 139(1), 185-193.

Schneider, B., & Gibb, K. S. (1997). Sequence and RFLP analysis of the elongation factor *Tu* gene used in differentiation and classification of phytoplasmas. *Microbiology*, 143(10), 3381-3389.

Sertkaya, E., Mutlu, C., Bayram, A., Bayram, Y., & Güçlü, Ş. (2010). Determination of population changes of *Laodelphax striatellus* (Fallen, 1826) and *Sogatella vibix* (Haupt, 1927) (Hemiptera: Delphacidae) by using different sampling methods in second crop maize in Diyarbakır province. *Türkiye Entomoloji Dergisi*, 34(2), 251–262.

Smart, C. D., Schneider, B., Blomquıst, L., Guerra, J., Harrison, N. A., ... & Kirkpatrick, B. C. (1996). Phytoplasma-specific PCR primers based on sequences of the 16S-23S rRNA spacer region. *Applied and Environmental Microbiology*, 62(8), 2988–2993.

Weintraub, P. G., & Beanland, L. (2006). Insect vectors of phytoplasmas. *Annual Review of Entomology,* 51(1), 91–111. doi:10.1146/annurev.ento.51.110104.151039.

Zhao, Y., Wei, W., Lee, I-M., Shao, J., Suo, X., & Davis, R. E. (2009). Construction of an interactive online phytoplasma classification tool, *i*PhyClassifier, and its application in analysis of the peach X-disease phytoplasma group (16SrIII). I*nternational Journal of Systematic Evolution and Microbiology*, 59, 2582–2593.

Figure 1. Disease symptoms associated with the presence of '*Ca*. P. solani' in maize. Top: maize reddening symptoms with red streak on vein of leaf and stem; bottom: empty corn cob and poor seed set formation from symptomatic maize plants.

Figure 2. Virtual RFLP patterns derived from *in silico* digestions of Tr-AcM-1, Tr-AcM-2, Tr-AcM-3 and Tr-AcM-4 strain sequences obtained from R16F2n/16R2 products (about 1.2 kb) and phytoplasma 16S rDNA gene fragments from strains enclosed in the 16SrXII subgroups with four key enzymes (*Alu*I, *Mse*I, *Bst*UI and *Hha*I). Red boxes show the identity in the profiles of the strains (*Alu*I and *Mse*I), green boxes show the difference of the strains using *Bst*UI and *Hha*I. MW: Marker, ΦX174 DNA profile digested with *Hae*III with the following fragments in base pairs from top to bottom: 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118; 72.

Figure 3. Phylogenetic tree constructed by neighbor-joining method inferred from the '*Candidatus* Phytoplasma' species nucleotide sequences of the 16S rDNA gene. The tree shows the relationships among the phytoplasma strains obtained from maize plants from Turkey (red squares) and some '*Candidatus* Phytoplasma' species available in GenBank. The tree was constructed using MEGA 7 software. GenBank accession number and ribosomal subgroups are reported on the right of the strain names. *A. laidlawii* was employed as outgroup to root the tree.

Figure 4. RFLP analysis of the fTufAY/rTufAY amplicons of the elongation factor Tu (tuf) gene with *Hpa*II. Only '*Ca*. P. solani' tuf-type b was detected. M, marker 1 kb DNA ladder; +, Con: positive control from 16SrXII-A subgroup; UdC, undigested amplified control.

Figure 5. Unrooted phylogenetic tree inferred from the '*Ca.* P. solani' strain nucleotide sequences of the *vmp1* gene. Minimum evolution analysis was carried out using the neighbor-joining method and bootstrap replicated 1,000 times. The full names of the phytoplasma strains included in the phylogenetic analysis are given in the Table 1. The GenBank accession number of each sequence is given in parenthesis; gene sequences obtained in the present study are indicated with red squares. Square brackets show the grouping obtained by digestion of the amplicons with the endonuclease *Rsa*I.

Figure 6. Virtual RFLP patterns of *vmp1* gene sequence amplified with the primer pair TYPH10F/R of strains Tr-AcM1, -2, -3 and -4 with the P7 strain from periwinkle from Lebano as control (GenBank

accession number AM992100). M: marker, ΦX174 DNA profile digested with *Hae*III with the following fragments in base pairs from top to bottom: 1,353; 1,078; 872; 603; 310; 281; 271; 234; 194; 118; 72.

Table 1. '*Ca*. P. solani' *vmp1* gene sequences available in GenBank used in the phylogenetic analysis.