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Detection of 'Candidatus Phytoplasma mali' and 'Candidatus Phytoplasma pronotum' in Apple Trees

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ABSTRACT

The apple orchards in Niğde, Turkiye were surveyed for '*Candidatus* Phytoplasma mali' associated with apple proliferation disease, and the suspicious samples were tested by PCR-RFLP methods. A comprehensive study was conducted which included sampling from a total of 19 orchards from four different districts. The samples were collected according to the major symptoms of phytoplasma disease which were foliar reddening, witches' brooms, leaf rosettes, yellowing, longer peduncles and development of undersized fruits. It was determined that six out of 62 plant samples were infected with phytoplasma. Also, two out of six infected samples were determined as infected by '*Ca.* P. mali' and unexpectedly four out of six infected samples were determined as infected by '*Ca.* P. prunorum' is associated with European Stone Fruit Yellows disease. Even if the incidence of the disease (9.7%) was low, the characterized phytoplasmas were considered a significant potential threat for these locations.

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Elma ağaçlarında '*Candidatus* Phytoplasma mali' ve '*Candidatus* Phytoplasma prunorum' teşhisi

ÖZET

Niğde ilindeki eki elma bahçelerinde elma çoklu sürgün hastalığına neden olan *'Candidatus* Phytoplasma mali' tespiti için survey yapılmıştır ve şüpheli örnekler PCR-RFLP yöntemiyle test edilmiştir. Dört farklı ilçedeki toplam 19 meyve bahçesinden örneklemeler yapılmıştır. Örnekler, fitoplazma hastalığının başlıca belirtileri olan yaprak kızarması, cadı süpürgesi, yaprak rozetleri, sararma, sapların uzaması ve meyvelerin cılız gelişmesi gibi belirtilere göre toplanmıştır. Toplam 62 bitki örneğinden altısının fitoplazma ile enfekte olduğu belirlendi. Ayrıca, enfekte olmuş altı örnekten ikisinin 'Ca. P. mali' ile enfekte olduğu ve beklenmedik bir şekilde enfekte olmuş altı örnekten dördünün Avrupa Sert Meyve Sarıları hastalığı ile ilişkili *'Ca* P prunorum' tarafından enfekte olduğu belirlenmiştir. Hastalığın görülme sıklığı (%9,7) düşük olsa bile, karakterize edilen fitoplazmaların bu lokasyonlar için önemli bir potansiyel tehdit olduğu değerlendirilmiştir.

Fitopatoloji

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INTRODUCTION

Apple (*Malus communis* L.) is a member of the genus *Malus* of the *Pomoideae* subfamily of the *Rosaceae* family in the *Rosales* order. Apple plays a significant part in the fruit production and economy of Niğde in Turkiye. The apple production in Niğde has significantly supplied the needs of domestic and foreign trade (Oğuz & Karaçayır, 2009). Although research on virus diseases in apples is quite common (Öztekin & Buzkan, 2012; Morca et al., 2021) there are limited studies on apple phytoplasma diseases in our country.

Phytoplasmas are phloem-inhabiting and cell wall-less bacterial plant pathogens. They are commonly

transmitted from infected plants to healthy ones by insect vectors. They interact with host plants and insect vectors, causing biological, morphological and physiological changes in them to survive in nature. As a result of these interactions, they cause significant economic losses in cultivated plants (Seemüller & Schneider, 2004). Phytoplasmas cause destructive damage in more than 700 plant species worldwide (Maejima et al., 2014). Infected plants by phytoplasmas exhibit several disease symptoms, and these symptoms include a purple or yellow discolouration of shoots and leaves, virescence (greening of petals), phyllody (transformation of floral organs into leaf-like formations), proliferation of shoots, witches' broom, stunting, general decline, and plant death (Bertaccini & Duduk, 2009).

Phytoplasmas are classified in terms of the sequence analysis of the 16S rRNA gene that has been widely used in classification because it is considerably conserved (Marcone et al. 1996). Classification of phytoplasmas is generally based on the amplification of the 16S ribosomal RNA gene by Polymerase Chain Reaction (PCR) and Restriction Endonuclease Enzyme Analysis (RFLP) of these PCR products. (Duduk &Bertaccini, 2011).

In fruit orchards, apple proliferation (AP), pear decline (PD) and European stone fruit yellows (ESFY) are economically important plant diseases associated with phytoplasmas (Seemüller &Schneider, 2004). AP's disease-related agent is 'Candidatus Phytoplasma mali', which is associated with the reduction in fruit sizes and the decrease of the market values of fruits by infecting the apple trees in almost all European countries. It has been reported transmitting with *Cacopsylla melanoneura* (Jarausch et al. 2019). Pear decline disease associated with phytoplasma is '*Ca.* P. pyri' was first discovered in North America as recently as 1945, but today, it is a primary biotic factor that has limited pear production in Europe, and transmitting psyllid vector *C. pyri* (Jarausch et al. 2019). ESFY disease-associated '*Ca.* P. prunorum' is infecting several stone fruits including apricots, peaches, almond and other stone fruit orchards, and *C. pruni* is the primary vector (Jarausch et al. 2019). All these phytoplasmas fall within the same 16SrX group.

'*Ca.* P. mali' is the associated agent of a severe proliferation disease affecting apple trees (Seemüller &Schneider 2004). The agent is closely related to '*Ca.* P. pyri' and '*Ca.* P. prunorum' in terms of phylogeny. Additionally, '*Ca.* P. mali' have infrequently been identified in stone fruits (Navratil et al., 2001; Mehle et al., 2007; Cieslinska & Morgas, 2011). '*Ca.* P. mali' has been observed in numerous European countries with ongoing apple production. In recent years, the presence of this disease has been confirmed in Turkiye (Canik &Ertunç, 2007; Sertkaya et al., 2008). This pathogen predominantly colonizes sieve tubes, typically near sections of plant sieve plates. Colonized sieve tubes may undergo necrosis during the summer. The modified phloem tissue then forms small, irregular sieve tubes that are subsequently invaded. While this pathogen is primarily found in functional sieve tubes, its dispersion can vary significantly in the stems and shoots of infected apple trees throughout the year (Seemüller et al., 1984). Late summer and autumn are active periods for the phytoplasma in stems and shoots. In winter, the phytoplasma transforms into stringlike structures after sieve tube degeneration, disappearing from the aerial parts of trees and passing into the roots, where numerous functional sieve tubes are present throughout the year. Recolonization of the phytoplasma in the stems and branches continues with the formation of new aerial sieve elements in April or May (Schaper & Seemüller, 1984).

Phytoplasma presence in fruit trees of Turkiye has been reported by visual observation of symptoms as well as by PCR-based analyses since 1999. The presence of the apple proliferation group phytoplasma (16SrX) agents has been reported in Turkiye (Çağlayan, 2023). This study aims to investigate apple orchards in Niğde province for AP disease using PCR-RFLP methods, to confirm the presence of AP disease in these locations, to contribute to Turkey's phytoplasma disease maps and to make recommendations for future studies on the subject.

MATERIAL and METHODS

Surveys and Plant Materials

The samples were randomly collected from apple trees exhibiting symptoms of AP and those without AP symptoms in selected apple orchards of Niğde (Figure 1), representing local orchards and cultivation regions, during October and November 2017 (Table 1). At the time of sample collection, both general and specific symptoms of AP were considered through the observation of the development of apple trees and fruits, assessing physiological conditions of shoots and leaves, and evaluating general physiological parameters in apple trees. The samples were collected by cutting 20 - 25 cm long shoots from symptomatic branches or various directions on randomly chosen apple trees from asymptomatic plants. The samples were placed in labelled plastic bags and stored at +4 °C for subsequent analysis procedures.

DNA Isolation from Plant Materials

DNA isolation of plant materials was performed by using 100 mg of leaf midribs crushing in mortars with liquid nitrogen, followed by the addition of 1 ml of a 2% CTAB (2% CTAB, 1.4 M NaCl, 20 mM EDTA and 500 mM Tris, 2% 2-mercaptoethanol) (Doyle &Doyle 1990) solution. The resulting homogenate was incubated at 60 °C for 30 min, followed by centrifugation at 15000

rpm for 10 min at 25 °C. The supernatant was washed once by phenol-chloroform-isoamyl alcohol (25:24:1) and once chloroform-isoamyl alcohol (24:1). After precipitation with cold isopropanol and washed with cold ethanol (70%), the pellet was suspended in 80-100 µl TE (10 mM Tris and 1 mM EDTA) solution.

PCR Analysis

DNA materials were analyzed by direct and nested PCR using primer pairs P1/P7 and R16F2n/R16R2, which amplify approximately 1800 bp and 1240 bp fragments, respectively, from the 16S rRNA gene sequence of phytoplasmas. (Deng & Hiruki, 1991; Lee et al., 1992, 1995). The PCR mixture included 1 µl of 20 ng/µl DNA, 5 µL 10×PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 0.01% gelatin) (Thermo Scientific) 1.5 mM $MgCl_2$, 0.25 µM each dNTPs, 0.4 µM each primer, 2 U Taq DNA polymerase (Thermo Scientific) in a total of 50 µl volume. The following amplification conditions were applied: pre-denaturation for 3 min at 94°C; amplification for 35 cycles of 94°C for 1 min denaturation, 57°C for 2 min (60°C for 1 min for nested PCR) annealing, 72°C for 3 min (1 min for nested PCR) extension, and a final cycle 72°C for 10 min. The amplified products were visualized under UV after electrophoresis of 1% agarose gel and staining with ethidium bromide.

Figure 1. The survey area of apple trees for phytoplasma diseases in Niğde province of Türkiye.
Şekil 1. Türkiye Niğde ili elma ağaçlarında fitoplazma hastalıkları yönünden araştırma alanı.

RLFP Analysis

The restriction endonucleases of *Tru1*I, *Rsa*I and *Ssp*I (Thermo Scientific) were used for RFLP analysis of the nested PCR products. The digestions were performed separately for each endonuclease overnight at 37°C for *Rsa*I and *Ssp*I, and 65°C for *Tru1*I. Phytoplasma-infected positive control plant materials ['*Ca.* P. prunorum' (ESFY), '*Ca.* P. mali' (AP) and '*Ca.* P. pyri' (PD) isolates] were kindly provided by Dr B. Schneider (Germany). The digested products were analyzed by electrophoresis using 2% agarose gels as described above.

RESULTS AND DISCUSSION

The main observed symptoms similar to the symptoms of AP disease in these survey studies were foliar reddening, leaf rosettes, yellowing, longer peduncles and development of undersized fruits (Figure 2). A total of 62 plant samples were collected from 19 orchards (Table 1). The amplicons of nested-PCR products showed as a result of electrophoresis that six samples formed the same size amplification profiles with the positive controls of ESFY, AP and PD around 1240 bp. The incidence of the disease was low 9.7%. Three samples (1, 7, and 13) were from Ulukışla location and the other three (27, 29, and 56) from the Central Districts of Niğde province.

RFLP analyses of samples 1, 7, 13, 27, 29 and 56 exhibited the expected digestion for 16SrX group phytoplasmas (Figure 3). The RFLP patterns revealed that the band profiles of these phytoplasma-infected samples matched those of positive controls for both '*Ca.* P. mali' and '*Ca.* P. prunorum' (Lee et al., 1995). The *Rsa*I RFLP pattern of samples 1 and 7 was the same with the positive control of '*Ca.* P. mali', and the samples 13, 27, 29 and 56 were the same with the positive control of '*Ca.* P. prunorum'. The *Ssp*I RFLP pattern also exhibited identical results. Samples 1 and 7 resulted in the restriction profiles of '*Ca.* P. mali', and the samples 13, 27, 29 and 56 resulted in the restriction profiles of '*Ca.* P. prunorum'. According to the RFLP analysis, none of the phytoplasma isolates was '*Ca.* P. pyri'.

The detection of ESYF in apple trees is attributed to vector transport and vegetative propagation as the main contributing factors. Specifically, '*Ca.* P. mali' is associated with inducing apple proliferation disease, predominantly affecting both cultivated and wild apple tree varieties. Nevertheless, there has been a report on different hosts, such as cherry (*Prunus avium*), apricot

Table 1. The apple samples were collected according to locations in Niğde province
Çizelge 1. Niğde ili lokasyonlarına göre toplanan elma örnekleri

District (*İlçe*)	Location (*Lokasyon*)	Number of orchards (*Bahçe sayısı*)	Number of apple trees* (*Elma ağacı sayısı*)
Ulukışla	Zıyıncak	2	2/9
	Kardeş Gediği	1	0/1
	Tatboğazı	1	0/1
	Tepeköy	1	0/1
	Acıpınar	1	1/2
	Alpağut	2	0/2
	Hüsniye	1	0/1
Bor	Kaynarca	1	0/6
Çamardı	Değirmenli	2	0/4
	İçmeli	1	0/6
	Bademdere	1	0/4
	Center	2	0/4
Centre	Sazlıca	2	1/12
	Central	1	2/9
Total (Toplam)		19	6/62

*infected/total sample.

Figure 2. Asymptomatic (no 1, 13, 27) and symptomatic (leaf rolling and reddening) (7, 29, 56) appearances of phytoplasma infected apples

Şekil 2. Fitoplazma ile enfekte elmalarda asimptomatik (no 1, 13, 27) ve simptomatik (yaprak kıvrılması ve kızarması) (7, 29, 56) görünümler

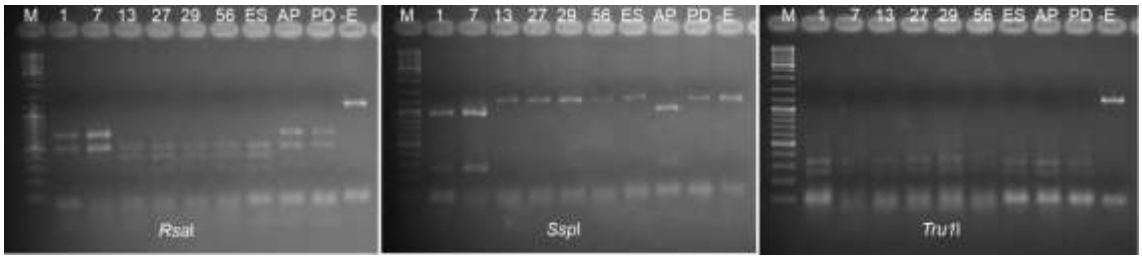

Figure 3. Restriction profiles of nested-PCR products amplified by using R16F2n and R16R2 primer pair formed after the digestion with *Rsa*I, *Ssp*I and *Tru1*I restriction enzymes. M: 100 bp DNA ladder mix (Thermo Scientific), phytoplasma positive controls ES: European stone fruit yellows, AP: Apple proliferation and PD: Pear decline, -E: Undigested amplicon

*Şekil 3. RsaI, SspI ve Tru1I restriksiyon enzimleri ile kesim sonrasında oluşturulan R16F2n-R16R2 primer çifti kullanılarak çoğaltılan nested PCR ürünlerinin fragment profilleri. M: 100 bp DNA ladder karışımı (Thermo Scientific), fitoplazma pozitif kontroller ES: Avrupa sert çekirdekli meyve sarılığı, AP: Elma çoklu sürgün ve PD: Armut yıkım, -E: Enzim eklenmeyen amplico*n.

(*P. armeniaca*) and plum (*P. domestica*) (Mehle et al., 2007). Additionally, the agent has been detected in hazelnut (*Corylus* spp.) (Marcone et al., 1996), pear (*Pyrus communis*), and Japanese plum (*Prunus salicina*) (Mehle et al., 2007). Cieślińska & Morgaś (2011) stated that '*Ca*. P. mali' and '*Ca*. P. pyri' were found to infect not only pome fruits but also stone fruits such as nectarines and cherries. While '*Ca*. P. mali' was identified in the nectarine cultivar (Super Queen), '*Ca*. P. pyri' was observed in the cherry cultivar (Kordia). Canik-Orel et al. (2019) reported that pear samples infected with 16SrX-A ('*Ca*. P. mali') and 16SrX-C ('*Ca*. P. pyri') subgroup phytoplasmas in mixed infections, while apricot samples showed the presence of 16SrX-B ('*Ca*. P. pronotum). They also identified the occurrence of phytoplasma, along with mixed infections involving 16SrX-C ('*Ca*. P. pyri') and 16SrX-A/16SrX-C, as well as 16SrX-C/16SrI (aster yellows). Although the possibilities of transmission of identified '*Ca*. P. mali' and '*Ca*. P. pronotum phytoplasmas in these locations with infected saplings and grafting or budding materials were considered most likely (Canik & Ertunç, 2007; Sertkaya et al., 2008), their potential vectors and alternative hosts should be investigated.

Kaya et al. (2016) stated that *Cacopsylla picta, C. melanoneura-C. affinis complex* and *C. pyri* individuals carry '*Ca*. P. mali' as well as '*Ca*. P. pyri'. Furthermore, the presence of *C. pruni* has been identified in Niğde in previous research and it has been confirmed in neighbouring provinces such as Adana and Mersin (Ulubaş Serçe, 2011, unpublished). Additionally, *C. pruni* has also been reported in Bursa province (Ulubaş Serçe et al., 2012).

The phytoplasma disease incidence in apple was determined as 9.68% in this study. Sertkaya et al., (2008) stated that they examined a total of 31 samples (28 symptomatic, 3 symptomless) from Adana, Niğde, and Mersin provinces in Turkiye, to investigate the presence of phytoplasma. The results of this research showed that six out of 31 tree samples consisting of two samples from Niğde and four samples from Adana were infected by '*Ca*. P. mali'. Based on the findings, the calculated disease incidence was 19.35%. Canik & Ertunç (2007) conducted a study in Isparta, Yalova and Ankara provinces of Turkiye to assess the presence and spread of '*Ca*. P. mali'. The researchers collected 201 samples from randomly selected apple orchards, encompassing both symptomatic and asymptomatic cases. The findings indicated that 8 out of the 201 samples were infected by '*Ca*. P. mali'. Consequently, the disease incidence was calculated as 3.9%. Even if this incidence of the disease is low, the fact that these pathogens were identified in these locations and able to be transmitted by psyllid vectors, should be considered as a significant potential threat for fruit tree production. Although the main pest of apple trees in Gülnar (Mersin) district of Turkey has been determined as *Cydia pomonella* (Sarı and Yıldırım, 2021), the prevalence of psylid vectors in apple orchards needs to be investigated.

CONCLUSION

In this research, the presence of '*Ca*. P. mali' associated AP disease was surveyed within the Niğde region. This study provides further evidence for the presence of '*Ca*. P. mali' in Turkiye and also provides updates about phytoplasma occurrence in Niğde province in apple orchards. Because of the categorization of these pathogens as an A2 pest by EPPO and the Plant Quarantine Regulation of Turkiye, apple growers have to be provided with phytoplasma-free saplings. Also, only this measure is not sufficient for the control of the disease because of the transmission of the disease by insect vectors. Although the detected phytoplasmas will likely be transmitted by infected seedlings, grafts or bud material in these locations, precautions should be taken by investigating their potential vectors and alternative hosts.

Author's Contribution

Ç. Ulubaş-Serçe[3] involved in the conception and design of the study. H. Meral[1] and M. Ekemen[2] surveyed and collected the samples. H. Meral conducted the molecular experiments, analysis and interpretation of the data. M. Ekemen drafted the article and Ç. Ulubaş-Serçe revised it critically for intellectual content. All authors have read and approved the final manuscript.

Conflict of interests/Competing interests

The authors declare that there is no conflict of interest.

Ethics approval

The authors declare that there is no ethical issue.

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