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ORIGINAL ARTICLE

Identification of phytoplasmas in stone fruit (*Prunus* sp.) and persimmon (*Diospyros kaki* L.) trees exhibiting leaf alterations and witches'-broom in Jordan

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Abstract

During field surveys conducted in 2020 in Jordanian orchards, phytoplasma-like symptoms (leaf yellowing/reddening and rolling, and witches'-broom) were observed in three stone fruit species (peach, European plum, sweet cherry) and persimmon. Molecular analyses identified phytoplasma strains belonging to the species '*Candidatus* Phytoplasma solani' (subgroup 16SrXII-A) as largely prevalent in stone fruit and persimmon symptomatic plants. Moreover, '*Ca*. Phytoplasma omanense' (16SrXXIX-B) was found in few European plum symptomatic plants. In previous studies, such phytoplasma strains were identified in other important crops (almond, pomegranate, and grapevine) and in several putative insect vectors, suggesting their complex ecology in Jordan. Further studies are needed to in-depth investigate the diffusion of phytoplasma-associated diseases of stone fruits throughout the Country, to clarify their aetiology, and to study their epidemiological pattern(s).

KEYWORDS
'*Candidatus* Phytoplasma omanense', '*Candidatus* Phytoplasma solani', 16S rRNA-encoding gene, symptoms

1 | INTRODUCTION

Phytoplasmas constitute a large group of plant pathogenic cell wall-less bacteria that inhabit the phloem tissue of infected plants and are plant-to-plant transmitted by insect vectors belonging to the families Cicadellidae, Cixiidae, Psyllidae, Delphacidae and Derbidae (Bertaccini et al., 2014; Weintraub & Beanland, 2006). They belong to the class Mollicutes, which includes bacteria with single membrane that have diverged from a Gram-positive ancestor (Zhao et al., 2005). Based on molecular and biological features, phytoplasmas have been classified into 49 species within the provisional genus '*Candidatus* Phytoplasma' (Bertaccini et al., 2022), and taxonomic groupings have also been established according to the similarity coefficients obtained by restriction fragment length polymorphism (RFLP) analyses on nucleotide sequence of 16S rRNA-encoding gene (Lee et al., 1998; Wei et al., 2008). Numerous agriculturally important plant diseases are associated with infection by phytoplasmas. The most common symptoms exhibited by phytoplasma-infected plants include virescence and phyllody, yellowing, flower sterility, proliferation of axillary buds resulting in witches'-broom, abnormal internode elongation and generalized stunting (Bertaccini et al., 2014).

Highly destructive phytoplasma-associated diseases affect many economically important *Prunus* species including almond (*Prunus amygdalus* Batsch), apricot (*Prunus armeniaca* L.), peach (*Prunus persica* L.), sweet cherry (*Prunus avium* L.) and plums (*Prunus domestica*

L. and other species). '*Ca*. Phytoplasma (*Ca*. P.) phoenicium', taxonomic subgroup 16SrIX-B and its variants, is associated with almond and peach witches'-broom in Lebanon, Iran and South Italy (Abou-Jawdah et al., 2003; Molino Lova et al., 2011; Nigro et al., 2020; Salehi et al., 2020; Zirak et al., 2021), and with apricot yellows in Iran (Salehi et al., 2018). '*Ca*. P. pruni', taxonomic subgroup 16SrIII-A, is associated with X-disease of peach and other stone fruits (mainly almond, apricot and sweet cherry) in United States and Canada (Davis et al., 2013; Uyemoto & Kirkpatrick, 2011; Wright et al., 2021). '*Ca*. P. prunorum', taxonomic subgroup 16SrX-B, is associated with European Stone Fruit Yellows (ESFY) disease in apricot, peach, plums, sweet and sour cherry in Europe (Fiore et al., 2018).

In the Middle East/North Africa (MENA) region, in addition to '*Ca*. P. phoenicium', '*Ca*. P. prunorum', '*Ca*. P. asteris', '*Ca*. P. trifolii' and '*Ca*. P. aurantifolia' were found associated with diseases in apricot, almond, peach, plum and sweet cherry (Khalifa et al., 2011; Khalifa & Fakhfakh, 2011; Orel et al., 2019; Zirak et al., 2010, 2021). In Jordan, stone fruits including peach, plum, almond, green and sweet cherry are very important exporting crops cultivated in the whole Country. More than 59,425 tons were exported to international markets in 2020 (MOA, 2021). Recently, Abu Alloush, Bianco, Busato, AlMahasneh, et al. (2023) reported the association of seven distinct '*Ca*. Phytoplasma' species with almond diseases in Jordan, and preliminary information on their putative insect vectors. However, few studies in limited locations were carried out focusing on phytoplasma-like diseases of other stone fruits in Jordan: '*Ca*. P. asteris' (subgroup 16SrI-B) was reported in association with peach yellowing and reddening (Anfoka & Fattash, 2004), and '*Ca*. P. solani' (subgroup 16SrXII-A) in association with plum yellowing and witches'-broom (Salem et al., 2020).

In the present study, a field survey was conducted in the whole Country to observe phytoplasma-like symptoms on stone fruits and to detect and type by molecular analyses the phytoplasmas infecting peach, plum, sweet cherry and persimmon.

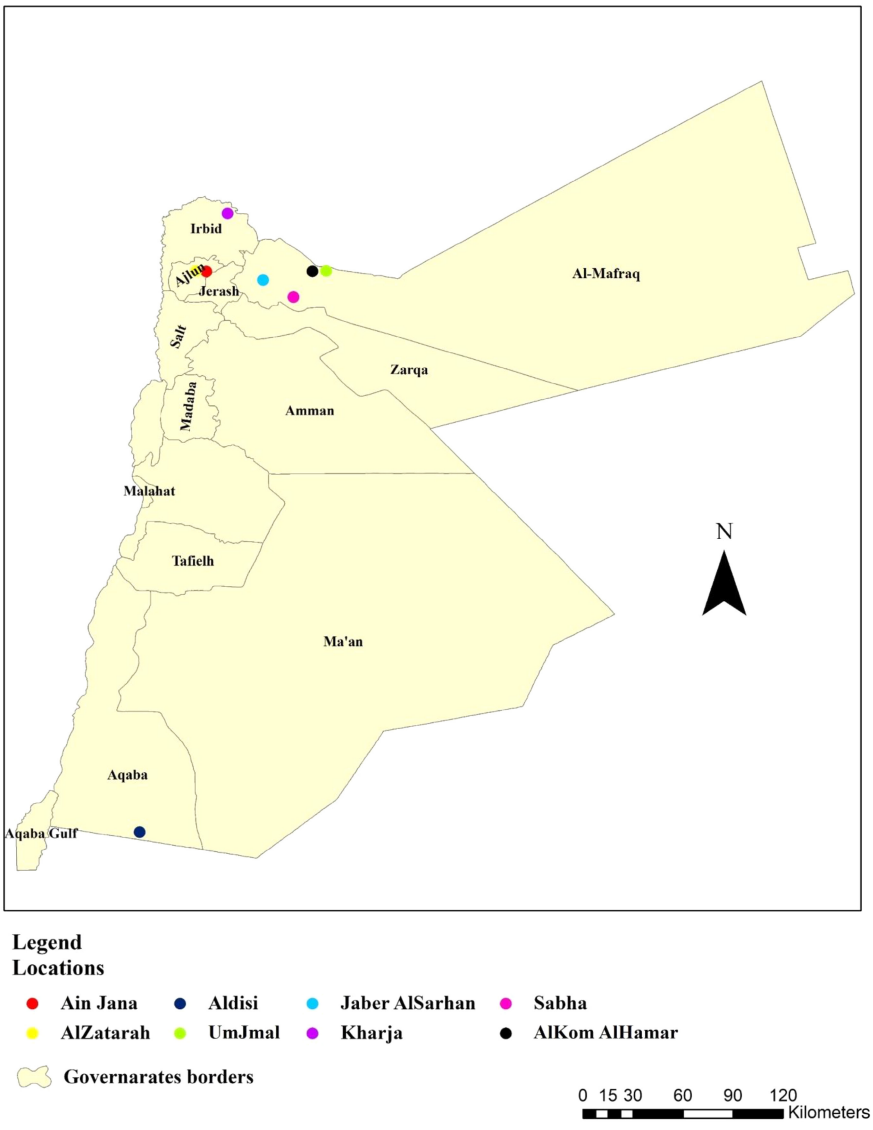

FIGURE 1 Map of regions surveyed for phytoplasma-like symptoms in stone fruit and persimmon tree orchards in this study.

2 | MATERIALS AND METHODS

2.1 | Field surveys, plant sampling and TNA extraction

From June to September 2020, field surveys for phytoplasma-like symptoms were conducted in Jordan in different stone fruits cultivation areas in the whole Country. Eight locations in the governorates of Irbid (Kharja), Ajloun (Ain Jana, AlZatarah), Al-Mafraq (Jaber AlSarhan, UmJmal, Sabha, AlKom AlHamar) and Aqaba (Aldisi) were surveyed (Figure 1). All selected orchards in Irbid and Ajloun governorates were rainfed, while those in Al-Mafraq and Aqaba were irrigated. All orchards were characterized by an intercropping system including stone fruits, grapevine and pome fruits. In each location, incidence of phytoplasma-like diseases was estimated as the percentage of symptomatic trees out of the observed ones. Leaf samples were collected from 68 symptomatic (25 peach, 33 plum, 10 sweet cherry) and 12 symptomless (four peach, six plum, two cherry) stone fruit trees. Moreover, six persimmon trees (five exhibiting phytoplasma-like symptoms and one symptomless) were collected during the survey (Table 1). Collected samples were transferred to the laboratories of National Agricultural Research Center, Baqaà, Jordan, and maintained at 4°C until total nucleic acid extraction.

Total nucleic acids (TNA) were extracted from the collected plants as previously described by Angelini et al. (2001) with some modifications. Leaf midribs and petioles (0.5 g) were ground in 3 mL of prewarmed 2% CTAB-based buffer in sterile mortars. Extracted TNA was washed by 0.3 mL of 70% ethanol, dissolved in 100 µL of TE-based buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), measured for quantity and quality by Nanodrop system and stored at −20°C until molecular analyses.

2.2 | Phytoplasma detection and classification

Nested PCRs were carried out to amplify the phytoplasma 16S rRNA-encoding gene using the primer pair P1/P7 (Deng & Hiruki, 1991; Schneider et al., 1995) followed by the primer pair R16F1/R16R0 (Lee et al., 1995). Reaction mixtures and reaction conditions were as previously described (Quaglino et al., 2009). TNAs extracted from healthy periwinkle and reaction mixtures devoid of TNAs were used as negative controls. No positive controls were utilized to avoid contamination risk. PCR products (6 µL) were analysed by electrophoresis on 1% (w/v) agarose gels in 1X TBE buffer, stained with Midori Green Easy (NIPPON Genetics EUROPE, Düren, Germany) and visualized on UV transilluminator.

Nested PCR products (F1/R0 fragment), amplified from plants, were sequenced in both strands by a commercial service (Eurofins Genomics, Germany). Nucleotide sequences were assembled by the Contig Assembling Program and trimmed to

TABLE 1 Phytoplasma-infected stone fruit and persimmon trees from locations surveyed in Jordan in this study.

Governorate	Location	Plant	No. of collected samples	No. of phytoplasma-infected samples
AlMafraq	Jaber AlSarhan	Symptomatic *Prunus persica* L.	15	5
		Asymptomatic *Prunus persica* L.	2	0
		Symptomatic *Prunus avium* L.	10	7
		Asymptomatic *Prunus avium* L.	2	0
		Symptomatic *Diospyrus kaki* L.	5	2
		Asymptomatic *Diospyrus kaki* L.	1	0
	UmJmal	Symptomatic *Prunus persica* L.	6	4
		Asymptomatic *Prunus persica* L.	1	0
	Sabha	Symptomatic *Prunus domestica* L.	10	6
		Asymptomatic *Prunus domestica* L.	2	0
	AlKom AlAhmar	Symptomatic *Prunus persica* L.	4	1
		Asymptomatic *Prunus persica* L.	1	0
Ajloun	Ain Jana	Symptomatic *Prunus domestica* L.	7	1
		Asymptomatic *Prunus domestica* L.	1	0
	AlZatarah	Symptomatic *Prunus domestica* L.	3	1
		Asymptomatic *Prunus domestica* L.	1	0
Irbid	Kharja	Symptomatic *Prunus domestica* L.	7	1
		Asymptomatic *Prunus domestica* L.	1	0
Aqaba	AlDisi	Symptomatic *Prunus domestica* L.	6	0
		Asymptomatic *Prunus domestica* L.	1	0
		Overall total	86	28

FIGURE 2 Symptoms observed on stone fruit and persimmon trees in Jordan during the survey carried out in this study. Witches'-broom and yellowing (a), yellowing, reddening and leaf rolling (b) observed on peach trees; yellowing observed on sweet cherry trees (c); witches'-broom and yellowing (d), witches'-broom (e), yellowing, reddening and leaf rolling (f) observed in plum trees; leaf scorch and rolling observed in persimmon (g).

the annealing sites of the nested PCR primer pairs in the software BioEdit, version 7.1.3.0 (Hall, 1999). Trimmed nucleotide sequences were aligned using the ClustalW Multiple Alignment program and analysed by Sequence Identity Matrix in the software BioEdit to estimate their genetic diversity. For attribution to '*Ca*. Phytoplasma' species, 16S rRNA-encoding gene nucleotide sequences, representative of the phytoplasma populations detected in this study, were aligned with those of the reference strains of the 49 '*Ca*. Phytoplasma' species previously described and checked for their sequence identity in the software. Species attribution was confirmed searching the species-specific signature sequences within the analysed F1/R0 nucleotide sequences. For group/subgroup attribution, 16S rRNA-encoding gene sequences were analysed by virtual RFLP using the online tool *i*PhyClassifier (Wei et al., 2008; Zhao et al., 2009). Nucleotide sequences of 16S rRNA-encoding gene of phytoplasmas, identified in the present study, and reference strains of '*Ca*. Phytoplasma' species were employed for phylogenetic analyses. The Minimum-Evolution method was employed using the Neighbour-Joining algorithm and bootstrap replicated 1000 times with the software MEGAX (Kumar et al., 2018).

3 | RESULTS AND DISCUSSION

3.1 | Phytoplasma-like symptoms observed in stone fruit and persimmon trees

During the field survey, yellowing, reddening, leaf rolling, and witches'-broom were observed on stone fruit trees, while leaf scorch and rolling in persimmon. In detail, in peach orchards in AlMafraq governorate, witches'-broom and yellowing were observed in Jaber AlSarhan and UmJmal, while yellowing, reddening and leaf rolling were observed in Alkom AlAhmar (Figure 2a,b). The disease incidence (percentage of symptomatic out of observed trees) ranged from 25% to 55%. The main symptoms exhibited by sweet cherry trees in AlMafraq governorate was yellowing (Figure 2c), with a disease incidence of around 60%. Concerning the symptoms exhibited by plum trees, witches'-broom and yellowing were observed in Sabha (AlMafraq) and Ain Jana (Ajloun) (Figure 2d), witches'-broom in Kharja (Irbid) (Figure 2e), and yellowing, reddening, and leaf rolling in AlZatarah (Ajloun) and Aldisi (Aqaba) (Figure 2f), with a disease incidence of around 55%, 45%, 15%, 25% and 20%, respectively. In persimmon, leaf scorch and rolling were observed in orchards

TABLE 2 Phytoplasmas identified in stone fruit and persimmon trees in Jordanian regions in this study.

Sample ID	Plant host	Location	Symptoms	Phytoplasma species	Identity % versus reference strain	16Sr subgroup (similarity coefficient)	Acc. No.
PP164	*Prunus persica* L.	UmJmal	Witches'-broom, yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	OR736053
PP165	*Prunus persica* L.	UmJmal	Witches'-broom, yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PP166	*Prunus persica* L.	UmJmal	Witches'-broom, yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PP147	*Prunus persica* L.	UmJmal	Witches'-broom, yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PP1191	*Prunus persica* L.	Jaber AlSarhan	Witches'-broom, yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PP1200	*Prunus persica* L.	Jaber AlSarhan	Witches'-broom, yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PP1202	*Prunus persica* L.	Jaber AlSarhan	Witches'-broom, yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PP1210	*Prunus persica* L.	Jaber AlSarhan	Witches'-broom, yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PP1225	*Prunus persica* L.	Jaber AlSarhan	Witches'-broom, yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PA110	*Prunus avium* L.	Jaber AlSarhan	Yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PA113	*Prunus avium* L.	Jaber AlSarhan	Yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PA117	*Prunus avium* L.	Jaber AlSarhan	Yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PA118	*Prunus avium* L.	Jaber AlSarhan	Yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PA119	*Prunus avium* L.	Jaber AlSarhan	Yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PA120	*Prunus avium* L.	Jaber AlSarhan	Yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PA122	*Prunus avium* L.	Jaber AlSarhan	Yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
DK186	*Diospyros kaki* L.	Jaber AlSarhan	Leaf scorch and rolling	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
DK188	*Diospyros kaki* L.	Jaber AlSarhan	Leaf scorch and rolling	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PP263	*Prunus persica* L.	AlKom AlAhmar	Yellowing, reddening, leaf rolling	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PD99	*Prunus domestica* L.	Sabha	Witches'-broom, yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PD104	*Prunus domestica* L.	Sabha	Witches'-broom, yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PD105	*Prunus domestica* L.	Sabha	Witches'-broom, yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PD106	*Prunus domestica* L.	Sabha	Witches'-broom, yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PD107	*Prunus domestica* L.	Sabha	Witches'-broom, yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PD108	*Prunus domestica* L.	Sabha	Witches'-broom, yellowing	'*Ca.* P. solani'	99.5	XII-A (1.00)	a
PD1075	*Prunus domestica* L.	Ain Jana	Witches'-broom, yellowing	'*Ca.* P. omanense'	99.6	XXIX-B (1.00)	OR736054
PD122	*Prunus domestica* L.	AlZatarah	Yellowing, reddening, leaf rolling	'*Ca.* P. omanense'	99.6	XXIX-B (1.00)	b
PD230	*Prunus domestica* L.	Kharja	Witches'-broom	'*Ca.* P. solani'	99.7	XII-A (1.00)	OR736055

Note: a, nucleotide sequences identical to OR736053; b, nucleotide sequence identical to OR736054.

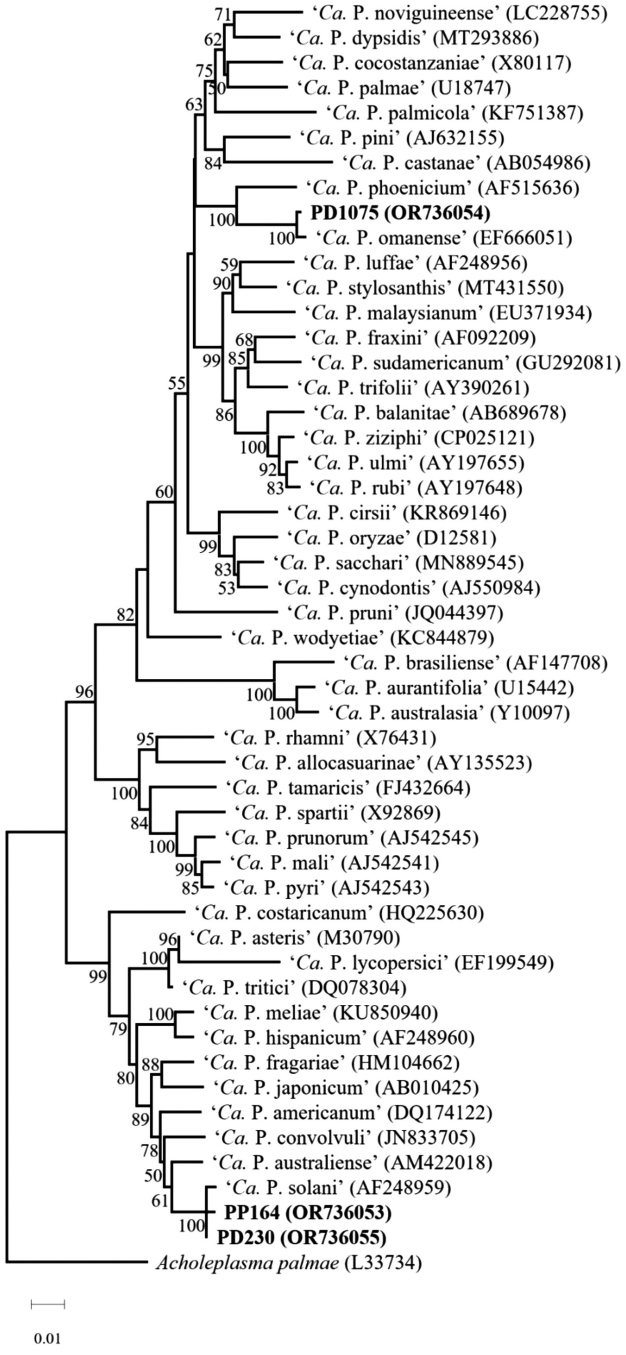

FIGURE 3 Phylogenetic tree based on the alignment of 16S rRNA-encoding gene nucleotide sequences of representative phytoplasma strains identified in stone fruit trees in Jordan (bold characters), and reference strains of previously described '*Candidatus* Phytoplasma' species. Evolutionary history was inferred using the Minimum Evolution (ME) method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbour-Interchange (CNI) algorithm at a search level of 1. The Neighbour-joining algorithm was used to generate the initial tree. All ambiguous positions were removed for each sequence pair (pairwise deletion option). *Acholeplasma palmae* (GenBank Acc. No. L33734) was used to root the tree.

located in Jaber AlSarhan (Figure 2g), with a disease incidence of around 60%. Most of such symptoms observed in stone fruits in Jordan were already reported in MENA countries (Orel et al., 2019; Zirak et al., 2010, 2021).

3.2 | Phytoplasma molecular detection

Nested PCRs allowed detecting the presence of phytoplasmas in 28 of 86 analysed plant samples. F1/R0 amplicons of the expected size (around 1370 bp) were obtained in 26 out of 68 symptomatic stone fruits trees (39.1%), and in two of five symptomatic persimmon trees (40%). In detail, phytoplasmas were detected in 10 of 25 (40%) symptomatic peach trees, 7 of 10 (70%) symptomatic sweet cherry trees, 9 of 33 (27.3%) symptomatic plum trees. No amplification was obtained in samples from symptomless trees (Table 1). Robustness of PCR reactions was proved by the absence of amplification in healthy periwinkle and reaction mixture devoid of TNA (negative control). Even if the incidence of phytoplasma-like symptoms was high in examined orchards, only 27.3%–70% of collected symptomatic stone fruit trees were found phytoplasma infected. This can be due to the uneven distribution of phytoplasmas in phloem tissues of infected plants (Constable et al., 2003), the possible low concentration of phytoplasma cells in plant tissues in the different sampling periods (Martini et al., 2011), and the possibility that observed symptoms are caused by other etiological agents or abiotic stresses.

3.3 | Phytoplasma classification and phylogeny

16S rRNA-encoding gene amplicon-derived chromatograms showed no evidence of double peaks, indicating the absence of intra-genomic heterogeneity or mixed infections (Zwolińska & Borodynko-Filas, 2021). According to 16S rRNA gene sequence identity versus the reference strains of '*Ca.* Phytoplasma' species and on the presence of species-specific signature sequences, the phytoplasma strains detected in 24 symptomatic stone fruit (10 peach, seven sweet cherry, seven plum) and two persimmon trees were attributed to the species '*Ca.* P. solani', while phytoplasma strains detected in two symptomatic plum trees were attributed to the species '*Ca.* P. omanense' (Table 2). In detail, 25 '*Ca.* P. solani' strains have identical 16S rRNA-encoding gene nucleotide sequence (GenBank Acc. No. OR736053), distinct from the reference strain STOL by seven single-nucleotide polymorphisms (SNPs) at positions 194 (C/T), 211 (C/T), 214 (C/T), 504 (T/A), 595 (A/G), 888 (C/T) and 1084 (T/C) from the annealing site of the primer R16F1. '*Ca.* P. solani' strain PD230 has 16S rRNA-encoding gene nucleotide sequence (GenBank Acc. No. OR736055) distinct from the reference

strain STOL by four SNPs at positions 504 (T/A), 595 (A/G), 888 (C/T) and 1084 (T/C) from the annealing site of the primer R16F1. '*Ca*. P. omanense' strains have identical 16S rRNA-encoding gene nucleotide sequence (GenBank Acc. No. OR736054), distinct from the reference strain IM-1 by five SNPs at positions 152 (G/A), 274 (T/C), 331 (C/T), 344 (G/A) and 712 (G/A) from the annealing site of the primer R16F1. Based on similarity coefficient obtained by comparison of virtual RFLP patterns, '*Ca*. P. solani' strains were attributed to taxonomic subgroup 16SrXII-A and '*Ca*. P. omanense' strains to subgroup 16SrXXIX-B (data not shown). Phytoplasma clustering in phylogenetic tree confirmed the attribution to phytoplasma species and taxonomic subgroups (Figure 3).

Phytoplasmas identified in symptomatic stone fruits and persimmon trees were found differentially distributed in the examined locations and associated with different symptoms. '*Ca*. P. solani' (16SrXII-A) was found in all AlMafraq locations (25 strains of 25) in association with peach witches'-broom and yellowing (UmJmal and Jaber AlSarhan) and peach yellowing, reddening and leaf scorch (Alkom), sweet cherry yellowing (Jaber AlSarhan), persimmon leaf scorch and rolling (Jaber AlSarhan), and plum witches'-broom and yellowing (Sabha), and in Irbid governorate (Kharja) in association with plum witches'-broom. '*Ca*. P. omanense' (16SrXXIX-B) was identified exclusively in Ajloun governorate in association with plum witches'-broom and yellowing (Ain Jana) and plum yellowing, reddening and leaf rolling (AlZatarah) (Table 2; Figure 2).

Remarkably, '*Candidatus* Phytoplasma' species identified in symptomatic stone fruit and persimmon trees were previously reported in Jordan in association with diseases of other important crops (grapevine, plum, peach) (Abu Alloush, Bianco, Amashah, Busato, et al., 2023; Abu Alloush, Bianco, Busato, Alkhawaldeh, et al., 2023; Abu Alloush, Bianco, Busato, AlMahasneh, et al., 2023; Anfoka & Fattash, 2004; Salem et al., 2013, 2020). Moreover, this is the first study reporting sweet cherry and persimmon infection by '*Ca*. P. solani', and plum tree infection by '*Ca*. P. omanense' in the Country. Several stone fruit phytoplasma-associated diseases, including European Stone Fruit Yellows (ESFY), peach X-disease and Peach Yellows Leaf Rolling (PYLR), are known to be very destructive in Euro-Mediterranean basin and in different parts of the world (Davis et al., 2013; Orel et al., 2019; Sabaté et al., 2014). None of such diseases were found in Jordan. Interestingly, in recent studies carried out in Jordan, several insects and additional host plants were found infected by '*Ca*. P. solani' and '*Ca*. P. omanense' (Abu Alloush, Bianco, Busato, Alkhawaldeh, et al., 2023; Abu Alloush, Bianco, Busato, AlMahasneh, et al., 2023), suggesting that the spread of such phytoplasmas, also in stone fruit and persimmon orchards, could be related to complex epidemiological patterns.

4 | CONCLUSION

This study evidenced natural phytoplasma infection of stone fruit crops including plum, peach and sweet cherry as well as persimmon in Jordan. The symptomatic trees exhibited several symptoms associated with infection by distinct '*Ca*. Phytoplasma' species. Further studies are needed to accurately survey the presence of phytoplasma-associated diseases of stone fruits and persimmon throughout the Country, to elucidate their aetiology, and to study their epidemiological pattern, including insect vectors and additional host (reservoir) plants.

FUNDING INFORMATION

No funding was received for conducting this study.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article.

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