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Xylella fastidiosa causes leaf scorch of pistachio (*Pistacia vera*) in Iran

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Summary. Since the early 2010s, pistachio (*Pistacia vera* L.) leaf scorch symptoms have been observed in orchards in several provinces in Iran. Seventeen of 83 symptomatic leaf samples from pistachio plants from 21 orchards were positive for the presence of *Xylella fastidiosa*, as detected by DAS-ELISA with *X. fastidiosa*-specific antibodies and by PCR assays with *X. fastidiosa*-specific primers. A Gram-negative bacterium similar to *X. fastidiosa* was isolated into solid media. DAS-ELISA and PCR confirmed the identity of the isolated bacteria as *X. fastidiosa*. Koch's postulates were fulfilled by artificially inoculating isolates obtained from pistachio showing leaf scorch to healthy pistachio and *Nicotiana tabacum* (cv. White Burley). Selected isolates induced leaf scorch symptoms when inoculated on tobacco and pistachio seedlings grown under greenhouse conditions. Early leaf scorch symptoms appeared approx. 2 months after inoculation of tobacco and approx. 3 months for pistachio. Reisolation of *X. fastidiosa* from inoculated and symptomatic plants and DAS-ELISA and PCR tests confirmed the identity of the re-isolated bacteria to be *X. fastidiosa*. On the basis of disease symptoms, pathogen isolation, pathogenicity tests and positive diagnosis by DAS-ELISA and PCR, *X. fastidiosa* is concluded to be the causal agent of pistachio leaf scorch in Iran. This is the first report of isolation and pathogenicity of *X. fastidiosa* in pistachio worldwide.

Keywords. Iran, isolation, DAS-ELISA, pathogenicity test, PCR.

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INTRODUCTION

Xylella fastidiosa Wells is a xylem-limited bacterium that can cause plant diseases in a wide range of plant hosts throughout the world. This fastidious bacterium is classified as a single species, although genetic studies support multiple subspecies (Schaad *et al.,* 2004). The pathogen is currently subdivided into four subspecies: *X. fastidiosa* subsp. *fastidiosa*, *X. fastidiosa* subsp. *multiplex*, *X. fastidiosa* subsp. *sandyi* and *X. fastidiosa* subsp. *pauca*, with a proposed fifth subspecies ('*X. fastidiosa* subsp. *morus*') still under scrutiny. The agent of leaf scorch affecting pear trees in Taiwan has recently been

found to differ genetically from *X. fastidiosa* to warrant classification as a novel species, denoted *Xylella taiwanensis* (Su *et al*., 2016). Since the first report in grape, *X. fastidiosa* has been isolated and identified from an increasingly large number of plant hosts, with or without symptoms, and is recognized to be the causal agent of various diseases (Baldi and La Porta, 2017). Diseases caused by this pathogen include Pierce's disease (PD) of grape, almond leaf scorch (ALS), alfalfa dwarf, citrus variegated chlorosis, leaf scorch of oak, pear leaf scorch and oleander leaf scorch (OLS) (Purcell, 2013), and the olive quick decline symdrom (Luvisi et al., 2017; Saponari *et al*., 2018).

According to the latest report from EFSA (EFSA, 2018), 563 plant species are reported to be infected by *X. fastidiosa*. These host include hundreds of plant genera in 82 botanical families. Although confirmed records of presence of the pathogen come from some European countries, field epidemic outbreaks occur only in southern Italy and in insular (Corsica) and continental (French Riviera) France and in the Balearic Islands and Alicante in mainland Spain (Saponari *et al*., 2018; Ramazzotti et al., 2018). In the European Union, several cultivated plants of high economic value (e.g. olive trees, almonds, cherries) or wide-spread ornamental plants (e.g. myrtle-leaf milkwort, oleander) have been identified as hosts of *X. fastidiosa*. Many other widespread plant species remain potential hosts in the European Union territory (EFSA, 2018).

Xylella fastidiosa was previously reported from symptomatic almond and grape plants in Iran, with identification based on graft transmission, isolation on culture media, pathogenicity tests, and positive reactions in DAS-ELISA and PCR assays specific for the bacterium. (Amanifar *et al.,* 2014; Amanifar *et al.,* 2016). In the last decade symptoms of leaf scorch and dieback have been observed in many pistachio orchards in regions of Iran. However, based on the available information, there are no reports of isolation and pathogenicity studies of *X. fastidiosa* in pistachio.

Pistachio is the fifth most important commercial nut crop in the world and has been cultivated in different countries including Iran, Turkey, other Mediterranean countries, and the USA. According to the Food and Agriculture Organization (FAO, 2012), approx. 85% of the world pistachio production currently comes from these countries. Iran is the biggest world exporter, with 40% production of world pistachio and having 61% of the world market (Amiri Aghdaie, 2009).

Although *X. fastidiosa* has already been identified by ELISA from pistachio samples from California, culture on media and PCR results did not confirm the presence of this bacterium (Costa *et al*., 2004). In the present study, we report the isolation of *X. fastidiosa* strains from symptomatic pistachio plants in Iran, and confirm its pathogenicity on this host for the first time.

MATERIALS AND METHODS

Sampling

Four pistachio growing provinces in Iran, Kerman, Khorasan Razavi, Qazvin, and Yazd, were visited during each summer and autumn from 2012 to 2017, and plants exhibiting leaf scorch symptoms were sampled. Eighty-three samples from 21 pistachio orchards were collected. Each sample was from one tree. The samples consisted of stems and leaves. The distance between two sampled orchards was at least 500 m. Two samples were collected from each hectare of each pistachio orchard where leaf scorch symptoms were found. The stem and leaf samples were placed in individual plastic bags and transferred to the laboratory. Each sample was divided into three subsamples for pathogen isolation and testing for *X. fastidiosa* using DAS-ELISA and PCR (Schaad *et al*., 2001; Bextine and Miller, 2004; Amanifar *et al.,* 2014).

Bacterial cultures

Tissue pieces (each of length 3–5 cm) of pistachio petioles and midribs from plants exhibiting leaf scorch symptoms were transferred to sterile plastic bags, surface sterilized by soaking for 3 min in 5% sodium hypochlorite solution and 3 min in 70% ethanol, and then rinsed three times for 5 min in sterile distilled water. The tissues were then transferred to new plastic bags containing 3 mL of sterile succinate-citrate-phosphate buffer (1 g L^{-1} disodium succinate, 1 g L^{-1} trisodium citrate, 1.5 g L^{-1} K_2HPO_4, 1 g L^{-1} KH_2PO_4, pH 7). After a 20 min incubation period at room temperature, 100 µL of the liquid were streaked onto each of the agar media; PD2 (4 g L^{-1} tryptone, 2 g L^{-1} soytone, 1 g L^{-1} trisodium citrate, 1 g L^{-1} disodium succinate, 0.5 g L^{-1} $MgSO_4$-$7H_20$, 1.5 g L^{-1} K_2HPO_4, 1 g L^{-1} KH_2PO_4, 0.01 g L^{-1} hemin chloride (0.1% in 0.05 N NaOH), 2 g L^{-1} bovine serum albumin, 15 g L^{-1} agar) (Davis *et al*., 1980); PW (4 g L^{-1} phytone peptone, 1.2 g L^{-1} trypticase, 0.5 g L^{-1} $MgSO_4$-$7H_20$, 1.5 g L^{-1} K_2HPO_4, 1 g L^{-1} KH_2PO_4, 0.01 g L^{-1} hemin chloride (0.1% in 0.05 N NaOH), 4 g L^{-1} L-glutamine, 6 g L^{-1} bovine serum albumin, 15 g L^{-1} agar) (Schaad *et al*., 2001); Xf-D1 (3 g L^{-1} L-glutamine, 1 g L^{-1} L-asparagine, 0.5 g L^{-1} L-cysteine, 1.5 g L^{-1} Trisodium citrate, 1.5 g L^{-1} Disodium succinate, 10 mL Hemin chloride (0.1% in 0.05%

NaOH), 3 g L^{-1} Potato starch, 15 g L^{-1} Agar) (Almeida et al., 2004); or nutrient agar (28 g L^{-1}) (Schaad *et al*., 2001). The petioles and midribs were also crushed in the plastic bags using a pair of sterile pliers, and 50 µL of extract from each sample were streaked onto culture media. The plates were incubated at 28°C for 4 weeks and examined with a stereomicroscope at weekly intervals for the presence of *Xylella*-like colonies (Wells *et al*., 1987; Chen *et al*., 2007). Seven to 20 d after plating on PD2 and PW media, small white colonies, if found, were re-streaked for three passages onto PD2 and PW media to ensure the purity of the strains. Isolates were stored in PD2 broth plus glycerol at -20°C. For Gram tests, bacterial cells were Gram stained and observed at 1000× magnification using phase contrast microscopy (Schaad *et al*., 2001). Colonies on two media cultures were tested by DAS-ELISA and PCR.

DAS-ELISA tests

Serological detection of *X. fastidiosa* by *PathoScreenR XF* kit was performed according to the manufacturer's instructions (Agdia, Inc.). DAS-ELISA was used to detect *X. fastidiosa* in symptomatic samples of pistachio. One gram of petiole or midrib tissue from each sample was surface sterilized by soaking for 5 min in 70% ethanol, rinsed twice for 3 min in sterile distilled water, transferred to a plastic bag with 3 mL of extraction buffer and crushed with a pestle at room temperature. The sap was loaded into wells coated with *X. fastidiosa*–specific antibodies. The cultures were tested as plant samples. For this purpose bacterial isolates cultured on PD2 and bacterial cells suspended in extraction buffer were also used as antigen. The optical absorbance of the samples was measured using a plate reader (KC4, v.3.1, Bio-Tek), at 630 nm. All samples were tested in duplicate wells, and the plates each included positive and negative controls. A suspension of an antigen of *X. fastidiosa* included in the ELISA kit was also used as the positive control, and a non-infected pistachio plant was used as a negative sample control. Samples with absorbance values above the average absorbance values of the known negative samples plus three times the standard deviation were considered positive for *X. fastidiosa* (Sutula *et al*., 1986).

DNA extractions

Total DNA was extracted from fresh pistachio samples using the Qiagen Plant DNAeasy mini kit (Qiagen, Inc.), according to the manufacturer's instructions. The cetyltrimethylammonium bromide (CTAB) method (Ausubel *et al*., 1992, Amanifar *et al*., 2014) was used to extract DNA from bacterial cells, which had been scraped from PD2 agar plates after 6 d incubation at 28°C. Five microliters of the DNA extracted from each culture was subjected to electrophoresis in 1% agarose gels. The gels were stained with ethidium bromide, and DNA bands observed with ultraviolet light were used to estimate DNA concentrations. The DNA solutions were each diluted to 5 ng μL^{-1} and stored at -20°C.

PCR assays

Three primer sets, RST31/RST33 (Minsavage *et al*., 1994) targeting the *RNA-Pol sigma-70 factor* gene, 272-1-int/272-2-int (Pooler and Hartung, 1995) targeting an *hypothetical protein HL*gene, and Dixon454fa/Dixon-1261rg (Chen *et al*., 1995) targeting the *16S rDNA* gene, were utilized for amplification of parts of the *X. fastidiosa* genome, and the resulting products were sequenced. PCR reactions were carried out using the TaKaRa TaqTM (Hot Start Version, Takara Bio Inc.) in 25 µL volumes. The components for the each PCR reaction included 12.5 µL of Master Mix (2 × Premix), 0.1 µM of each primer (1 µL) and 8 µL of water. Templates consisted of 300 ng of extracted DNA from each plant sample, extracted bacterial DNA, or a small portion of the bacterial colony suspended in distilled water for whole-cell PCR. The amplification programme consisted of an initial denaturation step at 95 °C for 5 min; 35 cycles of 95 °C for 45 s, annealing temperatures or 57°C for the *16S rDNA* gene, 56°C for the *hypothetical protein HL*gene for 30 s, or 55°C for the *RNA-Pol sigma-70 factor* gene for 30 s, and 72°C for 30 s; and a final elongation step of 72°C for 8 min. PCR products were separated by electrophoresis on 1% agarose gels run at 5 V cm^{-1} for 1 h, stained with ethidium bromide (10 µg L^{-1}), and visualized with UV light.

All collected plant samples and bacterial isolates were tested with PCR for detection of *X. fastidiosa*. Five plant samples and four bacterial isolates (for three primer sets) were sequenced and some sequences were submitted to GenBank. PCR products were purified and sent for sequencing by Macrogen. Sequences obtained from the *RNA-Pol sigma-70 factor*, *hypothetical protein HL*gene and *16S rDNA* genes were analyzed and some sequences were deposited in the GenBank database (International Nucleotide Sequence Database Collaboration) under accession numbers MG601055 (isolate), MG517438.1 (plant sample), MG458715 (strain raf8), MG732899 (isolate Pistachio-Raf83), MG732900 (isolate Pistachio-Raf83), MG732901 (isolate Pistachio-Raf5) for isolates and plant samples from pistachio.

Plant bioassays and pathogenicity tests

Bioassays were conducted on seedlings of *Nicotiana tabacum* (cv. White Burley) maintained in a greenhouse. After seeds germinated, the seedlings were kept at 25°C in an incubator until the two-leaf stage. The plants were then transferred to pots each containing a nutrient/soil growth medium and were then kept in a greenhouse at 27±6°C. Plants at the 4-leaf stage were inoculated with bacterial suspensions. Two isolates (Pistachio-Raf83 and Pistachio-8) of *X. fastidiosa* from pistachios (from two different regions) were used for inoculations. PD2 medium plus glycerol (100 µL each) containing bacteria that had been stored at -20°C was propagated on solid PD2 medium, and the cultures were incubated for 7 d at 28°C. Bacterial suspensions in sterile distilled water were precipitated at a velocity of 5,000 rpm (4,192 × g). Resulting pellets were resuspended in distilled water and a spectrophotometer was used to adjust bacterial concentrations to approx. 2×10^6 CFU mL^{-1} (absorbance of 0.4 at 600 nm). Two hundred microliters of bacterial suspension were injected into the main veins and petioles of two leaves of each plant using an insulin syringe (Francis *et al*., 2008). Inoculated plants were covered with plastic bags for 24 h. Control plants were inoculated with distilled water. For each isolate, three tobacco plants were inoculated. All inoculated plants were maintained in the greenhouse and fertilized with irrigation water. Plants were monitored for symptoms on a weekly basis for 3 months. Leaves of inoculated and control plants were assayed for *X. fastidiosa* infection 2 months after inoculation, using DAS-ELISA. Leaves were also used for re-isolation of *X. fastidiosa*. Re-isolated bacterial colonies were tested by PCR using RST31/RST33 primers, to confirm identification of the isolated bacterium as *X. fastidiosa* (Ledbetter and Rogers, 2009).

Pathogenicity tests were also conducted on seedlings of pistachio (cv. Badami) grown and maintained in a greenhouse. Based on the results of the bioassay tests on *N. tabacum*, Pistachio-Raf83 strain of *X. fastidiosa* caused more severe symptoms than Pistachio-8 strain in the tobacco leaves, so Pistachio-Raf83 was selected to for the pathogenicity test on pistachio seedlings. The strain was grown in liquid PD2 medium at 28°C with shaking (70 rpm) for 8 d. The resulting bacteria were centrifuged to obtain a pellet, which was resuspended in 5 mL of distilled water. A spectrophotometer was used to adjust bacterial concentrations to approx. 10^5 CFU mL^{-1} (absorbance of 0.3 at 600 nm). For each inoculation, a cell suspension drop (approx. 20 µL) was placed on the young stem of each non-infected pistachio plant (as confirmed by PCR), near a petiole. Each inoculated stem was pricked five times through the cell suspension using a syringe needle. Control plants were treated in the same way except that distilled water was used instead of bacterial suspension. Nine pistachio plants were inoculated with bacterial suspensions, and three were inoculated with distilled water. All plants were kept in a greenhouse (at 27±6°C) and fertilized with irrigation water. Plants were monitored for symptom development and recorded on a weekly basis for 1 year (Chang and Donaldson, 2009, Amanifar *et al*., 2014). Leaves of inoculated and control plants were assayed for *X. fastidiosa* infections 8 months after inoculation, using the commercial DAS-ELISA *PathoScreen* kit. Leaves were also used for re-isolation of *X. fastidiosa*. Re-isolated bacterial colonies were tested by PCR using RST31/RST33 and 272-1-int/272-2-int primers to confirm identification of the isolated bacteria (Ledbetter and Rogers, 2009).

RESULTS

Orchard survey

In pistachio orchards in Kerman, Qazvin, Yazd and Khorasan Razavi provinces, the first symptoms of pistachio leaf scorch appeared in August each year, as leaf discolourations. The tips and margins of leaves initially turned light gray-green, and this progressed towards the midribs during the summer (Figure 1). Marginal scorching of leaves began as early as July and continued to increase during the summer. Symptoms sometimes appeared first on one branch of a tree or a portion of one scaffold. Over the years, symptoms spread to larger areas of the trees and sometimes the entire canopies

Figure 1. Pistachio tree with leaf scorch symptoms on a branch, caused by *Xylella fastidiosa*.

were affected. Diseased trees bloomed and leafed out later than trees without symptoms, and they were less productive and had reduced terminal growth. Diseased trees usually survived for at least 3 years.

Bacterial cultures

Nineteen isolates of *X. fastidiosa* were obtained from 83 pistachio samples (Table 1). Bacterial colonies were observed on media 10 to 17 d after plating. The PD2 medium was more suitable than PW for bacteria isolations, though PW medium was a good growing medium. Isolates did not grow on nutrient agar. The size of isolated bacterial colonies on PD2 medium varied from 0.2 mm to less than 1 mm in diameter (Figure 2). Isolated colonies on PD2 medium were circular and white-opal in colour using reflected light from above. Bacterial cells from all isolates were determined to be Gram-negative. DAS-ELISA and PCR tests confirmed that the colonies recovered on both media were from *X. fastidiosa*.

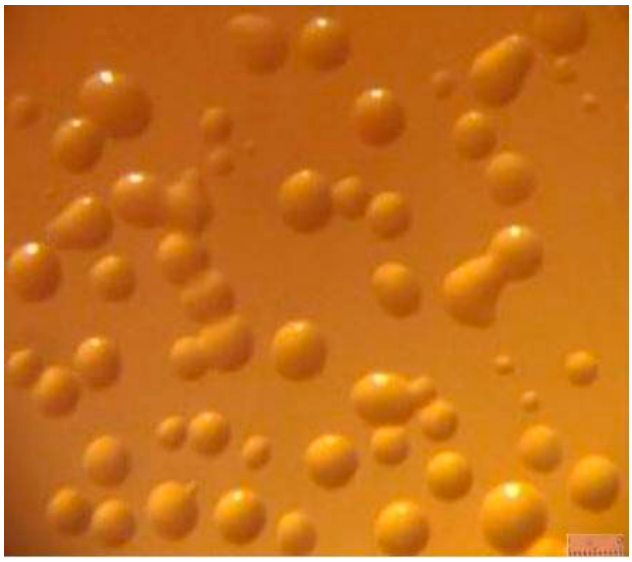

Figure 2. Colony morphology of a *Xylella fastidiosa* strain isolated from pistachio on PD2 agar medium, after incubation at 28°C after 12 days (Scale bar = 1 mm).

DAS-ELISA

Fifty-two of 83 pistachio plants tested positive for *X. fastidiosa* by DAS-ELISA (Table 1). Despite the presence of leaf scorch symptoms, not all samples tested positive for infection with *X. fastidiosa*. In Kerman and Yazd provinces 100% of the samples tested positive for the bacterium. Petiole and midrib extracts of pistachio trees that tested positive for *X. fastidiosa* had average (two replicates) absorbance values at 630 nm of 0.64 ± 0.33, compared with that of 0.124 ± 0.06 for the known negative control.

PCR assays

All three primers used effectively amplified their respective genes in the genome of *X. fastidiosa* from pistachio and culture colonies (Figure 3). Results of sequence analyses of the *RNA-Pol sigma-70 factor, 16S*

Table 1. Results of orchard survey for *Xylella fastidiosa* in symptomatic pistachio plant samples from Iran.

No. of plant samples[a]	No. of orchards[b]	Sampling month and year	Sampling province	Assay result		
				Culture (positive/tested)	DAS-ELISA (positive/tested)	PCR[c] (positive/tested)
10	2	September 2012	Kerman	2/10	4/10	4/6
4	1	August 2012	Qazvin	0/4	0/4	0/2
5	1	September 2012	Khorasan Razavi	1/5	3/5	1/3
11	3	September 2013	Kerman	3/11	11/11	2/5
6	2	September 2013	Khorasan Razavi	1/6	4/6	1/2
7	2	September 2014	Qazvin	0/7	0/7	0/3
24	5	September 2015	Kerman	5/24	17/24	4/7
4	1	October 2015	Khorasan Razavi	1/4	3/4	1/2
10	3	August 2016	Kerman	4/10	8/10	4/6
2	1	October 2017	Yazd	2/2	2/2	2/2

[a] Each sample was representative of an individual tree.
[b] The distance between two sampled orchards was at least 500 m.
[c] Tested by PCR using RST31/RST33, Dixon454fa/Dixon1261rg and 272-1-int/272-2-int primers.

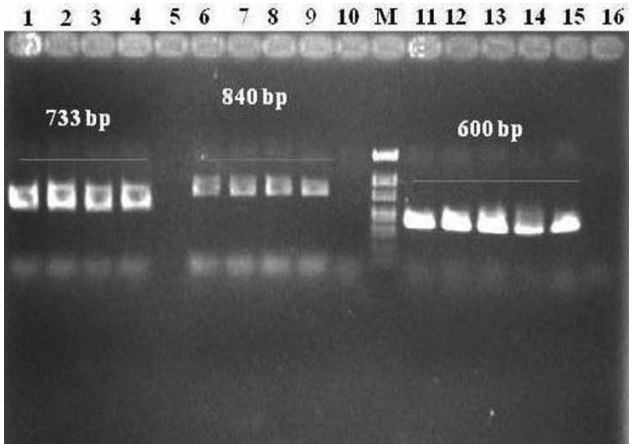

Figure 3. Gel electrophoresis pattern of PCR amplification products using *Xylella fastidiosa* specific primers. PCR products with primers RST31/RST33 from bacterial colonies isolated from pistachio (lane 1), from pistachio petioles (lane 2), from bacterial colonies isolated from grape (positive control, lane 3), from bacterial colonies isolated from almond (positive control, lane 4), from healthy pistachio petioles (negative control, lane 5). PCR products with primers Dixon454fa/Dixon1261rg from bacterial colony isolated from pistachio (lane 6), from pistachio petioles (lanes 7 and 8), from bacterial colonies isolated from almond (positive control, lane 9), from bacterial colonies isolated from grape (negative control, lane 10). PCR products with primers 272-1-int/272-2-int from bacterial colonies isolated from pistachio (lane 11), from pistachio petioles (lanes 12 and 13), from bacterial colonies isolated from almond (positive control, lane 14), from bacterial colonies isolated from grape (positive control, lane 15), from healthy pistachio petioles (negative control, lane 16). 100 bp DNA ladder (lane M).

rDNA, and *hypothetical protein HL* genes indicated that the isolates from pistachio were distinguishable from all the reference strains of *X. fastidiosa* subspecies in Genbank. In particular, nucleotide Blast analyses of *RNA-Pol sigma-70 factor, 16S rDNA* and *hypothetical protein HL* genes of pistachio isolates showed the greatest identity (96–100%) with those present in the Genbank (Table 2).

Plant bioassays and pathogenicity tests

Five days after *X. fastidiosa* inoculation, chlorosis symptoms appeared in tobacco leaf lamina. These symptoms gradually disappeared, and symptoms appeared in margins and tips of leaves. Early symptoms were leaf chlorosis and immediate mild necrosis, which gradually progressed in marginal leaf tissues to necrosis after 2 months (Figure 4, middle). Symptomatic plants were also each stunted, with dieback of branches (Figure 4, right). Control plants did not show any symptoms.

Six pistachio seedlings showed symptoms of leaf scorch under greenhouse conditions about 8 months after inoculation with bacterial cells (Figure 5). Early symptoms of the disease appeared about 3 months after inoculation (Figure 5). Control plants did not show any symptoms. Koch's postulates were proven using pistachio isolates inoculated onto pistachio seedlings.

DISCUSSION

None of the previous field surveys for *X. fastidiosa* in host plants has shown that pistachio is a natural host of this fastidious pathogen (Baldi and La Porta, 2017; EFSA, 2018; Saponari *et al*., 2018). We report the presence of *X. fastidiosa* in some regions of Iran in pistachio trees with leaf scorch symptoms, using three detection methods of culturing, DAS-ELISA and PCR. Scorching of leaf margins is the most common symptom caused

Table 2. Maximum nucleotide identity (%) of selected gene sequences between *Xylella fastidiosa* strains from pistachio and *X. fastidiosa* strains from other hosts present in Genbank. Respective accession numbers for isolates from pistachio are the following: *RNA-Pol sigma-70 factor* (MG601055), *16S rDNA* (MG517438.1, MG458715) and *Hypothetical protein* (MG732899, MG732900, MG732901). Accession numbers relative to strains are reported in order.

Gene	Pistachio isolate	Identity (%)	GenBank Accession No.	Host
RNA-Pol sigma-70 factor	Pistachio-Raf8	99	KT764083, KT764082	*Coffea arabica, Coffea arabica*
		98	CP000941, EU334069	*Prunus dulcis, Quercus palustris*
16S rDNA	Pistachio8	100	KF463301	*Prunus dulcis*
		99	DQ991190, CP020870.1, CP010051.1	*Carya illinoinensis*, *Olea europaea*, *Citrus aurantium*
	Pistachio-Raf3	100	KF463301	*Prunus dulcis*
		99	DQ991190.1	*Carya illinoinensis*
Hypothetical protein HL	Pistachio-Raf3	99	CP006740, EU021997, AE009442	*Morus* sp., *Persea americana, Vitis vinifera*
	Pistachio-Raf83	96	CP000941	*Prunus dulcis*

Figure 4. Healthy plant of tobacco (control, left), progressive development of tobacco leaf symptoms after inoculation with *Xylella fastidiosa*. Necrosis of leaves 40 days after inoculation (middle). Stunting of tobacco plant four months after inoculation (right).

by *X. fastidiosa*, but this symptom is not normally present until late in the growing season when temperatures are high, and plants are water-stressed (Hopkins, 1989). We observed severe symptoms of pistachio leaf scorch with increasing temperature and probably the maximum water demand by pistachio plants in August each year. The scorching symptoms, commonly induced by *X. fastidiosa,* result from blocking the host vascular systems. These symptoms can be confused with other factors such as drought, salt toxicity, or herbicide injury (Mircetich *et al*., 1976). Although *X. fastidiosa* was previously isolated and reported on the grape in Qazvin province of Iran (Amanifar *et al*., 2014), *X. fastidiosa* has not been detected in this province, although severe symptoms of leaf scorch have been observed in pistachio trees. The soil in these regions is salty, and these symptoms in Qazvin province are probably related to salt toxicity.

Disease progression in pistachio orchards during this study showed that severity increased from year to year and included the following stages (Figure 1): slight leaf scorch on individual host branches (first year), some branches with leaf scorch symptoms (second year), severe leaf scorch and leaf stunting (third year) and severe leaf stunting, dieback and death of affected trees (fourth year). Symptoms were fully developed by early September each year. Symptoms appeared first in single branches or limbs and spread throughout the entire canopie in the subsequent 3-4 years. Trees infected by *X. fastidiosa* eventually died.

These symptoms are similar to several leaf scorch diseases caused by *X. fastidiosa* on almond in Iran (Amanifar *et al*., 2016) and some other hosts (Hearon *et al*., 1980; Montero-Astua *et al*., 2008). Distribution of the disease in each orchard was irregular, often with single trees affected. This situation has also been observed in Iran in plantings of almond and grapevine with, respectively, leaf scorch and Pierce's disease (Amanifar *et al*., 2014; Amanifar *et al*., 2016), but mortality of pistachio trees was greater than in almond and grape.

Comparison of growth characteristics of *X. fastidiosa* isolates from pistachio with almond and grape isolates (Amanifar *et al.,* 2014) showed that colonies of *X. fastidiosa* from grape appeared on PD2 and PW culture media after approx. 7 d at about 28°C, but almond and pistachio isolates were slower growing appearing at least 10 d after culturing. All isolates from grape grew on the Xf-D1 and PD2 media. Almond and pistachio isolates did not grow on Xf-D1, but were able to grow on PD2 after 10 d. Maximum survival on PD2 media was 2 weeks after the appearance of bacterial colonies. We found differences in the colony morphology of grape, almond and pistachio isolates from Iran, specifically concerning colony margins which deserve further studies. Chen *et al*. (2007) showed that colony morphology has the potential to be used as a diagnostic tool for *X. fastidiosa* strain characterization.

Grape isolates of *X. fastidiosa* (Amanifar *et al*., 2014) did not amplify fragments in PCR using the Dixon454fa/Dixon1261rg primer set, but almond isolates (Amanifar *et al*., 2014) were amplified with this primer pair (Figure 3), Therefore, the pistachio isolates belong to a a subspecies multiplex, since these primers are specific for this subespecies (Figure 3).

Based on differences in *RNA-Pol sigma-70 factor, 16S rDNA* and *Hypothetical protein HL* genes sequencing and differences in biological and morphological traits of media cultures for Iranian isolates of *X. fastidiosa* it can be assumed we have two subspecies of this bacterium in Iran. Group I subsp. *fastidiosa* (grape isolates) and Group II subsp. *multiplex* (almond and pistachio isolates). However, further investigations are progressing for sequencing of more genes and specific phylogenetic analysis.

Figure 5. Pathogenicity tests: healthy plant of pistachio (uninoculated control, top left); initiation of pistachio leaf scorch symptoms 3 months after inoculation (top right); disease progress 1 year after inoculation under greenhouse condition (bottom).

DAS-ELISA was used as a conventional method for detection of *X. fastidiosa* in plants (Henneberger *et al*., 2004). Although PCR has been reported to be 100 times more sensitive than DAS-ELISA (Minsavage *et al*., 1994), in our tests with field plants it was not more sensitive. Other molecular techniques for *X. fastidiosa* identification/detection include PCR and PCR derivatives, including RFLP and RAPD analysis, as well are real-time PCR and loop-mediated isothermal amplification (LAMP). Extraction of *X. fastidiosa* DNA from culture and host species for PCR and related molecular analyses has been achieved from tissue by both standard commercial column kits and by basic CTAB or, in the case of cultures, Tris-EDTA-Sarkoysl techniques (EPPO, 2019).

About 62% of samples with typical leaf scorch symptoms were infected with *X. fastidiosa* as determined by DAS-ELISA and about 22% detected by culturing. From about 47% of the tissue samples tested, DNA of *X. fastidiosa* was extracted based on PCR results. Both DAS-ELISA and PCR have a potential for false positive and false negative reactions, which is always unclear, but one of the main causes is plant infection by other bacteria in addition to infection with *X. fastidiosa* (Sherald and Lei, 1991; Minsavage *et al*., 1994; Pooler and Hartung, 1995; Chen *et al*., 2007). In this study, we isolated other bacteria species in addition to *X. fastidiosa*, some of which were very slow to grow. These bacteria may interact with *X. fastidiosa*, which requires additional research.

The distribution range of *X. fastidiosa* is usually limited to tropical and subtropical areas, although leaf scorch diseases caused by *X. fastidiosa* also occur in colder climates, e.g. oak leaf scorch occurs as far north as Canada (Goodwin and Zhang, 1997) and almond leaf scorch occurs in regions with very cold winters in Iran (Amanifar *et al*., 2016). In this study, *X. fastidiosa* was detected from pistachio samples collected from arid-warm regions with cold winters of Iran.

Xylella fastidiosa is no longer a plant pathogen limited to a few countries in the Western Hemisphere, where its geographical distribution has expanded dramatically (Almeida and Nunney, 2015). One of the most relevant pending questions is what drives host specificity in this pathogen; in other words, why do pathogen genotypes cause disease in one plant species and not another, while still being able to colonize various plant species with different degrees of success but without inducing symptom expression (Almeida and Nunney, 2015). Our knowledge in Iran is still very limited about various aspects of this fastidious pathogen. Complementary studies are necessary to determine the molecular and biological characteristics of Iranian isolates of *X. fastidiosa* from different hosts and their host range and its insect vectors. Given the world-wide relevance of Iranian pistachio production and quality and the importance of the diseases caused by *X. fastidiosa* in fruit trees, it is necessary to deepen the knowledge on this pathogen in pistachio trees to better manage the disease.

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