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Detection and characterization of *Xylella fastidiosa* in Iran: first report in alfalfa (*Medicago sativa*)

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Summary. Bacterial pathogens, especially *Xylella fastidiosa* (*Xf*), are significant threats to agricultural productivity, affecting economically important crops. The recent detection of *Xf* in Europe and the Middle East, including Iran, has emphasized the urgency for comprehensive surveillance to assess and understand the genetic diversity and distribution of this pathogen. A comprehensive survey from 2019 to 2022 was carried out in Iran to investigate *Xf* occurrence. A total of 403 samples were collected from alfalfa, almond, citrus, cherry, grapevine, olive, and pistachio plantations. Using serological (DAS-ELISA) and molecular (PCR) techniques, *Xf* was detected in nine samples from grapevine, five from almond, and 18 from alfalfa, and these include the first records *Xf* infections in alfalfa in Iran. Multiprimer-PCR assays carried out on *Xf*-infected plants, using ALM1/ALM2, *XF*2542-L/*XF*2542-R, and *XF*1968-L/*XF*1968-R primers for subspecies and strain differentiation, showed that the isolates from almond were *Xf* subsp. *multiplex*, and those from alfalfa were *Xf* subsp. *fastidiosa*. The *Xf* subsp. *multiplex* infecting almonds belonged to *Xf* genotype II. Pathogenicity tests carried out using *Xf* subsp. *multiplex* and *fastidiosa* isolates showed that the pathogen caused symptoms on *Nicotiana benthamiana* plants within 20 d post-inoculation. This study emphasizes the requirement for continuous monitoring, to mitigate the impacts of *Xf* on Iranian agriculture, and to prevent widespread outbreaks of this pathogen in multiple crop types.

Keywords. Detection, isolation, pathogenicity test, symptomatology.

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INTRODUCTION

Plant diseases, particularly those caused by bacterial pathogens, continue to pose significant challenges to agricultural productivity (Nazarov

et al., 2020). Among these pathogens, *Xylella fastidiosa* (*Xf*) is an important vascular pathogen, affecting a wide range of economically important crops including grapevine, olive, citrus, coffee, and stone fruits, as well as numerous ornamental and forest species (Loureiro *et al*., 2023). The pathogen is primarily vectored by sap-feeding insects, including sharpshooters and spittlebugs (Avosani *et al*., 2024). Originating in South America, *Xf* has been responsible for several important plant diseases, including Pierce's disease (PD), olive quick decline syndrome (OQDS), almond leaf scorch (ALS), and citrus variegated chlorosis (CVC) (Picciotti *et al*., 2023). Recent emergence of *Xf* in Europe has raised concerns regarding the presence of multiple genotypes of the pathogen, and the vulnerability of diverse plant species to infections. Detection of the pathogen in the Middle East and western Asia has underscored requirements for comprehensive investigations and surveillance to understand its distribution, genetic diversity, and potential impacts on agriculture in these regions (Loureiro *et al*., 2023).

The first report on the presence of *Xf* in the Middle East and western Asia was in 2014 in Iran, where the pathogen was reported from symptomatic grapevines, almond and pistachio trees, 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; 2016).

In Iran, *Xf* was found in commercial almond orchards in Chaharmahal va Bakhtiari, West Azerbaijan and Semnan provinces (Amanifar *et al*., 2014). At the same time, in the Razavi-Khorassan province, a severe apricot decline syndrome was observed, which had been previously associated with phytoplasmas, but some plants also tested positive for *Xf* (Karimishahri *et al*., 2016). According to Amanifar *et al*. (2019), there are two subspecies of *Xf* in Iran, determined after gene sequencing and observations of differences in biological and morphological characteristics of bacterial colonies. These were subsp. *fastidiosa* isolated from grapes, and subsp. *multiplex* isolated from pistachios and almonds (Amanifar *et al*., 2016; 2014). *Xylella fastidiosa* can increase host range through horizontal gene transfer (HGT), enabling the bacterium to acquire genetic material from other organisms, potentially including genes associated with infection of new host plants (D'Attoma *et al*., 2020; Pierry *et al*., 2020, Woods *et al*., 2020). Genetic variation can also occur within *Xf* populations, from mutations and recombination, which give rise to variants with altered characteristics, potentially enhancing their ability to infect increased host ranges. These mechanisms, together with selective environmental pressures, can result in adaptation and expansion of *Xf* host range (O'Leary and Burbank, 2023).

Instances of leaf scorch and dieback symptoms, like those caused by *Xf* on several crop, have been reported in numerous alfalfa fields across regions of Iran.

Upon confirmation of the bacterium's presence within this country, it became important that routine surveillance efforts were initiated, aiming to mitigate occurrence and dissemination of *Xf* in previously unaffected areas, while also monitoring spread of the pathogen. Despite these observations, no investigations on the possible presence of *Xf* in these crops had been carried out. Therefore, the objective of the present study was to conduct an extensive survey to evaluate the presence of *Xf* in almond, grape, citrus, and alfalfa plants across different regions of Iran, to update knowledge of the distribution of *Xf* in this country.

MATERIALS AND METHODS

Study areas and collection of samples

A systematic sampling campaign was carried out in crops of different Iranian regions over the summer seasons from 2019 to 2022, in the regions of Qazvin, Isfahan, Chaharmahal Bakhtiari, Gilan, Zanjan, Tehran, and the central regions of Hormozgan and Kerman. In total, 403 samples were collected from different crop types, including alfalfa, almonds, cherry, citrus, grapevine, olive, and pistachio, showing symptoms recalling those caused by *Xf* (Figure 1). Each sample, which consisted of 4 to 6 cuttings/trees (up to 20 cm each), was kept in a closed plastic bag, labelled with relevant information (date, location, presence/absence of symptoms) (Table 1), and then kept in a cooling box during transport, brought to the laboratory and was conserved at 4°C before being analysed.

Serological detection (DAS-ELISA)

All samples underwent the double-antibody sandwich-enzyme linked immunosorbent assays (DAS-ELISA), using a polyclonal antibody kit (Agritest, Bari, Italy), according to the manufacturer's instructions. ELISA plates were coated with anti *Xf*-IgG and incubated at 37°C for 4 h. After washing, samples were loaded and incubated overnight at 4°C. Alkaline-phosphatase conjugated-anti *Xf*-IgG was added, and incubated at 37°C for 4 h. Absorbance was measured using a Multiskan FC microplate reader (ThermoFisher Scientific) at 405 nm. Positive reactions were identified if absorbance was three times greater than controls after 120 min.

Figure 1. Grapevine (a), alfalfa (b), and almond (c) plants infected with *Xylella fastidiosa* and showing symptoms of marginal leaf scorch, wilting of foliage, and withering of branches.

Table 1. Hosts, locations, sampling dates, and numbers of samples collected, for plants showing disease symptoms.

Host	Sampling province	Sampling date	Number of samples
Alfalfa	Chaharmahal va Bakhtiari	September 2021	3
		September 2022	6
	Isfahan	September 2021	3
		September 2022	6
Almond	Alborz	September 2022	12
	Chaharmahal va Bakhtiari	August\September 2021	11
		September 2022	35
	Isfahan	August\September 2021	8
		September 2022	54
Cherry	Isfahan	September 2022	15
Citrus	Hormozgan, Gilan kerman	September 2020	40
Grapevine	Alborz	August\September 2021	3
	Qazvin	August\September 2019	50
		September 2020	32
		August 2021	41
		August 2022	53
Olive	Gilan, Zanjan	December 2021	17
Pistachio	Tehran, Markazi	September 2021	14
		Total	403

Molecular detection (PCR)

DNA extraction was carried out using CTAB buffer (Hendson *et al*., 2001). For each sample 1 mL of homogenized extract was placed in a 2 mL microcentrifuge tube and heated at 65°C for 30 min, followed by centrifugation at 16,000× g for 5 min. Subsequently, 1 mL of the supernatant was carefully transferred into a fresh 2 mL capacity micro-centrifuge tube, and 1 mL of chloroform-isoamyl-alcohol (24:1) was added, mixed thoroughly, and centrifuged at 16,000× g for 10 min. Supernatant (700 µL) was then transferred to a 1.5 mL microcentrifuge tube, and approx. 0.7 volume of cold 2-propanol (490 µL) was added. After gentle mixing twice by inversion, the tubes were incubated at –20°C for 20 min. Subsequent centrifugation at 16,000× g for 20 min allowed for recovery of a pellet from each tube, which was washed with 1 mL of 70% ethanol and centrifuged again at 16,000× g for 10 min. The sample was then vacuum dried, and the pellet was resuspended in 100 µL of DNase/RNase-free water.

PCR assays were carried out using *Xf*-specific primers, *i.e*., RST31 (5'-GCGTTAATTTTCGAAGTGATTCGA-3') and RST33 (5'-CACCATTCGTATCCCGGTG-3') (Minsavage *et al*., 1994). PCR reactions were carried out in 20 µL final volumes each containing 4 µL of Bioline buffer [(5 mM dNTPs and 15 mM $MgCl_2$), 0.5 µL (10 pmoL μL^{-1}) of each primer, 0.2 µL Taq DNA polymerase (Lifetechnologies), 3 µL of total DNA template]. The cycle program used was 95°C for 5 min, followed by 40 cycles each at 95°C for 30 sec, 55°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 5 min. The assays were electrophoresed on 1.2% TBE agarose gel and were visualized under UV light after staining with GelRed dye (Biotium).

Biological detection (isolation)

Upon the confirmation of *Xf* DNA presence through one of the diagnostic techniques, isolations of *Xf* were carried out from positive almond and alfalfa plant samples. These were achieved through direct printing of twigs on buffered charcoal yeast extract (BCYE) agar plates (Wells *et al*., 1981). Twigs of length 8 to-10 cm were surface sterilized with 2% sodium hypochlorite for 2 min, were then rinsed in 70% ethanol for 2 min

followed by three rinses with sterile water. Each twig was then cut in half and pressed at one end with a plier, while the other end was gently pressed onto BCYE plates to make imprints. The plates were then closed and sealed with parafilm, incubated at 28°C for 4 weeks and regularly checked for the appearance of *Xf*-like colonies, which typically appeared as small, circular, and whitish yellow in colour.

Pathogenicity tests

The virulence potential of newly isolated *Xf* strains was assessed using a pathogenicity test. This involved *Xf* inoculations into stems of *Nicotiana benthamiana* plants, which are widely used as an experimental host for *Xf* (Lopes *et al*., 2000). One-month-old *N. benthamiana* plants were each inoculated with 50 µL of *Xf* suspension (OD_{600} = 0.32) prepared in phosphate buffered saline (PBS; pH 7.4, 0.01 M), using a 0.1 mL capacity insulin syringe. The plants were then maintained in a controlled environment at 28°C for 2 months and were visually inspected for development of characteristic symptoms of *Xf* (*i.e*., leaf scorch). The presence of *Xf* in the symptomatic *N. benthamiana* plants was confirmed through PCR assays using the RST31/33 primers.

Multiprimer-PCR specific detection of Xylella fastidiosa *subspecies*

The colonies presumed to be *Xf* were purified on BCYE plates, and the isolates were then subjected to PCR assays to verify their *Xf* nature, using *Xf*-specific primers RST31/RST33 as described above. Subsequently, all newly identified *Xf* strains, *i.e*., those found in infected plants and those identified from plates, were subjected to a multiprimer-PCR assays, using ALM1/ALM2, *XF*2542-L/*XF*2542-R, and *XF*1968-L/*XF*1968-R primers to differentiate the strains into the three *Xf* subsp., *fastidiosa*, *multiplex*, or *sandyi* (Hernandez-Martinez *et al*., 2006).

RESULTS

Serological and molecular detection of Xylella fastidiosa

The ELISA assay results yielded insights into the presence of *Xf* within the tested samples. Among the 176 grapevine samples tested, nine samples from Qazvin and Takistan provinces were positive for *Xf* infections. Of the 123 almond and 18 alfalfa samples tested, five from each plant type were found to be infected, with all the host plants originating from the provinces of Isfahan, Chaharmahal va Bakhtiari. These results were then verified through PCR assays using the *Xf*-specific RST31/RST33 primers, which confirmed the presence of *Xf* in all ELISA-positive samples, and its absence in the samples gave negative reactions (Figure 2). All the assessed olive, citrus, cherry and pistachio samples were found to be negative for *Xf*, based both on the ELISA and PCR assays.

Isolation of Xylella fastidiosa *from infected samples*

All almond and alfalfa samples that tested positive for *Xf* in ELISA and PCR assays were utilized for isolation of bacteria, using the printing method (Table 2). Two *Xf* isolates from alfalfa and one from almond were recovered on BCYE plates (Figure 3). All isolated bacterial colonies were further confirmed as *Xf* by PCR, using RST31/RST33 primers, and each isolate was triple-cloned before being stored in PBS supplemented with glycerol (50%) and maintained at -80°C.

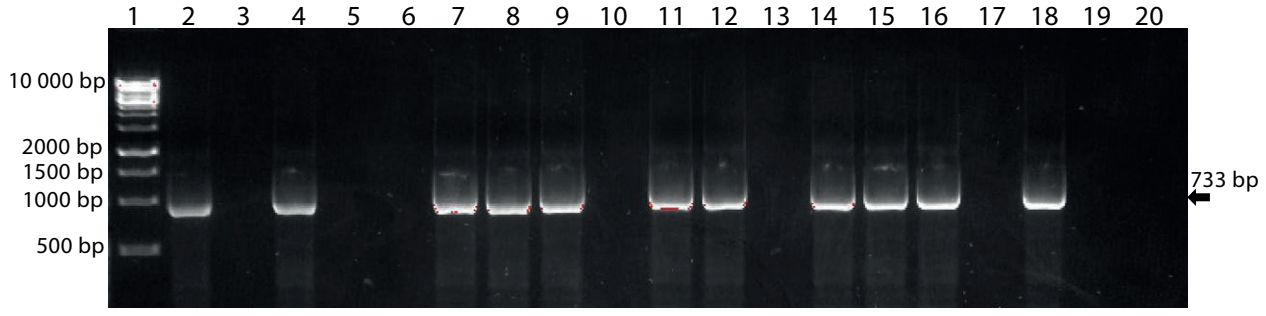

Figure 2. Agarose gel electrophoresis showing PCR amplified products using RST31/RST33 primers (733 bp). Representative samples from grapevine (lanes 2 and 4), alfalfa (lanes 7 to 9), and almond (lanes 11, 12, 14, 15 and 16), producing the expected amplicon, indicate presence of *Xf*. Lanes 3, 5, 6, 10, 13, 17 and 19 are for plant samples that tested negative. Lane 18 is for gDNA of *Xf* used as the positive control reaction. Lane 20 is for the negative control reaction. Lane 1 is the 1 Kbp DNA ladder.

Figure 3. BYCE media plates (4 weeks after preparation) showing bacterial colonies of *Xylella fastidiosa* isolated from plants of alfalfa (Q7A and Q257B) or almond (Q8B), infected with *Xylella fastidiosa*.

Table 2. Host plant species and geographical origins of the *Xylella fastidiosa* isolates obtained in this study.

Host plant	Region	Isolates	ELISA	PCR	Culture on BCYE
Alfalfa	Isfahan	Q7A	+	+	+
	Isfahan	Q23H	+	+	-
	Chaharmahal\Bakhtiari	Q58Z	+	+	-
	Chaharmahal\Bakhtiari	Q257B	+	+	+
	Chaharmahal\Bakhtiari	Q125P	+	+	-
Almond	Isfahan	Q55X	+	+	-
	Isfahan	Q57S	+	+	-
	Isfahan	Q25P	+	+	-
	Chaharmahal\Bakhtiari	Q47F	+	+	-
	Chaharmahal\Bakhtiari	Q8B	+	+	+

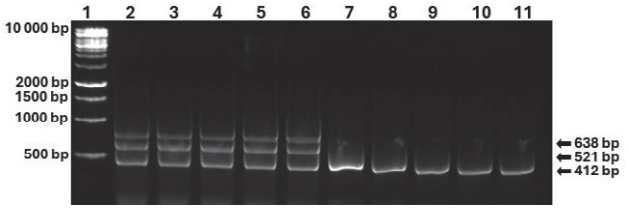

Figure 4. Agarose gel electrophoresis showing multiprimer-PCR amplified products. Amplification was achieved using ALM1/ALM2, *XF*2542-L/*XF*2542-R, and *XF*1968-L/*XF*1968-R primer pairs, with samples from almond (lanes 2 to 6) indicating infections by *Xanthomonas fastidiosa* subsp. multiplex, and samples from alfalfa (lanes 7 to 11) indicating infections by -*Xf* subsp. *Xf* subsp. *fastidiosa*. Lane 1: 1 Kbp DNA ladder.

Multiprimer-PCR assays

To differentiate the subsp. of the *Xf*-infected isolates from almond and alfalfa plants, a multiprimer-PCR assay was carried out, using the methods of Hernandez-Martinez *et al*. (2006). PCRs were with primers XF1968-L and XF1968-R amplifying a 638 bp fragment from oleander leaf scorch (OLS) strains but not from Pierces' Disease (PD) strains or almond leaf scorch (ALS) strains that belong to *Xf* subsp. *fastidiosa*. PCR with primers XF2542-L and XF2542-R amplify a 412 bp fragment from PD strains, but not from OLS strains. PCR with primers ALM1 and ALM2 produces a fragment of 521 bp from strains isolated from almond that belong to *Xf* subsp. *multiplex*.

The multiprimer-PCR results showed that when the *Xf* isolates from almond were tested, three bands of 412, 521, and 638 bp were obtained; whereas *Xf* alfalfa isolates uniquely yielded a 412 bp band (Figure 4), indicating that they belonged to genotype II of *Xf*, in contrast to the genotype I isolates, which produce only two bands in the PCR.

Pathogenicity tests

The pathogenicity tests showed that all isolates caused symptoms on *N. benthamiana* plants. At 20 days post inoculation (dpi), the *Xf*-infected tobacco plants had leaf margin and interveinal chloroses, and then scorch symptoms at 4 to 6 weeks after inoculation (Figure 5).

DISCUSSION AND CONCLUSIONS

Early detection of *Xf* infections is essential for effective management of this harmful plant pathogen. Emergence of *Xf* in new areas and failure to contain its spread in previously affected regions have become severe international problems (El Handi *et al*., 2022). To date, sev-

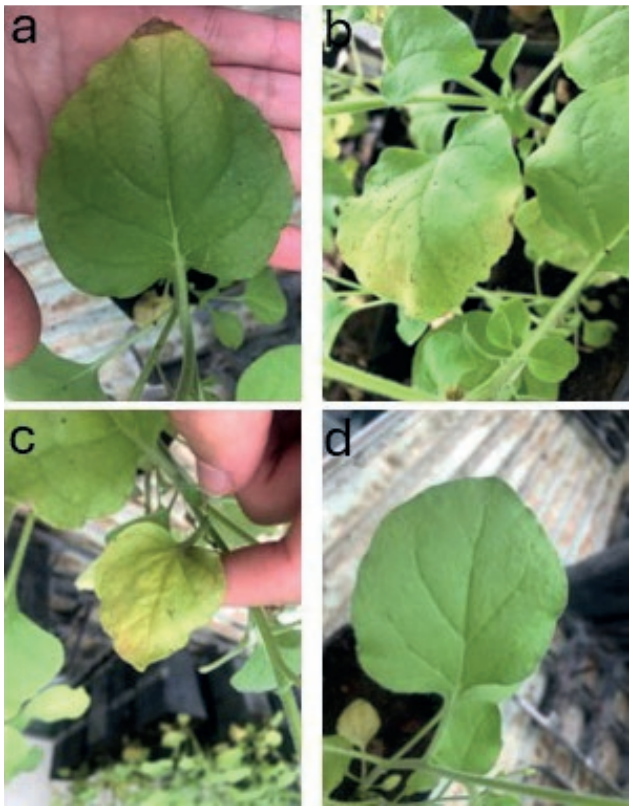

Figure 5. *Nicotiana benthamiana* plants inoculated with different isolates of *Xylella fastidiosa* sub sp. *alfalfa* [(a) isolates Q7A and (b) 257B)], and isolates from almond [(c) isolate Q8B], showing typical symptoms of *Xf* infections, including scorch of leaf margins, wilting of foliage, and withering of branches. (d) *N. benthamiana* plants inoculated with sterile water and showing no symptoms.

eral *Xf* subspecies have been isolated and identified from a wide variety of host plants (Loureiro *et al*., 2023). The broad distribution of this pathogen from the American continent to different parts of the world demonstrates its adaptability to various environmental conditions. As a result, the spectrum of host species vulnerable to this potentially serious pathogen continues to increase (Castro *et al*., 2021).

In Iran, the detection of new *Xf* subspecies could lead to important disease outbreaks, resulting in considerable socio-economic and agricultural challenges impacting local economies. To prevent these scenarios, it is important to monitor and counter the spread of *Xf*, and to accurately map its distribution, by identifying infested zones and developing effective control strategies to contain the pathogen (El Handi *et al*., 2022). This proactive approach is essential for limiting emergence of *Xf*, thereby safeguarding agricultural sustainability and economic stability. The present research extends previous studies conducted in Iran (Amanifar *et al*., 2019; 2016), aiming to update and monitor the presence of *Xf* in different Iranian regions and different host species. This study, which assessed 403 plant samples from several crop types and carried out during 4 consecutive years, detected, for the first time, *Xf* in alfalfa as a new host in this country. The results showed incidence ALS and PD in particular orchards, which occasionally resulted in withering symptoms on affected trees. Almond trees, as well vineyards Takestan City, in the Qazvin province, are noteworthy examples. Takestan City has the largest vineyards in Iran. Furthermore, isolation of *Xf* from alfalfa and almond hosts is a first record of this pathogen in Iran.

The results of multiprimer-PCR analyses of *Xf* isolates from alfalfa and almond in Iran, but not on those from grapevine which were previously characterized by Amanifar *et al*. (2016), demonstrated the presence of *Xf* belonging to subsp. *fastidiosa*, which was associated with alfalfa leaf scorch symptoms. The same method for identification of subspecies and strains was utilized by Hernandez-Martinez *et al*. (2006) for the differentiation of strains of *Xf* infecting grape, almond, and oleander. Similarly, in the present study, *Xf* subsp. multiplex isolates from almond were genotype II of *Xf*. Normally, strains of the *Xf* subsp. *multiplex* are less fastidious, can easily grow on artificial media, and produce mild ALS and PD symptoms (Almeida and Purcell, 2003). Isolation of *Xf* on artificial culture media, together with the results obtained from the pathogenicity test on *N. benthamiana*, are important for characterization of *Xf* strains in different hosts and crop varieties in Iran. The presence of *Xf* was observed in alfalfa fields located near almond trees affected by *Xf* infections.

At field scales, proximity between alfalfa fields and grapevine and almond plantations suggests a potential mechanism of leafhopper transfer between these plantations. Therefore, it is important that intensive insect surveys are carried out to identify potential vectors of *Xf* within and outside affected areas.

In conclusion, the swift detection of novel subspecies of *Xf* in Iran is important for effective outbreak management and mitigation of the diseases caused by this pathogen. The severe OQDS outbreaks in Italy demonstrate the necessity for proactive surveillance and rapid reaction strategies for managing these diseases in Iran. Implementing strict quarantine measures, deploying targeted control strategies, and encouraging collaboration among researchers, policymakers, and agricultural stakeholders, are all important components of proactive prevention of introduction and spread of new *Xf* subspecies in this country.

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AUTHOR CONTRIBUTIONS

D.G: writing of original draft, visualization, validation, methodology, formal analysis, and validation. N.H: writing, review and editing, visualization, supervision, resources, and administration. M.G.Z: investigation, visualization, and methodology. S.N: investigation, visualization, and methodology. K.E.H.: visualization, methodology, analysis, review, and editing. T.E: methodology, review and editing, visualization, supervision, resources, and funding acquisition. All authors read, revised, and approved the final manuscript.

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