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Continuous Pest Surveillance and Monitoring Constitute a Tool for Sustainable Agriculture: Case of *Xylella fastidiosa* in Morocco

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Abstract: Climate and trade changes are reshaping the cartographic distribution of lethal pervasive pathogens. Among serious emerging challenges is *Xylella fastidiosa* (*Xf*), a xylem-limited phytopathogenic bacterium that produces losses and damages to numerous crops of high economic and agronomic importance. Lately, this grave quarantine pathogen has expended its distribution by arriving to several European countries and infecting both wild and cultivated plants, and no cure has been identified so far. Countries without current outbreaks like Morocco, need to monitor theirs crops frequently because detecting diseases in the early stages may reduce the huge losses caused by *Xf*. For that purpose, inspections were managed in different regions in Morocco from March 2020 to July 2021 to assess the presence of *Xf* in several growing areas of vulnerable economic crops (i.e., almond, citrus and olive). To extend the likelihood of detection, hosts have been inspected and sampled randomly over different environments including symptomatic and asymptomatic plants. Each sample was screened for the existence of *Xf* by using the DAS-ELISA commercial kit, while, further analyses were carried out for doubtful samples, by PCR. Results of both tests did not show any positive sample in the investigated areas. This finding is an update on the *Xf* situation in Morocco and confirms that this country is still a free territory from this bacterium, at least in the monitored regions.

Keywords: DAS-ELISA; diagnosis; PCR; *Xylella fastidiosa*; sustainable agriculture; Morocco

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1. Introduction

The latest appearance and spread of *Xylella fastidiosa* (*Xf*) in the Euro-Mediterranean basin, have brought out some fragilities and criticalities of the quarantine system and the nursery sector. Given this, the early detection and continuous surveillance of *Xf* in Morocco constitute the key factors of sustainable management of this alien species with huge social and economic impacts, and ecological sustainability at the potential entry level of the biological invasion process of the bacterium.

Xf belongs to the top strange phytobacteria amongst the vascular bacteria. It is vectored by sap-feeding insects, infecting an extensive range of plant species [1]. The pathogen, which originates in South America, has hardly been investigated because of its association with catastrophic landscape damages [2] and devastating diseases altering the

major league crops like grapevine, olive, citrus, coffee, and stone fruits, besides numerous ornamental and forest species. It is the agent responsible for the famous Pierce's disease (PD), olive quick decline syndrome (OQDS) and citrus variegated chlorosis (CVC) [3]. Symptoms caused by infections with *Xf* are usually the outcome of the systemic occupation of the bacterium that can block the xylem vessels and lead to a gradual inability in water movements [4]. Several recent studies highlighted that an isolate of *Xf* subspecies *pauca* (ST53) has been destroying olive plantations in the Apulia region, among the leading olive production zones of Southern Italy [5]. Furthermore, the bacterium is distinguished by six subspecies which differ in biological and genetic properties [6]. Especially, worldwide movements of infected plants for commercial or panorama planting are most likely the major route that contributed to the propagation and establishment of *Xf*, external of the Americas, where it was known to be restricted until 20 years ago, while the presence of *Xf* was announced in Taiwan, Iran and in several outbreaks newly noticed in European countries [7]. The situation in the European territory raised serious concerns since different *Xf* genotypes have been reported, a broad list of plant species was found vulnerable to the infection, and spittlebug is the predominant and widespread European transmitter species up to now, found in European and Mediterranean countries [1]. The Moroccan climate could hardly be a limiting agent for the establishment of *Xf* and its vectors, notably in the littoral areas of northern Morocco. Climatic data per month on temperature and precipitation during the last 10 years in Morocco according to hikersbay.com/climate/Morocco (accessed on 12 December 2021) are suitable for the multiplication of *Xf*, for the development of the disease, and for the intense activity of the insect vectors. Consequently, they could represent a potential danger for the Moroccan plant patrimony (cultivated, forest, and ornamental plants) [8]. Furthermore, the geographical placement of Morocco, with its close proximation to Spain (13 km), and its commercial exchange with several European countries at the source of *Xf* (Spain, France, Italy) increase the potential risk of entry of *Xf* through infected plant materials/or insect vectors [9]. Morocco has a serious potential risk for the entry of *Xf* into its territory. According to Resourcetrade.earth (accessed on 12 December 2021) [10], Spain, a source country of *Xf*, constitutes the largest exporter of plants for planting (i.e., live plants, bulbs, roots and cut flowers) to Morocco. In the same period, Italy and France have exported 693 and 564 tons, respectively, of these potential host plants of the bacterium to the country. Furthermore, the importation from other European countries in which *Xf* has been intercepted or reported—Central and Southern Asia (Iran), Latin America and the Caribbean as well as Northern American countries, exporters of *Xf*—ranges from 13 to 280 Tons (average period of 2015–2019) as follows: Mexico (280 Tons), United States (275 Tones), Portugal (266 Tons), United Kingdom (242 Tons), Belgium (135 Tons), Germany (79 Tons), Iran (18 Tons), Argentina (17 Tons) and Brazil (13 Tons).

Moreover, the presence of potential insect vectors of *Xf* in Morocco like *Philaenus tesselatus* (the principal spittlebug registered with variable occurrences across the Moroccan country) increase the probability of the entering and establishment of *Xf* in Morocco [11].

Consequently, Morocco has introduced strict emergency measures, regularly updated aiming to prevent the potential introduction and spread of *Xf* into the country, and thus avoiding serious agricultural, environmental and social consequences [12]. In addition, surveys for *Xf* are now mandatory in Morocco, inspections, and diagnostic tests are also compulsory at consignments/place of production for the most susceptible species listed in the EU Decision 2017/2352.

Xf is a tardy-growing (fastidious) bacterium that needs special culture media, but some excellent selective media are available [13]. Additionally, symptom monitoring, isolation and culturing, observation and identification of *Xf* depend on various laboratory tests [14].

The focal point of serological approaches is the special properties of bacterial cell surface. For example, the enzyme linked immunosorbent assay (ELISA) [15] which is regularly employed in the case of *Xf* as a screening test for detection and has a high production capacity because of its easy and simple sample preparation. In fact, kits for

serological detection of the bacterium are provided by several companies. ELISA kits from Agritest (Italy) and Loewe (Germany) have been verified for grape, olives, citrus, almond, oak, oleander and other species [16]. In addition, direct tissue blot immunoassay (DTBIA) was newly reported, such as a replacement quick screening test, in order to detect *Xf* in olive samples [17]. Molecular techniques are more efficient compared to serological tests and they comprise conventional PCR [18] and numerous protocols of real-time PCR [19–21], and loop-mediated isothermal amplification (LAMP) [22]. Extraction of the bacterium DNA from plants is accomplished by the CTAB method or by standard commercial kits which can be performed manually or on automated platforms. In pest-free areas and buffer zones, molecular approaches are recommended for *Xf* detection because of their great sensitivity, as stated by the European Food Safety Authority [23].

The earliest detection of *Xf* infections is crucial to the management of this serious phytopathogen, thus, the aim of the present work was to conduct a large survey in order to assess the presence of *Xf* in olive, almond and citrus trees in different commercial groves in Morocco, to update Morocco's situation regarding *Xf*, and to update results obtained by the last monitoring in 2018. Furthermore, sustainable control measures of *Xf* must be done regularly because as it is known, Morocco belongs to the countries ranked at a high-risk level for the entry, establishment and spread of *Xf* [2].

2. Materials and Methods

2.1. Study Areas and Collection of Samples

Xf represents a serious threat to several crops in Morocco, thus, an inspection was managed from March 2020 to July 2021 nationwide, covering olive, almond and citrus planted areas. Overall, 51 commercial groves were visited where the typical symptoms of *Xf* (when presented) were inspected. A total of 1007 plants were randomly sampled and only twigs close to the symptomatic portion were collected (to avoid any false positive reaction being given by the symptomatic portion) as following: (1) 657 olive trees from five regions (Tanger, Béni Mellal, Marrakech, Errachidia, and Meknès), (2) 170 citrus trees collected in two regions (Azilal and Meknès) and (3) 180 almond trees from three regions (Meknès, Haouz, and Gharb,) (Table 1 and Figure 1). Each sample, which included 6–8 cuttings/trees (up to 20 cm/each) was kept in a closed plastic bag, labeled with information (date, location, presence/absence of symptoms, etc). In summer and on high temperature days, samples were kept in a cooling box during transport, and later, all samples were conserved at 4 °C in the laboratory before being analyzed.

Table 1. Crops, locations, number of groves and the collected trees.

Crop	Location	N of Groves	N of Collected Trees
Olive	Tanger	4	141
	Béni Mellal	5	110
	Marrakech	4	120
	Errachidia	5	170
	Meknès	4	116
Citrus	Azilal	5	80
	Meknès	10	90
Almond	Gharb	3	50
	Haouz	5	60
	Meknès	6	70
Total		51	1007

2.2. Sample Preparation

For either serological or molecular detection of *Xf*, 0.5–1 g of the plant tissue (leaf petioles and midribs excised from mature leaves) was recovered from different cuttings, representative of the entire sample.

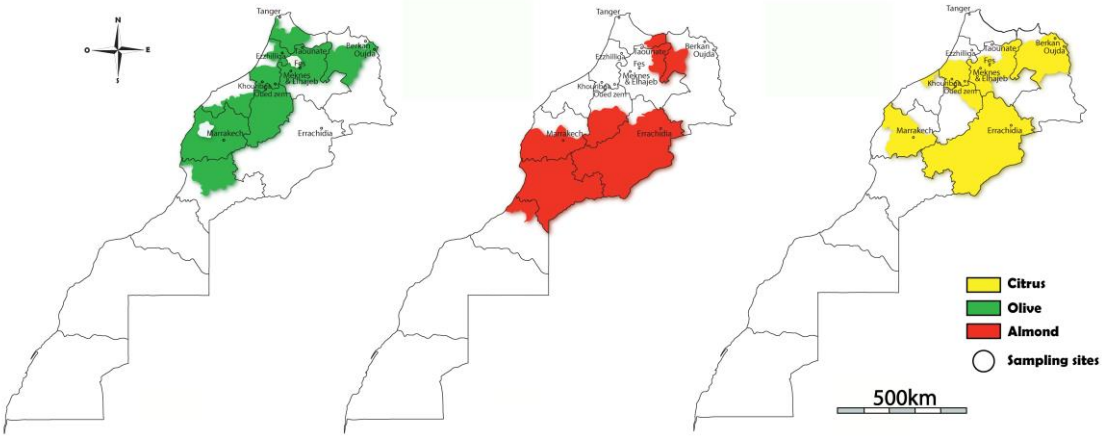

Figure 1. Locations of sampled groves in Morocco during the survey period (March 2020 to July 2021).

Prepared plant tissues were put in extraction bags (BIOREBA, Switzerland) and 5 mL of the extraction buffer was added per bag. Samples were then homogenized using the semi-automatic Homex 6 apparatus (Bioreba, Switzerland) and proceeded according to the extraction protocol developed by Loconsole et al. [24].

2.3. Testing Techniques

2.3.1. Serological Assay

All collected samples were checked by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) kit (Agritest, Italy) using a specific antibody and following the steps below: (i) coating the plate; the 96-well ELISA microplates were coated with 200 µL of anti *Xf* IgG diluted 1:200 in coating buffer and then incubated at 37 °C for 4 h. (ii) Antigen incubation and reaction's development; after washing the plates, samples were loaded onto the microplates and kept overnight at 4 °C, then the alkaline- phosphatase-conjugated-anti *Xf* IgG diluted 1:200 was added and plates were then incubated at 37 °C for 4 h prior to adding the substrate (1 mg/mL p-nitrophenyl-phosphate in diethanolamine buffer, pH 9.8). (iii) Absorbance reading; absorbance was calculated 4 times in a total of 3 h using a microplate reader (BioTek ELx 800 UV, Germany) at 405 nm. Positive reactions were determined if after 120 min the absorbance of a sample became 3 times more than the absorbance of the healthy controls [24].

2.3.2. Molecular Assay

- DNA isolation

DNA isolation was carried out using CTAB buffer [25] in a 2 mL micro-centrifuge tube, 1 mL of homogenized extract was put in and the sample was heated at 65 °C for 30 min and then centrifuged at 16,000× *g* for 5 min. In a new 2 mL micro-centrifuge tube, 1 mL of the supernatant was transferred, being careful not to transfer any of the plant tissue debris. 1ml of chloroform-isoamyl-alcohol (24:1) was added, and the sample was thoroughly blended by agitating and then centrifuging at 16,000× *g* for 10 min, then 700 µL of the supernatant was transferred to a 1.5 mL micro-centrifuge tube where 490 µL (approximately 0.7 volume) of cold 2-propanol was adjoined. After combining by upturning twice, the tube was incubated at −20 °C for 20 min. A further 20 min of centrifugation for the samples at 16,000× *g* allowed for the recovery of a pellet that was washed with 1 mL of 70% ethanol followed by extra centrifugation at 16,000× *g* for 10 min. Samples were vacuum dried, and the pellet was resuspended in 100 µL of DNase/ RNase—free water.

- PCR Primers and Cycling Conditions

For some of the samples that returned doubtful results using the ELISA test, the test for the existence of *Xf* in the DNA extracts was carried out by PCR test, using standard primers RST31/33, which are widely used in quarantine programs [26] for the detection

of the bacterium. PCR reactions were performed in 20 µL final volume adopting 0.5 µL for either forward and reverse primer, 3 µL of total DNA template and 4 µL of 5× GoTaq polymerase (Promega, Madison, WI, USA).

PCR conditions were: one denaturation step at 95 °C for 5 min followed by 35 cycles of: 30 s denaturation at 94 °C, 30 s for annealing at 55 °C and 40 s for elongation at 72 °C. The reaction was finally extended at 72 °C for 7 min and then amplified bands were observed on 1.2% TAE agarose gel. After electrophoresis, positive and negative samples were inspected [18].

3. Results and Discussion

The early detection of *Xf* infections is crucial to the management of this harmful plant pathogen worldwide [27]. *Xf* has been intensely investigated due its relation to devastating diseases, affecting several major crops such as grapevine, olive, coffee, citrus, and stone fruits, besides several forest and ornamental species [28]. The emergence of *Xf* in novel territory and the ineffective containment of its spread in territories where it previously established highlight the necessity to monitor the progress of large *Xf* outbreaks and to develop exhaustive pest management approaches [8]. Countries without current outbreaks, like Morocco, need to monitor their crops frequently because detecting diseases caused by *Xf* in their early stages may reduce the huge losses caused by pathogen later [29].

All gathered samples were evaluated for the existence/absence of *Xf* by utilizing an ELISA commercial kit (Agritest, Italy). The acquired outcomes did not reveal any positive sample. The ELISA examination was performed correctly. Indeed, the positive control provided with the kit reacted positively, while no color modification was noted with the negative control. Loconsole et al. [24] carried out various laboratory experiments, in which the reactivity and the response of various commercially accessible ELISA kits was paralleled, and revealed that the Loewe kit could detected a greater number of known positive samples with reactions happening in a period of two hours, pursuing manufacturer's instructions and employing the controls delivered with the kit. Therefore, this kit has been used widely to check the presence of *Xf* in several countries worldwide including the previous one in Morocco [30]. Furthermore, by using PCR in the present survey, no amplified DNA was acquired from any of the tested samples, validating the absence of the bacterium in our samples. Although, some positive reactions were expected from some samples, which resulted in doubt in ELISA, only positive controls in each PCR test generated the expected 733-bp amplicons. The employed primers (RST31/33) are broadly recognized for the detection of *Xf* in quarantine programs [31], along with other primers targeting the genomic region 16 S rDNA [31,32], which are more suitable for the proper detection of a bigger number of genetically heterogenous strains of *Xf* [20]. These results are taken as favorable proof, taking into account that *Xf* is absent in Morocco, relative to the surveyed tree crops which is consistent with other negative results obtained from different field surveys, recently carried out on the presence of *Xf* in some other countries such as from Jordan [33] and from Lebanon [5]. Nevertheless, recurrent sizeable surveys in various regions and on various potential host plants are required to hinder its ingress into the country [30]. It should be pointed that the attendance of leaf scorch symptoms that were noticed in many cases during the survey might have myriad origins, biotic or abiotic (salty winds, nutrient toxicity/deficiency, drought, frost damage, fungal pathogens, etc.). Although the risk presented this pathogen in variant hosts (maple, plane, oak . . . etc.) still needs to be evaluated, thus, plant health service authorities ought to alert presence of these hosts as well submit an avoidable risk.

4. Conclusions

Findings obtained in this investigation clearly indicated that *Xf*—up to this date—was not found in seven investigated areas (Tanger, Béni Mellal, Marrakech, Errachidia, Azilal, Meknès, Haouz, and Gharb) in Morocco, confirming the results obtained from the previous survey carried out during 2018. These results highlight the importance of control

measures adopted by phytosaniraty services in Morocco that could, up till now, prevent the introduction and spread of *Xf* in the country. Thus, frequent extended surveys in diverse regions and different host species, and the continious and accurate detection of *Xf* by rapid, sensitive, and reliable laboratory tests are required to avoid any entry of this pathogen into the country. Furthermore, sustainable control measures of *Xf* would include but are not limited to: (i) VSPP (voluntary certification program by the concerned stakeholders, mainly those in the nursery sector); (ii) screening more species and cultivars: promising results of the tolerant/resistant species cultivars as the case of Leccino and FS 17 in Italy); (iii) further research on heat treatment of plant propagation material; and, (iv) continuous surveillance and monitoring on vectors.

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