Review

Xylella fastidiosa: An Updated Review

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Abstract: *Xylella fastidiosa* (*Xf*) is classified as a quarantine pest due to its consequences on economically significant crops. Its main form of transmission in Europe is through the insect *Philaenus spumarius*. Due to climate change, populations of insect vectors became more extensive, resulting in the dissemination of the bacteria through longer periods, but destruction of these insects raises issues, due to their role in nature. Upon infection, *Xf* causes the occlusion of xylem vessels by bacterial aggregates, and tylosis production by the plant as a response to infection. Although symptomatic manifestations of Xf are often linked to water stress, a variety of plant species have been found to carry the pathogen without symptoms, making it all too easy to evade detection when relying on visual inspections. Beyond water stress, other conditions (individual plant resistance/tolerance, bacterial concentrations, transpiration rates, and interactions between subspecies) may be implicated in symptom development. A thorough understanding of how this disease develops, especially its capacity to spread from the initial focus and establish a systemic infection, is imperative. This review focuses on the *Xf* infection process, the development of symptoms, its spread within Portugal, and the actions that have been taken to counter it.

Keywords: Phytobacteria; insect vectors; *Philaenus spumarius*; Demarcated Zones; Olive Quick Decline Syndrome; Pierce's disease; almond leaf scorch disease; Phony Peach Disease

1. Introduction

Xylella fastidiosa (*Xf*) is a bacillary (rod-shaped) bacterium about 0.25 to 0.35 µm wide and 0.9 to 3.5 µm long, devoid of flagella [1]. It is an aerobic [2] Gram-negative [3] bacterium that multiplies in the xylem of various host plant species [4]. Since 1981 it has been considered a quarantine pest, on the EPPO A list, for affecting economically important agricultural crops as well as ornamental plants [5].

Xf belongs to the class *Gammaproteobacteria*, order *Lysobacterales*, and family Lysobacteraceae. The genus *Xylella* contains two species, *X. fastidiosa* and *X. taiwanensis* [6]. According to serological and phylogenetic studies, its strains have been divided into six subspecies: *X. fastidiosa* subsp. *fastidiosa* [7], *X. fastidiosa* subsp. *multiplex* [8], *X. fastidiosa* subsp. *pauca* [9], *X. fastidiosa* subsp. *sandyi*

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[10], and *X. fastidiosa* subsp. *morus* [11], of which only the first two have been validly published in the List of Prokaryote Names with Standing in Nomenclature. A further subspecies, *X. fastidiosa* subsp. *tashke* has also been reported once [12], but to date its genotype has not been detected again [13].

Due to their ability to infect a diverse range of hosts, including economically significant crops like grapevine, peach, almond, citrus, plum, and coffee, these organisms pose a threat to plant health and agricultural productivity [2]. Based on spatially explicit economic models, *Xf* presence could, in the next 50 years, have a direct impact on the profitability of olive cultivation, potentially resulting in a loss of revenue of 1.5 to 5.9 thousand million euros. Additionally, there may be severe indirect effects on the cultural heritage and landscape due to this disease [5].

It is imperative to understand the incidence of this bacteria and the corresponding risk factors, which are, to date, poorly understood. It is known that plant bacteria are spread by anthropogenic sources, by agricultural tools, through insect vectors, and by wastewaters, but the way their dissemination reaches agricultural crops is not properly understanded. Thus, this paper aims to provide an updated review of the available literature concerning this pathogen, and clarify the environmental flow of this bacteria.

2. Distribution in Europe

The first outbreak of this plant pathogen in the European Union was found in 2013, in olive trees near the town of Gallipoli, in the province of Lecce, in the Puglia region, Italy [14,15]. Analysis of the genetic of the subspecies found in this region indicates a close relationship to the subspecies of *Xf* present in Costa Rica. This finding suggests that the introduction of the bacteria in the Mediterranean area was likely due to the import of ornamental plants from that region [16]. Subsequently, the bacteria were detected in France (first outbreak in Corsica in 2015, Provence in 2019, and Occitania in 2020) on *Lavandula* spp., on *Myrtus communis* L., on *Salvia rosmarinus* Spenn., and on *Spartium junceum* L. [17]. In 2013, different European countries have reported the presence of infected coffee plants from Latin America (Mexico, Ecuador, Costa Rica, and Honduras) [6], suggesting that the global distribution of this agent continued to increase due to the movement of commodities, and plant materials.

In Spain the first outbreak occurred in the Balearic Islands in 2016 [18,19]. Three subspecies of the bacteria (*multiplex*, *fastidiosa*, and *pauca*) infected more than 20 plant species, including vine (*Vitis* spp.), almond (*Prunus* spp.), olive (*Olea europaea* L.), and fig (*Ficus* spp.) [20].

In December 2020, over 600 olive trees in the Balearic archipelago were found to be infected. By 2021, the total affected area had expanded to 2292 hectares, resulting in the destruction of more than 100,000 almond trees. In 2016, a single olive tree in Madrid tested positive for the multiplex subspecies of *Xf*. That same year, an ornamental plant nursery in Almeria (Andalusia) detected three specimens of *Polygala myrtifolia* L., although the bacterium has since been considered eradicated in this region [16].

3. Current distribution in Portugal

The introduction of *X. fastidiosa* subsp. *multiplex* in Portugal, occurred in 2019 in asymptomatic plants of *Lavandula dentata* L. [21]. Currently, in Portugal there are 18 *Xf* Demarcated Zones (DZ), as shown in Table 1, and 1 suppressed DZ in Tavira, in the Region of Algarve, where the disease was eradicated [22].

Table 1. Demarcated Zones (DZ) in Portugal (adapted from [22]).

Regions	Number of DZ	DZ designations
Northern	7	Oporto Metropolitan Area Sabrosa Alijó Baião

		Mirandela Mirandela II Bougado
Central	8	Castelo Novo Covilhã Fundão Gândaras Marrazes Monte Redondo Penamacor Póvoa de Midões
Lisbon and Tagus Valley	3	Lisbon Metropolitan Area Colares Palmela

These data show that Portugal is highly vulnerable to the emergence of the *Xf*, owing to its Mediterranean climate, which is marked by mild temperatures, frequent rainfall, and high humidity during winter, coupled with hot, dry summers. These conditions are ideal for the growth of the bacterium. The presence of insect vectors and preferred host plants, such as olive, grape, citrus, almond, oak trees, as well as ornamental plants, which are economically significant crops for the Portuguese agriculture, increase the risk of infection [3].

4. Transmission

The transmission of *Xf* occurs primarily through xylem-sucking insects [23]. The bacteria can be transmitted by any insect feeding on xylem, making all such insects potential vectors. Furthermore, the extensive planting of monocultures creates an environment that can facilitate the spread of the disease [16].

Over long distances, the spread of the bacteria is mediated by the transport of infected plants or by infectious insect vectors [2]. The list of these vectors is vast and includes 120 species from 4 families, which belong to the order *Hemiptera* [24], and mainly to the families *Cicadellidae* and *Aphrophoridae* [25].

In Europe, the insect *Philaenus spumarius* (Figure 1) is the most efficient vector for *Xf* [21]. So far, only *Philaenus spumarius* has been proven to transmit the bacterium in natural conditions in the EU. The probability of *P. spumarius* transmiting the bacterium was estimated with a median of 0.13 [26]. Other species in other *Auchenorrhynchan* families or *Cicadellidae* subfamilies have been tested for *Xf* transmission, always with negative results [27]. Some studies have found that some phloem-feeding insects can also acquire the bacteria, as they occasionally feed on xylem to replenish osmotic potential [28,29]. However, according to Cavalieri *et al*. despite being able to acquire the bacteria, they cannot transmit it to plants [30].

According to the latest studies, the list of potential insect vectors is continuously growing. More recently, other insect species, were also identified, in Europe, as competent vectors (such as *Philaenus italosignus*, and *Neophilaenus campestres*) but have only been shown to *possess* the capacity to acquire the bacterium in natural conditions, while their ability to transmit the bacterium to a new host plant still needs to be confirmed [2,26].

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Figure 1. An important vector of *X. fastidiosa* in Europe - *Philaenus spumarius* [21].

The potential presence and spread of *Xf* through seeds have not been extensively studied. However, PCR analysis has detected the bacteria in various parts of the seeds of oranges affected by *Citrus* variegated chlorosis (CVC) [31]. The researchers suggest that the seed coat may act as a reservoir for the bacteria and potentially contribute to the spread of the disease to new areas. However, Dalla *et al*. failed to detect *Xf* in plants obtained from seeds of CVC-affected fruits [32]. Cordeiro *et al*. also failed to detect *Xf* in orange seedlings propagated from seeds extracted from fruits with CVC symptoms. In seedlings of six lemon varieties, they also did not detect the bacteria or observed any of the CVC symptoms. Thus, it has been concluded that *Xf* is unlikely to be transmitted or spread by seeds from fruit of any citrus varieties grown in areas where CVC is endemic [33]. It is worth noting, however, that the studies conducted on the transmission of *Xf* through seeds were limited to small citrus samples. Therefore, further research is necessary to confirm the potential for transmission via seeds and to determine the actual risk of transmission in the field.

5. Hosts

Xf has a wide range of host plant species. The list of known plant hosts of European and non-European isolates is listed in Annex I to Implementing Regulation (EU) 2020/1201 and the plant genera and species identified as susceptible to subspecies of the bacteria anywhere in the world are listed in Annex II to Commission Implementing Regulation (EU) 2020/1201 (RE (UE) 2020/1201, 2020). According to data from EFSA's 2023 report, there are currently 679 plant species that have been identified as susceptible to the bacteria, corresponding to 304 genus and 88 families. In comparison with the previous database, update published in June 2022, 15 new species (and six genus) have been identified as Xf hosts. In the UE, five new plant hosts species were identified in Portugal, three in France, one in Italy and one in Spain [16].

In Portugal, the species *Vitis vinifera* (grapevine), *Olea europaea* L. (olive tree), *Nerium* L. (barley or oleander), *Prunus persica* (peach), *Prunus dulcis* (almond), *Citrus sinensis* (orange), *Quercus* sp., *Vinca* sp. L., *Malva* sp. L., *Sorghum* sp. L., *Catharanthus* sp., *Portulaca* sp. L., *Polygala myrtifolia*, *Westringia fruticosa*, *Acacia saligna*, *Spartium junceum*, *Rosmarinus* sp., *Myrtus comunis*, and *Rhamnus alaternos* are particularly noteworthy [35].

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6. Inoculation of the bacteria by the insect vector

Xf is naturally transmitted from one plant to another by insect vectors belonging to the or-der Hemiptera, mainly cycads, aphrophids and cercopids [21]. Most of the main European insect vectors belong to the Aphrophoridae family, that includes *Philaenus spumarius*, *Philaenus italosignus*, and *Neophilaenus campestris* [4,30].

The adult *Philaenus spumarius* (Figure 1) is a small insect, measuring between 5.3 and 6.9 mm in length, and displays a wide range of dorsal coloration patterns. Both nymphs and adults of this species feed on crude sap, which is low in sugar but high in water, amino acids, and mineral salts found in the xylem vessels. They use their modified mouthparts, the stylet, to access the sap, and it is here where the bacteria Xf can attach [36]. While feeding in the nymph stage, the insect secretes a mass of foam that serves as protection from predators and desiccation. This foam production begins within minutes of feeding and is produced from a fluid originating from the abdomen, along with a surfactant secreted by the epidermal glands of the seventh and eighth abdominal segments [37].

Based on their biological cycle (Figure 2), these insects spend the winter as eggs. After a diapause period of approximately 100 days, the eggs hatch in early spring. The nymphs then progress through five developmental stages over a period of 5-8 weeks, during which they remain covered by a protective mucilaginous foam [38]. Adults typically begin to emerge in April or May and start mating during early summer, after which they tend to remain in the surrounding vegetation [39]. During spring, the nymphs can be found in the weeds, while adults are typically found in the canopy from May through the summer. In autumn, the adults return to the weeds within the plot and surrounding areas, or to other plants in the vicinity [23].

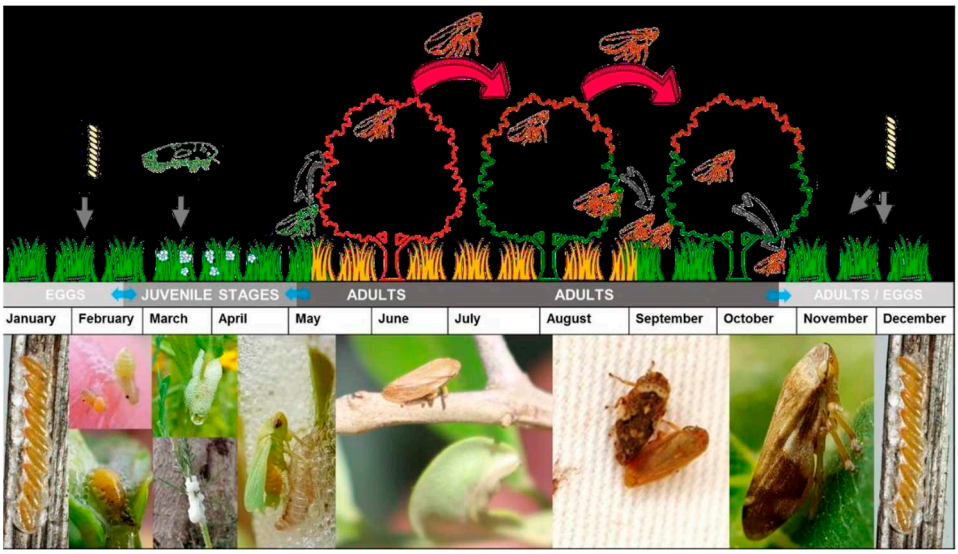

Figure 2. Biological cycle of *Philaenus spumarius* in Italy [23].

The insects acquire the bacteria by feeding on infected plants, and will subsequently host the bacterial cells themselves. They then proceed to release the pathogen into the transport system of host plants by inserting their stylet into the leaf petiole [3], from where the bacteria will spread to the xylem of the branches and stem [4]. Transmission of Xf does not re-quire an incubation period in the vector. The bacterium is persistently transmitted [21] by both nymphs and adults.

Once they have fed on the xylem of an infected plant, insects are able to immediately transmit the pathogen to healthy plants [6]. However, it has been discovered that nymphs lose their infectivity after ecdysis, or molting. During this process, there is an exchange of the buccal armor where the bacteria are attached, and they are subsequently eliminated [21,38]. To reacquire the bacteria, the new adults will need to feed on an infected plant.

When a vector feeds on an infected plant, the process of bacteria adhesion to the insect occurs. This process is directly regulated by the expression of the *rpfF* gene, which induces the synthesis of a diffusible signaling factor (DSF) [40] which, when detected by other bacterial cells, induces the

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expression of adhesins [41]. These adhesins are indispensable for the adhesion of the pathogen to the insect, and for the formation of a biofilm inside its body. Newman et al. found that bacteria with mutations in the *rpfF* gene are unable to produce DSF, which prevents biofilm formation on the insect vector, decreasing the bacteria's ability to colonize it. As a result, the transmissibility of the bacteria is reduced [42]. Killiny et al. found that, once established in a biofilm inside the insect, the bacteria are able to remain viable [41]. Additionally, both nymphs and adults, can retain the pathogen for several months after acquisition, allowing *Xf* to spread to plants far from the original infection, mostly by anthropogenic influences [40]. To accomplish this, *Xf* secretes a chitinase that is capable of digesting the inner surface of the insect vector's anterior gut [41,43]. Once an infected vector feeds, the bacteria detach from the foregut surface to enter the xylem of the plant. According to Killiny *et al*. the turbulence caused by ingestion is adequate to partially disaggregate the bacterial biofilm so that free cells can be injected into the plant [43].

Redak *et al*. also reported that the transmission process is highly efficient, as less than 200 viable bacterial cells in the gut of the vector are sufficient for producing infection [44]. Rapicavoli *et al.* also suggest that *Xf* can prevent the initial recognition by the plant, thereby delaying the triggering of the plant's immune response [45]. This may explain the effective way in which the bacteria establish themselves in their plant hosts.

7. Bacterial action in the xylem

The development of the disease in plants will now primarily depend on the bacteria's ability to move from the point of inoculation and establish a systemic population in the infected plants [3]. After inoculation, the bacterial cells multiply, forming a biofilm [3] composed bacterial cells, secreted nucleic acids, proteins, and exopolysaccharides (EPS) that can completely plug xylem vessels, blocking the transport of water and mineral salts [46].

After the local bacterial concentration increases, so does DSF, inducing as seen earlier, the expression of adhesins, with the subsequent formation of the first colonies on the inner walls of the xylem. During the formation of the first colonies, cell aggregation is controlled by a two-component regulatory system known as the phoP/phoQ system. This system can respond to the relatively harsh environment of the xylem, particularly to acidic pH, by inducing adaptive changes and protective phenotypes in the pathogen. These changes include the formation of cell aggregates that are better enabled to cope with environmental stress [47].

The synthesis and secretion of exopolysaccharides and biofilm are further under the control of the *Gum* genes [48]. According to Killiny *et al*., bacteria bearing mutations in this gene have impaired movement within the host plant [49]. On the contrary, mutants with the negative genetic regulator of *Gum*, PD1671, display a hypervirulent phenotype [50].

Biofilms are recognized for their ability to enhance the resistance of bacteria to stress and treatment. In the case of *Xf*, the formation of cell aggregates in biofilms, along with the plant's production of tyloses to isolate the pathogen, can contribute to its persistence and spread [51] causing occlusions in the vessels and decrease of water transport to the leaves, thereby compromising photosynthesis and transpiration rates [52].

8. Movement and distribution of the bacteria in the plant

As the xylem is responsible for transporting water from the roots to the leaves, *Xf* is capable of spreading along the vasculature, even against the flow of sap. This allows the bacteria to move effectively throughout the plant and cause systemic infections [4]. In this process, *Xf* needs to cross the xylem cells through the existing xylem pores (PMs). PMs, ranging from 5-20 nm in diameter, are composed of hemicellulose, cellulose microfibers, and pectins, and connect adjacent plant cells, forming a porous structure. They function to limit the passage of bacteria and air bubbles, protecting plants from embolisms [2]. The body size of *Xf* cells, on the other hand, is in the range 250-2400 nm [1]. It has been proposed that the induction of various cell wall degradation enzymes [52] allows for an increase in the pores between adjacent xylem vessels, thereby enabling bacteria to traffic from one vessel to the next [42]. Sun *et al*. also reported degrading activity in the cell wall of xylem membranes

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in infected vines, and increased risk of embolisms [52]. Montillon *et al*. revealed that the average proportion of occlusions in the varieties Leccino and Cellina di Nardo indicates that the bacteria exploited the PMs to spread systemically within the susceptible varieties. In these varieties, a clear degradation of the middle lamellae was observed, which allowed the bacteria to pass through. In contrast, this phenomenon was not observed in the resistant variety Leccino, which had intact lamellae [2]. During infections, decomposition enzymes produced by bacteria can degrade the components of the PMs, leading to an increase in their porosity.

This allows the bacteria to displace and diffuse along the xylem vasculature, which is a characteristic of their pathogenic virulence [53]. This fact may explain why some subspecies show higher virulence. As described by Chatterjee *et al*. virulence **in** Xf is associated with characteristics that allow it to move within and between xylem vessels [54]. Ionescu *et al*. linked the virulence of the bacteria to an environment with low pectin content and low DSF. This inhibits the adhesion of *Xf* to xylem walls, allowing it to spread rapidly without biofilm formation [55]. Benedictis *et al*. and Cardinale *et al*. reported notable differences in the distribution of occlusions between different twigs due to the erratic mode of colonization of the bacteria. Tyloses were found as responsible of the occlusions in twigs from older plants [51,56]**, w**hereas Montilon *et al*. reported that the occlusions observed in younger plants were composed of structures that are characteristic of an early event in the mechanism of pathogenesis [2]. Also, Lima *et al*. found that, in coffee bushes, the bacteria was distributed throughout the plant, confirming its downward translocation. Leite *et al*. observed that in plum plants, high concentrations occurred in the aerial part. Almeida *et al*. and He *et al*. also verified that the bacteria showed ascending and descending translocation, being found in the roots of citrus plants inoculated in the aerial part [57,58]. Cardinale *et al*., meanwhile, reported a low concentration of bacterial cells in vascular occlusions of Ogliarola and Salentina stems and a higher presence of bacterial aggregates in leaf petioles [56]. These variations may be related to the size of the xylem pores, which enlarge in diameter from the top of the branch downwards. As a result, the majority of the hydraulic resistance is concentrated in the lower portion of the branches, and pathogen invasion in the narrower vessels is less significant [59]. When examining the presence of *Xf* in embolized vessels, a smaller number of bacterial cells were found in the basal vessels, compared to those found in vessels of the apical part of the plant. This suggests that the bacteria have a functional preference for aerobic respiration [60]. As air bubbles are filtered through the small membrane pores at the top, the basal area of the plant is less oxygen**-**rich. That causes an accumulation of bacteria in the apical zones [61]. The age of the plant could also impact the translocation of the bacteria, with older plants experiencing faster movement due to changes in transpiration and anatomical differences in the constitution of the xylem [62]. Therefore, further research is necessary to determine the spatial and quantitative distribution of the bacteria on host plants and the seasonal dynamics of this pathogen.

9. Symptoms

To date, it is generally accepted that following the infection, water, and nutrient transport is impaired, due to the occlusion of xylem vessels by bacterial aggregates and the production of tylosis by the plant as a response to infection [2]. In infected grapevine and citrus, Goodwin *et al*. and Machado *et al*. found that impaired water and nutrient transport led to a drop-in photosynthesis rate, reduced transpiration rate, and high concentrations of abscisic acid, fructose, glucose, Ca^{2+}, and Mg^{2+}. They also observed low concentrations of Zn^{2+} and K^{2+}. Leaf senescence was associated with chlorosis, high levels of proline and abscisic acid, and increased stomatal resistance [63,64].

These mechanisms induce the emergence of the disease symptoms, from the apical organs to the roots [3]. The symptoms are, in general, associated with manifestations similar to those observed during water stress, as visualized in Figure 3: chlorosis in the marginal zone of the leaves, followed by necrosis with a yellowish halo around them, wilting, burning (necrosis) and, in more serious cases, death of the plant [21]. In some cases, it resembles mineral nutrient deficiency, such as marbling and chlorosis between veins. Depending on the plant species, irregular lignification of the bark, stunting, premature leaf fall, distortion, reduced size, and reduced fruit yield may also occur [6].

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Figure 3. *Xyllela fastidiosa* symptoms in an olive tree (author´s image).

Several important diseases, listed in Table 2, can also be associated with *Xf* infection, depending on the host and the observed symptomatology.

Table 2. Description of symptoms produced by *Xf* according to host plant (adapted from [6]).

Disease	Host	Symptoms
Pierce's Disease (PD)	Vine	Leaf wilting, yellow and red chlorosis with irregular distribution and dieback, green "islands" of healthy tissue and separation of leaf from petiole
Olive Quick Decline (OQD)	Olive	Leaf scorching and rapid decline of aging olive trees with progressive death from apical to root zone
Citrus variegated clorosis (CVC)	Citrus	Yellowish chlorotic spots with irregular borders beginning in the middle part of the crown and spreading over the entire plant
Oleander Leaf Scorch	Oleander	Yellowing of the leaves which is followed by characteristic scorching and necrosis of the apical, and marginal zone of the leaves
Bacterial Leaf Scorch	Oak	Irregular leaf scorching on oak trees, very evident in late summer and autumn, with pronounced apical discoloration with a red or yellow halo between burnt and green tissues, and the veins standing out yellow in apparently healthy areas
Almond Leaf Scorch	Almond	Irregular patterns of leaf necrosis causing leaf scorch leading to a clear decrease in productivity, a progressive mortality from the apical branches and finally death of the affected trees
Phony Peach Disease (PPD)	Peach	Branches with shorter internodes, smaller petiole length and leaf area and, in a more advanced stage of infection, senescence of the more mature leaves occurs, leaving the branch leafless or with a small number of leaves at its apex

10. Absence of symptoms

While in some hosts the infection induces visual changes, there are several species in which these bacteria colonize without causing symptoms [65]. Some authors suggest that in asymptomatic plants, these bacteria eventually die [65]. According to Bragard *et al*., the asymptomatic period after infection

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can vary greatly, ranging from 1 month in ornamental plants, to as long as 3-4 years in some hosts. This extended and variable asymptomatic period can hinder successful detection, especially when surveillance relies on visual inspection [66]. Some authors also mention that the most limiting factor in the manifestation of this disease are the weather conditions. Harsh winters limit the spread of the disease since the bacterium is sensitive to low temperatures [67]. The growth and survival of *Xf* in cell cultures *in vitro* are differentially influenced by extremely low or high temperatures [68]. According to McElrone *et al*., in times of severe drought, the symptoms of infection will be aggravated by increased water stress [69]. Therefore, it is expected that phytopa-thologies caused by the bacteria will increase in response to global climate change. However, some authors argue that occlusions are not responsible for symptomatology. Benedictis *et al*. found a similar number of occlusions in infected branches of Leccino olive trees compared to healthy trees of the same variety [51]. Queiroz-Voltan *et al*. observed variations in symptom severity for the same variety developed under the same soil and climate conditions, cultural treatments, and management [70]. It was concluded that external symptoms of water deficit cannot be solely at-tributed to *Xf* presence or absence. The response and manifestation of symptoms are influenced by various physiological and environmental factors, including differences in plant resistance or tolerance, varying concentrations of the bacteria, different transpiration rates, and occlusion capacity between plant subspecies. Hopkins *et al.* and Kadel *et al*. found that less infectious subspecies of the bacteria protects vines from more aggressive subspecies while showing decreased symptom manifestation [71,72].

11. Disease control measures

Undoubtedly, *Xf* is an emerging pathogen, and one of the most dangerous pests, with no available treatment. Eradicating the epidemic in an early stage has been successful through removal of infected plants when the bacteria was only sporadically detected. Currently, there are several attempts to control this disease, including implementing control measures on infected plants and using more sophisticated molecular methods. These control attempts can be grouped into four categories, depending on the target:

a) Control of infected plants;
b) Use of tolerant cultivars;
c) Use of products that affect bacterial development;
d) Control of insect vectors.

11.1. Control of infected plants

Currently, several efforts are being made to develop control measures to restrict the spread of this bacteria, by controlling infected plants. In the European Union, quarantine measures are regulated by Regulation (EU) No. 2016/2031 and phytosanitary measures, to prevent the introduction and spread of the bacteria within the European territory [73].

In Italy, the legislation mandates the division of the southeastern region into three areas, to enhance control of the disease. The infected area, where the disease is prevalent and cannot be eradicated, is designated as a "containment area" where infected plants must be uprooted. Within the "buffer area" (50 m radius around the infected tree), the uprooting of all surrounding plants is required [74]. In Portugal, following the guidelines stipulated in Implementing Regulation (EU) 2020/1201 and Regulation (EU) No. 2016/2031, once the presence of the bacterium is confirmed, measures must immediately be taken to prevent its spread and guarantee eradication [34]. In order to ensure the implementation and compliance with such measures, the national phytosanitary authority (DGAV), under Decree-Law No. 67/2020, of September 15th, establishes the demarcated zone, the measures for eradication of the bacteria and the restrictions on the movement of plants intended for planting in the Infection Zone and Buffer Zone [75,76].

Thus, as soon as an infected plant is found, a Demarcated Zone (DZ) is immediately established. A DZ (Figure 4) comprehends the Infected Zone (IZ) (including all susceptible plants within a 50 m radius around contaminated plants) and a surrounding Buffer Zone (BZ) (that includes all susceptible plants species within a 2500 m radius around the infected plant). In this Demarcated Zone (IZ+BZ

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total of 2550m radius around the infected plant) the following measures are established [21]: *In situ* destruction of the infected plants, as well as of others of the same species; *In situ* destruction of all plants of the species listed in Annex I and II in Implementing Regulation (EU) 2020/1201; A ban on planting in the Infected Zone of plants susceptible to the subspecies of the bacteria found in the demarcated area concerned, except under officially approved conditions of physical protection against the introduction of the bacteria by insect vectors; Prohibition of movement out of the Demarcated Zone and from the Infected Zone into the Buffer Zone of any plant, intended for planting, susceptible to the subspecies of the bacteria detected in the demarcated area; Prohibition of commercialization, in the Demarcated Area, in fairs and markets of any plant, intended for planting, susceptible to the subspecies of the bacteria detected in the demarcated area.

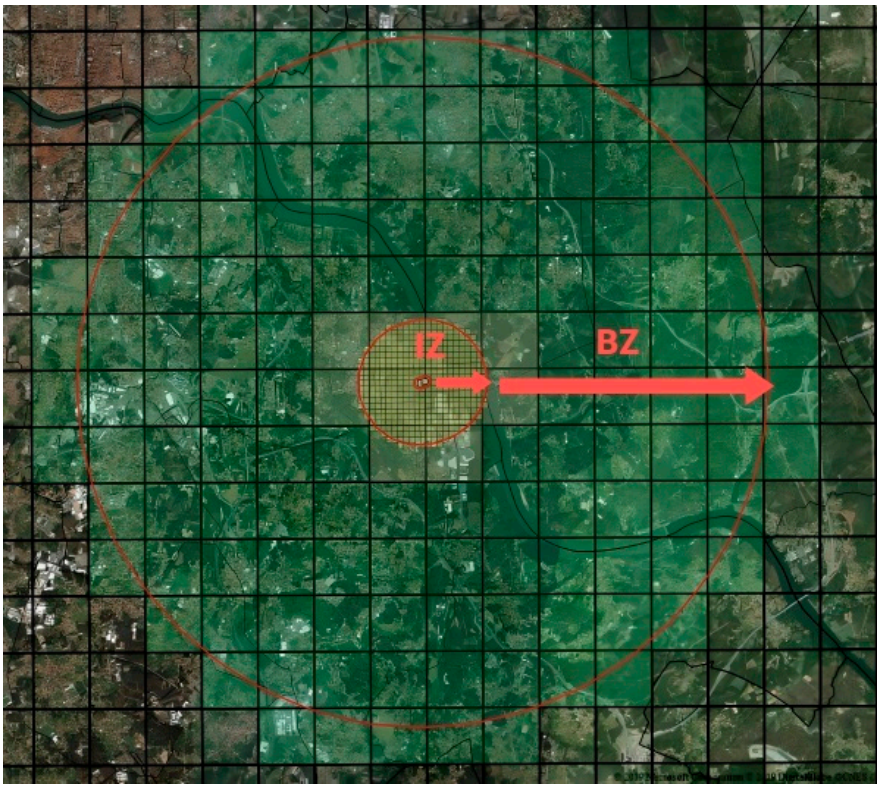

Figure 4. Demarcated Zone, which comprises the Infected Zone (IZ) 50m radius around infected plant and Buffer Zone (BZ) 2500m radius around infected plant (Adapted from [21]).

Pereira *et al*. identified key strategies for controlling the spread of insect vectors, including limiting the mobility of host plants, establishing safety barriers (buffer zones), and implementing mandatory certification and a phytosanitary passport for nurseries transporting plants between internal borders [3].

Aside from destroying and uprooting, pruning has also been experimented as a way to control the disease. Since the bacteria typically moves from branch terminals to the plant's trunk, pruning can eliminate the bacteria and provide temporary protection from reinfection by the vector. Furthermore, it can encourage the growth of new, uninfected branches. This method has already been tested in oleanders [77] citrus [78] coffee [79] grapevine [80] [81], almond trees [82] and olive trees [15]. However, according to *Bucci et al*., there is no conclusive evidence on the effect of pruning in containing any of the diseases caused by *Xf* [83].

Another method was proposed from early observations of the effects of frost on grapevines affected by Pierce's Disease. Feil *et al*. found that the bacteria are sensitive to low temperatures namely when infected grapevines were exposed to temperatures be-tween -8°C and -12°C [84]. Subsequently, a protocol for cold treatment was tested with promising results on 'Pinot Noir', Sauvignon, and 'Cabernet' vines affected by PD [85]. These authors took various varieties to four field sites in California with different winter temperatures to create a mathematical model for cold as a curative

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of vines infected with *Xf*. They found a direct correlation between control efficiency and cold locations. This simulation could help vineyard owners make management decisions regarding PD by choosing better geographical areas. However, the model is not yet validated, making pre-dictions or diagnosis speculative. Furthermore, it is uncertain if the results can be extend-ed to other plants since Amanifar *et al*. were unable to stop the infection by replicating this technique, and the bacteria survived in the roots of infected almond trees [86]. Moreover, the physiological/biochemical mechanism that underlie cold therapy is poorly under-stood. Identifying the factors responsible for eliminating *Xf* with this method is crucial to replicate them in other plants, and certificate their potential as a new approach to control the disease.

11.2. Use of tolerant cultivars

Another tested methodology involves screening for cultivars that are resistant and tolerant. The main concept is to study crop varieties that are more resistant, similar to the approach that has been used in the past for various pathogens. Some promising results have been achieved for grapevines [87] citrus [88] and olive trees [89]. However, it is important to note that tolerance to bacterial infection can diverge over time within the same plant, as it is influenced by intrinsic differences in structure, functional relationships, and plant response/defense mechanisms. Additionally, substituting one cultivar with another may not always be feasible without affecting the final product. Some plant varieties have shown resistance or tolerance to the bacteria in various studies. For instance, Sun et al. found that resistant grapevine varieties had a lower degree of xylem occlusion (20%), whereas susceptible varieties had a higher rate of occluded vessels (up to 60%) [52]. The list of tolerant and resistant plant genus and species is already reported. It is found that tolerant/resistant status is available for 72 plant species (with a total number of 713 records). The most studied genera are Vitis, Citrus and Prunus (417, 175 and 58 records, respectively), confirming the important economic value of these plant species [16]. Other investigations have been carried out in this context. In 2022, Surano et al. used electron microscopy to observe that Leccino olive trees exhibited greater resistance to infection symptoms compared to the Cellina di Nardo variety [46]. According to Petit et al. the symptoms of OQDs varied significantly among olive varieties. The study found that the less resistant varieties were less effective in producing tilosis, which enabled the bacteria to move within the vessels [4]. Montillon *et al*. found higher sensitivity in Salentina and Cellina di Nardo olive trees compared to Leccino varieties due to the presence of occlusions containing tyloses, gums, and pectin. However, no bacterial cell aggregates were detected [2]. Similarly, Cardinale *et al*. reported a low concentration of bacterial cells in vascular occlusions of the stems of Ogliarola and Salentina [56]. Benedictis *et al* found a higher number of occlusions in Cellina di Nardo and Ogliarola Salentina olive trees compared to the resistant variety Leccino [51]. Montillon *et al*. found higher sensitivity in Salentina and Cellina di Nardo olive trees compared to Leccino varieties due to the presence of occlusions containing tyloses, gums, and pectin. However, no bacterial cell aggregates were detected [74]. Mauricio *et al*. evaluated field resistance to *Xf* in 264 hybrids *of Citrus reticulata* × *Citrus sinensis* and pear orange. Non-infected plants were grafted with *Xf*-infected grafts. The authors observed that most hybrid progenies did not show symptoms of citrus variegated chlorosis (CVC) or detectable levels of the bacteria, while all pear orange seedlings were infected and showed CVC symptoms. The authors suggest that certain genes may be responsible for the hybrids' resistance to *Xf*, as their expression was significantly higher in the hybrid progenies [90]. These are promising studies that highlight the need to develop assays to test for tolerance to the bacteria.

11.3. Use of products that affect the development of the bacteria

In vitro and field studies have tested various chemicals, such as antibiotics, metal compounds, and natural products, to prevent infection. Benzothiadiazole, tested on tobacco plants, was found to be ineffective. Conversely, N-acetylcysteine, used as a fertilizer by Muranaka *et al*., showed promising results in improving symptoms, potentially due to its impact on bacterial biofilms [91]. Lacava *et al*. reported the in vitro antibiotic susceptibility of many *Xf* subspecies [92]. According to Amanifar *et al*., tetracyclines were found to be effective when injected into infected almond trees [86]. However, the

use of antibiotics in plants has been little studied and remains largely unknown. The use of antibiotics, when mixed with other agrochemicals, can promote a faster development of antibiotic resistance. This, associated with the consumption of raw food, can lead to the increase of antibiotic-resistant bacteria in humans and can cause a major public health problem, as referred by the "One Health approach" since it has human, animal and environmental impacts.

Dentamet, consisting of zinc (4%), copper (2%), and citric acid, has been widely evaluated as an effective treatment. Girelli *et al*. treated both resistant and susceptible olive trees with this biocomplex, obtaining significant modifications in the leaf metabolic extracts, such the increased of oleuropein. This is an important compound for plant protection and resistance against pathogens. The treatment also induced mannitol accumulation in leaves in response to infection, facilitating osmotic regulation. Additionally, endotherapy with Dentamet promoted the release of copper and zinc ions in the foliage, actively promoting the synthesis of the auxin hormone that stimulates plant growth [74]. Blonda *et al*. replicated the use of this complex. After spraying on olive tree canopies once a month from spring to early fall, he found that this fertilizer was able to provide relevant systemic activity, reducing both disease symptoms and the concentration of *Xf* cells inside the leaves [93]. However, Muranaka argues that bacterial biofilm formation is enhanced with these antimicrobial treatments [94]. The application of copper treatment leads to an upsurge in the prevalence of persistent cells within the biofilm. These bacterial cells exhibit suppressed metabolism and activity, enabling their survival in harsh environments and facilitating their transition into the persistent state [95,96].

Further *in vitro* investigations demonstrated that cecropin B (CB) exhibited bactericidal properties against multiple phytopathogenic bacteria, such as *Erwinia* spp., *Xanthomonas* spp., *Pseudomonas* spp., and *Clavibacter* spp. Grapevines that were transgenic and expressed cecropin B had only mild symptoms of infection when inoculated with *Xf*, and the bacteria spread slowly. The microbial activity and size of *Xf* colonies were reduced due to the decreased activity of CB [97].

In addition to antibiotics, metals, studies have also been reported, testing some natural substances produced by plants in response to *Xf*. Aldrich *et al*. and Maddox *et al*. reported an *in vitro* inhibitory activity of some compounds such as polyphenols, azadirachtin A, hesperidin (to a lesser extent), and radicinin [98,99] on the bacteria.

Azevedo *et al*. and Dourado *et al*. showed that certain endophytic microorganisms could reduce the virulence of *Xf* by competing with the pathogen or secreting substances that can modulate its virulence [100,101].

In a different line of research, Ahern *et al*. and Das *et al*. used specific phages capable of lysing *Xf in vitro*. However, their use in the field has not been evaluated [102,103]. Bacaari *et al*. investigated the effectiveness of endophytic bacteria when introduced into vines via stem punching. This method led to significant reductions in disease severity, indicating that these biological agents can reduce disease by inducing the expression of disease resistance. The strain used showed high efficacy in controlling Pierce's disease and can be easily applied through spray treatment as an eradication measure [104].

Research on essential oils (EOs) has also shown to be potentially useful in controlling this pathogen, as their efficacy against a wide range of pathogens and pests has been confirmed in vitro by several authors [105–107]. Santiago *et al*. investigated the action of sandalwood and patchouli essential oils and obtained promising results, since the oils exhibited antibacterial activity and could, therefore, be potentially used as natural sources for developing new pesticides [107]. Montesinos *et al*. tested the efficacy of eucalyptus essential oil against 11 phytopathogenic bacteria belonging to six different species. The study found that all phytopathogenic bacteria were susceptible to the oil, with *Xf* and *Xanthomonas fragariae* being the most affected. The bactericidal effect was particularly strong, with a lytic effect observed in three subspecies of *Xf* used in the study [108].

The application of the plant growth regulator abscisic acid (ABA) to infected 'Pinot Noir' and 'Cabernet Sauvignon' grapevines was described, including a foliar application. Pinot Noir vines treated with ABA showed a significant increase in the production of xylem sap phenolic compounds and healing effects when compared to control plants. The results demonstrated a positive correlation between ABA treatment and xylem sap phenolic compounds, indicating the antimicrobial properties of this compound [109].

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11.4. Control of insect vectors

In Portugal, a group of methods which involves the application of plant protection products that ensure safety for human health and the environment has been used for the control of insect vectors. Recently, an extraordinary authorization has been granted for the application of plant protection products containing acetamiprid, rape seed oil, and orange oil, which are expected to be effective for controlling vector insects [110]. According to Altamura *et al*., acetamiprid is highly toxic against *Philaenus spumarius* [111]. However, some authors suggest that this neonicotinoid does not have a significant impact on bacterial inoculation, as the vectors treated with this insecticide showed less vulnerability to it compared to other insecticides [38]. Bethke *et al*. also reported the effective insecticidal action of a neonicotinoid in reducing vector insects in California [112]. However, overuse of contact insecticides leads to the development of resistance in many pest species, and suppression of natural enemy populations [39]. According to Carolo, citrus oil showed a good effect when applied in high volume. However, it only worked on insects that were in nymphal states [113].

According to Dongiovanni *et al*. the use of orange oil significantly reduced the number of nymphs, indicating its potential to control juvenile populations [114]. However, its effectiveness may be limited to nymphs present in herbaceous plants and weeds, and it may not be usable in areas where the control of undergrowth plants is challenging or when the insects are in the adult stage. Additionally, Domenico *et al.* found that *P. spumarius* males and females were attracted or repelled by different concentrations of the same oil [115]. Research conducted by Lago *et al*. demonstrated the potential of using kaolin to serve as a protective barrier against insects, such as *Homalodisca vitripennis*, that can cause disease progression. The use of kaolin is known to repel the insects and reduce oviposition, leading to death. Furthermore, natural predators like birds and small lizards have been observed preying on *Cicadellinae* nymphs and adults while larvae coccinellids and lacewings attack postures. Taking this into consideration, it appears that an appropriate timing for testing new formulations as well as determining the volume of grout and number of applications may be necessary to construct an effective integrated pest management strategy that is also sustainable [116].

In the French Polynesia, Hoddle *et al*. tested the release of natural enemies of insect vectors, namely *Gonatocerus* sp. parasitoid. eggs. In this trial they observed that after 7 months the vector population decreased by 95% [117–119]. They also explored the isolation of specific viruses capable of decreasing bacterial adhesion to the insect, being potentially useful as biopesticides.

12. Conclusions

Understanding *Xf*, its vectors, and their relationship with the plant hosts, as well as recognizing symptoms in different hosts, is crucial to obtain a sustainable protection of plants. This review discusses the infection process, symptoms development, current distribution in Portugal, and actions taken to control the disease. Multiple solutions should be followed to reduce infection, namely reducing insect vectors, and using resistant plant varieties, are mentioned. The most effective control methods involve a combination of approaches, such as cultural measures and removal of host plants and insects. However, due to climate change, the populations of insect vectors become more extensive, resulting in the consequent dissemination of the bacteria through longer periods. The destruction of these insects raises the question regarding their role in nature. Simultaneously there are no specific products against them. Insects are the center of trophic chains, maintaining and regulating the population of most plants through pollinations and phytophagy, and, among other functions, are also involved in the recycling of organic matter. Therefore, insects are fundamental pieces for the maintenance of life. There is a need to deepen the knowledge about the consequences and effects of the decrease of these insects in the ecosystem.

Methods based on the introduction of endophytic microorganisms into the interior of plants, have also been reported. One of the main benefits caused by endophytes in the host plant is the promotion of growth. Simultaneously they can do a biological control diminishing or preventing the deleterious effects caused by pests and phytopathogens, reducing the use of pesticides. The literature studied indicates that endophytic microorganisms have an important role in plant development, so that such research leads to a promising future for agriculture and vegetable

cultivation. There is a need to deepen the knowledge regarding these methods, since it was observed that they have an inhibitory effect on *Xf* development.

Despite promising results in its control, *Xf* continues to spread and impact Europe's landscape, society, and cultural heritage. It is essential to have a clearer understanding of the interaction between the host plants, the pathogen and the favorable environment and establish the epidemiological significance, at a national level, of the infected plants that do not show symptoms and that present normal development. The detection of several species of asymptomatic plants shows the difficulty of knowing the time of infection. And because of that, these plants can be hosts of this bacterium without it causing them any damage. This fact raises the question "how long can these plants live with *Xf* without any damages?". At the moment, the control of this bacterium is carried out by applying the measures of EU Regulation No. 2016/2031, which consist in the destruction of infected plants. Considering the variability of plant responses to infection, it is necessary to implement these measures to effectively reduce the risk of spread. The non-existence of symptoms in bacteriologically positive plants may result, within the national survey, in the existence of false negative results that can cause the dissemination of the bacteria. Likewise, obtaining systematically negative samples can lead to underestimation of the expansion of the disease and cause its dispersion.

It is important to note thar the symptoms of the disease are severe, causing high plant mortality. This fact entails significant economic losses, for producers and consequently for our country. Urgent action is needed, including the creation of knowledge networks and research institutes to facilitate knowledge transfer and develop sustainable solutions for different crops, soil, and climate conditions.

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