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Is *Xylella fastidiosa* a serious threat to European forests?

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Abstract

The recent emergence of Olive Quick Decline Syndrome in Italy, caused by *Xylella fastidiosa*, has drawn attention to the risks posed by this vector-borne bacterium to important crops (especially fruit trees and grapevine) in Europe. Comparatively very little is known on actual and potential impacts of this pathogen in forests, in the native (North American) and introduced (European) regions, respectively. The present review aims to address important questions related to the threat posed by *X. fastidiosa* to European forests, such as: What are the symptoms, hosts and impact of Bacterial Leaf Scorch caused by *X. fastidiosa* on trees in North America? Which forest tree species have been found infected in the introduction area in Europe? How does *X. fastidiosa* cause disease in susceptible hosts? Are there any *X. fastidiosa* genotypes (subspecies and sequence types) specifically associated with forest trees? How is *X. fastidiosa* transmitted? What are the known and potential vectors for forest trees? How is disease affected by vector ecology? Is the distribution of *X. fastidiosa*, especially strains associated with trees, restricted by climatic factors? Is disease risk for trees different in forest ecosystems as compared to urban settings? We conclude by pointing to important knowledge gaps related to all these questions and strongly advocate for intensification of research about the *Xylella*-forest pathosystem, in both North America and Europe.

Introduction

The recent epidemics of Olive Quick Decline Syndrome (OQDS or CoDiRO) in Apulia, southern Italy has attracted much attention to *Xylella fastidiosa* in Europe (Saponari *et al.*, 2013; Almeida 2016). This

bacterium, previously only known from the Americas, its putative area of origin, has a very wide host range, reaching 650 plant species in 82 different plant families (EFSA 2018a, EFSA 2018b). Aside olive trees, it is considered a major threat to fruit crops such as grapevine, citrus, coffee, peach or almond (Rapicavoli *et al.* 2018, Sicard *et al.* 2018, EFSA 2018b, 2019). Comparatively very little is known about *X. fastidiosa* on forest trees, although the seminal work leading to the first description of the bacterium included strains isolated from American elm (*Ulmus americana*), American sycamore (*Platanus occidentalis*) and northern red oak (*Quercus rubra*), showing symptoms of what is now known as Bacterial Leaf Scorch (BLS) of shade and landscape trees (Wells *et al.* 1987; Lashomb *et al.* 2002, Sinclair & Lyon 2005, Seraldi 2007; Harris and Balci 2015).

Historically, *X. fastidiosa* was first associated with Pierce's disease (PD) of grapevine, a decline disease which has caused considerable damage in American vineyards from the late 19th century. The etiology of the disease remained unexplained for almost one century until the causal bacterium could eventually be cultured (Davis *et al.* 1978). It was later shown that strains causing PD formed a homogeneous group with other insect-vectored xylem-limited bacteria which had similar morphology and nutritional requirements (e.g. difficult to grow on synthetic media, hence "fastidiosa") and were associated with a range of diseases on various hosts, such as phony disease of peach or leaf scorch of almond. Based on DNA hybridization studies, serological and biochemical analyses, these bacteria were described as a new species in a new genus (Wells *et al.* 1987). *X. fastidiosa* was the first non-viral plant pathogen to have its complete genome sequenced (Simpson *et al.* 2000). Extensive samplings and phylogenetic analyses in the last decades have shown that *X. fastidiosa* is a genetically diverse species, which has been divided into several subspecies (EFSA 2018a, 2019; Denancé *et al.*, 2019). *X. fastidiosa* diseases have been reported mainly in regions with Mediterranean, tropical, and sub-tropical climates (EPPO 2019, EFSA 2018a, b, 2019). However, the bacterial leaf scorch of trees has been reported throughout the Eastern half of the United States, including in North-Eastern areas with cold winters (Lashomb *et al.* 2002).

The catastrophic OQDS epidemics in Apulia prompted intensive surveys in Italy and other European olive-producing countries. These surveys revealed the presence of *X. fastidiosa* in mainland Spain and the Balearic Islands, Tuscany, Italy, Portugal (as transient), Germany (outbreak considered as eradicated) and France, but generally on species other than olives (EPPO 2019, EFSA, 2019). For example, a large diversity of plants, including some trees (*Acer pseudoplatanus*, *Quercus ilex* and *Q. suber*) were found infected in natural settings in the island of Corsica (Denancé *et al.* 2017). Contrary to the Italian situation, with only one strain recently being associated with OQDS (Saponari *et al.*, 2019), a greater genetic diversity was found in *X. fastidiosa* strains in France and in Spain. The strains identified in these countries were of different sub-species, suggesting several introduction events (Denancé *et al.* 2017, Landa *et al.* 2020). In France, the date of introduction of *X. fastidiosa* was estimated between 1985 and 2001, by using an epidemiological model applied to disease data collected in Corsica (Soubeyrand *et al.* 2018). *X. fastidiosa* seems therefore well established in Europe at least in Corsica, Southern Italy (Strona *et al.* 2017), and the Balearic Islands (Moralejo *et al.* 2019).

So far, *X. fastidiosa* has been mainly seen as a threat to agriculture and horticulture, due to the severe diseases it causes on high value fruit crops (EFSA 2015, 2019) and, to a lesser extent, to ornamental plants and trees (Lashomb *et al.* 2002; Baldi & LaPorta 2017). No serious impact of *X. fastidiosa* has been reported in forests in its native range. However, the recent emergence of OQDS in Italy while little damage had been reported on olive trees in California (Krugner *et al.* 2014), illustrates that the impact of introduced strains of *X. fastidiosa* in new areas may be hardly predicted from what is known in other areas. The introduction and establishment of *X. fastidiosa* in some areas in Europe therefore raises the question of its potential future impact on European forests. In complement to previous general risk assessments (EFSA 2015, 2018b, 2019), the present review aims to address this question by: (1) providing an up-to-date and comprehensive summary of what is known about *X. fastidiosa* on forest trees, both in the native area (bacterial leaf scorch disease in North America) and in Europe; (2)

synthetizing the current understanding of the ecology, biology and genetics of *X. fastidiosa* and the epidemiology of diseases it causes, acquired on other pathosystems and most relevant to assess the threat to European forests; (3) identifying the knowledge gaps to assess the threat posed by *X. fastidiosa* to European forests and suggest research perspectives and practical recommendations.

1. Vulnerability of forest tree species

1.1 What are the symptoms of Bacterial Leaf Scorch of forest trees in North America?

In North America, *X. fastidiosa* has been associated with the disease referred as Bacterial leaf scorch (BLS) of shade and landscape trees (Sinclair and Lyon 2005). The attribution of the symptoms to a bacterium similar to that causing grapevine Pierce's disease was proposed for the first time in 1980, based on microscopic and immunological observations of symptomatic oak, elm and sycamore leaves (Hearon 1980, Sherald 2007). Koch's postulates, were later verified on red oaks (Chang & Walker, 1988). The symptoms of BLS typically occur as foliar burns or scorches, affecting the margin and interveinal areas of the leaf blade. Quite often, the necrotic area is separated from the green part by a yellow or red colored halo (Hammerschlag *et al.* 1986, Gould and Lashomb 2007) (Figure 1). The symptoms may be confused with drought effects or early senescence which may explain why they had previously been attributed to abiotic or viral causes (Hammerschlag *et al.* 1986, Sherald 2007, Purcell 2013).

Leaf symptoms in susceptible species usually appear in late summer and increase during the fall. In pin oak (*Quercus palustris*), symptoms appear earlier than in other oak species and early defoliation of infected trees is observed (Harris *et al.* 2014). BLS symptoms first appear in a single branch or part of the crown and then spread to the entire crown in several years (Hammerschlag *et al.* 1986, Gould and Lashomb 2007, Sherald 2007) (Figure 1). Parts of the tree affected one year remain so the following year (Hammerschlag *et al.* 1986). Positive detection of the bacterium in a tree is frequently associated with positive detection the following year (Barnard *et al.* 1998, Harris *et al.* 2014). Branches infected with the bacterium generally have a later budburst, with smaller leaves than in healthy trees (Zhang *et al.* 2011). The list of symptoms may also include decrease in fruit production, premature leaf abscission, stunting or reduced growth, decline in vigor, dieback, and eventually death (Sinclair & Lyon 2005; Hartmann *et al.* 1995; Barnard, 2009). Crown decline has been especially reported in pin oak and northern red oak, *Q. rubra* (Harris *et al.* 2014). In the same area, the distribution of the disease seems random, with some apparently healthy trees co-existing with highly symptomatic trees (Gould and Lashomb 2007).

In oaks, symptoms of bacterial leaf scorch are similar to those of wilting caused by *Bretziella* (*Ceratocystis*) *fagacearum* in North America ("oak wilt"). However, in the latter case, they appear earlier in the season and tree wilt occurs in a few months (Gould and Lashomb 2007), while decline of BLS-infected trees occurs over several years, often 5 to more than 10 (Hartman *et al.* 1995). In addition, unlike *Ceratocystis* wilt, trees with BLS do not exhibit brown or black stripes in the functional xylem (Sherald 2007). Leaves with browning (BLS) often remain on the tree (Sherald & Kotska 1992, Sinclair & Lyon 2005).

Figure 1 Symptoms of Bacterial Leaf Scorch on trees : close-up of leaf symptoms with the typical BLS discoloration on northern red oak (top left), elm (middle left), *Ginkgo biloba* (top right) ; general view of infected trees (pin oaks) showing early leaf senescence and drop (bottom)



1.2 Which forest tree species are affected in the native area (America)?

BLS disease of trees has been reported mostly in association with native American species of oak, elm and sycamore (Lashomb *et al.*, 2002). Oaks appear as an especially susceptible group, with at least 18 oak species reported as natural hosts in the *Lepidobalanus* (white oaks) and *Lobatae* (red oaks) sections (Gould & Lashomb 2007). The latter are the most frequently reported as affected by the disease (Barnard 2009), in particular pin oak and northern red oak (Sherald 2007, Mc Elrone 2008 *et al.*, Harris and Balci 2015). Disease reports on oaks came from many states in the eastern half of the United States: Florida, South and North Carolina, Tennessee, Oklahoma, Kentucky, Missouri, Indiana, Pennsylvania, New Jersey, New York, Washington DC (Sherald & Kotska 1992, Hartman *et al.* 1995, Sinclair & Lyon 2005, Sherald 2007, Nunney *et al.* 2013, Harris *et al.* 2014, Overall & Rebek 2015). There is no mention of the disease on oaks in California, although “oak-type isolates” (according to the terminology of Nunney *et al.* 2013; see below) are detected in other trees. Although most of the reports of *X. fastidiosa* in the USA referred to native tree species, Nunney *et al.* (2013) isolated a strain of *X. fastidiosa* from the European English oak (*Q. robur*) in Kentucky. The disease has been described in other broadleaf trees, such as mulberry (*Morus spp.*), red maple (*Acer rubrum*) or California buckeye (*Aesculus californica*) (Sinclair & Lyon 2005). A case of very high incidence of BLS was reported on London plane trees (*Platanus x acerifolia*), hybrids between the American sycamore *P. occidentalis* and the Eurasian oriental plane *P. orientalis*, in North Carolina (Lashomb *et al.*, 2002). Other broadleaved trees showing BLS symptoms and found infected by *X. fastidiosa* include ash (*Fraxinus spp.*) (McGaha *et al.*, 2007; Nunney *et al.*, 2013), *Alnus rhombifolia*, *Carya spp.*, *Cercis spp.*, *Juglans sp.* (Wong *et al.*, 2004; Hernandez-Martinez *et al.*, 2007; Yuan *et al.*, 2010; Melanson *et al.*, 2012; Nunney *et al.*, 2013) as well as *Fagus crenata*, as bonsai (Huang *et al.*, 2003). *Salix sp.* and *Eucalyptus sp.* have been reported as positive for *X. fastidiosa* but description of symptoms is missing (EFSA 2018a). The pathogen was reported as infecting a few gymnosperm species (Wong *et al.*, 2004; McGaha *et al.*, 2007) but these reports were not confirmed. In general, conifers are considered as potential non-hosts (EFSA 2019) and might be resistant to *X. fastidiosa* because they lack the vessels that the pathogen typically colonizes (McGaha *et al.*, 2007).

The full list of plant species naturally infected by *X. fastidiosa*, for which both symptoms and positive detection of the bacterium have been recorded, can be found in the EFSA database (EFSA 2018, Delbianco *et al.* 2019), which also includes partial reports, e.g. with only symptoms.

1.3 What is the impact of *X. fastidiosa* in forests in the native area?

Regardless of the tree species involved, infected trees do not die immediately but tree life is shortened and the aesthetic quality may be significantly reduced (Sherald and Kostka, 1992; Hartman *et al.*, 1995; Gould and Lashomb, 2007). This observation may provide a clue to interpret the reason why BLS on trees is mainly mentioned in urban areas, on ornamental trees (Sherald 2007, Zhang *et al.* 2011, Harris *et al.* 2014). In general, affected trees may decline to the point where they must be removed (Hearon *et al.*, 1980; Hartman *et al.*, 1995). Damage to ornamental trees can be significant (Harris & Balci 2015). In some cities in New Jersey, USA, 30% to 35% of red oaks showed symptoms (Gould & Lashomb 2007). The loss of value and the amount of replacement costs for older trees affected by this disease were estimated at \$8,000 per tree (Gould and Lashomb, 2005). In addition, *X. fastidiosa* has been reported to cause typical symptoms of leaf scorch on red oak and box-elder (*Acer negundo*) in a commercial nursery in Maryland (Huang, 2007), indicating that the disease may occur in nurseries and may affect the production and trade of plants intended for planting.

While most of the disease reports refer to urban areas and amenity trees, symptoms of BLS have been detected in severely declining oak trees (*Quercus spp.*) in several state forests in Delaware and New

Jersey since the beginning of 2000 (Griffiths, 2013). Hopkins and Purcell (2002) deemed that BLS could have a significant environmental and economic impact in the southeastern United States. However, only one relatively old study conducted in Florida suggested the presence of *X. fastidiosa* in both natural and urban environments on eight oak species, particularly on turkey oak (*Q. laevis*), the most frequent species in the area (Barnard *et al.* 1998). Nunney *et al.* (2013) reported that liquidambar is severely infected in urban areas of California, while there is no mention of symptoms in its natural range in the southeastern United States. Whether trees growing in urban areas may be more susceptible to the disease than trees in the forests remains an open question (see some hypotheses in a following section). One hypothesis could be an under-estimation of the impact of *X. fastidiosa* in forests. Despite a few characteristics, the symptoms can be easily confused with other foliar browning agents or factors and a reliable diagnosis can only be established on the basis of the detection of the bacterium. Molecular methods currently provide highly specific and sensitive ways to test a sample for *X. fastidiosa*, and have been continuously improved (Baldi & LaPorta 2017; Supplementary material) but detection is complicated by the irregular distribution of the bacteria in the plant. The difficulties of diagnosis can be illustrated by results of a survey in the central United States where a very low detection rate of 5% was obtained from trees apparently suffering from BLS (Adams *et al.* 2013), which could be explained by both misdiagnosis (incorrect assignment of symptoms to *X. fastidiosa*) or false negatives (true infection but not detected, due to insufficient sensitivity of the method or to inadequate sampling).

1.4 Which forest tree species have been found infected in the introduction area in Europe?

In Apulia, and also in Tuscany, the areas where OQDS has been observed, no forest trees were reported among more than 30 species other than the olive trees from which *X. fastidiosa* was recovered (EFSA 2018b, Saponari *et al.* 2019). In an intensive survey of forestry plants growing in nurseries, public and private gardens potentially exposed to high inoculum pressure in the Salento peninsula (Apulia), none of the tested species were found to be infected by *X. fastidiosa* (Potere *et al.*, 2015).

In France, intensive samplings following the OQDS emergence in Italy led to the first report of *X. fastidiosa* in 2015 from Corsica on *Polygala myrtifolia* and some other plant species (Denancé *et al.* 2017). The bacterium was later found in mainland France, on the French Riviera in a few urban areas. Since then, many different plant species have been sampled in these areas. Among more than 45,000 thousands samples analyzed, around 3% were found positive, in 49 different plant species, mainly shrubs but also in a few forest trees, *Acer pseudoplatanus*, *Prunus avium*, *Quercus suber* and *Q. ilex*, at low frequencies (<http://draaf.paca.agriculture.gouv.fr>, Denancé *et al.* 2017, Jacques unpublished results).

In Spain, especially in the Balearic Islands, 18 species have been recognized as hosts, among which the forest tree species *Fraxinus angustifolia*, *Juglans regia*, *Prunus avium* (EFSA 2018b).

Finally, among the 96 forest tree species (either European native or not) with significant abundance in European forests according to pooled National Forest Inventories (Mauri *et al.* 2017), 15 are listed as natural hosts of *X. fastidiosa* according to the EFSA database, among which nine have been recorded as infected in Europe (Table 1). Some species affected by BLS in America and largely present in Europe, such as *A. platanoides* or *Q. rubra*, were not found infected but they do not occur in the current geographical range of *X. fastidiosa* restricted to Mediterranean areas.

2. *X. fastidiosa* pathogenicity

2.1 Is *X. fastidiosa* a true plant pathogen?

Historically, *X. fastidiosa* has primarily been considered as a plant pathogen, in relation to the several emerging and devastating diseases it has caused on several agriculturally important crops such as grapevine and Citrus. However, research on the epidemiology of these diseases and surveys prompted

by these outbreaks have shown that *X. fastidiosa* can live as a harmless endophyte in many plant species (Gambetta *et al.* 2007, Chatterjee *et al.* 2008, Purcell 2013, Roper *et al.* 2019). This is especially the case in the putative native area of *X. fastidiosa* in America, where co-evolutionary processes with native plant species may be involved. For example, *Vitis rotundifolia* (Muscadine grape), which can be infected by *X. fastidiosa* with very few symptoms, is native to the southeastern region of North America and may have evolved resistance, contrary to *V. vinifera* (d'Attoma *et al.* 2019).

Based on currently available data, several authors suggested that the diseases caused by *X. fastidiosa* may be the exception rather than the rule, corresponding to encounters of particular bacterial genotypes with some host plants (Sicard *et al.* 2018; Roper *et al.* 2019). In many (most) cases, *X. fastidiosa* would behave as a plant commensalist.

2.2 How does *X. fastidiosa* cause disease in susceptible hosts?

After introduction by xylem sap-feeding insects, the bacteria may colonize and multiply in the vessels of stems, leaves and roots but their distribution is generally irregular, with accumulation occurring preferentially in some plant parts (Purcell and Saunders, 1999, Hopkins & Purcell 2002). Plants usually exhibit a long asymptomatic period (several months to years) after inoculation (EFSA 2019). Symptoms are generally thought to result mainly from water transport disruption after vessel occlusion by bacterial biofilms but also tyloses and gums produced by plants in reaction to the infection. The effect of several virulence factors is also likely involved (Ionescu *et al.*, 2014; Nascimento *et al.*, 2016, Gambetta *et al.* 2018), as it was shown that vines infected with *X. fastidiosa* exhibit unique symptoms, not produced in water-deficit treatments (Thorne *et al.* 2006). Pathogenesis and virulence factors have been especially well studied in the case of Pierce's disease of grapevine and were reviewed in Chatterjee *et al.* (2008), Rapicavoli *et al.* (2018) and Roper *et al.* (2019). Two main phases corresponding to plant colonization and insect acquisition are associated with different phenotypes of the bacterium. In the first phase upon inoculation of bacteria into the xylem vessels by vectors, bacterial cells are planktonic and move acropetally with the sap flow or make use of Type IV pili to circulate against the sap flow. These non-adhesive bacterial cells produce copious amounts of outer-membrane vesicles that prevent their attachment to xylem cell walls (Ionescu *et al.*, 2014). These vesicles carry a cargo of proteases and lipases that contribute to symptom development upon release of the cargo (Nascimento *et al.*, 2016). Secretion of endoglucanase (EngXCA) and cellobiosidase (CbhA) allows pit membrane degradation and circulation between xylem vessels (Chatterjee *et al.* 2008). During this systemic plant colonization phase or exploratory lifestyle, bacteria multiply and secrete the diffusible signaling factor (DSF) (Newman *et al.*, 2004). Accumulation of DSF up-regulates the secretion of adhesins enhancing cell stickiness and exopolysaccharide production, while genes involved in type IV pilus, polygalacturonase, and outer-membrane vesicle production are down-regulated. Consequently, in a second phase, large bacterial biofilms are formed, contributing to vessel occlusion; the flow of xylem sap is blocked, symptoms may appear (after a latent period of several weeks to months), and *X. fastidiosa* cells can be efficiently acquired and retained by insect vectors. It has been suggested that the balance between the two bacterial phenotypes (adhesive vs. non adhesive) is critical to explain commensalism or parasitism of *X. fastidiosa* in different host plants (Roper *et al.* 2019). Late recognition of the pathogen by the plant immune system, when it has already colonized a large part of the xylem system, could explain why downstream plant reactions, especially formation of tyloses, are ineffective to block the pathogen and eventually lead to extensive vascular blockage and possibly death in susceptible plants (Roper *et al.* 2019).

2.3 What are the observations concerning vascular colonization in forest trees?

In several species of oak, American sycamore and red maple, microscopic studies showed an accumulation of bacteria in the vessels of petioles and leaf veins (Hearon *et al.* 1980, Hartman *et al.*

1995). In a study on red and pin oak, the intensity of leaf symptoms appeared to be positively related to ELISA values in tested foliage, thus presumably indicative of larger *X. fastidiosa* populations (Harris *et al.* 2014). In red oak, *X. fastidiosa* was isolated from the sap of symptomatic tree branches, but only from August to January (Chang & Walker 1988). The bacterium was also detected in some cases from asymptomatic parts of infected trees or from completely asymptomatic trees (Harris *et al.* 2014). In sycamore, the bacterium was detected and isolated from sap collected from roots and stems almost all year round, with a generally higher frequency in stems (Henneberger *et al.* 2004). The bacteria present in the conductive tissues of the branches were believed to colonize the petioles and veins of the leaves at the time of bud break (Chang & Walker 1988, Henneberger *et al.* 2004, Zhang *et al.* 2011).

Mc Elrone *et al.* (2008) studied the functional and structural characteristics of xylem in different organs of both healthy and *X. fastidiosa*-infected trees of several American oak species. A high rate of embolism was observed in *X. fastidiosa* infected petioles, preceding a sharp decrease of hydraulic conductivity and occurrence of leaf symptoms in infected trees as season progressed. The occurrence of large pit membrane pores (greater than the diameter of individual *X. fastidiosa* cells) was demonstrated in several *Quercus* species and was suggested to contribute to the higher susceptibility to bacterial leaf scorch of these species by facilitating systemic colonization of the secondary xylem.

2.4 Interaction of *X. fastidiosa* and other stressors in trees

Since *X. fastidiosa* infection in susceptible plants results in some dysfunction of the vascular system, potentially affecting tree's ability to respond to water demand, it is not surprising that *Xylella* infection may be a contributing factor of decline in addition to other stress factors, especially drought (McElrone *et al.*, 2003). Hammerschlag *et al.* (1986) even suggested that the presence of BLS was an indicator of stress and Barnard (2009) questioned the primary pathogenicity of the bacterium for oaks. *Xylella* infections may act as a predisposing or aggravating factor associated with other decline factors (Sherald & Kotska 1992, Hartman *et al.* 1995, Hanneberger *et al.* 2004). Symptoms are generally expressed at the end of summer when water demand is the highest in plants (Chatterjee *et al.* 2008). A significant association was observed between *Xylella* infection and decline in *Q. laevis*, where shoots on *Xylella*-positive trees were approximately 38% shorter than those on paired *Xylella*-negative trees. However, the role of *X. fastidiosa* in oak decline was not firmly established and the authors also suggested a possible role of root rot caused by *Armillaria tabescens* (Barnard *et al.*, 1998).

Similarly, infection by *X. fastidiosa* on elms was suggested to predispose trees to attacks by elm bark beetles vectoring *Ophiostoma ulmi* and *O. novo-ulmi*, thereby favoring the establishment and spread of Dutch elm disease (Stipes and Campana, 1981). In a survey carried out in the District of Columbia, USA, the disease was about 12 times more common in scorch-affected elms than in other elms. Furthermore, over 40% of all cases of Dutch elm disease occurred in trees already affected by bacterial leaf scorch (Sinclair & Lyon 2005).

On American sycamore, leaf scorch is a chronic disease and it may take years before the occurrence of tree death (Gould and Lashomb, 2007). However, *X. fastidiosa*-infected sycamore trees may become attacked by secondary pathogens (e.g. *Botryosphaeria* spp.) causing cankers and xylem discoloration, eventually leading to death, similar to those caused by the vascular pathogen *Ceratocystis platani* (Britton *et al.*, 1998; Harrington, 2013). It should be noted that a number of Botryosphaeriaceae species have been recently identified in Europe in association with declining and dying London plane trees (Pelletteret *et al.*, 2017), suggesting that an interaction between *X. fastidiosa* and those fungal pathogens may be likely should *X. fastidiosa* spread to London plane trees in Europe.

In general terms, overall tree decline eventually leading to death may occur as a result of the interaction between *X. fastidiosa* infection and other secondary pathogens and pests whose establishment or activation is favored by the weakening of the tree caused by *X. fastidiosa*, including root rot pathogens, endophytic pathogenic fungi and bark beetles.

3. Are there any *Xylella fastidiosa* genotypes (sub-species and sequence types) specifically associated with forest trees?

Since the description of *X. fastidiosa* by Wells and colleagues in 1987, only one other species has been described in the genus *Xylella*. The species *X. taiwanensis* was proposed to account for the strains causing leaf scorch of nashi pear tree in Taiwan, formerly considered to be *X. fastidiosa* (Su *et al.* 2016). All other strains responsible for diseases on hundreds of different plant species share at least 95% of DNA identity altogether, satisfying the criteria for being placed in a unique species, *X. fastidiosa* (Denancé *et al.* 2019).

X. fastidiosa has been divided into several subspecies, based on both host-range and genetic/genomic data. Three subspecies are widely accepted: subsp. *fastidiosa*, subsp. *multiplex*, both taxonomically valid, and subsp. *pauca* (not yet formally valid in the absence of a type strain deposited in two international collections). Other subspecies have been proposed and this classification is not yet fixed (Almeida & Nunney 2015; Denancé *et al.* 2019). *X. fastidiosa* subsp. *fastidiosa* includes the agent of Pierce's disease on grapevine but also strains causing almond, alfalfa and maple leaf scorch. It may also include strains causing oleander and mulberry leaf scorch, that were proposed to form two other subspecies (subsp. *sandyi* and *morus*, respectively) but these subspecies failed to be recognized based on genomic data (Marcelletti and Scortichini, 2016, Denancé *et al.* 2019). *X. fastidiosa* subsp. *pauca* groups strains responsible for the olive decline in Italy and also strains associated with Citrus Variegated Chlorosis (CVC) and coffee leaf scorch (Almeida *et al.* 2008). *X. fastidiosa* subsp. *multiplex* has the widest host range (Nunney *et al.* 2013). It has been associated with almond leaf scorch and most of the documented reports on forest trees (cf Table 1; Schuenzel *et al.* 2005, Barnard 2009, Nunney *et al.* 2010). Within each sub-species, Sequence Types (ST) were further identified based on the polymorphism of seven housekeeping genes (Yuan *et al.*, 2010). A total of 87 STs have been described so far that include 39 STs within the *multiplex* subspecies (<http://pubmlst.org/xfastidiosa/> Last updated: 2019-03-06).

Only subspecies *multiplex* has been found in native American trees (Nunney *et al.* 2013), with the exception of one report on maple (*Acer* sp.) associated with subsp. *fastidiosa* (Yuan *et al.*, 2010). Within subspecies, the typing of isolates from various hosts and locations in the USA provides only limited evidence of strong host specificity. In a study with 143 isolates collected from various plants in California, Texas, Tennessee, Kentucky, Florida and Georgia, Nunney *et al.* (2013) showed a fairly high diversity with 32 STs identified within the subspecies *multiplex*. Most STs were isolated from several host species belonging to different botanical families. A cluster of strains (ST 08, 09, 24, 24, 37, 38, 39, 44, 45, 49, 51), described as the "oak type", were mainly associated with oaks and some other indigenous tree species (liquidambar, plane and elm), by contrast with two other clusters ("peach type" and "almond type") associated with fruit trees (almond, olive, peach, plum, apricot). Strains associated with oaks belonged to 4 STs (ST08, ST09, ST23 and ST44), but only two of these STs (ST09 and ST44) were strictly associated with oaks. Harris & Balci (2015) also found ST09 associated with oaks (*Q. palustris*, *Q. rubra*, *Q. coccinea*, *Q. phellos*), both in symptomatic and non-symptomatic tissues, in Washington DC. A few studies with reciprocal inoculations suggested that strains isolated from a given host are more aggressive on this host than on other species. This was shown for *X. fastidiosa* subsp. *multiplex* strains isolated from either pecan or sycamore that failed to induce strong symptoms on the other host, despite the strains were phylogenetically close (Sanderlin, 2017). Recently in an extensive study, Nunney and colleagues (2019) showed that *X. fastidiosa* subsp. *multiplex* strains are generally

specialized on their host of isolation while some hosts (plum and almond) can be infected by a large range of strains of this subspecies. Interestingly, two strains presenting the same ST displayed different host ranges, possibly explained by the occurrence of specialized genotypes within the same ST. Recently, it has been suggested that STs, at least ST6, would not be monophyletic (Landa *et al.* 2020).

None of the “oak type” strains (*sensu* Nunney *et al.* 2013) of subspecies *multiplex* associated with native American forest trees have been reported in Europe so far, despite extensive sampling campaigns organized in the *X. fastidiosa*-infected countries, mostly on symptomatic plants (EFSA 2018, 2019). It should however be noticed that the ST7-Griffin-1 strain isolated from a red oak in Georgia, USA (Chen *et al.*, 2013) was also isolated in Spain and in France. In Italy, the causal agent associated with OQDS in Apulia is *X. fastidiosa* subsp. *pauca* ST53 strain. The strain detected in Tuscany belongs to multiplex ST87 (Saponari *et al.* 2019). In Spain, the three main subspecies, i.e. *fastidiosa*, *pauca* and *multiplex*, have been detected, with one (ST1), one (ST80), and three (ST6, ST7 and ST81) STs, respectively (Landa *et al.* 2020, EFSA 2018). ST6, ST7 and ST79 of subsp. *multiplex*, ST53 of subsp. *pauca* and ST76 of subsp. *fastidiosa* were also found in France (Denancé *et al.* 2017).

It is remarkable to note that strains identified in Europe show a larger host range than usually reported in the native area for a single ST (Nunney *et al.* 2013). For example, ST53 has been detected on 34 plant species in Italy (Saponari *et al.*, 2019), not including *Q. ilex* which was found infected by this strain in France (Denancé *et al.*, 2017). The ST6 and ST7 strains have been reported on 54 plant species, including some forest tree species (*Acer pseudoplatanus*, *Prunus avium* and *Q. suber*) in France (Denancé *et al.* 2017). Several tree species were found infected by *X. fastidiosa* subsp. *multiplex* ST81 in Spain, including almond, olive and wild olive, plum and fig tree (*Ficus carica*), narrow-leafed ash (*Fraxinus angustifolia*), and acacia (*Acacia* sp.) (EFSA 2018b).

The basis for host specificity of *X. fastidiosa* strains, if any, is still mostly undetermined, whether it is an interaction with the host plant or vector genotype (Harris & Balci 2015). Recombination, within and among subspecies, is recognized to be the major driver of diversity in *X. fastidiosa*, potentially facilitating shifts to novel host plants (Nunney *et al.*, 2013, 2014; Gambetta *et al.* 2018; Denancé *et al.*, 2019; Vanhove *et al.* 2019, Landa *et al.* 2020). *X. fastidiosa* subsp. *multiplex* appears as the lineage that is most prone to recombination (Vanhove *et al.* 2019). For example, the emergence of coffee and citrus diseases in South America has been suggested to be associated with new pathogenicity acquired by the endemic subspecies *pauca* via inter-subspecific homologous recombination (IHR) with *X. fastidiosa* subsp. *multiplex* after the introduction of the latter in the region (Nunney *et al.* 2012). The ST53 lineage of subspecies *pauca* that is responsible for the outbreak of OQDS in southern Italy also shows evidence of IHR, with subspecies *fastidiosa* (Vanhove *et al.* 2019). The evolutionary history of *X. fastidiosa* is also driven by the acquisition of genetic material from heterologous origin, such as plasmids from other bacterial species or genomic regions of bacteriophage origin (Denancé *et al.*, 2019). These horizontal transfers may also contribute to adaptation, including to new hosts.

4 Transmission of *X. fastidiosa* by insect vectors

4.1 How is *X. fastidiosa* transmitted?

As far as it has been proven, any insect feeding predominantly from xylem is a potential vector of *X. fastidiosa* (Almeida 2016). However, recent experiments suggested that bacterial strain-vector or vector–plant interactions may influence bacterial acquisition, retention and inoculation by the insect (Esteves *et al.* 2019). *X. fastidiosa* is a unique organism in being persistent and propagative in its vectors, but non-circulative (Severin 1950; Purcell *et al.* 1979 ; Hill and Purcell 1995). Transmission is neither transstadial, i.e. the pathogen is lost at each molt (Purcell and Finlay 1979), nor transovarial,

i.e. from parent to offspring (Freitag 1951), beside apparently not requiring a latent period (or requiring a latent period shorter than one hour, i.e. between acquisition and transmission to another plant) (Purcell and Finlay 1979). Upon acquisition from an infected xylem vessel, bacterial cells are retained into the vector foregut. Bacterial populations inside the vector foregut even below detection thresholds (100 CFU/insect head) are enough for efficient inoculation to occur (Hill and Purcell 1995; Cornara *et al.* 2016). However, acquisition efficiency by vectors is likely favored by high bacterial load in the source plant (Almeida *et al.* 2005). High numbers of infective individuals staying on a plant increase the transmission probability and reduce the incubation time for symptoms appearance (Daugherty and Almeida 2009; Cornara *et al.* 2018; Cornara *et al.* 2019).

4.2 What are the known and potential vectors of *X. fastidiosa* on trees?

Insects that feed predominantly from xylem vessels belong to two superfamilies, i.e. Cercopoidea (froghoppers and spittlebugs) and Cicadoidea (cicadas), and one subfamily, i.e. Cicadellidae Cicadellinae (sharpshooters) of the order Hemiptera (Novotny and Wilson, 1997).

Most of the knowledge on *X. fastidiosa* epidemiology and vector-bacterium relationship comes from studies carried out on Pierce's Disease (PD) epidemics in California (USA), and CVC in Brazil; in both cases, sharpshooters play the key role in *X. fastidiosa* natural spread (Rapicavoli *et al.* 2018). A few studies have considered potential vectors of *X. fastidiosa* in trees in North America. The bacterium was detected in seven leafhopper species captured on elm and oak, some of which known as vectors of *X. fastidiosa* in other hosts, and three species of treehopper, not known to be competent vectors (Sherald 2007). Zhang *et al.* (2011) considering both insect infection and abundance, suggested that the sharpshooter *Graphocephala versuta* could be the main species involved in BLS spread in oaks in central New Jersey. *G. versuta* has also been putatively associated with the spread of *X. fastidiosa* in Oklahoma forest nurseries (Overall and Rebek 2015). Unfortunately, no transmission tests to forest trees to determine vectors' competence and efficiency have been performed, and most of the assumptions on BLS natural spread are based on candidate vectors sampling, estimates of their relative abundance, and molecular detection of *X. fastidiosa* in the insect. However, detection of *X. fastidiosa* in an insect does not imply that the insect can be a vector, since even phloem feeders may occasionally acquire the bacterium without being capable of transmitting it (Purcell 1980 ; Cavalieri *et al.* 2019).

While at least in North America some preliminary indications about possible vectors of *X. fastidiosa* to forest trees are available, very little is known regarding Europe. Few sharpshooter species are present in Europe and spittlebugs are deemed to play the key role in *X. fastidiosa* natural spread in European ecosystems (EFSA 2015, Almeida 2016, Cornara *et al.* 2019). The meadow spittlebug *Philaeus spumarius* (Figure 2) is currently considered the only relevant vector of *X. fastidiosa* across all the European outbreaks detected so far (Cornara *et al.* 2017a ; Cornara *et al.* 2017b ; Cruaud *et al.* 2018 ; Moralejo *et al.* 2019). Two other species, *Neophilaenus campestris* and *P. italosignus*, were found to be competent vectors of the bacterium on cherry, olive, and myrtle-leaf milkwort but only under experimental conditions (Cavalieri *et al.* 2019). In contrast with previous studies in the USA and Brazil (Paião *et al.* 2002; Krell *et al.* 2007), Cornara *et al.* (2020) suggested cicadas, at least the Mediterranean widespread species *Cicada orni*, have no or very negligible role in *X. fastidiosa* transmission to olive.

The meadow spittlebug *P. spumarius* has a very wide distribution in Europe, ranging from the Mediterranean to Lapland and it is a highly polyphagous species. It has the potential to live under different environmental conditions, from moist to relatively dry, as long as the host plant is actively growing and not subjected to severe water stress (Cornara *et al.* 2018). Oaks, such as *Quercus ilex* and *Q. crenata*, seem to be among preferred hosts of adults *P. spumarius* among woody plants (Di Serio *et al.* 2019 ; Bodino *et al.* 2020). Beside oaks, other forest trees have been reported as hosts for *P. spumarius*, as for example *Acer campestre* (Mazzoni 2005). Other xylem-feeders reported on forest trees in Europe, thus theoretically candidate vectors of BLS, include: *P. tarifa*, *Aphrophora alni* and *A.*

corticina, *Cercopis arcuata* and *Haematoloma dorsata*, whose adults can be found on oak plants; *Cicadella viridis*, whose adult females may lay their overwintering eggs on ash; *A. alni* and *N. campestris* that can be collected on elm; (Cornara *et al.* 2019; Bodino *et al.* 2020). Maple is reported as host of the sharpshooter *Graphocephala fennahi* (Sergel 1987). This latter species, native to Nearctic region and originally associated only with *Rhododendron* spp., extended its geographic range and host plant spectrum in Europe, and has been reported on several tree and shrub species (Arzone *et al.* 1986; Sergel 1987).

Figure 2 Meadow spittlebug, *Philaenus spumarius* : on the top, typical nymph spittle ; on the bottom, adult insect



4.3 How disease is affected by vector ecology

X. fastidiosa transmission is impacted by several factors, most of which are related to vectors biology, ecology, host-range and behavior (Redak *et al.* 2004; Sicard *et al.* 2018; Markheiser *et al.* 2020). For Pierce's (PD) disease in particular, it was shown that the timing of inoculation in the growing season, depending on vectors' phenology, ecology and life cycle, is critical for the establishment of host-plant chronic infections. A dramatic increase in PD severity occurred in California in the 1990s following the introduction of a new vector (i.e. *Homalodisca vitripennis*) from the South-Eastern States (Blua *et al.* 1999). This change in disease epidemiology was suggested to result from differences in the

feeding, flying and breeding behaviour between the native and introduced vectors (Hopkins & Purcell 2002; Almeida *et al.* 2005; Rapicavoli *et al.* 2018).

The life-cycle and ecology of *P. spumarius* has been particularly studied in relation to OQDS in Italy (for a detailed description of *P. spumarius* biology, ecology, phenology and life history refer to Cornara *et al.* 2018 and Bodino *et al.* 2019 and 2020). Eggs (the overwintering form) are laid close to the ground, usually on plant residue or directly on the basal portion of herbaceous plants between two apposed surfaces (e.g. stem and leaf sheath). Upon hatching in early spring, the nymphs crawl to reach a succulent herbaceous host, where they form the characteristic spittle (Figure 2), with a preference for plants belonging to the families Asteraceae and Fabaceae (Bodino *et al.* 2020). Adults, after emergence on herbaceous species, tend to disperse toward woody hosts (olive trees and wild trees such as oaks, lentisk, myrtle, etc.) in late spring. Once on infected olive trees, spittlebugs acquire *X. fastidiosa* and spread it from tree-to-tree within the olive orchard, and possibly to other trees and woody plants toward which they move for overwintering (Cornara *et al.* 2017a). In the end of summer, likely in response to shortening of photoperiod, spittlebugs go back to herbaceous hosts for oviposition (Cornara *et al.* 2017a, 2018). This dispersal trend from herbaceous plants to woody hosts and back to ground cover vegetation was found to be less pronounced in North-Western Italian olive orchards than in Apulia (Bodino *et al.* 2020). In Corsica, *Cistus monspeliensis* was reported as the main host plant for both nymphs and adults (Cruaud *et al.* 2018). Spittlebugs may likely not disperse over long distances during their lifetime: nymphs tend to remain close to the place where they hatched (Morente *et al.* 2018), and adults might just move few hundred meters during the search for overwintering hosts and oviposition places (Weaver and King, 1954; Cornara *et al.* 2018; Cornara *et al.* 2019). Hence, olive groves appear as very suitable habitats probably sustaining *P. spumarius* all along its life cycle with ground cover vegetation providing oviposition sites in fall and hosts for nymph development in spring while olive trees provide food, shelter and mating sites during summer (Santoiemma *et al.* 2019). Favorable habitats can potentially support high population levels of vectors, and ultimately, if *X. fastidiosa* is present, be associated to high probability of disease transmission. The landscape composition of the close surrounding of the olive groves (125–250 m) was suggested to play a significant role in *X. fastidiosa* epidemics by affecting vector populations. In agreement with *P. spumarius* preference for oaks, a species distribution model of *X. fastidiosa* based on occurrence data in Apulia identified evergreen and deciduous oak woodlands as the most favorable habitats among forested areas (Bosso *et al.* 2016b).

5 Is the distribution of *X. fastidiosa*, especially subsp. *multiplex*, restricted by climatic factors?

Most diseases caused by *X. fastidiosa* have been reported from regions with tropical, sub-tropical or Mediterranean climates. However, there are also reports from temperate and even continental areas (EFSA 2019). This wide distribution might be explained by the evolutionary history of the different subspecies in the Americas. Northernmost records of *X. fastidiosa* are always associated with subspecies *multiplex* (EFSA 2015). Phylogenetic and genomic studies suggest that *X. fastidiosa* subspecies may have evolved in different environments. In particular, the evolution of *X. fastidiosa* subsp. *multiplex* in a temperate environment in North America is supported by its rate of substitutions and observed nucleotide polymorphisms (Nunney *et al.* 2010). It probably diverged from more tropical forms related to *X. fastidiosa* subsp. *fastidiosa*, with a probable origin in Central America, and *X. fastidiosa* subsp. *pauca*, with a probable origin in South America (Nunney *et al.*, 2010; Nunney *et al.*, 2014). Observation and experimental data have provided evidence of the detrimental effect of low temperatures on survival of *X. fastidiosa*, especially for Pierce's Disease, with "winter (cold) curing" limiting the establishment of the disease in cold areas and of chronic infections in California (Lieth *et al.* 2011; Purcell 2013). However, experimental data is lacking to support subspecies *multiplex* or specific strains recovered from northern areas are more cold tolerant.

BLS in forest tree species is mostly associated with *X. fastidiosa* subsp. *multiplex* and has been reported in several regions with relatively cold winters, such as British Columbia, southern Ontario and Saskatchewan in Canada, north-eastern states of the USA, and in Argentina (Sherald 2007, Gould & Lashomb 2007, Harris *et al.* 2014). Adams *et al.* (2013) found that the northern limit of BLS in trees corresponded to the area where the average annual minimum temperatures over 10-24 years was between -18°C to -23°C. The survival of the bacterium in so cold conditions might be explained by the fact that the bacterium could overwinter in protected parts of the tree such as the trunk or roots (Chang & Walker 1988, Henneberger *et al.* 2004, Gould & Lashomb 2007, Adams *et al.* 2013). In plane trees, Henneberger *et al.* (2004) showed a very strong negative correlation between the accumulation of temperatures below -5°C and bacterial populations detected or isolated in the sap of the stems, while these temperatures were not being reached in the roots.

The EFSA Panel on Plant Health recently produced maps representing climatic suitability for establishment of *X. fastidiosa* in Europe, taking subspecies into consideration (EFSA 2019). Ensemble predictions were based on various SDM (Species Distribution Modelling) techniques, using available records in America and Europe. Subspecies *multiplex* had the widest climatic envelope compared with that predicted for subsp. *fastidiosa* or *pauca*, with suitable climatic conditions over a large part of the EU territory with the exception of central and northern Europe. The highest suitability for the three subspecies was found in Mediterranean and south Atlantic areas, characterized by warm temperatures during the summer-dry period, mild winter temperatures and well-defined rainy season. In an independent study, Godefroid *et al.* (2019) also predicted a large extent of climatically suitable areas in Europe, especially for subspecies *multiplex* and again mostly in circum-Mediterranean and Atlantic areas. Irrespective of climate change, the introduced strains have thus high potential for expansion. Additionally, new areas might be at risk under the ongoing climate change characterized by increasing minimum winter temperatures, since the three subspecies appear to be limited by cold (with subsp. *pauca* the more sensible and subsp. *multiplex* the less). However, too hot summer temperatures and precipitation regimes might become less favorable in other regions. Using several greenhouse scenarios, Bosso *et al.* (2016a) suggested that climate change would not affect the future potential distribution of *X. fastidiosa* in the Mediterranean basin.

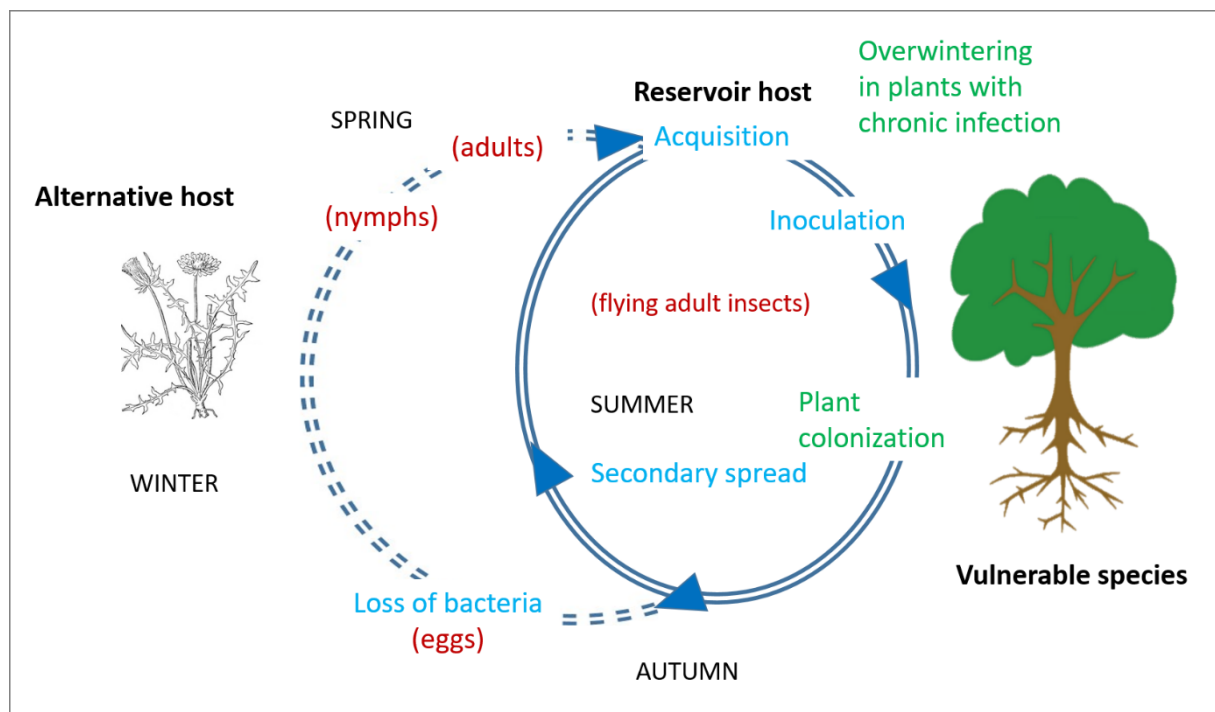
6 Is disease risk for trees different in forest ecosystems as compared to urban settings?

The fact that the BLS of trees in North America is mainly observed in urban areas while most affected tree species are native and also present in forests is not explicitly discussed in the literature. Nunney *et al.* (2013) and Harris *et al.* (2014) point to the lack of information on the presence of *X. fastidiosa* outside urban areas. The nature of the symptoms (leaf browning at the end of the season, early defoliation) could explain why they are more easily noticed for ornamental trees and overlooked in the forest environment. Landscape and amenity trees are daily observed by many people, thus BLS could be easily and more often noticed and reported in urban settings, while surveillance of forest trees is more sporadic. Some environmental factors could also contribute to the greater severity of the disease in urban environments, such as the existence of more frequent and intense stresses which could exacerbate the expression of symptoms (Barnard 2009, Nunney *et al.* 2013, Harris *et al.* 2014). The disease is often mentioned on trees that are already well developed or even old (McElrone *et al.* 2008) but can also affect trees shortly after planting (Sherald & Kotska 1992).

On the other hand, Sherald (2007) proposed that the highest prevalence of the disease in parks and gardens could be explained by ecological factors. For example, the presence of reservoir host species could be more commonly found in open areas than in forests. Similarly, some authors suggested that vectors of the disease could be particularly abundant in urban areas (Harris *et al.* 2014, Zhang *et al.* 2011). The abundance of vectors in residential landscaping might be favored by irrigation practices (Almeida *et al.* 2005) since sap-feeders are usually attracted by plants in a good hydric status (Yurtsever

2000). As indicated earlier, spittlebugs generally share their life-cycle between herbaceous plants and woody hosts (Figure 3). Parks with trees surrounded by well-watered garden plants and shrubs might constitute an especially favorable habitat for vectors, by sustaining their development during their life cycle. By contrast, closed-canopy forests with a poor understory may be less favourable for the nymphal stages, and would not allow the built-up of dense populations of *X. fastidiosa* vectors. The crucial role of understory vegetation has been demonstrated for the Saratoga spittlebug, *Aphrophora saratogensis*, which can be an important pest in young red pine plantations. Herbicidal treatments targeting overstory vegetation in pine plantations were shown to provide effective protection of susceptible pines from serious damage, by decreasing spittlebug populations (Linnane & Osgood 1976). Weed management was also suggested as a control measure reducing resident vector populations in infected olive and almond orchards in Italy and Spain (Dongiovanni *et al.* 2018, Bodino *et al.* 2019, Moralejo *et al.* 2019).

Figure 3 Hypothetical *Xylella fastidiosa* disease cycle in forest trees : processes indicated in blue are linked to insect (e.g. spittlebug) vectoring of the bacteria; processes in green are within plant; insect stages are indicated in brown. Here, the vulnerable species is a tree, both susceptible and economically or environmentally important. The alternative host is used by vectors for oviposition and nymph development. Acquisition of bacteria by vectors (emerging healthy adult insects) in spring is from chronically infected host plants (reservoir hosts), either the vulnerable species itself, other woody species or possibly the alternative host. Some vectors (in some species, or for a given species, according to climatic conditions) may overwinter as infected adults or make several generations per year. (see main text for further explanations)



Another possible factor explaining differences of disease risk between forest and urban trees (or fruit trees) could be linked to the genetic diversity of plant populations. Urban trees, and even more fruit trees and grapevine, largely belong to few vegetatively propagated cultivars/varieties. If a *X. fastidiosa* strain is introduced in a region where susceptible cultivars are widely planted, the transmission of this strain will be highly facilitated. Differences in cultivar susceptibility to *X. fastidiosa* have been documented in various plants, including grapevine (Rashed *et al.* 2013) and olive trees (Almeida 2016, D'Attoma *et al.* 2019). The epidemics of OQDS in the Apulia region was probably favored by the

encounter of a particular genotype of *X. fastidiosa* (belonging to ST53 of subsp. *pauca*) with widespread traditional olive cultivars (“Cellina di Nardo” and “Ogliarola salentina”, together representing 85% of olive trees in this region) which turned out to be highly susceptible, both in terms of bacterial population levels and expression of symptoms (D’Attoma *et al.* 2019), in a region with favorable environmental conditions (climate, ground cover, vector populations, etc...). More heterogeneous populations or landscapes, as generally occurs in European forests, might be comparatively less favorable for epidemic spread and evolution towards high virulence levels (Stukenbrock & Mc Donald 2008, Sicard *et al.* 2018). Moreover, homogeneous plantings, as may occur in tree alignments along streets and landscape settings, could also favor pathogen spread through root grafts, although there is no current evidence of such tree-to-tree transmission in tree species other than *Citrus* spp. and of any epidemiological significance of such mechanism (He *et al.* 2000).

Conclusions

Risk of *X. fastidiosa* disease emergence in forests

The recent “Update of the scientific opinion on the risks to plant health posed by *Xylella fastidiosa* in the EU territory” (EFSA 2019) concluded that “the spread of *X. fastidiosa* to forest areas could lead to impact on oaks, elms, maples and other tree species known to be affected in North America, but with high uncertainty to the level of this impact, especially for native European species absent in North America”. No new significant finding since then can notably change this statement.

The current situation of *X. fastidiosa* in North America may suggest a relatively low threat to forests since little damage has been reported in these ecosystems. This is despite American native forest tree species have been found susceptible to *X. fastidiosa*, especially to the subspecies *multiplex*, but disease reports and damage have been mostly restricted to urban or peri-urban environments, in parks and gardens. This moderate impact may reflect a long ecological and evolutionary history of *X. fastidiosa* with temperate forest ecosystems in North America (Almeida & Nunney 2015), a situation for which large-scale epidemics with severe impacts are not expected to occur (Jousimo *et al.* 2014). In particular, some level of resistance or tolerance to the bacterium may have evolved in native North American tree populations (Purcell 2013), resulting in a low frequency of highly susceptible genotypes and the necessity of stressful conditions for the expression of severe symptoms. The same author suggested that natural regulation of the bacterial populations by microbial antagonisms (resistance induced by low virulent strains or phages) could also be involved.

Extrapolating a low risk for European forests would however be too hasty. The history of emerging diseases associated with *X. fastidiosa* in different parts of the world, on different plants and with different subspecies consistently demonstrated that unpredictable host-*X. fastidiosa* interactions with high impact can occur as soon as a non-native component is included in the host-pathogen-vector system. The most common pathway leading to emergence of *X. fastidiosa* diseases has been the introduction of strains of the bacterium in new areas, through the movement of infected, potentially asymptomatic, plant material (Almeida and Nunney 2015, EFSA 2019). Examples include the introduction of *X. fastidiosa* subsp. *fastidiosa* in California in the late 19th century, possibly through the importation of coffee plants from Central America (Nunney *et al.* 2010). The Central American origin of the ST53 strain of *X. fastidiosa* subsp. *pauca* causing OQDS in Italy is strongly supported by pan-genomic studies (Giampetruzzi *et al.* 2017), although ST53 may not be native to Central America but originating from a single *pauca* genotype introduced from South America (Nunney *et al.* 2014). The scenario suggested for the emergence of CVC in Brazil involved a change in the virulence of the local native *X. fastidiosa* subsp. *pauca* strains that became able to infect Citrus after inter-subspecific recombination with introduced strains of *X. fastidiosa* subsp. *multiplex*, possibly originating from a contaminated plum plant (Nunes *et al.*, 2003; Nunney *et al.* 2012). Even in an area where the bacterium

is already present, subsequent introductions therefore represent a significant risk with the possibility of intra- or inter- subspecies recombinations changing the host range of the pathogen. Indeed, a characteristic of the bacterium which complicates risk prediction is its very wide actual and potential host range, albeit with no apparent phylogenetic signal. Contrary to the general rule observed in plant-pathogen interactions (Gilbert & Webb 2007) the known host range of *X. fastidiosa* cannot be used to make predictions of host jump to another plant species based on the phylogenetic relatedness with plants known as hosts (Nunney 2019). In the same line, Sicard *et al.* (2019) showed the absence of congruence between phylogenetic trees of the host plants and of the STs of the pathogen.

While introductions of pathogen strains and vectors (as shown for PD) clearly represent the main driver of *X. fastidiosa* disease emergence, the risk of establishment of the disease is strongly affected by environmental factors, especially climate and landscape features which drive pathogen survival, disease transmission and vector population levels (Sicard *et al.* 2018, EFSA 2019). *Xylella* pathosystems are typical examples illustrating the “epidemiological triangle” (here with additional layers of complexity leading to more than three components), i.e. the fact that the outcome in terms of disease and impact is the result of very specific interactions between host-pathogen-vector-populations and the environment, both biotic and abiotic (Almeida 2005). Compared to other situations where severe *Xylella* outbreaks occurred, European forests present some features potentially limiting risk, such as the occurrence of genetic and species diversity. However, diversity has not proven to be sufficient to limit the impacts of other introduced pathogens, especially with a large host-range, as exemplified by sudden oak death caused by *Phytophthora ramorum* in California, in very diverse woodlands (Rizzo *et al.* 2005). Moreover, as a vascular pathogen, the impact of *Xylella* may be strongly increased in trees already weakened by drought stress and other stressors, including pathogens and insect pests. Even if native European forest trees are not very susceptible to the strains that have been already introduced, *X. fastidiosa* may become an additional factor contributing to tree declines. Mediterranean ecosystems, with high climatic suitability for establishment of the bacterium (EFSA 2019, Godefroid *et al.* 2019) and potentially exposed to frequent drought stress can therefore be considered as higher risk areas. The fact that a low prevalence and no important impact has been observed on tree species, especially oaks (mainly represented by holm oak) in Corsica until now despite long presumed presence of *X. fastidiosa* may be a matter of hope. However, biological invasions are generally characterized by a lag phase corresponding to the build-up of populations until they reach levels associated with potential high impact (Aikio *et al.* 2009). Bacterial populations may not have reached sufficient levels in introduced areas in Europe to reveal the full range of susceptible species. This phenomenon is well exemplified by the history of *Phytophthora ramorum* in the United Kingdom, which was first only observed on ornamentals in nurseries, but after several years caused devastating epidemics in larch plantations, and has more recently emerged on chestnut (Brasier & Webber 2010; Webber 2017).

Knowledge gaps and recommendations.

Given the great uncertainty on potential impacts of *X. fastidiosa* on European forests, the first recommendation is to strictly apply quarantine, eradication and containment measures to avoid any new introduction or further spread of the pathogen (and potential alien vectors). Any introduced strain, including ST09 particularly associated with oaks in the USA, could represent a risk, in view of the unpredictable outcome of interactions with non-coevolved hosts and the potential of recombination with already present strains. Surveillance is also crucial and should be developed on forest and urban trees, especially oaks, and seedlings in nurseries. Since the *multiplex* subspecies, preferentially affecting forest tree species in America, shows lower sensitivity to cold than other subspecies, surveillance may be considered widely and not only focus the Mediterranean region. Improvement of detection methods, especially for trees, is still an important issue given the high concentration of tannins and difficulties linked to heterogeneous distribution of the bacterium in trees

(Supplementary material). The design of surveillance schemes could benefit from recent modelling approaches, as developed by White *et al.* (2017) and Martinetti and Soubeyrand (2019), while acquired surveillance data may help refining the models for more accurate predictions at a larger scale.

Many unknowns about the biology and epidemiology of the disease persist, especially as far as forest trees, especially European species, are considered. A deep understanding of the ecology of the disease, in a perspective of prevention, is essential when addressing risks to forests since control measures, both for the vector or the hosts, are much more limited or difficult to implement than in an agricultural context (Desprez-Loustau *et al.* 2015). Extrapolating knowledge from other pathosystems in the native area or other pathosystems in Europe (e.g. OQDS in Italy) may have strong limitations since *X. fastidiosa* diseases have appeared as the outcome of complex interactions between the bacterial strains, the vectors, the host plants and the environmental conditions (Almeida & Nunney 2015).

Processes of bacterial colonization within trees are still poorly studied. Understanding the factors conditioning the distribution of the bacterium in different parts of the tree is particularly important to assess the vulnerability of tree species and tree genotypes within species. The mortality of trees is likely related to the ability of *X. fastidiosa* to establish chronic infections, and thus successfully overwinter. Seasonal dynamics of bacterial colonization within trees, with potential survival in trunks and roots during winter, is a crucial process to be investigated, especially in cold areas. Specific response of *X. fastidiosa* strains to temperature, especially of *multiplex* subspecies from different geographical origins, should also be studied. The existence of anatomical features of xylem vessels affecting bacterial colonization and disease expression is an interesting field to be investigated. It is generally admitted that insect vectoring constitutes the only natural mean of *X. fastidiosa* spread. However, few studies, mostly on Citrus, have been conducted about possible horizontal (rootgrafts) or vertical (seed-to-seedling) transmission from tree-to-tree. *X. fastidiosa* subsp. *pauca* has been detected in different parts of the seed from CVC-affected sweet orange but no transmission of the bacterium to seedlings seems to occur (EFSA 2015). It would be of utmost importance to exclude (or confirm) possible transmission from root-graft or seeds for forest trees, in an epidemiological but also regulatory perspective. Generally speaking, a better knowledge of the distribution of the bacteria within trees could also improve sampling procedures for detection.

One of the most relevant and pending question relates to the susceptibility of European forest tree species to the different *X. fastidiosa* subspecies and sequence types, which clearly needs further investigation. This could start by screening a range of bacterial strains on a range of tree genotypes of different species, primarily focusing on oaks which appear to be especially susceptible in North America. This is, however, not an easy question since high virulence towards a particular host may be associated with a single strain as shown for OQDS, the emergence of which in Italy was unpredictable based on the few reports of *X. fastidiosa* in California (where only subsp *multiplex* was detected, with low impact). More generally, the determinants of host specificity and pathogenicity in *X. fastidiosa*, i.e. why a specific bacterial strain will cause severe disease on a particular host while being a harmless endophyte in another is not yet understood. We advocate that oaks be included as model plants, in addition to grapevine, to investigate pathogenesis in *X. fastidiosa*. Such studies may take advantage of the development of a miniaturized system to investigate systemic transcriptomic changes in oak during biotic interactions (Maboreke *et al.* 2016) and of the availability of a well annotated genome sequence (Plomion *et al.* 2018).

Another important field of investigation should focus on the vectors, since they are one of the major drivers of establishment and spread of *X. fastidiosa*. While since 2014 a large (but still insufficient) amount of data has been produced on the ecology of spittlebugs in European agroecosystems, almost nothing is known with regard to European forests. We consider mandatory to start filling this white sheet by carrying out surveys in forest habitats, starting by European regions affected by disease outbreaks, gathering data on vectors' presence, seasonal abundance and host preference, and vectors'

infection. Molecular methods allowing the detection of *X. fastidiosa* in insects have already provided interesting results but still require improvements (Cruaud *et al.* 2018). Such preliminary step should be followed by detailed studies on vectors' population dynamics, spatial distribution, dispersal, feeding habits and behavior on forest trees. Comparison of data gathered in different climatic conditions would permit to deepen our knowledge and refine models for risk assessment of BLS spread in European forests, eventually aimed at improving surveillance. These studies should proceed in parallel with transmission tests to forest trees found to be infected with *X. fastidiosa* (under controlled conditions for quarantine reasons). Knowledge on Apulian OQDS outbreak should not be generalized and applied to other pathosystems, since *Xylella*-pathosystems should be treated on a case-by-case basis. Therefore, besides *P. spumarius*, all the xylem-feeders should be investigated regarding their potential role in BLS epidemiology in European forests, including species that proved to be non-relevant in OQDS spread, as *Neophilaenus campestris* or *Cicada orni*. Of particular relevance, considering its distribution and host range, would be *Graphocephala fennahi*, a sharpshooter of American origin which has extended its host and geographical range in Europe during the last decades.

Current and future studies conducted in Europe may help understand aspects of BLS that have not been clarified in North America. However, some questions are currently difficult to address in a European context and experiments can only be carried out in quarantine facilities. Further investigations about BLS in North American forests, starting by the study of prevalence and severity of symptoms, are highly needed. Testing different hypotheses could explain why a lower impact has been reported so far in forests as compared to landscape trees. This could shed light on the underlying mechanisms, especially related to the ecology of vectors, the genetic variation in susceptibility of tree species and other natural disease regulation processes, such as microbial antagonisms, that could be of great interest to maintain or increase resilience of worldwide forests to *X. fastidiosa*.

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Table 1: European forest tree species reported as natural hosts of *X. fastidiosa* according to the EFSA database (EFSA 2018a, Delbianco *et al.* 2019)

Genus	species	Common name	Geographical origin	Natural infection*	Xf subspecies	comment
<i>Acer</i>	<i>A. platanoides</i>	Norway maple	EUR	USA, CAN	multiplex	Reports on 7 other <i>Acer</i> species in USA (multiplex + one <i>fastidiosa</i>)
	<i>A. pseudoplatanus</i>	Sycamore maple	EUR	FR	multiplex	
<i>Aesculus</i>	<i>A. hippocastanum</i>	European horse chestnut	EUR	CAN	unknown	BLS report - not retained in EFSA database
<i>Celtis</i>	<i>C. orientalis</i>		EUR-As			Infections on <i>C. occidentalis</i> in the USA (multiplex)
<i>Ficus</i>	<i>F. carica</i>	Common fig	As	USA, FR, SP	multiplex	Intercepted in Italy
<i>Fraxinus</i>	<i>F. angustifolia</i>	Narrow-leaved ash	EUR	SP	multiplex	Reports on 3 other <i>Fraxinus</i> species in Canada and USA (multiplex)
<i>Juglans</i>	<i>J. regia</i>	Common walnut	EUR-As	SP	<i>fastidiosa</i>	Reports on 2 other <i>Juglans</i> species in the USA (subsp unknown)
<i>Liquidambar</i>	<i>L. styraciflua</i>	American sweetgum	Am	USA	multiplex	
<i>Olea</i>	<i>O. europaea</i>	Olive	EUR	USA, ARG, BRA, IT, SP FR	multiplex, pauca	Mainly pauca in Europe, only multiplex in USA
<i>Platanus</i>	<i>P. orientalis</i>	Oriental plane	EUR			Numerous reports in USA on <i>P. occidentalis</i> (multiplex and 1 <i>fastidiosa</i>) BLS report on <i>P. x acerifolia</i> (London plane) not retained in EFSA database
<i>Prunus</i>	<i>P. avium</i>	Wild cherry	EUR-As	USA, IT, SP, FR	<i>fastidiosa</i> , multiplex, pauca	Also numerous reports on <i>P. dulcis</i> in America, and Europe
	<i>P. cerasifera</i>	Cherry plum	EUR-As	USA, FR	multiplex	
<i>Quercus</i>	<i>Q. ilex</i>	Holm oak	EUR	FR	pauca	Very few positive samples
	<i>Q. robur</i>	Pedunculate oak	EUR	USA	multiplex	single report
	<i>Q. rubra</i>	red oak	Am	USA	multiplex	Numerous reports ; also on other oak species
	<i>Q. suber</i>	Cork oak	EUR	FR	multiplex	single report
<i>Ulmus</i>	<i>U. glabra</i>	Scots elm	EUR	USA	unknown	Numerous reports on other <i>Ulmus</i> species in USA

*CAN = Canada, FR=France, SP= Spain, IT=Italy, ARG= Argentina, BRA = Brazil

Supplementary material : Diagnostic methods

Various reference documents are available concerning the diagnostic of diseases due to *X. fastidiosa*: (i) the International Plant Protection Convention's (IPPC) international standards on phytosanitary measures (ISPM) 27 Diagnostic protocols for regulated pests, in particular DP 25: *Xylella fastidiosa*, and ISPM 31 Methodologies for sampling of consignments (FAO, 2016, and 2018); (ii) the European and Mediterranean Plant Protection Organization (EPPO) diagnostic protocol for *X. fastidiosa* PM 7/24 (4) (EPPO, 2019); (iii) the EFSA pest survey card (Vos *et al.*, 2019); (iv) the Guidelines for the prevention, eradication and containment of *X. fastidiosa* in olive-growing areas (Catalano *et al.*, 2019).

EPPO PM7/24 protocol recommends in *X. fastidiosa* -free areas the use of two screening tests based on different biological principles or targeting different parts of the genome. In areas where *X. fastidiosa* is known to be present and in buffer zones, one positive test is sufficient to consider that a sample is infected. In vitro isolation is not recommended as a screening test because of poor isolation efficiency for *X. fastidiosa*.

The limit of detection of molecular tests being lower than that of serological tests, the use of molecular test is preferred for asymptomatic plant material, for insects, and for plant material from *X. fastidiosa*-free areas (EPPO, 2019). With *X. fastidiosa* forming biofilms, an additional ultrasonication step may help to disrupt biofilms and allow a better access of chemicals to bacterial cells to improve lysis (Dupas *et al.*, 2019; Bergsma-Vlami *et al.*, 2017). For PCR-based detection methods, inhibitory compounds from plant material (polyphenol content, polysaccharides) and from insects can be removed by chemical extraction methods such as CTAB-DNA extraction protocol (Francis *et al.* 2006) or partial dissection of insects to utilize only the head, where bacterial cells are located. Optimization of DNA extraction protocols is necessary when dealing with the small numbers of bacterial cells found in insect mouthparts (Bextine *et al.* 2004).

For the detection of *X. fastidiosa*, several conventional and qPCR tests have been validated (EPPO, 2019). These includes the conventional PCR test developed by Minsavage *et al.* (1994) and the real-time PCR tests described by Harper *et al.* (2010), Francis *et al.* (2006), Ouyang *et al.* (2013), Li *et al.* (2013), and Bonants *et al.* (2019). The tests of Harper *et al.* (2010) can also be used in a LAMP version (Yaseen *et al.*, 2015). Another isothermal amplification test proposed as an AmplifyRP XRT kit has been developed (Li *et al.*, 2016).

Identification of *X. fastidiosa* and assignation of subspecies can be performed directly on DNA extracted from plant material. The use of Multilocus Sequence Typing (MLST) analysis described by Yuan *et al.* (2010) is recommended by EPPO (2019) to identify strains. To be used directly on DNA extracted from plant material, slight modifications concerning Taq polymerase and primer concentration, and melting temperature were applied to the original protocol (Denancé *et al.*, 2019). Being based on end-point PCR, MLST-based identification has a higher limit of detection than the qPCR detection tests, also a nested version of the MLST-based identification has been proposed (Cesbron *et al.*, submitted). Sequencing of only two housekeeping genes is sufficient to assign a subspecies and detect possible recombinants. Subspecies assignment may also be performed by subspecies-specific molecular markers on isolated strains (Pooler and Hartung 1995, Hernandez-Martinez *et al.* 2006) or with multiplex qPCR test directly on extracted DNA from plant material (Dupas *et al.*, 2019).

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