



*Research article*

## **Histological analysis of *Xylella fastidiosa* infection in *Quercus pyrenaica* in Northern Portugal**

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**Abstract:** *Quercus pyrenaica* Willd thrives in the intermediate zone between the Mediterranean sclerophyllous and the temperate deciduous forest. In December 2022, the presence of the bacteria *Xylella fastidiosa* (*Xf*) was confirmed in samples collected from a *Quercus pyrenaica* located in Sabrosa, Vila Real, Portugal. Following *Xf* infection, the transport of water and nutrients is hindered due to the occlusion of xylem vessels. This loss of hydraulic conductivity may lead to vessel blockage and subsequent embolism formation. The objective of this study was to investigate the interaction between *Xf* and *Quercus pyrenaica* tissues, as well as the mechanism by which the bacteria can spread through the plant's xylem vessels, ultimately resulting in the formation of vascular plugs. At the time of the sample collection (10 months post-detection), symptoms of Bacterial Leaf Scorch (BLS) began

to appear. Examination of xylem vessels using both light and scanning electron microscopy (SEM) revealed the presence of various types of occlusions, predominantly tyloses. Additionally, fibrillar networks, gums, starch grains, and crystals were observed. The stem vessels exhibited significantly more occlusions compared to the leaves. Furthermore, individual bacterial cells were observed to be attached to the vessel wall. This implies that occlusions were primarily induced by tyloses and gums as a defensive response to the invasion of vascular pathogens, in addition to the pathogen itself. This study highlights the presence of starch grains in stems, which may function as a refilling mechanism, thereby preventing the loss of hydraulic conductivity in plants and potentially acting as a means to entrap the bacteria. These mechanisms exemplify the constitutive defense systems of the plant against *Xf*. Understanding the interaction between *Xylella fastidiosa* and *Quercus pyrenaica* is crucial, given that the latter species occupies nearly 95% of the natural distribution area of Portugal.

**Keywords:** histological analysis; tyloses; xylem; embolism; Bacterial Leaf Scorch

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## 1. Introduction

*Xylella fastidiosa* (*Xf*) is an aerobic, Gram-negative bacterium [1,2] known to proliferate within the xylem of a diverse set of host plant species [3,4]. Due to their capacity to economically infect important crops, they can induce various significant diseases and present a formidable threat to both plant health and agricultural productivity [3,5].

*Xylella fastidiosa* is naturally spread from one plant to another by insect vectors within the order Hemiptera, primarily including cicadas, aphids, and spittlebugs [6]. The majority of the primary European insect vectors belong to the Aphrophoridae family, which includes species such as *Philaenus spumarius*, *Philaenus italosignus*, and *Neophilaenus campestris* [4–7]. Insects acquire the bacteria by feeding on infected plants and subsequently introducing the bacteria into the transport system of the host plants by inserting their stylet into the leaf petiole, thus initiating their spread to the xylem of the branches and stems [4]. Following inoculation, the bacterial cells proliferate, creating a biofilm [2] capable of entirely obstructing the xylem vessels, thus impeding the transport of water and mineral salts [8].

Currently, it is widely acknowledged that the transportation of water and nutrients is hindered after infection. This is credited to the occlusion of xylem vessels by bacterial aggregates, along with the plant's production of gums and tyloses in reaction to the infection [3]. Consequently, typical disease symptoms such as chlorosis in the leaf margins, necrosis with a yellowish halo, wilting, and burning (necrosis), become evident [6].

*Xylella fastidiosa* has been identified in various plant species of commercial significance, notably in Italy, where it led to the demise of numerous centuries-old olive trees. However, several aspects of the disease are yet to be fully understood, as the mechanism of pathogenesis is still being investigated due to the intricate nature of the infection process [9].

Among the various diseases instigated by different subspecies of *Xf*, this study concentrates on the infection of *Quercus pyrenaica*. *Quercus pyrenaica* Willd., colloquially referred to as the Pyrenean oak, flourishes in the intermediate habitat between the Mediterranean sclerophyllous and the temperate deciduous forest [10]. Spanning approximately 600,000 hectares in Spain and 62,000 hectares in Portugal, *Quercus pyrenaica* Willd. occupies nearly 95% of its natural distribution area, making it one of the predominant oak species in the Iberian Peninsula [10,11]. In Portugal, the main concentration

of this species is noted in the Bragança region, covering approximately 40% of the total rangeland area [12]. Conversely, Pyrenean oak ecosystems are regarded as strategic environments for nature conservation aimed at sustaining and enhancing resources effectively [11].

The primary objective of this study is to delve into the intricate dynamics between *Xf* and *Quercus pyrenaica* tissues, aiming to unravel the mechanisms underlying the bacteria's propagation through the plant's xylem vessels, consequently resulting in the formation of vascular plugs. Moreover, our investigation aims to scrutinize the characteristics of tyloses and starch grains coexisting with the pathogen within xylem vessels, with the ultimate goal of shedding light on the sophisticated defense mechanisms employed by the plant to combat these bacterial invaders.

## 2. Materials and methods

The detection of *Xylella fastidiosa* in Portugal occurs through prospection. Prospecting for *Xylella fastidiosa* includes inspecting and carrying out the collection of samples and, in the event of their presence, implementing either control or eradication measures. In August 2022, 50 branches were randomly collected according to the General Directorate of Food and Veterinary (DGAV) protocol instructions, with 8-10 leaves in the four quadrants of a *Quercus pyrenaica* in Sabrosa, Vila Real, Portugal [13]. The components of the sample, leaves, and young stems from the tree were previously shaken, wrapped in newsprint paper, and placed in a plastic bag, carefully closed to protect the dispersion of possible insect vectors during transport. Then, after sample coding, the samples were sent directly to the Plant Health Laboratory from the National Institute of Veterinary Investigation (INIAV) for *Xylella fastidiosa* identification by polymerase chain reaction (PCR), according to the standard protocol 7/24 for *Xylella fastidiosa* from European and Mediterranean Plant Protection Organization (EPPO)[14].

In December 2022, the Plant Health Laboratory confirmed the identification of *Xylella fastidiosa* in *Quercus pyrenaica* located in Sabrosa, Vila Real. Following the confirmation of the bacterium's presence, this plant was selected for further sampling as it was the single positive specie in the northern region.

### 2.1. Plant sampling

For the morpho-anatomical analysis, leaves and young stems (n = 50) were collected in October from both *Xf*-positive and *Xf*-negative trees of *Quercus pyrenaica*. We did not include leaf petioles in our sampling protocol as our focus was primarily on assessing the impact of *Xylella fastidiosa* infection on the vascular tissues of the terminal stems and leaves midrib.

### 2.2. Histological Techniques (HT)

Histological analyses play a crucial role in the study of *Xylella fastidiosa* infection in *Quercus pyrenaica* trees by providing detailed insights into the interaction between the pathogen and its host at the cellular level. This information is essential to understand the disease dynamics and develop effective management strategies to mitigate its impact on forest ecosystems.

Histological Technique 1 (HT1): The purpose of HT1 is to obtain fresh, free-hand cross sections of the middle part of the leaf blade and young stems of *Quercus pyrenaica* immediately after harvesting.

These sections allow for quick examination of the plant tissue structure without extensive processing. The expected outcome of HT1 is to observe the general morphology and cellular structure of the plant tissue under a microscope.

Steps: In the field, fresh, free-hand cross sections (10  $\mu\text{m}$ ) of the middle part of the leaf blade and young stems of *Quercus pyrenaica* were made with the aid of a razor blade and a hand-microtome, immediately after harvesting. The sample cross sections were placed on a slide with a drop of distilled water and covered with a coverslip.

Histological Technique 2 (HT2): The purpose of HT2 is to enhance the transparency of fresh, free-hand cross sections using a solution of sodium hypochlorite, followed by dual staining with carmine and iodine green to visualize different tissue components. The expected outcome of HT2 is to observe the cellular structure of the plant tissue with an enhanced clarity and distinguish between different tissue components based on staining.

Steps: Moreover, some fresh, free-hand cross sections were clarified in a diluted solution of sodium hypochlorite to increase the transparency [15]. Following the attainment of a desirable level of transparency, the sections underwent a meticulous rinsing process using distilled water five times to remove excess sodium hypochlorite. Great care was taken to prevent any harm to the sections, as they had become exceptionally delicate during the clarification process. Following that, the handmade cross sections were subjected to a dual staining process using carmine and iodine green. First, they were treated with 0.4% alum carmine for 10 minutes, followed by rinsing with distilled water 5 times to remove excess carmine. Subsequently, the sections were stained with iodine green for 3–4 seconds [16]. After staining, the excess of green dye was eliminated through multiple washes with distilled water. This staining procedure resulted in the epidermis, parenchyma, and phloem elements appearing pink, while the sclerenchyma and xylem vessels appeared green.

After staining, the plant material was dehydrated in a graded ethanol series (70%, 80%, 90%, 95%, and 100%). The final clearing stage was executed using xylene, and the mounting process was finalized with Entellan (Merck, Darmstadt, Germany).

Alternative Histological Technique (HT3): An alternative histological technique was employed, wherein sections/parts of *Quercus pyrenaica* (leaf blades and young stems) were fixed in formalin-acetic acid-alcohol (FAA, 5:5:90 v/v) for 24 hours immediately after cutting in the field. HT3 is employed as an alternative method to either provide additional information or to overcome limitations of HT1 and HT2. It involves fixation, embedding, sectioning, staining, dehydration, clearing, and mounting to achieve a comprehensive visualization of the plant tissue structure. The expected outcome of HT3 is to observe the cellular structure of the plant tissue with a high resolution and detail, allowing for an in-depth analysis of the impact of *Xylella fastidiosa* infection.

Steps: After fixation of the plant material in the FAA solution for 24 hours immediately after cutting in the field, the plant material was dehydrated in a graded ethanol series (70%, 80%, 90%, 95%, and 100%). Samples were cleared by placing them in xylene for 1 hour; then, they were embedded overnight in liquid paraffin using a Leica EG1160 paraffin embedding station. After wax polymerization, the plant material was sectioned (5  $\mu\text{m}$ ) using a Leica RM 2135 Rotary Microtome and stretched on microscope slides. De-paraffinization was performed using xylene, and hydration was achieved by down-grading (100%–70%) the ethanol solutions. Staining with toluidine blue (0.1% for 7 minutes) preceded washing with water and new dehydration with ethanol. A final clearing step was performed with xylene, and mounting was completed in Entellan (Merck, Darmstadt, Germany).

### 2.3. Light microscopy

Specific parameters for light microscopy were set according to the desired magnification levels: 100x and 200x. The camera settings, such as exposure time, brightness, and contrast, were adjusted to optimize the image quality and clarity at each magnification level. Additionally, appropriate lighting conditions were ensured to minimize the glare and maximize the contrast for accurate visualization of the tissue structures.

Leaf and stem cross sections were observed under a light microscope Olympus IX 51 (Olympus Optical Co., GmbH, Hamburg, Germany) using an Olympus Colorview III camera and the software Cell\* (Soft Imaging System GmbH, Hamburg, Germany).

### 2.4. Scanning Electron Microscopy (SEM)

The investigation was conducted using a Scanning Electron Microscope (SEM) (SEM/ESEM FEI QUANTA-400) after preparation of the different samples (leaves and young stems). The SEM samples were prepared by affixing the plant tissue sections onto SEM stubs using an adhesive. The samples were directly mounted onto the stubs without an additional treatment or coating.

Images were acquired using a Low Vacuum Mode, maintaining a partial pressure inside the chamber of 1.33 mbar and an acceleration voltage of 30 KV, at various resolutions.

### 2.5. Quality control measures

Quality control measures were implemented throughout the sample collection, processing, and analysis stages to ensure the reliability and accuracy of the histological analysis of *Xylella fastidiosa* infection in *Quercus pyrenaica* in Northern Portugal. During sample collection, only healthy and visibly symptomatic plant specimens were selected to represent different stages of infection, thus minimizing the potential for sampling bias.

Moreover, the histological analysis was performed by trained personnel with expertise in plant anatomy and pathology to ensure an accurate interpretation of tissue structures and pathological changes associated with *Xylella fastidiosa* infection.

Overall, the implementation of rigorous quality control measures and careful consideration of potential biases helped to enhance the reliability and validity of the histological analysis in this study.

## 3. Results

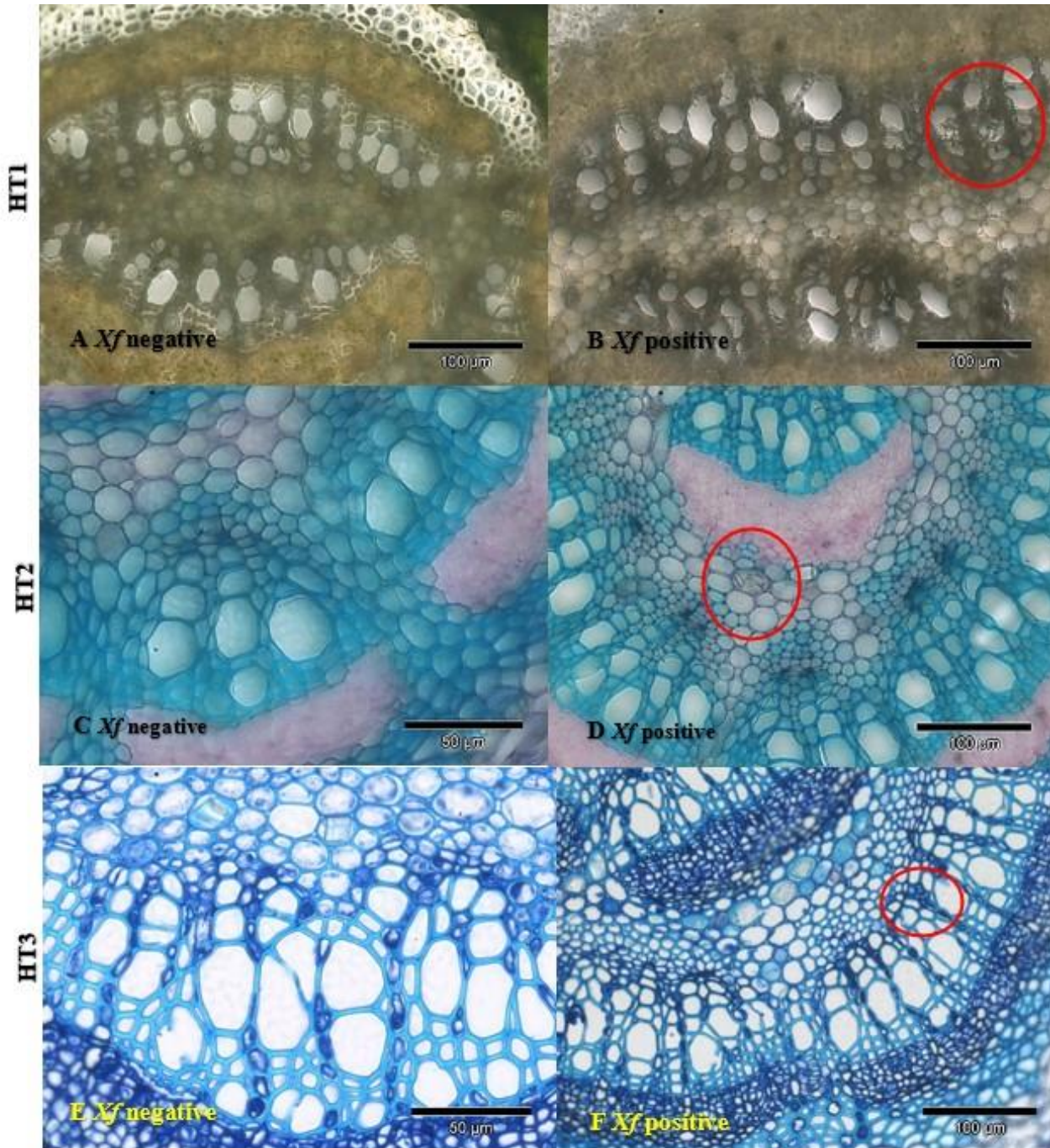
During the sample collection period (10 months post-detection), the symptoms of Bacterial Leaf Scorch (BLS) became apparent (Figure 1). These symptoms consisted of marginal leaf necrosis and chlorotic transition zones observed on leaves from the lower and middle sections of the oak. Moreover, most of the branches displayed symptoms of senescence.



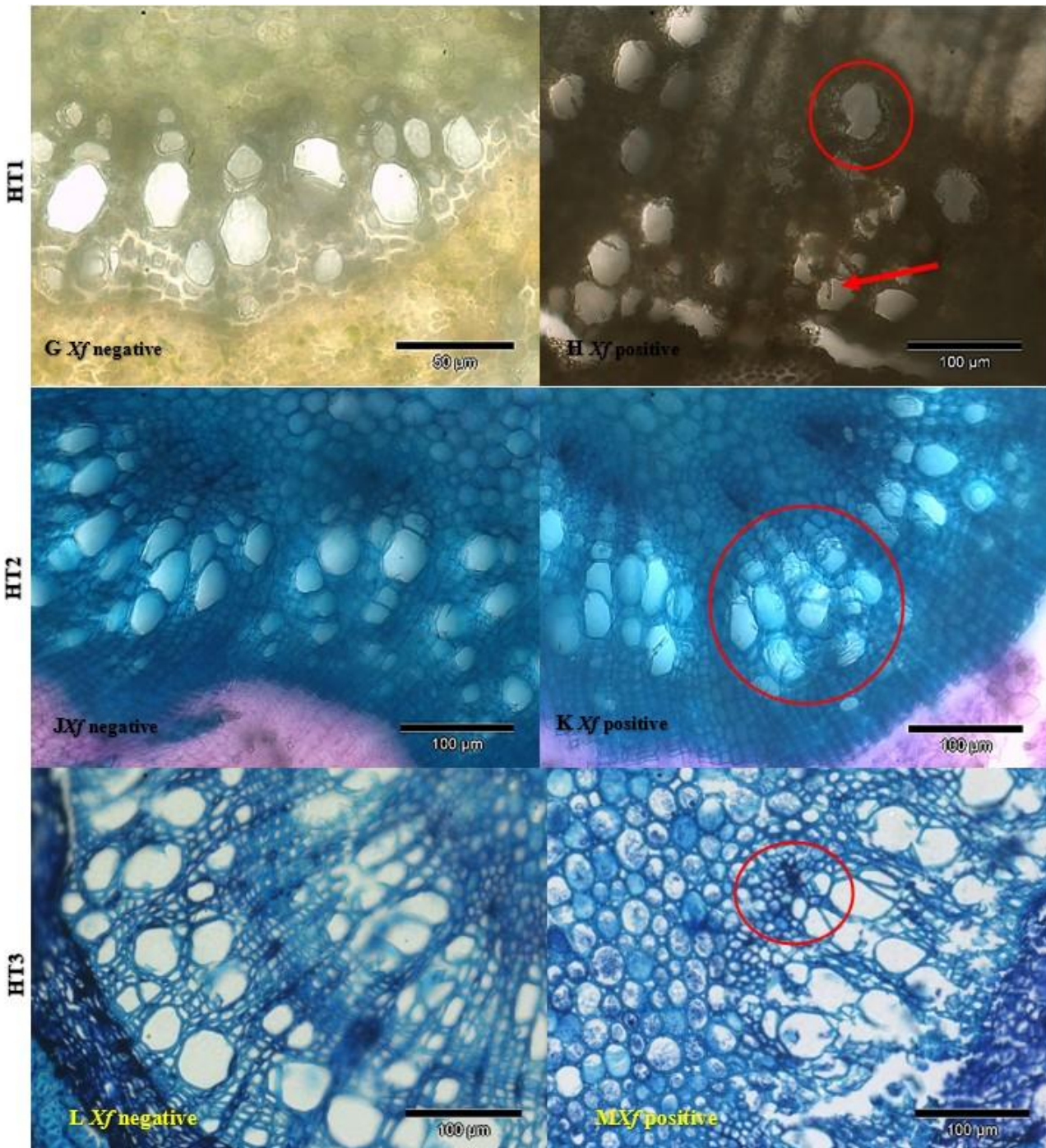
**Figure 1.** *Quercus pyrenaica* infected with *Xylella fastidiosa* 5 and 10 months after the positive result (author's image).

### 3.1. Types of occlusions observed

Examination of xylem vessels using SEM and light microscopy revealed the presence of different kinds of occlusions, mainly consisting of tyloses on *X. fastidiosa* positive plants (Figure 2B, 2D, 2F and Figure 3H, 3K, 3M) compared to negative ones. Fibrillar networks (Figure 3H), gums (Figure 4R), and calcium oxalate crystals (Figure 5) were observed on very rare occasions in infected plants. Numerous authors have also identified multiple forms of occlusion [17,18] Lin et al. confirmed significant disparities in xylem vessel occlusions, as well as variations in the types of occlusions, among four distinct *Vitis* genotypes [19]. Gzara et al. identified diverse forms of vascular occlusions, including tyloses, gels, starch grains, and gum deposits, by examining the stem cross-sections of five Tunisian grapevine cultivars using environmental scanning electron microscopy (ESEM) [20]. Using SEM, Fristschi et al. examined the stem internode and petiole tissues across four grape genotypes and revealed the presence of tyloses, fibrillar networks, and gum plugs within the lumens of tracheary elements in both control plants and those inoculated with *X. fastidiosa* [21]. Both De Benedictis et al. and Cardinale et al. noted that occlusions in twigs of field-grown infected olives primarily comprised of tyloses/gums and pectin gels [22,23]. On the contrary, Montilon et al. noted that occlusions were composed of gums or structures resembling callose. They did not detect the presence of tylose-like structures in the xylem vessels [3]. According to Sun et al., only one type of occlusion was observed [24].



**Figure 2.** Light microscopy images of transversal sections of the midrib of leaves of *Quercus pyrenaica*. In sections from *Xf* negative samples, vessels appeared almost free of occlusions; (B *Xf* positive) Presence of occlusions on xylem (surrounded by a red circle); (D *Xf* positive) In leaves' sections from infected samples, crystals were observed (surrounded by red circle); (F *Xf* positive) Presence of occlusions on xylem (surrounded by red circle).

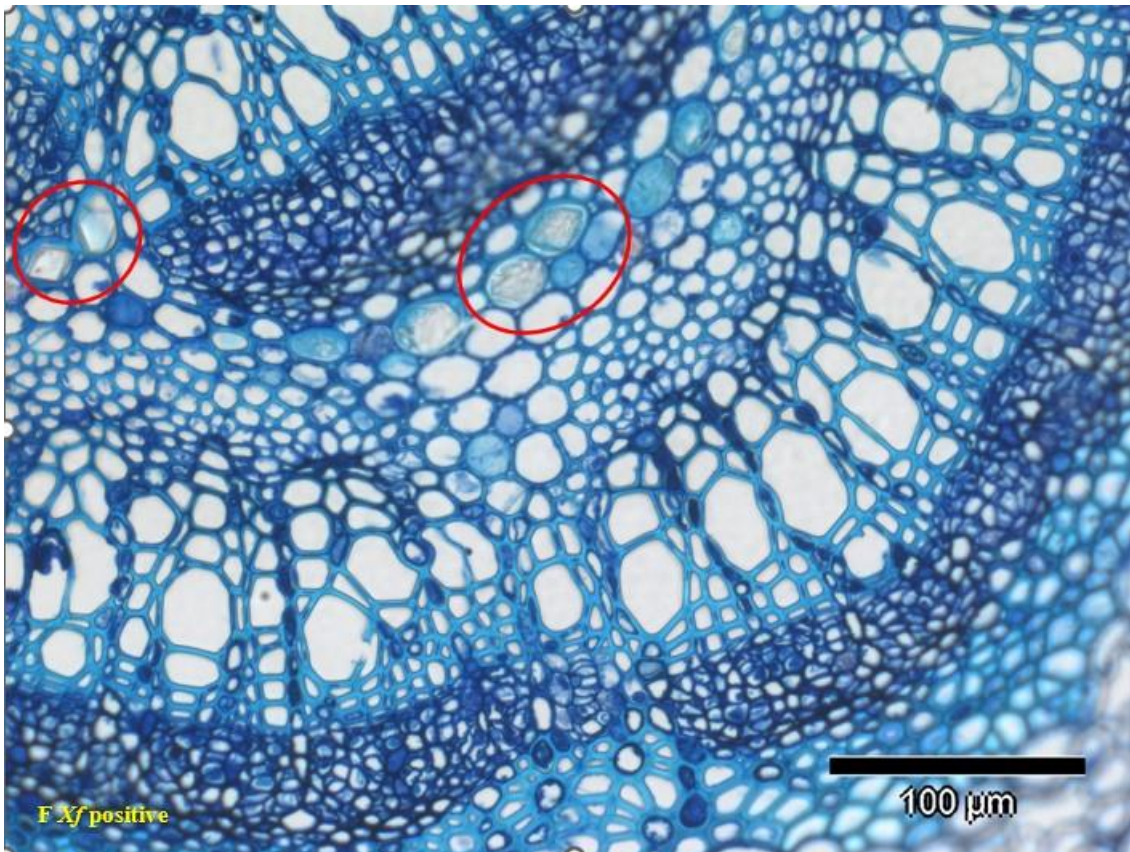


**Figure 3.** Light microscopy images of transversal sections of stems of *Quercus pyrenaica*. In sections from *Xf* negative samples, vessels appeared almost free of occlusions; (H, K, M *Xf* positive) Presence of tyloses (surrounded by red circle) and fibrillar network networks (indicated by red arrow).

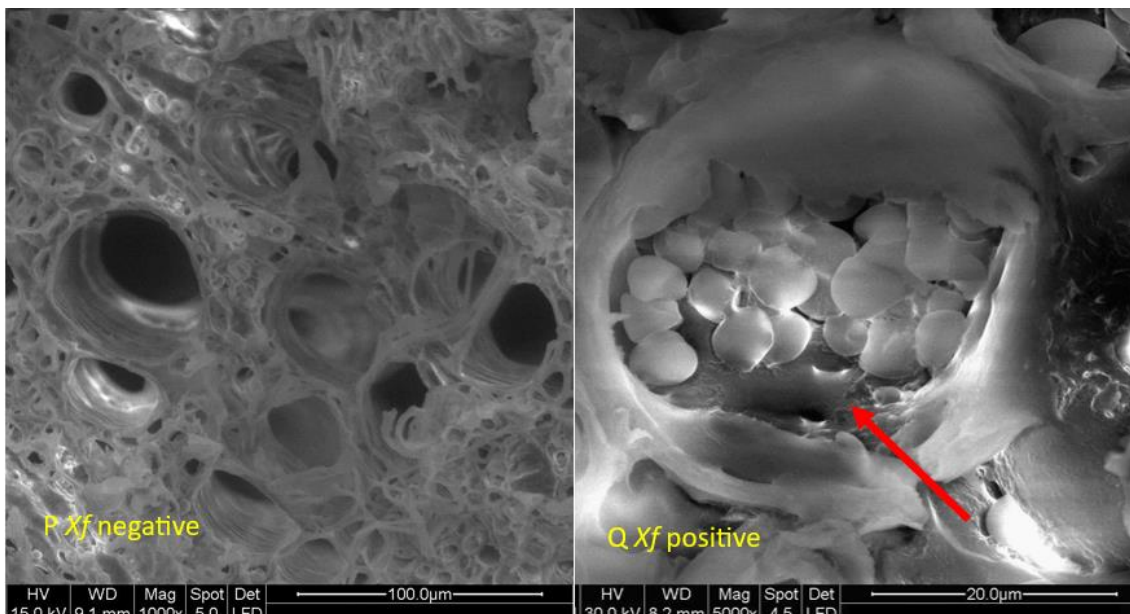




**Figure 4.** SEM microscopy images of transversal sections of stems of *Xf*-positive *Quercus pyrenaica*. (R) Presence of tyloses on xylem (indicated by blue arrow) and gum (indicated by red arrow); (S) SEM images of transversal sections of stems reveal a dense accumulation of starch grains (indicated by red arrow); (T) A bacterial cell was observed as an individual cell attached to the wall (surrounded by red circle).



**Figure 5.** Magnified detail of image 2F-*Xf* positive showing calcium oxalate crystals (surrounded by a red circle).

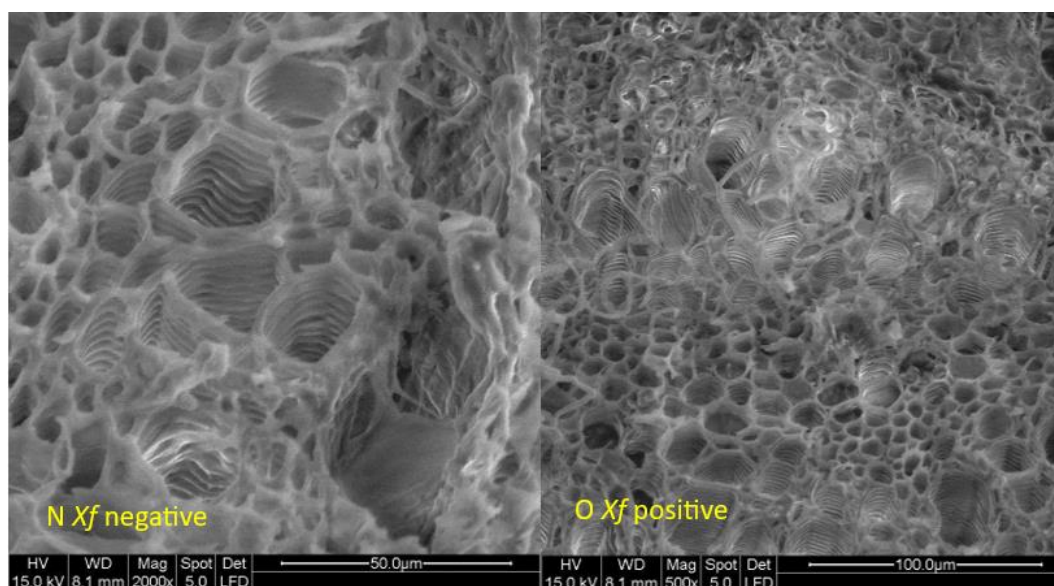


**Figure 6.** SEM images of transversal sections of stems appeared almost completely occluded with starch grains; Starch depletion in the xylem parenchyma, with intact PMs (indicated by red arrow).

In our research, the infected plants exhibited a high incidence of tyloses. This phenomenon may be associated with the development of air embolisms. Micco et al. found that petioles in oak trees infected with *X. fastidiosa* displayed increased embolisms before undergoing tylose production. These tyloses acted to seal affected vessels and redirected water to alternative vessels [25]. McElrone et al. detected embolisms in *Xf*-infected *Quercus rubra* and *Quercus palustris* before a notable decline in the petiole hydraulic conductivity (Ks), suggesting that embolism formation triggered hydraulic disruption, though it was not the sole cause of the occlusion [26]. Cochard and Tyree emphasized that the presence of air embolisms in *Quercus* vessels is a crucial prerequisite for the subsequent formation of tyloses in a particular conduit [27,28]. It can be inferred that the initiation of embolism formation triggers the development of tyloses in *Quercus pyrenaica*. This mechanism seals the affected vessels and redirects water to alternative pathways.

### 3.2. Distribution of occlusions

On the infected plants, the occlusions in the stem vessels, marked by the presence of tyloses, fibrillar networks, and gums (Figure 3 and Figure 4), were notably more abundant compared to occlusions in the leaves (Figure 2 and Figure 7). The leaves displayed tyloses and calcium oxalate crystals (Figure 2).



**Figure 7.** SEM images of transversal sections of leaves appeared with few occlusions.

Voltan et al. aimed to evaluate the blockage of xylem vessels in different parts of coffee plants. Among the plant organs studied, the stem showed the most significant obstruction of the xylem vessel elements, followed by the petiole, leaf blade, and root [29]. Stevenson et al. observed the development of anatomical symptoms in susceptible *Vitis vinifera* and noted that the leaf and petiole xylem were obstructed with gums and bacteria. In contrast, the stem xylem primarily showed occlusions with tyloses [30]. Additionally, Montilon et al. discovered significant differences in the distribution of occlusions among twigs [3]. This discrepancy in the distribution of occlusions can be explained by the unpredictable mode of bacterial colonization, which relies on the bacterium's ability to move within the xylem vessels.

Xylem tissue, which is the conduit for *X. fastidiosa* spread, can be classified into primary xylem and secondary xylem. The primary xylem is located in young organs (i.e., leaves, young stems, and roots), and is responsible for material transport and structural support; alternatively, the secondary xylem acts as a conductive and supportive tissue in more mature stems and roots [24]. The systemic dissemination of *X. fastidiosa* usually begins when introduced during the insect vector's feeding on an internode of a shoot. Subsequently, the pathogen moves both upward and downward along that shoot, utilizing stems containing substantial amounts of secondary xylem. The secondary xylem is characterized by numerous larger vessels featuring scalariform (ladder-like) pit membranes (and pit pairs) as the sole intervessel (I-V) pit membrane type. Conversely, the primary xylem comprises a restricted quantity of smaller vessels containing various types of I-V pit membranes [18]. These attributes of secondary xylem may ease the initial ingress, the subsequent progression of the pathogen, and the creation of vascular obstructions in stems containing substantial quantities of secondary xylem. Such traits could elucidate the findings in *Quercus pyrenaica*, thus shedding light on why occlusions were more common in the stems of this host compared to its leaves.

Conversely, Baccari et al. noted a greater occlusion in the petioles compared to stems in grapevines. In the context of grapes, it has been suggested that the heightened colonization of petioles could be attributed to their lower resistance in terms of their anatomy, characterized by clusters of xylem vessels that are shorter and narrower than those found in stems [31].

The successful systemic colonization of the xylem by *X. fastidiosa* is highly dependent on the pathogen's secretion of cell wall-degrading enzymes, as demonstrated by Roper *et al.* in 2007 [32]. During this process, *Xf* must traverse the xylem cells via the preexisting xylem pores known as pit membranes (PMs) [5]. PMs, which vary in diameter from 5 to 20 nm, are composed of hemicellulose, cellulose microfibers, and pectins, forming a porous network that connects adjacent plant cells. Their function is to impede the movement of bacteria and air bubbles, thus shielding plants from embolisms [3]. In contrast, the size of *Xf* cells ranges from 250 to 2400 nm [1]. A proposal suggested that the activation of diverse cell wall degradation enzymes [24,33] could lead to an expansion of pores between neighboring xylem vessels, thus facilitating the movement of bacteria from one vessel to another [34]. Sun and colleagues documented the deterioration of xylem membrane cell walls in infected vines. Meanwhile, Montilon and co-researchers demonstrated the systemic spread of bacteria through PMs in susceptible olive tree cultivars. These vulnerable varieties exhibited notable degradation of the middle lamellae, which facilitated bacterial transmission [3,24]. Throughout infections, bacterial decomposition enzymes can break down the constituents of the PMs, resulting in a heightened porosity. The impact of these enzymes' activity was recently examined by Fanton and Brodersen in grapevines [35]. Additionally, Pérez-Donoso et al. confirmed the pivotal role of these enzymes in diminishing the integrity of pit membranes. As a result, the inter-vessel movement of *X. fastidiosa* is likely to require enzymatic disruption of the PMs, markedly increasing the susceptibility of vessels to cavitation [36]. Montilon et al. reported that electron microscope examinations unveiled *Xf*'s utilization of PMs for systemic dissemination. After being infected by *Xf*, the PMs experience degradation, thus indicating the involvement of enzymes that break down bacterial cell walls [3].

In our investigation, we did not observe degraded middle lamellas, which is a condition that would enable bacteria to traverse adjacent vessels through degraded pit membranes. Instead, we identified vessels with intact PMs (Figure 6Q), which likely maintained the impermeability of these structures to the bacteria. Therefore, our results provide evidence for the hypothesis, particularly established in the grapevine/PD pathosystem, that the impairment of xylem conductivity acts as an initial event in the

pathogenesis mechanism of the hosts [37,38]. As per Mc. Elrone et al., an embolism probably occurs due to the deterioration of PMs during the colonization of new petiole xylem [39]. Our findings indicated that *Quercus pyrenaica* possesses xylem vessels with intact PMs and a substantial coating of lignin, likely rendering them more resistant to bacterial migration. It can be hypothesized that in *Quercus pyrenaica*, bacteria are recognized and confined within the primary xylem, thus prompting the secretion of defense chemical compounds such as tyloses. We can deduce that the stem morphology acts as a preexisting barrier that prevents bacterial movement in *Quercus pyrenaica*, partially accounting for its slower colonization compared to other host plants.

### 3.3. Presence of bacterial cells

In our study, we observed a bacterial cell attached to the vessel wall as an individual entity (see Figure 4T). Bacterial cells attach to the vessel wall and cluster within a biofilm matrix through proliferation. This matrix is comprised of nucleic acids, proteins, humic substances, and exopolysaccharides (EPS). These biofilms act as a protective barrier for bacterial communities, providing resilience against dehydration, host defenses, and various stresses. Furthermore, biofilms enhance virulence by enabling the coordinated expression of pathogenicity genes through quorum sensing [40]. Traditionally, it is believed that the biofilm serves as the primary factor responsible for obstructing water movement in *X. fastidiosa*-infected plants. However, research has revealed that vessel occlusions also arise from the active responses of host plants to the presence of the bacterium, thus serving as a defense mechanism [30,40,41].

De Benedictis et al. noted that occlusions primarily consisted of tyloses, gums, and pectin gels rather than aggregates of *Xf* cells [23]. In a similar vein, Cardinale et al. noted a sparse presence of bacterial cells within vascular occlusions [22]. In the study conducted by Ingel et al., *X. fastidiosa* cells initially evaded detection and were not observed in the xylem vessels of infected vines. However, as symptoms of Pierce's disease advanced, *X. fastidiosa* bacteria became readily visible in approximately 10% of the vessels, appearing both as individual cells and as small cell aggregates along the lateral walls of the xylem vessels [23].

In many vessels, it appears that a localized bacterial presence is not necessary for tylose development.

During their observations, Falsini et al. noted a pink/violet matrix either devoid of bacterial cells or with degenerated ones. This observation led to the hypothesis that the matrix could be generated by the host plant as a defense mechanism against bacterial dissemination [42]. Voltan et al. noted no significant variances in the presence of the bacterium, leading them to conclude that other physiological or environmental stress factors likely contributed to the plant's response [29]. Ingel et al. found that most vessels with tyloses did not contain bacterial cells. This finding implies that direct, localized recognition of *X. fastidiosa* was not the primary factor that triggered tylose formation [37]. In Pierce's disease in a grapevine, a notable proportion of colonized vessels in the infected leaves were discovered to be unobstructed. Rather, they housed small colonies or individual cells, indicating that vessel blockage was not an intentional colonization strategy utilized by the pathogen [41]. Micco et al. linked the dynamic process of obstruction formation to a wound repair program activated in response to vascular pathogen invasion [25]. Perez-Donoso et al. observed that internodes contained a significantly higher number of vessels occluded by tyloses compared to vessels containing *X. fastidiosa*. This indicates that a localized bacterial presence is not necessary for tylose development in many vessels. It suggests that a signal originating from vessels colonized by *X. fastidiosa* induces

tylose formation in vessels where the bacteria are not detected [36].

It seems that the relatively low counts of bacteria in infected plants are unlikely to cause the reduction in the water transport capacity and the consequent external disease symptoms observed in *X. fastidiosa*-infected plants [24,34]. Additionally, Sabella et al. posited that the decline in hydraulic conductivity might arise from vessel blockages due to both bacterial activity and plant responses, potentially triggering an embolism. The ability of infected plants to detect an embolism and engage mechanisms to restore the hydraulic conductivity could impact the severity of the disease symptoms [43]. Our findings corroborate those of the previously mentioned authors. The discovery of a single bacterium in *Quercus pyrenaica* suggests that tylose formation was a response to the invasion of vascular pathogens.

#### 3.4. Xylem Anatomical characteristics as a host defense mechanism

Alves et al. showed that the percentage of xylem vessels colonized by bacteria could also be affected by the anatomical features of the host [44]. Previous studies have hypothesized about the impact of the vessel diameter on a plant's vulnerability to xylem-dwelling pathogens, with some cases indicating a resistance in plants possessing shorter and narrower vessels. In a study examining the vascular anatomy of 12 grape varieties susceptible to *X. fastidiosa*, Chatelet et al. noted that tolerant host plants displayed narrower vessels. They proposed that this narrowing could hinder the movement of bacteria within the xylem vessels [45]. Sun et al. performed a quantitative assessment of vascular occlusions in grapevine genotypes susceptible and resistant to Pierce's Disease. They pinpointed tyloses as the predominant occlusive form, notably prevalent in larger vessels. While tylose occlusions didn't completely stop the systemic spread of the pathogen, they noticeably prevented water conduction, thereby accelerating the progression of disease symptoms in susceptible genotypes [24]. Similarly, Pouzoulet et al. documented an elevated susceptibility to the vascular pathogen *Phaemoniella chlamydospora* in grapevines with larger vessel diameters. This heightened susceptibility was linked to enhanced fungal compartmentalization within wider vessels. Moreover, larger vessels displayed a higher prevalence of tyloses and occlusions, thus fostering a favorable environment for pathogen proliferation while concurrently diminishing the plant's hydraulic conductivity. The processes involved in the refilling of xylem vessels were recognized as pivotal elements in conferring resistance to vascular wilt diseases [46].

Anatomy alone may not fully explain the observed differences, as there is a possibility that the composition of the xylem fluid may also influence the colonization [47]. The ionic composition of xylem fluid can either augment or diminish hydraulic conductance within the vessels. Higher concentrations of monovalent ions in the xylem fluid may elevate hydraulic conductance, whereas divalent cations may reduce it [44]. The role of calcium and magnesium has been suggested to elucidate the attraction of *X. fastidiosa* to other cells or the xylem walls. [48]. The anatomical characteristics of the xylem and the overall chemical composition of the fluid may also impact the ability of these cells to either form large aggregates or persist longer as small aggregates and free cells.

#### 3.5. Starch grains depletion as a host defense mechanism

In this study, the presence of starch grains in the stems was documented (see Figure 6Q and Figure 4S). The presence of starch grains could be linked to a reduced photosynthetic activity. It has been proposed that the initiation and expansion of tyloses within the vessel lumina depend on the

development of a sufficient turgor pressure in xylem parenchyma cells, surpassing the yield threshold of the cell wall structures of PMs [30]. This mechanism and the development of increasing xylem tension gradients during subsequent transpiration cycles could account for a large number of embolisms [28]. In a reaction, plants typically close their stomata, intending to lower the transpiration rates and mitigate the risk of cavitation. Nevertheless, this adaptive reaction carries a price, resulting in a significant decline in photosynthesis and the overall plant growth [49]. When confronted with a reduced photosynthetic activity, the nearly stagnant plant growth triggers the accumulation of carbon in the shape of starch grains within the xylem rays of the stem. Amid severe water stress episodes, plants can utilize this stored starch to support crucial processes such as respiration, metabolism, and defense mechanisms [50]. Nevertheless, persistent water stress arising from a growing number of vessel obstructions can lead to a sustained decline in photosynthesis. In the absence of a sufficient carbon supply over an extended period, the depletion of starch reserves occurs as the demand for carbon necessary for survival surpasses the available resources [50]. This phenomenon is known as carbon starvation and, when combined with hydraulic failure from chronic water stress, can cause leaf shedding, disruption of vascular transport, and plant death [37].

Pouzoulet et al. recorded a notable accumulation of starch reserves in grapevine cultivars resistant to the relevant stressor [46]. Moreover, Valtaud et al. documented significant quantities of starch grains in leaves originating from non-infected canes, which they considered as an initial defense mechanism [51]. As per Nardini et al., the depolymerization of starch seems to be triggered during xylem refilling [52]. Nardini et al. have advanced the osmotic hypothesis as a mechanism for reinstating hydraulic conductivity. According to this hypothesis, an active process occurs where embolized vessels are refilled with water. The impetus for this refilling is created by enriching the sap with solutes, which is comprised of inorganic and organic substances (like proteins and polysaccharides), as well as sugars derived from the hydrolysis of starch. The breakdown of starch in xylem parenchyma cells yields soluble sugars, which are then liberated into the vessels, thus generating an osmotic flow of water into their lumens [52]. Additionally, Ingel et al. employed X-ray computed microtomography and machine-learning techniques to demonstrate that *X. fastidiosa* triggers a notable depletion of starch in xylem-ray parenchyma cells. This suggests that a signaling mechanism originating from bacteria-inhabited vessels enables a systemic response to *X. fastidiosa* infection [37]. Montilon *et al.* discovered that the resistant cultivars employ a more effective strategy for xylem refilling, as evident from the presence of starch grains in the vessels of infected plants [3].

Starch hydrolysis is a recognized strategy for replenishing xylem vessels that have experienced cavitation. It involves the dense accumulation of starch grains in the xylem parenchyma [43]. The reduction in starch is likely linked to the provision of carbohydrates to synthesize the cell wall materials necessary for tylose formation. This starch depletion, coupled with the downregulation of the photosynthetic machinery, results in chronic carbon deficiencies. Additionally, hydraulic failure caused by obstructions in the later stages of the disease restricts the water supply to the leaves, prompting stomatal closure and decreased photosynthesis due to reduced carbon dioxide diffusion. As a result, thirst and starvation are likely intertwined and contribute to the eventual demise of plants succumbing to infection [37].

Through an SEM-EDX analysis on stem cross-sections of infected plants, Sabella et al. revealed an accumulation of starch grains in xylem vessels. These findings imply that this species may have the ability to initiate more efficient refilling mechanisms, thus restoring the hydraulic conductivity of vessels [43]. Additionally, Masrahi et al. observed a notable abundance of starch grains in the xylem

parenchyma of the lianas *Cocculus pendulus* and *Leptadenia arborea*. This profusion of starch grains has the potential to lower the solute potential of the xylem sap, thereby facilitating the upward flow of water. This suggested strategy could assist plants in remedying air-filled vessels and surviving in the water deficit of their demanding habitat. Additionally, the presence of starch granules within the fibers of grapevine xylem suggests that they play a role in generating an osmotic driving force for the refilling process [53]. Likewise, Bucci et al. detailed the prevalent presence of starch granules observed in the vessels of *Schefflera macrocarpa* and *Caryocar Brasiliense*. They proposed that these starch granules might augment vascular water uptake by osmotically elevating active solutes [54]. Our observations of starch grains align more closely with the depiction by Nardini *et al* [52]. The presence of starch grains in *Quercus pyrenaica* may act as a refilling mechanism, thus preventing the loss of plant hydraulic conductivity and potentially serving to entrap the bacteria.

Another notable finding is the consistent absence of bacteria in the living cells of cambium and phloem tissues. This supports the hypothesis that *Xf* predominantly thrives when surrounded by deceased cells throughout its lifecycle.

#### 4. Conclusions

In this investigation, the presence of *Xylella fastidiosa* was identified through a PCR analysis conducted at the INIAV Plant Health Laboratory. The infected *Quercus pyrenaica* plant was naturally found in the outbreak area of Sabrosa, Vila Real. Observations using LM and SEM revealed that the host responded to the infection by generating tyloses, fibrillar networks, gums, and starch grains.

Despite our understanding of the interactions between *Xylella fastidiosa* and the xylem, vascular occlusions remain a prevalent occurrence in plants affected by this vascular disease. The discovery of a solitary bacterium in *Quercus pyrenaica* implies that occlusions are primarily induced by tyloses and gums as a defensive response to the invasion of vascular pathogens, in addition to the pathogen itself.

The factors determining whether parenchyma cells produce one type of occlusion over the other, and whether these inclusions contribute differently to disease resistance or susceptibility, remain unclear. However, we can infer that the presence of secondary xylem in *Quercus pyrenaica* stems may facilitate the initial entry of the pathogen.

Our findings indicate that *Quercus pyrenaica* exhibits xylem vessels with intact PMs and a nearly complete lignin coating, rendering them less permeable to bacterial migration. Given the oak's limited symptom manifestation and the detection of a lone bacterium in *Quercus pyrenaica*, it can be inferred that the formation of tyloses may impede the pathogen's spread from the initial entry points.

This study highlights the presence of starch grains in stems. Starch grains in *Quercus pyrenaica* may function as a refilling mechanism, preventing the loss of hydraulic conductivity in plants and potentially acting as a means to entrap the bacteria.

These mechanisms exemplify the plant's constitutive defense systems against *Xf*. According to the EFSA data, there were two *Quercus ilex* reported as resistant to *Xf* [55]. However, the *Quercus pyrenaica* in question was destroyed in October 2023, one year after detection, showing almost no symptoms. Understanding the interaction between the host plants, the pathogen, and the environment is crucial. Immediate action is imperative, including streamlining permissions for field-based scientific research within our nation. This will enable the transfer of knowledge and the development of sustainable solutions tailored to various crop types, soil compositions, and climate variations.



## Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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## Conflict of interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

## Authors contributions

Conceptualization, T.L., B.G., I.C., P.P.; methodology: T.L., B.G., I.C., P.P.; validation: T.L., B.G., I.C.; formal analysis: A.M., B.G., I.C., P.P.; investigation: T.L. and L.S.; data curation: T.L. and L.S.; writing original draft preparation: T.L. and L.S.; writing, review and editing: T.L., B.G., I.C., P.P., A.M. All authors have read and agreed to the published version of the manuscript.

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