

Article

Xylem Sap Mycobiome in Grapevine Naturally Infected with *Xylella fastidiosa*. A Case Study: Interaction of *Xylella fastidiosa* with *Sclerotinia sclerotiorum*

A. Perelló ^{1,*}, A. Romero-Munar ², I. Martínez ⁴, A. Busquets ³, M. Cañellas ², B. M. Quetglas ², R. Bosch ², J. Vadell ², C. Cabot ² and M. Gomila ²

¹ UCA-FICA-CONICET Pontificia Universidad Católica Argentina, Facultad de Ingeniería y Ciencias Agrarias Av. Alicia Moreau de Justo 1300, CABA, Buenos Aires, Argentina.

² Department of Biology. University of the Balearic Islands, Cra. Valldemossa, km 7,5, 07122 Palma, Spain

³ Scientific-Technical Services. Universitat de les Illes Balears, Cra. Valldemossa, km 7,5, 07122 Palma, España.

⁴ CIDEFI-CONICET FCA y F-UNLP, Buenos Aires, Argentina.

* Correspondence:

Abstract: Grapevine (*Vitis vinifera*) is an economically crucial crop for agriculture, especially in Mediterranean regions. *Xylella fastidiosa* subsp. *fastidiosa* (*Xff*), a bacterium responsible for serious plant diseases as Pierce's disease, poses a growing threat to viticulture. This study aims 1) to explore the diversity of culturable fungal endophytic communities present in the grapevine sap of naturally *Xff*-infected grapevines in the field and 2) to study the interaction between a pathogenic fungus identified in the xylem sap with *Xff*. Xylem sap was collected from vines of cv. Cabernet Sauvignon in a vineyard in Mallorca, Spain. The microbial communities were analyzed using traditional culture-depending techniques for fungal identification. Beneficial species identified included *Aureobasidium pullulans* and *Rhodotorula mucilaginosa*, which have antagonistic activities against fungal species associated with grapevine trunk diseases (GTDs). Nonetheless, the pathogenic fungi *Sclerotinia sclerotiorum* (*Ss*) and some members of the Dematiaceae taxonomic group like *Phoma* complex, *Cladosporium* sp, and *Alternaria alternata* were also identified. A similar diversity of fungal species was found in plants that tested negative and positive for the presence of *Xff*. Despite the small samples size used, these preliminary results suggest a potential complex interaction between *Xff* and the sap endophytic microbiome. In addition, the interaction between *Xff* and *Ss* was further studied in grapevines artificially inoculated with *Xff* under controlled conditions. Interestingly, the results showed a synergistic effect, as *Xff*-inoculated grapevines were more vulnerable to *Ss* infection. This study provides novel insights into the fungal endophytic communities associated with *Xff* in grapevines-infected plants, and highlights some potential interactions among the bacteria and the sap microbiome components. During microbial interference, depending on the edaphoclimatic and crop managing conditions, while some of the identified mycobiota members could prove beneficial yielding plants more tolerant to *Xff*, others could be detrimental for grapevines as *Xff* could accelerate fungal diseases.

Keywords: endophytic mycobiome; grapevine; *Xylella fastidiosa*; cross-kingdom interactions; synergism

1. Introduction

Grapevine (*Vitis vinifera*) is one of the most widely cultivated fruit crops with a great economic impact on the agriculture industry. This crop is part of the landscape of the entire Mediterranean region where viticulture is of high economic relevance, whether for fruit fresh consumption, dried fruits or for wine production. In Mallorca, the history of wine dates back to the Romans who introduced the cultivation of grapevines to the island and ever since, wine has continued to be produced with varying degrees of success. Among the most recent disturbing events experienced by the viticulture industry on the island was the detection in 2016 of *Xylella fastidiosa*, a quarantine organism in the European Union. *Xylella fastidiosa* subsp. *fastidiosa* (Xff) [1] is the causal agent of Pierce's Disease (PD), one of the most destructive diseases for viticulture in affected regions and has led to substantial economic losses for the wine industry [2]. Moreover, the incidence of PD under climate change conditions is expected to increase for the Mediterranean regions likely to severe summers and milder winters that would favor the spread of this disease [3].

Xylella fastidiosa is a gram-negative, xylem-confined bacterium, extremely slow-growing in culture. Natural transmission occurs via insects feeding suctorially on xylem sap, with an efficiency that varies among vector species [4].

The bacterium overwinters in the xylem of the host plants as well as in weeds [5]. and do not kill the hosts until later stages of its life cycle. Moreover, this causal agent can also live asymptotically as endophyte [2,4]. In susceptible grapevines, the bacterium produces occlusions in the xylem vessels reducing the plant's hydraulic conductivity [6]. The strategy of grapevines to constrain Xff infection involves, among others, the formation of tyloses, a defense response also deployed against grapevine trunk diseases (GTDs) [7].

Few studies to date have focused on the interaction of Xff with the xylem sap endophytic community. Changes in the bacterial population and disease symptoms expression in Xff-infected grapevines were reported [8]. Moreover, different components of the microbiome in Xff-infected grapevines with antimicrobial activity and plant growth promoting potential have been reported recently. Furthermore, few reports on the potential synergic effects that the microbiome could exert on Xff virulence are available. Along this line, the presence of the endophytic N-fixing *Methylobacterium* increased the symptoms caused by Xff subsp. *paucum* in *Citrus sinensis* is mentioned [9]. However, interactions between Xff and fungal pathogens have not been documented. Thus, the studies of these interactions considering that coexistence Xff and these fungal pathogens in the same host plant are important, as they may influence the epidemiological scenario of plant diseases.

On the other hand, *Sclerotinia sclerotiorum* (Ss) is a devastating necrotrophic fungus affecting over 500 plant species worldwide [10]. It belongs to the *Sclerotiniaceae* family and is characterized by white cottony mycelium and melanized sclerotia, of which can remain viable in soil for up to 10 years [11]. Infection occurs through two germination pathways: carpogenic germination, where sclerotia release airborne ascospores that land on plant tissues, germinate, and penetrate using decaying material as a nutrient source [12]; and myceliogenic germination, in which sclerotia germinate directly, forming hyphae that infect the plant stem base or produce new sclerotia in the absence of a host [13,14]; This fungus was reported on grapevine causing shoot blight in Chile [15,16,17,18]. Additionally, this fungus was reported among the endophytic mycobiota associated with *Vitis vinifera* in the Iberian Peninsula [19] but not as a pathogen causing visible symptoms on that crop. In 2021, grapevines cv. Callet growing in a commercial vineyard located northeast of the island of Mallorca showed severe symptoms of canker and shoot blight during spring and early summer, with a 70% incidence [20]. The presence of Ss was also confirmed as a component of the mycobiome of the xylem sap of the vineyard sampled in this current study.

Taking the above into consideration, due to the lack of information and data on grapevine endophytic communities in Mallorca, this work aimed to (1) preliminary

explore the diversity of the fungal culturable endophytic community of sap grapevine that occupy an ecological niche that overlaps with *Xff*, focusing on candidates for biological control of the bacterium or potential phytopathogens associated with fungal diseases interacting with *Xff* on grapevine plants (2) as to acquire new knowledge on the interaction among *Xff* co-occurring with the phytopathogenic fungi *S. sclerotiorum*.

2. Results

2.1. Fungal Microbial Diversity Recovered from the Sap of Grapevine Plants

The fungal identification is shown in **Figure 1**. These results indicated the different groups of microbiota diversity in xylem sap of grapevines cv. Cabernet Sauvignon recovered. Most of the endophytic fungi recovered belonged to the Phylum Ascomycota with two main groups: yeast-like and filamentous (mycelial). These species can be considered to belong to different functional groups, true endophytes, beneficial saprophytes, opportunistic or not, and latent pathogens associated with trunk's diseases.

Regarding the first group, the yeast-like fungi, the morphocultural and microscopic analysis shown the presence of single-celled, spherical or elliptical spores, 3–15 µm in size, which could give rise to the formation of pseudohyphae (yeasts) when the sprouted cell does not separate from the mother cell. On Sabouraud agar, pale and opaque, mucilaginous colonies developed, with some species with characteristic pigments, although they were generally cream, pink or dark in color. Microscopically, most of the yeast species differed very little and thus physiological tests are necessary for their complete identification. Among them, *Aureobasidium pullulans*, *Rhodotorula mucilaginosa* and other yeast-like fungi that were recovered from the samples, stand out for their potential use in future biological control tests that could position them as promising antagonists to modulate the impact of GTDs.

The second group, the filamentous fungi, belong to the Phylum Ascomycota, the Deuteromycetes's group (Imperfect Fungi) which show only asexual reproduction and several types of vegetative spores. Among them, *Penicillium* spp. complex, along with *P. chrysogenum*, were recovered from the sap of grapevines that had tested positive or negative for the occurrence of *Xff*. Moreover, some fungi of potential risk, specifically those previously mentioned as pathogens for grapevine like *Sclerotinia sclerotiorum*, *Cladosporium* sp and *Phoma* complex and *Alternaria alternata* were also identified.

Depending on their presence in the xylem sap of plants that tested positive or negative for the occurrence of *Xff*, the differences in the fungal diversity complex recovered are shown in **Figure 1**. Qualitative-quantitative differences in the structure of the microbiome recovered were found to show a higher relative diversity in the sap samples of plants that tested positive for the presence of *Xff* compared to those samples that tested negative to the bacteria. A total of 13 different groups/complex of fungi were recovered; however, some of these, like *P. chrysogenum*, *A. alternata*, *Cladosporium* sp. only appeared in plants that had tested negative to the presence of *Xff*, while *S. sclerotiorum*, yeast-like, *Phoma* complex and *A. pullulans* appeared associated in plants that tested positive to the bacteria. The rest of the microorganism isolated were shared by both groups (*Aspergillus* sp., *Botryosphaeria* complex, Coelomycetes, *Penicillium* spp., *Phaeoacremonium/Phaeomoniella* sp., *Phomopsis/Diaporthe* complex, *Rhodotorula mucilaginosa*). Related to the Dematiaceae complex group, the morphocultural analysis has shown a great presence of Coelomycetes, with conidia formed in closed or partly closed fruiting structures with type pycnidial conidiomata e.g *Phoma* complex clade (**Figure 1**). Interestingly, in addition to those species belonging to the Dematiaceae complex identified here, the other taxonomical members of the Coelomycetes group like *Phomopsis/Diaporthe* complex and *Cladosporium* spp. were

identified by morphobiometrical features and their identities need to be corroborated by molecular techniques.

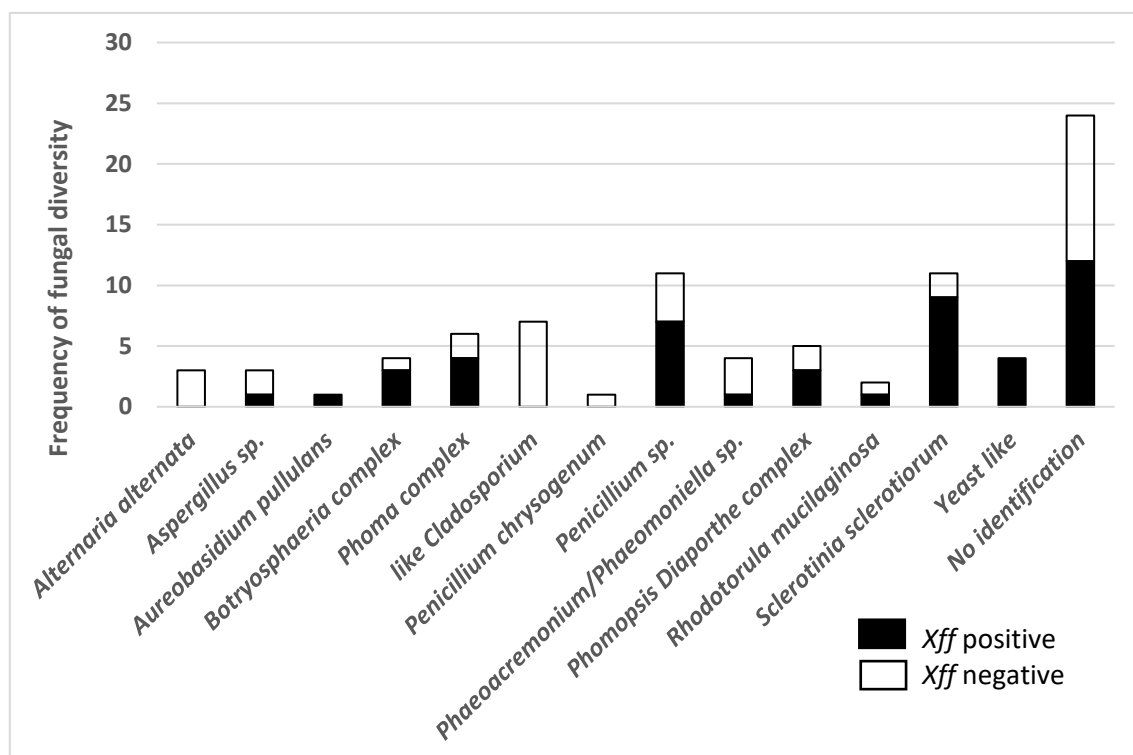


Figure 1. Mycobiome diversity in the xylem sap collected from a vineyard in Mallorca, Spain. Data from 86 fungi isolates, obtained from two plants that tested positive and three plants that tested negative for the occurrence of *Xff*. *No identification*: fungal genus unclassified and referred to as unidentified taxon.

2.2. Interaction Between *Sclerotinia sclerotiorum* (Ss) and *Xylella fastidiosa* (Xff) on Grapevine Plants Under Greenhouse-Controlled Conditions

After the artificial infection with Ss, the symptoms observed 7 days post inoculation (dpi) were necrosis in the stems, which evolved in an elongated and extended shape up and down from the initial point of infection, as the disease progressed. As a result of the necrotic lesion, many shoots broke and fell prematurely at 5 dpi. Other symptoms recorded were necrosis of the leaf petioles, epinasty and, as a consequence, leaf wilting of the compromised leaves in the shoots with symptoms (**Figure 2, 3**).

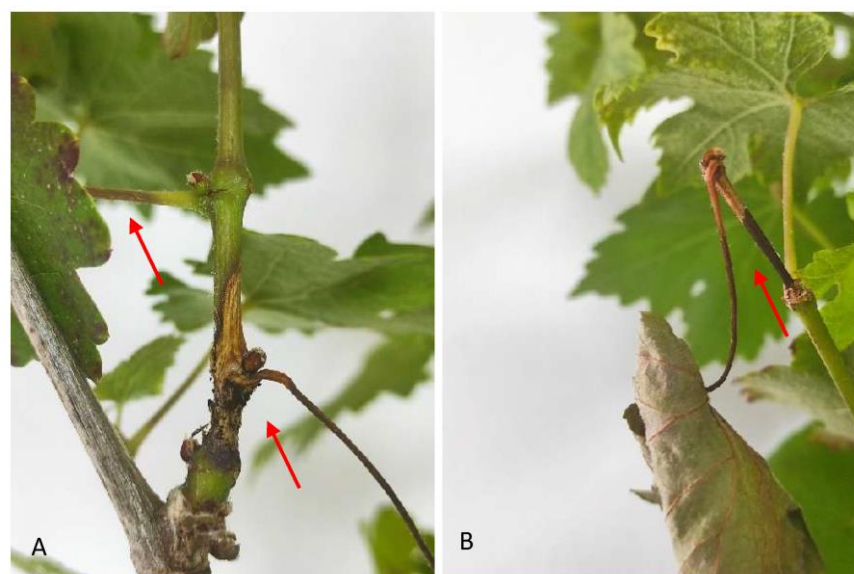


Figure 2. Symptoms of *S. sclerotiorum* in Cabernet Sauvignon grapevines at 7 dpi. **(A)** Broken stems from the point of infection because of the injury. Necrotic lesions (arrows), brown in color, with darker edges extended along the stem that remained green, compromising petioles that were totally or partially necrotic and therefore led to the wilting of the leaves in the affected shoot **(B)** detail of necrotic petiole and leaf wilting.



Figure 3. Symptoms of *Ss* in *C. Sauvignon* grapevine at 7 dpi **(A)** Epinasty and necrosis in the stem with total or partial decay of the shoot or its leaves. **(B)** Detail of the necrotic lesion in a young shoot fractured due to spontaneous breaking. **(C)** Epinasty and distal wilting in terminal shoot's leaves.

Significant differences were found between *Ss* and *Ss+Xff* treatments for the average number of infective lesions on stems from the initial point of inoculation (5:8 vs 8:8). A significant higher initial inoculation points developed in necrotic lesions in the combination *Ss+Xff*, in comparison with the *Ss* treatment (**Figure 4 A**). Also, significant differences in the length of the necrotic lesions (16.88 cm vs 46.05 cm) and the number of necrotic petioles/plant (18 vs 27) between *Ss+Xff* and *Ss* were found (**Figure 4 B-C**). The *Xff* control plants and healthy control without inoculation plants (Mock), did not registered necrotic symptoms. It should be highlighted, the greater disease intensity over time and the faster progress found in the combination *Xff-Ss* compared to *Ss*.

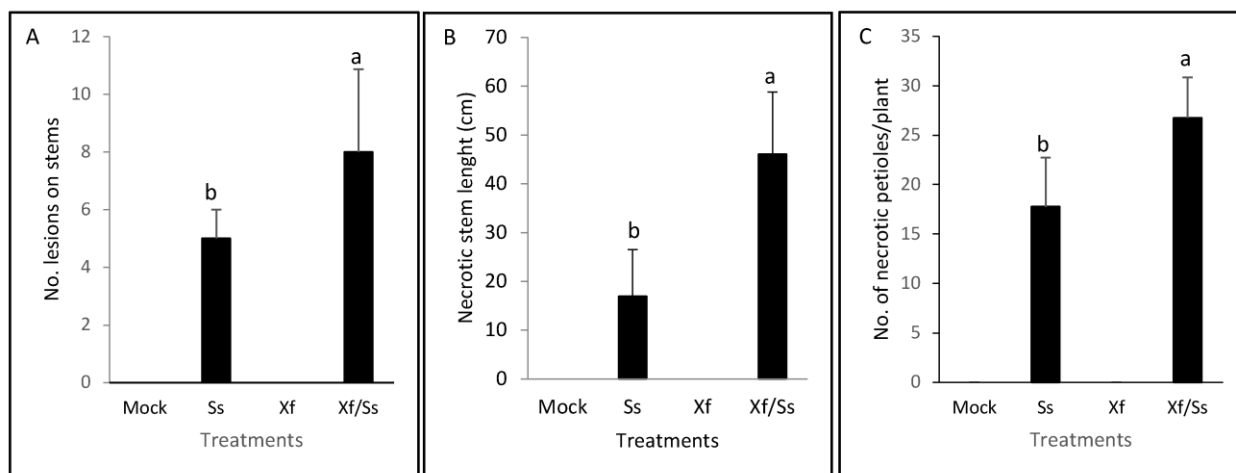


Figure 4. Symptoms on grapevine plants control (Mock), infected with *Sclerotinia* (Ss), *Xylella* (Xff) and the combination (Ss+Xff). **(A)** Number of necrotic lesions on stems; **(B)** necrotic stem length and **(C)** number of necrotic petioles per grapevine plant analyzed. Measurements of symptoms were performed at 7 dpi. Different letters indicate statistically significant differences ($p < 0.05$).

In the **Figure 5**, the biplot generated from the principal component analysis (PCA), indicated that the first two principal components (F1 and F2) explain 98.14% of the total variability of the data, with a predominance of the first component (87.52%). This suggests that most of the variation in the data is captured by the F1 axis. The three variables studied (number of necrotic petioles, number of lesions on stems, and total length of the lesions) are closely correlated, since their arrows point in similar directions. These variables mainly contribute to the first component (F1), indicating that F1 summarizes the combined effect of these measurements. Moreover, the Xff-Ss treatment (plants with both infections) is strongly associated with high values in all variables analyzed, being in the same direction as the arrows. The *S. sclerotiorum* (Ss) treatment shows a moderate response, partially separating from the control and the plants with *Xylella fastidiosa* (Xff). Mock (control) and Xff (only *Xylella*) are in the left quadrant, indicating that they present low values in the measured variables. Thus, the biplot suggests that co-infection (Xff-Ss) generates a greater impact on the three parameters evaluated, while Mock plants and with Xff show low levels of necrosis. This shows a synergic effect between *Sclerotinia* and *Xylella* on the severity of symptoms (**Figure 5**).

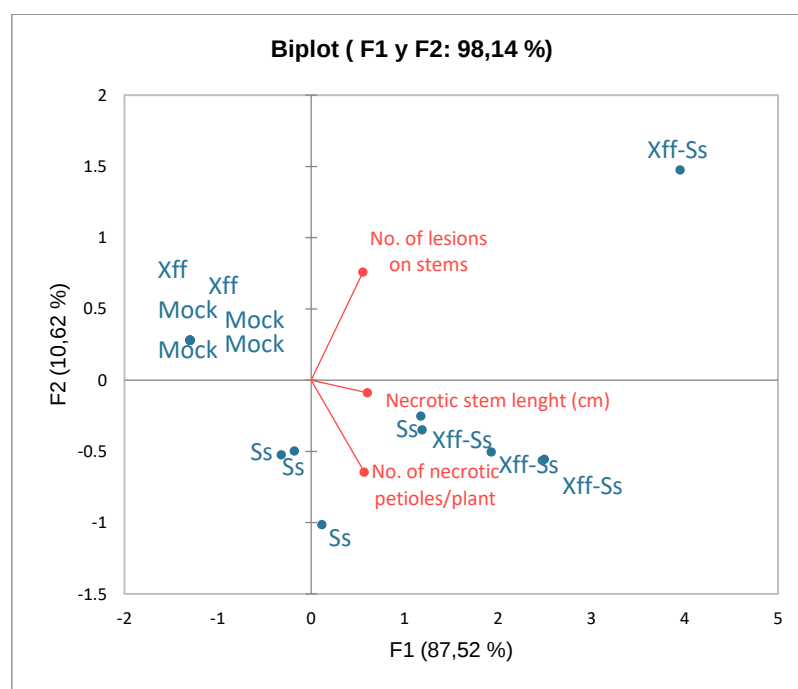


Figure 5. PCA biplot graph representing the three variables measured (number of necrotic petioles, number of lesions on stems, and total length of the lesions) in the interaction *Xff-Ss* on grapevine Cv. Cabernet Sauvignon.

The graphic representation of the total accumulated disease measured as the area under the disease progress curve (AUDPC) is shown in **Figure 6**. Analysis of the data indicate a relative higher accumulated disease- assessed as stem necrotic lesions length (cm) at three progressive times of observations during the experiment-, registered in the combination *Xff-Ss* (A) in comparison with the AUDPC in the treatment *Ss* (B). Moreover, a faster velocity to increase of symptoms was shown in the first treatment (A).

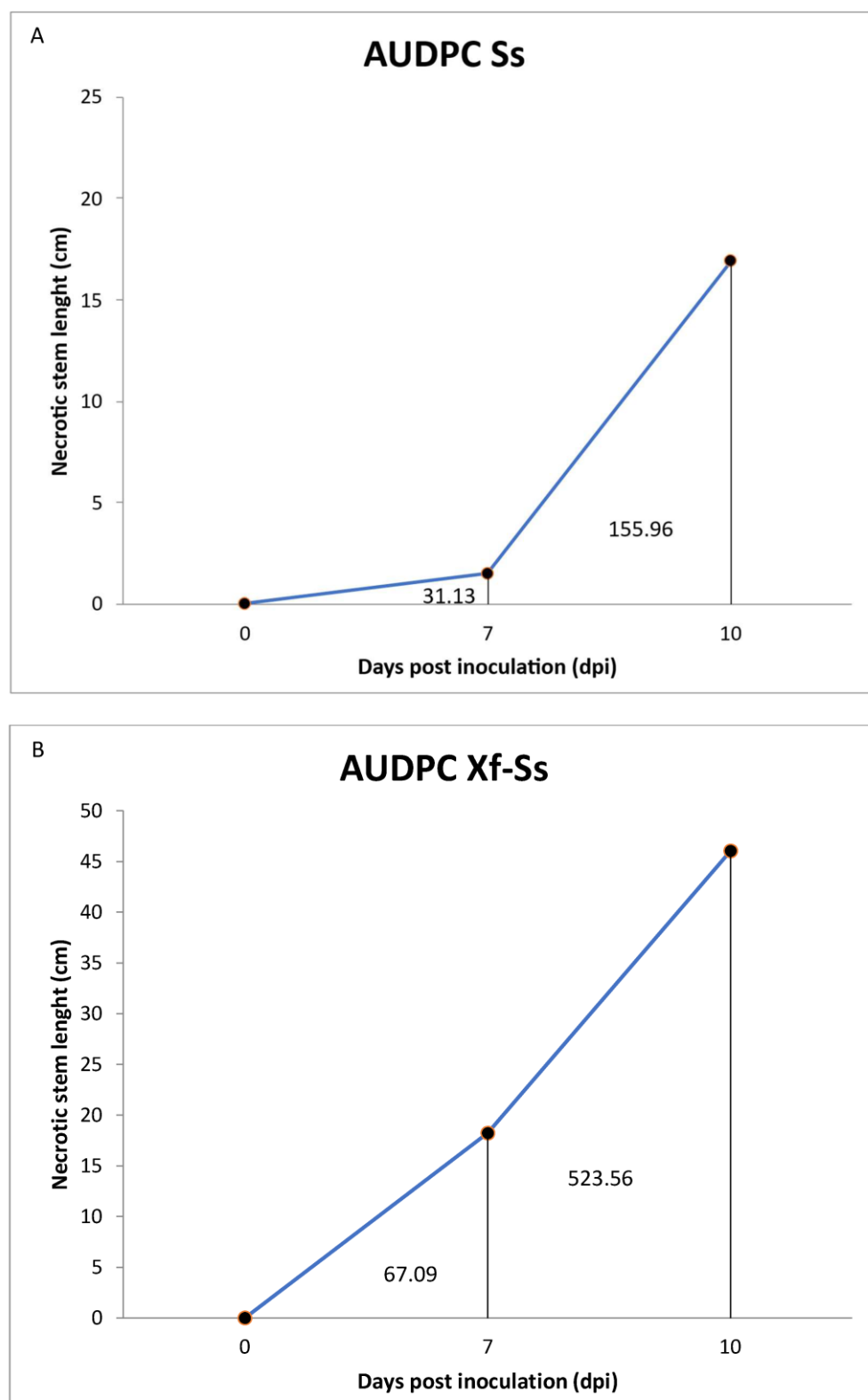


Figure 6. AUDPC. Graphic representation of the total accumulated disease measured as the area under disease progress curve in both treatments: *Xff*-Ss (A) and Ss (B). Data obtained from three evaluation diseases time points.

Although the type of symptoms recorded was the same in both treatments (Ss and *Xff*+Ss), a significantly greater intensity of symptoms and aggressiveness of *Sclerotinia* stands out for plants previously infected with *Xff* (Figure 7).



Figure 7. Symptoms on Cabernet sauvignon grapevine plants at 7 dpi in Mock, *Ss*, *Xff* and *Ss+Xff*. Detail of grape bunches and stem necrosis, stem breakage, wilting and falling of leaf petioles caused by *Ss* in plants inoculated with *Xff* (below).

Moreover, changes were detected in the stomatal conductance of plants affected by *Xff* in the presence of *Ss*. Our results shown a different response induced by the *Ss* and *Xff* with respect to control plants. According to our results, grapevine plants subjected to stress caused by *Ss* showed highly significant values of stomatal conductance ($450 \text{ mmol m}^{-2}\text{s}^{-1}$) compared to the control plants ($200 \text{ mmol m}^{-2}\text{s}^{-1}$). These high values were also reflected in the interaction of the fungus with *Xff* in concomitant infections ($350 \text{ mmol m}^{-2}\text{s}^{-1}$). The lowest stomata opening value was induced by *Xff* ($140 \text{ mmol m}^{-2}\text{s}^{-1}$) although without statistically significant differences with control plants. Regarding chlorophyll content, non-significant differences were found among treatments (**Figure 8**).

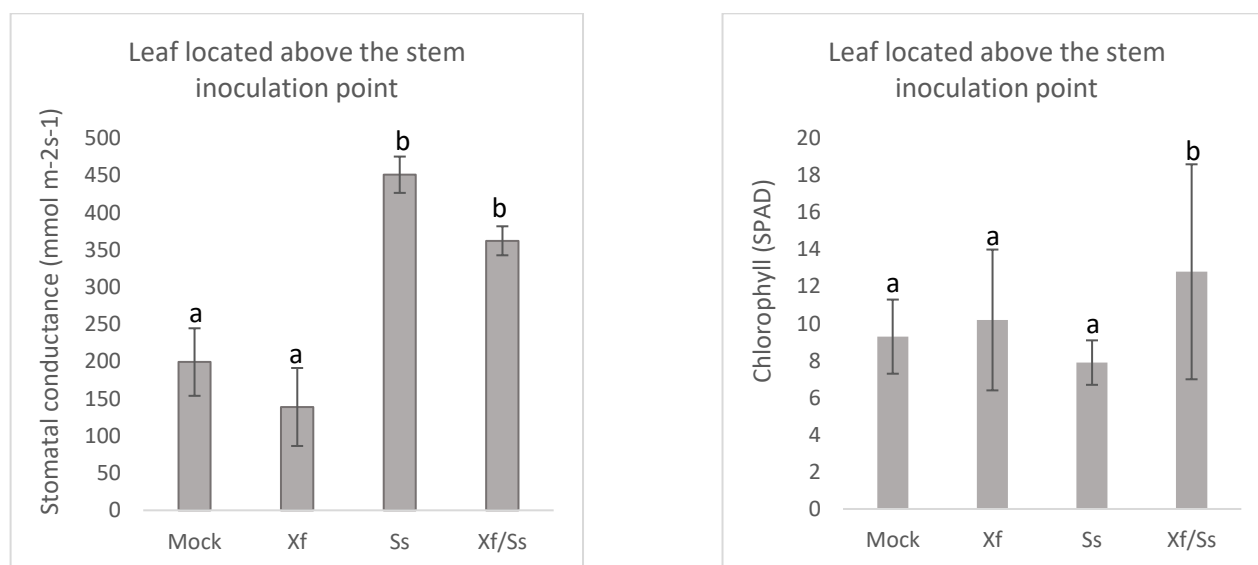


Figure 8. Stomatal conductance and chlorophyll content on grapevine leaves. Treatments: Control (Mock), *Xylella* (*Xff*), *Sclerotinia* (*Ss*) and *Xylella fastidiosa* + *Sclerotinia sclerotiorum* (*Xff/Ss*). The bars represent the average of three measurement per treatment with their respective standard deviation. Different letters Indicate statistically significant differences ($p < 0.05$).

3. Discussion

Vitis vinifera hosts a complex of endophytic microorganisms that interact among themselves and within the plant. Those microorganisms can be beneficial, neutral or pathogenic to the plant, although the nature of their interactions is unknown in most cases [27, 28]. Some of these microorganisms are even considered as natural biocontrol agents due to their ability to protect the plant against phytopathogens and reinforce the natural plant defenses [21].

In previous studies a great diversity of endophytes belonging to different taxa has been documented on grapevine plants [22, 23]. In this line, in viticulture and oenology the complex of the microbiota present is recognized by a major imprint on the regional local "terroir" [29]. Moreover, the endophytic community involved during the wine's early fermentation stages- which is partially determined by the endophytic plant-borne yeast and bacteria present- is linked to the geographical origin that reflects the features of different winegrowing regions. In this sense it should be noted that recent studies have highlighted the contribution of the native vine microbiota in the winemaking process of wines from a particular region [30]. Here, the study of the culturable mycobiota associated with *Xff* under field conditions was challenged by the high variation in the bliding time among plants, and by the final volume of collected sap, which, in most cases, was not enough to continue the study. Eventually, different fungi in addition to the yeast like fungus were identified from the sap of five plants, three of then identified as positive in *Xff*. These

microorganisms recovered included both beneficial and potential pathogenic fungi of grapevine. Regarding the group of beneficial fungi, some of them could be of agronomical interest for integrated pest management (IPM). Along this line, *Rhodothorula mucilaginosa* and other yeast-like microorganisms, and *Aureobasidium pullulans* also isolated here, have been previously reported as promised antagonist for the biocontrol of grapevine trunk diseases (GTDs) [31]. Moreover, biocontrol activity for the management of bitter rot in grapes by *A. pullulans* has been reported [32]. On the other hand, yeast and yeast-like microorganisms have been investigated as biological control of grape diseases, focusing on the dynamics of the indigenous yeast populations present during the spontaneous fermentation of grape must into wine. [31].

Among the most worrisome fungi identified were the presence of GTD-associated fungi, nowadays considered another important health biotic risk for viticulture. The necrotrophic pathogenic fungus identified was *S. sclerotiorum*, causes shoot rot in grapevines, usually in spring time when temperatures are mild. [20]. In the same way, other genus -like *Alternaria*, *Phoma*, *Cladosporium*, *Diaporthe/Phomopsis*- also recovered in this study, may become pathogens when proper conditions occur. Several studies indicate that species of *Alternaria* are responsible for causing berry rots, raising molds and rots, as well as, pedicel and rachis diseases of grapevine [33]. Moreover, *Alternaria alternata* is described as a postharvest pathogen and causing leaf spot [34]. Regarding *Phoma* complex, the fungus was previously identified as pathogen causing diseases like the decline and death of young grapevines [35, 36].

According the preliminary analysis of the fungal identity analyzed here, it is highlighted that, some fungal taxa belonging to the Dematiaceous Coelomycetes group overlap at the genus and species level given the similar morphology among many of the isolated taxa, - small spherical conidia, or ellipsoids without septa, or with one to several septa, conidiomata pycnidial -, and also similar characteristics of the colonies - dark, felted - therefore, further confirmatory molecular techniques are required for an exhaustive accurate diagnosis of the members of each isolated group. Along this line, some reports [37], compared the biodiversity of fungi in *Vitis vinifera* by both, traditional and molecular approach, to obtain a better resolution in species identification, richness and of the distribution patterns of microbes. These researchers concluding that a combination of both approaches (i.e. traditional and culture-independent) is needed for proper evaluation. In agreement, in our work, the traditional technique using morpho biometrical analysis of colonies and conidia was useful as a first preliminary exploratory approach of the grapevine endophytic fungal diversity, but not sufficient for the complete identification of the microbiota recovered from the sap samples. Therefore, a molecular complementary analysis is needed to give accuracy to discriminate among identity of similar complex fungal.

Differences in the fungal communities of Xff positive and negative plants were found, with these communities showing the different richness, depending on the presence of the bacterium. These findings could contribute to the understanding of the roles played by each fungal group in the stability and functioning of its respective ecosystem.

Some groups recovered here e.g *Botryophacteria* complex (Hypocreales), *Phaeoacremonium/ Phaeomoniella* sp (Togniniales) and taxonomic members of the Dematiaceous group with pycnidial conidiomata (Coelomycetes like *Phoma* complex, *Phomopsis/Diaporthe* complex) are compatible with pathogens of the grapevine trunk causing GTDs that parasitize the xylem of plants, block xylem vessels and therefore prevent sap flow (tracheomycosis or hadromycosis) and thus, causing plant decline or deterioration. The main symptoms produced by trunk diseases, which overlap between the causal agents and Xff symptoms, are a decrease in the productivity of the plant, the death of the arms, progressive deterioration, graft failure, shoot death, foliar symptoms such as chlorosis and necrosis, white rot of the trunk and necrosis with dark wedge-shaped discoloration on the trunk [38].

There is little information regarding the outcomes of the interaction between *Xff* and other microbiome components in co-occurring infections. Few synergic effects have been studied about how different pathogenic microorganisms can contribute to the cause of a crop disease through synergistic interactions that are triggered during co-infections [39,40]. Previous work by Araujo et al. (2002) [9] found that the presence of the endophyte *Methylobacterium* had a synergistic effect causing an increase in the occurrence and intensity of symptoms induced by *Xff* subsp. *pauca*, in *Citrus sinensis*. In this work, we focused on the interaction between a fungal pathogen found in the xylem sap, *Ss*, which was recently registered on grapevines in Mallorca causing shoot blight and *Xff* [20]. The infection of grapevines by *Xff* yielded plants more vulnerable to *Ss*, which could have been the result of a detrimental effect of *Xff* on the plant's defense systems. However, *Xff* lacks a Type III secretion system, that suppresses the host plant defense responses [41]. We hypothesize that the increased virulence of *Ss* in *Xff*-infected grapevines could rather be due to a debilitated plant's metabolism caused by the bacteria which development obstructs the xylem vessels, negatively affecting among others stomata opening, carbon assimilation and plant growth [42]

Regarding the physiological parameters examined, *Ss* effects prevailed on those triggered by *Xff* infection. The two pathogens exerted an antagonist effect on the movement of the stomata. Regarding the stomatal closure induced by *Xff*, it has been suggested that the colonization of xylem vessels by *Xff* and the presence of tyloses, deployed by the plant as a defense mechanism to try to isolate the bacteria, decreases the xylem vessels hydraulic conductivity causing water deficit [42]. Furthermore, the triggering of ABA signaling, which was previously mentioned to cause stomatal closure, could also have an antagonistic effect on the host defense responses [43]. Moreover, stomatal closure decreases the rate of photosynthesis and transpiration, preventing evaporative cooling and consequently increasing leaf temperature [44, 45].

On the other hand, according to Guimarañes and Stotz [46], the effect of *Ss* on the stomata movement could be attributed to an oxalic acid-mediated stomatal opening that could cause foliar dehydration by disturbing guard cell function altering guard cell osmoregulation and interfering with abscisic acid (ABA)-induced stomatal closure. The latter could at least partly explain why our results showed that the opening effect triggered by *Ss* prevailed on the closure response induced by *Xff* infection as the fungus activity would have cancelled the ABA effect on stomata.

To conclude, this preliminary screening of the biodiversity complex of sap grapevine endophytes under *Xff* infection and the symptoms developed under concomitant infection of the bacterium and the fungus *Ss*, underscores the importance of taking into consideration the microbiome-pathogen interaction when plant disease studies are designed under field conditions. The results here also alert about the underdiagnosed and underestimated components of microbiota, especially fungi co-isolated with bacteria, which are often considered irrelevant as they supposedly do not alter the outcome of the infection.

4. Materials and Methods

4.1. Experimental Design

To study the biodiversity of sap grapevine fungal endophytes in *Xff*-affected vineyards, a survey was conducted in a commercial vineyard located in typical wine production region in the island of Mallorca (Spain) in 2020. Selection of grapevines was performed based on the analysis of a total of 50 grapevines cv. Cabernet Sauvignon analyzed from that place in summer in 2019 to identify *Xff*-positive plants, according to protocols shown on section 4.2.

4.2. PCR Assays to Test the Presence of *Xf*

DNA extraction from plant extracts was performed from leaf veins and petioles, and xylem sap using an EZNA HP Plant Mini kit (Omega-Biotek) following the manufacturer's instructions, as described in the EPPO protocol (EPPO, 2016) [47]. The presence of *Xff* was assessed by real time PCR using two specific protocols with primers XF-F/XF-R and the TaqMan probe XF-P (Harper et al. 2010) and primers HL5/HL6 and a TaqMan probe (Francis et al. 2006) using an Applied Biosystems QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, Massachusetts, USA) [48,49]. All samples were analyzed by triplicate.

4.3. Sap Collection

The sap samples were collected in the vineyard during grapevine bleeding. Ten plants of Cabernet-Sauvignon were selected nonetheless sufficient xylem sap to continue the study was obtained only from 5 plants. To collect the bleeding sap, the remaining canes left after pruning were cut off 2-3 cm, and a few mL of sap were allowed to drop to clean the cut before attaching a collecting tub to the cut end. Sap samples were kept at 4°C until use. Sap aliquots were used to confirm the presence of *Xff*, according to protocols shown on the previous section. One hundred µL of each sap sample were plated on Sabouraud agar and incubated for 7 days at 30°C.

4.4. Fungal Identification

The microorganisms were identified using conventional methods that involved isolating and cultivating them on artificial media and then classifying them according to their taxonomy. Cultivable fungi were isolated and incubated in Petri dishes with PDA 2% medium or Sabouraud medium for 3-7 days in a growth chamber at 25°C, 12 h of light and 12 h of darkness.

Each of the different morphologically identified colonies was transferred through a small agar disk (about 5 mm²) of the growing fungus to a fresh 60 mm diameter PDA plate. The obtained colonies were grouped and numbered according to their morphological characteristics, based on shape, form, size, growth time, border, surface, opacity, pigmentation, and the shape and size of the fungal fruiting bodies, spores, and hyphae. Additionally, shoot tissues and material collected with visible symptoms were placed in a moist chamber, and direct observation of leaf symptoms were carried out.

With the use of specific keys, microorganisms derived from colonies were identified by microscopic examination of mycelia and spores, morphobiometric traits, and cultural features. A group-level taxonomy classification was carried out, which considered the identification of endophytes which are beneficial microorganisms, and the risk genera linked to pathogenic fungus on wood trees that have not been previously reported in Mallorca on grape plants, as well as genera that are still poorly known despite their importance as plant pathogens. Representative cultures were deposited at the UIB culture collection. The current name of the microorganism was used according to Index Fungorum from 2018 [50].

4.5. Inoculation Assays to Test the Interaction *Xylella fastidiosa* subsp. *fastidiosa* (*Xff*) with *Sclerotinia sclerotiorum* (*Ss*)

The manifestation of symptoms in plants infected with *Xff* interacting with the fungal pathogen *Ss*, recently identified as a new potential biotic adversity for grapevines at Mallorca [20], was examined.

Two-years-old potted grapevine plants cv. Cabernet Sauvignon were used. A year earlier, half of the plants had been inoculated with the strain of *X. fastidiosa* subsp. *fastidiosa*

RTA821. The inoculum was prepared from 10-days colonies grown on BCYE agar. A milky solution was prepared using Ringer's solution to achieve a suspension of approximately 108 cell ml⁻¹, which was immediately used, as it tended to precipitate. Inoculations were performed at the lowest node of each branch, where a 10 µl droplet was applied using a needle inoculation technique by pin pricking until complete absorption of the drop was observed. The other half of plants were mock inoculated using 10 µl of Ringer's solution.

Four treatments were set up in tetraplicate: 1) non-infected plants (Mock) 2), plants previously infected with *Xff*, 3) plants inoculated with *Ss*, 4) plants previously infected with *Xff* and inoculated with *Ss*.

Sclerotinia artificial inoculations were carried out in 8 points of 2 branches/plant and treatment for fungal infection. The inoculum consisted of placing agar discs with the fungus's actively growing mycelium on the fresh plant wounds and then covering this inoculation zone with parafilm. Each inoculated branch was covered with nylon bags for 48 h to prevent desiccation and increase humidity. The length and number of branches with rot symptoms and the number of petioles showing rot symptoms were measured for 7 days post infection (dpi). Regarding morphological measurements of disease evolution, the number of lesions on the stems, the length of necrotic lesions and the number of necrotic petioles, were measured in each plant, twice per week. The AUDPC was calculated according to Madden et al. (2007) [51].

4.6. Chlorophyll SPAD and Stomatal Conductance

Plant disease progression was assessed by physiological and morphological measurements. At physiological level, leaf total chlorophyll concentration using a portable chlorophyll meter (SPAD Model CL-01, Hansatech Instruments) and stomatal conductance (gs) measured using a Leaf Porometer were measured on leaves located above and below the *Ss* inoculation point. Measurements were performed once a week, from 10 to 13 h.

4.7. Statistical Analysis

The statistical software InfoStat 2020 was used for data analysis [52] for the one-way ANOVA and Tuckey's test ($p < 0.05$). For the interaction assay, a CPA analysis was performed using the XLSTAT software.

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