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# European Grapevine Cultivars and ~~Rootstock~~ Show Differential Resistance to *Xylella fastidiosa* Subsp. *fastidiosa*

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**Abstract:** Several *Xylella fastidiosa* subsp. *fastidiosa* (ST1) strains that cause Pierce's ~~D~~isease were isolated from well-known grapevine cultivars in Spain. In this study, we applied an approach to assess PD susceptibility among different *Vitis vinifera* cultivars and rootstocks. This method incorporated disease severity, disease progression and ~~potential water~~ from the stem xylem. In total, 24 popular cultivars and 5 rootstocks were selected for this study. The virulence of the *Xff* strain XYL 2055/17 was significantly higher than that of strain XYL 2177/18. However, while this difference in strain virulence did not seem to modify the susceptibility profiles of the cultivars, disease severity could be climate dependent. This work established two significantly different groups of European cultivars of grapevine characterized by high and low susceptibility to *Xff* ST1: cultivars with high susceptibility, including reference cultivars such as Tempranillo and Tempranillo Blanco and cultivars with high resistance such as Hondarrabi Zuri and Cabernet Sauvignon. Cultivar susceptibility was independent of the rootstock on which they were grafted. No conclusive data were found regarding the potential of water loss as an early detection test prior to ~~the symptomatic period~~. This study provides a ~~reference~~ framework with which to advance ~~susceptibility cultivar~~ studies under different environmental conditions.

**Keywords:** Pierce's ~~D~~isease; *Xylella fastidiosa*; grapevine; Scholander pressure chamber; ~~AUDPC; rAUDPC; severity index;~~ quarantine plant pathogen; water potential

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## 1. Introduction

*Xylella fastidiosa* (*Xf*) [1] is a Gram-negative bacterium and a vascular plant pathogen with an extremely large host range [2]. To date, this species has been found to infect 690 plant species classified under 306 genera and 88 families (EFSA, 2023; Sicard, 2018; Trkulja, 2022 [2–4]. Major plant hosts (https://gd.eppo.int/taxon/XYLEFA/hosts (accessed on 08 October 2023)) include two ornamental species (*Nerium oleander* and *Polygala myrtifolia*) and four major crops: citrus, coffee, almond, and grapevine (*Vitis vinifera*). The diverse diseases caused by *Xf* have significant economic impacts and agricultural management consequences [5]. Since 2019, *Xf* has been included as a *Priority Pest* under European Regulations (Regulation (EU) 2019/1702). One of the characteristics that makes *Xf* a very dangerous pathogen, besides its very high genetic plasticity, is its ability to be transmitted by likely all species of the two main xylem-feeder insect groups: leafhoppers (Cicadellidae subfamily Cicadellinae) and spittlebugs (Cercopoidea, families Aphrophoridae, Cercopidae, and Clastopteridae) [3].

From a taxonomic point of view, *Xf* is a complex species with several subspecies and sequence types (ST). *X. fastidiosa* subsp. *fastidiosa* (*Xff*), which belongs to ST1causes Pierce's Disease (PD) and diverse syndromes in several plant hosts [6-7]. PD was first described in California in 1892, where it remains a significant problem for the grape industry. A devastating PD outbreak in the Temecula Valley in Southern California during the late 1990s was associated with the establishment of a very efficient vector in the area, the glassy-winged sharpshooter (*Homalodisca vitripennis*), which prompted an intense investigation of the pathogen and the development of programs to manage PD and the vectors involved [3]. Control of this disease is difficult and, despite significant projected savings of around USD 200 million under the PD Control Program [8], a corresponding increase in efficacy will require the deployment of diverse improved strategies that should include the management of water stress and appropriate plant resistance [6, 9].

The main factors causing PD symptoms appear to be related to water stress and the blockage of xylem vessels by the bacterial biofilm, as well as the resulting production of tyloses and gums by the plant, which cause hydraulic dysfunctions leading to desiccation and plant death within a few years [10,11]. Subsequently, characteristic leaf symptoms such as marginal necrosis, necrotic leaves, "matchstick" leaves, petiole abscission, green islands on stems, and irregular cluster ripening become apparent. However, the development of symptoms may not be entirely due to ~~the~~ vessel~~'s~~ occlusion. Instead, the pathogenesis may be more complex and occur starting from the earliest stages of infection, before the colonization of vessels [12, 13]. The time period between inoculation and the appearance of symptoms in plants varies depending on the plant species and age and occurs between three and four months, extending beyond a year after the initial infection [13], with longer periods observed in woody plants compared to herbaceous plants [14]. The symptoms typically begin in late summer or early autumn when environmental conditions are generally hot and dry and plants are subjected to ~~consequent~~ water stress and other physiological responses, which vary in different varieties [15, 16].

Several techniques are available to assess the water status of plants via physiological indicators. These methods include measuring stomatal conductance, leaf and stem water potential, relative water content, stem and fruit diameter, and sap flow [17, 18]. Leaf water potential is recognized as one of the most important indexes to evaluate the water status of plants, providing high theoretical value and important information for multiple applications to quantify critical physiological processes, including drought responses [19, 20]; thus, leaf water potential is a good indicator of water stress [21]. Predawn leaf water potential (PLWP) and stem water potential (SWP) were found to be simple and precise indicators for assessing grapevine water status [22–24] and subsequent same-day spatial comparisons [25]. Drought tolerance is known to be determined in part by factors related to water transport within the plant, and there is a direct relationship between hydraulic conductivity and xylem physiological status [26]. The data provided by this technology suggest early infection indicators that may be related to PD resistance factors. Moreover, to avoid the use of destructive technology, as in the case of Scholander´s pressure chamber, ~~the data obtained for the grapevine in an open field, although used for other purposes,~~ were correlated with data provided by other nondestructive and portable devices [27].

There is a great diversity of grapevine cultivars that ~~may~~ have been bred in different geographical areas and are often adapted to different soil and climatic conditions, frequently leading to physiological differences among them [28]. PD resistance has only been identified in several wild grape species endemic to the Americas, including *V. arizonica/candicans* and *Muscadinia rotundifolia*, whereas all assayed European *V. vinifera* genotypes are susceptible to this disease [29-32]. Recent research has compared PD tolerance/resistance within the three major Eurasian pedigree lineages and provides a benchmark for PD susceptibility levels for some of the most widespread table and wine grape cultivars [33]. The complexity of the genomic architecture of resistance to the bacterium and the role of climate in shaping this resistance [32] have also required the use of additional research efforts to evaluate a larger number of *V. vinifera* cultivars from different

genetic pools under different environmental conditions. These studies obtained information on the vascular and anatomical characteristics of these grapevine cultivars to develop tools for assessing and predicting the PD susceptibility ~~of different cultivars~~ [10].

In 2013, the first European outbreak in olive trees *(Olea oleander)* occurred in the Italian region of Apulia; this outbreak was associated with a disease previously known as Olive Quick Decline Syndrome (OQDS) and caused by *Xf* subsp. ~~*Pauca*~~ [34]. New outbreaks were reported in other European countries in the following years and involved different *Xf* subspecies and numerous plant hosts [35]. ~~In~~ the Balearic Islands of Spain, *Xff* was first reported in October 2016 to cause PD and also infect other hosts [7]. Since the first occurrence of PD in Europe, the presence and spread of this bacterium in Mediterranean crops and plant species in the natural and urban landscapes have become a major risk. For this reason, Italy, France, and Spain are now taking specific phytosanitary measures aimed at the eradication or containment of this disease. These measures are based on exhaustive surveys and monitoring PD symptoms to achieve a quick removal of problematic vines. This method of management could significantly impede *Xf* transmission in healthy vine populations.

The recent outbreaks of PD in Europe have driven the pursuit of new and more effective control strategies for both PD and other diseases caused by *Xf*, resulting in the proposal of promising and innovating methods that could facilitate crop sustainability with little to no economic, social, or environmental risks [9]. These methods include techniques conferring systemic resistance in vines [36], the use of synthetic peptides by endotherapy [37], and behavioral manipulation for the control of insect vectors [38]. Nevertheless, to achieve the appropriate management of PD in Europe, it will be necessary to evaluate both the aggressiveness of the different *Xff* isolates and the resistance responses of the numerous clones and cultivars of *Vitis* planted in this continent. Furthermore, given the expected variability in PD epidemiology dependent on agro-climatic factors and the expression of pathogenicity factors [33, 39], these studies should be conducted under standardized climatic conditions and correlated with the agro-climatic variability of viticultural areas.

The main objective of this work is to describe the severity of PD using a local inoculum and grapevine cultivars and rootstocks; evaluate disease progression under different climatic conditions; and establish the relationship between symptomatology, xylem pressure changes, and the detection of the bacterium using a standardized molecular methodology. The 24 grapevine cultivars and 5 rootstocks selected in this study are among the most economically relevant in the Spanish wine market. These cultivars were chosen to provide data that could allow the implementation of clonal selection processes. The disease severity, water potential, and time course of PCR detection were studied under four different weather environments, including a climate-controlled greenhouse and open field conditions.

## 2. Materials and Methods

### *2.1. Terminology*

Grapevine plants for new plantations are normally marketed as grafted. The same variety can be grafted onto different rootstocks to achieve better adaptation to the soil, phenological development, or resistance to endemic pests. In this study, European grapevine varieties (*Vitis vinifera* subsp. *vinifera*) grafted onto different rootstocks were evaluated. To avoid discrepancies in terminology, a variety grafted onto the rootstock is referred to as the "*Cultivar*". Plants corresponding to varieties used as rootstock and belonging to different species of the genus *Vitis* were also studied; the term used in this work to designate such species is "*Rootstock*".

### *2.2. Plant Material and Facilities*

24 *Cultivars* and 5 *Rootstocks* [40, 41] were selected based on their representativeness in the Spanish Protected Designations of Origin (DOs) or Protected Geographical Indications (PGIs) [28] to evaluate their resistance and disease progression.

During the years 2019, 2020, and 2021, three trials were carried out ~~on~~ an open field (see the climate ~~parameters~~ in Figure 1). One additional trial in a greenhouse was carried out in 2021. The plants were grown under controlled conditions with 70% relative humidity; a day/night temperature of 25 °C ± 2 °C and 18 °C ± 2 °C, respectively; and the absence of additional artificial lighting. The trials were conducted from May to November, ensuring a minimum daily light period of 16 h during the first four months after inoculation.

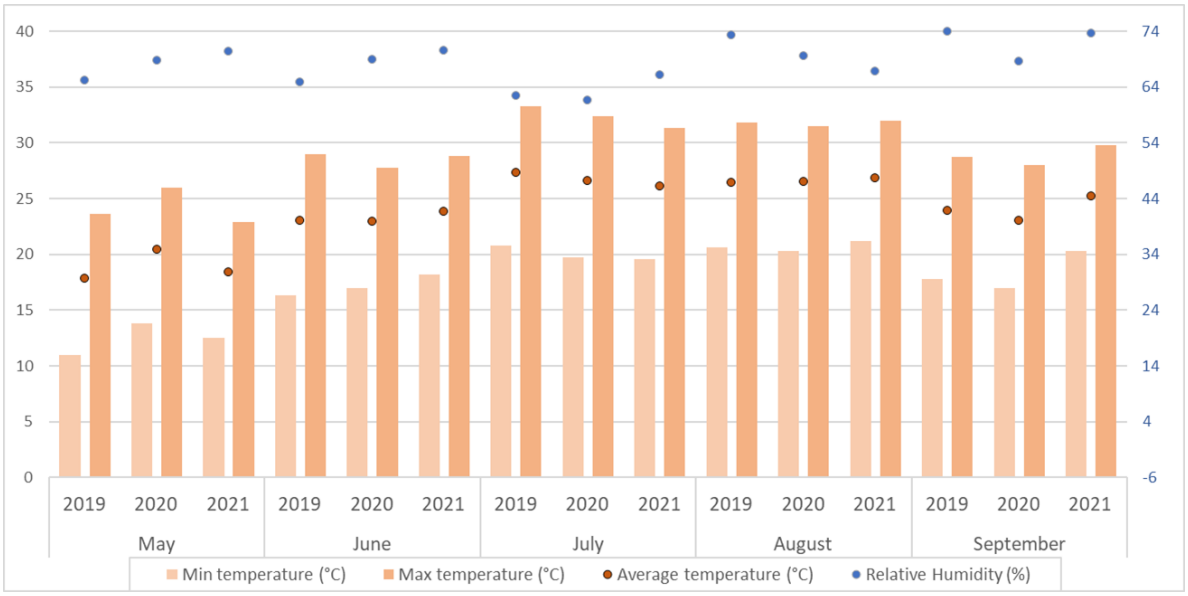



**Figure 1.** Monthly average, maximum and minimum temperature (°C), and relative humidity (%) for the three years and the evaluation period (from June to September) in which the field trials were conducted to evaluate the *Cultivar*'s susceptibility to *Xff*.

Pre-grafted and rooted plants (one-bud cuttings) from the nursery were kept at 4 °C before planting. The plants were potted (6 L pots) into a mixture of peat and siliceous sand (3:1). The peat (pH 6.0 and 90% organic dry matter) was supplemented with calcium carbonate (7 g/L) and NPK14-10-18 fertilizer (1.5 g/L). The plants were selected for the trial if they showed the same phenotypic profile after a period of 20–30 days.

The greenhouse was accredited as a temporal confinement greenhouse for *Xff* tasks; NEIKER´s facilities are located in this area on the northern peninsula of Spain (42°51′10.2″ N 2°37′29.7″ W). The trials using an open field were conducted in Palma de Mallorca, Spain, at the Balearic Governmental Center for Agricultural Improvement "SEMILLA" (39°35′23.5″ N 2°39′51.1″ E). The plants were randomly distributed in 12-plant rows along an insect-proof net tunnel and exposed to environmental temperatures.

### *2.3. In Planta Evaluation of PD Susceptibility*

Inoculations were performed as follows: *Xff* ST1 strains (IVIA5770, XYL 2055/17 and XYL 2177/18) were isolated from *Vitis vinifera* on the Balearic Islands. These strains were previously characterized for several scientific grapevine tests [42, 43, 29, 7]. The bacteria were cultured on a ~~BCYE~~ solid medium at 28 °C for 7–10 days. A bacterial suspension was prepared in phosphate-buffered saline (PBS) and adjusted to $OD_{600}$ = 0.3, resulting in a final concentration of approximately $1 \times 10^8$ CFU.ml$^{-1}$. Next, 10 µL of that suspension was used to inoculate the plants following a general procedure consisting of the pin-prick method [44, 45]. In total, 9 out of 12 plants per genotype were inoculated, while the remaining 3 plants were mechanically injured in the same way as those prepared in PBS but without an inoculum.

The Severity Index was assessed on a scale of 0 to 5, as previously described by Su et al. in 2013 [46], based on the number of affected and symptomatic leaves, as follows: asymptomatic leaves (0), 1–2 symptomatic leaves (1), 3–4 symptomatic leaves (2), 5–7 symptomatic leaves (3), 8–10 symptomatic leaves (4), and more than 10 symptomatic leaves (5). Based on these data, it was possible to calculate the disease progression expressed as the absolute area under the disease progression curve (AUDPC). This value was then transformed by calculating the relative AUDPC (rAUDPC) through dividing by the maximum AUDPC ~~from~~ the duration of ~~the~~ data recording [47].

### *2.4. Detection of Xf*

Each leaf was taken between 3 and 5 nodes above the inoculation point. Only petioles and veins were used for DNA extraction, amounting to a minimum of 0.5 g fresh tissue. The CTAB protocol for DNA extraction was applied [48]. Real-time PCR (RTi-PCR) for *Xf* detection was carried out using the primers XF-F: 5′-CAC GGC TGG TAA CGG AAG A-3′, XF-R: 5′-GGG TTG CGT GGT GAA ATC AAG-3′, and XF-P: 5′-FAM-TCG CAT CCC GTG GCT CAG TCC-BHQ-1-3′ [48,49], using TaqMan universal master mix 2x (Applied Biosystems). Final optimized reaction conditions were as follow: RTi-PCR reactions were done in 20 µl reaction volumes containing 10 µl of 2X TaqMan universal master mix (Applied Biosystems), 300 nM *Xf* sense (XF-F) and antisense (XF-R) primers, 100 nM 6′FAM/BHQ-labeled XF-P probe, ultra-pure bovine serum albumin (BSA) at 300 ng/ µl (Invitrogen) and 2 µl of total DNA template. Optimal thermocycling conditions were as follows: 50ºC for 2 min and 94ºC for 10 min, then 40 cycles for 10 s and 62ºC for 40 s. using Quant Studio TM 5 model QS5STD. All samples were amplified in duplicate. Threshold values were applied automatically by the QuantStudio™ Design and Analysis Software (Applied Biosystems). Positive amplification was determined by a crossing threshold (Ct value < 38 cycles). Bacterial re-isolation was accomplished from the same tissue sample RTi-PCR analysed. The recovered colonies were scraped from BCYE growth medium and resuspended in potassium phosphate buffer, prior to being assessed by RTi-PCR.

### *2.5. Water Potential (Ψ) Assessment*

~~The~~ Ψ was assessed only in the greenhouse trial using the Scholander pressure chamber (PMS Instrument Company, Albany, NY, USA). The equipment was set up using a pressure of 3 bar in the $N_2$ gas cylinder and a gas flow rate of 5 bar in the equipment. Measurements were performed systematically between 9:30 and 11:30 am, two hours after sunrise, as recommended by Knipfer et al. (2020) [50]. Leaves were cut from positions 2, 3, or 4 above the inoculation point, depending on phenological development, using a scalpel blade. Immediately after excision, the petiole was placed in the Scholander chamber, and the pressure equilibrium technique described by Castander et al. (2020) [51] was used to measure Ψ in MPa.

### *2.6. Statistical Analysis*

~~The~~ rAUDPC and water potential (Ψ) were analyzed in each trial to assess the ~~variable resistance among the response variables~~. All ~~the~~ rAUDPC data were normalized via square root transformation and then subjected to analysis of variance (ANOVA), followed by Fisher's Least Significant Difference (LSD) test (R software, version 4.2.2). Differences at $p < 0.05$ were considered significant. Post-hoc analysis with Tukey's test, T-test, descriptive data in boxplots, and Pearson correlation coefficients using average trait values were also obtained via the free Jamovi software version 2.3.28.

## 3. Results

### *3.1. Pierce's Disease Susceptibility in Vitis Vinifera Cultivars and Rootstocks*

During 2019, we evaluated the susceptibility of 24 different grapevine *Cultivars* and 5 *Rootstocks* to two different strains of *Xff* (XYL 2055/17 and XYL 2177/18) that were previously isolated in the same geographical area where the trial was conducted (Balearic Islands). The rAUDPC data were recorded during the period between 8 ~~WPI~~ (2 months) and 16 WPI (4 months), which corresponded to a maximum AUDPC of 280 u² for calculating the rAUDPC. Inoculated plants in which the bacterium was not detected via RT-PCR were not considered for an rAUDPC assessment.

We observed significant differences in the susceptibility of *Cultivars* and *Rootstocks* to each of the two strains of *Xff* (Figure 2A). The average rAUDPC for all *Cultivars* combined was significantly higher for strain XLY 2055/17 (0.58) than for strain XYL 2177/18 (0.46), indicating the higher virulence of strain XLY 2055/17 (Figure 2A). In this case, the correlation between the rAUDPC values of both *Xff* strains was high, with a significant coefficient of q = 0.903 ($p < 0.05$). The significant differences between *Xff* strains and significant correlation indicate that one of the factors involved in cultivar resistance were determined by the bacterial genotype. This relation could be established under a linear regression ($R^2$ = 0.8156) expressed as (Figure 2B):

$$\text{rAUDPC}_{\text{XYL 2177/18}} = 0.8437\ \text{rAUDPC}_{\text{XYL 2055/17}} - 0.0266.$$

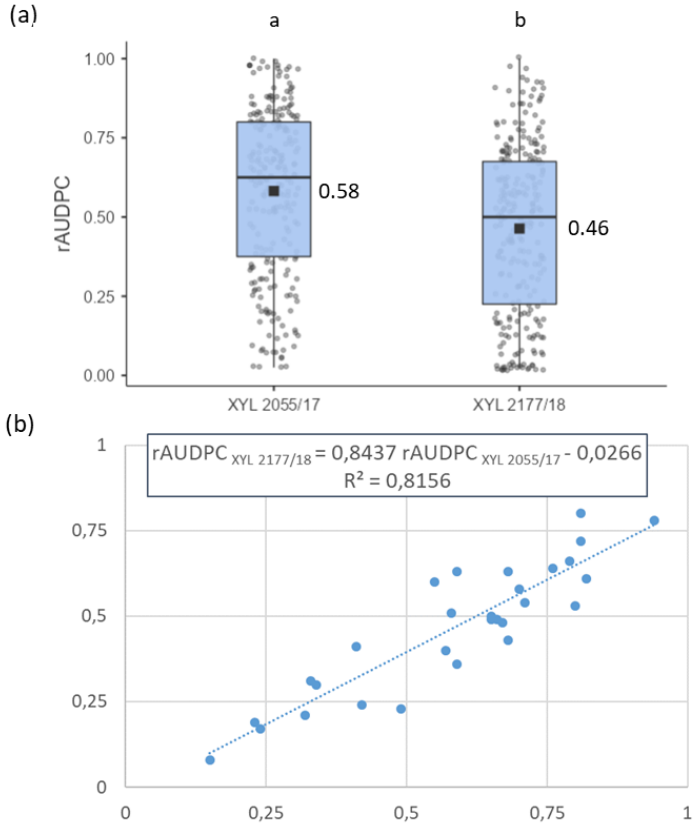
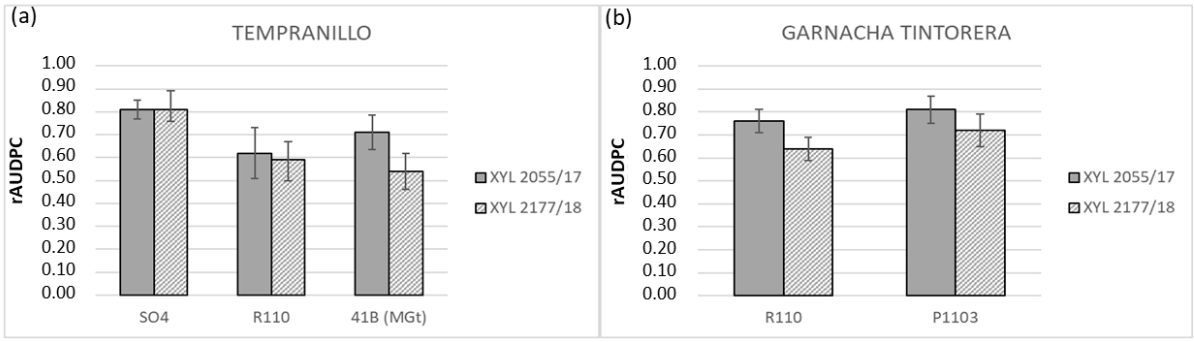



**Figure 2.** Descriptive and statistical parameters for the evaluation of grapevine *Cultivars'* susceptibility to *Xff* strains based on an estimation of the rAUDPC recorded from 8 WPI to 12 WPI for 24 *Cultivars* and 5 *Rootstocks* artificially inoculated with XYL 2055/17 and XYL 2177/18. **(a)** ~~The figure refers to the statistical and descriptive parameters:~~ Each boxplot reports the second and third quartiles, with median (line) and mean values (dot) indicated as squares and points showing all values considered. The *Xff* strains followed by different letters~~, which~~ indicate significant differences among the mean rAUDPC values based on T-test ($p < 0.05$). **(b)** Linear approach for modelling the relationship between *Xff* strains and their coefficient of determination ($R^2$).

Two *Cultivars* studied were grafted onto three different *Rootstocks*. However, this grafting did not appear to produce variation in the intrinsic susceptibility of the grafted *Cultivar*. Disease progression, expressed as the rAUDPC values for Tempranillo and Garnacha Tintorera, was independent of the rootstock upon which the plants were grafted (Figure 3). Therefore, hereafter, we refer to each *Cultivar* independently from the *Cultivars* that were grafted.




(c)

| ANOVA trAUDPC | Sum of Squares | df | Mean Square | F | p |
|---|---|---|---|---|---|
| *Cultivar* | 0.274 | 9 | 0.0304 | 2.02 | 0.05 |
| Residuals | 1.129 | 75 | 0.0150 | | |

**Figure 3.** Mean rAUDPC values obtained for Tempranillo **(a)** and Garnacha Tintorera **(b)** *Cultivars* grafted onto different *Rootstocks* (SO4, R110, 41 B, and P1103). The plants were artificially inoculated with two *Xff* strains: XYL 2055/17 (solid grey) and XYL 2177/18 (grey grid). The data were recorded over one year (2019) in an open field over 8 weeks, from 2 months (8 WPI) to 4 months (16 WPI). Error bars represent the standard error of the mean. **(c)** The statistical parameters shown in the table show no significant differences among all the *Cultivars*.

To establish the resistance levels to *Xff*, a new trial was repeated under the same conditions the following year (2020) through inoculation with only the most virulent strain, XYL 2055/17, according to the results ~~previously~~ obtained the year prior (Figure 3). We observed significant differences among *Cultivars* and *Rootstocks* in their individual susceptibility to the pathogen. The *Cultivars* Tempranillo Blanco, Tempranillo RJ43, Tempranillo RF78, Tempranillo, Malvasía, Monastrel, Mencía, Garnacha Tintorera, and Macabeo yielded the highest rAUDPC values, with no significant differences among them. Conversely, Pedro Ximenez, Albariño, Verdejo, Pinot Noir, Hondarrabi Zuri, H. Beltza, and Cabernet Souvignon presented the highest resistance to *Xff* (Figure 4A).

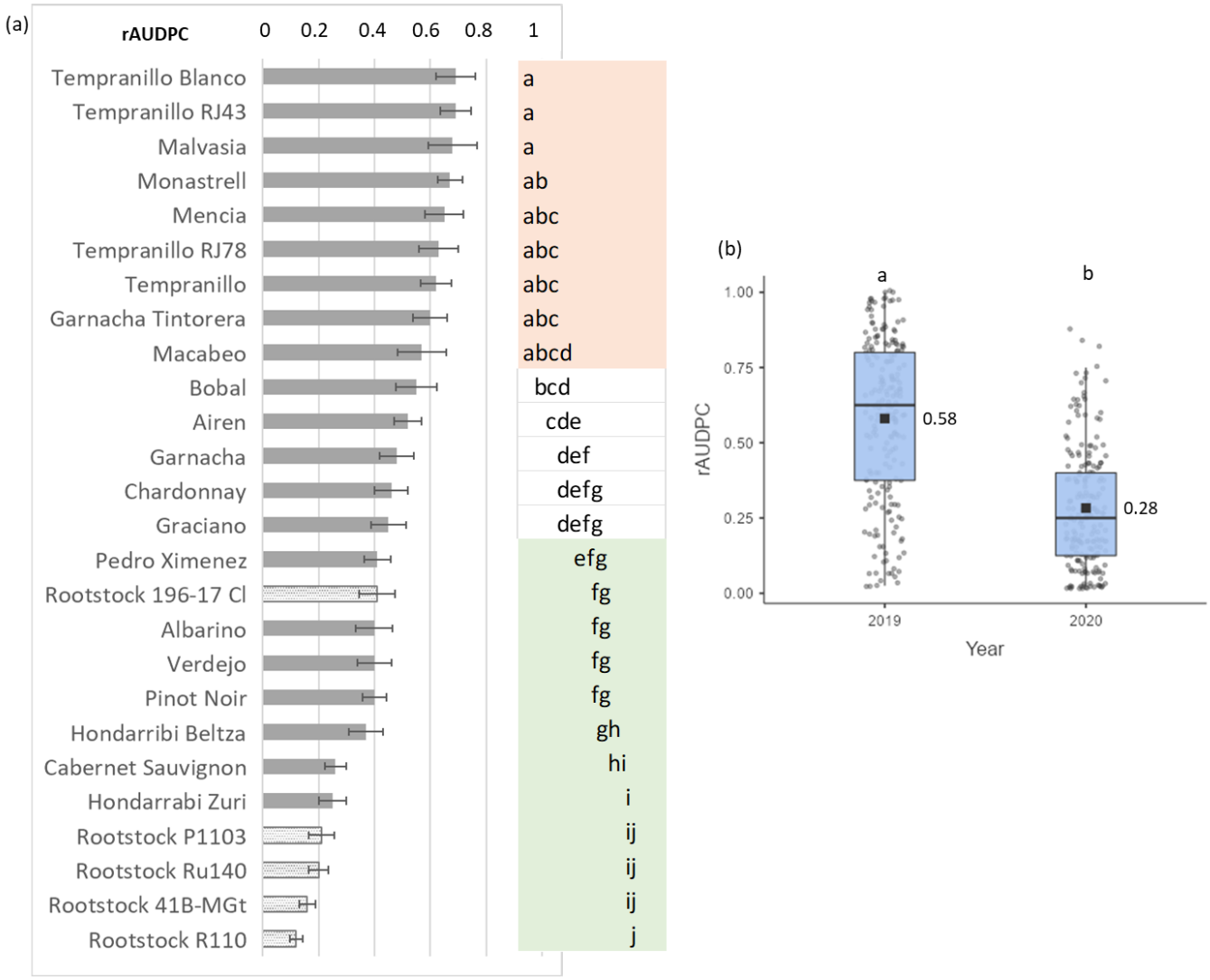



**Figure 4.** (**a**) ~~The~~ mean rAUDPC values obtained for *Cultivars* and *Rootstocks* artificially inoculated with *Xff* ST1 XYL 2055/17, recorded over two consecutive years (2019 and 2020) for 8 weeks, from 2 months (8 WPI) to 4 months (16 WPI). Error bars represent the standard error of the mean. *Cultivars* followed by the same letter indicate non-significant differences among rAUDPC values based on Fisher's LSD test ($p < 0.05$). The most susceptible cultivars with the highest rAUDPC values are marked in red ~~and were found to be the most resistant~~, while cultivars with the lowest values are marked in green. The number of inoculated plants per *Cultivar* considered to obtain the mean rADPC from two ~~replicas (~~years ~~2019 and 2020)~~ varied from 7 to 18. Inoculated plants in which the bacterium was not detected by RTi-PCR were not considered for the rAUDPC assessment. (**b**) Descriptive and statistical parameters in the rAUDPC by the year in which the trial was carried out, 2019 and 2020. Each boxplot reports the second and third quartiles, with median (line) and mean values (dot) indicated as squares and points showing all values considered. The year followed by different letters~~, which~~ indicate significant differences among the mean rAUDPC values based on T-test ($p < 0.05$).

In this study, the five most common *Rootstocks* upon which European varieties are grafted were shown to be less susceptible to *Xff* than most commercial *Cultivars* of *V. vinifera*. This result was essentially confirmed under our conditions because all *Rootstocks* (R110, 41B-MGt, Ru140, P1103, and 196-17 Cl) presented very low susceptibility to the pathogen, with R110 yielding the lowest rAUDPC among all plants assayed for *Xff* (Figure 4A).

Notably, we observed significant differences between the years (Figure 4B). This statistical result indicates that the factors involved in grapevine varietal resistance could be dependent on climate.

### *3.2. Disease Severity and Disease Progression (rAUDPC) under Open Field and Controlled Greenhouse Conditions*

To better estimate and establish the degree of susceptibility, it is necessary to evaluate plants during several campaigns or under different climate conditions. Accordingly, we repeated the assays for one additional year. However, due to operational limitations, we decided to perform the inoculations with only 5 *Cultivars* that showed different levels of susceptibility to *Xff* in previous field trials together with 1 *Rootstock.* In this case, we used the *Xff* ST1 strain IVIA 5770, the only strain that current regulatory restrictions allow to be manipulated outside of demarcated areas in Spain.

During 2021, two new trials were carried out in parallel, one in the same location as the two previous trials (Balearic Islands) and the other under controlled conditions in a greenhouse located in a different climatic area of northern Spain. Special care was taken to ensure that the plants for both trials belonged to the same grafted lot and were maintained without fertilizer or water restrictions.

The results obtained in this new trial carried out in 2021 using a restricted number of *Cultivars* and *Rootstocks* confirm those obtained in the two previous years. All the *Cultivars* and *Rootstocks* were susceptible to *Xff*, in this case, against the strain *Xff* IVIA 5770, with clear pathogen resistance depending on climatic conditions. The rAUDPC values were significantly higher in four of the five *Cultivars* tested: Tempranillo, Garnacha, Pinot Noir, and Chardonay (Figure 5). The *Cultivars* in which the rAUDPC values were not significatively different between field and greenhouse were Hondarrabi Zuri and *Rootstock* R110 (Figure 5). Both *Cultivars* belonged to the group with the lowest rAUDPC values recorded in previous field trials, as shown in Figure 4A.

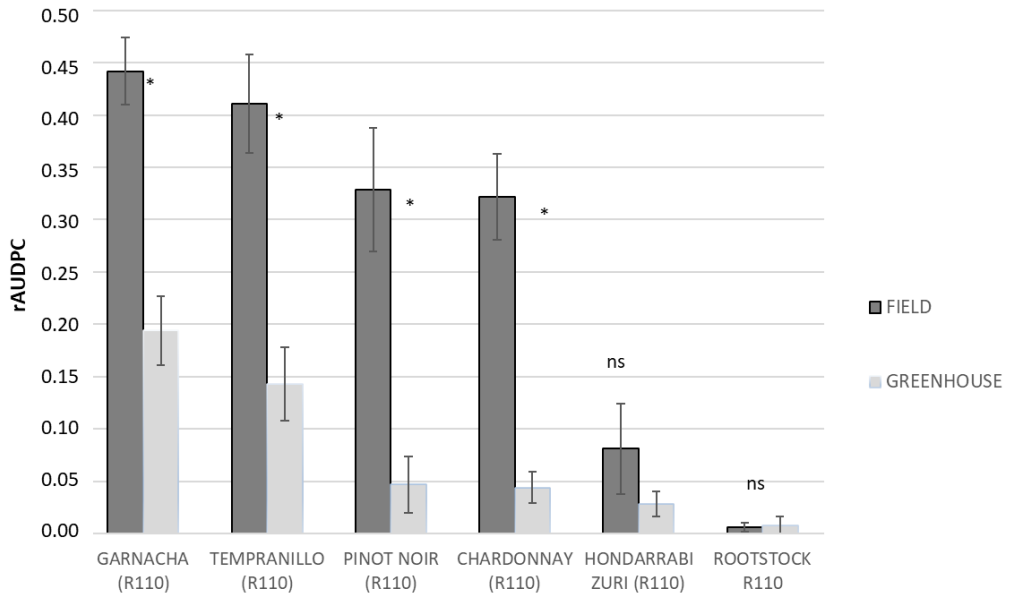



**Figure 5.** Mean rAUDPC values obtained for 5 *Cultivars* and 1 *Rootstock* artificially inoculated with *Xff* IVIA 5770, recorded over one year (2021) in 2 locations under both an open field (FIELD) and controlled conditions (GREENHOUSE). The rAUDPC was calculated based on the SI progress for 8 weeks, from 2 months (8 WPI) to 4 months (16 WPI). Error bars represent the standard error of the mean. Only infected plants and inoculated plants with the bacterium detected via RTi-PCR were considered for rAUDPC assessment. The number of inoculated plants per *Cultivar* considered to obtain the mean rADPC was 9. Asterisks indicate the level of significance: *, $p < 0.05$; ns, not

significant) based on T-test between the means values obtained in Field (dark grey) and Greenhouse (light grey) for each *Cultivar*.

Four months (16 WPI) after inoculation, the SI recorded under field conditions exceeded level 3. However, under greenhouse conditions, this level was not exceeded in any of the *Cultivars* studied (Figure 6). In both the greenhouse and the field, it was possible to observe significant differences in the level of *Cultivar* susceptibility to *Xff* (rAUDPC). However, in the greenhouse, the average of this indicator did not clearly discriminate resistance levels between the *Cultivars* studied. Only Tempranillo yielded a significantly different rAUDPC than that recorded in *Rootstock* R110 (Figure 6).

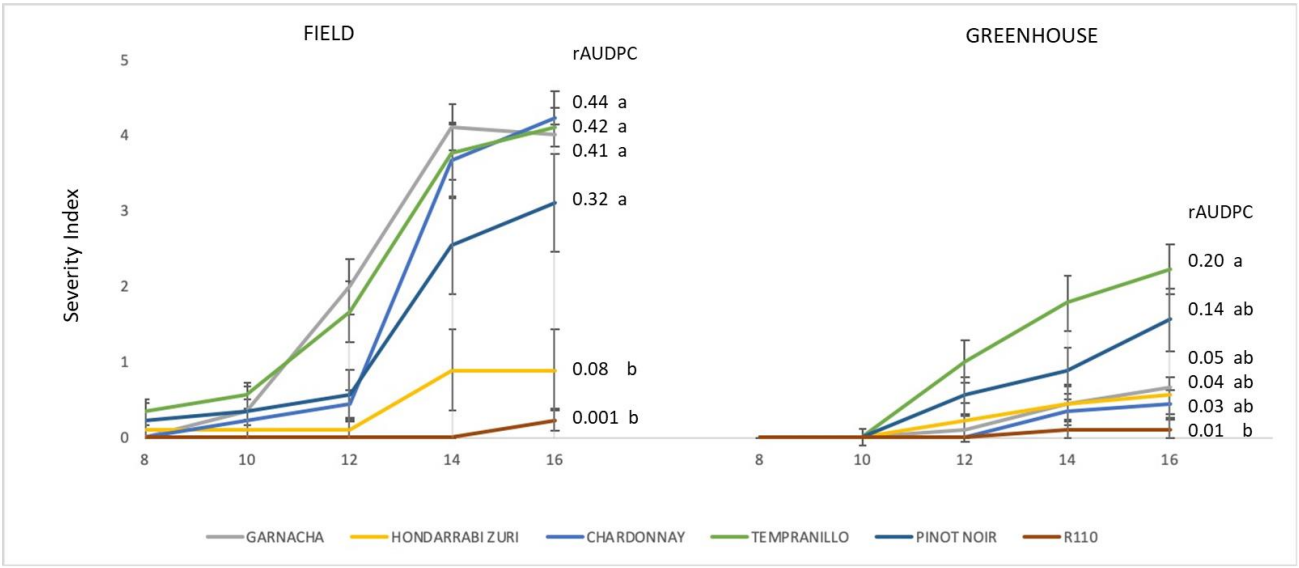



**Figure 6.** Disease progression in an open field (Field) and greenhouse-controlled conditions (Greenhouse) from 8 to 16 WPI. The Severity Index (SI) was assessed on a scale of 0–5 described by Su et al. (2013) [46] based on the number of symptomatic leaves, from asymptomatic to symptomatic leaves ((0) to 1–2 (1), 3–4 (2), 5–7 (3), 8–10 (4), and >10 (5), respectively). The plants were artificially inoculated with the *Xff* IVIA 5770 strain. Values are the means of 13 replicates, and error bars represent the standard error. The rAUDPC derivates from the time progression of the SI are shown in the boxes. Cultivars followed by the same letter indicate non-significant differences between the rAUDPC values based on Fisher's LSD test ($p < 0.05$).

Importantly, after inoculation, the asymptomatic period was shorter in the open field than that in the greenhouse. Asymptomatic to symptomatic ~~transmission~~ occurred between 10 and 12 WPI in the greenhouse and ~~was able to occur~~ after 8 WPI in the open field (Figure 6).

~~Without considering the average rAUDPC values obtained in all of the trials carried out in this work to evaluate resistance to *Xff*,~~ Table 1 shows the maximum SI recorded in the inoculated plants, in both the field and the greenhouse. Two months after inoculation (16 WPI), all *Cultivars* of *Vitis vinifera* tested in the field recorded the maximum value of SI (5). Only two of the five evaluated *Rootstocks* (41 B-MGt and R110) did not obtain the same maximum SI value. In the greenhouse, no *Cultivar* yielded the maximum SI value (5), and only Tempranillo reached level 4 (Table 1).

**Table 1.** Maximum Severity Index value (max. SI) recorded in ~~some of~~ the plants of each *Cultivar* and *Rootstock* evaluated in all of the trials carried out in this study at 8 and 16 WPI (max. SI 8 WPI/max. SI 16 WPI). The first line and the columns below it show the year in which the trials were carried out, the second line shows where the trials were conducted ~~(in open field—"Field" or under controlled conditions in a greenhouse—"Greenhouse")~~. The third line corresponds to the *Xff* strain artificially inoculated (XYL2055/17, XYL2177/18, and IVIA5770) in 24 grapevine *Cultivars* and 5 *Rootstocks*. The *Rootstock* column indicates the rootstock on which each cultivar was grafted.

| *Cultivar* | *Rootstock* | 2019 | | 2020 | 2021 | |
|---|---|---|---|---|---|---|
| | | Field | | | | Greenhouse |
| | | XYL 2177/18 | XYL 2055/17 | XYL 2055/17 | IVIA 5770 | IVIA 5770 |
| Airen | R110 | 3/5 | 4/5 | 2/5 | - | - |
| Albarino | R110 | 2/5 | 5/5 | 1/3 | - | - |
| Bobal | R110 | 4/5 | 3/5 | - | - | - |
| Cabernet Sauvignon | R110 | 1/5 | 2/5 | 1/5 | - | - |
| Chardonnay | R110 | 5/5 | 5/5 | 1/5 | 1/5 | 0/1 |
| Garnacha | R110 | 4/5 | 5/5 | 3/5 | 1/5 | 0/2 |
| Garnacha Tintorera | R110 | 5/5 | 5/5 | 3/5 | - | - |
| Garnacha Tintorera | P1103 | 5/5 | 5/5 | 3/5 | - | - |
| Graciano | R110 | 2/5 | 4/5 | 1/5 | - | - |
| Hondarrabi Zuri | SO4 | 3/5 | 3/5 | 1/2 | 1/5 | 0/2 |
| Hondarribi Beltza | 196-17 Cl | 2/5 | 3/5 | 0/3 | - | - |
| Macabeo | R110 | 3/5 | 3/5 | - | - | - |
| Malvasia | R110 | 3/5 | 4/5 | - | - | - |
| Mencia | R110 | 4/5 | 4/5 | - | - | - |
| Monastrell | R110 | 2/5 | 3/5 | - | - | - |
| Pedro Ximenez | R110 | 2/5 | 3/5 | 2/5 | - | - |
| Pinot Noir | R110 | 1/5 | 3/5 | 1/5 | 3/5 | 0/3 |
| Tempranillo | R110 | 4/5 | 4/5 | 1/5 | 3/5 | 0/4 |
| Tempranillo | SO4 | 4/5 | 5/5 | 3/5 | - | - |
| Tempranillo | 41B-MGt | 4/5 | 5/5 | 1/5 | - | - |
| Tempranillo Blanco | R110 | 5/5 | 5/5 | 3/5 | - | - |
| Tempranillo RJ43 | R110 | 3/5 | 5/5 | 4/5 | - | - |
| Tempranillo RJ78 | R110 | 3/5 | 5/5 | 3/5 | - | - |
| Verdejo | R110 | 3/5 | 2/5 | - | - | - |
| Rootstock 196-17Cl | | 3/5 | 3/5 | 1/5 | - | - |
| Rootstock 41B-MGt | | 2/3 | 2/4 | 1/4 | - | - |
| Rootstock P1103 | | 1/5 | 1/5 | 1/3 | - | - |
| Rootstock R110 | | 1/2 | 1/2 | 2/2 | 0/1 | 0/1 |
| Rootstock Ru140 | | 1/5 | 1/5 | 1/3 | - | - |

### *3.3. Water Potential Progression during Xff Infection and Disease Development*

To associate PD symptomatology and bacterial detection with the effects of infection on the water status of the plants, the Ψ differences between treatments (control and inoculated) were also analyzed for 19 weeks, from 2 WPI to 21 WPI, in plants growing under controlled conditions in the greenhouse. ~~The~~ Ψ varied as the phenological stage of the plants progressed, but significant differences between inoculated and non-inoculated plants were observed from 12 WPI onwards (Figure 7). However, this result was not clearly established in all tested *Cultivars*. In two of the five cultivars (Garnacha and Pinot Noir), significant differences were not found, even at 21 WPI (Figure 8A). In the *Rootstock* R110 and Hondarrabi Zuri, which both presented the lowest severity index in the greenhouse (Figure 5), it was possible to find significant differences in Ψ between inoculated and healthy plants (Figure 8A). These results suggest no relationship between the severity of the disease and variation in water potential, at least in some *Cultivars*. On the other hand, at 12 WPI, the first period with significant differences in Ψ between inoculated and control plants, 41% of inoculated plants already indicated positive bacterial detection, as shown in Figure 8B.

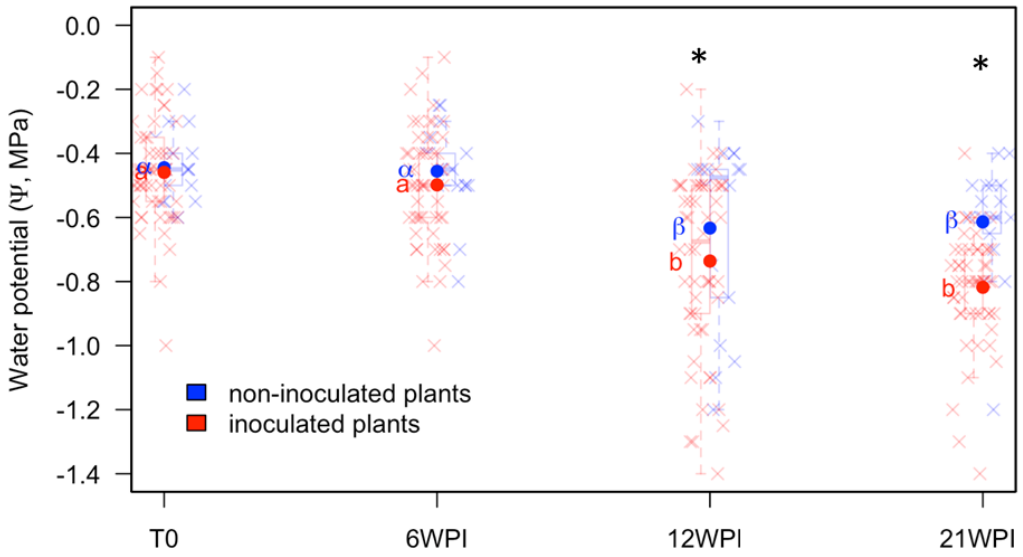
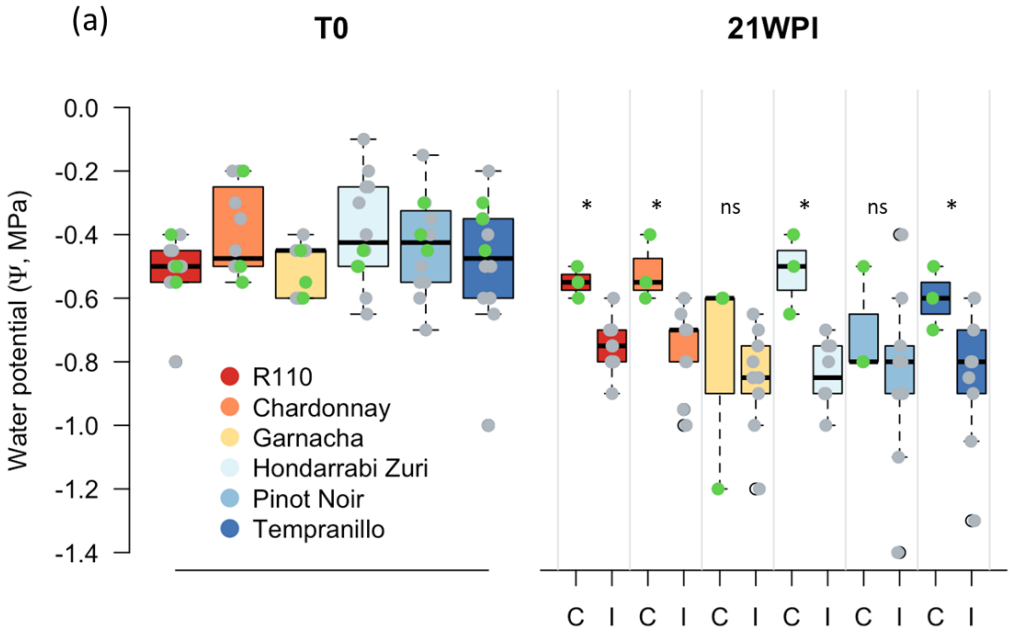



**Figure 7.** Water potential variation over time under greenhouse-controlled conditions. Latin letters correspond to the inoculated plants, while Greek letters represent the non-inoculated plants. The asterisks indicate significant differences ($p < 0.05$) by T-test between inoculated and non-inoculated plants at each time point: T0 (before inoculation), 6, 12, and 21 WPI. ~~The water potential (Ψ) is expressed in MPa.~~ Individual measurements for each plant are shown with "×" signs. The plants were artificially inoculated with *Xff* IVIA 5770.




(b)

| | (%) RTi-PCR+ | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2 WPI | 4 WPI | 6 WPI | 8 WPI | 10 WPI | 12 WPI | 14 WPI | 16 WPI | 18 WPI | 21 WPI |
| R110 | 0 | 0 | 0 | 22 | 22 | 22 | 22 | 33 | 89 | 100 |
| Chardonnay | 11 | 11 | 44 | 44 | 44 | 44 | 44 | 44 | 78 | 100 |
| Garnacha | 0 | 11 | 11 | 22 | 22 | 22 | 44 | 56 | 78 | 100 |
| Hondarrabi Zuri | 0 | 22 | 22 | 22 | 22 | 33 | 33 | 44 | 78 | 100 |
| Pinot Noir | 0 | 44 | 44 | 44 | 44 | 44 | 67 | 78 | 78 | 100 |
| Tempranillo | 0 | 44 | 44 | 44 | 44 | 78 | 89 | 100 | 100 | 100 |
| Total | 2 | 22 | 28 | 33 | 33 | 41 | 50 | 59 | 84 | 100 |

**Figure 8.** Progression of the water potential (Ψ) and RT-PCR detection of *Xff* IVIA 5770 artificially inoculated in grapevine plants. The trial was carried out under controlled conditions in a greenhouse. (**A**) Ψ recorded before (T0) and after 21 WPI for artificial inoculation with *Xff* IVIA 5770 in 5 grapevine *Cultivars* and 1 *Rootstock* (R110) and 21 WPI under greenhouse-controlled conditions. Each boxplot reports the second and third quartiles, with median values (line) in the square and points showing outliers (green for non-inoculated (C) and grey for inoculated plants (I). The asterisks indicate significant differences ($p < 0.05$) by T-test between inoculated and non-inoculated plants at 21 WPI (**B**) Percentage of artificially inoculated plants in which the bacterium was detected via RTi-PCR from 2 to 21 WPI.

## 4. Discussion

As previously described, strains of *Xff* ST1, isolated for the first time in the Balearic Islands, were able to cause disease in some of the main grapevine varieties planted in different Spanish regions [29]. The 24 *Cultivars* studied in this work were found to be susceptible. However, it was also possible to clearly establish different levels of resistance ~~between~~ cultivars. The results obtained agree with those previously published, showing different degrees of resistance in certain ~~numbers of~~ grapevine cultivars, most likely influenced by their different pedigrees (linked to their shared centers of domestication) and xylem anatomical features [33] or an engineered innate immune defense in grapevines [52].

Notably, the two determining factors of grapevine cultivar resistance to *Xff* in this study were the bacterial genotype (*Xff* strain) and environmental climate. The virulence of the *Xff* strain XYL 2055/17 was significantly higher than that of strain XYL 2177/18. ~~Thus, disease severity could be dependent on climate.~~

Plant–pathogen interactions are multifaceted processes mediated by the pathogen- and plant-derived molecules. Thus, the differential virulence between strains is well

known. In this work, we detected this condition ~~for the first time~~ in two strains of *Xff* isolated independently in the same geographical area over consecutive years, which could corroborate the results of the genetic variability of *Xff* isolated in the Balearic Islands recently presented by Dr. Landa's research team in 2022 [7]. Despite the differences in virulence found between the two strains in this study, resistance was highly correlated between cultivars. Thus, strain virulence does not appear to modify the susceptibility profiles.

In the absence of a greater number of trials under different agroclimatic conditions, this work established, for the first time, two significantly different groups of European cultivars of *Vitis vinifera* ~~were~~ characterized as having high and low susceptibility to *Xff*. The results indicate that Tempranillo and Tempranillo Blanco can be considered as reference cultivar (variety) with high ~~resistance~~ compared to Hodarrabi Zuri and Cabernet Sauvignon with lower susceptibility.

The results obtained in this work demonstrate that the *Rootstock* commonly used to graft commercial varieties of grapevine are also susceptible to PD caused by *Xff* European isolates. However, the level of susceptibility was significantly lower than that of most commercial *Cultivars*. Several studies showed that rootstocks affect scion ~~genotypes~~ in many different ways. Rootstocks, for example, can influence scion vigor and phenology and confer differential tolerance to drought and disease ~~prone conditions~~ in various crops [53, 54]. However, the low susceptibility found in the *Rootstocks* studied was not found to confer lower susceptibility among the cultivars. Our results indicate that the susceptibility of one grapevine cultivar to PD could be independent of the rootstock on which the cultivars were grafted ~~and that the level of susceptibility of *Cultivars* grafted onto the same rootstock could possess significantly different levels of susceptibility~~.

To ensure higher control over environmental variables, it is common to evaluate cultivars for their resistance to pathogens in growth chambers or greenhouses before evaluations under field conditions. However, the susceptibility ratings under ~~both~~ conditions were previously found to show either a significant correlation [55, 56] or no correlation [57]. Additionally, in certain cases, greenhouse evaluations tend to overestimate susceptibility [58]. In the end, screening for resistance to pathogens could be more accurate when conducted in open fields or greenhouses, depending on the expression of plant-defense-related genes after bacterial inoculation or the presence of climatic conditions more conducive to bacterial growth [59]. Therefore, because inoculation with a quarantine pathogen such as *Xff* is more practical in controlled facilities, we evaluated the correlation between plant susceptibility to *Xff* among five cultivars and one *Rootstock* in a greenhouse and in the field. The results showed that disease progression was significantly slower in the greenhouse than in the open field. Consequently, the rAUPC found in grapevines grown in this facility was no greater than 0,25 units from 2 to 4 months after inoculation. This result is not surprising since in other pathogenicity models of *Xff* in greenhouses, disease severity did not reach the levels found in the open field, as in the case of *Xff* in southern highbush blueberry (*Vaccinium* sp.) [60] and almond [43].

Under the current disease scenario, ~~*Xf*~~ can handle tasks with ~~infected~~ plant material only under confined, approved conditions to avoid spread of the disease, denoted as temporal confinement stations for quarantine pathogens (Regulation (EU) 2016/2031). Biosafety greenhouses are the only facilities that can be used for this purpose. Taking into account our results, it would be possible to discriminate between highly and moderately susceptible cultivars with reference to Tempranillo *Cultivars*. Tempranillo grafted onto Rootstock R110 was able to reach a severity index of 4 after 16 WPI, as shown by our results. Together with Tempranillo, Pinot Noir (both grafted onto R110) presented the earliest symptoms. The first symptoms were recorded at 12 WPI. This result confirms our selection of Chardonnay and Pinot Noir varieties as indicator plants in the pathogenicity tests and suggests the inclusion of the Tempranillo and Garnacha varieties in standards for the Diagnostic Protocol of *Xff*.

The differences in varietal susceptibility levels highlighted in this work under open field conditions, together with previous work showing that disease incidence and severity may be related to agricultural management, suggest that PD in Europe could be, in the future, managed as a chronic disease as it is now managed in ~~other~~ American winegrowing areas [32]. Several other results support this theory, including ~~the clearly lower severity of PD in organic versus conventional grapevine management and the distance from the original focus (Moralejo, 2019 [39]),~~ the positive results recently found when using innovative synthesized chemical treatments [37, 42], the efficacy of foliar-applied biological treatments [61], insect vector deterrence [62], and others [9]. We should also consider the wide variety of biostimulants appearing on the fertilizer and agro-sanitary markets whose contributions to vineyard health in different agroclimatic grapevine areas remain to be studied [63, 64].

It is well known that PD in ~~European and American~~ grapevine ~~varieties are~~ related to a decrease in water conductance, water potential, and hydraulic conductance [10, 11, 65]. Compared to healthy leaves developed under the same conditions, *Xf*-infected plants showed a reduction in available water and nutrients resulting in wilting and death due to stress. This result was corroborated using the Scholander test in woody plants [12]. In this study, we were able to associate this physiological effect with the onset of symptomatology and *Xff* infection. Based on these results, it is not easy to consider this parameter as an early detection test for *Xff* infection prior to the symptomatic period. The differences in water potential started to become significantly distinct between healthy and infected plants once the bacteria were detectable via standard RTi-PCR. However, ~~as the Scholander test is a destructive technology that provides results similar to those of other non-destructive and portable devices [25, 27], the assessment of water potential could be an alternative indicator for *Xff* infection in open fields.~~ We cannot ignore these new and non-destructive technologies as they create the possibility to survey, and potentially geo-reference, large numbers of plants in real time.

Undoubtably, grapevine agro-management in novel environments coupled with *Cultivar* resistance will play a determining role in PD control in Europe. Indeed, such methods are already being prioritized in other crops [66]. Considering the data presented in this work, several agro-climatic parameters should be tested to enhance breeding programs and establish a discriminative threshold for cultivar selection. At the same time, we should explore new technologies for the early detection of differential physiological responses and phenotyping of foliar disease severity under controlled conditions [67, 68].

## 5. Conclusions

The 24 European *Vitis vinifera* cultivars studied in this work, representing more than 70% of the cultivated area in Spain, are susceptible to the PD caused by current *Xff* ST1 strains detected in Europe. After carrying out trials over three consecutive years, we obtained consistent results indicating two significantly different groups of ~~susceptibility whose levels of~~ resistance may not be influenced by the rootstock upon which the plant is grafted. Importantly, *Cultivar* susceptibility is clearly influenced by the environmental conditions under which the plants are grown.

~~The differences found in water potential alongside the first symptoms, as well as the detection of the bacteria in plant tissues, open the door to using mobile and non-destructive devices to detect new foci of this disease by analyzing a larger number of plants in real time.~~

Tempranillo and Tempranillo Blanco could be included as references in breeding programs for *Xff* as indicators of highly susceptible C*ultivars*, since under field and greenhouse conditions, these varieties presented the largest SI and rAUDPC values four months after inoculation.

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