Increase in ring width, vessel number, and $\delta^{18}\text{O}$ in olive trees infected with *Xylella fastidiosa*

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Abstract

Xylella fastidiosa (*Xf*) is a bacterium that causes plant diseases in the Americas. In Europe, it was first detected on the Salento Peninsula (Italy), where it was found to be associated with the Olive Quick Decline Syndrome (OQDS). Here, we present the results of the first tree-ring study of infected and uninfected olive trees of two different cultivars, one resistant and one susceptible, to establish the effects induced by the spread of the pathogen inside the tree. Changes in wood anatomical characteristics, such as an increase in the number of vessels and in ring width, were observed in the infected plants of both the cultivars Cellina di Nardò (susceptible to *Xf* infection) and Leccino (resistant to *Xf* infection). Thus, whether infection affects the mortality of the tree or not, the tree shows a reaction to it. The presence of occlusions was detected in the wood of both four-year-old branches and the tree stem core. As expected, the percentage of occluded vessels in the *Xf*-susceptible cultivar Cellina di Nardò was significantly higher than in the *Xf*-resistant cultivar Leccino. The $\delta^{18}\text{O}$ of the four-year-old branches was significantly higher in infected trees of both cultivars than in non-

33 infected trees, while no variations in $\delta^{13}\text{C}$ were observed. This suggests a reduction in leaf
34 transpiration rates during infection and seems to be related to the occlusions observed in rings of the
35 four-year-old branches. Such occlusions can determine effects at leaf-level that could influence
36 stomatal activity. On the other hand, the significant increase in the number of vessels in infected trees
37 could be related to the tree's attempt to enhance water conductivity in response to the pathogen-
38 induced vessel occlusions.

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40 **Keywords**

41 Dendrochronology; wood anatomy; stable isotopes; tylosis; *Olea europaea* L.

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64 1. Introduction

65 A sudden decline in olive (*Olea europaea* L.) trees has been observed since the early 2010s on the
66 Salento Peninsula (southeastern Italy), and is now known as the Olive Quick Decline Syndrome
67 (OQDS). This disease is caused by the bacterium *Xylella fastidiosa* (*Xf*) subsp. *pauca* strain ‘De
68 Donno’, a pathogen that occurs naturally in the Americas. Its presence in Italy represents the first
69 confirmed record in Europe (Martelli et al., 2015). Olive trees are infected with bacteria by xylem-
70 feeding insects, which results in the rapid spread of the disease (Martelli et al., 2015). The exact
71 mechanisms of pathogenesis are not yet fully described, but it seems that the hydraulic system of the
72 host tree changes its structure according to the interaction between the pathogen and the tree. Theories
73 about the nature of this interaction include xylem blockage by bacterial aggregation, phytotoxins
74 produced by the bacteria, gums and tyloses produced by the tree that may decrease xylem
75 conductance, or a combination of all these factors (Newman et al., 2003; Roper et al., 2007; Pérez-
76 Donoso et al., 2007; Stevenson et al., 2004). According to Cardinale et al. (2018), occlusions in
77 vessels of plants infected by *Xf* are built up by tyloses combined with a bacterial biofilm. While the
78 general symptoms of OQDS are well documented (Martelli et al., 2015), the behaviour of bacteria in
79 the xylem hydraulic network of the olive tree is poorly understood. Vascular pathogens are among
80 the most difficult plant pathogens to study because they live deep inside their host plants, making the
81 exploration of their biology difficult. Actions to control these plant pathogens are rarely reliable; apart
82 from controlling the vector, the most efficient long-term strategy is to understand the mechanisms
83 underlying plant resistance to the pathogen (Yadeta and Thomma, 2013). Trees protect themselves
84 from the consequence of lesions or infection by walling off the damage or by compartmentalizing the
85 pathogen. Variations in wood anatomical characteristics can therefore represent an adaptive structural
86 solution adopted by the tree in order to optimize both its performance and its chances of survival
87 against external threats (Shigo, 1984; Kaus et al., 1996; Kroken et al., 2008; Smith, 2008; Renzi et
88 al., 2012; Tulik et al., 2017). The critical role played by lignin in *Xf* resistance of the olive cultivar
89 Leccino has already been demonstrated by Sabella et al. (2018); they suggested that the cell walls of

90 Leccino infected plants (enriched in lignin) became more specialized in order to limit the ability of
91 the *Xf* to degrade pit membrane and to move into the adjacent vessels. Trees record signals of past
92 environmental conditions and their reaction to trends and events in their rings. Here, for the first time,
93 tree rings were studied to understand OQDS in the *Xf*-susceptible cultivar Cellina di Nardò and in the
94 *Xf*-resistant cultivar Leccino (Luvisi et al., 2017; Saponari et al., 2017). Tree-ring characteristics such
95 as ring width and vessel size and density provide information about tree physiology and vitality.
96 Changes in ring-width patterns have been used to understand the impact of different fungal pathogens
97 on tree vitality (Cherubini et al., 2002; Vezzola et al., 2019; Pouzoulet et al., 2017). Thus, tree rings
98 and their anatomical traits might be used to establish the spread of pathogens inside the tree (Fonti et
99 al., 2010; Renzi et al., 2012). In particular, carbon $\delta^{13}\text{C}$ and oxygen $\delta^{18}\text{O}$ isotopes in wood tissue may
100 provide insights into stomatal activity, transpiration, photosynthesis rates, and water-use efficiency
101 (Farquhar et al., 1982; Barbour et al., 2007).

102 In this study, wood anatomical, dendrochronological, and tree-ring carbon and oxygen stable isotopes
103 analyses were carried out in naturally infected plants of the *Xf*-susceptible cultivar Cellina di Nardò
104 and of the *Xf*-resistant cultivar Leccino. The main aim of this work was to see if any *Xf*-induced
105 changes in wood anatomical characteristics (vessel size, ring width, and presence of occlusions)
106 occurred, and, if so, to determine if they were related to the temporal development of *Xf* infection.

107 **2. Materials and methods**

108 **2.1 Study area and plant details**

109 We selected two orchards near the city of Lecce (Parco Naturale Regionale Bosco e Paludi di Rauccio,
110 40°27'24.0"N 18°09'43.8"E, Apulia, Italy) in which olive trees (25–35 years) of two different
111 cultivars, i.e., cultivar Leccino and cultivar Cellina di Nardò, were agronomically managed in a
112 similar manner over the last 5 years. Phytosanitary treatments were carried out according to EU
113 Decision 2015/789, which is required to control the main insect vector, i.e., a splitfly (*Philaenus*
114 *spumarius*). Orchards were monitored weekly to detect outbreaks of insects or other pests. No

115 irrigation was carried out. The presence of *Xf* was assessed in trees that showed OQDS symptoms
116 since 2015 using real-time PCR (Harper et al., 2010). Evaluations of disease severity was carried out
117 by visual inspections of the plants every 2 months after pruning (from February 2015 to August 2018),
118 following Luvisi et al. (2017). In both *Xf*-positive (*Xf*-p) and *Xf*-negative (*Xf*-n) samples, plants were
119 monitored and tested for some of the most common pathogens, including *Colletotrichum*
120 *gloeosporioides*, *Mycocentrospora cladosporioides*, *Spilocaea oleagina*, and *Pseudomonas*
121 *savastanoi* pv. *savastanoi*. Diagnostic tests (real-time PCR) were carried out on leaves or woody
122 chips, according to protocols reported in the literature, for *Verticillium dahliae* (Bilodeau et al., 2012),
123 *Colletotrichum* spp., *C. acutatum* and *C. gloeosporioides* (Garrido et al., 2009), *Phaeomoniella*
124 *chlamydospora* (Martín et al., 2012), *Phaeoacremonium aleophilum* and *P. parasiticum* (Aroca et al.,
125 2008; Carlucci et al., 2015), *Botryosphaeria dothidea* (Romanazzi et al., 2009), *Diplodia seriata*
126 (Martín et al., 2014), and *Phytophthora* spp. (Drenth et al., 2006; Cacciola et al., 2001). Apart from
127 some sporadic evidence of *C. gloeosporioides*, *S. oleagina*, and *P. savastanoi* pv. *Savastanoi*, both
128 *Xf*-p and *Xf*-n trees tested negative for other pathogens. Samples were not collected from plant organs
129 showing symptoms of pathogens other than *Xf*.

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131 **2.2 Core sampling, sample preparation, and analyses**

132 In 2017, tree stem cores from *Xf*-p (n=3 per cultivar) and *Xf*-n (n=3 per cultivar) olive trees were
133 sampled. The cores were taken from each tree using an increment borer as described by Grissino-
134 Mayer (2003). In the laboratory, the cores were dried at room temperature and prepared using a sliding
135 core-microtome (Reichert, Germany) (Gärtner and Nievergelt, 2010). We identified annual rings
136 under a stereomicroscope (Wild M3Z, Leica, Germany) using standard dendrochronological
137 procedures by dating them from bark to pith (Stokes and Smiley, 1968). Intra-annual density
138 fluctuations (IADFs) are often formed in olive trees, making dating very difficult since they are
139 difficult to distinguish from real ring boundaries (Cherubini et al. 2003, 2013; Battipaglia et al. 2010,
140 2016; Gea-Izquierdo et al. 2012). For this we used microsections that enable a more secure

determination of annual rings. Wood anatomical inspections of density fluctuations allows differentiating them from ring boundaries, which simplifies the detection of annual rings especially in Mediterranean species (Gärtner et al., 2015). These microsections were not suitable for further analyses because the occlusions of interest are removed from the sections due to their thickness of 20 μm only. Therefore, ring-by-ring images (5X magnification) were captured directly from the core surface using a digital video camera (EC3, Leica Microsystems, Wetzlar, Germany) connected to a transmitted light microscope (Orthoplan, Ernst Leitz, Wetzlar, Germany). Digital photos with a fivefold magnification were analysed and, to simplify subsequent measurements, vessel lumen was manually edited by using Adobe Photoshop (Adobe Systems Incorporated, USA); occluded vessels were colored red, un-occluded vessels remained white. Every cross-section was divided into four parts to avoid optical distortion, to achieve faster measurement and to provide a better measurement accuracy.

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2.3 Branch sampling, sample preparation, and wood anatomical analyses

In 2018, four-year-old branches from *Xf-p* (n=3 per cultivar) and *Xf-n* (n=3 per cultivar) olive trees (cultivar Cellina di Nardò and Leccino) were sampled. Following Blum (1893), a 4% aqueous formaldehyde solution was used as an antiseptic and fixative to preserve the tissue morphology, which was examined using standard histological techniques. Samples were prepared from radial sections of branches previously cut into pieces approximately 2 cm long in order to enable them to fit into the microtome. Thin sections (13-15 μm thick) were then cut perpendicular to the growth axis from each piece with a sliding WSL Lab microtome (modified Reichert-type, Gärtner et al., 2015). The thin sections were stained with a safranin and astrablue mixture (50:50), dehydrated with ethanol (75%, 96%, and 100 %), rinsed with xylol, and finally embedded in Canada balsam (Gärtner and Schweingruber, 2013). Images (5X magnification) were captured using a digital video camera (EC3, Leica Microsystems, Wetzlar, Germany) connected to a transmitted light microscope (Orthoplan, Ernst Leitz, Wetzlar, Germany). For each thin section, several images were captured and later stitched

167 together using the PTGui software (New House Internet Services B.V., Rotterdam, NL) in order to
168 obtain a panorama image of the radial section of the entire branch (von Arx et al., 2016). Then, for
169 each observable year (2015 to 2018), the number of xylem vessels, the lumen area (μm^2) of un-
170 occluded vessels, and the longitudinal/transversal diameter ratio (L/T ratio) were determined using
171 the image analysis software WinCell (Regent Instruments Inc., Québec, Canada). Lumen of the
172 occluded vessels was manually edited by using Adobe Photoshop (Adobe Systems Incorporated,
173 USA); and individually counted to obtain the percentage of xylem vessel occlusions. Ring widths
174 were measured on all samples using Photoshop's calibrated measuring tool.

175

176 **2.4 Stable isotope analyses**

177 Part of the branches used for the wood anatomical analysis was also used to analyse the carbon and
178 oxygen stable isotope composition of the wood. For each biological replicate ($n=3$), each branch was
179 separated into four pieces at different intervals and for each piece, each single tree ring was split with
180 a scalpel and each wood sample was ground using a centrifugal mill (Mixer Mills, MM 200, Retsch,
181 Haan, Germany). After milling, aliquots of 9.5-11.0 mg were packed into porous bags and used for
182 cellulose extraction, as described in Battipaglia et al. (2008). Aliquots of 1.0 – 1.2 mg of cellulose
183 were weighed in silver capsules and pyrolyzed to CO at 1450°C (PYRO-cube, Elementar, Hanau,
184 Germany) for subsequent C and O isotope analysis by an isotope ratio mass spectrometer (Delta Plus
185 XP, Thermo, Bremen, Germany). The results are presented in delta notation as $\delta^{13}\text{C}$ or
186 $\delta^{18}\text{O} = (\text{R sample}/\text{R standard}) - 1 \times 1000(\text{‰})$, relative to the Vienna Pee Dee Belemnite (VPDB) for
187 carbon and the international Vienna Standard Mean Ocean Water (VSMOW) for oxygen. The
188 standard deviation for the repeated analysis of an internal standard (commercial cellulose) was better
189 than $\pm 0.2 \text{‰}$ for carbon and $\pm 0.3 \text{‰}$ for oxygen.

190 The calibration versus VPDB was done by measuring IAEA USGS-24 (graphite) and IAEA-CH7
191 (polyethylene). For carbon, the calibration versus VSMOW was done using IAEA-C3 cellulose and
192 other inter-comparison standards (Saurer et al., 1998).

193 2.5 Statistical analysis

194 Lumen length, width, area, L/T ratio (n=3), ring width, and data about the number and percentage of
195 vessels occluded in the four-year-old branches (n=3) and in stem cores (n = 3) for each year were
196 subjected to 2-way ANOVA, with the two cultivars (Leccino vs. Cellina) and the two health statuses
197 (*Xf*-p vs. *Xf*-n) as factors, followed by Tukey-HSD (honestly significant difference) post-hoc tests (p
198 < 0.05). Vessel number for each year was subjected to the Student t-test, as were data of carbon
199 ($\delta^{13}\text{C}\%$) and oxygen ($\delta^{18}\text{O}\%$) stable isotopes (n=3) for each cultivar. Disease severity data were not
200 normally distributed (Shapiro-wilk test, $p \leq 0.05$), so the nonparametric Kruskal-Wallis test was used
201 to determine overall differences in means. Graphs were made using Prism (GraphPad Software, San
202 Diego, USA).

203

204 3. Results

205 3.1 Vessel traits in four-year-old branches of plants naturally infected with *X. fastidiosa*

206 Observation of micro sections simplified the identification of the annual rings, both in branches and
207 in trunk core samples (see Figure S1 available as Supplementary Data at *Tree Physiology* Online).
208 Tree-ring analyses of naturally *Xf*-p trees of the cultivars Leccino and Cellina di Nardò revealed no
209 significant differences in average lumen length, lumen width, lumen area, or L/T
210 (longitudinal/transversal diameter) ratio compared to *Xf*-n samples (Figure 1). Furthermore, no
211 significant differences were found between the two cultivars. The average vessel length and width of
212 all four-year-old branches in the two cultivars (in both *Xf*-n and *Xf*-p plants) were $35.33 \pm 3.34 \mu\text{m}$
213 and $26.68 \pm 2.55 \mu\text{m}$, respectively (Figure 1a and 1b). The average vessel lumen area in all four-year-
214 old branches of the two cultivars (in both *Xf*-n and *Xf*-p plants) was $706.96 \pm 144.03 \mu\text{m}^2$ (Figure 1c).
215 Tree orientation can be influenced by several stress factors during wood maturation. The magnitude
216 of maturation stress differs between longitudinal and tangential directions (Clair et al., 2013), so the
217 L/T ratio was also calculated. However, no statistically significant difference was observed between

218 the two cultivars and with respect to the health status (Figure 1d).

219 Compared to *Xf*-n trees of both cultivars, *Xf*-p trees of both cultivars showed a higher number of
220 vessels per ring area (mm²) (Figure 2). *Xf*-p trees of the cultivar Leccino showed a 63% increase in
221 the number of vessels in 2016 and a further increase of 36% in 2018. An even bigger increase was
222 observed in the year 2017, when the number of vessels in the *Xf*-p trees increased 163% (Figure 2a).
223 *Xf*-p trees of the cultivar Cellina di Nardò showed increases of 165%, 80%, and 186% in the years
224 2015, 2016, and 2017, respectively (Figure 2b).

225

226 **3.2 Ring widths in four-year-old branches**

227 Trees of both cultivars responded to *Xf* infection by increasing annual ring widths (Figure 3). In *Xf*-p
228 trees of the cultivar Leccino, the increases in ring width were 3.2 (2015), 2.5 (2016), 2 (2017), and
229 1.5 (2018) times as great as those of the *Xf*-n trees. In the cultivar Cellina di Nardò, the increase in
230 ring width was statistically significant in the years 2015 and 2017, with increases 3 and 1.5 times
231 those of healthy plants (Figure 3). Between the healthy plants of the two cultivars, there were no
232 significant differences in the ring-width measures, with an average ring width of $778.92 \pm 378.83 \mu\text{m}$
233 for the cultivar Leccino and $846.50 \pm 357.41 \mu\text{m}$ for the cultivar Cellina di Nardò (Figure 3). There
234 was no significant difference between the *Xf*-p plants of the two cultivars; in the years 2015, 2016,
235 and 2017, and the average ring width was $1643.00 \pm 860.31 \mu\text{m}$ for Leccino and 1491.16 ± 676.96
236 μm for Cellina di Nardò (Figure 3). In the year 2018, ring width in the cultivar Leccino was
237 significantly greater (3.4 times) than in the cultivar Cellina di Nardò (Figure 3).

238

239 **3.3 Occlusions in four-year-old branches and stem cores**

240 Both cultivars showed a high number of occlusions in the oldest rings of the infected four-year-old
241 branches (Figure 4a). The cultivar Cellina di Nardò showed a significantly higher percentage of vessel
242 occlusions in the years 2015 and 2016 than did the cultivar Leccino (Figure 4a).

243 The presence of vessel occlusions in both cultivars over the years from 2010 to 2017 was analysed
244 (Figure 4b and 5). As expected, infected samples from the susceptible cultivar Cellina di Nardò
245 showed a significantly higher percentage of vessel occlusions than the resistant cultivar Leccino over
246 this period (Figure 4b).

247

248 **3.4 Stable isotopes analysis**

249 To verify the correlation between the anatomical characteristics of the wood and tree physiological
250 responses, past photosynthesis rates and stomatal activities were analysed using $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ stable
251 isotopes in tree rings (for the period 2015-2018) (Figure 6). A significant increase in $\delta^{18}\text{O}$ was
252 observed in *Xf*-p plants of both the cultivars in the years 2015 and 2016. Leccino showed an increase
253 in $\delta^{18}\text{O}$ of 5.02% in 2015 and 3.35% in 2016, while Cellina di Nardò showed an increase of 5.33% in
254 2015 and 6.29% in 2016 (Figure 6). No variation in $\delta^{13}\text{C}$ was observed.

255

256 **4. Discussion**

257 In literature (Tulik et al., 2017; Renzi et al., 2012; Pearce et al., 1996) is well known that plants
258 affected by vascular pathogens could exhibit changes in vessel size (usually reduced vessel size) to
259 slow the pathogen colonization of compromised tissues. In this study, tree-ring analyses of naturally
260 *Xf*-infected olive tree cultivars Cellina di Nardò (susceptible) and Leccino (resistant) revealed no
261 significant changes in vessel characteristics (length, width, area, and L/T ratio) when compared with
262 *Xf*-n plants (Figures 1). The lack of changes in vessel size could indicate the existence of a
263 considerable time lag between the first appearance of *Xf* symptoms on leaves, that in our case became
264 visible in 2015, and the first changes in wood anatomy. Further research is required to understand if
265 this time lag exists or if changes in vessel size, indeed, don't accompany the *Olea europaea*
266 constitutive response to the *Xf* infection. In general, changes in ring width, vessel size, and vessel

density are also associated with changes in environmental conditions (e.g., drought or defoliation) that may contribute to the development of *Xf* infection (Corcuera et al., 2004; Fonti et al., 2010). The vessel numbers in *Xf*-p trees of both cultivars increase from 2015 through 2018, with higher average values in *Xf*-p plants as compared to those in *Xf*-n plants (Figure 2). The increase in vessel number could be related to the interconnectedness of the vascular system. Specifically, a higher number of vessels may help maintain sufficient water conductivity if the plant is suffering from pathogen-induced drought stress (Carlquist and Hoekman, 1985; Hacke and Sperry, 2001). A previous study by Sabella et al. (2019) found that the resistant cultivar Leccino is characterized by a vessel frequency (number of vessels per mm²) significantly higher than that of the susceptible cultivar Cellina di Nardò. Other studies have speculated on the role of vessel diameter as a factor influencing a tree's susceptibility to xylem-dwelling pathogens. Several cases have been described in which resistance is related to shorter and smaller vessels (Pouzoulet et al. 2017; Chatelet et al., 2011; Elgersma, 1970). Vessel number shows a high mean sensitivity to environmental conditions and biotic and abiotic stress factors (Corcuera et al., 2004). According to Corcuera et al. (2004), tree-ring width is highly correlated with the mean number of vessels; our data confirm these observations. In *Xf*-p plants of both cultivars, mean ring-width values from 2015 to 2018 show a temporal trend that is positively correlated ($r = 0.66$; $p\text{-value} = 0.019$) with the trend recorded for mean vessel numbers (Figures 3 and 2). Thus, our data suggest that vessel number and ring width are the two variables that the tree can more readily modify in response to the *Xf* disease. This observation was made by Corcuera et al. (2004), who described it as a response to other environmental factors in evergreen plants with diffuse-porous wood. De Micco et al. (2008) classify the wood of olive trees as diffuse-porous, a trait that was confirmed during our observations (Figure 5).

Anatomical observations of the wood from both the 4-year-old branches and the stem cores of the *Xf*-p and *Xf*-n plants of the two analysed cultivars revealed vessel occlusions since 2010 (the last observable year). This suggests that the vessels were already occluded in 2010, although a later

occlusion of the previously formed vessels cannot be excluded (Figures 4a and 4b). Observed vessel occlusions were significantly higher in *Xf*-p plants of the cultivar Cellina di Nardò, which showed an increasing trend from 2010 to 2018 (Figure 4b). However, the 4-year-old branches of the same cultivar exhibited a decreasing trend from 2015 to 2018 (Figure 4a). Vessel occlusions observed in wood infected with the bacterium *Xf* (Figure 5) are similar to those occurring at the margin of fungal decay lesions, i.e., vessel occlusions by tylosis only (Pearce, 1996). Using magnetic resonance imaging, Perez-Donoso et al. (2007) demonstrated that the loss of xylem water transport in *Xf*-inoculated grapevines is caused by the presence of tyloses in the lumen of vessels, that occlude water conduits. According to the literature, *Xf* infection included host-derived vascular occlusions, such as tyloses, the formation of which requires extensive plasticity and reorganization of the plant cell wall (Perez-Donoso et al., 2007). Using transcriptome profiling, Giampetruzzi et al. (2016), showed that both cultivars react to the presence of *Xf* by a strongly alteration of transcripts related to cell wall remodelling. Tyloses are generally considered to be a primary defence against vascular pathogen attacks because they contribute to stopping the transportation of the pathogen within xylem vessels (Clérivet et al., 2000). Resistant trees use tyloses to trap vascular pathogens, but if tylose formation affects too many vessels, it can result in drought stress (Yadeta and Thomma, 2013). In the resistant plants, tyloses form much faster and the pathogen is quickly isolated; on the contrary, in the susceptible plants, tylose formation achieves slowly so, not only, the plants cannot trap the pathogen but they continue to develop tyloses by occluding a lot of vessels (Yadeta and Thomma, 2013). Sun et al. (2013) demonstrated that tyloses form in grapevines susceptible to Pierce's Disease (PD), with over 60% of vessels becoming fully occluded. In contrast, tyloses occurred in less than 20% of vessels in PD-resistant vines. The number of vessels forming tyloses in our study was higher in the stem cores than in the 4-year-old branches (Figure 4a and 4b). Also, the development of tyloses induced by pruning is faster in the basal region of the stem than in the apical region, as shown in *Vitis vinifera* shoots (Sun et al., 2006). This process may have an evolutionary explanation, as most vascular wilt pathogens are soil-borne and enter their hosts through the roots. In response, plants create physical

318 barriers to prevent the spread of the pathogen through their tissues, according to the CODIT
 319 (Compartmentalization Of Decay In Trees) model (Yadeta and Thomma, 2013; Shigo, 1984).

320 Cardinale et al. (2018) suggest that occlusions may appear as gums or bacteria biofilms in the
 321 branches. These occlusions seem to correlate with the carbon ($\delta^{13}\text{C}$) and oxygen ($\delta^{18}\text{O}$) isotopes data
 322 (Figure 6). Measurements of the carbon ($\delta^{13}\text{C}$) and oxygen ($\delta^{18}\text{O}$) isotope composition of plant tissues
 323 provide important insight into how water-use efficiency relates to photosynthesis and transpiration
 324 (Flanagan and Farquhar, 2014). The C isotope ratio actually reflects leaf internal CO_2 concentration,
 325 which is driven by the balance between photosynthesis and stomatal conductance to CO_2 , not
 326 transpiration directly. This balance does not seem to have been affected by the presence of *Xf*, as there
 327 was no difference in $\delta^{13}\text{C}$ between *Xf*-n and *Xf*-p of the two cultivars. However, a statistically
 328 significant increase in the oxygen ($\delta^{18}\text{O}$) isotope was recorded in *Xf*-p trees of both cultivars in the
 329 years 2015 and 2016. Oxygen exists in two common stable forms, i.e., ^{18}O and ^{16}O . The isotope ratio
 330 in tissues is determined by the isotope ratio of soil water and evaporation processes because water
 331 molecules containing ^{16}O evaporate faster than water molecules containing ^{18}O (Flanagan and
 332 Farquhar, 2014). The leaf water isotope enrichment, which is later transferred to organic materials, is
 333 mainly controlled by the relative humidity of the atmosphere. If all environmental conditions are the
 334 same (as is the case for our *Xf*-n and *Xf*-p trees), the effect of higher transpiration rates becomes
 335 important because it dilutes the leaf water enrichment. Trees transpiring at a higher rate take up more
 336 depleted soil water, and therefore have relatively lower $\delta^{18}\text{O}$ values in their tissues (Barbour et al.,
 337 2007). Anyway, if higher transpiration occurs because of higher stomatal conductance, then leaf water
 338 enrichment is smaller (Farquhar et al., 2007). The obtained data seem to indicate a reduction in
 339 transpiration rates in leaves of *Xf*-p of both Cellina di Nardò and Leccino. These results are in
 340 agreement with data reported in the literature. Vascular pathogens that target the xylem usually cause
 341 a reduction in water flow, inducing a leaf water deficit that can cause stomatal closure and lower
 342 transpiration (McElrone et al., 2003).

Combining the results for the two isotopes, it seems that photosynthesis and transpiration were reduced to a similar degree in *Xf*-p trees (as reflected by the unchanged $\delta^{13}\text{C}$). It is also interesting to note that there was a significant correlation ($r = 0.78$, $p\text{-value} = 0.021$) between the mean tree-ring $\delta^{18}\text{O}$ recorded in the years 2015 and 2016 (Figure 6) and the higher number of occlusions observed in the same years (Figure 4a). As reported in previous studies, there is a positive relationship between the number of occluded vessels and the induced reduction of hydraulic conductance in petioles and stems with symptoms of leaf scorch (Newman et al., 2003, Alves et al., 2004). Our data suggest an increase in the occurrence of leaf scorch symptoms according to the observed disease severity distribution on the canopies (see Table S1 available as Supplementary Data at *Tree Physiology* Online). Scorched spots can reduce leaf conductance and render stomata non-functional in necrotic areas. It is important to note that the phytosanitary treatments for the control of the *Xf* insect vector (*Philaenus spumarius*) were introduced according to the EU Decision 2015/789 and became mandatory in Apulia from the 2016. So, it is plausible to suppose that in the years 2015 and 2016 (before of the phytosanitary treatments) the size of the vector population found in field exposed plant branches to a major inoculum pressure if compared to the next years in which population abundance has been conditioned largely by the application of the pesticides (Santoemma et al., 2019).

359

360 **Conclusion**

This study is the first to use dendrochronological methods to study how *Xylella fastidiosa* infection affects olive tree wood formation and stable isotopic composition. Tree rings can provide information that is useful for understanding how trees respond to infection. The increase in ring width and the number of vessels in the *Xf*-p trees of both the Leccino and Cellina di Nardò cultivars indicates that trees respond to *Xf* infection by altering their anatomical characteristics and producing more radial growth. The significantly higher number of occlusions observed in the stem cores of the cultivar

367 Cellina di Nardò could be caused by tyloses that are not able to block the spread of the pathogen.
368 Instead, by occluding many vessels, the tyloses aggravate the *Xf* symptoms. The occlusions observed
369 in the wood seem to be related to the variation of $\delta^{18}\text{O}$ observed in the tree rings and thus with the
370 regulation of transpiration rates in leaves. Further studies on the nature and composition of these
371 occlusions and on the distribution of bacteria in the tree are needed to better understand the interaction
372 between the *Xf* bacterium and the olive tree.

373 **Supplementary data: Figure S1.** Examples of a) branch and b) trunk core micro sections with the
374 identification of the annual rings (dark arrows). **Table S1.** Disease severity distribution on the
375 canopies of the two cultivars Cellina di Nardò and Leccino (three *Xf*-p plants per cultivar). Data are
376 expressed as mean disease severity scores based on a visually assessed scale from 0 to 3: 0 =
377 symptomless; 1 = leaf scorching on few branches or few desiccated branches affecting the portion of
378 the canopy; 2 = leaf scorching on several branches or desiccation affecting a large part of the portion
379 of the canopy; and 3 = canopy with desiccated branches uniformly distributed (Luvisi et al., 2017).
380 Observations were assessed from February 2015 to August 2018, every two months.

381

382 **Conflict of interest**

383 The authors declare no conflicts of interest.

384

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387 *fastidiosa* (Screen-X).

388

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392 **Author Contributions**

393 P.C., A.L., H.G. and E.S. conceived and designed the experiments. S.M., E.S. and M.V. did the
394 sampling. E.S. and M.V. performed health analysis. H.G. and S.M. performed sample preparation
395 and wood anatomical analyses. S.M. and M.S. performed stable isotope analyses. E.S., A.L. and P.C.
396 analysed data and prepared figures. E.S. wrote the manuscript. A.L., P.C. and L.D.B. critically
397 reviewed the manuscript. All authors read and approved the final manuscript.

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623 **Figure legends**

624

625 **Figure 1.** Xylem vessel a) lumen length (μm), b) lumen width (μm), c) lumen area (μm^2) and d) L/T
626 (lumen longitudinal/transversal diameter) ratio in *X. fastidiosa*-negative (*Xf*-n) and *X. fastidiosa*-
627 positive (*Xf*-p) four-year-old branches from Leccino and Cellina di Nardò olive tree cultivars, in
628 different years (2015, 2016, 2017, 2018). Values are the means $\pm$ standard deviation ($n = 3$). Results
629 for each year were analyzed by two-way ANOVA.

630

631 **Figure 2.** Number of vessels per ring area (mm^2) in *X. fastidiosa*-negative (*Xf*-n) and *X. fastidiosa*-
632 positive (*Xf*-p) four-year-old branches of a) Leccino and b) Cellina di Nardò olive tree cultivars, in
633 different observable years (2015, 2016, 2017, 2018). Values are the means $\pm$ standard deviation ($n =$
634 3). The significant differences were highlighted according to Student t-test (* $P < 0.05$; ** $P < 0.01$;
635 *** $P < 0.001$).

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637 **Figure 3.** Ring width in *X. fastidiosa*-negative (*Xf*-n) and *X. fastidiosa*-positive (*Xf*-p) four-year-old
638 branches of Leccino and Cellina di Nardò olive tree cultivars, in different years (2015, 2016, 2017,
639 2018). Values are the means $\pm$ standard deviation ($n = 3$). Results for each year were analyzed by
640 two-way ANOVA followed by Tukey-HSD post hoc test. Different letters correspond to statistically
641 different means.

642

643 **Figure 4.** Percentage (%) of vessel occlusions in *X. fastidiosa*-negative (*Xf*-n) and *X. fastidiosa*-
644 positive (*Xf*-p) Leccino and Cellina di Nardò olive tree cultivars a) four-year-old branches, results are
645 presented for rings of different years (2015, 2016, 2017, 2018) and b) olive tree cores, results are
646 presented for rings of different years (2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017). Values are
647 the means $\pm$ standard deviation ($n = 3$). Results for each year were analyzed by two-way ANOVA
648 followed by Tukey-HSD post hoc test. Different letters correspond to statistically different means.

649 **Figure 5.** Images (5X magnification) of tree rings from cores of *X. fastidiosa*-negative (*Xf*-n) and *X.*
650 *fastidiosa*-positive (*Xf*-p) plants of the Leccino and Cellina di Nardò cultivars. Images show the
651 presence of more vessel occlusions in *Xf*-p Cellina di Nardò than in *Xf*-p Leccino. *Scale bar:* 100 µm.

652
653 **Figure 6.** Carbon ($\delta^{13}\text{C}\text{‰}$) and oxygen ($\delta^{18}\text{O}\text{‰}$) isotope ratio behavior in rings of different years
654 (2015, 2016, 2017, 2018) from *X. fastidiosa*-negative (*Xf*-n) and *X. fastidiosa*-positive (*Xf*-p) trees of
655 the cultivars Leccino and Cellina di Nardò. Each year value represents the mean $\pm$ standard deviation
656 of the different pieces of branch from different biological replicates (n=3). The significant differences
657 are highlighted according to Student t-test (*P < 0.05; **P < 0.01; ***P < 0.001).

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Figure 1.

Figure 2

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***Xf-n* Cellina di Nardò**

***Xf-p* Cellina di Nardò**

***Xf-n* Leccino**

***Xf-p* Leccino**

740

741 **Figure 5.**

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Figure 6.

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Figure S1.