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1 **RESEARCH PAPER**

2

3 **TITLE**

4 Esca grapevine disease involves leaf hydraulic failure and represents a unique  
5 premature senescence process.

6

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26

27 **Key words**

28 Hydraulic failure, magnesium deficiency, senescence, tyloses, vascular disease, *Vitis*  
29 *vinifera* L..

30

31 **Running head**

32 Esca: a unique senescence process in grapevine

## 33 **ABSTRACT**

34 Xylem anatomy may change in response to environmental or biotic stresses. Vascular  
35 occlusion, an anatomical modification of mature xylem, contributes to plant resistance  
36 and susceptibility to different stresses. In woody organs, xylem occlusions have been  
37 examined as part of the senescence process, but their presence and function in leaves  
38 remain obscure. In grapevine, many stresses are associated with premature leaf  
39 senescence inducing discolorations and scorched tissue in leaves. However, we still  
40 do not know whether the leaf senescence process follows the same sequence of  
41 physiological events and whether leaf xylem anatomy is affected in similar ways. In  
42 this study, we quantified vascular occlusions in midribs from leaves with symptoms of  
43 the grapevine disease esca, magnesium deficiency, and autumn senescence. We  
44 found higher amounts of vascular occlusions in leaves with esca symptoms (in 27%  
45 of xylem vessels on average), whereas the leaves with other symptoms (as well as  
46 the asymptomatic controls) had far fewer occlusions (in 3% of vessels). Therefore, we  
47 assessed the relationship between xylem occlusions and esca leaf symptoms in four  
48 different countries (California in the US, France, Italy, and Spain) and eight different  
49 cultivars. We monitored the plants over the course of the growing season, confirming  
50 that vascular occlusions do not evolve with symptom age. Finally, we investigated the  
51 hydraulic integrity of leaf xylem vessels by optical visualization of embolism  
52 propagation during dehydration. We found that the occlusions lead to hydraulic  
53 dysfunction mainly in the peripheral veins compared to the midribs in esca  
54 symptomatic leaves. These results open new perspectives on the role of vascular  
55 occlusions during the leaf senescence process, highlighting the uniqueness of esca  
56 leaf symptoms and its consequence on leaf physiology.

57

## 58 **INTRODUCTION**

59 The enormous anatomical diversity of flowering plants is a visual representation of  
60 their evolution and adaptation to different living environments (Crang et al. 2018).  
61 Plants can modify the anatomy of their cells, tissues and organs based on different  
62 environmental conditions and in response to abiotic and biotic stresses (e.g. Bortolami  
63 et al. 2019, Trueba et al. 2022). Xylem vascular occlusions are considered a functional  
64 anatomical trait that result in response to a broad range of environmental and biotic  
65 factors (De Micco et al. 2016). They can be caused by gels (or gums), formed by  
66 amorphous extracellular materials (Rioux et al. 1998), and tyloses, which are  
67 expansions of parenchyma cells inside the vessel lumen (Zimmermann 1979).  
68 Vascular occlusions have been identified during senescence process induced by  
69 different causes, such as tissue aging (Dute et al. 1999, Salleo et al. 2002), wounding  
70 (Sun et al. 2007, 2008), flooding (Davison and Tay 1985), and vascular diseases  
71 (Pouzoulet et al. 2019, Mensah et al. 2020). In general, vascular occlusions are formed  
72 to hydraulically isolate specific regions of the plant, contributing to wound or pathogen  
73 compartmentalization (Pearce 1996), and increasing decay resistance and  
74 mechanical support during heartwood formation (De Micco et al. 2016). The  
75 consequences of these occlusions have been largely studied in woody perennial  
76 organs, where they are thought to contribute to plant resistance to vascular pathogens  
77 (Venturas et al. 2014, Park and Juzwik 2014, Rioux et al. 2018). However, they  
78 sometimes can lead to decreased hydraulic conductivity (McElrone et al. 2010, Deyett  
79 et al. 2019, Mensah et al. 2020, Fanton et al. 2022), and many theoretical articles have  
80 hypothesized that excessive production of vascular occlusions during disease could

81 lead to lethal impairment in plant water transport (Fradin and Thomma 2006, Yadeta  
82 and Thomma 2013, Oliva et al. 2014).

83 In leaves, xylem occlusions have been studied during natural autumn senescence  
84 (Salleo et al. 2002) and induced premature senescence by the bacterium *Xylella* spp.  
85 (Fritschi et al. 2008, Choat et al. 2009), but their functions or effects on leaf physiology  
86 are still unknown. In addition, published reviews on leaf senescence (defined here as  
87 the degradation of the cellular structures and transition in cellular metabolism, Woo et  
88 al. 2013) have focused on molecular mechanisms, rather than the anatomical and  
89 physiological changes that occur in the xylem (Lim et al. 2007, Schippers et al. 2015),  
90 probably because vascular occlusions are frequently observed at the late stages of  
91 the senescence process (Chaffey and Pearson 1985). In this context, exploring the  
92 underlying origins of xylem occlusions and their effects on plant physiology may  
93 improve our understanding of their functional role in leaf senescence resulting from  
94 different environmental factors.

95 Recently, we have associated vascular occlusions with *in vivo* quantification of xylem  
96 hydraulic failure in midribs, petioles, and stems during the development of leaf  
97 symptoms caused by the grapevine trunk disease esca (Bortolami et al. 2019, 2021a).  
98 The wood-infecting fungal pathogens damage the host's vascular system, through  
99 enzymatic decomposition of woody cells/tissues and/or production of phytotoxin  
100 metabolites (Lecomte et al. 2012, Gramaje et al. 2018). Strikingly, vascular occlusions  
101 in esca symptomatic leaves or stems were observed at a distance from pathogen  
102 infection localized in the trunk or wounds (Bortolami et al. 2019, 2021a). This fatal  
103 disease impacts grape yield and quality worldwide (Mondello et al. 2018), and while

104 its severity appears to have a relationship with climate (Bortolami et al. 2021b), the  
105 mechanisms behind leaf (and plant) death are largely unknown (Claverie et al. 2020).  
106 In grapevine, premature or age-dependent leaf senescence induced by different  
107 stressors leads to similar physiological responses and leaf symptom phenotypes that  
108 are sometimes hard to distinguish. Esca leaf symptoms consist of partial and total leaf  
109 discoloration and scorching, followed by leaf fall (Calzarano et al. 2014), similar to  
110 some mineral deficiencies and naturally occurring autumn senescence. Moreover, a  
111 decrease in stomatal conductance was observed during autumn senescence (Douthe  
112 et al. 2018, Gowdy et al. 2022), premature senescence induced by magnesium  
113 deficiency (Rogiers et al. 2020), and esca (Bortolami et al. 2021b). Therefore, we  
114 hypothesized that grapevine leaves affected by different stresses would be associated  
115 with similar responses of xylem anatomy, namely, the presence of vascular  
116 occlusions. In this study, we compared midrib vascular occlusions during leaf symptom  
117 development from esca versus leaf senescence induced by magnesium deficiency  
118 and autumn in grapevine (*Vitis vinifera* L.). In addition, we focused on esca leaf  
119 symptoms, comparing occlusion production in leaves from esca-infected vineyards  
120 across different climatic regions and cultivars. Finally, we followed the progression of  
121 vascular occlusions during esca symptom development and, through the use of non-  
122 invasive visualization of embolism, we quantified the xylem hydraulic integrity in the  
123 blade.

124

## 125 **MATERIALS AND METHODS**

### 126 **Plant material and symptoms of senescence**

127 To quantify xylem occlusions during different senescence types, we sampled leaves  
128 from *V. vinifera* cultivars (cv) in the Vitadapt experimental plot (van Leeuwen et al.  
129 2019). This plot includes 52 cultivars grafted onto the Selection Oppenheim 4 (SO4)  
130 rootstock and planted in 2009 in the Bordeaux region (France, 44°47'23.8"N  
131 0°34'39.7"W). As presented in Table 1, in August 2018, we sampled seventeen healthy  
132 (control) leaves, twenty-two esca symptomatic leaves, and six magnesium-deficient  
133 leaves from five grapevine varieties. In October 2018, we sampled from the same  
134 varieties twenty-one leaves with phenotypes of autumn senescence (Table 1).  
135 Examples of the sampled induced senescence leaf symptoms are presented in Fig.  
136 S1 for a white (Chenin) and a red (Castets) cultivar. Single leaves presented partial or  
137 total discoloration, or scorched tissue in between the major veins, and we were able  
138 to distinguish the different stresses by the whole-plant symptom pattern. Esca leaf  
139 symptoms were identified by the so-called 'tiger-stripe' leaves, as described in  
140 Lecomte et al. (2012), in the entire length of the stems. We identified the magnesium-  
141 deficiency phenotype in plants with leaves with discolorations only located in the lower  
142 part of the canopy and with no scorching. We identified autumn senescence in leaves  
143 with total or partial discoloration at the end of the growing season.

144 To further explore leaf xylem anatomy during esca symptom development,  
145 symptomatic and healthy (control) leaves were sampled from eight cultivars (Castets,  
146 Chenin, Grenache, Mourvedre, Sangiovese, Sauvignon blanc, Tempranillo, and  
147 Tempranillo blanco) from different countries (France, Italy, Spain, and California in the  
148 United States). The exact number of replicates per country and variety is presented in  
149 Table 1. In France, we sampled thirty-nine leaves from five cultivars of the Vitadapt plot  
150 in August 2018 as described above. In Italy (Carassai, Ascoli Piceno Province,  
151 43°02'18.07"N 13°39'39.41"E), leaves were sampled in June 2019 from two *V. vinifera*



152 cultivars , planted in 1989, grafted onto Kober 5BB rootstock. In Spain (La Rioja region,  
153 42°26'19.7"N 1°49'30.5"W), leaves were sampled in late August 2019 from three *V.*  
154 *vinifera* cultivars, planted in 2013, grafted onto the 110 Richter (110R) rootstock. In  
155 California (in the North Coast regions, 39°00'15.1"N 122°51'08.3"W), leaves were  
156 sampled in August 2020 from one *V. vinifera* cultivar , planted in 2000, grafted onto  
157 the Teleki 5C (5C) rootstock.

158 To explore the evolution of occlusion formation over time during esca symptom  
159 development, we monitored esca leaf symptoms and sampled leaves on eight potted  
160 plants from June (day of the year, doy=172) to September 2018 (doy=262). The  
161 experimental setup is described in Bortolami et al. (2019). Briefly, 30-year-old *V.*  
162 *vinifera* cv Sauvignon blanc vines, grafted onto the Millardet de Grasset 101-14 (101-  
163 14 MGt) rootstock, were uprooted from the field in February 2018 and transferred into  
164 20L pots. We sampled, over the experimental season 2018, forty-five leaves from  
165 three control asymptomatic plants and five esca symptomatic plants. Leaf sample size  
166 was as follows: eight control, thirteen asymptomatic leaves before the plant expressed  
167 esca symptoms, nine asymptomatic leaves from plants with symptomatic leaves, and  
168 fifteen esca symptomatic leaves. In September 2017 and 2018, we brought control  
169 and symptomatic plants to synchrotron SOLEIL (PSICHE beamline), and scanned leaf  
170 midribs with X-ray microCT, as described in Bortolami et al. (2019). The same midribs  
171 analyzed in Bortolami et al. (2019) are reported on here, but we report only novel data  
172 on the percentage of occluded vessels quantification (in number), and not the  
173 theoretical loss of hydraulic conductivity (PLC).

174

175 **Light microscopy and occlusion quantification**

176 On each leaf, a one-centimeter section of midrib was cut at one centimeter from the  
177 petiole point. Samples collected in France were directly put in a FAA solution  
178 composed of 0.64% paraformaldehyde, 50% ethanol, 5% acetic acid, and 44.36%  
179 water (v/v). After one to three days in the FAA solution on a shaker at 90 rpm, samples  
180 were dehydrated using a graded series of alcohol (80%, 100%, 100% for 30' each)  
181 and stored at 4 °C until analysis. Samples collected in California, Italy, and Spain were  
182 put directly in 80% alcohol, mailed to France and stored at 4°C until analysis. After  
183 one night in the FAA solution, samples were dehydrated using the same graded series  
184 (80%, 100%, 100% v/v for 30' each). Samples were then embedded using a graded  
185 series of LR White resin (Agar scientific, 33%, 50%, and 66% LR White v/v in ethanol  
186 solution for 120' each, and 100% three times for 7 hours each). Finally, samples were  
187 polymerized in capsules at 60 °C for 24-48 hours. Transverse sections of 1.8 to 2 µm  
188 thickness were cut using an Ultracut S microtome (Reichert) equipped with a glass  
189 knife at the Bordeaux Imaging Center, a member of the France Bio Imaging national  
190 infrastructure (ANR-10-INBS-04). Cross sections were stained with 0.05% (w/v)  
191 Toluidine Blue O. Stained sections were dried and photographed with a DS-Fi3  
192 camera (Nikon, France) mounted on a stereo microscope SMZ1270 (Nikon, France).  
193 By the use of Toluidine blue O dye, we identified the nature of vascular occlusions,  
194 which includes gels (i.e. amorphous extracellular material, mainly composed by  
195 pectins and polysaccharides, Fig. 1B, D) and tyloses (i.e. expansions of the neighbour-  
196 vessels parenchyma cells, Fig. 1B, D).

197 Xylem vessels were identified and quantified in one entire cross-section per leaf using  
198 imageJ (Schneider et al. 2012) and categorized as empty vessels or occluded vessels  
199 (presenting gel or tyloses). In addition, we observed a deposition of crystals in vessels  
200 of 93% of the samples and thus quantified their presence. To confirm that vascular

201 occlusions were equally distributed within a sample (i.e. in the one-centimeter  
202 segment), we quantified vascular occlusions in three cross-sections along the  
203 segment (i.e., subsamples) for 29 (over the one-hundred seventy-seven analyzed)  
204 midribs. We found that the presence of occlusions did not significantly differ among  
205 cross-sections within a sample ( $F_{55,12498}=0.97$ ,  $P=0.54$ , treating repetition, sample  
206 identity, and their interaction as fixed effects in an independent linear model, with a  
207 binomial distribution for presence/absence of an occlusion, in SAS using the GLIMMIX  
208 procedure).

209

#### 210 **Non-invasive optical determination of xylem functionality**

211 To investigate the extent to which esca impacts xylem functionality and leaf embolism  
212 resistance, the onset and propagation of embolism were evaluated in four leaves from  
213 three different control asymptomatic plants and in eleven leaves from four different  
214 esca symptomatic plants, using the optical vulnerability (OV) technique (Brodrigg et  
215 al. 2016) during a dry-down experiment. In every leaf the region of interest (i.e. the  
216 scanned area) included the midrib (at 3-5 cm from the apex of the leaf) in the middle  
217 of the scan and approximately 10x3 cm around it. The experiment took place over two  
218 weeks (in September 2019) on > 2-m long stems of potted *V. vinifera* cv Sauvignon  
219 blanc plants uprooted in February 2019 from the same plot as the one used for the  
220 leaf midrib sampling for occlusion quantification (INRAE, Bordeaux). In the early  
221 morning, stems were cut under water at their base, put inside dark plastic bags with  
222 humid paper and rapidly transported to the lab. Once at the lab, the abaxial side of  
223 intact leaves was fixed on a scanner (Perfection V800 Photo, EPSON, Suna, Japan)  
224 using a transparent glass and adhesive tape. Brightness and contrast as well as leaf

225 scanned area were adjusted to optimize visualization of embolisms. Imaging of  $991 \pm$   
226  $90 \text{ mm}^2$  (average  $\pm$  SE) of each leaf was automatically performed every 5 min  
227 throughout plant dehydration using a computer automation software (Autolt 3). Upon  
228 completion, the stacks of images, consisting of 1,800 to 2,000 scans per leaf, were  
229 analyzed to highlight embolism events revealed as changes in light transmission  
230 through the leaf xylem. Analyses were carried out using ImageJ software and following  
231 instructions from <http://www.opensourceov.org>. Briefly, total embolism was quantified  
232 by subtracting pixel differences between consecutive images (i.e. pixel values that did  
233 not change resulted in a value of zero). In these series, white pixels represented leaf  
234 embolism. Noise was removed using the ImageJ outlier removal, and pixel threshold  
235 was used to extract embolism from any background noise remaining. The embolism  
236 area per image was calculated as the sum of non-zero pixels on the total scanned  
237 surface ( $\text{mm}^2$ ) or on green leaf surface only ( $\text{mm}^2$ , excluding the symptomatic tissue).  
238 We considered the veins that embolized as functional (i.e. containing water under  
239 tension), while the remaining veins were considered as non-functional (i.e. containing  
240 any combination of air, occlusion, or static water). As stems dehydrated, stem water  
241 potential ( $\Psi_{\text{stem}}$ ) was simultaneously monitored every 30' using psychrometers (ICT  
242 Internationale, Armidale, NSW, Australia), which were installed in the middle of the  
243 stem prior to the start of the experiment (i.e. when stems were fully hydrated). The  
244 accuracy of psychrometer readings was assessed periodically with a Scholander  
245 pressure bomb (SAM Precis, Gradignan, France), using leaves adjacent to the  
246 scanned ones and that had been covered for at least 2h with aluminum foil and  
247 wrapped in a plastic bag.

248

249 **Esca leaf symptom severity**

250 Each leaf was photographed before sampling for the leaf xylem anatomy and OV  
251 measurements during esca symptom development. We quantified the percentage of  
252 green tissue on the upper face of each leaf using ImageJ in order to estimate leaf esca  
253 symptom severity. On samples used for OV measurements, this estimation was done  
254 on the scanned area only, not on the whole leaf surface. We first detected the green  
255 hue ranges on RGB pictures of control-asymptomatic leaves using the color threshold  
256 in ImageJ, and then used these green ranges to select and quantify the green pixels  
257 in each photo. We finally reported these values to the total leaf surface and obtained  
258 a percentage of green tissue per leaf or area analysed. In every cultivar we covered  
259 the esca symptom severity spectrum (from low to high percentage of green tissue) as  
260 much as possible in order to avoid sampling bias.

261

## 262 **Statistical analysis**

263 We investigated whether induced senescence types and cultivars are associated with  
264 the probability of occlusion formation in xylem vessels using multiple generalized linear  
265 mixed models to compare control versus esca symptomatic plants (fixed effect). Prior  
266 to analysis the data (bounded between 0.0001 and 1) were transformed using a logit  
267 function as appropriate for analyzing proportions (Warton and Hui 2011). In order to  
268 use the logit function the lowest value of 0.0001 was assigned to samples with no  
269 presence of occlusions. We investigated whether esca symptomatic and control  
270 leaves presented different response in embolized surface during decreasing  $\Psi_{\text{Stem}}$   
271 using a generalized linear mixed model with the plant treated as random effect and  
272 the interaction between symptom presence and  $\Psi_{\text{Stem}}$  as a fixed effect. The  
273 relationships between the percentage of occluded vessels and esca symptom severity

274 (expressed in percentage of leaf green tissue) were tested using linear regression  
275 models. The effect of the age of symptom on the amount of vascular occlusion was  
276 tested with a general linear mixed model on symptomatic leaves, using the sampling  
277 date as fixed effect, and the plant as random effect. All statistical tests were performed  
278 using the SAS software (SAS 9.4; SAS Institute). We used the GLIMMIX procedure  
279 for generalized linear mixed models, and the REG procedure for regression analyses.  
280 The normality of the response variables was tested using a Kolmogorov-Smirnov test  
281 implemented in the UNIVARIATE procedure, prior to analyses.

282

## 283 **RESULTS**

### 284 **Anatomical diversity of vascular occlusions and presence of crystals**

285 In each sample, we categorised the vessels as occluded by gels and/or tyloses, with  
286 depositions of crystals, or with an absence of any structure (Fig. 1). Crystals (both  
287 druse and prismatic) were present inside some vessel lumen (in 15% of vessels on  
288 average, Fig. 1C). These crystals were more often present in samples from which  
289 occlusions were absent (on average, 21% of vessels with crystals in control leaves,  
290 versus 10% in esca symptomatic leaves, Fig. S2). We showed that control  
291 (asymptomatic) leaves (almost) never had occlusions (in 2% of vessels on average,  
292 Fig. 2-4), and that under microCT X-ray scans (data from Bortolami et al. 2019) and  
293 optical visualizations (Fig. 5) they were almost fully functional. This relationship  
294 suggests that crystals are likely present in functional rather than occluded vessels (see  
295 supplemental discussion SD1 for details). Consequently, to evaluate the impact of  
296 different stresses on xylem anatomy and water transport, we reported here only the

297 percentages of occlusions, and not of crystals, in different experiments and  
298 comparisons.

299

### 300 **Vascular occlusions in senescing leaves**

301 To investigate the presence of occlusion during the autumnal or premature leaf  
302 senescence induced by different causes, we compared midribs from control, esca  
303 symptomatic, magnesium deficient, and autumn senescent leaves (Fig. 2). The  
304 amount of vessel occlusions in midribs significantly differs among the four leaf  
305 categories ( $F_{3,62}=19.4$ ,  $P<0.0001$ ). Midribs in esca-symptomatic leaves presented the  
306 highest percentage of occluded vessels ( $27\% \pm 4$ , average  $\pm$  SE) comparing to  
307 autumn-senescent leaves ( $4.0 \pm 1\%$ ), control leaves ( $2.9 \pm 1\%$ ) and magnesium-  
308 deficient leaves, which had the lowest percentage of occluded vessels ( $0.53 \pm 0.5\%$ ).  
309 However, two autumn-senescent midribs, that were statistical outliers, had over 15%  
310 occluded vessels (Fig. 2).

311

### 312 **Vascular occlusions in esca symptomatic leaves from different cultivars and** 313 **countries**

314 We quantified the percentage of occluded vessels in esca symptomatic and control  
315 (asymptomatic) leaf midribs from four different countries (France, Italy, California in  
316 the USA, and Spain) in eight cultivars: Castets, Chenin, Grenache, Mourvedre,  
317 Sangiovese, Sauvignon blanc, Tempranillo, and Tempranillo blanco (Fig. 3, Fig. S3,  
318 Table S1). Our results show that esca symptomatic leaves presented a significantly  
319 higher presence of occlusions compared to controls ( $F_{1,93}=85.93$ ,  $P<0.0001$ ), see  
320 Table S1 for the detailed comparisons within varieties and countries. Cultivars had

321 significantly different levels of occlusions among the symptomatic samples  
322 ( $F_{7,48}=13.55$ ,  $P<0.0001$  Fig. 3A). However, the variability was high, ranging from no  
323 occlusion in symptomatic Grenache midribs to  $52 \pm 5\%$  of occluded vessels in  
324 symptomatic Tempranillo blanco midribs (Fig. 3A). Leaf symptom severity (quantified  
325 as the percentage of green leaf tissue) had no significant effect on the leaf occlusion  
326 level within symptomatic leaves, except for Sangiovese (Fig. 3B, Table S2).  
327 Nevertheless, the most occluded leaves (above 40%) had the most severe symptoms  
328 (less than 40% of green tissue), and among the mildly symptomatic leaves (more than  
329 60% of green tissue) the occlusions rarely occupied more than 30% of the vessels  
330 (Fig. 3B). When considering the control leaves (entirely green) in the statistical  
331 analysis, the percentage of occluded vessels was significantly related to the  
332 percentage of leaf green tissue ( $R^2=0.31$ ,  $P<0.0001$ ).

333

#### 334 **Vascular occlusion evolution during esca symptom appearance**

335 During one experimental season, we monitored esca leaf symptoms on eight potted  
336 plants from June (doy=172) to September 2018 (doy=262, Fig. 4). The symptoms  
337 developed at the same time in the season; hence, using 'doy' as a good comparison  
338 to evaluate the effect of symptom age on occlusion formation. Using light microscopy,  
339 we found that control plants always had low levels of occlusions inside their midribs,  
340 as was the case for the majority of asymptomatic leaves both before and after  
341 symptoms appeared on other leaves on the same plant (<7% of occluded vessels, Fig.  
342 4). Ten out of fifteen symptomatic leaves had a higher level of occlusions (>14%)  
343 compared to controls, as well as two leaves before symptom appearance, and one  
344 asymptomatic leaf from a symptomatic plant (Fig. 4). Overall, we found a significant



345 effect of the presence of esca symptoms on the amount of vessel occlusions (i.e.  
346 comparing the four leaf categories in Fig. 4,  $F_{3,35}=9.36$ ,  $P=0.0001$ ), but there were no  
347 changes in the occlusion level over time within the symptomatic leaves ( $F_{5,5}=1.19$ ,  
348  $P=0.43$ ), suggesting that occlusions do not increase with symptom age. Scanning  
349 midribs with X-ray microCT, we found higher average occluded vessels (compared to  
350 light microscopy) both in control and symptomatic midribs ( $15.2 \pm 4\%$  and  $62.7 \pm 7\%$ ,  
351 average  $\pm$  SE, respectively, Fig. 4).

352

### 353 **The hydraulic consequences of xylem occlusions**

354 Using a non-invasive optical vulnerability technique, we were able to quantify the  
355 embolism spread in control and esca symptomatic leaf veins (Fig. 5, 6). At the end of  
356 dehydration, when no supplemental embolism formation was detected, we summed  
357 all the embolism events within a sample (red vessels, Fig. 5). We found that almost all  
358 the veins embolized in control leaves (Fig. 5 A-D), whereas the veins did not embolize  
359 in wide portions of the leaf blade in esca symptomatic leaves (Fig. 5 E-M), especially  
360 in between the main veins where the leaf was yellow and scorched. In two  
361 symptomatic leaves no embolism event was observed (Fig. 5 N, O). In most  
362 symptomatic leaves, the primary and secondary order veins readily embolized,  
363 whereas the tertiary or above order veins did not. Plotting the embolism spread versus  
364 the decrease in stem water potential, we found that control leaves had a final  
365 embolized area of  $25.1 \pm 7 \times 100 \text{ pixels mm}^{-2}$  on the entire scanned area, compared  
366 to  $5.2 \pm 2 \times 100 \text{ pixels mm}^{-2}$  in symptomatic leaves (Fig. 6A). When only the green  
367 surface of the leaves was considered (i.e. excluding the symptomatic discolored and  
368 scorched tissues; Fig. 6B), control leaves had the same embolized area evolution

369 (they were completely green), whereas symptomatic leaves had a final embolized area  
370 of  $13.0 \pm 4 \times 100$  pixels  $\text{mm}^{-2}$  (Fig. 6B). We found a significant effect of esca both  
371 considering the whole scanned surface ( $F_{2,456}=219.05$   $P<0.0001$ ) or only the green  
372 area ( $F_{2,456}=128.80$   $P<0.0001$ ). In control leaves, embolism events were detected  
373 between -1.9 and -3.3 MPa (Fig. 6A, B), whereas in symptomatic leaves these events  
374 appeared across a larger range of  $\Psi_{\text{Stem}}$  (i.e. between -1.3 and -4.9 MPa, Fig. 6A, B).  
375 Finally, it is worth noting that the total embolized area of symptomatic leaves was  
376 almost always less ( $21 \pm 0.1\%$ ) than half that of the control leaves (noted with the  
377 horizontal line in Fig. 6B).

378

## 379 **DISCUSSION**

380 In this study, we highlight the uniqueness of esca leaf symptoms compared to other  
381 induced senescence processes. We demonstrated that only esca symptomatic leaves  
382 had significant levels of occluded vessels in their midribs compared to those of leaves  
383 with magnesium deficiency or undergoing autumn senescence. We investigated esca  
384 leaf symptoms in detail and demonstrated that the percentage of occlusion: (i) varied  
385 among cultivars; and (ii) were similar among symptomatic leaves collected throughout  
386 the season (i.e. occlusions did not increase over time). Finally, using the optical  
387 vulnerability (OV) technique, we explored the hydraulic integrity in esca symptomatic  
388 leaves. Our finding of non-functional vessels across large portions of esca  
389 symptomatic leaf blade, compared to the highest density of functional xylem vessels  
390 in the midrib, suggests that vascular occlusions are localized near the scorched and  
391 discolored areas of the leaves in between the major veins and at the margins of the  
392 leaf.

393

394 **Esca, a unique senescence process**

395 Comparing different leaves with different senescence types, esca leaf symptoms had  
396 the highest levels of vessel occlusion. Both magnesium deficiency and autumn  
397 senescence types share in common with esca a leaf chlorosis and a decrease in  
398 stomatal conductance (Salleo et al. 2002, Brodribb and Holbrook 2003 for autumn  
399 senescence, Tränkner et al. 2016, Rogiers et al. 2020 for magnesium deficiency, and  
400 Lecomte et al. 2012, Bortolami et al. 2021b for esca). Given that occlusions were  
401 absent from leaves with magnesium deficiency, it is likely that the impacts of  
402 magnesium deficiency on leaf gas exchange are not related to a loss of leaf xylem  
403 hydraulic conductivity. During autumn senescence, occlusions have been detected in  
404 parts of the leaf we did not examine, namely the petioles and the stem-petiole junction  
405 (Chattaway 1949, Salleo et al. 2002, De Micco et al. 2016). Indeed, during autumn,  
406 the hydraulic conductivity decreases in petioles, but not in leaf blades (Salleo et al.  
407 2002). This difference in the spatial distribution of occlusions suggests that the visible  
408 similarities between esca-symptom leaves and autumn senescence (described above)  
409 are due to different mechanisms. In the most prominent hypothesis of esca leaf  
410 symptom formation, toxins are translocated from the fungal biomass (the pathogens  
411 reside in the woody tissues) out to the leaves through the vascular system (Claverie  
412 et al. 2020). Previously, using microCT X-ray we showed that more basal organs  
413 (stems, Bortolami et al. 2021a) are less occluded than distal organs (petioles and  
414 midribs, Bortolami et al. 2019). Here we show that occlusions are more frequent in the  
415 leaf blade than in the midribs (Fig. 5), supporting the hypothesis that fungal toxins are  
416 expected to accumulate, and thus initiate a host response in the form of xylem  
417 occlusions, where the water flow ends (i.e. peripheral veins). In contrast, during

418 autumn senescence the hormones that initiate the senescence process are  
419 synthesized *in situ* (Schippers et al. 2015), suggesting a more local response in the  
420 vasculature (for example, only at the abscission point in petioles).

421

## 422 **Exploring the vascular occlusions and hydraulic integrity during esca leaf** 423 **symptoms**

424 Comparing esca symptomatic leaves among cultivars and countries, we found  
425 significant levels of occlusion in the majority of the symptomatic leaves analyzed. It  
426 has been previously demonstrated that Sauvignon blanc vines (30 year-old plants  
427 transplanted from the vineyard into pots and grown in a greenhouse) suffer from  
428 occlusion-driven hydraulic failure during esca leaf symptom expression (Bortolami et  
429 al. 2019, 2021a). Here, we demonstrated that occlusions (and subsequent hydraulic  
430 failure) are also associated with esca in the field, and this finding is not particular to  
431 one specific climate or cultivar (or to our previous greenhouse experiment). However,  
432 the range of occlusion levels among cultivars is noteworthy and suggests differences  
433 between genotypes regarding the extent of vascular occlusion. This is consistent with  
434 the fact that, during other vascular diseases, occlusions can be considered beneficial  
435 up until the point at which they impact plant function. In the first case, occlusions  
436 efficiently compartmentalize pathogens and limit the spread of infection within a plant  
437 (Clérivet et al. 2000, Rioux et al. 2018, Pouzoulet et al. 2020); in the second case,  
438 occlusions interfere with water movement, generating hydraulic failure with detrimental  
439 consequences for the plant (Sun et al. 2013, Mensah et al. 2020). When a xylem  
440 vessel is occluded, we cannot separate these two consequences. Especially in the  
441 case of esca, where the subsequent reduction in water flow should reduce the spread

442 of pathogen-derived toxins as well, while suppressing xylem functionality by hydraulic  
443 failure. More studies are needed to test this hypothesis and relate vascular occlusion  
444 rates to plant mortality relative to the degree of esca susceptibility in each cultivar.

445 Following occlusions in leaves from symptomatic plants throughout the season, we  
446 observed that esca symptomatic leaves had a higher proportion of occluded vessels  
447 than asymptomatic leaves from the same plants and healthy leaves from control  
448 plants. Moreover, levels of occlusions were similar throughout the season, indicating  
449 that the number of occlusions does not increase over time, but is coincident with the  
450 onset of symptoms. In three out of twenty-two samples, occlusions were present in  
451 asymptomatic leaves from plants with other symptomatic leaves (light and dark green  
452 dots above 10% in Fig. 4). At the same time, we also observed that visual symptoms  
453 were sometimes (in five out of fifteen samples) associated with low levels of occlusions  
454 (red dots in Fig. 4). These results are also consistent with the comparison among and  
455 within cultivars, where the level of occlusions was not related to symptom severity (Fig.  
456 3B). We could explain this variability by the fact that we analyzed 2  $\mu\text{m}$ -thick cross-  
457 sections on midribs, which can reach 20 cm in length, and as such, we may have  
458 underestimated occlusions. Indeed, if we quantified more non-functional vessels using  
459 microCT X-ray it is also because we could explore 1cm volume in each sample,  
460 increasing the probability of observing occlusions. The comparison with the optical  
461 embolism visualization supports this hypothesis, as the midribs are not uniformly  
462 functional (or non-functional) along their length. From optical visualizations, we  
463 considered each vein with an embolism event during dehydration (i.e. containing water  
464 under tension) as functional. We observed that midribs are the most functional parts  
465 in symptomatic leaves (Fig. 5) and that many regions of leaf blade were already non-  
466 functional before we started the dry-down experiment. We can conclude that

467 occlusion-driven hydraulic failure may have a stronger impact on the total leaf  
468 hydraulic conductivity than what was previously estimated by X-ray scans in midribs  
469 (Bortolami et al. 2019). Moreover, even when only the remaining green asymptomatic  
470 part of the leaves was considered, we quantified that the relative embolized surface  
471 was lower in symptomatic leaves compared to controls, reinforcing the hypothesis that  
472 vascular occlusions strongly affect the leaf xylem functionality of grapevines with esca.

473

## 474 **Conclusions**

475 In this study, we showed that esca leaf symptoms are associated with anatomical  
476 modifications that are unique to its proper premature leaf senescence process. Indeed,  
477 no vascular occlusions were detected in leaves with magnesium deficiency or autumn  
478 senescence. This result reinforced the particularity of esca over other induced  
479 senescence types, highlighting the need to understand the underlying physiological  
480 (and pathological) mechanisms of esca pathogenesis. Importantly, this work confirms  
481 that vascular occlusions in leaves during esca are associated with hydraulic failure in  
482 the leaf blade. The different cultivar (and climatic) susceptibility in occlusion formation  
483 during esca could also open new perspectives regarding the use of occlusion  
484 quantification to characterize plant resistance to the disease.

485

## 486 **Supplementary data**

487 The following Supplementary Data are available for this article:

488 **Table S1.** Effect of esca leaf symptoms on the presence of occluded vessels in *Vitis*  
489 *vinifera*.

490

491 **Table S2.** Linear relationships between the percentage of occluded vessels and esca  
492 symptom severity (expressed in percentage of green tissue per symptomatic leaf,  
493 control leaves were excluded from the analysis) for each *Vitis vinifera* cultivar.

494

495 **Figure S1.** Pictures of the three senescence types from a white cultivar (Chenin, A,  
496 C, E, G) and a red (Castet, B, D, F, H) *Vitis vinifera* cultivar.

497

498 **Figure S2.** Relationship between percentage of xylem vessels with crystals and  
499 vessels with occlusion (examples in Fig. 1) for each sample in *Vitis vinifera*.

500

501 **Figure S3.** Occluded vessels [%] from different cultivars and different countries in *Vitis*  
502 *vinifera* midribs collected from leaves with (from light red to dark red) and without (blue,  
503 all cultivars combined) esca symptoms.

504

505 **Supplemental Discussion SD1.** Crystals in xylem vessels.

506 **Supplementary References**

507

508 **Conflict of interest**

509 None declared.

510

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513 (FranceAgriMer and CNIV) within the PHYSIOPATH project (program Plan National  
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516

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522 method implementation and the SOLEIL synchrotron facility (HRCT beamline  
523 PSICHE) for providing the materials and logistics.

524

## 525 **Author's contributions**

526 G.B., C.E.L.D., G.A.G., designed the experiments;

527 G.B., K.B., D.G., G.R., conducted the leaf sampling;

528 N.F. conducted the histological observations;

529 L.J.L., S.D. conducted the non-invasive optical visualization of embolism, and  
530 analyzed the images;

531 G.B. analyzed the images from light microscopy, and analyzed the data;

532 G.B., C.E.L.D., G.A.G. wrote the article; all authors edited and agreed on the last  
533 version of the article.

534

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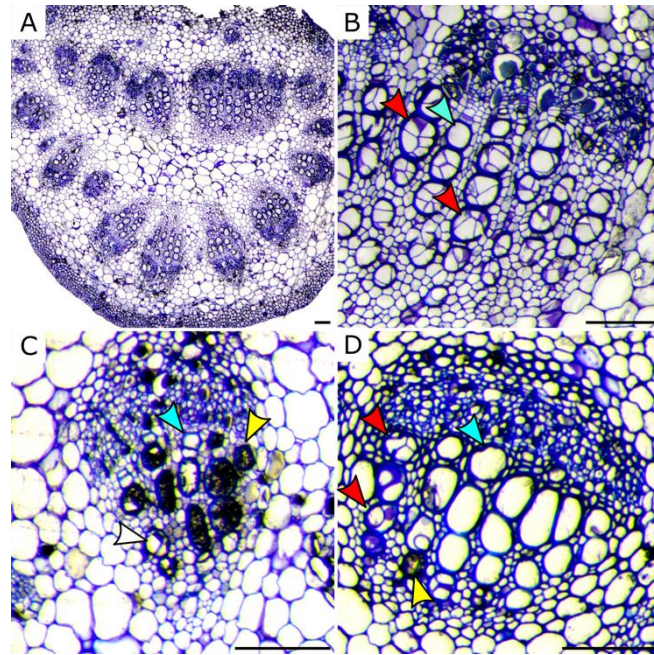
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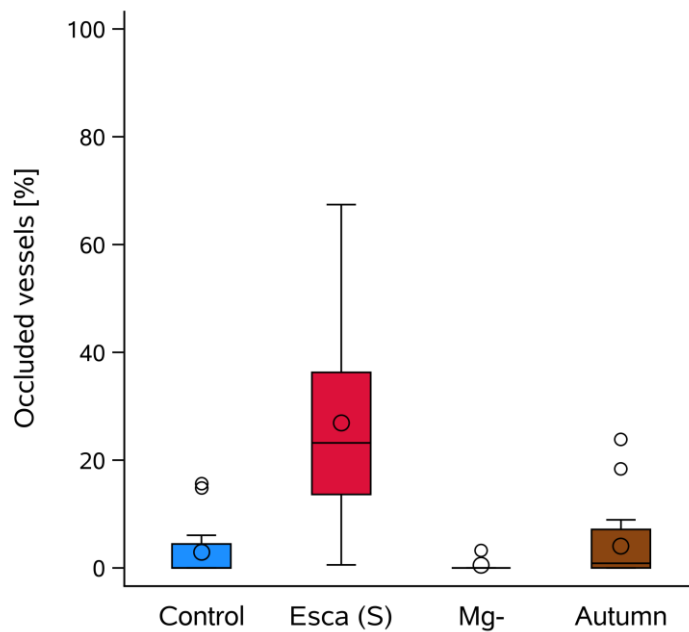
670

671 **FIGURES**



672

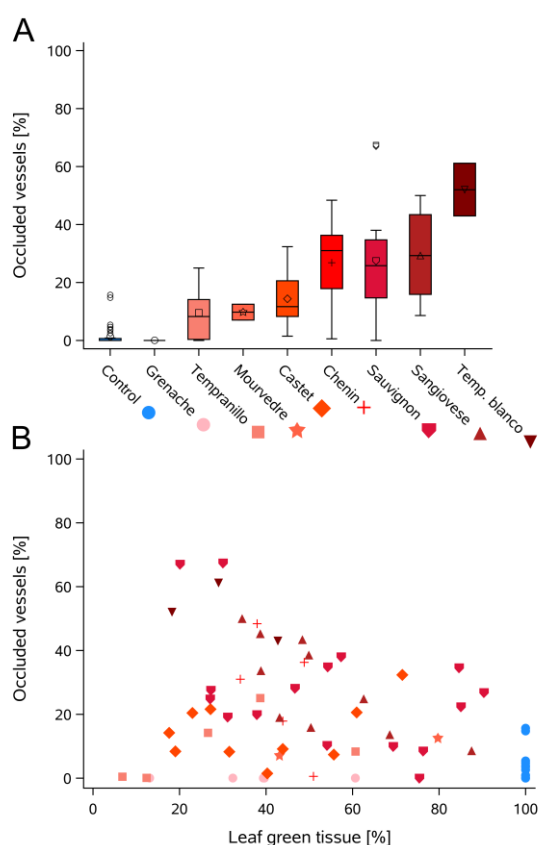
673 **Figure 1.** Xylem vessel anatomy in *Vitis vinifera* midribs. In whole midrib transverse  
674 cross-sections (A), vessels were classified according to: the absence of any structure  
675 in their lumina (i.e. apparently functional vessels; blue arrowheads, B-D); the presence  
676 of crystal depositions when druse crystals were covering the vessel surface (yellow  
677 arrowheads, C, D), or when prismatic crystals were identified (white arrowhead, B);  
678 the presence of occlusion when gel pockets and/or tyloses were observed in their  
679 lumina (red arrowhead, B, D). Scale bars = 100µm.



680

681 **Figure 2.** Percentage of occluded vessels in *Vitis vinifera* midribs of control leaves and  
 682 leaves from three types of leaf symptom (esca leaf symptoms, magnesium deficiency,  
 683 Autumn senescence). Colors correspond to midribs from: healthy leaves (control)  
 684 (blue, n=17), esca symptomatic (red, n=22), magnesium deficiency (gray, n=6) and  
 685 autumn senescence (brown, n=21) leaves. Boxes and bars show the median,  
 686 quartiles, and extreme values. Larger circles within boxes correspond to means and  
 687 smaller circles outside boxes to outlier values.

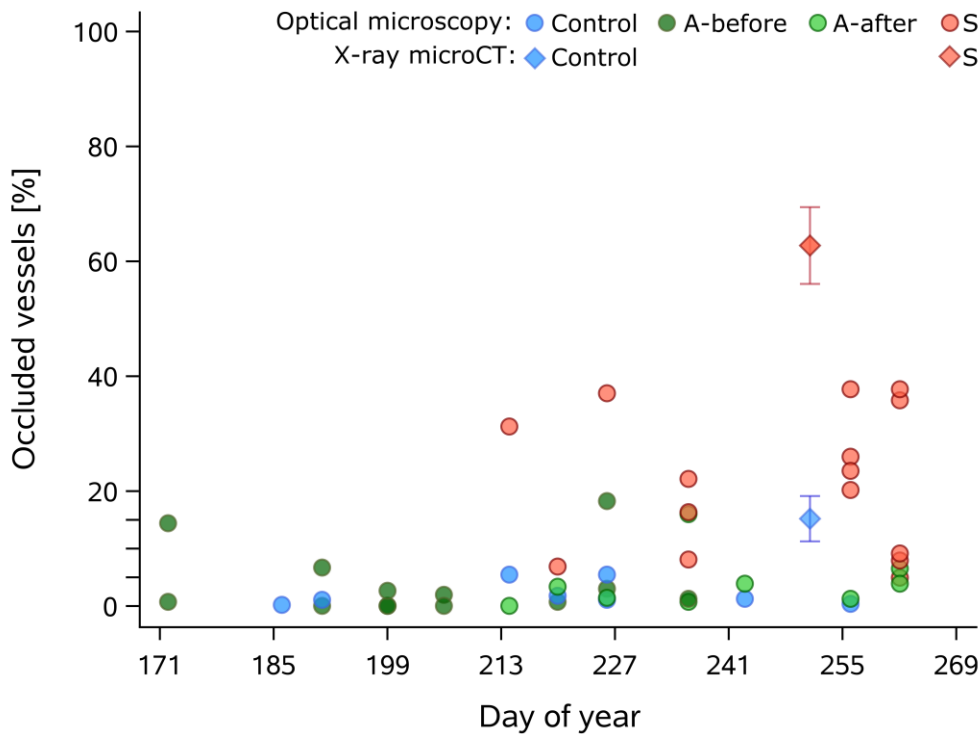




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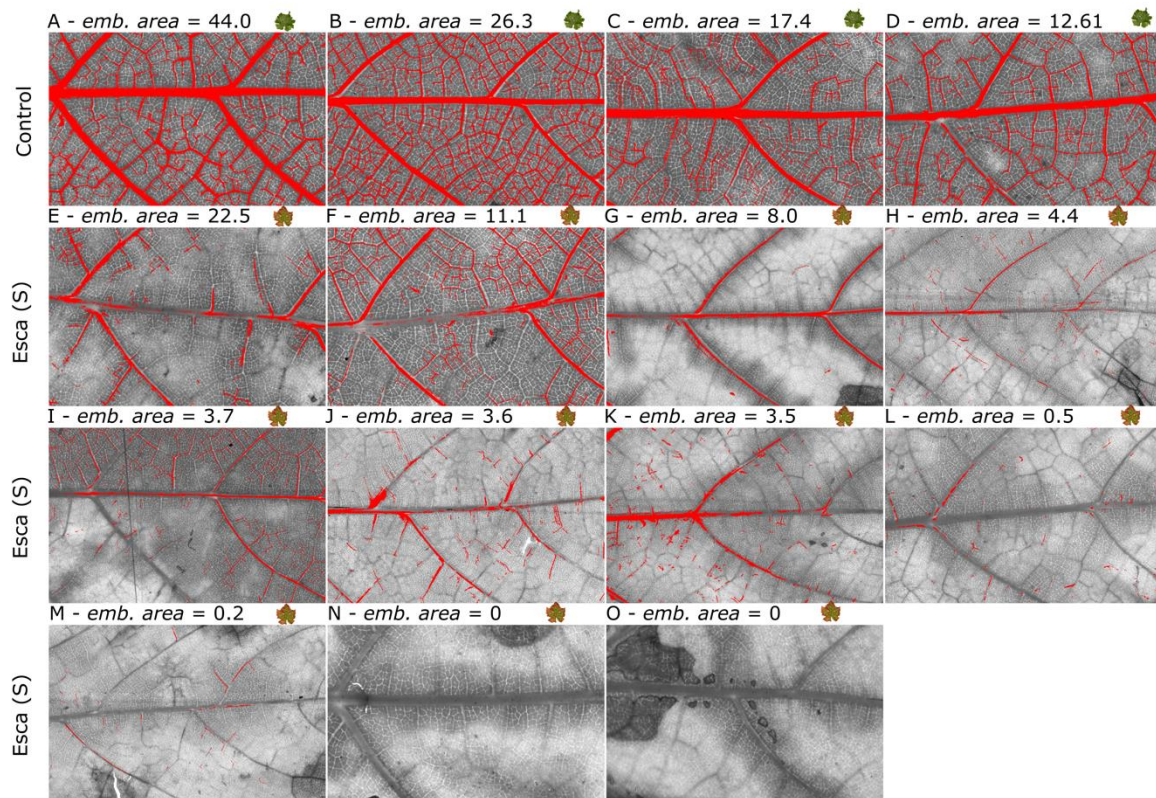
689 **Figure 3.** Occluded vessels [%] in different cultivars from different countries in *Vitis*  
690 *vinifera* midribs of leaves with (red shades) or without (blue) esca leaf symptoms. (A)  
691 Percentage of occluded vessels in midribs from control leaves (blue, all control leaves  
692 from all cultivars and countries combined) and esca symptomatic (from light red to  
693 dark red, all countries combined) leaves. Boxes and bars show the median, quartiles,  
694 and extreme values. Larger symbols within boxes correspond to means and smaller  
695 symbols outside boxes to outlier values. The statistical differences are presented in  
696 the text. An additional analysis between each cultivar and its specific control per  
697 country is presented in Table S1. (B) Relationship between the percentage of  
698 occluded vessels and esca symptom severity (expressed in percentage of green  
699 tissue per leaf). Control leaves were excluded from the statistical analysis but are  
700 represented on the graphics as blue filled circles for illustration. The colors and

701 markers are the same as panel A: circles for Grenache, squares for Tempranillo, stars  
 702 for Mourvedre, diamonds for Castets, plus for Chenin, pentagons for Sauvignon blanc,  
 703 upside-triangles for Sangiovese, and downside-triangles for Tempranillo blanco. The  
 704 regressions for each cultivar are presented in Table S2, where a significant  
 705 relationship was found only for Sangiovese.  
 706



707  
 708 **Figure 4.** Evolution of occluded vessels [%] over the season (July to September) in  
 709 *Vitis vinifera* cv Sauvignon blanc midribs. Symbols correspond to single leaves from  
 710 well-watered control plants (blue, n=8), asymptomatic leaves before esca symptom  
 711 appearance (dark green, n=13), asymptomatic leaves (light green, n=9) and esca  
 712 symptomatic (red, n=15) leaves from plants with symptomatic leaves. Circles  
 713 correspond to optical microscopy observations; diamonds correspond to X-ray  
 714 microCT scan analyses (data from Bortolami *et al.*, 2019). In X-ray microCT

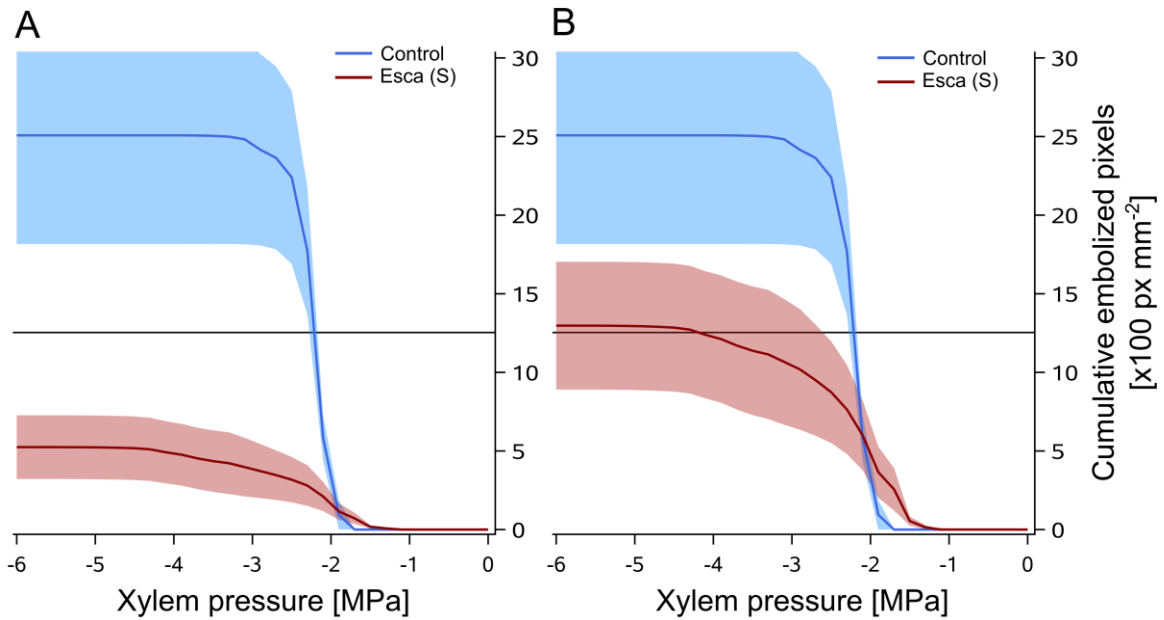
715 quantification, symbols correspond to means and bars to  $\pm$  SE (n=8 control in blue,  
716 and n=13 esca leaves in red).



717

718 **Figure 5.** Visualization of embolism formation during dehydration of *Vitis vinifera* cv  
719 Sauvignon blanc leaves from control and esca symptomatic leaves. Each panel  
720 corresponds to a leaf blade from control leaves collected on asymptomatic control  
721 plants (A-D) and esca symptomatic leaves (E-O). Red veins correspond to veins that  
722 embolized at the end of dehydration. The total cumulative embolized area (emb. area)  
723 is indicated for each sample (x 100 pixels mm<sup>2</sup>).

724



725

726 **Figure 6.** Embolism formation in leaf blade from *Vitis vinifera* cv Sauvignon blanc from  
 727 control (blue) and esca symptomatic (red) leaves during dehydration. **(A)** Sum of  
 728 embolized pixels on the total (green and symptomatic) scanned surface over  
 729 decreasing xylem pressure. **(B)** Sum of embolized pixels on the green scanned area  
 730 (i.e. excluding symptomatic discolored portions) over decreasing xylem pressure.  
 731 Lines (moving averages over 0.2 MPa) represent the average evolution of embolized  
 732 areas (x100 pixels mm<sup>-2</sup>) during decreasing xylem pressure (MPa). The bands  
 733 represent the SE for each group, and the horizontal black lines correspond to 50 % of  
 734 embolized area for control leaves.

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741 **TABLE**

742 **Table1.** Sample size for vessel occlusion quantification in *Vitis vinifera* leaves  
 743 collected in four different countries, eight varieties, an different senescence types.

Region	Plantation year	Control asymptomatic	Esca symptomatic	Magnesium deficient	Autumn
France	2009	Castets n=5 Chenin n=2 Mourvedre n=3 Sangiovese n=4 Sauvignon blanc n=3	Castets n=5 Chenin n=5 Mourvedre n=2 Sangiovese n=5 Sauvignon blanc n=5	Castets n=2 Chenin n=2 Mourvedre n=2	Castets n=5 Chenin n=3 Mourvedre n=7 Sangiovese n=1 Sauvignon blanc n=5
Italy	1989	Sangiovese n=3 Sauvignon blanc n=3	Sangiovese n=5 Sauvignon blanc n=4	-	-
Spain	2013	Castets n=3 Tempranillo n=3 Tempranillo blanco n=1 Grenache n=1	Castets n=5 Tempranillo n=5 Tempranillo blanco n=3 Grenache n=5	-	-
California (US)	2000	Sauvignon blanc n=8	Sauvignon blanc n=7	-	-

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