

1 **Effect of mycoviruses on the virulence of *Fusarium circinatum***
2 **and laccase activity.**

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21 **Abstract**

22

23 Laccase enzymes (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) play a major role
24 in the degradation of phenolic compounds such as lignin. They are common in fungi and
25 have been suggested to participate in host colonization by pathogenic fungi. Putative
26 mycoviruses have recently been isolated from the causal agent of pine pitch canker
27 disease, *Fusarium circinatum* Nirenberg & O'Donell. In this study, the effects of single
28 and double mycoviral infections on laccase activity, growth rate and pathogenicity were
29 investigated in fourteen *F. circinatum* strains. Extracellular laccase activity was analyzed
30 by the Bavendamm test, image processing and a spectrophotometric method. Mycelial
31 growth, *in vivo* pathogenicity and seedling survival probability were also determined in
32 Monterrey pine (*Pinus radiata* D. Don) seedlings. The findings showed that (i) mycelial
33 growth of isolates from the same fungal population was homogeneous, (ii) the presence
34 of mycovirus appears to increase the virulence of fungal isolates, (iii) co-infection (with
35 two mycoviruses) caused cryptic effects in fungal isolates, and (iv) laccases embody a
36 possible auxiliary tool in fungal infection. The prospects for biocontrol, the adaptive role
37 of *F. circinatum* mycoviruses and the importance of laccase enzymes in host colonization
38 are discussed.

39

40 **Keywords:** Biocontrol, image analysis, multicopper oxidases, pine pitch canker disease,
41 ssRNA.

42

43

44 1. Introduction

45

46 Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belong to the multicopper
47 oxidase group of enzymes and are specialized in catalyzing the oxidation of phenolic
48 substrates by reduction of O₂ to H₂O. Laccases are common in eukaryotes, including
49 fungi, and have been widely studied in the phylum Ascomycota [1]. These enzymes
50 (molecular weight around 60-70 kDa) are usually extracellular and show a high degree
51 of specificity for degrading polyphenol substrates such as lignin [2]. They play an
52 essential role in nutrient turnover (mainly nitrogen and carbon) in nature, due to their
53 capacity to degrade lignocelluloses in forest soil and litter, and they are abundant in
54 saprophytic fungi [3]. Laccases may also play an important role in host colonization by
55 pathogenic fungi as they can damage host tissues, thus favouring fungal infection [4,5].
56 Additionally, they have important applications in industry (e.g. textile and paper
57 industries) as well as in bioremediation and environmental biotechnology [6].

58

59 The fungus *Fusarium circinatum* Nirenberg & O'Donnell is the causal agent of pine pitch
60 canker disease. This invasive necrotroph is considered the most important pathogen of
61 pine seedlings in several countries around the world and particularly affects conifers such
62 as Monterrey Pine (*Pinus radiata* D. Don) and *Pseudotsuga menziesii* (Mirb.) Franco
63 [7,8]. It can infect branches, stems, seeds, cones and roots in host trees of any age,
64 causing pre- and post-emergence damping-off in seedlings (mortality rates up to 90%)
65 and severe damage and reduced growth in adult trees [9]. Pine pitch canker fungus is
66 widespread throughout the world and has been reported in Mexico, USA, Haiti, South
67 Africa, Japan, Korea, Southern Europe and South America [10]. The pathogen spreads
68 via the movement of contaminated material (seeds, wood, nursery seedlings, etc.) as
69 well as via air- and soilborne spores and insect vectors [11] and via damage to trees
70 caused by storms or human activities [12]. The disease is expected to spread rapidly in
71 the future, and it has been estimated that approximately 10 million hectares of native
72 pine forest and plantations in the EU are potentially endangered [13].

73

74 Several management measures and treatments for controlling *F. circinatum* have been
75 suggested: application of adaptive silviculture programmes [14], selection of particular
76 species for planting [8], treatment of seeds with hot water [15], addition of hydrogen
77 peroxide to irrigation water [16] and biocontrol techniques involving bacteria [17] or other
78 fungal species [18]. However, although some of these techniques are potentially useful,
79 new methods of biocontrol focused on field and nursery application are required.

80

81 Mycoviruses (viruses that infect fungi) are common in many fungal species, including
82 some plant pathogens [19]. Fifteen families of mycoviruses have been described: these
83 include single-strain RNA viruses which sequence serves as template for RNA-
84 dependent RNA polymerase (RdRp) (ss(+)RNA), viruses that require the intervention of
85 RNA replicase to copy their genome into positive sense (ss(-)RNA) and also viruses with
86 double-strain RNA (dsRNA) and single-strain DNA (ssDNA) [20,21]. The effects of
87 mycoviruses on fungi vary from induction of a cryptic state to increase the capacity of
88 host to produce disease (hypervirulence). Although only a few mycoviruses reduce the
89 virulence of their host (hypovirulence), this kind of viruses is of particular interest for
90 biocontrol purposes [22]. One of the best known examples of virus-mediated
91 hypovirulence is that involving chestnut blight (causal agent *Cryphonectria parasitica*
92 (Murrill) M. E. Barr). *Cryphonectria hypovirus 1* (CHV-1, *Hypoviridae*), which is one of
93 the four *Hypovirus* spp. that infects the fungus, has shown good results in biocontrol
94 treatment and has been shown to reduce fungal virulence (decreased mycelial growth
95 and sporulation rate) [23,24]. Other mycoviruses hosted by pathogenic fungi have also
96 been identified as promising organisms for biological control [25,26].

97

98 Changes in laccase activity in fungi have been reported in relation to mycoviral infection
99 [27,28]. Laccase activity may also be altered in pathogenic fungi in the presence of
100 mycoviral infection, and reduced enzymatic activity may be associated with lower
101 virulence [29–31]. Three mycoviruses hosted in mitochondria that infect *F. circinatum*
102 have recently been identified as putative members of Narnaviridae (genus *Mitovirus*) and
103 designated *Fusarium circinatum* mitovirus 1, 2-1 and 2-2 (FcMV1, FcMV2-1 and FcMV2-
104 2) [32]. Although little is known about the effects of these mycoviruses, any of them that
105 reduce laccase activity could potentially be used to develop a biocontrol technique to
106 treat pine pitch canker disease.

107

108 In this study, we hypothesized that *F. circinatum* isolates infected by mycoviruses would
109 show differences in laccase activity relative to isolates not infected by viruses. We also
110 expected to observe a positive correlation between laccase activity and host
111 pathogenicity. To our knowledge, this is the first study focusing on this topic in relation
112 to pine pitch canker disease. The objectives of this study were (i) to analyze the possible
113 effects of mycoviruses FcMV1 and FcMV2-2 on laccase activity in *F. circinatum*; (ii) to
114 investigate the variations in laccase activity, growth rates and infection development in
115 relation to mycovirus presence, and (iii) to evaluate the relationship between enzyme
116 activity and pathogenicity in Monterrey pine seedlings.

117 2. Material and Methods

118

119 2.1. Selection of isolates

120

121 Seven isolates of *F. circinatum* were obtained from two different locations in northern
122 Spain (Asturias and Cantabria) where wild-types of this fungus are commonly infected
123 by mycoviruses as previously reported [10]. Two monosporic cultures for each isolate
124 were selected, and the presence of mycoviruses was confirmed according to Álvarez et
125 al. [32]. The mating type (MAT) of each isolate was previously investigated [8] (Table 1).
126 Briefly, isolates FC104 and FC072 were free of mycovirus and isolates FC104v and
127 FC072v (i.e. of the same strains) were infected with FcMV1 ("v" indicates infection with
128 mycovirus). Isolate FC070v was also infected with FcMV1 and isolate FC070w was
129 infected with both FcMV1 and FcMV2-2 ("w" indicates co-infection). Isolates FC020,
130 FC035 and FC042 were free of mycovirus and FC020v, FC035v and FC042v were
131 infected with FcMV2-2. Finally, isolate FC221 was free of mycovirus and isolate FC221w
132 was co-infected with both mycoviruses. FcMV2-1 was not present in the evaluated
133 isolates.

134

135 2.2. Bavendamm test.

136

137 Seven samples of each isolate were cultured in Bavendamm medium to enable
138 estimation of the level of extracellular laccase activity. The fungal isolates were grown in
139 darkness at 25° C in specific media containing 0.50% w/v tannic acid, 1.50% w/v malt
140 extract and 2% w/v agarose. Tannic acid and malt-agarose solutions were prepared with
141 distilled water and autoclaved separately before being mixed together; the pH was
142 adjusted to 4.50 with NaOH 10M [31,33]. The global intensity of the enzymatic reaction
143 was evaluated after incubation for five days, and the change in color of the media (from
144 whitish to dark brown) was assessed according to the following qualitative scale: (-) non
145 appreciable reaction, (+) slight reaction or (++) intense reaction (Fig. 1).

146

147 2.3. Monitoring for mycelial growth.

148

149 In parallel to the Bavendamm test, photographs of the Petri dishes containing the fungal
150 isolates were taken every day for five days with a Canon EOS 550D camera (white backlit
151 screen as background and constant light). The photographs were processed using
152 ImageJ 1.48v [34] in order to quantify the area affected by enzymatic reaction (i.e. brown
153 area over whitish medium) [35,36]. The mean area affected by enzymatic reaction (S)

154 and mean growth of the isolate (G; calculated as the mean value of colony size increase
155 between two consecutive observations) were measured daily.

156

157 2.4. Laccase activity.

158

159 The *F. circinatum* isolates were cultured for one week in Bavendamm medium. Three
160 plugs (5x5 mm) comprising mycelia and medium were then removed from the edge of
161 each isolate and transferred to 1.50 ml tubes. Aliquots (1.50 ml) of twice-autoclaved
162 distilled water (4° C) were added to the plug samples to extract crude extracellular
163 laccase. After incubation for thirty minutes at room temperature, the tubes were
164 centrifuged for three minutes at 10⁴ g and the supernatant was extracted. The laccase
165 activity was assayed after adding 0.80 ml of 2.50 mM 2,6-dimethoxyphenol (DMP, broad
166 spectrum enzyme substrate) to 0.20 ml of the crude laccase in 100 mM phosphate buffer
167 (pH 6.90) at 37° C [37]. The absorbance of samples was measured at 468 nm and 25°
168 C in a LAN OPTICS (2000-2100) spectrophotometer [38]. Absorbance was measured
169 immediately and five minutes later. Finally, the increase in absorbance was calculated
170 as an absolute value for the measurement period (ΔA_{0-5}).

171

172 2.5. *In vivo* pathogenicity.

173

174 To test *in vivo* the ability of each strain to cause disease (pathogenicity), the isolates
175 were inoculated into 405 one-year-old nursery seedlings of Monterrey pine (i.e. 27
176 replicate seedlings per isolate and 27 control seedlings). A small incision was made two
177 centimeters above the root collar and 10 μ l of spore suspension (10⁶ spores/ml of distilled
178 water) was inoculated into the wound. In control seedlings, an incision was made in the
179 same way, but distilled water only was inoculated into the wound. The wound was
180 covered with Parafilm® for one week. The treated and control seedlings were held
181 separately in plant growth chambers at 25° C with a 16h photoperiod. The seedlings
182 were watered three times a week throughout the study period, with equal amounts of
183 distilled water.

184

185 After one week, the visual severity of symptoms in each plant were assessed every two
186 days during a period of 15 days, according to the following scale: 0 = healthy plant, 1 =
187 necrosis only at the point of inoculation and healthy foliage, 2 = necrosis >2 cm beyond
188 the point of inoculation, 3 = needles wilting and appreciable dieback and 4 = dead plant
189 [39] (Fig. 1). Finally, the area under the disease progress curve (AUDPC) was calculated
190 as the sum of the area of the corresponding trapezoids as previously described [8].

191

192 2.6. Statistical analysis.

193

194 All analyses were performed with R software [40]. The Kruskal-Wallis rank sum tests
195 were carried out with the “Agricolae” package [41] to analyze the variation in S, G, ΔA_{0-5}
196 and AUDPC values according to two different factors: isolate (14 strains, Table 1) and
197 mycovirus presence (evaluated as follows: not infected (\emptyset); infected with FcMV1 or
198 FcMV2-2; and co-infected with both mycoviruses). Dunn’s test [42] was applied for post-
199 hoc analysis of data, with “DescTools” package [43]. The Pearson's product-moment
200 correlation [44] was also calculated for (a) G and ΔA_{0-5} , (b) mean values of AUDPC and
201 ΔA_{0-5} for each isolate, and (c) the G and S variables. Survival analysis based on the non-
202 parametric Kaplan-Meier estimator [45] was carried out with “Survival” package [46].
203 Survival curves were created with the “Survfit” function and the differences between the
204 curves were tested with the “Survdiff” function.

205

206 <<Insert Figure 1 around here>>

207 3. Results

208

209 3.1. Bavendamm test and mycelial growth.

210

211 All isolates showed an intense response in the Bavendamm test (Table 1). The mean
212 value of S was $491.27 \pm 27.85 \text{ mm}^2$ (standard error). The Kruskal-Wallis rank sum test
213 revealed significant differences in S between isolates ($X^2= 37.45$; d.f.= 13; $P= <0.01$) but
214 not in relation to mycovirus presence ($X^2= 0.94$; d.f.= 3; $P= 0.81$). Isolate FC104 yielded
215 the highest value of S (mean value $724.22 \pm 31.21 \text{ mm}^2$), which was significantly different
216 from the values yielded by other isolates, including FC104v ($P= <0.01$). FC042 and
217 FC070v resulted in the lowest S values, without significant differences between them
218 ($P= 0.47$). The S values produced by these isolates and the non-infected pairs (FC042
219 and FC070) were not significantly different ($P= 0.15$; $P= 0.21$, respectively) (Fig. 2). The
220 G and S variables were closely correlated ($t= 19.04$; d.f.= 96; $P= <0.01$; $\rho= 0.88$).

221

222 The isolates grew quickly, and the mean G value was $227.28 \pm 16.83 \text{ mm}^2/\text{day}$. Growth
223 did not vary significantly in relation to mycovirus presence ($X^2= 2.13$; d.f.= 3; $P= 0.54$)
224 and it also did not differ significantly between isolates ($X^2= 22.27$; d.f.= 13; $P= 0.05$).

225

226 <<Insert Table 1 and Figure 2 around here>>

227

228 3.2. Laccase activity.

229

230 Laccase activity (expressed as $\Delta A_{0.5}$) differed significantly in relation to the isolate ($X^2=$
231 22.54 ; d.f.= 13; $P= 0.04$), whereas the presence of the mycovirus did not have a
232 significant effect ($X^2= 1.92$; d.f.= 3; $P= 0.58$). Of the fungal isolates infected with
233 mycovirus, FC042v produced the greatest increase in the absorbance, which was
234 significantly different from that produced by the same isolate not infected with the
235 mycovirus, which yielded the lowest absorbance increase (FC042, $P= <0.01$). Likewise,
236 $\Delta A_{0.5}$ also differed significantly between FC104 and FC104v ($P= 0.01$) (Fig. 3) but the
237 correlation between G and $\Delta A_{0.5}$ was not significantly different ($t= -0.88$; d.f.= 96; $P= 0.37$;
238 $\rho= -0.09$).

239

240 <<Insert Figure 3 around here>>

241

242

243 3.3. Pathogenicity *in vivo*.

244

245 The values of AUDPC obtained in relation to the different treatments varied significantly
246 depending on the isolate ($X^2= 98.90$; d.f.= 14; $P= <0.01$). The highest AUDPC value was
247 obtained for FC072v and it was significantly different from that obtained for its pair FC072
248 ($P= 0.02$). The lowest value was obtained for seedlings infected with FC042 (mean value
249 36.92 ± 2.17) and was significant different from the values corresponding to the other
250 isolates ($P= <0.03$, in all cases) (Fig. 4).

251

252 <<Insert Figure 4 around here>>

253

254 The AUDPC also varied significantly in regard to viral infection ($X^2= 25.75$; d.f.= 3; $P=$
255 <0.01). The value was higher in all plants infected by *F. circinatum* isolates than in control
256 seedlings, as expected (<0.01 , in all cases). The AUDPC values were higher in FcMV1-
257 infected fungi than in non-infected ($P= <0.01$) and co-infected isolates ($P= 0.02$), but
258 there were no significant differences between FcMV2-2 infected isolates ($P= 0.11$).
259 There were no significant differences between co-infected isolates and either isolates
260 infected with FcMV2-2 only ($P= 0.16$) or non-infected isolates ($P= 0.40$) (Fig. 5). The
261 correlation between AUDPC and ΔA_{0-5} as average values for each isolate were almost
262 statistically significant ($t= 2.13$; d.f.= 13; $P= 0.05$; $\rho= 0.50$).

263

264 <<Insert Figure 5 around here>>

265

266 Survival analysis revealed significant differences between treatments ($X^2= 94.50$; d.f.=
267 4; $P= <0.01$) (Fig. 6). The survival probability of seedlings was significantly lower in plants
268 inoculated with isolates infected with FcMV1 than in the virus-free isolates ($X^2= 11.10$;
269 d.f.= 1; $P= <0.01$). FcMV2-2 presence in fungi did not produce any differences in plant
270 host survival relative to non-infected isolates ($X^2= 3.30$; d.f.= 1; $P= 0.06$). No differences
271 were found in seedlings survival probability between isolates infected with FcMV1 or
272 FcMV2-2 ($X^2= 1.50$; d.f.= 1; $P= 0.22$). Likewise, survival probability was not different in
273 plants inoculated with co-infected strains in respect of non-infected isolates ($X^2= 0.40$;
274 d.f.= 1; $P= 0.52$).

275

276 <<Insert Figure 6 around here>>

277

278 4. Discussion

279

280 The study findings indicate that laccase activity and the area affected by enzymatic
281 activity were fairly homogeneous in most of the fungal isolates. Only isolate FC104
282 yielded a higher S value than the other strains. This isolate differed from the others in
283 geographical origin (Asturias region) and in mating type (MAT 1) [47]. The apparent
284 similarity in the S value for other isolates may be related to the low genetic variability
285 among isolates from the Cantabrian population, in which only MAT 2 has been identified
286 (Table 1). The observed differences seem to support the theory that suggests punctual
287 introductions of the fungus in the Iberian Peninsula and a subsequent wide dissemination
288 of the clonal population [48,49].

289

290 Growth rate and area affected by enzymatic reaction were closely correlated. Thus, the
291 colored area measured by image analysis may be considered as an acceptable
292 indication of colony development, as S was mainly limited to the area occupied by the
293 colony. This method based on pixel colorimetry has proved useful and reliable for
294 establishing chromatic differences between mycelia and media.

295

296 Infection with a single mycovirus led to higher fungal pathogenicity and lower survival of
297 seedlings infected by *F. circinatum* isolates. FcMV1 infection was associated with higher
298 AUDPC values and lower survival than the other treatments, and FcMV2 caused a slight
299 increase in the fungal virulence and a non-significant decrease in the survival relative to
300 the virus-free isolates. In view of these findings, neither of these mycoviruses appear
301 useful for biocontrol purposes (such as with CHV-1 in chestnut blight [24]) because of
302 their lack of capacity to promote hypovirulence in the host. On the other hand, although
303 both FcMV1 and FcMV2-2 were associated with a reduction in survival relative to control
304 seedlings, the AUDPC values increased by <20% relative to virus-free isolates, and this
305 increment was only significant in FcMV1 (Fig. 5). Furthermore, mycelial growth did not
306 vary in relation to mycovirus presence. Taking all this into account, we concluded that
307 neither FcMV1 nor FcMV2-2 induced hypervirulence in their fungal hosts. However
308 further studies are needed to confirm it. Co-infection resulted in similar AUDPC values,
309 plant survival probability and colony growth rates as in the fungal isolates free of
310 mycovirus. This finding contrasts with a previous report of hypovirulence in *C. parasitica*
311 isolates (lower sporulation and mycelial growth) caused by simultaneous infection of
312 CHV-1 and Mycoreovirus 1 (MYRV-1, *Reoviridae*) [50]. In a study involving
313 *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & De Not., isolates infected with
314 *Botryosphaeria dothidea* chrysovirus 1 (BdCV1) and *Botryosphaeria dothidea* partitivirus

315 1 (BdPV1) showed slower growth rate and lesions were shorter when the fungus was
316 simultaneously infected by both mycoviruses, suggesting a hypovirulent effect of this
317 multi-viral infection [51]. Simultaneous infection with two putative member of
318 *Partitiviridae* also caused a strong reduction in laccase activity in *Botrytis cinerea* Pers.
319 isolates, and the enzymatic activity was lower than in single infection and wild-type [30].
320 In the present study, co-infection of fungal isolates with FcMV1 and FcMV2-2 did not
321 induce hypovirulence, although further studies focusing on the synergistic effect of
322 mycoviruses within their hosts are required.

323
324 A previous study reported the presence of mycoviruses in Iberian isolates of *F.*
325 *circinatum* but not in South African isolates [10]. It is therefore possible that members of
326 the Iberian population of *F. circinatum* host mycoviruses because the fungi initially
327 introduced in Spain was harbouring viruses at that time [49]. On the other hand, this type
328 of mycovirus may play an adaptive role in non-native regions where the fungus has
329 recently been introduced, apparently improving host resilience [52]. This approach would
330 explain the observed virulence in strains infected with mycovirus and is consistent with
331 the hypothesis supporting ancient co-evolution between mycovirus and fungi, mainly
332 mediated by horizontal transmission of viruses (through mycelial fusion rather than the
333 spread to progeny) [21,53].

334
335 Extracellular laccase activity did not vary depending on mycovirus presence. Moreover,
336 mycelial growth rate was not related to enzymatic activity. In contrast, laccase activity
337 varied between isolates and seemed to be related to pathogenicity. Enzymatic activity
338 was only lower in isolates FC221, FC020 and FC104 (significantly lower in the case of
339 FC104) when they were infected, supporting the idea that the mycoviruses do not cause
340 hypovirulence [27]. By contrast, a strong reduction in laccase activity (indicated by the
341 Bavendamm test reaction and $\Delta A_{0.5}$) was observed in *C. parasitica* isolates infected by
342 dsRNA mycovirus in a study in which isolates that did not produce laccase were identified
343 as hypovirulent strains by a complementary pathogenicity test [31]. In a study of laccase
344 production in *Ophiostoma ulmi* (Buisman) Nannf. and *Ophiostoma novo-ulmi* Brasier
345 (causal agent of Dutch Elm Dieback) differences between the two species were observed
346 [4]. Thus, the less aggressive *O. ulmi* showed lower or even null laccase activity than the
347 more pathogenic *O. novo-ulmi* (0-0.20 U ml⁻¹ vs 0.12-0.34 U ml⁻¹ respectively). In the
348 aforementioned study comparing two strongly related species with different
349 pathogenicity, the authors proposed laccases as a useful tool for overcoming tree
350 defences. Similarly, higher values of enzymatic activity were obtained for virus-free
351 strains of *Diaporthe ambigua* Nitschke ($\Delta A_{0.5}$ for mycovirus-free strains: 0.11-0.17; $\Delta A_{0.5}$

352 ₅ of mycovirus-infected strains: 0.01-0.02) and the Bavendamm test was negative in
353 infected and less virulent strains [37]. Similar conclusions have been reached for *B.*
354 *cinerea* [29]. However, enhanced laccase activity has also been observed in hypovirulent
355 strains of *B. cinerea*, in a study in which the authors concluded that this enzyme was not
356 important in the virulence of the pathogen [26]. The values obtained in the present study
357 were higher than those reported in previous studies (mean value of $\Delta A_{0-5} = 0.17 \pm 0.03$),
358 suggesting intense extracellular laccase activity and ruling out hypovirulence in the
359 isolates.

360

361 *F. circinatum* does not possess specialized infection structures such as apressoria or
362 haustoria. Production of extracellular cell wall-degrading enzymes is therefore expected
363 to be higher in this necrotrophic species [54]. As *F. circinatum* initially colonizes the host
364 by occupying intercellular spaces, it has been suggested that the fungus would
365 segregate extracellular enzymes in order to degrade the cell wall to obtain nutrients from
366 plant cells [55]. Other enzymes (e.g. cutinases) and mycotoxins (e.g. bauvericin) have
367 been identified as important substances in plant infestation by *Fusarium* species [56],
368 indicating the involvement of an enzymatic complex in host colonization. Laccase may
369 thus enhance fungal pathogenicity by making cellulose accessible to other enzymes [57]
370 and probably acts as an initial infection tool enabling *F. circinatum* to overcome tree
371 defenses. This is supported by the findings of a study involving laccase production by
372 *Heterobasidion annosum* (Fr.) Bref. [5], in which the authors also suggested complex
373 uses of laccases during fungal infection. In summary, we conclude that laccases may be
374 important in early host colonization. Nevertheless, complete characterization of these
375 enzymes (chemical structure, molecular weight, suitable thermic and pH range, kinetic
376 constants, etc.) is required [58] for a better understanding of their metabolism and their
377 participation in the development of pine pitch canker disease.

378

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380

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388

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389 **6. References**

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563 **Tables and figures**

564

565 Tables

566

567 Table 1. Data and results of tests of *Fusarium circinatum* isolates (seven isolates, two
568 monosporic cultures/isolate): origin; host (Pp: *Pinus pinaster* Aiton, Pr: *Pinus radiata*);
569 mating-type (MAT); mycovirus presence (FcMV1/FcMV2-2); intensity of Bavendamm
570 test reaction (B.t.; qualitative scale: -, +, ++); area affected by enzymatic reaction (S);
571 mycelial growth (G); increase of absorbance in five minutes (ΔA_{0-5}) and area under the
572 disease progress curve (AUDPC). Mean values and standard error (SE) are shown. (*)
573 Source of data: [47].

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Isolate	Origin	Host	MAT	FcMV1	FcMV2-2	B.t.	S (mm ²) ± SE	G (mm ² /day) ± SE	ΔA _{0.5} ± SE	AUDPC ± SE
FC104v	Asturias*	Pp*	1*	✓	-	(++)	524.98 ± 55.15	226.21 ± 35.50	0.09 ± 0.04	44.33 ± 1.75
FC072v	Cantabria	Pr	2	✓	-	(++)	519.32 ± 44.11	252.71 ± 22.62	0.08 ± 0.02	43.35 ± 1.52
FC070v	Cantabria	Pr	2	✓	-	(++)	387.36 ± 45.70	174.91 ± 27.79	0.24 ± 0.06	46.46 ± 1.44
FC070w	Cantabria	Pr	2	✓	✓	(++)	443.03 ± 51.04	224.61 ± 28.30	0.14 ± 0.04	45.40 ± 1.34
FC221w	Cantabria*	Pr*	2*	✓	✓	(++)	542.83 ± 36.29	229.24 ± 26.57	0.15 ± 0.03	43.37 ± 1.36
FC020v	Cantabria	Pr	2	-	✓	(++)	480.60 ± 38.29	220.02 ± 27.82	0.10 ± 0.03	46.29 ± 1.56
FC035v	Cantabria	Pr	2	-	✓	(++)	503.56 ± 43.11	229.89 ± 21.44	0.13 ± 0.04	47.12 ± 1.22
FC042v	Cantabria	Pr	2	-	✓	(++)	447.40 ± 34.71	229.67 ± 22.57	0.32 ± 0.07	49.03 ± 1.63
FC104	Asturias*	Pp*	1*	-	-	(++)	724.22 ± 31.21	332.74 ± 13.01	0.23 ± 0.04	45.14 ± 1.31
FC072	Cantabria	Pr	2	-	-	(++)	504.00 ± 47.13	199.99 ± 34.72	0.10 ± 0.05	41.79 ± 2.14
FC221	Cantabria*	Pr*	2*	-	-	(++)	515.79 ± 46.29	233.29 ± 25.94	0.44 ± 0.15	45.24 ± 1.54
FC020	Cantabria	Pr	2	-	-	(++)	482.57 ± 35.56	231.64 ± 22.50	0.08 ± 0.01	36.92 ± 2.17
FC035	Cantabria	Pr	2	-	-	(++)	417.13 ± 27.38	202.97 ± 14.82	0.14 ± 0.07	45.11 ± 1.32
FC042	Cantabria	Pr	2	-	-	(++)	384.91 ± 15.46	203.73 ± 12.62	0.07 ± 0.02	44.33 ± 1.45

Figures

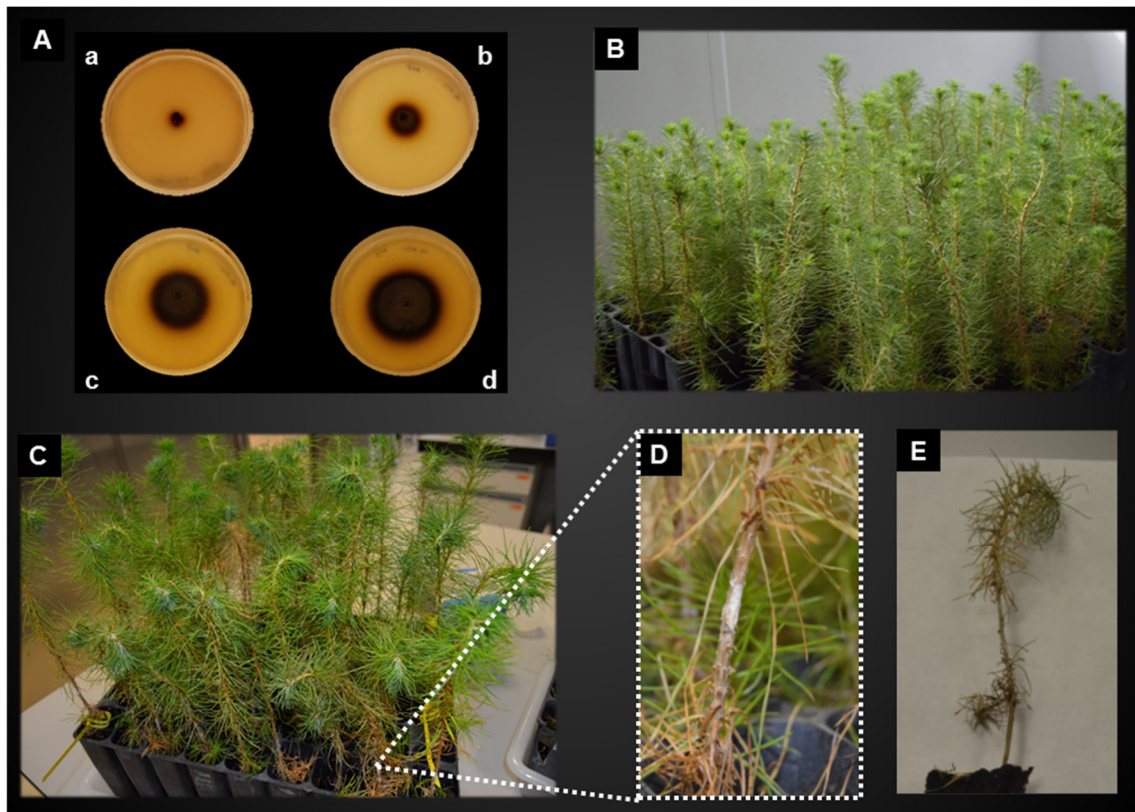


Fig. 1. Scheme of the study. A: Bavendamm test progress at four different moments: 24 h (a), 48 h (b), 72 h (c) and 96 h (d) after culture (isolate shown: Fc072). B: Control *Pinus radiata* seedlings on the 13th day of pathogenicity test. C: *Pinus radiata* seedlings inoculated with Fc072v (foreground) and Fc072 (background) on the 13th day of pathogenicity test. D: Detail of resin surrounding the point of inoculation. E: Detail of dead seedling showing the symptomatology of pine pitch canker damping-off.

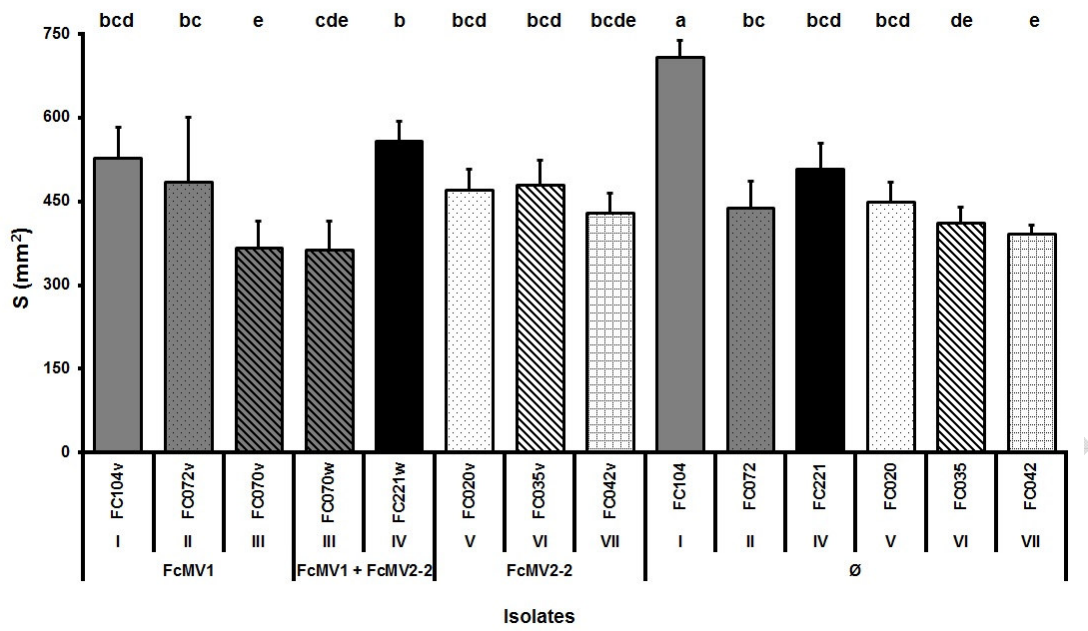


Fig. 2. Area affected by enzymatic reaction during the five days of the assay (S) for each fungal isolate. Small letters (a–e) denote significant differences (Dunn’s test, $P = <0.05$). (Ø): mycovirus-free isolates. Comparisons between pairs of isolates are indicated by color of plot and roman numbers (I–VII). Median values and standard error are shown.

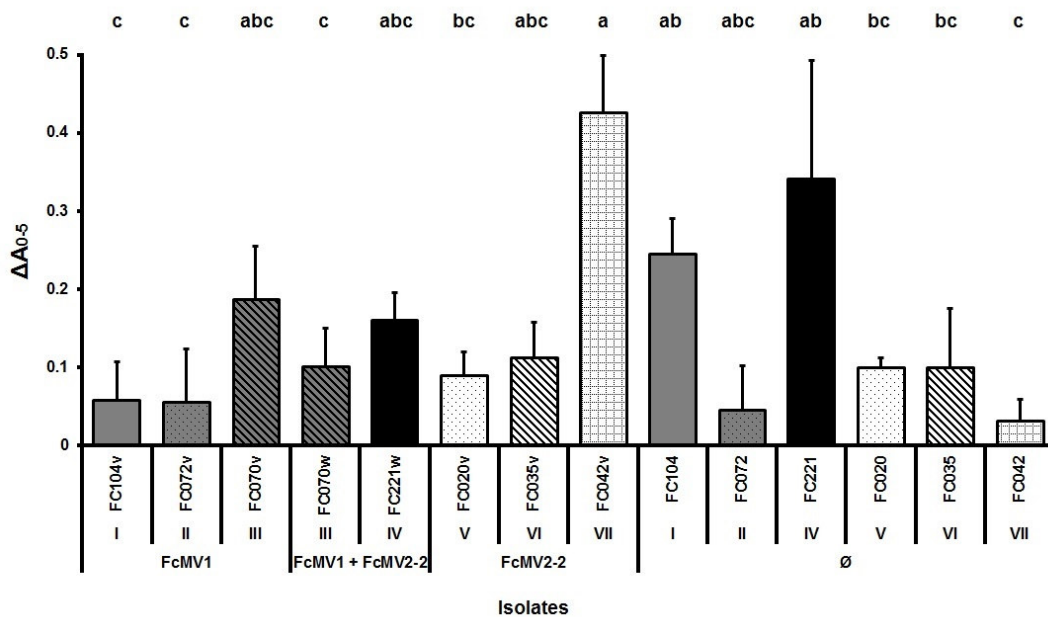


Fig. 3. Extracellular laccase activity (ΔA_{0-5}) in the different isolates. Small letters (a–c) denote significant differences (Dunn’s test, $P = <0.05$). (Ø): virus-free isolates. Comparisons between pairs of isolates are indicated by color of plot and roman numbers (I–VII). Median values and standard error are shown.

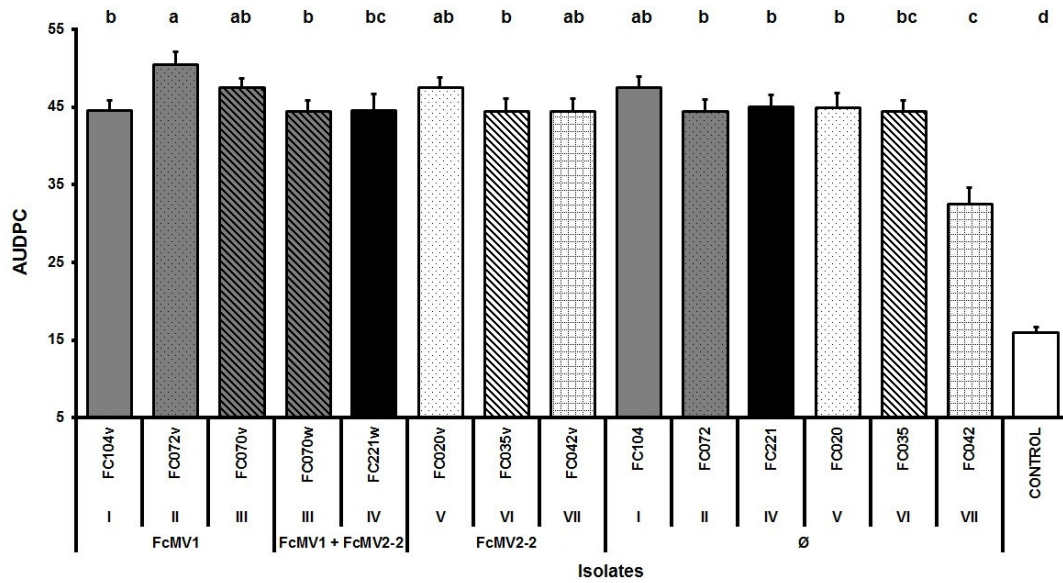


Fig. 4. Area under the disease progress curve (AUDPC) for the different fungal isolates. Small letters (a–d) denote significant differences (Dunn’s test, $P = <0.05$). (∅): virus-free isolates. Comparisons between pairs of isolates are indicated by color of plot and roman numbers (I–VII). Median values and standard error are shown.

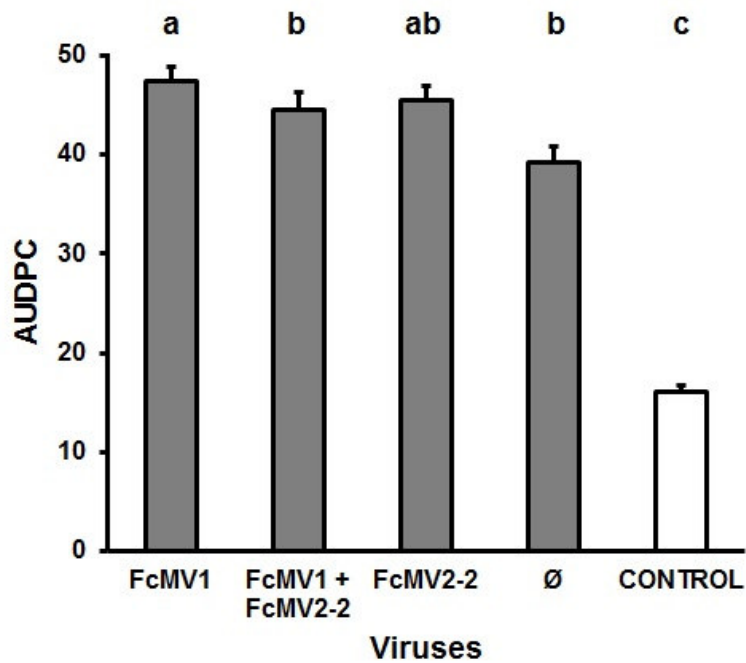


Fig. 5. Area under the disease progress curve (AUDPC) in relation to mycovirus presence. Small letters (a–c) denote significant differences (Dunn’s test, $P = <0.05$). (∅): virus-free isolates. Median values and standard error are shown.

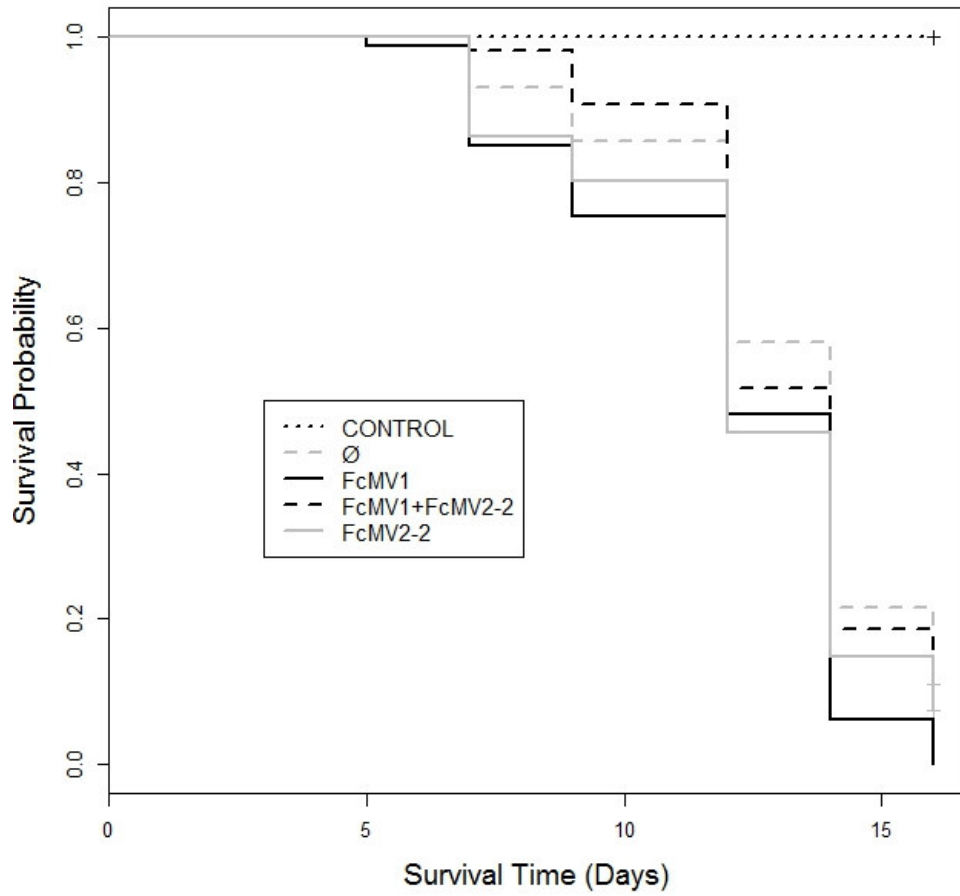


Fig. 6. Plot of survival probability determined using the Kaplan-Meier estimate of the survival function for Monterrey pine (*Pinus radiata*) seedlings infected with *Fusarium circinatum* in relation to mycovirus presence. (Ø): mycovirus-free isolates.