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

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Abstract

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

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

1. Introduction

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belong to the multicopper oxidase group of enzymes and are specialized in catalyzing the oxidation of phenolic substrates by reduction of O_2 to H_2O. Laccases are common in eukaryotes, including fungi, and have been widely studied in the phylum Ascomycota [1]. These enzymes (molecular weight around 60-70 kDa) are usually extracellular and show a high degree of specificity for degrading polyphenol substrates such as lignin [2]. They play an essential role in nutrient turnover (mainly nitrogen and carbon) in nature, due to their capacity to degrade lignocelluloses in forest soil and litter, and they are abundant in saprophytic fungi [3]. Laccases may also play an important role in host colonization by pathogenic fungi as they can damage host tissues, thus favouring fungal infection [4,5]. Additionally, they have important applications in industry (e.g. textile and paper industries) as well as in bioremediation and environmental biotechnology [6].

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

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

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

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

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

2. Material and Methods

2.1. Selection of isolates

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

2.2. Bavendamm test.

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

2.3. Monitoring for mycelial growth.

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

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

2.4. Laccase activity.

The *F. circinatum* isolates were cultured for one week in Bavendamm medium. Three plugs (5x5 mm) comprising mycelia and medium were then removed from the edge of each isolate and transferred to 1.50 ml tubes. Aliquots (1.50 ml) of twice-autoclaved distilled water (4º C) were added to the plug samples to extract crude extracellular laccase. After incubation for thirty minutes at room temperature, the tubes were centrifuged for three minutes at 10^4 g and the supernatant was extracted. The laccase activity was assayed after adding 0.80 ml of 2.50 mM 2,6-dimethoxyphenol (DMP, broad spectrum enzyme substrate) to 0.20 ml of the crude laccase in 100 mM phosphate buffer (pH 6.90) at 37º C [37]. The absorbance of samples was measured at 468 nm and 25º C in a LAN OPTICS (2000-2100) spectrophotometer [38]. Absorbance was measured immediately and five minutes later. Finally, the increase in absorbance was calculated as an absolute value for the measurement period ($\Delta A_{0\text{-}5}$).

2.5. *In vivo* pathogenicity.

To test *in vivo* the ability of each strain to cause disease (pathogenicity), the isolates were inoculated into 405 one-year-old nursery seedlings of Monterrey pine (i.e. 27 replicate seedlings per isolate and 27 control seedlings). A small incision was made two centimeters above the root collar and 10 µl of spore suspension (10^6 spores/ml of distilled water) was inoculated into the wound. In control seedlings, an incision was made in the same way, but distilled water only was inoculated into the wound. The wound was covered with Parafilm® for one week. The treated and control seedlings were held separately in plant growth chambers at 25º C with a 16h photoperiod. The seedlings were watered three times a week throughout the study period, with equal amounts of distilled water.

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

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192 2.6. Statistical analysis.

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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, $\Delta A_{0\text{-}5}$
196 and AUDPC values according to two different factors: isolate (14 strains, Table 1) and
197 mycovirus presence (evaluated as follows: not infected (Ø); 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 $\Delta A_{0\text{-}5}$, (b) mean values of AUDPC and
201 $\Delta A_{0\text{-}5}$ 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>>

3. Results

3.1. Bavendamm test and mycelial growth.

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

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

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

3.2. Laccase activity.

Laccase activity (expressed as $\Delta A_{0\text{-}5}$) differed significantly in relation to the isolate (X^2= 22.54; d.f.= 13; P= 0.04), whereas the presence of the mycovirus did not have a significant effect (X^2= 1.92; d.f.= 3; P= 0.58). Of the fungal isolates infected with mycovirus, FC042v produced the greatest increase in the absorbance, which was significantly different from that produced by the same isolate not infected with the mycovirus, which yielded the lowest absorbance increase (FC042, P= <0.01). Likewise, $\Delta A_{0\text{-}5}$ also differed significantly between FC104 and FC104v (P= 0.01) (Fig. 3) but the correlation between G and $\Delta A_{0\text{-}5}$ was not significantly different (t= -0.88; d.f.= 96; P= 0.37; ρ= -0.09).

<<Insert Figure 3 around here>>

3.3. Pathogenicity *in vivo*.

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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 $\Delta A_{0\text{-}5}$ as average values for each isolate were almost
262 statistically significant (t= 2.13; d.f.= 13; P= 0.05; ρ= 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>>
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4. Discussion

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

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

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

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

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

Extracellular laccase activity did not vary depending on mycovirus presence. Moreover, mycelial growth rate was not related to enzymatic activity. In contrast, laccase activity varied between isolates and seemed to be related to pathogenicity. Enzymatic activity was only lower in isolates FC221, FC020 and FC104 (significantly lower in the case of FC104) when they were infected, supporting the idea that the mycoviruses do not cause hypovirulence [27]. By contrast, a strong reduction in laccase activity (indicated by the Bavendamm test reaction and $\Delta A_{0\text{-}5}$) was observed in *C. parasitica* isolates infected by dsRNA mycovirus in a study in which isolates that did not produce laccase were identified as hypovirulent strains by a complementary pathogenicity test [31]. In a study of laccase production in *Ophiostoma ulmi* (Buisman) Nannf. and *Ophiostoma novo-ulmi* Brasier (causal agent of Dutch Elm Dieback) differences between the two species were observed [4]. Thus, the less aggressive *O. ulmi* showed lower or even null laccase activity than the more pathogenic *O. novo-ulmi* (0-0.20 U ml^{-1} vs 0.12-0.34 U ml^{-1} respectively). In the aforementioned study comparing two strongly related species with different pathogenicity, the authors proposed laccases as a useful tool for overcoming tree defences. Similarly, higher values of enzymatic activity were obtained for virus-free strains of *Diaporthe ambigua* Nitschke ($\Delta A_{0\text{-}5}$ for mycovirus-free strains: 0.11-0.17; $\Delta A_{0\text{-}}$

$_5$ of mycovirus-infected strains: 0.01-0.02) and the Bavendamm test was negative in infected and less virulent strains [37]. Similar conclusions have been reached for *B. cinerea* [29]. However, enhanced laccase activity has also been observed in hypovirulent strains of *B. cinerea*, in a study in which the authors concluded that this enzyme was not important in the virulence of the pathogen [26]. The values obtained in the present study were higher than those reported in previous studies (mean value of $\Delta A_{0\text{-}5}$= 0.17 ± 0.03), suggesting intense extracellular laccase activity and ruling out hypovirulence in the isolates.

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

5. Acknowledgments

E. J. Muñoz-Adalia is in receipt of grants from the European Social Fund and from the Consejería de Educación de Castilla y León (JCyL) (ORDEN EDU/1083/2013). This study was supported by the Ministerio de Economía y Competitividad under Project: AGL2012-39912. Isolates FC104, FC104v, FC221 and FC221w were provided by the Instituto Agroforestal Mediterráneo (Universidad Politécnica de Valencia). The authors thank Milagros de Vallejo and Juan Blanco (Gobierno de Cantabria) for their help in carrying out the study.

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Tables and figures

Tables

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

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

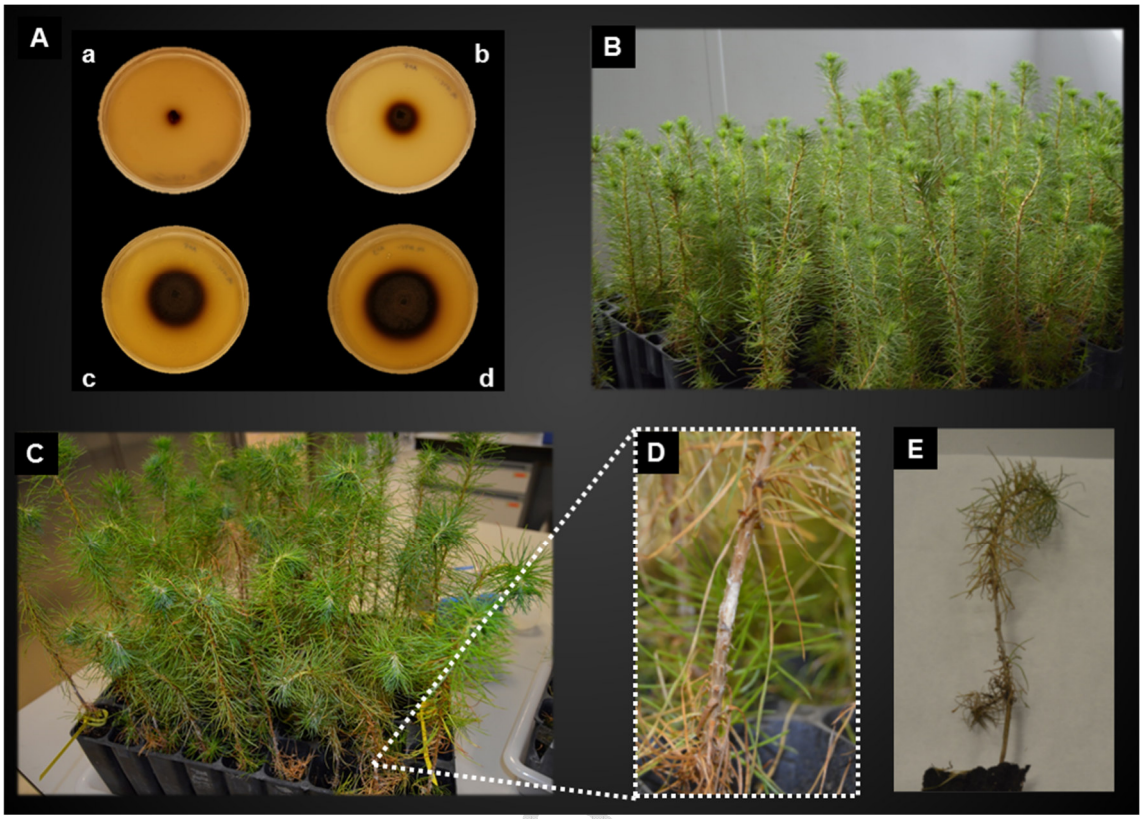

Fig. 1. Scheme of the study. A: Bavendamm test progress at four different moments: 24 h (a), 48 h (b), 72 h (c) and 96 h (d) after culture (isolate shown: Fc072). B: Control *Pinus radiata* seedlings on the 13th day of pathogenicity test. C: *Pinus radiata* seedlings inoculated with Fc072v (foreground) 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.

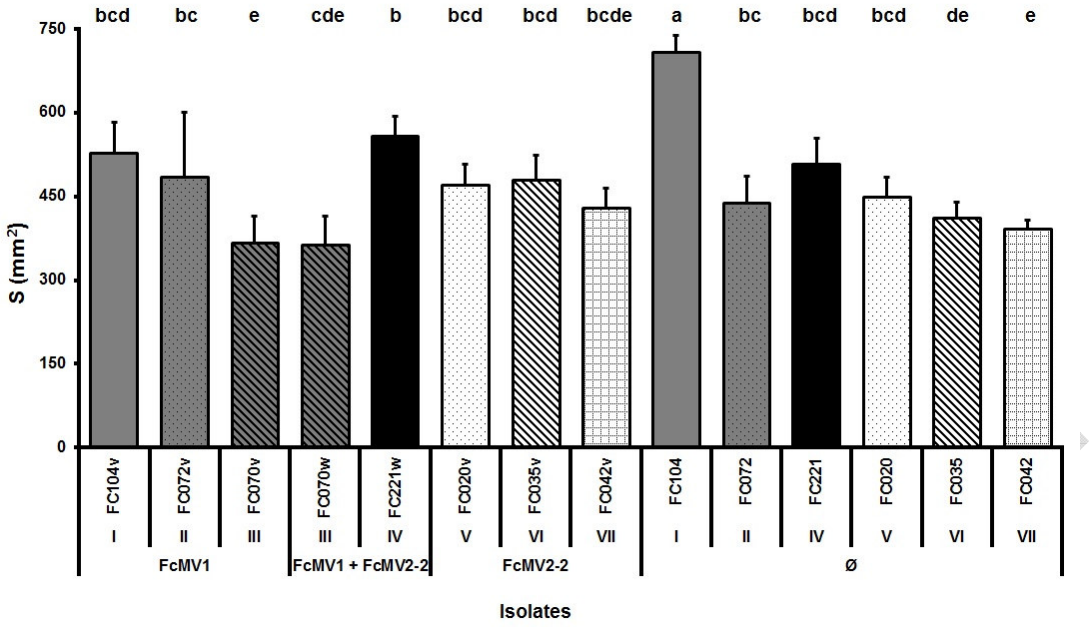

Fig. 2. Area affected by enzymatic reaction during the five days of the assay (S) for each fungal isolate. Small letters (a–e) denote significant differences (Dunn´s test, P= <0.05). (Ø): mycovirus-free isolates. Comparisons between pairs of isolates are indicated by color of plot and roman numbers (I-VII).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

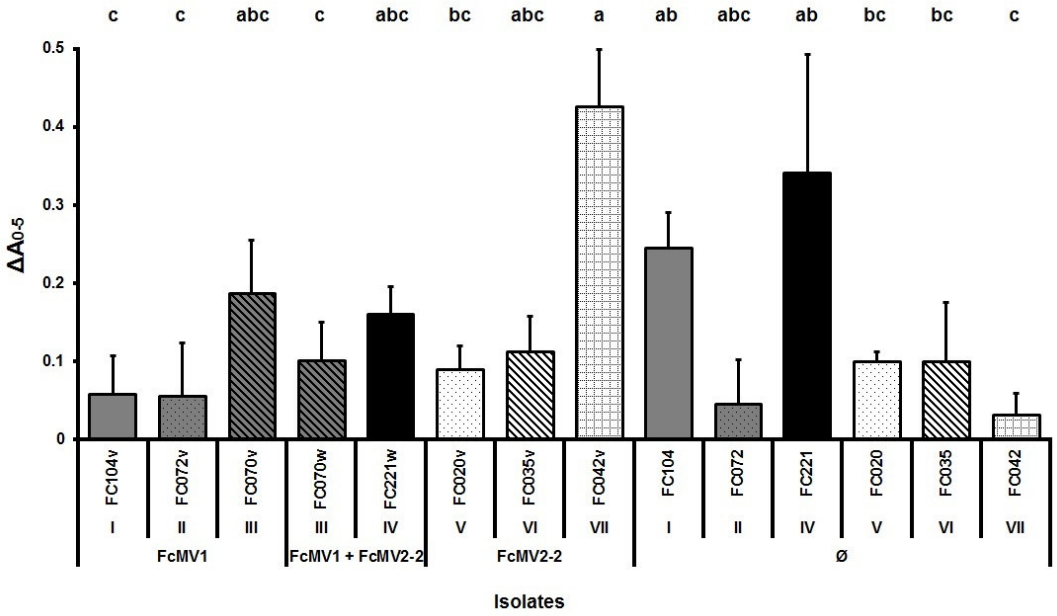

Fig. 3. Extracellular laccase activity ($\Delta A_{0\text{-}5}$) in the different isolates. Small letters (a–c) denote significant differences (Dunn´s test, P= <0.05). (Ø): virus-free isolates. Comparisons between pairs of isolates are indicated by color of plot and roman numbers (I-VII). Median values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

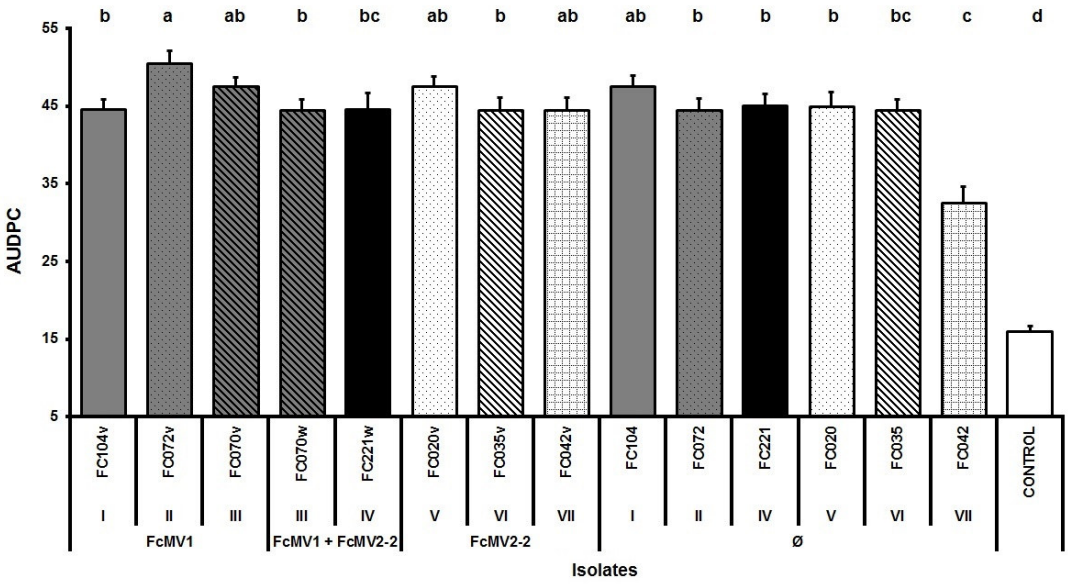

Fig. 4. Area under the disease progress curve (AUDPC) for the different fungal isolates. Small letters (a–d) denote significant differences (Dunn´s test, P= <0.05). (Ø): virus-free isolates. Comparisons between pairs of isolates are indicated by color of plot and roman numbers (I-VII). Median values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

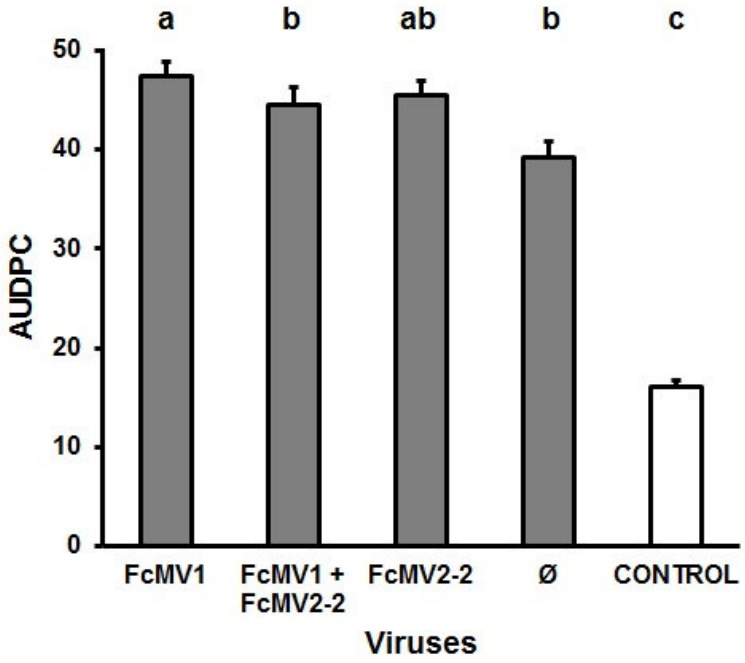

Fig. 5. Area under the disease progress curve (AUDPC) in relation to mycovirus presence. Small letters (a–c) denote significant differences (Dunn´s test, P= <0.05). (Ø): virus-free isolates. Median values and standard error are shown.

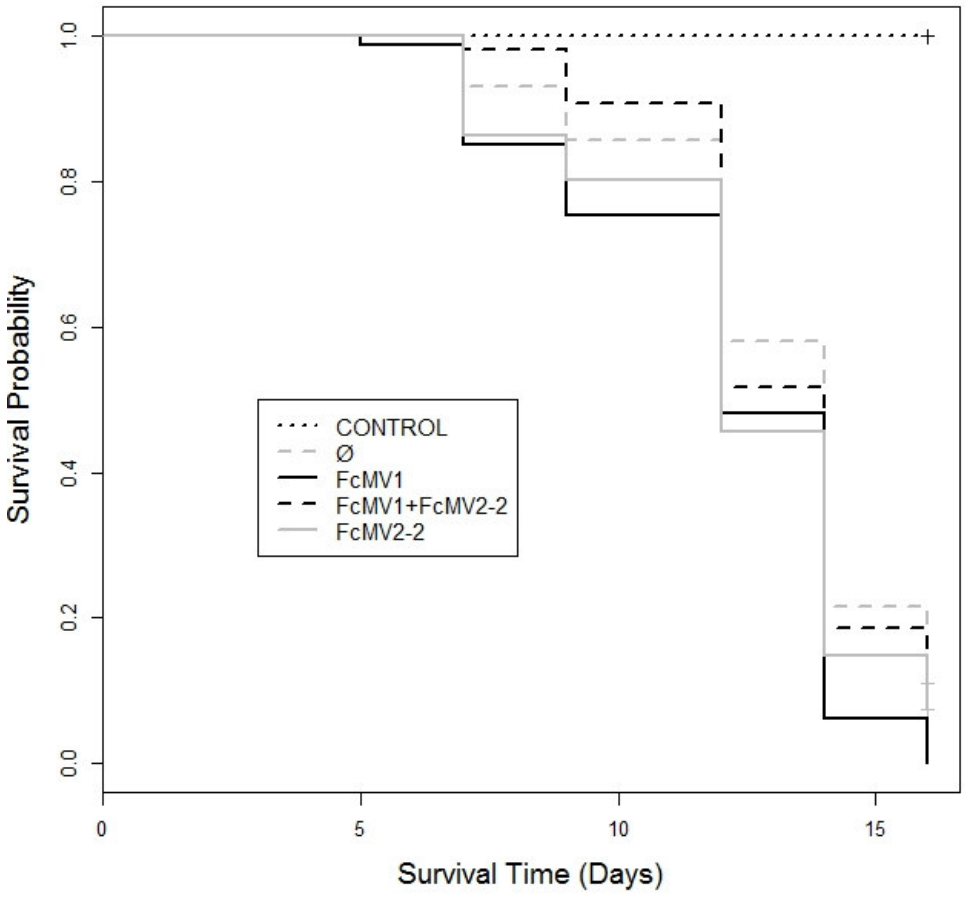

Fig. 6. Plot of survival probability determined using the Kaplan-Meier estimate of the survival function for Monterrey pine (*Pinus radiata*) seedlings infected with *Fusarium circinatum* in relation to mycovirus presence. (Ø): mycovirus-free isolates.