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SHORT COMMUNICATION

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Effect of mycoviruses on growth, spore germination and pathogenicity of the fungus *Fusarium circinatum*

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Abstract

Aim of the study: To assess the impact on two mycoviruses recently described in *F. circinatum* mitovirus 1 and 2-2 (FcMV1 and FcMV2-2) on i) mycelial growth, ii) spore germination and iii) relative necrosis.

Material and methods: Fourteen monosporic strains of *F. circinatum* (one of each pair infected with mycoviruses and the other without them) of the pathogen with and without viruses were selected for the assay. The statistical analysis, were a linear mixed model of analysis of variance considering one between-subjects factor (isolate) and one within-subjects factor with four levels (1=without viruses, 2=only virus FcMV1, 3=only virus FcMV2-2 and 4=both viruses).

Main results: Colony growth rates of *F. circinatum* isolates were significantly reduced in presence of mycoviruses (p=0.002). The spore germination was also reduced in the *F. circinatum* isolates containing mycovirus as compared to mycovirus-free isolates (p<0.001). No significant differences in lesion lengths caused by *F. circinatum* were found in relation to the presence/absence of mycovirus (p<0.61).

Research highlights: Reduction of the percentage of spore germination in the isolates of *F. circinatum* with mycovirus infections, as compared to free isolates, provides indications of reduction of metabolic activity and plant physiology are discussed. The lack of significant differences found in the length of the lesions caused by *F. circinatum* with respect to the presence/absence of mycovirus, indicates that further studies with a larger number of variables are required

Additional keywords: pine pitch canker; hypovirulence; biological control; forest pathology.

Abbreviations used: FcMV (*Fusarium circinatum* Mitovirus); PDB (potato dextrose broth); PPC (pine pitch canker).

Authors´ contributions: Conceived, designed the experiment and performed the experiments: JAFP, EJMA, PMA and JJD. Analyzed the data: VP. Wrote the paper: JMG, JAFP and JJD. All authors reviewed the paper.

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Introduction

Fusarium circinatum (teleomorph *Gibberella circinata* Nirenberg & O'Donnell) is an ascomycetous fungus that causes pine pitch canker (PPC) disease in *Pinus* spp. and other conifers such as *Pseudotsuga menziesii* (Barnard & Blakeslee, 1987; Dwinell *et al.*, 1998). The pathogen was first detected in 1945 in *Pinus virginiana* Mill in the southeastern United States (Hepting & Roth, 1946). Since then, it has continued to expand throughout the world and has been found infecting different conifer species in Mexico (Guerra-Santos, 1998), Haiti (Hepting & Roth, 1953), Japan (Kobayashi & Muramoto, 1989), South Africa (Viljoen *et al.*, 1994, Coutinho *et al.*, 2007), Chile (Wingfield *et al.*, 2002), Korea (Cho & Shin, 2004), Uruguay (Alonso & Bettucci, 2009), Colombia (Steenkamp *et al.*, 2012) and, more recently, in Brazil (Pfenning *et al.*, 2014). In Europe, the pathogen has been also recorded in Spain (Dwinell, 1999; Landeras *et al.*, 2005), France

(EPPO, 2006), Portugal (Bragança *et al.*, 2009) and Italy (Carlucci *et al.*, 2007).

PPC fungus causes severe symptoms in adult trees (*e.g.* wilting and reduced growth) with frequent formation of cankers producing abundant amounts of resin that usually appears on the trunk or thicker branches. The lesions damage pine trees and make them susceptible to windthrow and can even kill the trees by interrupting the sap flow (Hepting & Roth, 1946). The fungus also causes damping off in seedlings, leading to mortality rates of up to 100% (Martinez-Álvarez *et al.*, 2014a). This necrotrophic fungus is thus considered the most important pathogen of pine seedlings on a global scale (Wingfield *et al.*, 2008).

The presence of the pathogen in Spain has caused high economic losses because of the ban on planting susceptible species, as well as the high costs of monitoring the disease and implementing treatments aimed at eradication (BOE, 2006, 2010). However, no effective treatments have been development in the field and efforts have mainly focused on nursery treatments (Gordon *et al.*, 2015). Biological control is emerging as one of the most promising options for limiting the effects of the disease (Sánchez-Fernández *et al.*, 2013).

The use of mycoviruses is considered one of the most promising strategies for biological control of forest diseases (Muñoz-Adalia *et al.*, 2016a). In fact, some mycoviruses can be used as biocontrol agents by promoting hypovirulence in their host, with the best-known case in forest pathology being the chestnut blight caused by *Cryphonectria parasitica* (Carey *et al.*, 2005; Zamora *et al.*, 2012). Mycovirus infections can lead to induction of a cryptic state (asymptomatic), reduction of mycelial growth, change in the colour of the colony or even alteration of the specific structure formation (Rodríguez-García *et al.*, 2014). The effects of mycoviruses on spore production and pathogenesis have also been widely reported (Kazmierczak *et al.*, 1996; Wang *et al.*, 2014, Zamora *et al.*, 2016), indicating the potentially damaging effects of the viruses on plant health. Three different species of the genus *Mitovirus* 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) (Martínez-Álvarez *et al.*, 2014a; Vainio *et al.*, 2015; Muñoz-Adalia *et al.*, 2016a).

The main aim of the study was to evaluate how mycoviruses affect the pathogenicity of *F. circinatum.* For this purpose, we assayed colony growth, spore germination and pathogenicity of isolates on *Pinus radiata* D. Don seedlings.

Material and methods

Fungal isolates

Seven *F. circinatum* isolates were selected for the study. Two paired monosporic cultures (mycovirus-

Table 1. Results of tests of *Fusarium circinatum* isolates (seven isolates, two monosporic cultures/isolate): origin; mycovirus infection (- =absence of mycovirus and + =presence of mycovirus); host (Pp=*Pinus pinaster*, Pr=*Pinus radiata*); MAT: mating-type; spore germination (SG), expressed as a percentage; area of the fungal colony (AFC) in mm^2; relative necrosis length (LRN) in mm.

Isolate[1]	Origin	FcMV1	FcMV2-2	Host	MAT	SG	AFC	LRN
Fc104v	Asturias*	(+)	(-)	Pp*	1*	4.20 ± 0.94	1090.68 ± 183.75	31.03 ± 1.54
Fc104	Asturias*	(-)	(-)	Pp*	1*	13.53 ± 0.94	1608.14 ± 347.37	31.37 ± 1.37
Fc221w	Cantabria	(+)	(+)	Pr	2	5.20 ± 0.94	1461.61 ± 96.68	26.90 ± 1.53
Fc221	Cantabria	(-)	(-)	Pr	2	5.86 ± 0.94	1660.21 ± 303.85	27.29 ± 1.37
Fc020v	Cantabria	(-)	(+)	Pr	2	5.20 ± 0.94	1506.60 ± 477.91	26.79 ± 1.36
Fc020	Cantabria	(-)	(-)	Pr	2	5.20 ± 0.94	1514.91 ± 174.51	26.07 ± 1.37
Fc035v	Cantabria	(-)	(+)	Pr	2	4.86 ± 0.94	962.73 ± 439.77	26.16 ± 1.36
Fc035	Cantabria	(-)	(-)	Pr	2	4.70 ± 0.94	1624.41 ± 179.50	26.07 ± 1.37
Fc042v	Cantabria	(-)	(+)	Pr	2	4.86 ± 0.94	1316.76 ± 239.81	23.94 ± 1.36
Fc042	Cantabria	(-)	(-)	Pr	2	6.53 ± 0.94	1673.33 ± 134.92	24.03 ± 1.37
Fc070v	Cantabria	(+)	(-)	Pr	2	5.53 ± 0.94	1566.74 ± 220.94	26.06 ± 1.54
Fc070w	Cantabria	(+)	(+)	Pr	2	3.03 ± 0.94	956.49 ± 252.60	26.45 ± 1.53
Fc072v	Cantabria	(+)	(-)	Pr	2	2.53 ± 0.94	1677.04 ± 199.26	26.81 ± 1.54
Fc072	Cantabria	(-)	(-)	Pr	2	8.70 ± 0.94	1605.00 ± 293.38	28.14 ± 1.37
FcCa06	Cantabria	(-)	(-)	Pr	2	68.83 ± 1.53	1541.62 ± 101.44	--

[1]v=strain infected with mycovirus; w=co-infection with two strains mycoviruses. *Source of data: Pérez-Sierra *et al*. (2007).

infected and mycovirus-free) were used per isolate (Table 1). The presence of different mycovirus strains in each isolate was reported in a previous study by our research group (Vainio *et al.*, 2015). In the present study, the presence or absence of mycoviruses (FcMV1 and/or FcMV2-2) was confirmed according to Martínez-Álvarez *et al.* (2014b). For in vitro experiments, an isolate (FcCa06) previously confirmed as mycovirus-free and pathogenic (Martínez-Álvarez *et al.*, 2012, 2014a) was also used.

In vitro experiments: mycelial growth and spore germination

Mycelial growth of *F. circinatum* isolates was evaluated on Petri dishes containing PDAS medium (potato dextrose agar and 0.5 mg/L streptomycin). A 4-mm mycelial plug of the pathogen was placed in the centre of the plate. The plates were sealed with Parafilm© and kept in darkness at 25ºC for seven days (Leslie & Summerell, 2006). Seven replicates of each isolate were used in the assays. The growth of the colonies (along 2 perpendicular axes) was evaluated daily, and the total area of growth was calculated by the following equation:

$$\text{Area} = \pi/4\ (D_1 \times D_2),$$

where D= diameter. A sample of 100 µL of conidial suspension of *F. circinatum* isolates was grown in Potato Dextrose Broth (PDB) supplemented with 5 g/L streptomycin sulphate (PDBS) (Martinez-Alvarez *et al.*, 2012). Three flasks were prepared for each isolate. The spores were incubated for 6 hours under stirring at 180 cycles/min at 25° C in darkness and at 60% humidity. Three aliquots (each 10 µL) of the spore suspension were removed from each flask for evaluation of germination (200 conidia in each sample, *i.e.* a total of 1,800 conidia were scored). The spore suspensions were examined under a light microscope (Nikon E600) with a 40x lens. A hemocytometer was used to count the number of germinated spores as the grid helped to avoid counting the same spore more than once.

In vivo experiments: pathogenicity tests

Pathogenicity tests were performed with two-year-old seedlings of *P. radiata* from Galicia, Spain. The plants were raised individually in root trainers (cells of 200 mL) and incubated in a growth chamber at 21º C with a 16-h photoperiod, following a completely randomized design.

Inoculations were carried out by the stem inoculation technique (Martínez-Álvarez *et al.*, 2016). U-shaped wounds were cut into the bark of the seedlings, at 5-7 cm above ground, with a sterile scalpel (Correll *et al.*, 1991). The conidia suspension was obtained from *F. circinatum* isolates cultured for seven days in PDB at 25ºC and darkness. Aliquots (100 µL) of spore suspension (adjusted to 10^6 conidia/mL using a hemocytometer) were inoculated into the wound, which was covered with Parafilm© to prevent drying (Martínez-Álvarez *et al.*, 2014a). Twenty-seven seedlings were inoculated for each isolate tested. Control seedlings (n=27) were prepared in the same way with sterilized distilled water instead of spore suspensions.

The total length of the seedlings, the root collar diameter and the length of necrotic damage were measured in all seedlings. In order to obtain a more accurate view of the extent of the necrosis, the stems were split lengthways with a scalpel. To avoid the effect of the size of the seedlings, the relative necrosis was estimated as the ratio between length of the necrotic lesion and total length of the seedling.

Statistical analysis

Mycelial growth. For the statistical analysis, we used a linear mixed model of analysis of variance with repeated measurements, considering four between-subject factors and one within-subject factor with seven levels. The mathematical formulation of the model was as follows:

$$Y_{in(jkl);t} = \mu + \alpha_j + \beta_{k(j)} + \gamma_{l(j)} + \beta\gamma_{kl(j)} + \\ + \delta_{i(jkl)} + \tau_t + \alpha\tau_{jt} + \beta\tau_{kt(j)} + \gamma\tau_{lt(j)} + \\ + \beta\gamma\tau_{klt(j)} + \delta\tau_{it(jkl)} + \varepsilon_{in(jkl);t}$$

where j=0 (experimental unit with an isolate) or 1 (control without an isolate); k=0 (isolate without virus 1) if j=1 or k=1 (isolate with mycovirus1) if j=0; l=0 (isolate without virus 2) if j=1 or l=1 (isolate with mycovirus 2) if j=0; i=1,..,15 for the isolates with j=k=l=0 if i=1,..,5, j=k=0 and l=1 if i=6,..,9, k=1 and j=l=0 if i=10,..,13, j=0 and k=l=1 if i=14, j=1 and k=l=0 if i=15; n=1,..,7 for the replicates of the experimental units and t=2,..,7 for the repeated measurements (from day 2 to day 7).

Spore germination. For the statistical analysis, we used a linear mixed model of analysis of variance with repeated measurements, considering four between-subject factors and one within-subjects factor with three levels. The mathematical formulation of the model was given as follows:

$$Y_{in(jkl);t} = \mu + \alpha_j + \beta_{k(j)} + \gamma_{l(j)} + \beta\gamma_{kl(j)} + \\ + \delta_{i(jkl)} + \tau_t + \alpha\tau_{jt} + \beta\tau_{kt(j)} + \gamma\tau_{lt(j)} + \\ + \beta\gamma\tau_{klt(j)} + \delta\tau_{it(jkl)} + \varepsilon_{in(jkl);t}$$

where j=0 (experimental unit with an isolate) or 1 (control without an isolate); k=0 (isolate without mycovirus 1) if j=1 or k=1 (isolate with mycovirus 1) if j=0; l=0 (isolate without mycovirus 2) if j=1 or l=1 (isolate with virus 2) if j=0 ; i=1,..,15 for isolates with $j=k=l=0$ if i=1,..,5, $j=k=0$ and $l=1$ if i=6,..,9, k=1 and $j=l=0$ if i=10,..,13, j=0 and $k=l=1$ if i=14, j=1 and $k=l=0$ if i=15; n=1,..,3 for the replicates of the isolates and t=1,2,3 for the repeated measurements (1 for 6 hours, 2 for 12 hours and 3 for 24 hours).

Relative necrosis. For the statistical analysis, we used a linear mixed model of analysis of variance with three between-subject factors. The mathematical formulation of the model was given as follows:

$$Y_{in(kl)} = \mu + \beta_k + \gamma_l + \beta\gamma_{kl} + \delta_{i(kl)} + \varepsilon_{in(kl)}$$

where k=0 (isolate without mycovirus 1) or 1 (isolate with mycoirus1); l=0 (isolate without mycovirus 2) or 1 (isolate with mycovirus 2); i=1,..,14 for the isolates with $k=l=0$ if i=1,..,5, k=0 and l=1 if i=6,..,9, k=1 and l=0 if i=10,..,13, and $k=l=1$ if i=14; n=1,..,27 for the replicates. Finally, we used an individual pairwise t-test for all comparisons between the least square means. All tests were carried out with SAS® 9.4 statistical software (Foundation SAS 9.1.3, 2008).

Results and discussion

Effect of *Fusarium* mycoviruses on colony growth

In vitro growth rates of the *F. circinatum* isolates varied significantly depending on the presence/absence of mycoviruses (F=6.03, p=0.002). In particular, *F. circinatum* isolates infected with micoviruses, both single infections with FcMV1 and FcMV2-2 (t=2.39, p=0.02 and t=3.63, p<0.001, respectively) and co-infection with FcMV1 + FcMV2-2 (t=4.27, p<0.001), yielded lower growth rates than mycovirus-free *F. circinatum* isolates (Fig. 1A). There were no significant differences between mycovirus-infected *F. circinatum* isolates in relation to the type of mycovirus (FcMV1, FcMV2-2 or co-infection) (Fig. 1A). A reduction in the mycelial growth of different pathogens infested with mycoviruses has been reported to be related to hypovirulence in fungi in several studies (Bottacin *et al.*, 1994; Ahn & Lee, 2001; Castro *et al.*, 2003; Robin *et al.*, 2010; Rodríguez-García *et al.*, 2014; Zheng *et al.*, 2014; Yu *et al.*, 2015). Nevertheless, the effect on mycelial growth may also be determined by the species (Vainio *et al.*, 2012; Hyder *et al.*, 2013), isolate (Hunst *et al.*, 1986; Rodríguez-García *et al.*, 2014; Yu *et al.*, 2015) temperature (Vainio *et al.*, 2010; Bryner & Rigling, 2011; Romeralo *et al.*, 2012; Zamora *et al.*, 2016) or the type of mycovirus (*Cryphonectria parasitica* CHV1, mycovirus hypovirulences). These factors, among others, may explain the inconsistencies in the findings of different studies. The conditions of the experiment appear to be related to the effect of the virus, and use of the same stains as those used by Muñoz-Adalia *et al.* (2016b) thus failed to yield any effect of mycoviruses on the mycelial growth of *F. circinatum*.

Effect of *Fusarium* mycoviruses on spore germination

A large reduction in spore germination was observed in the mycovirus-infected isolates of *F. circinatum,* relative to mycovirus-free isolates (F=28.21, p<0.001). Again, mycovirus-infected isolates, both single infections with FcMV1 and FcMV2-2 (t=28.76, p<0.001 and t=13.18, p<0.001, respectively) and co-infection with FcMV1 + FcMV2-2 (t=8.81, p<0.001), yielded lower germination rates than mycovirus-free

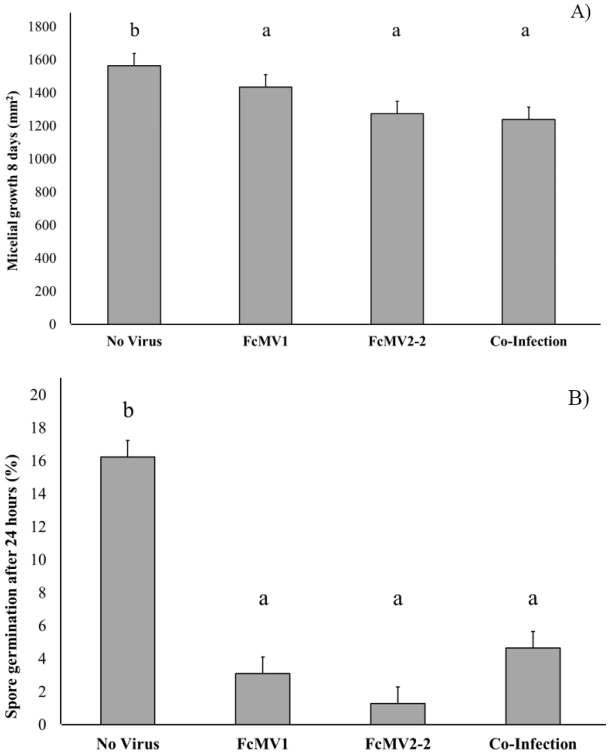

Figure 1. Effect of mycovirus infection on mycelial growth, mm² (A) and on germination, % (B) in *Fusarium circinatum*. Data were analyzed by the restricted maximum likelihood (REML) for fixed effects. Lowercase letters (a-b) denote significant differences (T-Student test, p <0.05). Values shown are means and standard errors.

Table 2. Variation in the length (mm) of the necrotic lesion (%) *Pinus radiata* D. Don, in plants inoculated with isolates of *Fusarium circinatum* infected with mycoviruses (FcMV1, FcMV2-2, and co-infection) relative to healthy control plants inoculated with sterile distilled water. The data were analyzed by the restricted maximum likelihood (REML) for fixed effects.

Group	Relative necrosis (%)
No virus	28.14 ± 1.37 a
Co-infection	26.67 ± 1.53 a
FcMV1	27.96 ± 1.54 a
FcMV2-2	25.62 ± 1.36 a

Lowercase letters (a) denote significant differences (Student's t test, $p<0.05$). Values shown are means and standard errors.

isolates (Fig. 1B). No significant differences were observed in relation to the mycovirus. Some studies have demonstrated that the presence of hypovirulence-causing mycoviruses also reduces sporulation (Kazmierczak *et al.*, 1996, Moleleki *et al.*, 2003; Robin *et al.*, 2010; Zamora *et al.*, 2016). Thus, Ihrmark *et al.* (2004) observed that the frequency of germination of basidiospores was reduced by the presence of mycoviruses. However, to our knowledge no specific research has been carried out to study the effect of mycoviruses on spore germination in ascomycetes.

Effect of *Fusarium* mycoviruses on pathogenicity

The pattern observed in the *in vivo* experiment was not consistent with the findings of the *in vitro* assays (both mycelial growth and spore germination). In fact, no significant differences in the length of lesions caused by *F. circinatum* were found in relation to the presence/absence of mycovirus (F=0.62, $p<0.61$) (Table 2). Although necrosis has traditionally been considered an indicator of the disease progress, because *F. circinatum* kills stem tissue during colonization (Glazebrook *et al.*, 2005), recent research has demonstrated that *F. circinatum* can colonize the plants without causing necrosis, at least at the early stages of infection (J. Martín-García, unpubl. data). Further studies should therefore be carried out at different temperatures and with other techniques, such as fluorescence (Oßwald *et al.*, 2014) and molecular methods (Bodles *et al.*, 2006), to determine the damage caused beyond the visible symptoms.

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