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Effect of putative mitoviruses on *in vitro* growth of *Gremmeniella abietina* isolates under different laboratory conditions

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Abstract

Mitoviruses have been found in several forest pathogens (i.e. *Cryphonectria parasitica, Gremmeniella abietina*), and because they have been shown to reduce the virulence of host fungi there is a growing interest in studying their use as a biocontrol. This study was carried out to test the effect of temperature (5°C, 15°C, 25°C and 35°C), pH (4, 5, 7 and 9) and osmotic potential (–0.6, –1.2, –1.8 and –2.4 MPa) on the mycelial growth of seven *G. abietina* isolates under controlled laboratory conditions. Four of the isolates hosted mitoviruses and three of them did not. During the experiment, mycelial growth was recorded every week for a period of 8 weeks. Results showed no differences in growth behavior between mitovirus infected and non-infected isolates when placed under different pH modifications. However, the mitovirus-infected isolates presented larger mycelial growth than the mitovirus-free ones when at the fungi's optimal growing temperature of 15°C. When growing at certain osmotic potentials (–0.6 and –1.8 MPa) a reduction in growth of the mitovirus-infected isolates was observed. The results of this experiment suggest that mycelial growth among non-infected isolates and isolates naturally infected by mitovirus vary under different culture conditions, thus providing further insight into the effects of mitovirus on *Gremmeniella abietina* isolates.

Key words: mitoviruses; Scleroderris canker; in vitro; biological control; *Gremmeniella abietina*; dsRNA.

Resumen

Efecto de posibles mitovirus en el crecimiento *in vitro* de aislados de *Gremmeniella abietina* bajo diferentes condiciones de laboratorio

Los mitovirus son virus exclusivamente fúngicos que han sido aislados de algunos patógenos forestales (i.e. *Cryphonectria parasitica, Gremmeniella abietina*) y ya que pueden reducir la virulencia del hongo existe un creciente interés por su posible papel como agentes de control biológico. Se ha llevado a cabo un estudio para evaluar el efecto de la temperatura (5°C, 15°C, 25°C y 35°C), el pH (4, 5, 7 y 9) y el potencial osmótico (–0.6, –1.2, –1.8, –2.4 MPa) en el crecimiento micelial de siete aislados de *G. abietina* bajo condiciones controladas de laboratorio. Cuatro de los aislados albergaban mitovirus y tres de ellos no. Durante el experimento, el crecimiento micelial fue registrado semanalmente hasta completar 8 mediciones. Los aislados infectados con mitovirus presentaron mayor crecimiento micelial que los no infectados a la temperatura de crecimiento óptimo del hongo de 15°C. No se observaron efectos de la presencia de mitovirus entre los aislados infectados y los no infectados en los tratamientos de modificación del pH. Cuando se modificaron los potenciales osmóticos se observó una reducción del crecimiento micelial de los aislados infectados con mitovirus en los potenciales osmóticos de –0.6 y –1.8 MPa. Los resultados de este experimento sugieren que la presencia de los mitovirus afecta al crecimiento micelial del hongo bajo distintas condiciones de laboratorio. Este estudio proporciona un conocimiento más profundo de los efectos de las infecciones víricas en aislados españoles de *Gremmeniella abietina*.

Palabras clave: mitovirus; chancro de Scleroderris; *in vitro;* control biológico; *Gremmeniella abietina;* ARNdc.

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Introduction

Gremmeniella abietina (Lagerberg) Morelet (anamorph *Brunchorstia pinea* (P. Karsten) Höhnel) is a pathogenic fungus which has caused destruction in plantations and natural conifer forests in Northern and Central Europe, North America, and Japan (Yokota, 1975; Dorworth, 1979; Kaitera and Jalkanen, 1992) producing symptoms such as stem cankers and shoot dieback (Donaubauer, 1972). This fungus has been divided into three races: European, North American and Asian. Within the European race three biotypes have been determined based on the length of spores, number of septa, disease symptoms, and molecular markers. There is biotype A (LTT, large tree type), biotype B (STT, small tree type) and alpine biotype (Uotila, 1983; Hamelin *et al*., 1993; Hellgren and Hogberg, 1995; Kaitera and Jalkanen, 1996; Hantula and Muller, 1997). In Europe, the fungus mostly affects genera *Picea* spp. and *Pinus* spp. although it has also been found on genera *Abies* and *Larix*. In Spain, it presence on *Pinus pinaster* was first reported in 1929 (Martínez, 1933) and later on *Pinus halepensis* in 1999 (Santamaria *et al*., 2003). Notwithstanding, it has only been isolated from symptomatic *Pinus halepensis* trees. The symptoms observed generally consist of dry needles, branches with some distortion of terminal twigs and eventual dieback or death of the trees (Santamaria *et al.,* 2003). Spanish *G. abietina* is currently recognized as part of the European race (Santamaria *et al*., 2005) and has recently been related to biotype A, although it has a unique genotype (Botella *et al*., 2010).

Mycoviruses, which are obligate parasites of fungi, are widespread in all major taxonomic groups of plant pathogenic fungi (Ghabrial and Suzuki, 2009; Pearson *et al*., 2009). They are transmitted through hyphal anastomosis and/or fungal sporulation (Zhang *et al*., 2010). Fungal viruses differ in their genomes, which can contain DNA, double-stranded (ds) RNA or single-stranded (ss) RNA genomes (Pearson *et al*., 2009). Eight families and one genus are currently described in the International Committee on Taxonomy of Viruses (ICTV) (2011): *Chrysoviridae*, *Endornaviridae*, *Hypoviridae*, *Narnaviridae*, *Barnaviridae*, *Partitiviridae*, *Reoviridae* and *Totiviridae* and genus *Rhizidiovirus* (Hausner *et al.*, 2000; Zhang *et al*., 2010). Mycoviruses usually produce latent infections in nature, affecting sometimes the host's phenotype and/or its growth (Aoki *et al*., 2009). Symptoms produced by the presence of mycoviruses may vary from zero to severe effects on host physiology and may lead to attenuation (hypovirulence) or enhancement of fungal virulence (hypervirulence) (Ghabrial and Suzuki, 2009). Because some viruses are capable of reducing virulence of fungal pathogens they can potentially be used for control of fungal diseases (McCabe *et al*., 1999; Boland 2004; Zhang *et al*., 2010). However, they must fulfill two requirements in order to be suitable for biological control: firstly, have the ability to decrease the fitness of the pathogenic fungus and secondly, transmit the dsRNA efficiently enough to be maintained in a large proportion of the pathogen population (McCabe *et al*., 1999).

Members of genus *Mitovirus* are only found in fungi and belong to the family *Narnaviridae* (Ghabrial and Suzuki, 2009). They lack true virions, and have a (+) ssRNA genome of approximately 2.5 kb (Boland, 2004). Mitoviruses have been recorded in several phytopathogenic fungi such as *Cryphonectria parasitica* (Polashock and Hillman, 1994; Polashock *et al.*, 1997), *Ophiostoma novo-ulmi* (Brasier, 1983; Rogers *et al*., 1987), *Sclerotina homoeocarpa* (Deng *et al.*, 2003; Deng and Boland, 2004), *Helicobasidium mompa* (Osaki *et al.*, 2005), *Chalara elegans* (Park *et al.*, 2006) and *Botrytis cinerea* (Castro *et al.,* 2003; Wu *et al.*, 2007). In most cases, the presence of mitoviruses is associated with reduction of fungal pathogenicity (Ghabrial and Suzuki, 2009; Wu *et al.*, 2010). Members of the genus *Mitovirus* have also been isolated in *G. abietina* (Tuomivirta and Hantula, 2003) which, in the Spanish population, has recently been discovered to host (Botella *et al.*, 2012a).

Reduction of virulence could be related, among other reasons, to anomalous mycelial growth in the fungal pathogen caused by mitoviruses (Ghabrial and Suzuki, 2009; Pearson *et al.* 2009). However mycelial growth is also influenced by environmental and cellular conditions such as temperature, pH and osmotic potential. Temperature limits mycelial growth and production of fruiting bodies in most fungi while the pH determines availability of elements such as nitrogen, calcium and magnesium among others (Carlile *et al.*, 2001). Osmotic potential has also been identified as an important parameter in the ecology and growth of phytopathogenic fungi (Davis *et al.*, 2000). For example, a decrease in the potential produces a reduction in fungal growth due to the subsequent energy increase needed to maintain the swelling of the hyphal cells (Lira-Méndez and Mayek-Pérez, 2006). In general, the effect of the mitovirus could be combined with the effects of these environmental parameters and therefore modify fungal behaviour.

Although some strains of *G. abietina* have been shown to host dsRNA mycoviruses, the effect these agents have on the virulence of this problematic phytopathogenic fungus has not yet been investigated. Accordingly, the main objective of the present study has been to evaluate the effect of the occurrence of viral dsRNA molecules (the replicative form of *Mitovirus)* on the *in vitro* mycelial growth of *G. abietina* isolates under different temperature, pH and osmotic potential conditions.

Materials and Methods

Fungal material

To develop this study seven Spanish isolates of *G. abietina* were chosen: four isolates were naturally infected by putative mitoviral molecules (P3-12, 00P-07, Hon 3-3 and P1-12) and three were not (Hon 9-2; P1-8 and VAI-13) (Botella *et al.*, 2010). All isolates were selected based on previous studies developed in our laboratory in which RT-PCR and sequencing techniques confirmed the presence or absence of mitoviruses (Botella *et al.*, 2012a, 2012b). The isolates were previously stored in 15% glycerol at –80º C and were reactivated on modified orange serum agar medium (MOS-agar; Müller *et al.*, 1994) before performing the experiment. Thus, four weeks before the experiment fungi isolates were sub-cultured in MOS medium and kept in the dark at 15°C in order to obtain sufficient amounts of mycelium.

Mycelial growth

At the bottom of every Petri dish containing 20 ml MOS medium two perpendicular lines were drawn, and a 1mm squared piece of mycelium from each isolate was placed over the intersection of both lines. Mycelial growth was measured weekly for a period of 8 weeks. The response variable was the growth area calculated by the following formula: Area = $\pi/4$ ($d_1 \times d_2$) where d_1 and d_2 were the two diameters measured along the lines.

Culture conditions for monitoring mycelium growth

The effect of mitovirus infection on mycelial growth under different laboratory conditions was the main focus of this study. Three experiments were conducted, each taking into account a separate factor: changes in temperature, pH or osmotic potential. Within each experiment four variations were tested: four temperatures (5°C, 15°C, 25°C and 35°C), four pH values (4, 5, 7 and 9) and four osmotic potentials (–0.6, –1.2, –1.8 and –2.4 MPa). The effect of temperature on mycelial growth was investigated by placing Petri dishes in several stoves at 5°C, 15°C, 25°C and 35°C. To examine the effect of pH, HCl or KOH 1N was added to MOS medium until the pH required was reached. All these Petri dishes were placed in the dark at 15°C since it is the optimal temperature for fungal development (Santamaria *et al.,* 2004). Finally, in order to evaluate the effect of different osmotic potential on mycelial growth, different concentrations of KCl (250, 500, 750 and 1000mM) were added to MOS medium in order to reach the osmotic potential (ψ_π) values of –0.6 MPa, –1.2 MPa, –1.8 MPa and –2.4 MPa (Lira-Méndez and Mayek-Pérez, 2006). Petri dishes were incubated at 15°C ± 1°C in continuous darkness. Four repetitions of each combination "isolate × treatment" were completed.

DsRNA extractions

Fungal mycelium of mitovirus-infected isolates from significative treatments was incubated in MOS medium covered with cellophane for two weeks. Mycelia were first freeze-dried and then ground for 20 minutes into a fine powder. DsRNA was extracted following a modified version of the protocol described by Morris and Dodds (1979). The dsRNA presence in every isolate was verified by electrophoresis. Samples were loaded in a 1% agarose gel, which contained 1x TAE buffer and GelRed™ 10,000X. The test was run in a 1x TAE buffer during 60 min at 90V/30 cm, and immediately afterwards observed under UV light and photographed. The marker used to estimate the lengths of the dsRNA molecules was λ-DNA Hind III – ΦX174Hae III (DyNAzyme™).

Statistical analysis

All statistical analyses were done with SAS program (SAS Institute Inc., 2004). The response variable in all models was growth area (mm^2). A repeated-measures ANOVA for every treatment was calculated by means of Repeated Procedure by SAS to test the effect of the time on the mycelial growth of the isolates. In this case,

the growth areas of every week were used as responses variables. Furthermore, for every experiment (temperature, pH and osmotic potential) a model was calculated to evaluate the effect of the putative presence of mitovirus (yes/no), the treatments (4) and their interactions by a two-way analysis of variance. A significance of 95% was taken in all of the analyses. A Tukey HSD test was used on means of factors when significant differences were found in the ANOVA model. Before the analyses were performed, normality, linearity and homocedasticity for the residuals were probed with Shapiro-Wilk test and graphical procedures.

Results

Effect of temperature

A significant effect of time on the colony growth area ($p < 0.001$) was observed. Although the interaction between time and mitovirus presence was significant ($p = 0.017$) as well as the interaction between time and temperature ($p < 0.001$), only the effect of temperature and mitovirus presence on mycelial growth at the end of the experiment (eight weeks after plating) is shown in Table 1. The average growth from mitovirus-infected and mitovirus-free isolates is shown in Figure 1 at 5°C (A), 15°C (B), 25°C (C) and 35°C (D) throughout the eight weeks. Growth at 25°C was minimal and there was no growth at 35°C. Mean growth area was significantly different among mitovirus-infected and mitovirus-free isolates ($p = 0.0030$), temperatures ($p < 0.001$) and their interactions ($p < 0.001$). According to the Tukey test, the largest colony areas were found at 15°C whereas the smallest were found at 35°C. The overall mean colony size of mitoviruses-infected isolates was significantly bigger than that of the mitoviruses-free ones. When temperatures were considered separately, significant differences among mitoviruses-infected and mitoviruses-free isolates were found only at 15°C ($p = 0.0043$), the temperature that produced the most growth.

Effect of pH

A significant effect of time was observed on the growth area in the pH experiment ($p < 0.001$) and in its interaction with the pH treatments ($p < 0.001$) but not in the mitovirus presence ($p = 0.7265$). Average growth from mitovirus-infected and mitovirus-free isolates is shown in Figure 2 at pH 4 (A), pH 5 (B), pH 7 (C) and pH 9 (D) throughout the eight weeks. Only the data from the effect of pH values on mycelial growth at the end of the experiment (week 8) is shown in Table 2. The growth area was affected by the pH value ($p < 0.001$), but it was neither affected by the mitovirus presence ($p = 0.9459$) nor their interaction ($p = 0.2753$). The largest mycelial growth for all samples was observed at pH 4 while the smallest was shown at pH 9. No differences were shown between mitovirus-infected and mitovirus-free isolates in any pH treatment.

Effect of osmotic potential (ψ_π)

In this experiment, time also affected the growth ($p < 0.001$) and interacted as well with the osmotic potential ($p < 0.001$) and the mitovirus presence ($p < 0.0447$). Data taken in the eight week showed the greatest differences (Table 3). The average growth of mitovirus-infected and mitovirus-free isolates is shown in Figure 3 at –0.6MPa (A), –1.2MPa (B), –1.8MPa

Table 1. Mycelial growth (mm²) after 8 weeks at different temperatures. Mean value ± standard error (SE). Treatments tagged with * presented significant differences among isolates

Mitovirus[1]	Temperature				Total[2]
	5°C	15°C*	25°C	35°C	
Infected	2.41 ± 0.16 a[3] B[4]	12.08 ± 0.51 a A	0.254 ± 0.50 a B	0.196 ± 0.00 a B	3.73 ± 0.25 a
Mitovirus-free	2.51 ± 0.19 a B	7.30 ± 0.59 b A	0.223 ± 0.59 a C	0.196 ± 0.00 a C	2.56 ± 0.29 b
Total[5]	2.45 ± 0.63 B	10.02 ± 0.38 A	0.249 ± 0.39 C	0.196 ± 0.00 C	

[1] If the isolate was naturally-infected with mitovirus. [2] Average growth when combinig all the temperatures together. [3] Different letters in the same column show values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test). [4] Different letters in the same row show values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test). [5] Average growth when combinig all the isolates together.

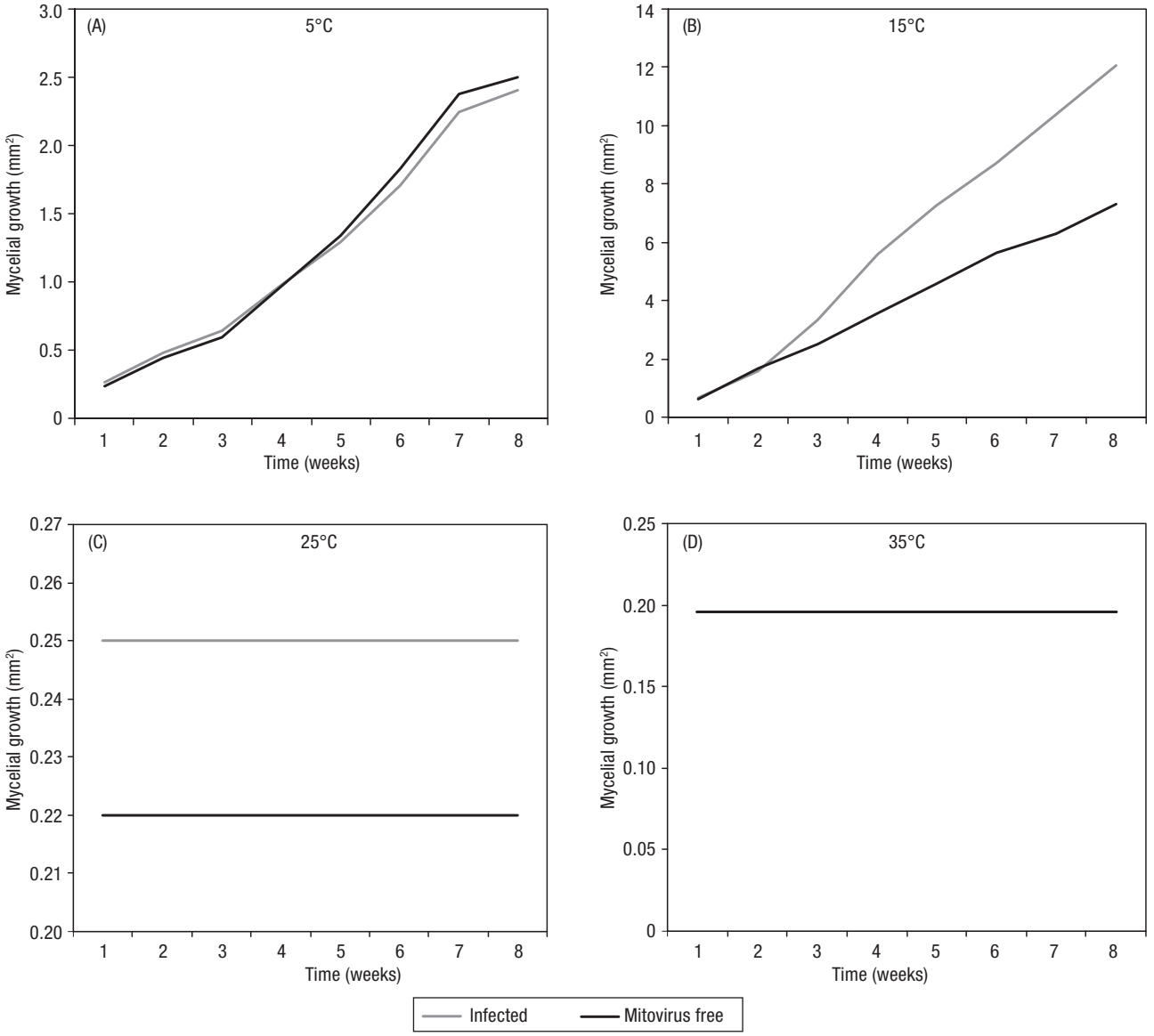

Figure 1. Average growth from mitovirus-infected and mitovirus-free isolates at 5°C (A), 15°C (B), 25°C (C) and 35°C (D) over the eight weeks.

Table 2. Mycelial growth (mm²) after 8 weeks at different pHs. Mean value ± standard error (SE)

Mitovirus[1]	pH value				Total[2]
	pH 4	pH 5	pH 7	pH 9	
Infected	17.28 ± 1.84 a[3] A[4]	9.96 ± 0.97 a B	9.75 ± 0.88 a B	6.62 ± 0.43 a B	10.90 ± 0.58 a
Mitovirus-free	20.25 ± 2.13 a A	8.92 ± 1.12 a B	8.23 ± 1.02 a B	6.45 ± 0.50 a B	10.96 ± 0.66 a
Total[5]	18.55 ± 0.88 A	9.51 ± 0.88 B	9.10 ± 0.88 B	6.55 ± 0.88 B	

[1] If the isolate was naturally-infected with mitovirus. [2] Average growth when combinig all the pH values together. [3] Different letters in the same column show values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test). [4] Different letters in the same row show values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test). [5] Average growth when combinig all the isolates together.

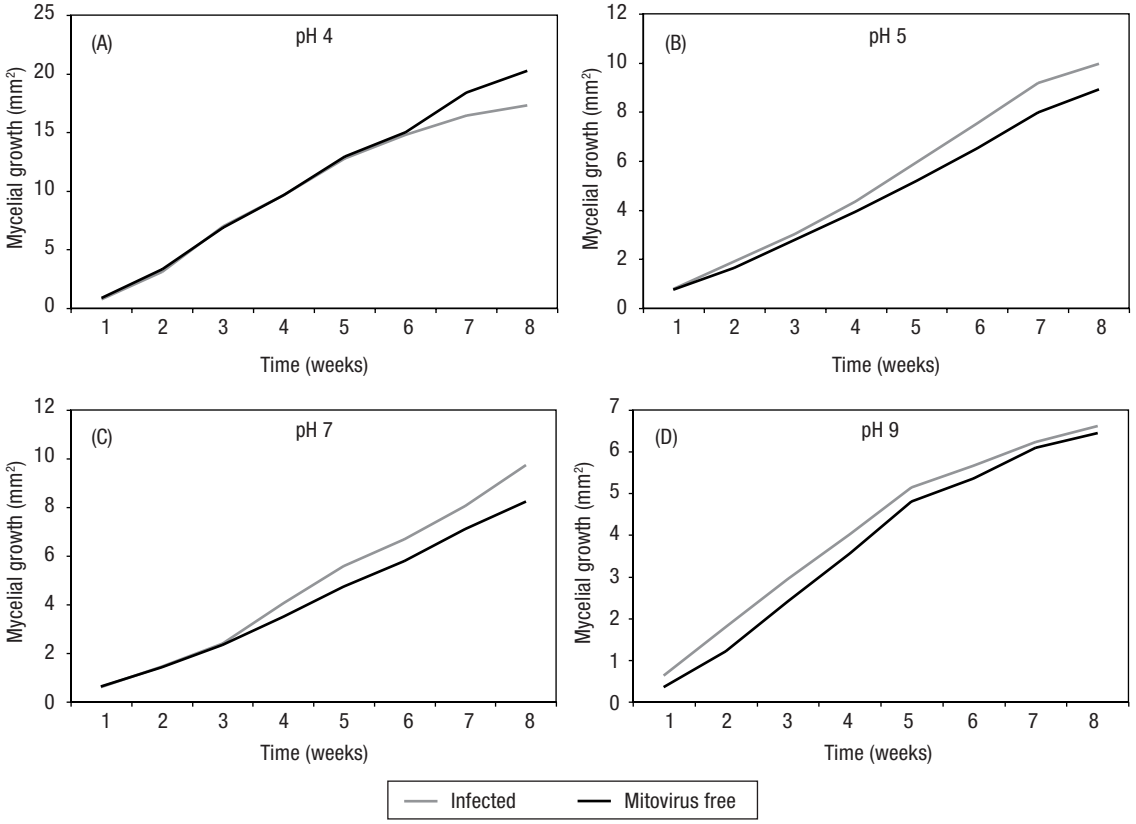

Figure 2. Average growth from mitovirus-infected and mitovirus-free isolates at pH 4 (A), pH 5 (B), pH 7 (C) and pH 9 (D) over the eight weeks.

(C) and –2.4MPa (D) throughout the eight weeks. The model was significative ($p = 0.027$) although it was not the mitovirus presence ($p = 0.1378$) nor osmotic potential ($p = 0.0805$), but the interaction was significative ($p = 0.0034$), that is, the effect of mitovirus presence was different among the different osmotic potentials. When osmotic potential was considered separately at ψ_π of –0.6 MPa ($p = 0.0167$) and at –1.8 MPa ($p = 0.0387$), mitovirus-free isolates presented a higher mycelial growth than the mitovirus-infected ones which did not happen at the osmotic potentials of –1.2 MPa ($p = 0.7515$) and –2.4 MPa ($p = 0.1004$).

DsRNA banding patterns

The presence of the different putative mitoviruses was confirmed by dsRNA extraction and gel electrophoresis after significant treatments were carried out

Table 3. Mycelial growth (mm²) after 8 weeks at different osmotic potentials. Mean value ± standard error (SE). Treatments tagged with * presented significant differences among isolates

Mitovirus[1]	pH value				Total[2]
	–0.6 MPa*	–1.2 MPa	–1.8 MPa*	–2.4 MPa	
Infected	7.66 ± 0.79 b[3] AB[4]	9.24 ± 0.49 a AB	6.47 ± 0.71 b B	9.96 ± 0.77 a A	8.33 ± 0.35 a
Mitovirus-free	10.74 ± 0.80 a A	9.00 ± 0.56 a AB	8.82 ± 0.82 a AB	7.96 ± 0.89 a B	9.13 ± 0.40 a
Total[5]	8.98 ± 0.53 A	9.14 ± 0.53 A	7.48 ± 0.53 A	9.10 ± 0.53 A	

[1] If the isolate was naturally-infected with mitovirus. [2] Average growth when combinig all the osmotic potentials together. [3] Different letters in the same column show values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test). [4] Different letters in the same row show values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test). [5] Average growth when combinig all the isolates together.

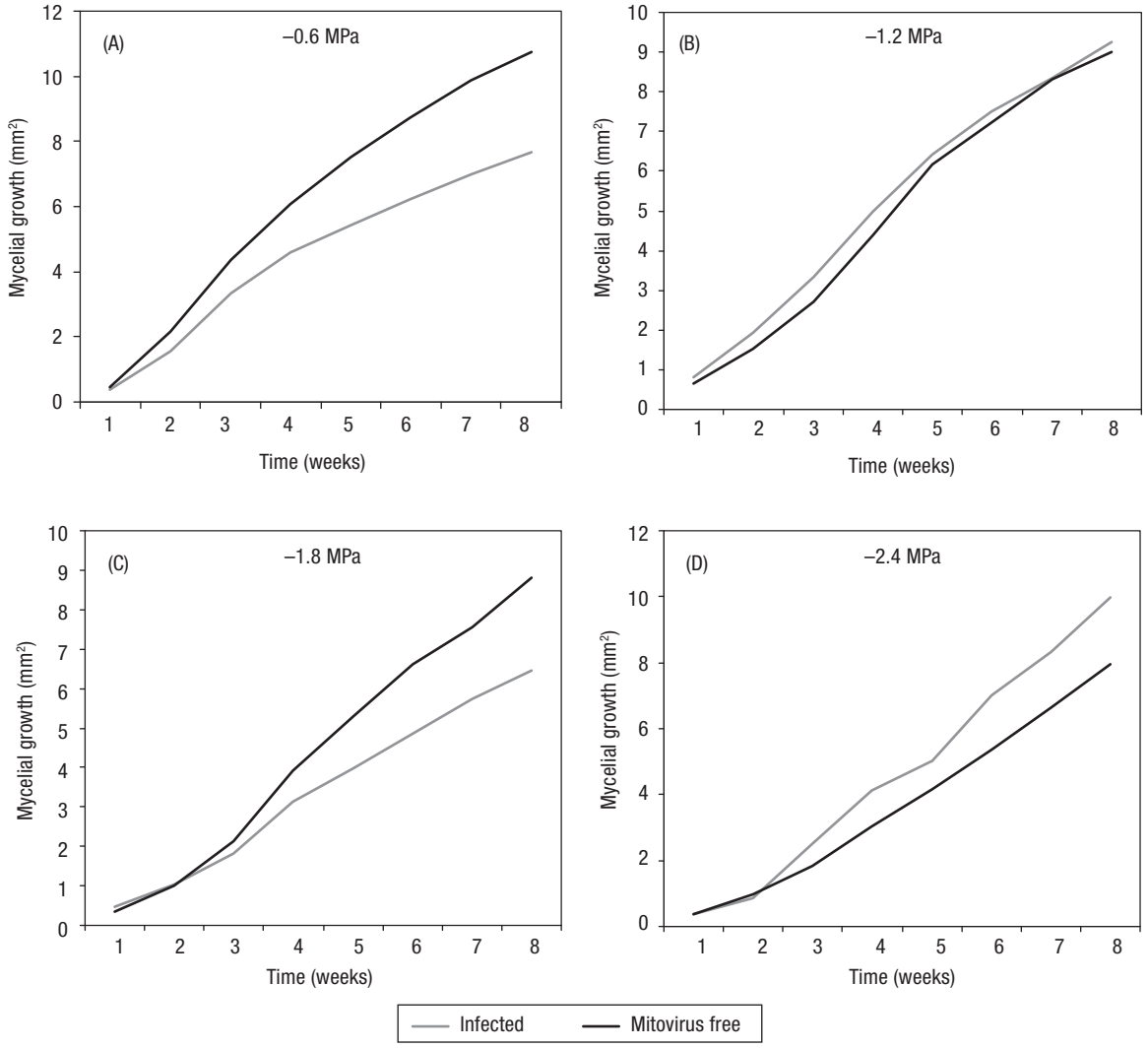

Figure 3. Average growth from mitovirus-infected and mitovirus-free isolates at –0.6 MPa (A), –1.2 MPa (B), –1.8 MPa (C) and –2.4 MPa (D) over the eight weeks.

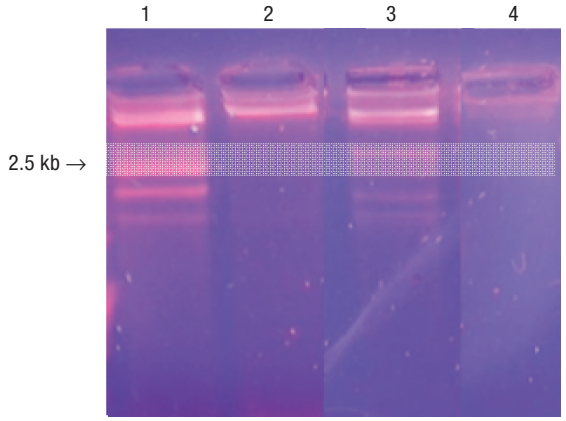

Figure 4. A GelRed-stained 1% agarose gel showing the dsRNA banding patterns. Lane 1, P3-12 (–0.6MPa); lane 2, Hon 3-3 (–0.6MPa); lane 3, P3-12 (–2.4MPa); lane 4, Hon 3-3 (–1.8MPa).

(Figure 4). Isolate P3-12 was found to maintain a 2.5 kb band despite receiving the treatments of ψ_π –0.6 MPa and –2.4 MPa. Conversely, the 2.5 kb band was not sustained in isolate Hon3-3 after treatments of ψ_π –0.6MPa and –1.8 MPa. These results suggested that putative mitovirus occurrence is not affected equally by similar osmotic potential and therefore KCL concentrations. In addition, dsRNA bands that appeared in P3-12 suggested the occurrence of other putative mycoviruses, which did not seem to be affected by the different treatments either. According to the size of the bands and the previous work developed in the laboratory (Botella *et al.,* 2010) they possibly belonged to genera *Totivirus* (*ca* 6kb) and *Partitivirus* (three bands of *ca* 1-2 kb).

Discussion

Mycelial growth depends on the temperature of the environment. In our study, all the isolates showed an optimal growth at 15°C, which was in accordance with Santamaría *et al.* (2004) who demonstrated that Spanish isolates of *G. abietina* had the best growth at this particular temperature. Furthermore, the presence of mitovirus seemed to have a significant effect on *G. abietina* isolates at its optimal growing temperature of 15°C because the isolates with mitovirus present had higher mycelial growth than isolates without mitoviruses. This increase in the mycelial growth of our isolates could be related to a higher virulence of the pathogen since, in general terms, a suppression of mycelial growth has been reported to be closely associated with hypovirulence of fungi (Ghabrial *et al.*, 2009; Pearson *et al.,* 2009) although it could also be related to other factors (e.g., poor sporulation).

Heat tolerance was previously observed in several fungi among virus-infected and virus-free isolates (Marquez *et al.*, 2007; Herrero *et al.,* 2011) but in our study neither mitovirus-infected nor mitovirus-free isolates were able to endure the heat (few isolates hardly grew at 25°C and no growth was observed at 35°C). Marquez *et al.* (2007) observed that plants inoculated with the virus-infected wild type isolate of *Curvularia protuberata* R.R. Nelson and Hodges, with presence of the virus named CThTV, tolerated soils temperatures as high as 65°C for two weeks whereas plants inoculated with the virus-free isolate of the fungus dried-up and became chlorotic. Light evidence of heat tolerance was also observed in *Tolypocladium cylindrosporum* W. Gams due to the different behaviors displayed between virus-infected and virus-free isolates at 30°C (Herrero *et al.,* 2011).

The pH value determines the availability of elements such as nitrogen, calcium and magnesium, among others, taken up by the fungus. In other fungi the effects of viruses have been shown to undergo variations when the composition of substrates, and therefore the availability of elements, differ. Nevertheless, Van Diepeningen (2006) observed that abundance of available nutrients in rich medium could mask viral effects on *Aspergillus* isolates. In our study no statistical differences were shown between mitovirus-infected and mitovirus-free strain growth under any treatment variation. Fungal cellular activity measured by means of growth and metabolism rates tend to decrease if the fungi are grown at different pH values from their optimal (Perez *et al.,* 2000). According to our results, highest mycelial growths of all the isolates were observed at the initial pH 4, which is consistent with the general statement that most fungi will grow properly over a broad pH range on the acidic side of neutrality, i.e., pH from 4 to 7 (Carlile *et al.*, 2001). Nevertheless, it is known that several species of isolates are able to modify the initial pH of the media in order to stabilize the acidity or alkalinity of the substrate (Carlile *et al.*, 2001; Vazquez Garcia *et al.,* 2002).

In our study, there wasn't any clear evidence that a decrease in osmotic potential produced a reduction of mycelial growth as previously observed in other fungal species (Imolehin *et al.*, 1980; Lira-Mendez and Mayek-Perez, 2006; Palmero *et al.,* 2008; Armengol *et al.,* 2011). A reduction of the growth of the mitovirus-infected isolates was observed at –0.6 MPa and –1.8 MPa which can be linked to a decrease in the virulence of the isolates. Changes in behavior were also observed in isolates with and without viral infection when growing at certain osmotic potentials for *Monosporascus cannonballus* (Armengol *et al.,* 2011).

Plant pathologists have been interested for a long time in mycoviruses (and among them, the mitoviruses) because of their potential use as biological control agents (Pearson *et al.* 2009). Although many viruses produce no obvious phenotypic changes, it is reasonable to assume that many virus infections will have some effect on growth (McCabe *et al.* 1999). The results from this experiment suggest that the presence of mitoviruses affects mycelial growth under different culture conditions as previously observed (Vainio *et al.* 2010). Nevertheless, the differences in growth among isolates may be also having been due to a genetic influence (Zharare *et al.*, 2010) a possibility not tested here due to not working with genetically similar strains. Previous studies have shown that viruses found in many fungi, e.g., *Cryphonectria parasitica, Fusarium graminearum* or *Botrytis cinerea,* produce several phenotypic changes such as reduction in growth and sporulation of the fungal strains they infect (Chu *et al*., 2002; Boland, 2004; Van Diepeningen *et al*., 2006; Robin *et al*., 2010; Wu *et al*., 2010; Zhang *et al*., 2010). In our study, the isolates growing at osmotic potential medium of –0.6 MPa and –1.8 MPa also showed a reduction of the mycelial growth. However, in the virus-infected *Fusarium oxysporum* strains when growing on PDA only slight morphological alterations were evident (Lee *et al.,* 2011). Furthermore, it has been observed that

several *Cryphonectria parasitica* virus-infected strains grow as well as virus free isolates on most artificial media although they are incapable of producing grilling cankers on chestnut trees and sporulate poorly (McCabe *et al.* 1999). In other cases, the presence of dsRNAs did not cause any fungal specific symptoms, such as reduced mycelial growth (Aoki *et al.,* 2009). In some *Alternaria* spp. species there was no correlation between the radial growth of isolates and the presence of the dsRNAs (Zabalgogeazcoa, 1998). In contrast to hypovirulent interactions, there is evidence that some mycoviruses are beneficial to their hosts. Tan *et al.,* (2007) observed statistically significant differences in *in vitro* growth rates of virus-infected versus uninfected isolates, with the infected cultures growing more rapidly. In our results, an increase of the mycelial growth was observed at treatment 15°C, the optimal growing conditions of *G. abietina*.

This study provides additional knowledge on the effects of mitovirus infection on *G. abietina* isolates. However, further research including other virulence-associated parameters such as sporulation rates and *in vivo* virulence are recommended to establish an association between mycovirus infection and fungal virulence in Spanish *G. abietina* isolates. The development of a biocontrol protocol may create opportunities for biological control of this disease.

Conclusions

In our study mycelial growth depended on the treatment and the presence of mitoviruses. The presence of mitoviruses did not reduce mycelial growth of *Gremmeniella abietina* at its optimal growing temperature of 15°C. No effects of the occurrence of mitoviruses were shown among the mitovirus-infected and the mitovirus-free ones at any pH value. When growing at certain osmotic potentials (–0.6 and –1.8 MPa) a reduction in the growth of the mitovirus-infected isolates compared to the mitovirus-free ones was observed. Further research including other virulence-associated parameters is recommended.

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