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Effect of putative mitoviruses on growth of *Gremmeniella abietina* isolates *in vitro* and on its pathogenicity on *Pinus halepensis* seedlings

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Efecto de posibles mitovirus sobre el crecimiento de aislados de *Gremmeniella abietina in vitro* y en su patogenicidad en plántulas de *Pinus halepensis*

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Abstract

Mitoviruses have been found in several forest pathogens (e.g. *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 ºC, 15 ºC, 25 ºC and 35 ºC), 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 and to observe the effect of the presence of mitoviruses in the pathogenity of *G. abietina* isolates inoculated to *Pinus halepensis* seedlings. Four of the isolates hosted mitoviruses and three of them did not. During the *in vitro* experiment, mycelial growth was recorded every week for a period of 8 weeks. In the greenhouse experiment, once the seedlings started to show symptoms, disease severity was recorded during 5 weeks. At the end of the experiment, plants were carried to laboratory and necrosis length was measured in all of them. Results from *in vitro* experiment showed that the mitovirus-infected isolates presented larger mycelial growth than the mitovirus-free ones when at the fungi's optimal growing temperature of 15 ºC. However, no differences in growth behavior were observed between mitovirus infected and non-infected isolates when placed under different pH modifications. When growing at certain osmotic potentials (-0.6 and -1.8 MPa) a reduction in growth of the mitovirus-infected isolates was observed. In the greenhouse experiment, larger necrosis lengths were observed in the plants inoculated with mitovirus infected isolates. The results of this experiment provide further insight into the effects of mitovirus on *Gremmeniella abietina* isolates.

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

Resumen

Los mitovirus son virus exclusivamente fúngicos que han sido aislados de algunos patógenos forestales (e.g. *Cryphonectria parasitica, Gremmeniella abietina)* y puesto 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 ºC, 15 ºC, 25 ºC y 35 ºC), el pH (4, 5, 7 y 9) y el potencial osmótico (-0.6, -1.2, -1.8 y -2.4 MPa) en el crecimiento micelial de siete aislados de *G. abietina* bajo condiciones controladas de laboratorio y para observar el efecto de la presencia de mitovirus en la patogenicidad de los aislados de *G. abietina* inoculados en plántulas de *Pinus halepensis*. Cuatro de los aislados albergaban mitovirus y tres de ellos no. Durante el experimento llevado a cabo en laboratorio, el crecimiento micelial fue registrado semanalmente hasta completar 8 mediciones. En el experimento llevado a cabo en el

invernadero, a partir de que las plántulas mostraron síntomas, se midió la severidad de la enfermedad una vez por semana, durante 5 semanas. Al finalizar el experimento, las plántulas fueron llevadas al laboratorio, donde se midió la longitud de la necrosis producida por el patógeno. Los resultados del experimento *in vitro* mostraron que los aislados infectados con mitovirus presentaron mayor crecimiento micelial que los que no infectados en la temperatura de crecimiento óptimo del hongo de 15 ºC. 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 comparación con los no infectados en los potenciales osmóticos de -0.6 y -1.8 MPa. En el experimento efectuado en el invernadero, la longitud de las necrosis encontradas en plántulas con aislados infectados por mitovirus fueron mayores que las que presentaron las plántulas inoculadas con aislados sin mitovirus. Este estudio proporciona un conocimiento más profundo de los efectos de las infecciones víricas en aislados españoles de *Gremmeniella abietina*.

Palabras clave: mitovirus, Scleroderris canker, *in vitro*, *in vivo*, control biológico, *Gremmeniella abietina*, dc ARN.

1. 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: 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.

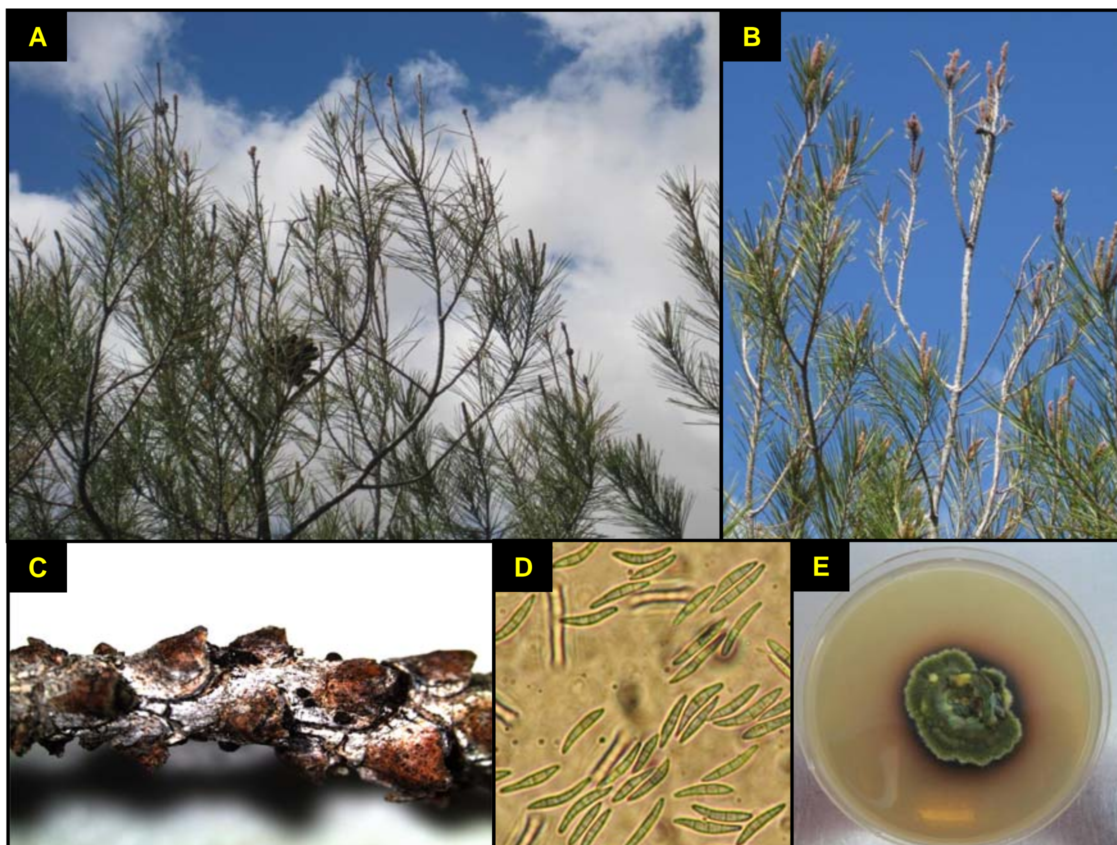

Figure 1a *Gremmeniella abietina* symptoms in Spanish stands: dry needles, crown defoliation; **b** Terminal twig distortion; **c** Fruiting bodies; **d** Conidia; **e** Mycelium in pure culture.

The symptoms observed generally consist of dry needles, branches with some distortion of terminal twigs and eventual dieback or death of the trees (Figure 1) (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 (Fig 2b) (Zhang *et al*., 2010). Fungal viruses differ in their genomes, which can contain DNA, double-stranded (ds) RNA or single-stranded (ss) RNA genomes (Fig 2a) (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, to have the ability to decrease the fitness of the pathogenic fungus and secondly, to 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, 2003). 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*., 2011).

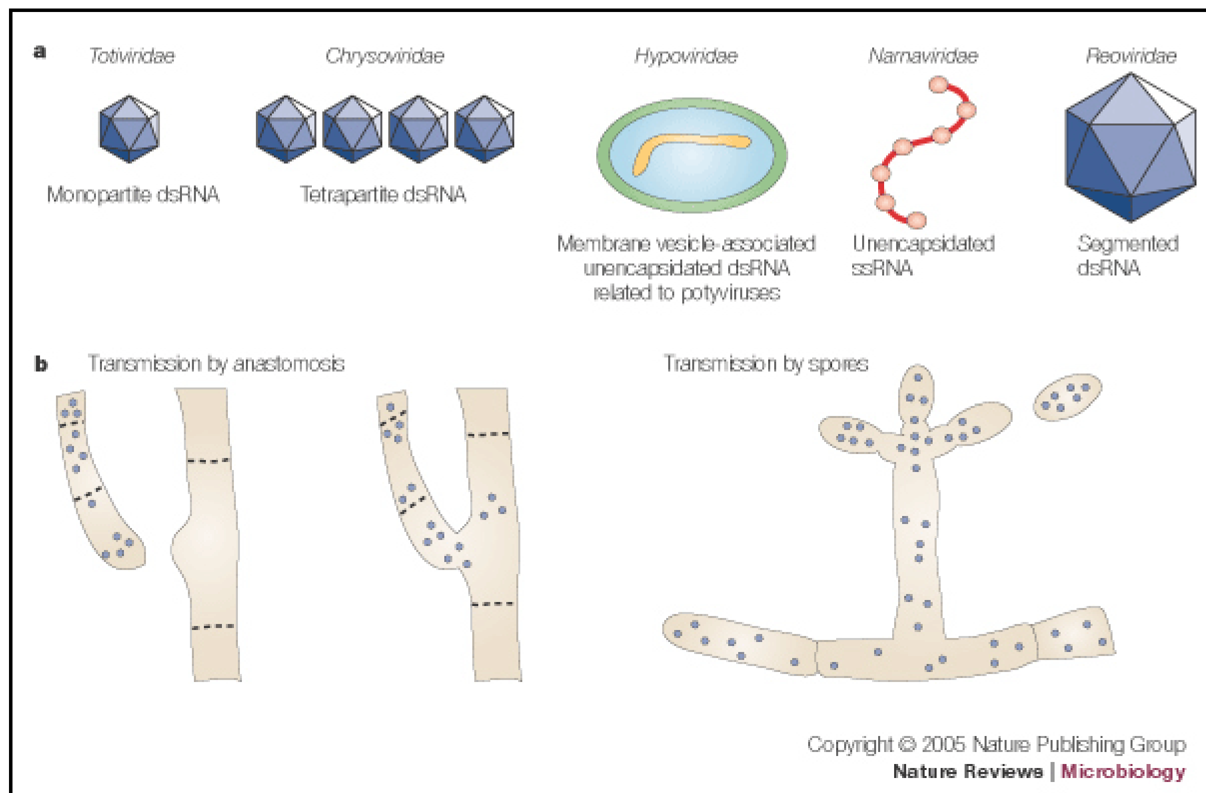

Fig 2a Taxonomic families represented by mycoviruses that are associated with hypovirulence of plant pathogenic fungi are shown with the virus structure and genome composition. **b** Mycoviruses are not infectious by an extracellular route. Transmission is restricted primarily to intracellular routes that include anastomosis (fusion of hyphae) or during the formation of spores (Nuss, 2005)

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.*, 2011). 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 behavior.

2. Objetives

Although some strains of *G. abietina* have been shown to host dsRNA mycoviruses, the effect these agents have on the virulence of this phytopathogenic fungus has not yet been investigated. Accordingly, the main objectives of the present study were:

i. 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
ii. To observe the effect of the presence of mitoviruses in the pathogenity of *G. abietina* isolates inoculated on *Pinus halepensis* seedlings

3. Materials and Methods

3.1 Fungal material

To develop this study seven Spanish isolates of *G. abietina* (Table 1) 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 -80 º C and were reactivated on modified orange serum agar medium (MOS-agar medium; 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 ºC in order to obtain sufficient amounts of mycelium.

Table 1: Isolates used in the experiment ([1]according to Botella *et al*. 2010).

Isolate	Mitoviral molecules[1]	Origin	Province	Year of isolation
00P-07	Yes	Valle de Cerrato	Palencia	2001
VAI-13	No	Villalba de los Alcores	Valladolid	2003
Hon 3-3	Yes	Hontoria	Palencia	2007
Hon 9-2	No	Hontoria	Palencia	2007
P1-8	No	Valle de Cerrato	Palencia	2007
P1-12	Yes	Valle de Cerrato	Palencia	2007
P3-12	Yes	Valle de Cerrato	Palencia	2007

3.2 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 (Fig 3a, 3b). 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 x d_2) where d_1 and d_2 were the two diameters measured along the lines.

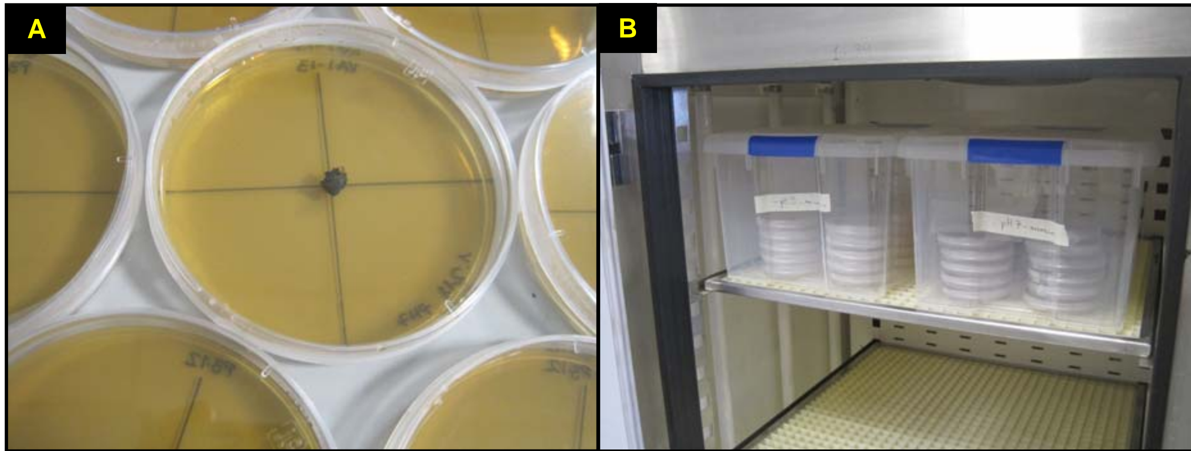

Fig 3a Petri dishes used in the experiments with the two perpendicular lines. **b** Boxes where the Petri dishes were stove in.

3.3 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 ºC, 15 ºC, 25 ºC and 35 ºC), four pH values (4, 5, 7 and 9) and four osmotic potentials (-0.6, -1.2,-1.8 and -2.4MPa). The effect of temperature on mycelial growth was investigated by placing Petri dishes in several stoves at 5 ºC, 15 ºC, 25 ºC and 35 ºC. 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 ºC 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 ºC ± 1 ºC in continuous darkness. Four repetitions of each combination “isolate x treatment” were completed.

3.4 DsRNA extractions

In order to know if mitoviral molecules remain present after the treatments, extractions of dsRNA were carried out in significative treatments (Fig 4a). Fungal mycelium of mitovirus-infected isolates 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 (Fig 4b). Samples were loaded in a 1% agarose gel, which contained 1x TAE buffer and GelRed^{TM} 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^{TM}).

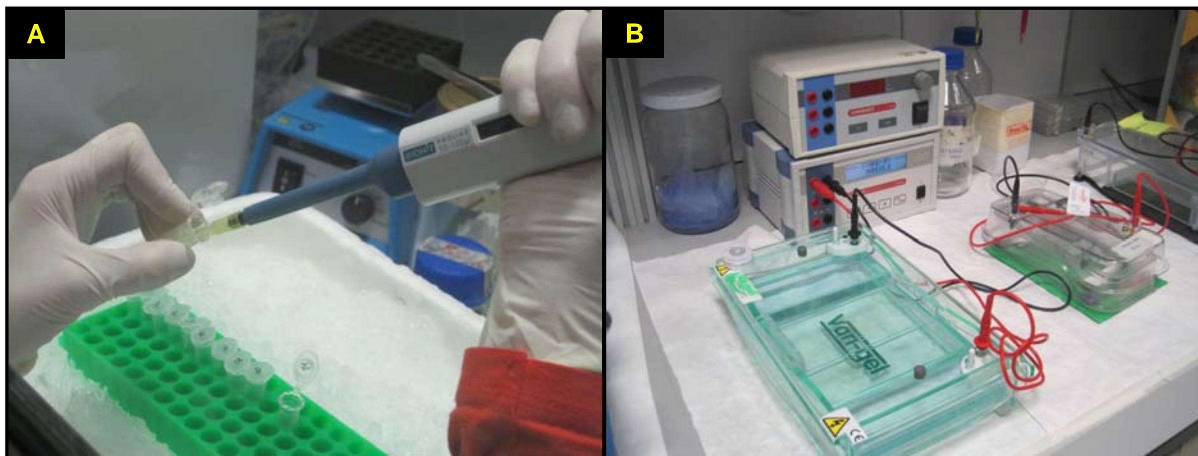

Fig 4a dsRNA extraction process **b** Gel electrophoresis apparatus.

3.5 In vivo pathogenicity tests

Pathogenicity tests were carried out in the greenhouse using 1-year old seedlings of *Pinus halepensis* and the same fungal isolates used in the laboratory experiment. Plant material was provided by the Serranillo nursery (Ministry of Agriculture, Food and Environment). Seedlings were placed in trays leaving spaces among them to avoid contact. In order to perform the inoculation, a wound was made with a sterile scalpel at 10 cm below the shoot apex. A 0.5 cm diameter piece of MOS with *Gremmeniella abietina* mycelium was placed on each wound and covered with Parafilm (Fig 5). Fifteen repetitions of every combination were made and 15 plants were used as controls, thus a total of 120 plants were inoculated.

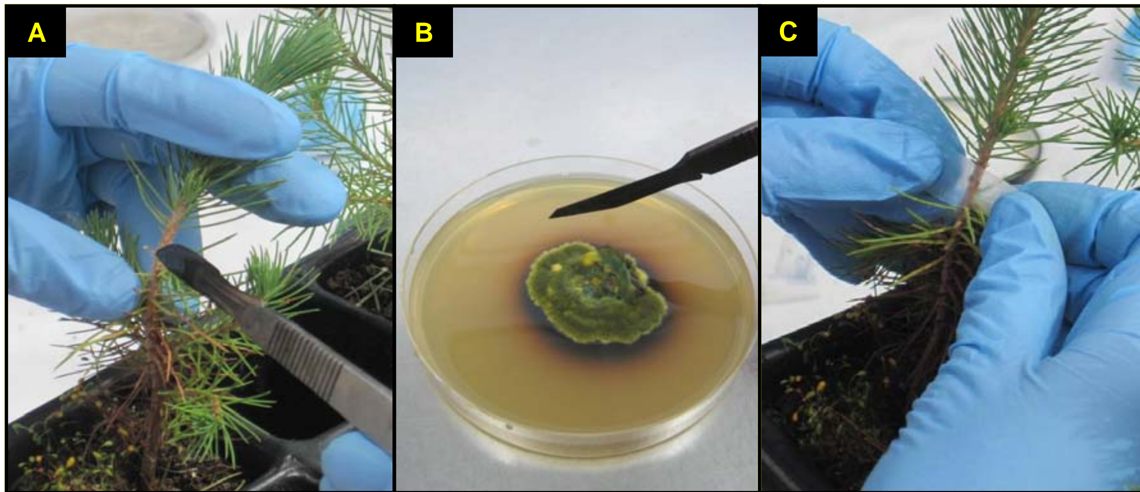

Fig 5 Followed steps to inoculate the fungus: **a** Wound with the scapel; **b** Piece of *G. abietina*; **c** Cover with Parafilm.

Two and a half months after inoculations, some of the plants started to show symptoms of decline. A visual evaluation was made over 5 weeks to measure the disease severity according to the following scale: 0, symptomless; 1, chlorosis; 2, advanced chlorosis; 3, dieback; 4, necrotic; 5, dead (Fig 6). Afterwards, plants were cut and carried to the laboratory in order to measure the necrosis produced by the pathogens and the plant length. Relationship among necrosis and total length was defined as relative necrosis length and was used, with the severity index, as response variable in the statistics analyses. In order to verify if *G. abietina* was the fungus producing the necrosis (Koch postulates), in half of the symptomatic seedlings samples of 1cm were cut, surface-sterilized and placed in Petri dishes with MOS media at 15 ºC for several weeks for reisolation.

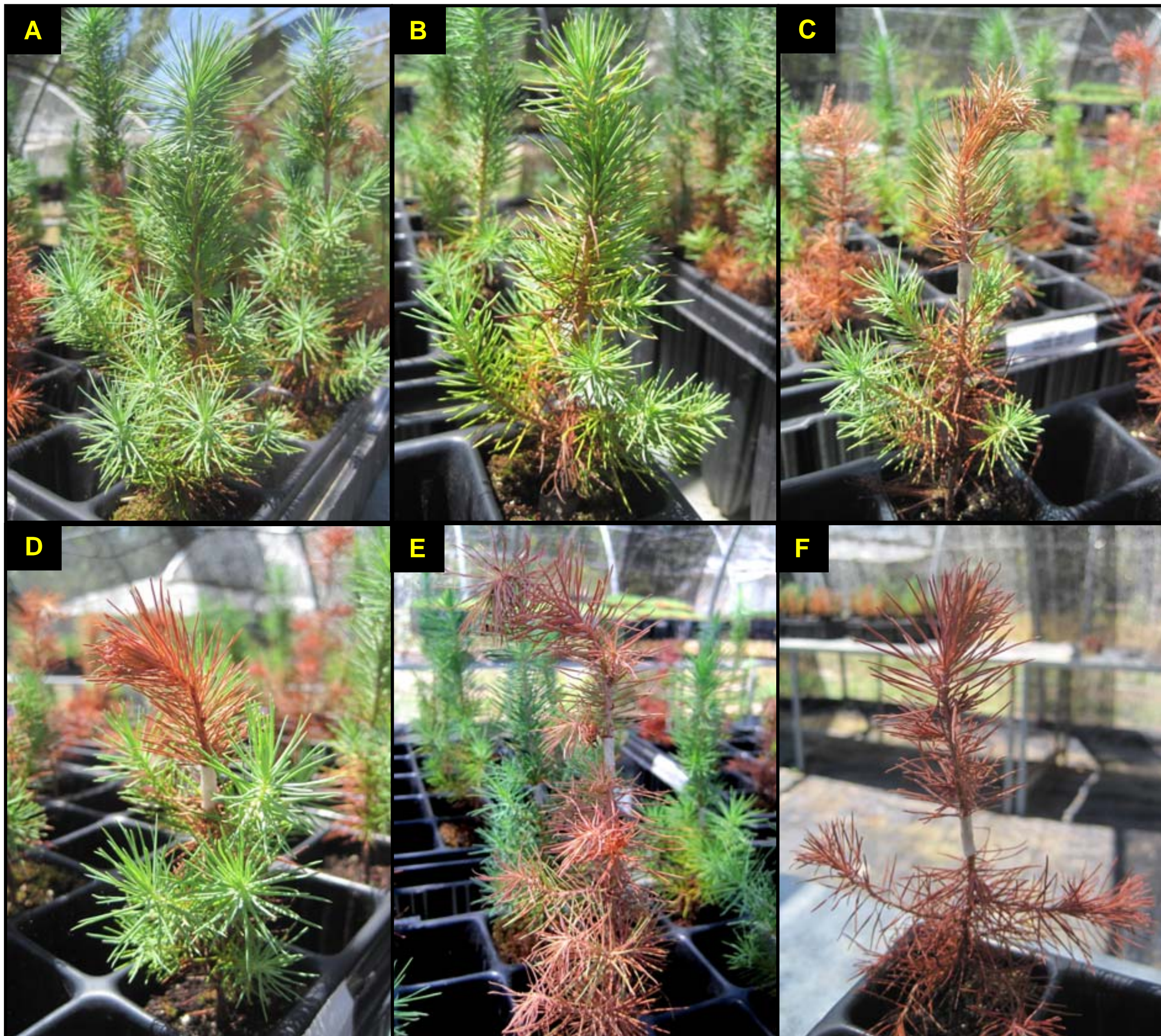

Fig 6 Severity index **a** Symtomless; **b** Chlorosis; **c** Advanced chlorosis; **d** Dieback; **e** Necrotic; **f** Dead.

3.6 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 from pathogenicity tests were also analyzed with SAS program. Two models were made to evaluate the presence of mitoviruses (yes/no): first, severity index was used as response variable and second, relative necrosis length. In all the analysis a 95% of significance was considered. Normality, linearity and homocedasticity for the residuals were probed with Shapiro-Wilk test and graphical procedures. Since data did not fulfill these requirements, they were analyzed with a non-parametric test (the two-sample median test).

4. Results

4.1 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 2. The average growth from mitovirus-infected and mitovirus-free isolates is shown in Figure 7 at 5 ºC (A), 15 ºC (B), 25 ºC (C) and 35 ºC (D) throughout the eight weeks. Growth at 25 ºC was minimal and there was no growth at 35 ºC. 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 ºC whereas the smallest were found at 35 ºC. 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 ºC ($p=0.0043$), the temperature that produced the most growth.

Table 2: Mycelial growth (mm^2) after 8 weeks at different temperatures. Mean value ± standard error (SE). Treatments tagged with * presented significant differences among isolates. [1]If the isolate was naturally-infected with mitovirus. [2]Average growth when combining 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 combining all the isolates together.

Mitovirus [1]	**Temperature**				**TOTAL[2]**
	5 º C	**15 º C ***	**25 º C**	**35 º 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	

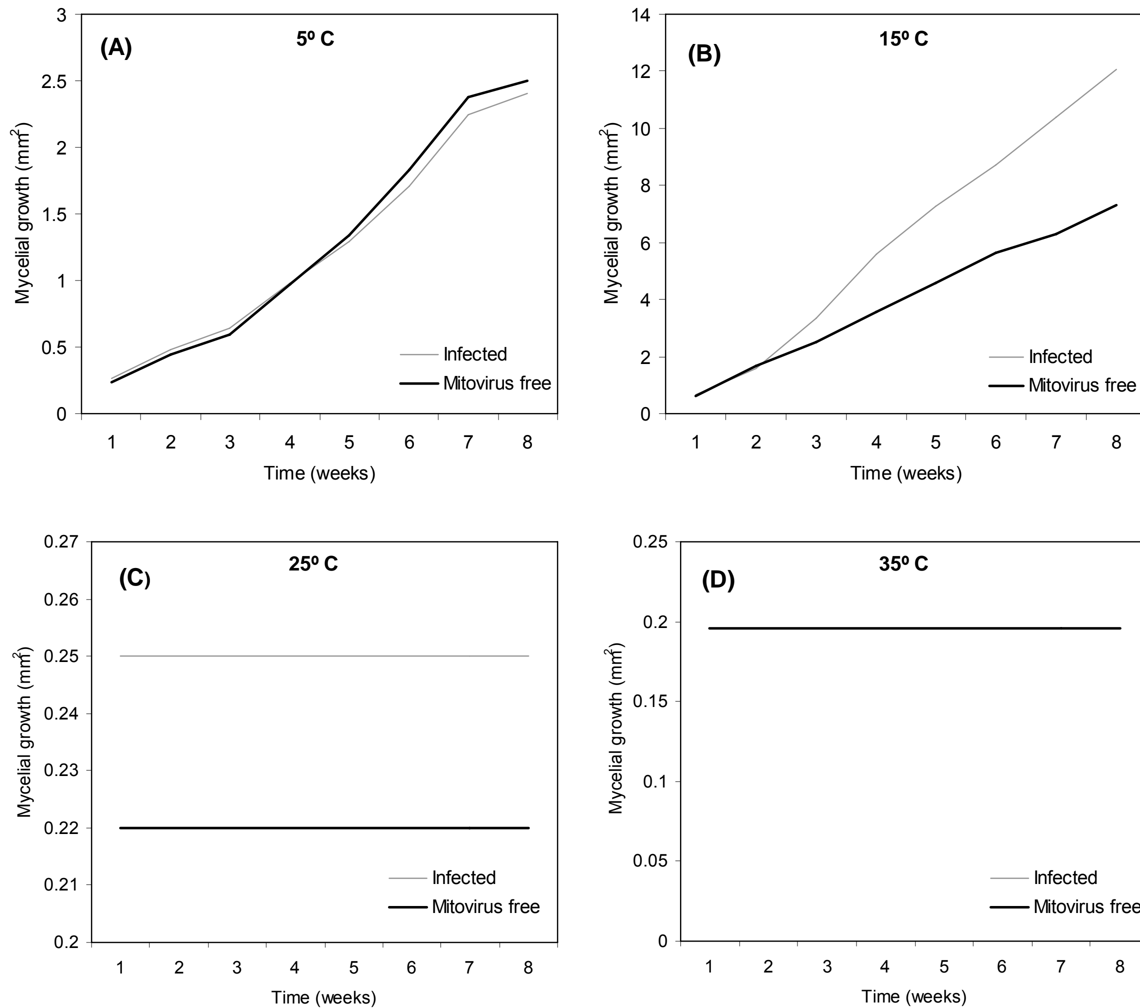

Fig 7 Average growth from mitovirus-infected and mitovirus-free isolates at 5 ºC (**a**), 15 ºC (**b**), 25 ºC (**c**) and 35 ºC (**d**) over the eight weeks.

4.2 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 8 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 3. 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.

Table 3: Mycelial growth (mm²) after 8 weeks at different pHs. Mean value ± standard error (SE). [1]If the isolate was naturally-infected with mitovirus. [2]Average growth when combining 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 combining all the isolates together.

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	

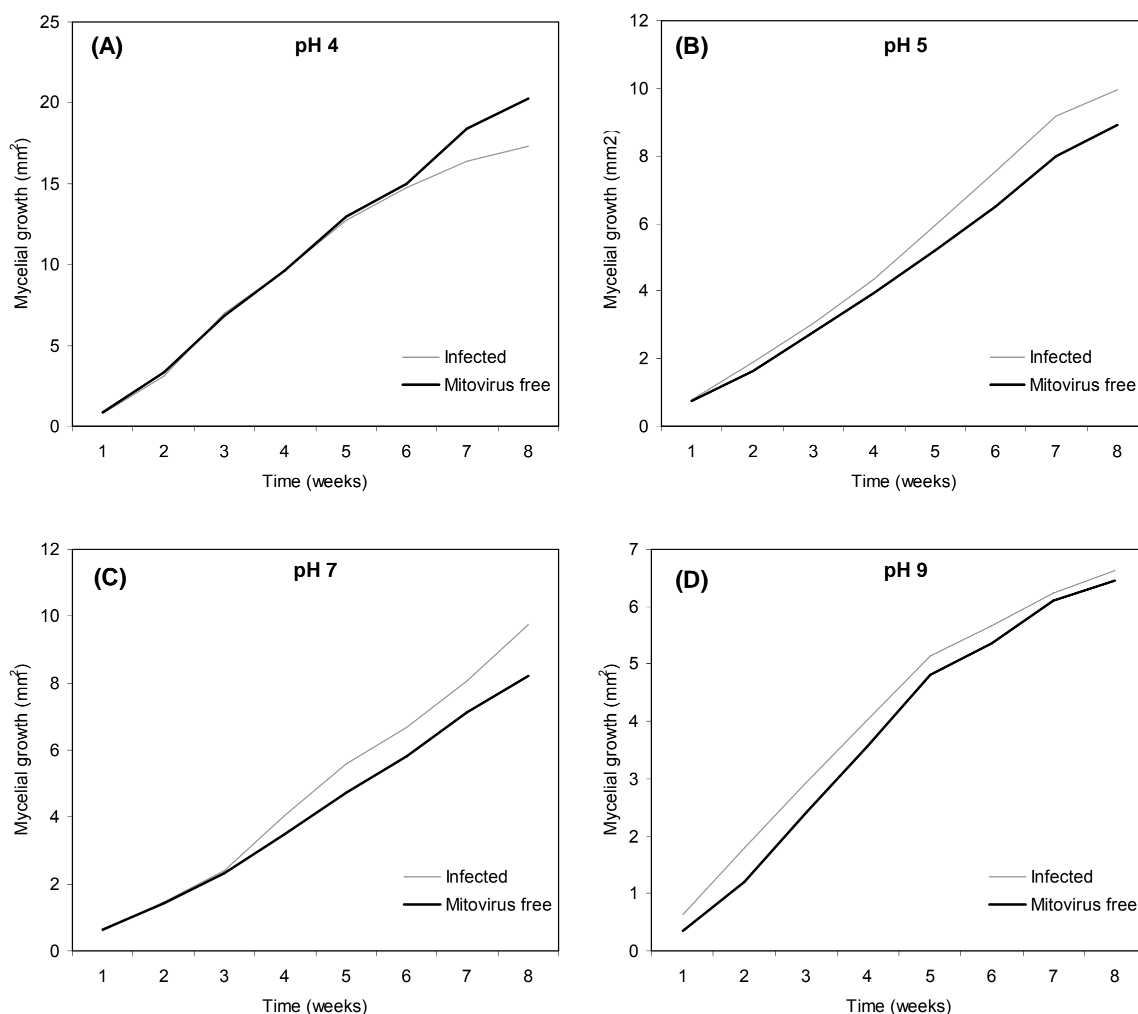

Fig 8 Average growth from mitovirus-infected and mitovirus-free isolates at pH 4 (**a**), pH 5 (**b**), pH 7(**c**) and pH 9 (**d**) over the eight weeks.

4.3 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 4). The average growth of mitovirus-infected and mitovirus-free isolates is shown in Figure 9 at -0.6MPa (A), -1.2MPa (B), -1.8MPa (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 -1.2MPa (p=0.7515) and -2.4MPa (p=0.1004).

Table 4: Mycelial growth (mm²) after 8 weeks at different osmotic potentials. Mean value ± standard error (SE). Treatments tagged with * presented significant differences among isolates. [1]If the isolate was naturally-infected with mitovirus. [2]Average growth when combining 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 combining all the isolates together.

Mitovirus [1]	**Osmotic potential**				**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	

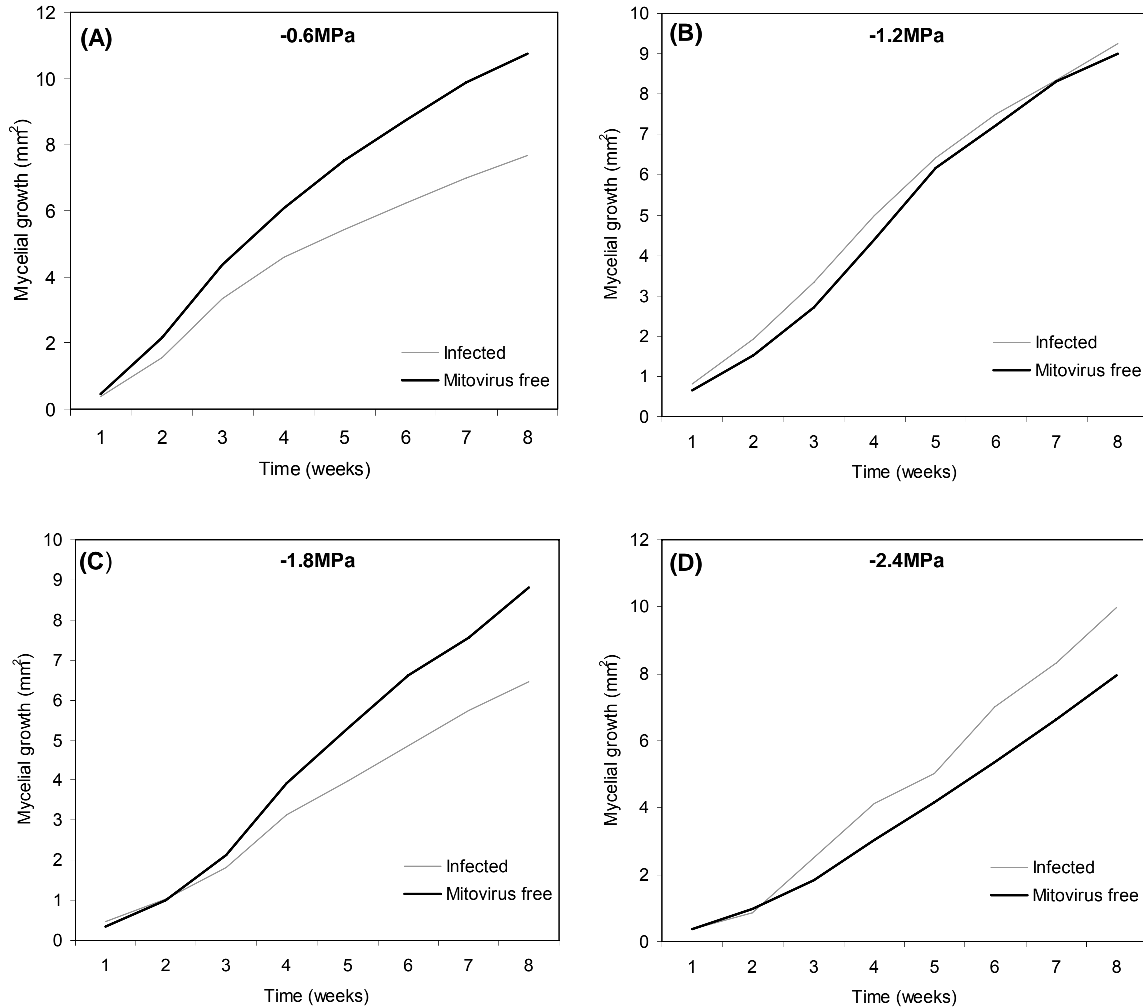

Fig 9 Average growth from mitovirus-infected and mitovirus-free isolates at -0.6MPa (**a**), -1.2MPa (**b**), -1.8MPa (**c**) and -2.4MPa (**d**) over the eight weeks.

4.4 DsRNA banding patterns

The presence of the different putative mitoviruses was confirmed by dsRNA extraction and gel electrophoresis after significant treatments were carried out (Fig 10). Isolate P3-12 had a 2.5 kb band in treatment 15 ºC (lane 7) and was found to maintain this band despite receiving the treatments of ψ_π -0.6MPa (lane 1) and -2.4 MPa (lane 10). It happened the same with isolates 00P-07 and P1-12: treatments of ψ_π -0.6MPa (lane 3, lane 4), -2.4 MPa (lane 11) and temperature of 15 ºC (lane 5). Conversely, the 2.5 kb band was not sustained in isolate Hon3-3 after treatments of temperature 15 ºC (lane 6), ψ_π -0.6MPa (lane 2) and -1.8MPa (lane 8) and in isolate P1-12 after treatment of ψ_π -1.8MPa (lane 9). 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).

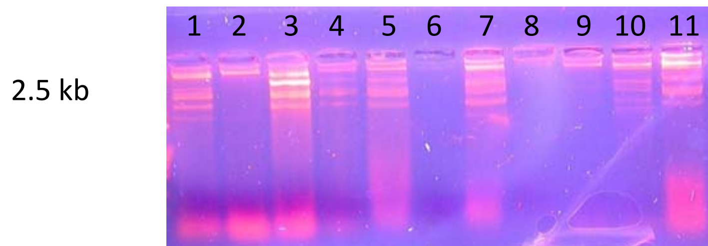

Fig 10 A GelRed-stained 1 % agarose gel showing the dsRNA banding patterns. Lane 1, P3-12 (-0.6MPa); lane 2, Hon 3-3 (-0.6MPa); lane 3, 00P-07 (-0.6MPa); lane 4, P1-12 (-0.6MPa); lane 5, P1-12 (15 ºC); lane 6, Hon 3-3 (15 ºC); lane 7, P3-12 (15 ºC); lane 8, Hon 3-3 (-1.8MPa); lane 9, P1-12 (-1.8MPa); lane 10, P3-12 (-2.4MPa); lane 11, 00P-07 (-2.4MPa).

4.5 In vivo pathogenicity tests

The average relative necrosis length (Fig 11) from seedlings inoculated with mitovirus infected and from non-infected isolates is shown in Figure 12. The necrosis length of seedlings inoculated with mitovirus-infected isolates was significantly larger (p=0.0291) than the necrosis from seedlings which were inoculated with non-infected isolates. In the second model, severity index was used as response variable, but it was not significative with a 95% level of significance. Seedlings inoculated with mitovirus infected isolates had a higher disease severity that the non-infected isolates but it was not statistically significant (Fig 13). *Gremmeniella abietina* was re-isolated from 35% of the symptomatic seedlings that were inoculated.

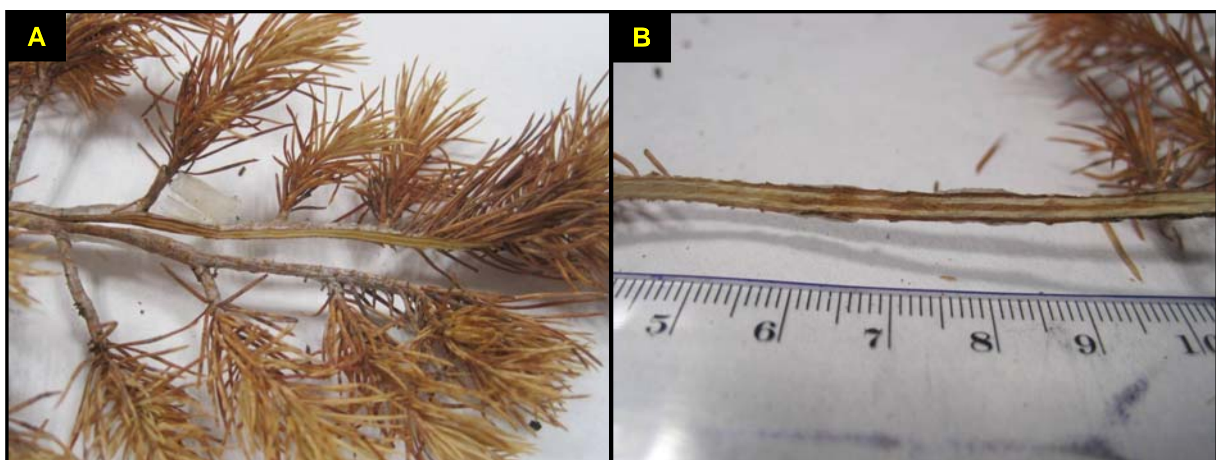

Fig 11a Plants were cut with a scalpel. **b** Necrosis length was measured.

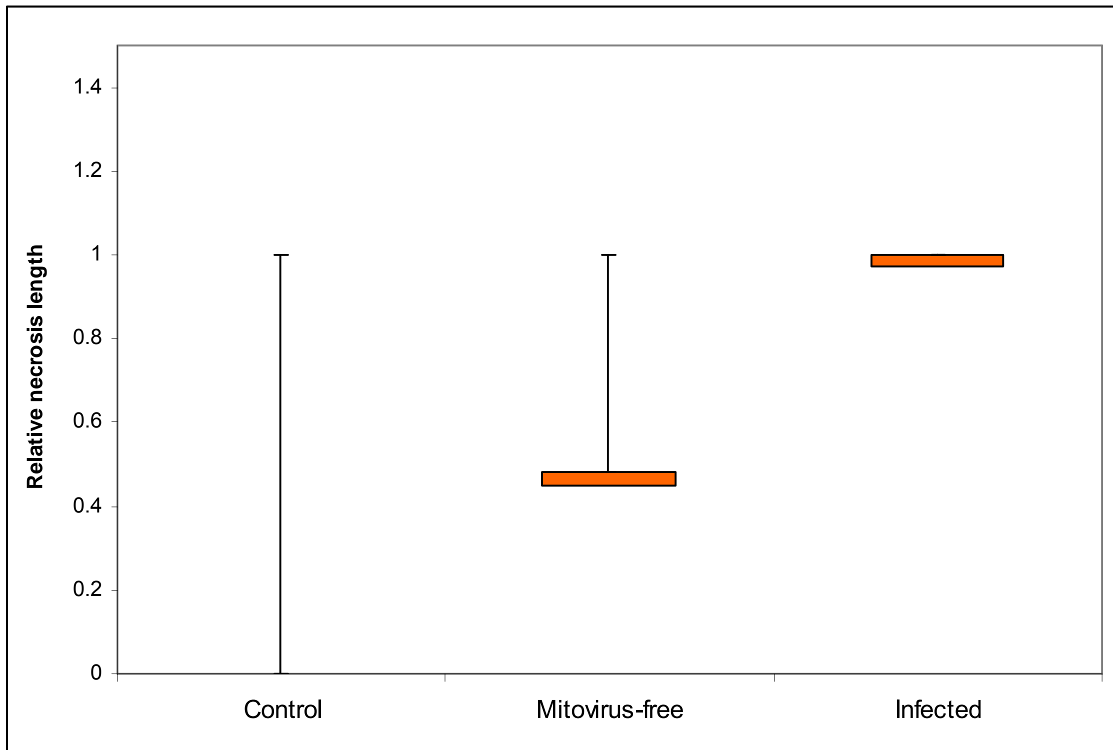

Fig 12 Box plot for control seedlings and seedlings inoculated with mitovirus infected and mitovirus-free isolates. Bars and boxes represent: maximum, 3rd quartile, median, 1st quartile and minimum.

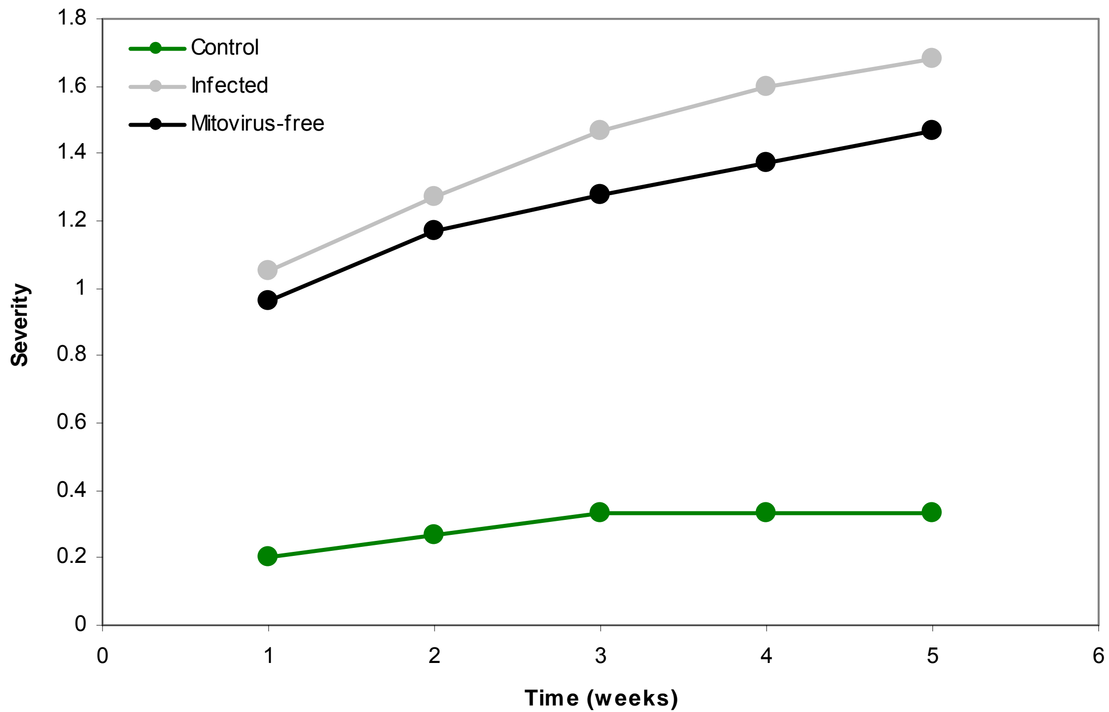

Fig 13 Mean severity index of each group (control, infected and mitovirus-free) over the 5 weeks.

5. Discussion

Mycelial growth depends on the temperature of the environment. In our study, all the isolates showed an optimal growth at 15 ºC, 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 ºC 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 ºC and no growth was observed at 35 ºC). 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 65ºC 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 ºC (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. 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).

Concerning *in vivo* pathogenicity test, mitovirus' infection resulted in any statistically significant difference in visual disease severity among seedlings. Necrosis length of seedlings inoculated with mitovirus-infected isolates was significantly larger than the necrosis from seedlings which were inoculated with non-infected isolates, that is, mitovirus infected isolates did not reduce the pathogen aggressiveness, which is the most desirable feature in control of plant pathogenic fungi (Xu *et al.* 2005). This behavior could suggest an advantageous to the infected isolates and could lead to a hypervirulence of the pathogen. However additional studies are required to understand the role of this mitovirus in *G. abietina* features.

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 have been due to a genetic influence (Zharare et al., 2010) a possibility not tested 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; Chu *et al*., 2011). 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 s*trains 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 ºC, the optimal growing conditions of *G. abietina*. Although no visual disease severity statistically significant difference was noticed among plants, larger necrosis lengths were recorded in those seedlings infected with mitovirus isolates, suggesting a possible hypervirulence produced by the mitoviruses' presence.

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 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.

6. 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 ºC. 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. No differences were found in visual disease severity among plants however larger necrosis lengths were observed in the plants inoculated with mitovirus infected isolates, suggesting a possible hypervirulence produced by the mitoviruses' presence. Further research including other virulence-associated parameters is recommended.

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References

Aoki N, Moriyama H, Kodama M, Arie T, Teraoka T, Fukuhara T. 2009. A novel mycovirus associated with four double-stranded RNAs affects host fungal growth in *Alternaria alternata*. Virus Res 140, 179-187.

Armengol J, Alaniz S, Vicent A, Beltran R, Abad-Campos P, Perez-Sierra A, Garcia-Jimenez J, Ben Salem I, Souli M, Boughalleb N. 2011. Effect of dsRNA on growth rate and reproductive potential of *Monosporascus cannonballus*. Fungal Biol 115, 236-244.

Boland G, 2004. Fungal viruses, hypovirulence, and biological control of Sclerotinia species. Can J Plant Pathol 26, 6-18.

Botella L, Tuomivirta TT, Kaitera J, Navarro VC, Diez JJ, Hantula J. 2010. Spanish population of *Gremmeniella abietina* is genetically unique but related to type A in Europe. Fungal Biol 114, 778-789.

Botella L, Tuomivirta TT, Hantula J, Diez JJ. 2012a. Presence of viral dsRNA molecules in the Spanish population of *Gremmeniella abietina*. Journal of Agricultural Extension and Rural Development 4 (9), 211-213.

Botella L, Tuomivirta TT, Vervuur S, Diez JJ., Hantula J. 2012b. Occurence of two different species of mitoviruses in the European race of *Gremmeniella abietina* var *abietina*, both hosted by the genetically unique Spanish population. Fungal Biol http://dx.doi.org/10.1016/j.funbio.2012.05.004

Brasier CM.1983. A cytoplasmically transmitted disease of *Ceratocystis ulmi*. Nature 305, 220-223.

Carlile, M.J.; Watkinson, S. C.; Gooday, G.W., 2001. The Fungi 2nd ed. Academic press, London, UK.

Castro M, Kramer K, Valdivia L, Ortiz S, Castillo A. 2003. A double-stranded RNA mycovirus confers hypovirulence-associated traits to *Botrytis cinerea*. FEMS Microbiol Lett 228, 87-91.

Chu Y, Jeon J, Yea S, Kim Y, Yun S, Lee Y, Kim K. 2002. Double-stranded RNA mycovirus from *Fusarium graminearum*. Appl Environ Microb 68, 2529-2534.

Davis, D J.; Burlak C., Money, N. P., 2000. Osmotic pressure of fungal compatible osmolytes. Mycol Res 104 (7), 800-804.

Deng, F., Xu, R. Boland, G. J. 2003. Hypovirulence associated double-stranded RNA from *Sclerotinia homoeocarpa* is conspecific with *Ophiostoma novo-ulmi* mitovirus 3a-Ld. Phytopathology 93, 1407-1414.

Deng, F., Boland, G.J. 2004. A satellite RNA of *Ophiostoma novo-ulmi* mitovirus 3a in hypovirulent isolates of *Sclerotinia homoeocarpa*. Phytopathology 94, 917-923.

Donaubauer, E. 1972. Distribution and hosts of *Scleroderris lagerbergii* in Europe and North America. Eur J Forest Pathol 2, 6-11.

Dorworth CE. 1979. Influence of inoculum concentration on infection of red pine seedings by *Gremmeniella-abietina*. Phytopathology 69, 298-300.

Ghabrial S.A, Suzuki N. 2009 Viruses of plant pathogenic fungi. Annu Rev Phytopathol 47, 353-384.

Hamelin R, Ouellette G, Bernier L. 1993. Identification of *Gremmeniella-abietina* races with Random Amplified Polymorphic DNA markers. Appl Environ Microb 59, 1752-1755.

Hantula J, Muller MM, 1997. Variation within *Gremmeniella abietina* in Finland and other countries as determined by Random Amplified Microsatellites (RAMS). Mycol Res 101, 169-175.

Hausner, G., Belkhiri, A., Klassen, G.R. 2000. Phylogenetic analysis of the small subunit ribosomal RNA gene of the hyphochytrid *Rhizidiomyces apophysatus*. Can J Botany 78, 124-128.

Hellgren M, Hogberg N, 1995. Ecotypic Variation of *Gremmeniella-abietina* in Northern Europe - Disease patterns reflected by DNA variation. Can J Botany 73, 1531-1539.

Herrero N, Perez-Sanchez R, Oleaga A, Zabalgogeazcoa I, 2011. Tick pathogenicity, thermal tolerance and virus infection in *Tolypocladium cylindrosporum*. Ann Appl Biol 159, 192-201.

International Committee on Taxonomy of Viruses Database (ICTV) In: Virus taxonomy, Classification and nomenclature of viruses, Ninth Report (2011) Ed: King, A.M.Q., Adams,M.J., Carstens, E.B. and Lefkowitz, E.J. Elsevier, USA.

Imolehin ED, Grogan RG, Duniway JM.1980. Effect of temperature and moisture tension on growth, sclerotial production, germination and infection by *Sclerotinia minor*. Phytopathology 70, 1153–1157

Kaitera J, Jalkanen R, 1992. Disease history of *Gremmeniella-abietina* in a *Pinus-sylvestris* Stand. Eur J Forest Pathol 22, 371-378.

Kaitera J, Jalkanen R, 1996. In vitro growth of *Gremmeniella abietina* isolates (European race) at different temperatures. Scand J Forest Res 11, 159-163.

Lee K, Yu J, Son M, Lee Y, Kim K, 2011. Transmission of *Fusarium boothii* mycovirus via protoplast fusion causes hypovirulence in other phytopathogenic fungi. PloS ONE 6, e21629.

Lira-Méndez, K., Mayek-Pérez, N. 2006. Potencial osmótico variable en el crecimiento in vitro y la patogenicidad en frijol (*Phaseolus vulgaris* L.) de *Fusarium* spp. Rev Mex Fitopatol 24 (2), 88-97.

Marquez LM, Redman RS, Rodriguez RJ, Roossinck MJ. 2007. A virus in a fungus in a plant: three-way simbiosis required for themal tolerance. Science 315, 543-515.

Martínez J. 1933. Una grave micosis del pino observada por primera vez en España. Bol Soc Española de Historia Natural 33, 25-29.

McCabe P, Pfeiffer P, Van Alfen N. 1999. The influence of dsRNA viruses on the biology of plant pathogenic fungi. Trends Microbiol 7, 377-381.

Morris T.J., Dodds J.A. 1979. Isolation and analysis of double-stranded-RNA from virus-infected plant and fungal tissue. Phytopathology 69, 854-858.

Müller Mm, Kantola R, Kitunen V (1994) Combining sterol and fatty acid profiles for the characterization of fungi. Mycol Res 98, 593–603.

Nuss D.L. 2005. Hypovirulence: Mycoviruses at the fungal-plant interface. Nat Rev Microbiol 3, 632-642.

Osaki H, Nakamura H, Nomura K, Matsumoto N, Yoshida K. 2005. Nucleotide sequence of a mitochondrial RNA virus from the plant pathogenic fungus, *Helicobasidium mompa* Tanaka. Virus Res 107, 39-46.

Palmero D, de Cara M, Iglesias C, Ruiz G, Tello JC. 2008. Effects of water potential on spore germination and viability of *Fusarium* species. J Ind Microbiol Biotechnol 35,1405–1409.

Park Y, Chen X, Punja ZK. 2006. Molecular and biological characterization of a mitovirus in *Chalara elegans* (*Thielaviopsis basicola*) Phytopathology 96, 468-479.

Pearson MN, Beever RE, Boine B, Arthur K. 2009. Mycoviruses of filamentous fungi and their relevance to plant pathology RID D-3988-2011. Mol Plant Pathol 10, 115-128.

Perez J, Martinez B, Rivas E, Diaz MA. 2000. Efecto del pH sobre la germinación conidial, crecimiento y esporulación de *Didymella bryoniae* (Awersw) Rehn. Rev Protección Veg Vol 15 (3), 185-187.

Polashock JJ, Hillman BI. 1994. A small mitochondrial double-stranded (ds) RNA element associated with hypovirulent strain of the chesnut blight fungus and ancestrally related to yeast cytoplasmic T and W dsRNAs. Proc Natl Acad Sci U.S.A. 91, 8680-8684.

Polashock J.J, Bedker PJ, Hillman BI.1997 Movement of a small mitochondrial double-stranded RNA element of *Cryphonectria parasitica*: ascospore inheritance and implications for mitochondrial recombination. Mol Gen Genet 256,566-571.

Robin C, Lanz S, Soutrenon A, Rigling D.2010. Dominance of natural over released biological control agents of the chestnut blight fungus *Cryphonectria parasitica* in south-eastern France is associated with fitness related traits. Biol control 53, 55-61.

Rogers HJ, Buck KW, Brasier CM. 1987. A mitochondrial target for doubled-stranded RNA in diseased isolates of the fungus that causes Dutch elm disease. Nature 329: 558-560

Santamaria O, Pajares JA, Diez JJ. 2003. First report of *Gremmeniella abietina* on *Pinus halepensis* in Spain. Plant Pathol 52, 425-425.

Santamaria O, Pajares JA, Diez JJ. 2004. Physiological and morphological variation of *Gremmeniella abietina* from Spain. Forest Pathol 34, 395-405.

Santamaria O, Alves-Santos FM, Diez JJ. 2005. Genetic characterization of *Gremmeniella abietina* var. *abietina* isolates from Spain. Plant Pathol 54, 331-338.

SAS Institute INC. SAS/STAT®. 2004. User’s Guide. Version 9.1. Cary, NC: SAS Institute Inc. USA.

Tan CMC, Pearson MN, Beever RE, Parkes SL. 2007. Why Fungi Have Sex? Abstract: XIVth International Botrytis Symposium, Cape Town, South Africa. October 21th-27th.

Tuomivirta T, Hantula J. 2003. *Gremmeniella abietina* mitochondrial RNA virus S1 is phylogenetically related to the members of the genus *Mitovirus*. Arch Virol 148, 2429-2436.

Uotila A. 1983. Physiological and morphological variation among Finish *Gremmeniella abietina* isolates. Commun Inst For Fenn 119, 1-12.

Van Diepeningen A, Debets A, Hoekstra R. 2006. Dynamics of dsRNA mycoviruses in black Aspergillus populations. Fungal Genet Biol 43, 446-452.

Vainio,E. J.; Korhonen,K; Tuomivirta,T.T.; Hantula,J., 2010 A novel putative partitivirus of the saprotrophic fungus *Heterobasidion ecrustosum* infects pathogenic species of the *Heterobasidion annosum* complex. Fungal Biol 114, 11–12, 955-965

Vazquez-Garcia A, Santiago-Martinez G, Estrada-Torres A. 2002. Influencia del pH en el crecimiento de quince cepas de hongos ectomicorrizógenos. Anales del Instituto de Biología, UNAM, Serie Botánica 73(1), 1-15.

Wu M, Zhang L, Li G, Jiang D, Hou MS, Huang HC. 2007. Hypovirulence and double stranded RNA in *Botrytis cinerea.* Phytopathology 97, 1590-1599.

Wu M, Zhang L, Li G, Jiang D, Ghabrial S. 2010. Genome characterization of a debilitation associated-mitovirus infecting the phytopathogenic fungus *Botrytis cinerea*. Virology 406, 117-126.

Xu P, Fang C, Mannas J P, Feldman T, Sumner LI W, Roossinck M J. 2008. Virus infection improves drought tolerance. New Phytol 180, 911-921.

Yokota, S. 1975. Scleroderris canker of todo-fir in Hokkaido, Northern Japan. III. Dormant infection of the causal fungus. Eur J Forest Pathol 5, 7-12.

Zabalgogeazcoa I, Benito EP, García Ciudad A, García Criado B, Eslava AP. 1998. Double-stranded RNA and virus-like particles in the grass endophyte *Epichloë festucae*. Mycol Res 102, 914-918.

Zhang L, De Wu M, Li GQ, Jiang DH, Huang HC. 2010. Effect of Mitovirus infection on formation of infection cushions and virulence of *Botrytis cinerea*. Physiological and Mol Plant Pathol 75, 71-80.

Zharare, G. E., Kabanda, S. M., & Poku, J. Z. 2010. Effects of temperature and hydrogen peroxide on mycelial growth of eight pleurotus strains. Sci Hortic-Amsterdam 125(2): 95-102.