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Vertical Transmission of *Fusarium circinatum* Mitoviruses FcMV1 and FcMV2-2 via Microconidia

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Abstract: Pine Pitch Canker disease, caused by the pathogenic fungus *Fusarium circinatum*, affects conifer species worldwide. However, the virulence of the pathogen may be affected by the presence of mycoviruses. The aim of this laboratory-based study was to investigate the probability and rate of transmission of *F. circinatum* mitoviruses FcMV1 and FcMV2-2 via microconidia. Ten isolates of mitovirus-infected *F. circinatum* were subcultured to produce a total of 100 single-spore colonies (ten replicates per isolate). The total RNA and cDNA obtained from each spore isolate (monosporic culture) were amplified by PCR with specific primers for detection of *F. circinatum* mitoviruses FcMV1 and FcMV2-2. The mitoviruses were detected in a high percentage of the individual spore isolates (between 60% and 100% depending on the fungal isolate). However, the probability of transmission was not statistically significantly associated with either the *F. circinatum* isolate or the viral strain. A high proportion of transmission via microconidia is critical for development of a biological control program against Pine Pitch Canker (PPC) disease in forests. However, further studies are needed to establish the effect of these mitoviruses on the virulence of *F. circinatum*.

Keywords: Pine Pitch Canker disease; hypovirulence; mitovirus; microconidia; biological control

1. Introduction

The ascomycete fungus *Fusarium circinatum* Nirenberg et O'Donnell (teleomorph *Gibberella circinata* Nirenberg et O'Donnell) is an important pathogen of conifer species worldwide. It causes Pine Pitch Canker (PPC) disease, which leads to reduced growth of adult trees in forest plantations, resinous bleeding cankers on trunks and large branches, and death of trees due to girdling [1]. It also has detrimental effects in nurseries [2]. Although the pathogen has serious economic and ecological impacts on nurseries and pine plantations throughout the world [3,4] no method of controlling PPC has yet been developed. The role of biological control in reducing the impact of the pathogen is crucial within a framework of integrated management of the disease. The EU Council Directive 2009/128/EC has introduced new legislative provisions to achieve the sustainable use of pesticides and member states should give priority to non-chemical methods of plant protection and pest management [5]. Chemical control approaches may have deleterious impacts on biodiversity, a negative effect on pathogen resistance to the fungicide, and harmful consequences to non-target fungi [6]. Thus, biological control offers several advantages over chemical control [7], since it is considered less toxic to humans and to the environment, and microbial organisms may control resistant pests and reduce the possibility of development of further resistance [8].

Viruses that infect fungi, i.e., mycoviruses, are widespread in all major taxonomic groups of plant pathogenic fungi [9,10]. Mycoviruses are currently classified on the basis of their genome diversity as follows: linear double-stranded RNA (dsRNA); linear positive-sense single-stranded RNA (+ssRNA); linear negative-sense ssRNA (-ssRNA); and circular ssDNA [11]. Mycoviruses may have no effect on the host, may cause phenotypic changes or affect the growth or physiology of the host, possibly leading to attenuation (hypovirulence) or enhancement of fungal virulence (hypervirulence) [9]. As potential biological control agents, mycoviruses must fulfill the following requirements: they must be capable of lowering the fitness of the pathogenic fungus that they infect, and they must be able to transmit the dsRNA efficiently enough to be maintained in a large proportion of the fungal population [12].

Mycoviruses are commonly transmitted by hyphal anastomosis (horizontal transmission), with cytoplasmic exchange occurring between compatible isolates [10], or by fungal sporulation (vertical transmission). However, the efficiency of virus transmission varies depending on spore type (asexual/sexual) and species [13,14]. Members of the genus *Mitovirus* (family *Narnaviridae*, +ssRNA) have only been found in filamentous fungi, in which they are restricted to the mitochondria [11]. They occur in several phytopathogenic fungi and in some cases their presence is associated with reduced fungal pathogenicity [9]. The Spanish population of *F. circinatum* has recently been found to harbor several members of the genus *Mitovirus*: *Fusarium circinatum* mitovirus 1 (FcMV1, length 2419-bp) and two strains of *Fusarium circinatum* mitovirus 2 (FcMV2-1 and FcMV2-2, length 2193 and 1973-bp, respectively) [15]. These mitoviruses are common in *F. circinatum* isolates from northern Spain. They have been shown to be polymorphic [16] and their viral genome has recently been studied [17]. The main aim of the present study was to investigate how the probability and rate of transmission of these mitoviruses via microconidia vary in relation to the fungal isolate and strain of virus (FcMV1 and FcMV2-2). A further aim of the study was to examine the possible correlation between the transmission rate (%) of the isolates and other phytopathological variables, such as germination and mycelial growth, as previously determined in Muñoz-Adalia et al. [18] and Flores-Pacheco et al. [19].

2. Materials and Methods

2.1. F. circinatum Isolates

Ten mitovirus-infected isolates, both belonging to mating types of the Spanish fungal population (MAT-1 and MAT-2), were used in the present study. The main features of the isolates are described in Table 1: three of the isolates harbored FcMV1, four harbored FcMV2-2, and three harbored both mitoviruses. The isolates were cultivated on potato dextrose agar (PDA, Scharlab S.L.) for seven days in daylight at room temperature (approx. 25 °C). Ten single-spore (monosporic) cultures were then obtained from each isolate through the streak-plate method: several drops of sterile distilled water (SDW) were spread on a Petri dish containing mycelia of the fungus; then a sterile inoculation loop was used to systematically streak the solution over the exterior of the agar in a Petri dish to obtain isolated colonies of the fungus. After 24 h, germinating spores were separated under a microscope and placed individually in a Petri dish with PDA and grown for ten days until extraction of total nucleic acids.

Table 1. Virus positive isolates of *F. circinatum* used to obtain single-spore cultures. Isolate, code; Host, from which the sample was isolated; Region and Locality (village); Mating type, MAT-1 or MAT-2; FcMV1, FcMV2-2, presence (+) or absence (−) of mitovirus infection.

Sample	Host	Region	Locality	Mating Type	FcMV1	FcMV2-2
FcCa6	*Pinus radiata*	Cantabria	Comillas	MAT-2	+	−
072	*P. radiata*	Cantabria	Comillas	MAT-2	+	−
Fc104	*P. pinaster*	Asturias	Unknown	MAT-1	+	−
020	*P. radiata*	Cantabria	Cabezón de la Sal	MAT-2	−	+
FcCa4	*P. radiata*	Cantabria	Cabezón de la Sal	MAT-2	−	+

Table 1. *Cont.*

Sample	Host	Region	Locality	Mating Type	FcMV1	FcMV2-2
035	*P. radiata*	Cantabria	Cabezón de la Sal	MAT-2	–	+
042	*P. radiata*	Cantabria	Cabezón de la Sal	MAT-2	–	+
FcCa1	*P. radiata*	Cantabria	Rionansa	MAT-2	+	+
FcCa70	*P. radiata*	Cantabria	Comillas	MAT-2	+	+
Fc221	*P. radiata*	Cantabria	Unknown	MAT-2	+	+

2.2. Total RNA Extraction and PCR

Total nucleic acids were isolated following the protocol described by Vainio et al. [20] to quantify the vertical transmission of mitoviruses to the spore cultures. A reverse transcription polymerase chain reaction (RT-PCR) [21,22] was then applied with the aim of obtaining complementary DNA (cDNA). The cDNA was used as a template for PCR with the following virus-specific primers: FMC1F1 (5′-CGTGGATTAAAACCCACAAA-3′), FMC1Rev1 (5′-TGGTAATCTACCATAGCAATTAYTC-3′), FMC3F1 (5′-GAYAGAACTTTTACTCAAGATCC-3′) and FMC3Rev1 (5′-ATTCATCTYTTGGCAAATTCATA-3′) [16]. The primer pair FMC1F1/FMC1Rev1 was specific to FcMV1, whereas FMC3F1/FMC3Rev1 was used to detect FcMV2-2. The amplification conditions were as follows: 10 min at 95 °C, followed by 37 cycles of 30 s at 95 °C, 45 s at 53 °C, 2 min at 72 °C; and a final extension of 7 min at 72 °C. The presence or absence of mitoviruses was confirmed by electrophoresis of the PCR products on 1.2% agarose gels (stained with GelRed® 10.000×) in 1× TAE buffer and visualized under UV light. A GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to estimate the lengths of the cDNA molecules.

2.3. Statistical Analysis

Binary logistic regression analysis was used to test whether the categorical factors *F. circinatum* isolate and strain of virus contributed significantly to the probability of transmission of the viruses. A generalized linear model (binomial family) was applied using the GLM function in R software. For this purpose, we carried out analysis of deviance used to indicate how well the model fits the data, i.e., a measure of discrepancy between observed and fitted values [23]. Four models were evaluated, with transmission of viruses as the dependent variable and isolate (FcCa6, 072, Fc104, 020, FcCa4, 035, 042, FcCa1, FcCa70, Fc221), virus (FcMV1 and FcMV2-2) and their interactions as independent variables (Table 3). The difference in the deviance between the null model and the isolate and virus models provides a test for the gross effect of the factors [23]. The statistical significance of the effects was assessed by the p-value (with $p < 0.05$ indicating significant effect). These analyses were performed with the R software version 3.4.3 [24].

The correlation between additional growth and germination variables, due to the small sample size (n = 7) and non-normal distribution of the data, was explored with a non-parametric correlation matrix of the results of transmission rates and the additional variables of seven Spanish isolates (072, Fc104, 020, 035, 042, FcCa70 and Fc221), as proposed by Muñoz-Adalia et al. [18] and Flores-Pacheco et al. [19]. The following variables were included in the analysis: (1) spore germination (%); (2) area of fungal colony (mm^2), measured by the growth of the fungus on PDAS (potato dextrose agar plus 0.5 mg/L streptomycin) for seven days; (3) mycelial growth on Bavendamm's medium containing tannic acid (mm^2/day), measured for five days using photographic methods based on pixel colorimetry (image processing with ImageJ 1.48v); (4) relative necrosis length (mm) produced by *F. circinatum* isolates in *P. radiata* seedlings; and (5) area under the disease progress curve (AUDPC). These analyses were performed using the "Hmisc" package in R software [25].

3. Results

The transmission of the virus' strains was confirmed by the visualization of the PCR products by gel electrophoresis (Figure 1). The transmission rate of every viral strain (%) was high in most of the experiments, varying from 60% to 100% depending on the *F. circinatum* isolate (Figure 2).

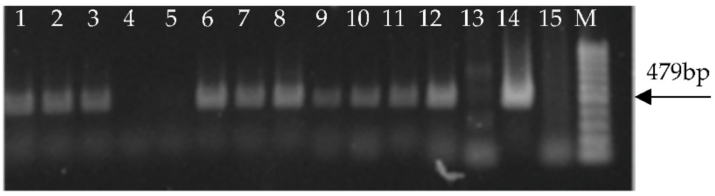

Figure 1. Presence of mitovirus confirmed by gel electrophoresis. Lanes 1–3, 6–13 positive samples; Lanes 4–5 negative samples; Lane 14, positive control; Lane 15, negative control (water and PCR mix), M, marker (GeneRuler 100 bp DNA Ladder).

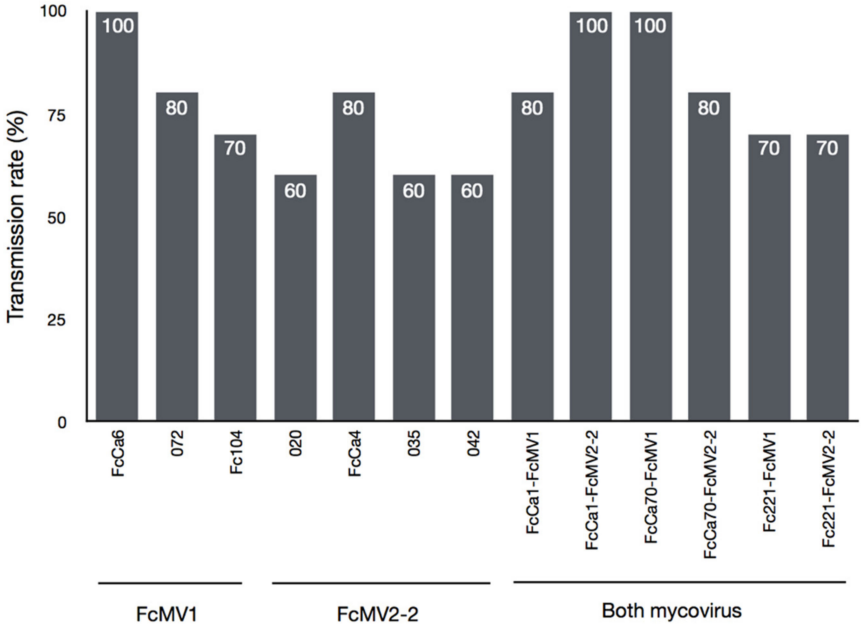

Figure 2. Transmission rate (%) of every strain of the virus in the *F. circinatum* isolates under study.

The rate of vertical transmission of mitoviruses was not correlated with any of the variables analyzed (Table 2), including the mycelial growth of the isolates, either on normal PDAS media, or on modified Bavendamm's medium. We found significant negative correlations among spore germination and mycelial growth rate on Bavendamm's medium ($r = -0.75$, $p = 0.05$) and relative length of necrosis and AUDPC ($r = -0.8$, $p = 0.02$).

Table 2. Spearman correlation matrix for the variables under study. Correlations were considered significant at $p < 0.05$ (shown in bold type).

	Transmission Rate	Area of Fungal Colony [1]	Spore Germination [1]	Relative Length of Necrosis [1]	Area under the Disease Progress Curve [2]	Mycelial Growth [2]
Transmission rate	1					
Area of fungal colony	$r = 0.64$ $p = 0.13$	1				
Spore germination	$r = 0.07$ $p = 0.89$	$r = 0.16$ $p = 0.73$	1			
Relative necrosis length	$r = 0.24$ $p = 0.60$	$r = -0.04$ $p = 0.94$	$r = -0.36$ $p = 0.42$	1		
Area under the disease progress curve	$r = -0.54$ $p = 0.21$	$r = -0.5$ $p = 0.25$	$r = 0.31$ $p = 0.50$	**$r = -0.82$** **$p = 0.02$**	1	
Mycelial growth	$r = -0.21$ $p = 0.66$	$r = -0.14$ $p = 0.76$	**$r = -0.75$** **$p = 0.05$**	$r = 0.04$ $p = 0.94$	$r = -0.14$ $p = 0.76$	1

[1] Source of data: Flores-Pacheco et al. [19]; [2] Source of data: Muñoz-Adalia et al. [18].

In the present study, we found that the probability of transmission of FcMV1 was 0.833 ± 0.048 (mean ± standard error) and that of FcMV2-2 was 0.729 ± 0.053. We applied a logistic model to explore whether the probability of transmission depended significantly on the type of virus or the isolate. Inclusion of isolate, virus and their interactions in the model did not greatly reduce the deviance and none of the variables had significant effects (Table 3). The probability of transmission did not therefore depend on either the strain of mitovirus ($p = 0.16$), on the isolate ($p = 0.59$), or the interaction of factors ($p = 0.99$).

Table 3. Deviance in models of transmission of mitoviruses by *F. circinatum* isolates and strain of virus. The name of the model, a descriptive notation, the formula for the linear predictor, the residual deviance (or goodness of fit likelihood ratio chi-squared statistic), the residual degrees of freedom (Res df), and the Akaike Information Criterion (AIC) are shown.

Model	Notation	Logit (π_{ij})	Res Deviance	Res df	AIC
Null	ϕ	η	138	129	
Isolate (i)	i	$\eta + \alpha_i$	135.92	128	139.92
Virus (v)	v	$\eta + \beta_j$	123.06	120	143.06
Additive	i + v	$\eta + \alpha_i + \beta_j$	123.06	119	145.06
Saturated	i × v	$\eta + \alpha_i + \beta_j + (\alpha\beta)_{ij}$	117.06	117	143.06

4. Discussion

The transmission rates of viral strains recorded in our study were high in most of the isolates. A high rate of transmission was expected, as mitoviruses FcMV1 and FcMV2-2 are associated with the mitochondria that the offspring inherit from the parents [17]. The high transmission rates of mycoviruses are consistent with those reported for asexual spores of other fungal species such as *Ustilaginoidea virens* [26] and *Epichloë festucae* [27] whereas for other pathogens variable rates of virus transmission were observed, e.g., Heterobasidion annosum [13] and CHV1 hypovirus infecting *Chryphonectria parasitica* [28–30]. In some species, usually ascomycetes, there appear to be barriers to the transmission of viruses during sexual reproduction and the formation of sexual spores [27]. While no virus-transmission was observed via sexual spores in *C. parasitica* [31] and of the root rot pathogens *Helicobasidium mompa* and *Rosellinia necatrix* [32], wide-ranging rates of transmission were reported for *H. annosum*, [33], *H. parviporum* [21], and *Lentinula edodes* [34]. The lack of repetition of the

experiment limit us to draw conclusions about the variance and reproducibility of our methodology and to estimate standard errors on the transmission rate. Experiments of less replicates made at different points of time are recommended for future studies to gain more statistical representation.

In our study, the rate of vertical transmission of mitoviruses was not correlated with any of the variables analyzed (germination, growth, necrosis). This result is not consistent with previous findings using a quantitative genetics approach, in which an association between the fitness of the host and its vertically transmitted parasites was expected [35,36]. It is likely that the persistence of either horizontally or vertically transmitted infections are not favored if infection produced by mycoviruses leads to a reduction of host fitness and hence has negative implications for a fungal host population [37].

Whether the presence of *F. circinatum* mitoviruses causes hypo or hypervirulence of the host pathogen is an interesting point of discussion. Previous in vitro studies showed contradictory results; total growth of the fungus on PDAS and spore germination was significantly reduced by the presence of mitoviruses FcMV1 and FcMV2-2 [19] whereas mitovirus-infected isolates did not show different extracellular laccase activity or mycelial growth rate (mm^2/day) on Bavendamm's medium [18]. For in vivo studies, where the mitovirus infected and mitovirus-free isolates were inoculated on young seedlings, there were different results as well. While Muñoz-Adalia et al. [18] observed that FcMV1 led to higher fungal pathogenicity and lower survival of seedlings, Flores-Pacheco et al. [19] obtained that there were no significant differences in the necrotic lesions caused by the pathogen irrespective of whether it was infected with the mitoviruses. In view of the above, it can be concluded that to date, the presence of mitoviruses has no clear pattern in the behavior of the Spanish isolates of the pathogen. One possible explanation could be that the presence of same mycovirus may have different effect on their host, depending on ecological and environmental conditions as previously reported [14,38]. Another hypothesis could be that mycoviruses are no longer active or pathogen's parasites and remain in the mitochondria as symbionts, producing no effects on the host. However, this seems unlikely since Muñoz-Adalia [17] found evidences of viral replication detected by high-throughput sequencing such as antisense vsRNA reads. In other Fusarium species, the presence of mycoviruses has not been related to any morphological changes, although it was observed that *Fusarium graminearum* virus 1 (FgV1) causes hypovirulence [39]. Other studies have shown that the location of mitoviruses in the mitochondria of a host such as *Botritis cinerea* may cause ultrastructural malformations that lead to hypovirulence [40,41]. Mycoviruses may or may not alter fitness, sporulation, and pathogenicity in the host. They can also cause antagonistic interactions between the host and mycorrhizal or saprotrophic fungi [38], tolerance to high concentrations of salt in the growth medium [42], and thermal tolerance [43,44]. Although not studied here, these aspects may be of interest in future research.

The results of our study indicated that the probability of transmission did not depend on either the strain of mitovirus or on the isolate. These results contrast with those obtained for *C. parasitica* [30,36] in which vertical transmission was influenced by the fungal isolate and viral strain. This could be explained by the low genetic diversity of the Spanish population of *F. circinatum* due to its recent introduction and low rate of sexual reproduction [45,46] as almost all clonal isolates are expected to behave similarly and have similar transmission probabilities. The possible dependence of the transmission of *F. circinatum* virus also depends on external factors such as temperature, osmotic potential, and pH. The influence of these factors is also of interest for future studies.

Mycoviruses do not usually have extracellular stages in their life cycles and they are generally strongly dependent on their fungal host for intracellular transmission, with some exceptions [47–49]. In fungal anastomosis, isolates of the same species are not always compatible, even in the same population, because of differences in vegetative compatibility groups (VCGs) and mating types [14], resulting in vegetative incompatibility and provoking programmed cell death [47]. Some of the genes that restrict fungal anastomosis and virus transmission have been identified [50–53]. To date, both the genetic diversity and diversity of the VCGs of the Spanish population of *F. circinatum* have been found to be low and suitable for virus transmission, although this could change if the mating

types reproduced sexually [54]. Although the classic hypothesis considers that the existence of the VCGs lowers the probability of movement of viruses between different species, there are some exceptions to this, and transmission of viruses between incompatible isolates or different species has been observed [13,48,55–58]. This suggests that viruses can move from their fungal host to new hosts [42]. Other studies have indicated that in vitro assays may underestimate the transmission of viruses, especially horizontal transmission, and their ability to overcome the barrier of fungal somatic incompatibility [48,59]. Lee et al. [60] showed that it was possible to transmit viruses between different species in the laboratory via protoplast fusion, whereas Ikeda et al. [61] demonstrated that zinc chloride treatment helped the transmission of the mycovirus because it inhibited heterogenic incompatibility in *R. necatrix*. In the Spanish population of *F. circinatum*, natural transmission between other species was studied by isolating other fungi in which the disease is commonly established, although no virus was detected in any other fungal species [62]. Although there is no clear pattern in the geographical distribution of mycoviruses hosted by *F. circinatum* in Spain [17], it is likely that the fungi that were introduced in Spain were harboring viruses at that time [18]. However, whether the mitoviruses were acquired when the fungus established in Spain or if they came along with the fungus when it was introduced represents a hypothesis that remains unknown. The transmission of viruses may also be influenced by insect vectors; for example, the natural transmission of mycovirus CHV1 by corticolous mites that feed on the virus-infected mycelium highlights the potential use of these arthropods in the natural biological control of chestnut blight [63]. Further research is recommended to clarify whether other methods of vertical and horizontal transmission, including vector-mediated transmission, are possible.

5. Conclusions

One of the requirements for the use of mycoviruses as effective biological control agents is a high rate of virus transmission. The study findings indicated a high rate of transmission of mitoviruses between asexual spores. However, the probability of transmission was not significantly associated with the pathogen isolate or type of virus. The mechanisms whereby viruses are transmitted within populations of pathogens are complex and represent an important topic of research. The study findings also highlight the need for further studies on the horizontal and vertical transmission of mitoviruses, the role of these viruses in *F. circinatum* virulence, the factors involved in the transmission, and the potential use of mitoviruses in managing fungal diseases.

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