INTERACTIONS
VIRUS - FUNGUS - INSECT
IN PINE PITCH CANKER DISEASE

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TESIS DOCTORAL:

INTERACCIONES VIRUS – HONGO – INSECTO EN LA ENFERMEDAD DEL CHANCRO RESINOSO DE LOS PINOS

INTERACTIONS VIRUS – FUNGUS – INSECT IN PINE PITCH CANKER DISEASE

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Interactions virus – fungus – insect in pine pitch canker disease

Interacciones virus **–** hongo **–** insecto en la enfermedad del chancro resinoso de los pinos

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"Consider a rotting tree stump in a forest. You and I casually walking past it on a trail would not give it more than a passing glance. But wait a moment. Walk around the stump slowly, look at it closely- as a fellow scientist. Before you, in miniature, is the equivalent of an unexplored planet".

Edward O. Wilson

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1. Abstract / Resumen

1.1. Abstract

The ascomycetous fungus *Fusarium circinatum* Nirenberg & O'Donell is the causal agent of pine pitch canker disease (PPC), one of the most devastating forest diseases worldwide. PPC has been reported in fourteen countries throughout America, Africa, Asia and Europe. So that, it is though that more than ten million hectares of pine forests and plantations are threatened by this disease in Europe. *F. circinatum* is mainly considered a necrotroph when it infects conifers via stem, however its ecological role as an endophyte in several herbaceous plants as well as a possible saprophyte in litter have been recently reported. The overall aim of this Thesis was to analyze the interactions that take place among members of the PPC pathosystem. For this purpose, we carried out six scientific works focused on the study of relationships between three main elements of the PPC: viruses, fungi and insects. Firstly, a review about the use of fungal viruses (mycoviruses) in biocontrol of forest diseases was elaborated. Secondly, the complete genome of *F. circinatum* was used as query for *in silico* annotating five candidate genes putatively related with fungal virulence. The proposed annotation was based in the comparison with previously characterized genes in other *Fusarium* species and gene ontology. Fungus-virus interaction was addressed in a third study by high throughput sequencing of RNA. In this work, we studied the anti-viral response performed by the fungus against three previously described *Mitovirus* spp. (FcMV1, FcMV2-1 and FcMV2-2). As a result, new data about FcMV2-2´s genome were provided in addition to new insights about the RNA silencing process in the fungus. In a fourth study, deletion mutants of *F. circinatum* lacking the earlier annotated gene *Fcrho1* were generated. Our results revealed not lethal effects of gene deletion even though *Fcrho1* was an effective regulator of vegetative growth *in vitro*. Otherwise, we evaluated the effect of single and co-infection by FcMV1 and FcMV2-2 in laccase enzyme production, mycelial growth and fungal pathogenicity in *Pinus radiata* D. Don seedlings. The results of this article showed a main cryptic effect of virus infection in the fungus although FcMV1 significantly increased *in planta* virulence of its host. This study also proved a rather high rate of extracellular laccase activity in *F. circinatum*. On a larger scale, we studied the mycobiota phoretically associated with two bark beetles (Coleoptera; Scolytinae) considered as main forest pests (*i.e. Tomicus piniperda* L. and *Tomicus destruens* Wollaston) in both infected and asymptomatic pine stands. Fungal community showed a moderate diversity and was dominated by *Sydowia polyspora* (Bref. & Tavel) E. Müll, a fungus considered as an endophyte, a saprophyte as well as a pathogen of conifers. This Thesis provided novel information about ecological interactions between trees, insects and filamentous fungi under an approach based on a network analysis. The results

show the high complexity of interactions virus-fungus-insect in the pathosystem of the PPC and provide information on forest pathology from the point of view of virology applied to biocontrol as well as on cell biology and ecology of communities applied to the protection of forests.

Key words: Ecological network, forest health, fungal pathogenicity, *Fusarium circinatum*, gene annotation, mycoviruses, pathosystem, *Pinus radiata*, RNA silencing and *Tomicus* spp.

1.2. Resumen

El hongo ascomicete *Fusarium circinatum* Nirenberg & O'Donell es el organismo causante de la enfermedad del chancro resinoso de los pinos, una de las enfermedades forestales más devastadoras del mundo. Esta patología ha sido detectada en catorce países de América, África, Asia y Europa. En este último continente, se considera que más de diez millones de hectáreas de pinares y repoblaciones podrían estar en riesgo de ser afectadas por este patógeno. De forma general, *F. circinatum* ha sido descrito como un hongo necrotrofo durante la infección en el tallo de las coníferas, si bien actúa como endófito en numerosas especies herbáceas y se piensa que puede permanecer como saprófito en la hojarasca del bosque. El objetivo principal de esta Tesis se centró en analizar las interacciones que se establecen entre los diversos componentes del patosistema del chancro resinoso de los pinos. Para ello, se llevaron a cabo un total de seis trabajos científicos centrados en el estudio de las relaciones que tienen lugar entre tres elementos principales: virus, hongos e insectos. En primer lugar, se elaboró un trabajo de revisión sobre el uso de virus fúngicos (micovirus) en el control biológico de enfermedades forestales. Por otra parte, se empleó el genoma completo de *F. circinatum* para identificar *in silico* cinco genes plausiblemente relacionados con la virulencia del hongo. Para llevar a cabo la anotación fue necesario el uso de la información existente sobre genes de patogenicidad en otras especies de *Fusarium,* así como métodos de ontología génica. La interacción hongo-virus fue atendida en un tercer estudio mediante técnicas de secuenciación masiva de ARN, observándose la respuesta antiviral del patógeno durante la infección de tres especies de *Mitovirus* previamente descritos (FcMV1, FcMV2-1 y FcMV2-2). Como resultado de esta investigación, se aportó nueva información referente al genoma del micovirus FcMV2-2 así como sobre los posibles mecanismos celulares empleados por el hongo como defensa ante la presencia de ARN vírico. En un cuarto trabajo se procedió a la deleción de uno de los genes previamente anotados (*Fcrho1*) y se estudiaron los efectos fenotípicos de dicha supresión génica tanto *in vitro* como *in vivo*. Los resultados mostraron la ausencia de un efecto letal tras la eliminación del gen, aunque *Fcrho1* resultó un regulador efectivo del crecimiento vegetativo *in vitro*. Adicionalmente, se evaluaron los efectos de la infección sencilla y de la coinfección de FcMV1 y FcMV2-2 en la producción de enzimas lacasas, en el crecimiento micelial y en la patogenicidad *in vivo* de *F. circinatum* a fin de caracterizar sus consecuencias durante la colonización de plántulas de *Pinus radiata* D. Don. Dicho trabajo mostró un efecto mayoritariamente críptico de la infección por parte de ambos virus, si bien FcMV1 incrementó significativamente la virulencia en planta de su hospedante. Asimismo, este estudio demostró una elevada actividad lacasa extracelular en *F. circinatum*. A una escala mayor, se analizó la comunidad fúngica transportada por dos especies de escolítidos (Coleoptera, Scolytinae) causantes de plagas forestales: *Tomicus piniperda* L. y

Tomicus destruens Wollaston tanto en pinares afectados por la enfermedad como asintomáticos. La comunidad de hongos estudiada resultó moderadamente diversa y fuertemente dominada por *Sydowia polyspora* (Bref. & Tavel) E. Müll, un hongo descrito como endófito, saprófito y patógeno de coníferas. El estudio de dicha comunidad aportó nueva información sobre las relaciones ecológicas que tienen lugar en la naturaleza entre los árboles, los insectos y los hongos filamentosos bajo una aproximación de red ecológica. Los resultados de esta Tesis ponen de manifiesto la complejidad de las interacciones virus-hongo-insecto y aportan nuevos datos sobre la enfermedad del chancro resinoso de los pinos en el ámbito de la virología destinada al control biológico, la biología celular y la ecología de comunidades aplicada a la protección de las masas forestales.

Palabras clave: Anotación génica, *Fusarium circinatum,* micovirus, patogenicidad fúngica, patosistema, *Pinus radiata,* sanidad forestal, red ecológica, silenciamiento del ARN y *Tomicus* spp.

2. Introduction

2.1. The pathosystem: a network of interactions

In nature several biological domains include taxa with plant pathogenic behaviour, being bacteria, fungi and protists especially important in forest health. In the kingdom Fungi, the main clades including phytopathogenic taxa are the phyla Ascomycota and Basidiomycota (subkingdom Dikarya), Glomeromycota (formerly Zygomycota) and class Deuteromycetes (Hibbett *et al.*, 2007; Lanier *et al.*, 1978; Robert *et al.*, 2005). Filamentous fungi are considered one of the most important challenges for crop and forest protection worldwide. More specifically, invasive forest pathologies have become more important in recent times as they have been widespread as a result of globalization (Wingfield *et al.*, 2015). These pathologies have an important incidence either at ecological or socioeconomic levels worldwide, thus threatening the conservation of natural resources and biodiversity as well as reducing economical yield of forestry.

The interaction between a plant and a fungus becomes a disease when environmental conditions enable the development of alterations in the normal state of the host (susceptible state) attributable to the fungus in a virulent state. This phenomenon has traditionally been summarized in the "disease triangle" in which the corners represent the pathogen, the host and the environment, although it also can be considered a "disease tetrahedron" if human activity is included as a fourth vertex (Zadoks & Schein, 1978). Disease can be differentiated from simple parasitism since the damage is extensive and persistent (Gilbert, 2002). Among pathologies that affect woody plants, damping-off diseases are particularly important in nurseries, while cankers, root rot, wilt and dieback are among the most important diseases in forests and plantations (Butin, 1995).

The classic concept of a "pathosystem" (Robinson, 1976) represents the idea of a subsystem formed by at least one pair of elements (*i.e.* one plant host and one parasite) harboured by a higher ecosystem. This concept has been broadly used in plant pathology in the past; however, more recent interpretations have increased the members or nodes in the systems and have described the pathosystem as a combination of a plant host, a pathogen and a vector species (Eigenbrode *et al.*, 2018). In addition, the complexity of biological interactions that take place in the field extends the above-mentioned three roles of the system involving other nodes that participate more or less directly, positively or negatively in the development of a disease. Some examples of these types of multiple associations have been reported in the scientific literature and include pathosystems formed by virus-fungus-plant (Ahn & Lee, 2001), fungus-phoretic

mite-insect-plant host (Moser *et al*., 2010) and fungus-protist-plant host (Halo *et al*., 2018). The pathogenic role could be played by more than one species in a single system, thus increasing the complexity of the interaction; nevertheless, some additional elements may participate by attenuating or even increasing the virulence of the pathogen (*e.g.* viruses that reduce the aggressiveness of a fungus; see below). Host density may also affect the virulence of the pathogen and the presence or absence of a secondary host could trigger changes in the disease development. In the same way, the occurrence of carriers and vectors strongly modulates the interaction between nodes in the system as a smaller population of vectors may reduce the severity of the disease. Finally, abiotic factors (*e.g.* nutrient availability or drought-induced stress) usually determine the degree of susceptibility of hosts and thus act as predisposing factors (Termorshuizen, 2016). Hence, the study of a specific forest pathology requires consideration of these complex interactions for a better understanding of the aetiology of the disease.

The interactions within the pathosystem have implications on a larger scale. Thus, the outbreak of a specific forest disease may entail changes in *e.g.* species distribution, tree age structure and biodiversity. This approach is addressed by the landscape pathology which studies the interactions among disease and the rest of the ecosystem, including abiotic patterns and multi-level ecological processes (Holdenrieder *et al*., 2004). In this regard, forest management (*i.e.* woodland fragmentation, substitution of susceptible species, etc.) may significantly affect some important aspects of forest health such as the availability of a suitable host, connectivity between host populations or abundance of vectors. Phytopathogens can also act as regulatory elements in plant communities as they can reduce the dominance of their host, favouring the fitness of other taxa and therefore acting as a stabilizing agent (*sensu* Mordecai (2011)). By contrast, in an environment inhabited by taxa with different degrees of susceptibility, the disease may promote destabilization and thus favour the abundance of tolerant species over others. Such considerations synthesize the idea of the pathosystem as a network in which interactions between nodes range in a continuum that affects and is affected by the surrounding environment.

This doctoral research focuses on the pine pitch canker (PPC, formerly PPCD) pathosystem, with special emphasis on the virus-fungus-insect interactions (Figure 1). More specifically, the Original Articles that form this Thesis (referred by their roman numbers in the text) are organized in increasing order of complexity of interactions. Original Article I reviewed the knowledge of mycoviruses and their use as biocontrol agents in forest health. Original Article II reports a study of the causal agent of PPC (*Fusarium circinatum* Nirenberg & O'Donell) in which five new candidate genes were identified using the complete fungal genome as query.

Original Article III addresses the single interaction between the previously mentioned nodes (*i.e.* virus and fungus) and centres on fungal response during mycoviral infection. Original Article IV reports investigation of the fungus-plant interaction when a single fungal gene was deleted, and Original Article V considers the complex interactions between virus, fungus and plant host. On a larger scale, the study reported in Original Article VI investigated the multiple interactions between fungi, vector *sensu lato* (or carrier) insects and pine hosts in stands infected with or threatened by PPC.
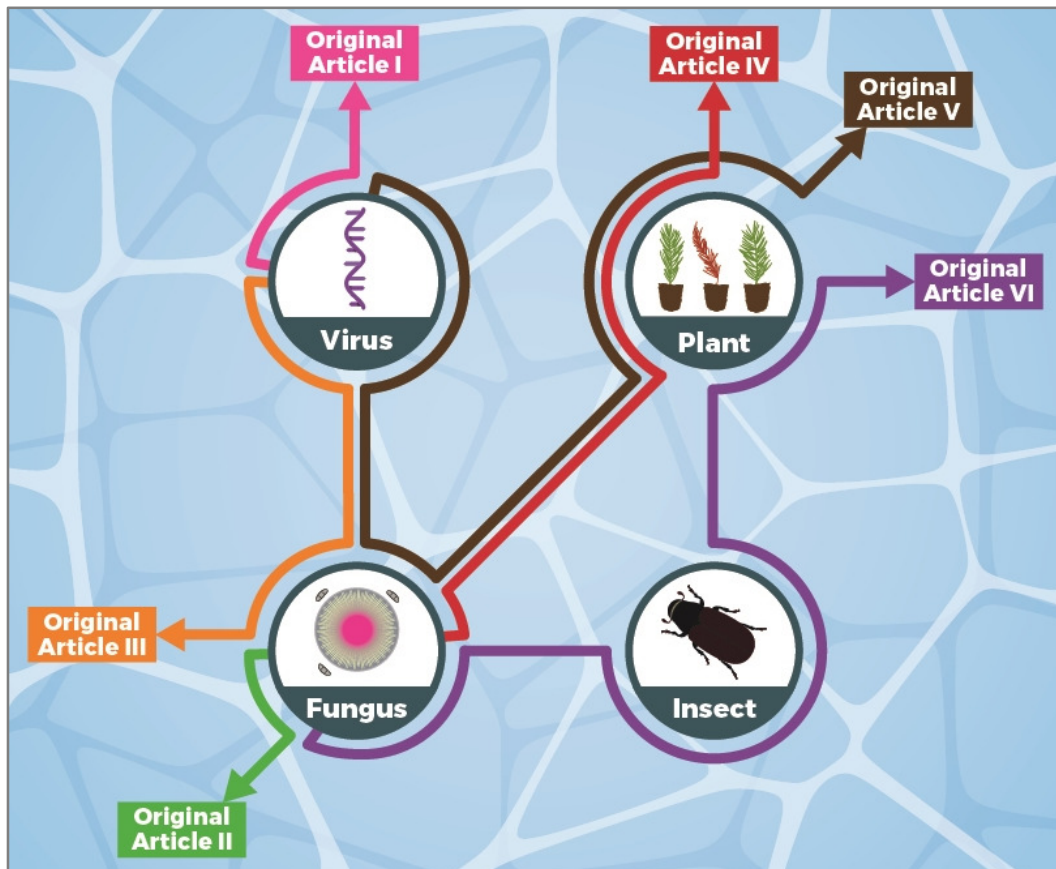
Figure 1. Outline of the pathosystem under study. The concept map includes all Original Articles forming part of the Thesis.

2.2. Fungus – plant interactions in the pine pitch canker pathosystem

The fungus *F. circinatum* (Ascomycota; Nectriaceae; teleomorph: *Gibberella circinata* Nirenberg & O'Donell) is the organism that causes one of the most devastating forest disease worldwide: the pine pitch canker. The fungus is thought to be native to Mexico and the south-eastern Unites States (Figure 2) although it is also possible that it spread from Mexico to the USA (Gordon *et al*., 2015) where the species was first reported in 1946 (Hepting & Roth, 1946). The PPC fungus was reported at that range as associated with chronic infections and causing occasional outbreaks in *Pinus* spp. plantations and seed orchards (Aegerter *et al*., 2003; McCain *et al*., 1987). The fungus has been introduced in fourteen countries throughout the

world and has been detected in Africa (South Africa), American non-native range (Haiti, Chile, Uruguay, Colombia and Brazil), Asia (Japan and South Korea) and Europe (Spain, France, Italy and Portugal) (Figure 2). In Europe, this pathogen is included in the A2 list of quarantine pests developed by the European and Mediterranean Plant Protection Organization (EPPO), and it has been estimated that more than 10 million hectares of pine forests and plantations are endangered by this fungus (COST Action FP1406, 2014). In addition, the current scenario of global warming could facilitate the natural spread of PPC in the mid-term along the Iberian Peninsula, south-western France and southern Italy according to Möykkynen *et al.* (2015). Conversely, the models suggested that central and northern European countries may be free of the disease unless an eventual introduction of the pathogen takes place.
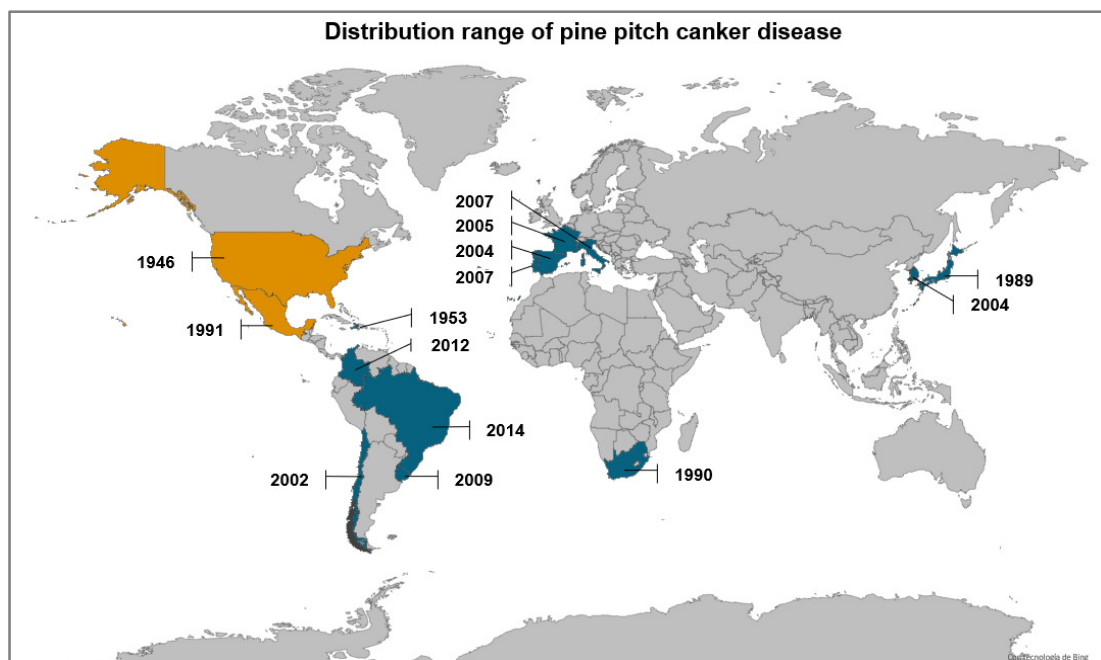
Figure 2. Distribution range of *Fusarium circinatum*. Dates refer to those cited in first reports. Map up-dated in March 2018. Countries shaded in orange: Possible native range.

Macroscopically, *F. circinatum* has hairy white mycelium that become coloured when mature ranging from pale-pink to purple in potato-dextrose agar culture medium. The morphological diagnosis is usually based on microscopic features of colonies growing in Spezieller Nährstoffarmer agar medium. Under these conditions, it is possible to note the coiled sterile hyphae (circini) and the absence of chlamydospores. *F. circinatum* mainly reproduces asexually in field conditions (Gordon *et al*., 2006a) by producing either micro- or macroconidia, even though microconidia are rather abundant. These single-cell spores are oval, ovoid or allantoid in shape and are produced in mono- and polyphialides. By contrast, macroconidia are curved, slender and usually have three septa. Macroconidia are produced in orangish sporodochia, which are difficult to observe in culture conditions (Leslie & Summerell, 2006).

The main spread pathways of this fungus are via air- and soilborne spores, vector insects *sensu lato* (see below) and anthropogenic activities (*e.g.* movement of asymptomatic plants, use of contaminated pruning tools, etc.). Recent studies have provided new insights into spore dispersion by wind: Dvořák *et al.* (2017) found that spores could be dispersed at least 1 km by wind of speed 5 m / s. The study also showed an almost constant presence of spores throughout the year in Spain, in contrast to previous findings indicating an important level of seasonality (Garbelotto *et al.*, 2008). According to Dvořák *et al.* (2017), low temperatures and low surface humidity in the host also favour spore release. Regarding soilborne spores, Serrano *et al.* (2017) performed a long-period study in which spore survival was evaluated in pine branches, needles and soil. These researchers failed to re-isolate *F. circinatum* either from vegetal material or from soil after 28 months period and 8 months respectively. In consequence, litter and soil did not appear to be important as long-term reservoirs of inoculum, probably due to the absence of long-resting structures such as chlamydospores.

The genome of this phytopathogen includes eleven chromosomes and shows a median total length of 439486 Mb (Wingfield *et al*., 2012). The presence of a twelfth chromosome was discussed in relation to AFLPs analysis reported by De Vos *et al.* (2014), although this chromosome is known to be meiotically unstable and can be deleted or duplicated in strains of other *Fusarium* spp. Hence, the National Center for Biotechnology Information (NCBI) currently includes eleven defined chromosomes in the public database. Annotation of the genome is not complete, and further research is required to identify encoding genes related to virulence factors, as Slinski *et al.* (2016) reported that virulence is an inheritable phenotypic character. Regarding population genetics, it is thought that *F. circinatum* isolates infecting pine stands in northern Spain are genetically homogeneous and the number of vegetative compatibility groups (VCG or vc) is low (Iturritxa *et al*., 2011; Pérez-Sierra *et al*., 2007). Conversely, VCGs are diverse in the natural range of the pathogen (Wikler & Gordon, 2000). Berbegal *et al.* (2013) studied the population structure of the pathogen in Spain and found only two multilocus genotypes consistent with the geographical distribution of infected stands. These considerations support the idea of two different introductions of the pathogen in Spain.

The PPC fungus can infect more than sixty species of *Pinus*, although Monterey pine (*Pinus radiata* D. Don) has been shown to be the most susceptible host. Other conifers can become infected by this fungus, being Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) quite vulnerable (Gordon *et al*., 2006b). The appearance of resin-soaked cankers on the trunk and thick branches is the most evident symptom of the disease in mature trees. Resin bleeds abundantly from cankers and covers the affected area (Figure 3). These lesions can occasionally girdle the stem or branches predisposing the tree to die by breaking during

windstorms (Wingfield *et al.*, 2008). In addition, deformations caused by PPC drastically reduce the value of timber making the use of logs for the wood industry impossible. Other symptoms are mortality of cones and female flowers as well as wilting and crown dieback caused by water flow disruption. *F. circinatum* also causes severe damping-off, wilting and tip dieback in seedlings. The fungus damages seedlings either in a pre-emergence state or after the appearance of germlings, representing an important threat in forest nurseries where mortality rates can reach up to 90% (Gordon *et al.*, 2015; Martínez-Álvarez *et al.*, 2014).
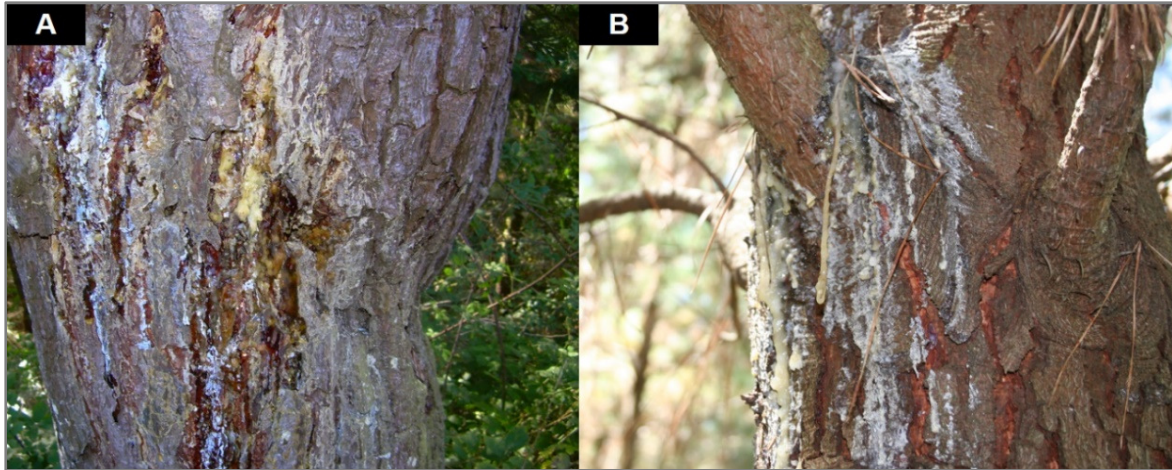
Figure 3. PPC symptomatology in mature trees. A: resin-soaked canker in trunk; B: resin bleeding in branches and stem. Images reproduced by courtesy of the Forest Pest and Diseases Laboratory (University of Valladolid).

F. circinatum is described as a necrotroph in conifers, *i.e.* the fungus progresses as it necrotizes healthy tissues of the host and it consumes the nutrients made accessible during the infection (García-Guzmán & Heil, 2014). The fungus lacks specific structures for infection such as haustoria or appressoria (Mendgen *et al.*, 1996) and it is thought that the presence of injuries facilitates access of the fungus to the potential host (Gordon & Reynolds, 2017). Martín-Rodrigues *et al.* (2013) studied the infection process in *P. radiata* and described the spatial colonization pattern of the fungus. The first phase consisted of intercellular colonization of medullary rays, phloem and cortex in order to access plant reserves of polysaccharides and lipids. In this period, which lasts around one week after inoculation, the fungal biomass increases greatly due to spore germination and hyphal growth. At later stages of infection, the PPC fungus spreads vertically along the stem through the cortex and phloem periphery, resin ducts and axial tracheids in xylem and the parenchyma of pith. Biomass accumulation then decreases and becomes stable as the first symptoms appear. These authors also report that resin production as a host response does not seem to negatively affect *F. circinatum*. These findings suggest a significant role of cell wall-degrading enzymes, such as cellulase, cutinase,

endopolygalacturonase and laccase, during the host colonization, which deserves further research. On the other hand, some species of the genus *Fusarium* are well-known producers of mycotoxins, such as beauvericin, enniatins, fusaproliferin and moniliformin (Jestoi, 2008). Specifically, it is known that *F. circinatum* segregates beauvericin (Fotso *et al.*, 2002), which has toxic effects in humans and livestock. Although the phytotoxicity of this compound has been demonstrated (Paciolla *et al.*, 2004), its importance in fungal infection and overcoming the host´s defence mechanisms is not known. On the other hand, Martín-Rodrigues *et al.* (2015) studied pine root infection by *F. circinatum*. These researchers found that the fungus penetrates the roots, without need for natural injuries, and subsequently colonizes phloem and xylem to sporulate inside tracheids. In the same study, some mycelial morphologies were observed (*i.e.* mycelial strands, runner hyphae and hyphopodia-like structures, among others), while only the finest branches and roots were severely damaged. Recent studies also showed that the fungus can remain in a latent state in asymptomatic pines (Hernández-Escribano *et al.*, 2018a; Swett *et al.*, 2018). Consequently, the relationship between the PPC fungus and the pine host could be mainly biotrophic (*i.e.* microorganisms that penetrate living host tissues to cause damage) when infection starts in roots (or branches under certain conditions) being able to become necrotrophic during stem colonization.

The hemi-biotrophic behaviour of *F. circinatum* in herbaceous plants has been reported (Gordon & Reynolds, 2017) along with possible saprophytic behaviour in conifer debris (Serrano *et al.*, 2017). This ascomycetous fungus has been characterized as an endophyte (*i.e.* a microorganism that asymptomatically inhabits healthy tissues of the host) of grasses (Swett *et al.*, 2014) and maize (*Zea mays* L.) (Swett & Gordon, 2015). In the latter, *F. circinatum* colonizes cortical and epidermal tissues intercellularly without causing damage. Thus, Swett and Gordon (2015) considered that this fungus may be an endophyte of herbaceous plants that eventually colonize susceptible pines as a necrotroph, and grasslands thus become an important reservoir of inoculum. Hernández-Escribano *et al.* (2018b) found *F. circinatum* in leaves and stems of five herbaceous species in infected pine stands. They also reported the possible vertical transmission from mature plants to offspring, thus increasing the complexity of the interactions of PPC with understory plants.

PPC causes severe economic losses and ecological alterations worldwide; nevertheless, measures for effective control of the disease are already being developed. Several control methods have been assayed under laboratory and greenhouse conditions. Thus, both hot water and hydrogen peroxide treatments have proven suitable for reducing seed contamination (Agustí-Brisach *et al.*, 2012; Berbegal *et al.*, 2015). The use of suppressive composts (López-López *et al.*, 2016) and essential oils (Iturritxa *et al.*, 2017) has also shown positive results.

The participation of living organisms in disease prevention (*i.e.* biological control) has also shown promising results, and bacteria (Iturritxa *et al*., 2017; Soria *et al*., 2012) and other fungi (Martínez-Álvarez *et al*., 2012, 2016) have been tested for their ability to control *F. circinatum*. Despite these advances, there is not yet an effective method available for controlling the disease in forests and plantations, which strongly limits management of the disease in the field. Environmentally friendly methods (*i.e.* biological control together with integrated forest management) are therefore the most promising and desirable alternatives for reducing the negative impact of *F. circinatum* (Bezos *et al*., 2017).

2.3. Virus – fungus interactions: mycoviruses and biocontrol

Viruses are non-cellular entities with an extrachromosomal phase and whose genomes are formed by DNA or RNA molecules that require a host for replication to occur (Jagdale & Joshi, 2018). They have been traditionally described as obligate parasites as they need the cell machinery of an organism to multiply and spread to new hosts. Nevertheless, novel approaches have highlighted that viruses establish complex ecological interactions with their hosts that range from strict parasitism (*e.g.* virus that causes spotted wilt in plants) to mutualism (*e.g.* viruses that suppress anti-feeding compounds in plants favouring their insect host). These interactions are flexible, and they can vary from beneficial to detrimental depending on environmental conditions (Fraile & García-Arenal, 2016; Roossinck, 2015a,b)

The first report of a virus infecting a fungus (mycovirus) appeared in 1962 and involved cultured *Agaricus bisporus* (J.E. Lange) Imbach as the host (Hollings, 1962). Mycoviruses seem to be ubiquitous in nature and they infect most fungal clades, including yeast, filamentous fungi and edible mushrooms (Bozarth, 1972; Pearson *et al*., 2009). The evolutionary relationship between mycoviruses and fungi remains under discussion. One of the most accepted hypothesis is based on an ancient co-evolution between fungi and mycoviruses supported by the use of mitochondrial genetic code by some viruses or the need for fungal enzymes for capsid protein processing. Another hypothesis postulates that mycoviruses may have jumped from plant hosts to fungal endophyte hosts more recently. This idea may be supported by the close phylogenetic similarity between some mycoviruses and plant viruses (Pearson *et al*., 2009).

The study of mycoviruses has advanced greatly in the past few decades, resulting in the description of a total of sixteen families, although some taxa remain unclassified. The families are grouped by the type of nucleic acid molecules comprising the viral genome. Seven families include members formed by double-stranded RNA (dsRNA: *Chryso-*, *Endorna- Megabirna-*,

Partiti-, *Quadri-*, *Reo-* and *Totiviridae*), while five additional families include RNA viruses in a positive state, *i.e.* ready for replication without previous participation of RNA replicase (ss(+)RNA: *Alphaflexi-*, *Barna-*, *Gammaflexi-*, *Hypo-* and *Narnaviridae*). The taxonomic position of four families is currently unclear and is formed by negative single-stranded RNA viruses (ss(-)RNA; *Mycomononegaviridae*), reverse transcribing RNA viruses (ss(+)RNA-RT; *Metaviridae* and *Pseudoviridae*) and circular single-stranded DNA viruses (unassigned) (Ghabrial *et al.*, 2015).

Mycoviruses have attracted the interest of plant pathologists, as some of them are able to promote weakness state (hypovirulence) in their fungal hosts (Xie & Jiang, 2014). The best-studied case of virus-based biocontrol (or virocontrol) is the interaction between Cryphonectria hypovirus 1 (CHV-1; *Hypoviridae*) and the causal agent of chestnut blight (*Cryphonectria parasitica* (Murrill) M.E. Barr). Infection with this mycovirus greatly reduces the *in planta* virulence, negatively affects conidiation and alters colonial morphology and mycelial growth (Zamora *et al.*, 2017). This *Hypovirus* sp. has been satisfactory used to control chestnut blight in Europe, even though its use was not successful in North America due to the high diversity of VCGs in fungal populations (Milgroom & Cortesi, 2004). Briefly, the process consists of inoculating CHV-1-infected isolates in chestnut trees (*Castanea* spp.) damaged by *C. parasitica*, belonging either inoculated or local wildtype strains to the same VCG. When a heterokaryon forms, mycoviruses are transferred from hypovirulent to virulent strains. As a result of mycelial fusion and horizontal viral transmission, only healing cankers appear in trunk resulting in tree recovery by callus formation over the lesion (Heiniger & Rigling, 1994) . The successful use of CHV-1 to control *C. parasitica* in field conditions has popularized consideration of mycoviruses as promising biocontrol agents for plant protection.

Three putative members of the genus *Mitovirus* (*Narnaviridae*) have been identified as infecting *F. circinatum* (Martínez-Álvarez *et al.*, 2014). All of these are ss(+)RNA viruses, lack capsids and replicate inside the mitochondria of the fungus. More specifically, *Fusarium circinatum* mitovirus 1 (FcMV1) is 2419 nucleotides in length and has a single open reading frame (ORF) that encodes an RNA-dependent RNA polymerase (RdRp) 731 amino acids in length. Two variants of Fusarium circinatum mitovirus 2, *i.e.* FcMV2-1 and FcMV2-2, were also identified by these authors. These strains were found to be respectively 2193 and 1973 nucleotides in length and to have a single ORF that encodes RdRp of 724 and 596 amino acids in length. Vertical transmission of FcMV1 and FcMV2-2 via microconidia was studied by Romeralo *et al.* (2018) who found that these mitoviruses infect asexual spores in a rate of 60-100%. These authors also reported that the probability of viral transmission was independent of either the mitovirus strain under consideration or the fungal isolate. In this regard, viral

prevalence in Spanish isolates of the PPC fungus has been described as moderate-high (Vainio *et al.*, 2015). This research also suggested variations in viral abundance between fungal populations throughout northern Spain. So that, isolates of *F. circinatum* from Cantabria region seem to frequently harbour mitoviruses (*e.g.* infection rates reached 83.3% and 85.7% in Rionansa and Comillas, respectively) whereas other areas such as Basque Country did not provide positive detections of the studied *Mitovirus* spp. The apparently common association between mycoviruses and *F. circinatum* in this non-native range, provides a suitable framework for studying molecular and cellular effects of viral infection in the PPC fungus.

2.4. Fungus – insect interactions: pathogens in phoretic communities

Insects are one of the most diverse and successful animal groups. Some of these establish complex interactions with fungi, and ambrosia and bark beetles (Coleoptera; Scolytinae) are considered a paradigmatic case of holobionts (*i.e.* animals that establish complex mutualisms and symbioses with microbes) (Six, 2013). Most members of the Scolytinae are secondary pests that colonize stressed or dying trees, although populations of some species may increase to epidemic level enabling them to colonize and kill healthy hosts. These outbreaks have severe ecological impacts, mainly in boreal forests, in which economic losses may be drastic (Popa *et al.*, 2012; Vega & Hofstetter, 2015). This negative effect in forestry revenues was also reported by Amezaga (1992) in northern Spain, where damage caused by the pine shoot beetle (*Tomicus piniperda* L. Col.; Scolytinae. Figure 4) reached around 57 million euros in 1989.

Bark beetles usually carry diverse communities of filamentous fungi in their guts and on their exoskeletons (phoretic associations) (Jankowiak & Kolařík, 2010; Giordano *et al.*, 2013; Romón *et al.*, 2014); in fact, some genera have cuticular structures termed mycangia that facilitate the transport of fungal propagules as well as maintain the required conditions (*e.g.* moisture, temperature, etc.) for spore survival (Balachowsky, 1962; Six, 2012). These transported fungi can benefit insects in multiple ways by providing food for larvae, taking part in the detoxification of plant-response compounds or providing access to nutrients stored in woody tissues (Klepzig & Six, 2004). Mutualistic fungi are not the only guild present in mycobiota carried by bark beetles, and some entomopathogens, endophytic commensals or phytopathogens can appear in those communities (Six & Wingfield, 2011). On the other hand, the life cycle of bark beetles is highly compatible with being able to act as vectors of forest diseases. More specifically, some beetles breed in the trunks of weakened trees (*e.g.* damaged by mycosis) and eventually become loaded with fungal spores. Emerging adults fly to the crown of healthy hosts to perform the maturation feeding and in some cases to hibernate.

They can thus inoculate spores of phytopathogenic fungi into a new host and consequently propagate the disease (Santini & Faccoli, 2014). Despite their small body size, bark beetles can fly long distances; for instance, *Dendroctonus ponderosae* Hopkins and *Dendroctonus pseudotsugae* Hopk. have been reported to travel 1000-2500 m in a flight season (Robertson *et al*., 2009; Withrow *et al*., 2013). Regarding bark beetles distributed throughout Iberian Peninsula, *Ips sexdentatus* Börner can disperse up to 4000 m (Jactel, 1991), whereas *T. piniperda* usually covers shorter distances (*i.e.* ≤ 400 m), although some individuals are able to disperse up to 2000 m (Barak *et al*., 2000). These considerations together with the exponential population dynamics of this insects indicate them as one of the most effective vectors *sensu lato* of forest diseases.

Figure 4. Side view of *Tomicus piniperda* L. (Coleoptera, Scolytinae).

F. circinatum is known to establish phoretic associations with a wide range of invertebrates, including molluscs and arthropods (*i.e.* crustaceans and insects) (Storer *et al*., 2004). In its native range, the PPC fungus is putatively vectorized by several species of bark beetles such as *Conophtorus radiatae* Hopk., *Ips mexicanus* Hopk., *Ips paraconfusus* Lanier, *Ips plastographus* LeConte, *Pityophthorus carmeli* Swaine and *Pityophthorus setosus* Blackman. In addition, the dry twig beetle *Ernobius punctulatus* (Col.; Anobiidae) has also been proposed as vector of the disease (Brockerhoff *et al*., 2016). Outside the American range of the pathogen, Romón *et al.* (2007, 2008) studied the phoretic associations between PPC and some members of Curculionidae in northern Spain, observing that the fungus was frequently isolated from the weevil *Brachyderes incanus* L. (Col.; Entiminae) and the bark beetles *Hylastes attenuatus* Erichson, *Hylurgops palliatus* Gyllenhal, *Hypothenemus eruditus* Westwood, *I. sexdentatus*, *Orthotomicus erosus* Wollaston and *Pityophthorus pubescens* Marsham. The species showing the closest phoretic relationship with the fungus were *P. pubescens* (phoresy rate of 25%), *B. incanus* (14.28%) and *H. palliatus* (11.96%) followed by

the other taxa studied (phoresy rates < 9% in all cases). The interaction between *P. pubescens* and *F. circinatum* was studied in detail by Bezos *et al.* (2016), who observed infrequent fungus-insect interactions (≤ 2.04% phoretic association; 0.75% fungal recovery from galleries) in contrast to previously reported results. These findings suggest that associations between phytopathogens and bark beetles are variable and may be driven by complex environmental factors.

The pine shoot beetle (*T. piniperda*; Figure 4) is native of Eurasian region and selects numerous species of *Pinus* as well as *Picea* spp. and *Larix* spp. as hosts (Långström *et al.*, 2002). In the Iberian Peninsula, *T. piniperda* mainly inhabits pine woodlands in central and northern areas and is sometimes found in sympatry with the species *Tomicus destruens* Wollaston. Unlike the pine shoot beetle, *T. destruens* predominantly occurs in the Mediterranean Basin where it can cause severe damage in thermophilic forests (Gallego *et al.*, 2004; Vasconcelos *et al.*, 2006). *T. piniperda* can co-occur with *Tomicus minor* Hartig which also has a Palearctic range and mainly colonizes damaged trees (Gallego *et al.*, 2004). Regarding feeding habits, *T. piniperda* feeds on shoots of healthy pines during its maturation feeding stage, although it is considered a secondary pest in relation to trunk infestation in Europe (Annila *et al.*, 1999; Cedervind *et al.*, 2003). By contrast, this insect is an aggressive pest of pine plantations in the USA where it was firstly detected in Ohio in 1992 (Haack & Poland, 2001). This bark beetle is a well-known carrier of plant pathogens and of blue stain fungi (Silva *et al.*, 2015; Solheim *et al.*, 2001) whereas its possible association with *F. circinatum* was initially considered weak (Romón *et al.*, 2008). Later research by Bezos *et al.* (2015) provided new insights into the participation of *T. piniperda* in the PPC pathosystem. These authors showed that *T. piniperda* should be considered a possible vector of *F. circinatum* in northern Spain as it complies with Leach´s postulates (Leach, 1940). They showed that *T. piniperda* carries viable spores of the pathogen (phoresy rate reached ~4% over three years of sampling) in natural habitats, that it colonizes either healthy or infected trees and that its feeding habits inside the current shoots may facilitate contact between *F. circinatum* and susceptible hosts. These findings provided a new framework of interactions in which the role of bark beetles in the epidemiology of PPC should not be neglected as they connect two key elements of the system (*i.e.* host and pathogen) with other relevant elements such as the fungi within their mycobiota. Hence, *Tomicus* species should not be neglected as key-factor in the epidemiology of PPC, considering their widespread distribution and their ability to explore different habitats. Furthermore, they create feeding wounds in healthy pines and switch between different trees during their life cycle, connecting two key nodes of the system (*i.e.* host and pathogen) with other relevant elements such as the fungi within their carried mycobiota.

3. Objectives

3.1. General objective

Pine pitch canker represents one of the most important threats to pine forests, plantations and nurseries worldwide. Study of the factors involved in the development of the disease is required due to the high economic and ecological impact of *F. circinatum*. In this respect, factors ranging from the molecular basis of fungal virulence to ecological associations with other organisms in the ecosystem should be addressed to advance the understanding of the aetiology of PPC. The overall aim of this doctoral research was to investigate *F. circinatum* at biological and ecological level, with a special focus on how it interacts with the three *Mitovirus* species hosted by this pathogen as well as the relationship with the plausible insect vector *T. piniperda* and its associated mycobiota.

3.2. Specific objectives

[1] To evaluate the potential of mycoviruses based on the existing knowledge as well as the successful treatments involving them in the biocontrol of forest diseases (I).

[2] To further characterize *F. circinatum* at genomic level by annotating pathogenicity-related genes using the complete genome of the fungus as query (II).

[3] To investigate the anti-viral response of *F. circinatum* through the RNA silencing process during infection of FcMV1, FcMV2-1 and FcMV2-2 (III).

[4] To analyze the phenotype consequences of deleting a single gene related to a cell signalling pathway in the PPC fungus (IV).

[5] To study the *in vitro* and *in planta* phenotypic effects of viral infection with FcMV1 and FcMV2-2 (V).

[6] To examine the mycobiota associated with *T. piniperda* both in pine stands infected with *F. circinatum* and in asymptomatic stands in order to investigate the ecological interactions between fungi, insects and host trees (VI).

4.1. Field and microbiological methods

4.1.1. Sample preparation and culture techniques

4.1.1.1. Field sampling

► Collection of pine shoots in two pine stands in northern Spain (VI).

4.1.1.2. Handling of plant material

► Seedlings acclimation and handling (IV & V).

4.1.1.3. Culture methods and measurement of colony variables

► Solid media (III, IV, V & VI).
► Liquid media (IV & V).
► Measurement of colony growth and shape (IV, V & VI).
► Observation of colonies by light microscopy (IV & VI).

4.1.1.4. Sporal suspensions

► Preparation of spore suspensions and adjustment of concentration (IV & V).
► Obtaining spore suspensions by sonication (VI).

4.1.2. Enzyme extraction and analysis

► Extraction of extracellular laccase enzymes and measurement by the Bavendamm test, photography (pixel colourimetry) and spectrophotometry (V).

4.1.3. Inoculation techniques and assessment of symptoms

► Stem inoculation and measurement of symptomatology. Use of area under disease progress curve (AUDPC) method (IV & V).

4.1.4. Processes involving bacteria

► Measurement of the density of bacterial colonies by spectrophotometry (IV).
► Transformation of fungal cells mediated by *Agrobacterium tumefaciens* (Smith & Townsend) Conn. (ATMT) (IV).
► Electroporation of *A. tumefaciens* (IV).
► *Escherichia coli* Escherich transformation with DNA construction (IV).

4.2. Molecular biology

4.2.1. DNA and RNA extraction

► DNA and RNA extraction (IV & VI), total RNA extraction (III), plasmid DNA extraction (IV).

4.2.2. Polymerase chain reaction (PCR) and reverse transcription-polymerase chain reaction (RT-PCR)

► Amplification of specific genomic regions by PCR and RT-PCR (III, IV & VI).

4.2.3. Separation of DNA and RNA fragments

► Electrophoresis in agarose gel (III, IV & VI).

4.2.4. Generation of deletion mutant

► Generation of deletion mutant using a modified version of OSCAR protocol (IV).

4.2.5. Sanger sequencing

► Single read sequencing of DNA (III & VI).

4.2.6. Next generation sequencing

► High throughput sequencing of small RNAs (III).

4.3. Identification of organisms and viruses

4.3.1. Eukaryotic organisms

4.3.1.1. Fungi

► Use of fungal isolates from the collection belonging to the Laboratory of Forest Pests and Diseases (University of Valladolid) and previously identified by morphological and molecular methods (III, IV & V).

► Identification of fungal taxa in relation to morphological features and operational taxonomical units (OTUs) formation (VI).

► Validation of OTUs by fingerprinting by PCR amplification of M13 minisatellite (VI).

► Molecular identification of fungal taxa by PCR amplification of internal transcribed spacer (ITS) region (VI).

4.3.1.2. Insects

► Molecular identification of *T. piniperda* and *T. destruens* by PCR amplification of the cytochrome oxidase I (*COI*) gene (VI).

4.3.2. Mycoviruses

4.3.2.1. Mitovirus *species*

► Use of fungal isolates from the collection belonging to the Laboratory of Forest Pests and Diseases (University of Valladolid) and previously identified as infected by mycoviruses or mycovirus-free (III & V).

► Molecular identification of Fusarium circinatum mitovirus 1, 2-1 and 2-2 (FcMV1, FcMV2-1 and FcMV2-2 respectively) by specific primer pairs and RT-PCR (III).

► Identification of FcMV1, FcMV2-1 and FcMV2-2 by sequence analysis (III).

4.4. Ecological analysis

4.4.1. Ecological indicators

► Taxonomic richness (observed and estimated), diversity and evenness (Shannon and Simpson indices), dominance (Camargo´s index) and similarity between sampled communities (Sorensen index) (VI).

4.4.2. Sampling effort evaluation

► Sample-based rarefaction curves (VI).

4.4.3. Phoretic association between insects and fungi

► Phoresy index (PI) (VI).

4.4.4. Ecological network analysis

► Network analysis including connectance, mean number of links per taxa and tripartite graph (VI).

4.4.5. Co-occurrence analysis

► Computing co-occurrence frequencies by pair of taxa (observed and expected) and identification of positive / negative / random associations (VI).

4.5. Statistical analysis

4.5.1. Non-parametric tests

► Kruskal-Wallis rank sum test (IV, V & VI).

► Pearson's product-moment correlation (V).

4.5.2. Post-hoc analysis

► Dunn´s test (IV, V & VI).

4.5.3. Survival analysis

► Survival function obtained with Kaplan-Meier non-parametric estimator (V).

4.5.4. Peak analysis

► Local maxima identification through histograms (III).

4.6. Bioinformatics

4.6.1. Primer design

► Forward and reverse primers design and validation along query genome (III).
► Specific primers design for mutant deletion formation (IV).

4.6.2. Sequence comparison

► Maximum identity match searching using nucleotide (BLASTn and MegaBLAST) and protein (BLASTp and BLASTx) sequences as queries in the NCBI GenBank database (II, III & VI).

4.6.3. Managing of sequences

► Sequence trimming (II, III & VI).
► Pairwise alignment of nucleotide (method: CluscalW) and protein (method: BLOSUM matrix) sequences (II & III).
► Nucleotide sequences translation into proteins (II & III).
► Sequence mapping along query genome (II & III).
► Dendrogram construction by Neighbour-joining as statistical method, bootstrap method as a test of phylogeny and Kimura2 as substitution method (VI).

4.6.4. De novo *assembly*

► *De novo* assembly of NGS dataset by selecting different read size, k-mer size and specific assembling scripts (III).

4.6.5. Gene annotation and ontology

► Gene annotation analysis using the NCBI GenBank database (II).
► Gene ontology analysis using the GO database (II).
► Identification of open reading frame and flanking regions in a target gene (IV).

5. List of Original Articles

This Thesis is based on the Original Articles[i] listed below (I-VI):

Original Article I

Title: The use of mycoviruses in the control of forest diseases

Authors: E. Jordán Muñoz-Adalia, M. Mercedes Fernández and Julio J. Diez

Journal: Biocontrol Science and Technology 26 (5), 577-604

Publication date: 2016

Reference: doi:10.1080/09583157.2015.1135877

Original Article II

Title: *In silico* annotation of five candidate genes associated with pathogenicity in *Fusarium circinatum*

Authors: E. Jordán Muñoz-Adalia, Mercedes Fernández, Brenda D. Wingfield and Julio J. Diez

Journal: Forest Pathology 48 (3), e12417

Publication date: 2018

Reference: doi:10.1111/efp.12417

Original Article III

Title: Characterization of small RNAs originating from mitoviruses infecting the conifer pathogen *Fusarium circinatum*

Authors: E. Jordán Muñoz-Adalia, Julio J. Diez, M. Mercedes Fernández, Jarkko Hantula and Eeva J. Vainio

Journal: Archives of Virology 163 (4), 1009–1018

Publication date: 2018

Reference: doi:10.1007/s00705-018-3712-2

Original Article IV

Title: The *Fusarium circinatum* gene *Fcrho1*, encoding a putative Rho1 GTPase, is involved in vegetative growth but dispensable for pathogenic development

Authors: E. Jordán Muñoz-Adalia, M. Carmen Cañizares, Mercedes Fernández, Julio J. Diez and M. Dolores García-Pedrajas

Publication status: Manuscript

Original Article V

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

Authors: E. Jordán Muñoz-Adalia, J. Asdrúbal Flores-Pacheco, Pablo Martínez-Álvarez, Jorge Martín-García, Mercedes Fernández and Julio J. Diez

Journal: Physiological and Molecular Plant Pathology 94, 8-15

Publication date: 2016

Reference: doi:10.1016/j.pmpp.2016.03.002

Original Article VI

Title: *Sydowia polyspora* dominates fungal communities carried by two *Tomicus* species in pine plantations threatened by *Fusarium circinatum*

Authors: E. Jordán Muñoz-Adalia, Antonio V. Sanz-Ros, J. Asdrúbal Flores-Pacheco, Jarkko Hantula, Julio J. Diez, Eeva J. Vainio and Mercedes Fernández

Journal: Forests 8, 127

Publication date: 2017

Reference: doi:10.3390/f8040127

► [i] Citation styles in each Original Article are standardized through the Thesis. Numeration of tables and figures is correlative and independent in each article.

6.1. Fungal viruses as a node in the pathosystem: Original Article I

Uso de micovirus en el control de enfermedades forestales

Resumen

En la actualidad se han descrito alrededor de quince familias de micovirus si bien solo el 80% de ellas han sido catalogadas a nivel taxonómico. Los micovirus pueden agruparse en función de su genoma, el cual puede estar formado por cadenas simples o dobles de ARN o bien, por cadenas sencillas de ADN. Los efectos que los micovirus provocan en sus hospedantes van desde el estado críptico (asintomático) hasta el desarrollo de hiper- o hipovirulencia. La transmisión horizontal de estos virus depende de la presencia de grupos de compatibilidad vegetativa (vc) y tipos de apareamiento (MAT) en la población de sus posibles hospedantes. El control biológico basado en el uso de micovirus de la enfermedad del chancro del castaño (causada por el hongo *Cryphonectria parasitica*) ha resultado exitoso y por ello se considera un modelo en la gestión de la sanidad forestal. Para conseguir esta clase de herramientas de control biológico aplicables en otras enfermedades del arbolado se requiere un conocimiento profundo de la sintomatología viral y de sus vías de transmisión. El presente estudio se centra en la revisión del conocimiento existente sobre la aplicación de micovirus en el manejo de enfermedades de plantas leñosas de interés forestal, así como en sus perspectivas de futuro.

Palabras clave: control biológico, grupos de compatibilidad vegetativa (vc), hipovirulencia, protección forestal, transmisión de virus y virocontrol.

The use of mycoviruses in the control of forest diseases

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Abstract

Fifteen families of mycoviruses have been described and 80% of these catalogued. However, their evolutionary relationship with fungi is not clear. The mycovirus genome can be formed by single or double-stranded RNA or single-stranded DNA. The effects of mycoviruses range from the induction of a cryptic state (asymptomatic) to promotion of hyper- or hypovirulence in the host. Horizontal transmission of mycoviruses is determined by the presence of different vegetative compatibility types and mating types. Biocontrol of chestnut blight (*Cryphonectria parasitica*) has been found to be a successful mycovirus-based treatment and is considered a model in forest disease management. Development of this type of biological control tool for use in other forest pathologies requires a sound knowledge of viral symptomatology and transmission. The present review focuses on the application of mycoviruses and the prospects for future use in the biological control of forest diseases as well as on advances in mycovirus-applied research in forestry, landscape and culture of woody plants.

Keywords: biological control, forest protection, hypovirulence, vc types, virocontrol, virus transmission.

1. Introduction

Viruses that infect fungi, *i.e.* mycoviruses, are frequent in the subkingdom Dikarya (phyla Ascomycota and Basidiomycota), phyla Blastocladiomycota and Neocallimastigomycota (formerly Chytridiomycota) and Glomeromycota (formerly Zygomycota) (Herrero *et al.*, 2012; Hibbett *et al.*, 2007). Most fungal genera, ranging from microscopic yeasts to the more evolved edible mushrooms, have been described as hosts of mycoviruses (Hammond *et al.*, 2008; Lim *et al.*, 2005; Magae, 2012; Ro *et al.*, 2007; Schmitt & Breinig, 2006; Stielow *et al.*, 2011; Strauss *et al.*, 2000). This also applied to filamentous fungi that cause plant diseases.

Despite the apparent abundance of mycoviruses in nature, research on these infective agents is relatively scarce. Some recent studies have attempted to uncover the biological mechanisms that drive viral infection, replication and transmission in fungi and the ecological and management implications. As a result, agroforestry researchers have discovered the potential use of these viruses in biocontrol, with special attention given to mycoviruses that confer hypovirulence (weakened state) in their pathogenic hosts.

In this article, we review studies concerning the use of mycoviruses to control devastating forest diseases. Our main goal is to provide background information about biocontrol based on fungal virus research as well as on the degree to which different protection strategies are being implemented.

2. General aspects of mycoviruses

2.1. Taxonomy, diversity and biology

More than 250 fungus-related viral sequences have been identified and sequenced according to National Center for Biotechnology Information (NCBI, 2014; Xie & Jiang, 2014), resulting in 22 genera divided among 15 families according to the list published by the International Committee on Taxonomy of Viruses (ICTV, 2014) (Figure 1). Nevertheless, 20% of mycoviruses have not yet been catalogued (Pearson *et al.*, 2009; Van Regenmortel *et al.*, 2010).

Mycoviruses usually replicate in the cytoplasm, although some (*e.g. Mitovirus* sp.) replicate in mitochondria of the host species (Göker *et al.*, 2011; Milgroom & Hillman, 2011). Structurally, mycovirus genomes contain one or more open reading frames (ORFs) that encode proteins required for virus replication and sometimes for capsid synthesis. The molecular size of mycovirus genomes varies somewhat, *e.g.* Rosellinia necatrix quadrivirus 1 (RnQV1) segments range in size from 3.70-4.90 kbp with a single ORF (Chiba *et al.*, 2009), while the

maximum size of Chalara elegans RNA Virus 1 (CeRV1) has been reported to be 5.31 kbp in length and contain three ORFs (Park *et al*., 2005). Other mycoviruses may be longer, *e.g.* Cryphonectria hypovirus 1 (CHV-1) is 12.70 kbp in length and has at least two ORFs (Allemann *et al*., 1999; Shapira *et al*.,1991). Overall, the size of genome ranges between the extremes of *Partitiviridae* viruses (1.4-2.4 kbp and a single ORF) and *Hypoviridae* viruses (~9-13 kb and two overlapping ORFs); in addition, some families such as *Alphaflexiviridae* may contain several more or less overlapping ORFs (*e.g.* Botrytis virus X: ~7.0 kb and five ORFs) (Ghabrial *et al*., 2015). In some cases, small RNA molecules may also occur as satellite elements associated with the main genome particles (*e.g.* 0.9-1.4 kb elements associated with 3.7-5.0 kbp mycovirus genome in basydiomicetous yeast *Xanthophyllomyces dendrorhous*; anamorph: *Phaffia rhodozyma* (Flores *et al*., 2015)).
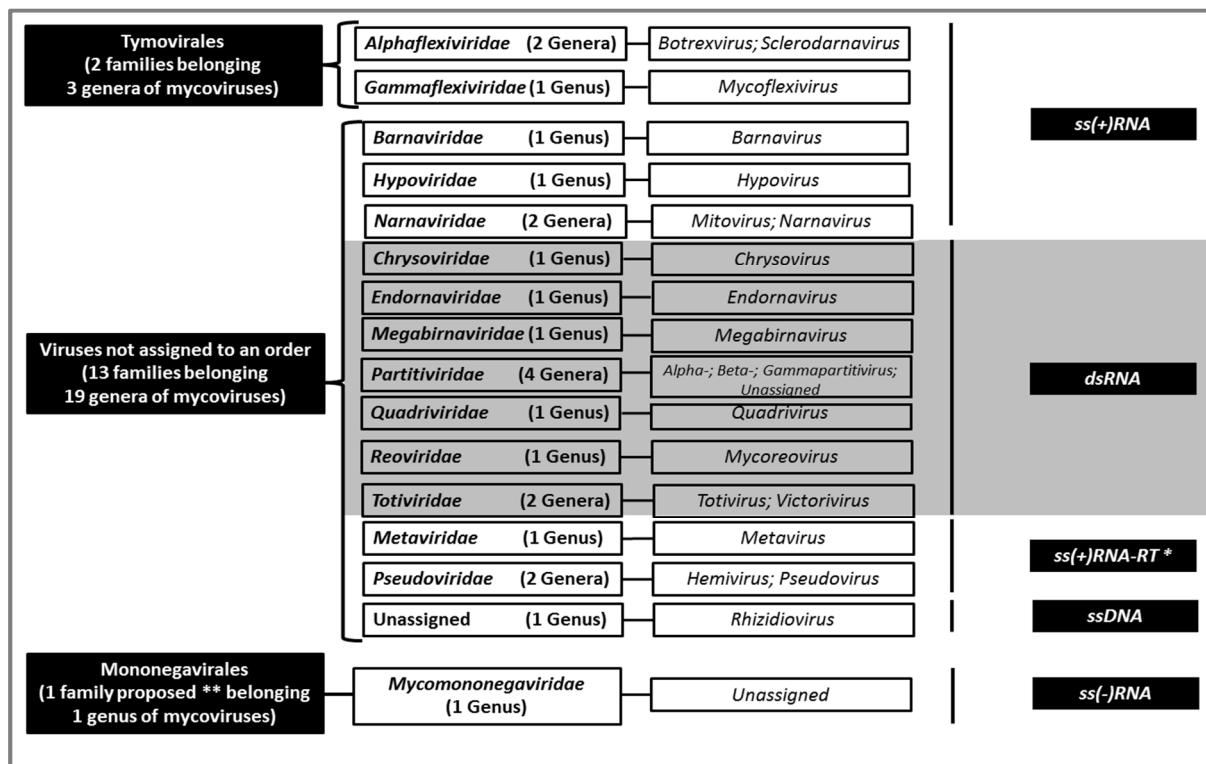
Figure 1. General taxonomy of mycoviruses according to ICTV classification criteria, Virus Taxonomy 2014 Release. *Classification under consideration; **Family proposed by Ghabrial *et al*. (2015).

Mycoviruses can be differentiated on the basis of molecular structure. Thus, seven families possess double-stranded RNA (dsRNA) genomes, and six families have single-stranded RNA (ssRNA) genomes. The latter are further divided into two subcategories: five families have ss(+)RNA genomes and one family has a ss(-)RNA genome (Figure 1). The mycoviruses belonging to ss(+)RNA families possess viral RNA with the same base sequence as mRNA. The functions of the RNA are similar to mRNA during replication, serving as a template for

protein synthesis such as RNA-dependent RNA polymerase (RdRp) or capsid. On the other hand, ss(-)RNA mycoviruses require participation of RNA replicase for their single strain genome to be transcribed into positive sense RNA. Only a few mycoviruses are formed by single circular molecules of DNA (ssDNA) (Ghabrial *et al*., 2015; Pearson *et al*., 2009).

The evolutionary relationship between mycoviruses and their hosts remains unclear. Two main hypotheses have been proposed. Briefly, one hypothesis is based on ancient co-evolution of mycoviruses and fungi whereby the speciation of viruses is closely related to vertical transmission (see below), and the asymptomatic presence of mycoviruses may denote a long period of coexistence between viruses and fungi. This would explain the complex relationships between host species and mycoviruses, which range between severe disadvantage to the host (antagonism) and mutualism where the infected host obtains some benefit under certain conditions, as suggested in other viral associations (Botella *et al*., 2015; Roossinck, 2015a, 2015b). The other hypothesis suggests the eventual transfer of viruses from plants to saprophytic or pathogenic fungi. In this case, viral transmission may take place during co-existence of fungal endophytes with plants, and small differences detected even within mycovirus families can be explained by a recent change of host (Chiba *et al*., 2011; Ghabrial, 1998; Liu *et al*., 2010; Pearson *et al*., 2009).

2.2. Transmission of mycovirus

The mechanism of viral transmission is another important aspect of viral biology. Mycoviruses can be transmitted in three ways: by horizontal, vertical or extracellular transfer. Horizontal transmission takes place when a mycovirus colonises a new host through hyphal contact and subsequent mycelia fusion (anastomosis) between individuals during heterokaryon formation (mediated by a self / non-self recognition system). Nevertheless, isolates of the same species are not always compatible, even in the same population. In this type of transfer, different vegetative compatibility groups (vc types or VCGs) play a special role, sometimes restricting movement of the virus (Leslie, 1993). Heterokaryon formation is genetically controlled by a specific *het* or *vic* loci. Heteroallelism in the *het* locus is not possible, resulting in reduction in cell lysis or mycelial growth (Saupe, 2000). At the same time, the presence of different mating types (MAT´s) in fungal populations makes transmission more complex (Coppin *et al*., 1997; Milgroom & Hillman, 2011).

In vertical transmission, mycoviruses commonly infect asexual spores. Nevertheless, prevalence rates may vary significantly between species, *e.g.* in *Heterobasidion annosum* only 3% of conidia are infected (Ihrmark *et al*., 2002) in contrast to 100% infection in *Cryphonectria parasitica* (Ding *et al*., 2007). Fungal viruses can also colonise sexual spores, infecting a new

generation of the host: 8-13% dsRNA infected ascospores of *Magnaporthe grisea* (Chun & Lee, 2009) whereas 10-84% dsRNA infected basidiospores of *H. annosum* (Ihrmark *et al*., 2004). However, in a more recent study, lower vertical transmission of *Heterobasidion parviporum* to basidiospores (8.3%) was observed in a spruce forest (Vainio *et al*., 2014). The authors of the latter study suggested that continuous spore load in stumps may be related to the high rate of infected basidiospores, in contrast to low rates of infection in standing trees, as previously reported. It is now considered that the predominant route of viral transmission is via asexual spores, and vertical transmission has not been reported to occur in many fungal species (Carbone *et al*., 2004; Milgroom & Hillman, 2011).

Extracellular transmission, in which purified viral particles of *Sclerotinia sclerotiorum* hypovirulence-associated DNA virus 1 (SsHADV-1) infected extracellularly virus-free protoplasts, intact hyphae and hyphal fragments of white mould fungus (*Sclerotinia sclerotiorum*) either *in vitro* (PDA culture) or *in vivo* (leaves of infected plants) has recently been described (Yu *et al*., 2013). These authors also mentioned that purified viral DNA did not infect mycelia or fungal protoplasts, suggesting that whole viral particles are needed for extracellular infection.

On a larger scale, transmission of mycoviruses between species has also been reported (Lee *et al*., 2011; Liu *et al*., 2003; Vainio *et al*., 2011a), opening up new research lines focusing on the genetic, evolutionary and ecological factors involved in transmission.

2.3. Hypovirulence process

The effects of mycoviruses infection can range from cryptic symptoms (asymptomatic) to the promotion of hypervirulence, through variations of colonial morphology and inducement of colour changes (Ghabrial & Suzuki, 2009). In fact, the same mycovirus can have different effects on their host depending on ecological conditions (Hyder *et al*., 2013). One phenomenon caused by mycoviruses, especially interesting for agroforestry science, is hypovirulence. Only a few mycoviruses reduce spore production, causing slow mycelial growth or less aggressive invasion in pathogenic hosts, making viruses effective in biocontrol (Milgroom & Hillman, 2011; Nuss, 2005) or virocontrol (Chiba *et al*., 2010). In this sense, hypovirulence have been proved according to Koch´s postulates using infectious cDNA of *C. parasitica* (Chen & Nuss, 1999) and *S. sclerotiorum* (Marzano *et al*., 2015), hyphae infection of *Sclerotinia* spp. using viral particles (Yu *et al*., 2013) and protoplast infection using dsRNA (Chiba *et al*., 2013; Hillman *et al*., 2004; Lee *et al*., 2011). Hence, knowledge about mycovirus-mediated hypovirulence is improving biocontrol strategies in many cases of agroforestry health (see next section).

Both hyper- and hypovirulence are strongly related to the presence of specific viruses, even in co-infection. Four dsRNA mycoviruses have been detected in *Nectria radicicola* (anamorph: *Cylindrocarpon destructans*) (Ahn & Lee, 2001). Removal of one virus, L1 (6.0 kbp), caused a reduction in virulence of the fungus, while later reinfection through anastomosis recovered the virulence of the isolate. Detailed laboratory studies complemented with pathogenicity field assays are essential for developing virocontrol techniques.

One challenge in plant pathology and the use of mycoviruses is the antiviral response of fungi or RNA silencing. When viruses infect healthy cells, dicer-type nucleases initiate a response that produces viral RNA processed segments (sRNAs). The RNA-induced silencing complex then identifies homologous sequences on mRNA and subsequently degrades sRNAs (Dang *et al*., 2011; Hammond *et al*., 2008; Nuss, 2011; Schumann *et al*., 2010; Tauati *et al*., 2014; Yaegashi, *et al*., 2013b). In a study attempting to clarify this evolutionary relationship, Segers *et al*. (2007) found symptomatic differences between hypovirulence-mycovirus infected *C. parasitica* isolates. The use of *C. parasitica* strains in which RNA silencing genes were disrupted enabled identification of genes coding for particular dicer and argonaute-like proteins as required elements in antiviral response (Sun *et al*., 2009; Zhang & Nuss, 2008).

3. Mycoviruses in forest diseases: case studies

3.1. Cryphonectria parasitica

C. parasitica is the causal agent of chestnut blight, a severe disease that causes widespread damage in North America, where it infects American chestnut (*Castanea crenata*), in Europe, where it infects the European chestnut (*Castanea sativa*) and in Asia, where it colonises Asian species of chestnut (*C. crenata* and *Castanea mollissima*). The disease is characterised by damage to cambial tissues and the appearance of cankers. These cankers tend to girdle the stem, killing the trees (Milgroom & Cortesi, 2004).

Many ss(+)RNA mycoviruses have been identified in *C. parasitica*, four of them belonging to the genus *Hypovirus*. Cryphonectria hypoviruses 1-4 (CHV-1, CHV-2, CHV-3 and CHV-4) have been reported in different parts of the northern hemisphere (Hillman *et al*.,1994; Shapira *et al*., 1991; Smart *et al*., 1999). In relation to dissemination, transmission in conidia has been reported as highly variable, ranging from 0% to 100%. Transmission through ascospores has not been observed in nature (Ding *et al*., 2007). However, the presence of mycovirus in ascospores of field-released transgenic strains of fungi ranged between 30% and 50% depending on culture conditions (Anagnostakis *et al*.,1998).

The best known example of a mycovirus that causes hypovirulence is CHV-1. When CHV-1 infects *C. parasitica* it causes weakness, reducing mycelial growth and sporulation. Infected fungi are only capable of forming superficial (healing) cankers on stems, and the trees can therefore survive the disease. Other symptoms of the presence of CHV-1 in isolates include changes in colony morphology and colour (Peever *et al*., 2000; Rigling *et al*.,1989). CHV-1 originally occurred in Europe (Italy and France) and Asia (Japan, China and Korea) but was later introduced into the USA (Allemann *et al*., 1999; Liu *et al*., 2002). Five genetically characerised subtypes of CHV-1 have been identified: F1 and F2 (from France), I (Italy), D (Germany) and E (Spain) (Allemann *et al*., 1999; Gobbin *et al*.,2003; Zamora *et al*., 2014). CHV-1 is now considered an important biocontrol tool in Europe.

CHV-2 and CHV-3 are both common in North America. However, although CHV-2 occurs in native *C. parasitica* in Asia (Hillman *et al*., 1992; Peever *et al*., 1998), CHV-3 is only present in the USA (Michigan) (Peever *et al*., 1997). Both CHV-2 and CHV-3 have proved useful in biocontrol as they induce hypovirulence in American forests and plantations. The mycovirus most commonly associated with chestnut blight in American forests (CHV-4) is traditionally considered to induce a cryptic state and is therefore not useful for biocontrol purposes (Enebak *et al*., 1994; Linder-Basso *et al*., 2005).

Mycoreovirus 1 (MyRV-1) (*Reoviridae*) has been identified in hypovirulent strains of chestnut blight fungus (Suzuki *et al*., 2004). Viral transmission of this *Mycoreovirus* sp. to sexual spores has been reported (Deng *et al*., 2007), and reovirus-infected isolates have been shown to produce mature perithecia and viable ascospores, which in turn host MyRV-1. Other mycoviruses belonging to the *Reoviridae* and *Narnaviridae* families -respectively Mycoreovirus 2 (MyRV2) and Cryphonectria mitovirus 1 (CpMV1)- have also been identified (Hillman & Suzuki, 2004). This fungus can host many *Reoviridae*, *Partitiviridae*, *Totiviridae* and *Megabirnaviridae* mycoviruses that usually infect other fungi (Eusebio-Cope *et al*., 2015).

3.2. Ophiostoma novo-ulmi

Dutch elm disease (DED), caused by *Ophiostoma ulmi* and *O. novo-ulmi*, was the most devastating disease affecting elms (*Ulmus* spp.) in Europe during the 20th Century (some 30 million elms were killed in the UK) (Brasier, 2001; Potter *et al*., 2011). These fungi cause death of the tree by vessel cavitation due to fungal growth in the xylem. Two pandemics have occurred. In the first, which began in the 1910s, *O. ulmi* spread through Europe causing severe damage to adult trees and later spread to North America. In the 1950s, two subspecies of *O. novo-ulmi* (Euro-Asian race: *O. novo-ulmi* subsp. *novo-ulmi*; and the North American race: *O.*

novo-ulmi subsp. *americana*) caused high mortality in European and American forests. In both cases, bark beetles (Coleoptera, Scolytinae) played an important role as vectors of the disease (Brasier & Kirk, 2010; Brasier, 1976, 1991; Santini & Faccoli, 2014).

In relation to the presence of mycoviruses, the d-factor has been identified as a cytoplasmically transmitted agent. It is characerised as a dsRNA virus, causing a reduction in fungal growth in wounds made by feeding bark beetles and in amoeboid colony morphology as well as lower vigour and growth rates and low conidial viability (Brasier, 1986; Sutherland *et al.*,1997). Thirteen dsRNA mycoviruses with similar symptoms to the d-factor were later identified as being responsible for infection of a specific isolate called Ld (Cole *et al.*, 1998; Doherty *et al.*, 2006; Hong *et al.*,1999; Hong *et al.*,1998a, b). The complete genomes of *O. novo-ulmi* mitoviruses (OnuMV1a, OnuMV1b, OnuMV3a, OnuMV3b, OnuMV4-Ld, OnuMV5-Ld and OnuMV6-Ld) have been sequenced and RdRp sequences for OnuMV1a, OnuMV1b and OnuMV3b have also been established (Hintz *et al.*, 2013).

In addition, other *Ophiostoma* species have been demonstrated to harbour mycoviruses. *Ophiostoma minus* (causal agent of blue stain in pine wood), and the saprophyte *Ophiostoma quercus* hosts viruses belonging to the *Totiviridae* and *Partitiviridae* families (respectively Ophiostoma minus totivirus (OmV) and Ophiostoma quercus partitivirus 2 (OPV2) (Doherty *et al.*, 2007). A distant relationship between OPV2 and Ophiostoma partitivirus 1 (OPV1) was suggested (Doherty *et al.*, 2007). OPV1, which was previously detected in the pathogenic fungus *Ophiostoma himal-ulmi* (Crawford *et al.*, 2006), it is not currently used in biocontrol.

3.3. Heterobasidion annosum

H. annosum s.l. is one of the most destructive fungi in the northern hemisphere. It is the causative agent of root disease in many coniferous species (*Abies* spp., *Calocedrus decurrens*, *Juniperus* spp., *Larix* spp., *Picea* spp., *Pinus* spp., *Pseudotsuga menziesii*, *Sequoiadendron giganteum*, *Thuja plicata* and *Tsuga heterophylla*) as well as in some broadleaf species (*Betula*, *Fagus* and *Populus* species) (Garbelotto & Gonthier, 2013; Gonthier & Thor, 2013). This fungal infection causes the death of trees (especially on pines and junipers), severe root and butt rot, general decay and decreased diameter growth in boreal forest and plantations, making it a major threat to timber production and the forest industry. Infection can occur in two ways: primary infection is caused by airborne basidiospores, while secondary infection takes place through colonisation of mycelia after contact with roots or grafting between infected and healthy trees (Asiegbu *et al.*, 2005; Thor *et al.*,2005; Tokuda *et al.*, 2011; Woodward *et al.*,1998).

Additionally, dsRNA mycoviruses in P and S types of *H. annosum* (Heterobasidion partitivirus P (HaV-P) and Heterobasidion annosum virus (HaV)) have been partially sequenced (Ihrmark *et al*., 2001). The authors included these mycoviruses in *Partitiviridae* and reported that *H. annosum* s.l. harbours dsRNA viruses at a frequency of approximately 15% in Europe and western Asia. A new putative member of *Partitiviridae*, Heterobasidion partitivirus 3 (HetPV3), was subsequently detected in Chinese strains of *Heterobasidion ecrustosum* (Vainio *et al*., 2010). In a later study, a new dsRNA virus belonging to *Partitiviridae* and designated Heterobasidion partitivirus 2 (HetPV2) clearly formed a subcluster with HaV-P due to their genomic similarities (Vainio *et al*., 2011b). In addition, three new putative viruses, also included in *Partitiviridae*, were catalogued and subsequently named Heterobasidion partitivirus 1 (HetPV1), HetPV4 and HetPV5 (Vainio *et al*., 2011a). These authors proposed a close genetic relationship between HetPV1 and HaV, while the two other viruses were found to be more similar to mycoviruses associated with Heterobasidion parviporum partitivirus Fr110B and other disease-associated viruses.

Another three partitiviruses have been identified more recently: Heterobasidion partitivirus 6, 7 (HetPV6 and HetPV7 respectively) (Vainio *et al*., 2012, 2013c) and Heterobasidion partitivirus 8, strain 1 from *Heterobasidion irregulare* (HetPV8-ir1) (Vainio *et al*., 2013a). All are taxonomically distant from all other *H. annosum* s.l. viruses. HetPV6 resembles Fusarium graminearum virus 4 (FgV4), with around 40% of protein level sequence similarities, while HetPV8-ir1 shares only 32% of RdRp similarities with HaV-P and 33% RdRp similarities with HetPV2 (Vainio *et al*., 2010, 2013a, 2013b). A recent study showed that four different viral species may be present in the same plot affected by *H. parviporum* (Vainio *et al*., 2014). Three of these were provisionally assigned to HetPV6 and two possible congeneric strains of *Betapartitivirus* sp., named HetPV2-pa1 and HetPV7-pa1, were also identified.

3.4. **Gremmeniella abietina**

Many coniferous tree species (mainly *Picea*, *Pinus*, *Abies* and *Larix* species) in Northern and Central Europe, North America and Japan host the fungus *Gremmeniella abietina* (anamorph: *Brunchorstia pinea*), leading to the appearance of stem cankers and shoot dieback and causing severe damage in woods and plantations when weather conditions are favourable. Three races of this fungus (European, North American and Asian) have been catalogued. The European race is subdivided into three biotypes (A, B and alpine) (Botella *et al*., 2010; Donaubauer, 1972; Hamelin *et al*., 1996; Kaitera & Jalkanen, 1992; Romeralo *et al*., 2012; Santamaría *et al*., 2005; Senn, 1999), although the taxonomy is currently under revision (Romeralo pers. com.).

Three families of dsRNA mycoviruses have been detected in this forest pathogen: Gremmeniella abietina mitocondrial RNA virus S1 (GaMRV-S1, *Narnaviridae*) (Tuomivirta & Hantula, 2003a); Gremmeniella abietina RNA virus L1 (GaRV-L1, *Totiviridae*); and Gremmeniella abietina RNA virus MS1 (GaRV-MS1, *Partitiviridae*) (Tuomivirta & Hantula, 2003b), with a high frequency of occurrence; *e.g.* the mycoviruses have been detected in 89% of Spanish isolates (Botella *et al*., 2012) and in 50% of Turkish isolates (Aday *et al*., 2012). In addition, three mycoviruses were found together infecting the same isolates of *G. abietina* var. *abietina* type A (Tuomivirta & Hantula, 2005). Later, Botella *et al*. (2012) reported the absence of mitoviruses in biotype B from Turkey, biotype A from North America and European Alpine biotype. On the contrary, biotype A from Finland and Spain hosted mycoviruses. Specifically, Spanish populations hosted two mycoviruses (GMV1 and GMV2) in high proportion (74% of isolates hosted dsRNA). These authors discussed the possible factors determining presence and transmission of mitoviruses between fungal races and highlighted the role of asexual reproduction in virus widespread. In fact, the higher proportion of mitovirus presence was detected in Spain where only asexual reproduction has been reported. Regarding the high presence and the low genetic variability detected in GMV2 in Spanish isolates, the researchers suggested a possible recent host switch and a subsequent adaptation to these new conditions. The findings of recent RdRp sequencing studies support the idea of a low degree of genetic variation in *G. abietina* mitoviruses in the European population (Botella *et al*., 2014).

3.5. Fusarium circinatum

Pine pitch canker is a virulent disease caused by *Fusarium circinatum* (teleomorph: *Gibberella circinata*) in many pine species and in Douglas fir (*Pseudotsuga menziesii*) worldwide. Infections have also been observed to cause significant damage in *Abies alba*, *S. giganteum*, *Larix decidua* and *Picea abies* (Martínez-Álvarez *et al*., 2014a). The pathogen was first detected in the southeastern USA and Mexico (where it is probably endemic) and then in Haiti, South Africa, Chile, France, Korea, Spain, Italy, Japan, Portugal, Uruguay and Brazil (Aegerter *et al*., 2003; Enebak & Stanosz, 2003; Gordon *et al*., 2006; Martínez-Álvarez *et al*., 2012; Pfenning *et al*., 2014). This fungus causes dieback in trees due to the formation of bleeding and resinous cankers on trunk and branches. Moreover, *F. circinatum* frequently causes death and damping-off of seedlings through both pre- and post-emergence infection, making such infections a significant threat to nurseries and afforestations (Aegerter *et al*., 2003; Wingfield *et al*., 2008).

Three putative *Mitovirus* spp. (*Narnaviridae*) were recently identified in *F. circinatum* isolates from *Pinus radiata* in northern Spain and named Fusarium circinatum mitovirus 1, 2-1 and 2-2

(FcMV1, FcMV2-1 and FcMV2-2) (Martínez-Álvarez *et al*., 2014b). The genetic structure of the mycoviruses hosted by *F. circinatum* isolates from Spain and South Africa has also been studied (Vainio *et al*., 2015). Only Spanish isolates were found to host mycoviruses, which showed very similar sequence variants (>95% similarity). Indeed, a high rate of asexual spore transmission of mycoviruses (ranging between 70% and 100%) has been preliminary observed (Bezos *et al*., 2015), indicating the potential use of the mycoviruses as biocontrol agents.

3.6. Botryosphaeria *spp.*

Botryosphaeria spp. commonly occur as endophytic fungi in healthy hosts, but may become virulent when their host is subjected to environmental stress or physical damage (Burgess *et al*., 2006; Smith, Crous, Wingfield *et al*., 2001; Smith *et al*., 1996). Despite its taxonomic complexity, *Botryosphaeria dothidea* (anamorph: *Fusicoccum aesculi*) is cited as the causal agent of stem and branch cankers on apple trees (*Malus domestica*), ring spot on pear trees (*Pyrus communis*) and dieback and stem cankers on eucalyptus trees (*Eucalyptus* spp.) among many other woody species (Brown-Rytlewski & McManus, 2000; Slippers & Wingfield, 2007). *Eucalyptus* sp. is one of the most common trees planted in commercial and clonal forestry at an international level. Eucalyptus dieback and cankers are of special interest in forest science because of the reduced growth, offspring failure and adult tree death caused by the pathogen (Pérez *et al*., 2010). The gummy exudation produced in cankers also makes the wood less valuable, causing significant economic losses in the forest industry (Rodas *et al*., 2009).

Two dsRNA mycoviruses were recently detected in non virulent isolates of *B. dothidea* infecting *Pyrus pyrifolia* (Wang *et al*., 2014). These researchers reported Botryosphaeria dothidea chrysovirus 1 (BdCV1) as a new member of *Chrysoviridae* and also identified Botryosphaeria dothidea partitivirus 1 (BdPV1). Although BdPV1 was included in *Partitiviridae*, the capsid proteins of the mycovirus do not show significant similarity to any other capsid proteins. Analysis of the RdRp sequence also suggests the inclusion of this mycovirus in a new *Partitiviridae* clade (with 39% RdRp similarity to the most closely related *Chrysovirus* sp.).

3.7. Hymenoscyphus fraxineus

Ash dieback is an invasive disease caused by the fungus *Hymenoscyphus fraxineus* (synonym: *Hymenoscyphus pseudoalbidus*; anamorph: *Chalara fraxinea*). The fungus infects *Fraxinus* spp. with notable incidence in common ash (*Fraxinus excelsior*) and narrow-leafed ash (*Fraxinus angustifolia*). This pathogen has been spreading in Europe since the 1990s and causes severe damage in forests (pure or mixed stands), nurseries and urban green areas

(Hietala *et al.*, 2013; Kowalski, 2006; Timmermann *et al.*, 2011). It has also been cited in East Asia and Japan infecting *Fraxinus mandshurica* and *Fraxinus chinensis* (Gross *et al.*, 2014). The fungus infects ash trees of all ages, causing rapid crown dieback in adult trees, cankers and bark lesions on stem and twigs, and also leaf wilt. The disease frequently causes the death of young trees a few years after infection. However, it may become a chronic disease in older trees, reducing the tree's defences against other pathogens and pests or environmental factors (Gross *et al.*, 2014; Kowalski & Holdenrieder, 2009; Timmermann *et al.*, 2011).

A new ssRNA mycovirus that infects this pathogenic fungus was recently discovered (Schoebel *et al.*, 2014). The authors proposed inclusion of the virus in the genus *Mitovirus* (*Narnaviridae*) and named it Hymenoscyphus fraxineus mitovirus 1 (HfMV1). They noted the possibility of rapid genetic divergence based on their findings of large differences in the strains isolated in Switzerland, Poland, Germany, Lithuania and Japan. They hypothesised that the similarities between Swiss and Japanese strains may denote a European pathogen introduction across infected host material from Asia. Moreover, the prevalence of this mycovirus was high (90% in Swiss isolates according to Schoebel *et al.* (2014)), supporting the most accepted hypothesis of predominance of vertical transmission via ascospores.

3.8. Other fungal pathogens in woody plants

Botrytis cinerea (teleomorph *Botryotinia fuckeliana*) causes grey mould disease in more than 200 crops species over the world, including farmland crops, ornamental species and fruit crops such as grapes (*Vitis vinifera*), pear trees, raspberries and blackberries (*Rubus* spp.) (Rodríguez-García *et al.*, 2014; Williamson *et al.*, 2007). The presence of different genera of mycovirus in this fungus has been widely reported (Castro *et al.*, 2003; Potgieter *et al.*, 2013; Rodríguez-García *et al.*, 2014; Wu *et al.*, 2007; Zhang *et al.*, 2010). These studies highlight the wide diversity of viruses that this fungus is able to host and which provide a wide range of opportunities for research in the field of fungal virology. Another three mycoviruses that infect *Botrytis* sp. have recently been sequenced: Botrytis virus F (BVF, *Gammaflexiviridae*), Botrytis virus X (BVX, *Alphaflexiviridae*) and Botrytis porri RNA virus 1 (BpRV1, dsRNA virus) (Xie & Jiang, 2014).

Verticillium dahliae and *Verticillium albo-atrum* are both causal agents of *Verticillium* wilt disease. They have been cited in a broad range of hosts and more than 200 species, including bushes and trees (Schall & Davis, 2009; Smith, 1965). Specifically, *V. dahliae* can infect economically important woody crops such as gooseberry (*Ribes grossularia*), apricot (*Prunus armeniana*), olive (*Olea europea*), quince (*Cydonia oblonga*) and roses (*Rosa* spp.), as well as

other species of ecological interest such as maple (*Acer palmatum*), sycamore (*Acer pseudoplatanus*), raspberry, honeysuckle (*Lonicera* sp.) and broom (*Cytisus scoparius*). *V. albo-atrum* causes damage to the tree of heaven (*Ailanthus altissima*), striped maple (*Acer pennsylvanicum*), yellow poplar (*Liriodendron tulipifera*) and other landscape species (Morehart *et al*.,1980; Schall & Davis, 2009; Smith, 1965). Some studies have demonstrated the presence of mycoviruses in these pathogenic fungi. For example, a *Chrysovirus* sp. named Verticillium dahliae chrysovirus 1 (VdCV1) was identified by Cao *et al.* (2011). A novel member of the family *Partitiviridae* was identified in *V. albo-atrum*: Verticillium albo-atrum partitivirus 1 (VaaPV1) (Cañizares *et al*., 2014), although no details were provided about the pathogenic effect of the mycovirus in its fungal host.

Some opportunistic fungal pathogens of *Pinus* spp., such as *Diplodia pinea* (synonym: *Sphaeropsis sapinea*) and *Diplodia scrobiculata* (Smith *et al*., 1996), also host mycoviruses. Two dsRNA mycoviruses have been identified in *D. pinea*: Sphaeropsis sapinea RNA virus 1 and 2 (SsRV1, SsRV2 respectively; *Totiviridae*) (Preisig *et al*.,1998); and one in *D. scrobiculata*: Diplodia scrobiculata RNA virus 1 (DsRV1; related to *Chrysoviridae*) (De Wet *et al*., 2008, 2011).

Another pathogenic fungus of interest in agroforestry is the causal agent of root rot disease, *Rosellinia necatrix* (anamorph: *Dematophora necatrix*). The interest is due to the pathogenicity of the fungus in several woody species *e.g.* apple, olive, grape and poplar (*Populus* spp.) (Pérez-Jiménez, 2006). Many families of mycoviruses are known to infect this fungus, *e.g. Chrysoviridae*, *Quadriviridae*, *Partitiviridae*, *Reoviridae* and *Totiviridae* (Xie & Jiang, 2014). Two dsRNA mycoviruses have also been associated with hypovirulence: Rosellinia necatrix megabirnavirus 1 (RnMBV1), included in a new family of mycoviruses (*Megabirnaviridae*) and Rosellinia necatrix partitivirus 2 (RnPV2) (Xie & Jiang, 2014).

4. Future perspectives for use of mycoviruses in biocontrol

As already mentioned, many fungi hosted by forest, horticultural and ornamental species harbour mycoviruses to a greater or lesser extent (Table 1). Although many of these have not yet been found to be of use for biocontrol purposes, many of them provide new opportunities for research in forestry science. Despite the promising outlook, the use of mycoviruses in biological control is limited by the need for detailed analysis of (a) the symptoms associated with mycovirus-caused hypovirulence, (b) transmission mechanisms and biological and ecological conditions, (c) treatment effectiveness in the field and (d) subsequent persistence in the host population.

4.1. Identification of factors leading to hypovirulence: research in progress and lessons learned

The best known example of a disease managed by mycoviruses is chestnut blight. In Europe, CHV-1 has been used to induce hypovirulence (Robin & Heiniger, 2001) with goods results in field inoculation trials (Juhásová *et al*., 2005; Robin *et al*., 2000; Zamora *et al*., 2014). CHV-1 and CHV-3 have been used with less success in the USA than in Europe, with natural hypovirulence being reported in Michigan (Milgroom & Cortesi, 2004). For other pandemics such as DED, mycoviruses infecting *O. novo-ulmi* appear promising for biocontrol, because of the symptoms that they cause in host isolates, such as slow mycelial growth, abnormal or amoeboid colony formation, reduction in asexual spore production, low cytochrome oxidase level and formation of mitochondrial DNA plasmids (Hong *et al*., 1999).

In relation to the application of biocontrol in diseased forests in boreal areas, no clear relationship between viral presence and fungus growth rate was observed in *H. annosum* s.l. (Vainio *et al*., 2010). However, significant variations in growth and changes in the effects of virus were observed in relation to the culture conditions. The effect of HetPV6 infection in relation to multiple variables (geographical, culture conditions and host) has been investigated in four *Heterobasidion* species (Vainio *et al*., 2012). No significant differences in growth were found in *H. parviporum* (*in vivo* and *in vitro*) or *H. annosum* (*in vivo*). However, significantly increased mycelial growth was observed in infected *H. annosum* cultures (laboratory assays condition: 6º C and 15º C culture on MOS agar plates). Consequently, these results do not support a possible use of HetPV6 in virocontrol, although HetPV6 is very frequent in fungal populations and apparently does not interfere in subsequent viral infection (Vainio *et al*. 2013b).

Mycoviruses may eventually be used as tools in the management of invasive diseases, for example in ash dieback. Although Hymenoscyphus fraxineus mitovirus 1 does not show harmful effects in its host, future perspectives for its application in biocontrol are promising because of the phylogenetic position of this mitovirus relative to others that are known to cause hypovirulence (Schoebel *et al*., 2014). In fact, HfMV1 is closely related to *Cryphonectria cubensis*, *S. sclerotiorum* and *Helicobasidium mompa* mitoviruses.

Several *Totiviridae*, *Chrysoviridae* and *Partiviridae* mycoviruses have been identified in *Fusarium graminearum* (Lee *et al*., 2011; Yu *et al*. 2011). More specifically, a mycovirus described in *F. graminearum* infecting maize in Korea (named Fusarium graminearum virus 1-DK2; FgV1-DK2) is capable of reducing mycelial growth and sporulation, decreasing

mycotoxin production and increasing pigmentation (Chu *et al*., 2002). In a later study addressing this topic, a mixed infection of two dsRNA viruses was reported, with no changes in mycelial morphology but with a high rate of transmission in conidia and ascospores (30-100%) (Chu *et al*., 2004). A recent study identified a new mycovirus associated with hypovirulence in *Fusarium virguliforme* and closely related to *F. graminearum* mycoviruses (Marvelli *et al*., 2014). Moreover, two new putative mycoviruses belonging to the *Mitovirus* genus have been described in *Fusarium coeruleum* isolates, in addition to one new *Alphapartitivirus* sp. in *Fusarium solani* f. sp. *pisi* (Osaki *et al*., 2015). Mycoviruses infecting in *F. coeruleum* are closely related to FcMV1, which opens up a new line of phylogenetic research. Together these results encourage the continued study of hypovirulence induced by mycoviruses in *Fusarium* spp. (with special focus on *F. circinatum*) whose use in biocontrol may prove to be a profitable consequence of in-depth studies of this species.

Grey mold, caused by *B. cinerea*, is being investigated by various research groups around the world because of the global importance of this disease. The rare formation of multicellular penetration structures (infection cushions) and decreased mycelial growth are probably caused by hypovirulence induced by mycoviruses (especially Botrytis cinerea mitovirus 1 (BcMV1), main mycovirus implied in hypovirulence process) as suggested by Rodríguez-García *et al*. (2014); Wang *et al*. (2014) and Zhang *et al*. (2010). These advances are very encouraging in agroforestry technology and are leading the way to the development of new treatments in the control of tree diseases, at least for incipient infections, thus possibly reducing economic and ecological damage.

4.2. Mycoviruses transmission and biological conditions

The existence of vegetative incompatibility is a major limitation in virocontrol, due to the instability of hyphal fusion between fungi that have not the same vc type. In the case of *C. parasitica*, fungal viruses can be transferred thought anastomosis among different vc types (0.13-0.50 transmission rates between CHV-1 strains differentiated by one or two vegetative incompatibly genes), although slowly and in less proportion (3-4%) (Cortesi *et al*., 2001; Liu & Milgroom, 1996; Peters *et al*., 2012). This limitation in biocontrol may be reduced with more knowledge about *vc* types at the population level. Papazova-Anakieva *et al*. (2008) studied CHV-1 transmission between vc types in Macedonia, where only five vc types were detected and high rates of transmission between isolates with predominance in one direction were found. So that, *vic* genes for this species has been characerised (Choi *et al*., 2012; Zhang *et al*., 2014) enabling multilocus PCR assays development in order to analyse incompatibility genes profiles in field populations of fungus (Short *et al*., 2015).

Table 1. Summary of mycoviruses of agroforestry interest.

Fungus	Main hosts	Mycoviruses	Family	References
Cryphonectria parasitica	*Castanea* spp.	CHV-1; CHV-2; CHV-3; CHV-4; MYRV-1	*Hypoviridae; Reoviridae*	Hillman *et al.* (1994); Linder-Basso *et al.* (2005); Shapira *et al.* (1991); Smart *et al.* (1999); Suzuki *et al.* (2004)
Ophiostoma novo-ulmi	*Ulmus* spp.	OnuMV1a; OnuMV1b; OnuMV1c; OnuMV2; OnuMV3a; OnuMV3b; OnuMV4-Ld; OnuMV5-Ld; OnuMV6-Ld; OnuMV7-Ld; DsRNA01_ORF; DsRNA02_ORF	*Narnaviridae*	Hong *et al.* (1998a,b, 1999); Doherty *et al.* (2006); Hintz *et al.* (2013)
Heterobasidion annosum complex	*Various*	HaV; HaV-P; HetRV1; HetRV2; HetRV3; HetRV4; HetRV5; HetRV6; HetRV7; HetRV8; HetPV2-pa1; HetPV7-pa1	*Partitiviridae*	Ihrmark et al. (2001) Vainio et al. (2010, 2011a,b, 2012, 2013c, 2014)
Gremmeniella abietina	*Pinus spp., Picea spp., Abies spp., Larix spp.*	GaMRV-S1; GaRV-L1; GaRV-MS1	*Narnaviridae; Totiviridae; Partitiviridae;*	Tuomivirta and Hantula (2003a,b)
Fusarium circinatum	*Pinus* spp.; *Pseudotsuga menziesii*	FcMV1; FcMV2-1; FcMV2-2	*Narnaviridae*	Martínez-Álvarez *et al.* (2014b)

Table 1. (Cont.).

Fungus	Main hosts	Mycoviruses	Family	References
Botryosphaeria dothiea	*Pyrus* spp., *Malus* spp., *Eucalyptus* spp.	BdCV1; BdPV1	*Chrysoviridae; Partitiviridae*	Wang *et al.* (2014)
Hymenoscyphus fraxineus	*Fraxinus* spp.	HfMV1	*Narnaviridae*	Schoebel *et al.* (2014)
Botrytis cinerea	Various	BcMV1	*Narnaviridae*	Wu *et al.* (2010)
Verticillium dahliae	Various	VdCV1	*Chrysoviridae*	Cao *et al.* (2011)
Verticillium albo-atrum	Various	VaaPV1	*Partitiviridae*	Cañizares *et al.* (2014)
Diplodia pinea	*Pinus* spp.	SsRV1; SsRV2	*Totiviridae*	Preisig *et al.* (1998)
Diplodia scrobiculata	*Pinus* spp.	DsRV1	*Chrysoviridae-related*	De Wet *et al.* (2011)
Rosellinia necatrix	Various	RnMBV1; RnPV2	*Megabirnaviridae; Partitiviridae*	Chiba *et al.* (2009, 2013)

Zamora *et al.*, (2012) studied vc types and mating types involved in this disease in the region of Castilla y León (Spain) and 11 vc types were identified. Two of these accounted for 88% of *C. parasitica* in the sampled population. Five of the remaining vc types were scarce (<10 isolates / vc type). In relation to the mating types present in *C. parasitica*, two mating types were found: MAT-1 was the most frequent and MAT-2 was only present in two of the provinces studied. It was concluded that the low diversity of vc types may explain the low incidence of MAT-2, supporting the idea that the fungus mainly undergoes asexual reproduction. However, the presence of two mating types in the same area could increase vc type diversity in an scenario where sexual reproduction eventually dominates. Elaborating a complex database of vc types among different CHV subtypes involves a large sampling effort, especially in areas with a high diversity of subtypes (>130 vc types in China: Wang *et al.*,1991), but could greatly improve biocontrol against chestnut blight disease. Similarly, the main problem in relation to the use of RnMBV1 (causal agent of hypovirulence process on *R. necatrix* under laboratory conditions) for biocontrol purposes is the presence of a diverse fungus population (with numerous vc types) leading to the prevalence of sexual spores over anastomosis (Chiba *et al.*, 2009). The possibility of observing variations in the hypovirulence phenomenon caused by environmental conditions and genetic intervention was also suggested (Chiba *et al.*, 2009).

The long term transmission of virus between incompatible isolates of *R. necatrix* was studied in apple trees (Yaegashi *et al*., 2013a). After 2-3 years, both strains of fungus originally inoculated (one virus-free and other infected by dsRNA element called N10) and their hybrids were detected in trees. Moreover, isolates of both lineages (initially infected and non-infected) contained mycovirus, despite the vegetative incompatibility. The number of viral particles increased during the study period and six new mycovirus sequences were identified. The authors suggested the possible role of mycoparasitic fungi and mycophagous invertebrates as vectors involved in virus transmission thus enabling the vc types restrictions to be overcome.

More detailed knowledge of the virus transmission process and vc types is needed in the case of *O. novo-ulmi*, especially in regions where vc types are limited, *e.g.* Canada (Hintz *et al*., 2013). Such conditions may be favourable for carrying out field assays. In the case of *F. circinatum*, the low vc type diversity detected in many locations such as Spain (Iturritxa *et al*., 2011; Pérez-Sierra *et al*., 2007) and other regions where recent introduction of the pathogen is plausible may be suitable for implementing biocontrol treatments. For example, the three previously mentioned mitoviruses (FcMV1, FcMV2-1 and FcMV2-2.) have been identified in Spanish isolates of *F. circinatum* belonging to the both local mating types, and it has been suggested that the occurrence of these mitoviruses is not restricted by the mating type compatibility (Vainio *et al*., 2015). Therefore, if any of the three recently identified mycoviruses (Martínez-Álvarez *et al*., 2014b) were found to cause hypovirulence, inoculation treatments could be implemented as in the European chestnut blight technique.

Only three different vc types of *V. dahliae* have been identified in ornamental woody plants in Illinois (USA) (Chen, 1994). The lower diversification in the population was suggested to be related to the eventual establishment of the fungus in nurseries with subsequent dispersion. The presence of virus in less aggressive fungal isolates and high affinity in vc types suggests that the use of VdCV1 or VaaPV1 for biocontrol purposes is feasible. Indeed, VdCV1 has been isolated in non-defoliating strains of fungus (Cao *et al*., 2011). Nevertheless, these mycoviruses have not been shown to induce hypovirulence. Similarly, in a study of vc types involved in ash dieback in the UK, strong vegetative incompatibility was found between isolates from the same population (Brasier & Webber, 2013). The authors concluded that the low degree of compatibility may be caused by the genotype heterogeneity as a result of the well-known dominance of sexual reproduction in the species (Gross *et al*., 2012, 2014). The mycoviruses that infect this pathogen are known to be genetically diverse (estimated nucleotide reposition rate 0.16) and able to infect sexual spores (Schoebel *et al*., 2014). The low compatibly between isolates may preclude their use in biocontrol. However, rapid changes

in the mycovirus genome and the infrequent role of ascospores as virus vectors imply new opportunities in virocontrol research for this invasive disease.

Regarding inter-specific transmission of mycoviruses, the high level of genetic similarity between HetPV1 strains (98% in polymerase sequence) isolated from different species of *Heterobasidion* (*Heterobasidion australe* and *H. parviporum*) infecting the same host suggests that mycovirus transmission is frequent in this fungal complex in nature (Vainio *et al*., 2011). This is also supported by the findings of laboratory studies with *Heterobasidion* spp., which demonstrated inter- and intraspecific transmission via anastomosis (Ihrmark *et al*., 2002; Vainio *et al*., 2010). Furthermore, the possibility of protoplasmic transmission of mycoviruses in *Fusarium boothii* was analysed (Lee *et al*., 2011). These authors used the protoplast fusion method to inoculate FgV1-DK21 into *F. graminearum*, *Fusarium asiaticum*, *Fusarium oxysporum* f. sp. *lycopersici* and *C. parasitica*. They showed that this method could be used for inter- and intraspecific virus transmission and reported changes in colony morphology caused by mycovirus presence, even in fungi with no known hypovirulence related to FgV1-DK21. The survival rate of tomato plants (*Solanum* sp.) infected with mycovirus-treated *Fusarium* spp. was higher (71.7%) than in virus-free isolates (23.3%). In *C. parasitica*, FgV1-DK21 was effectively transmitted via *F. boothii* protoplast, and the virulence was lower than in virus-free and CHV-1 infected isolates. These results have clear implications for the development of management strategies in the medium term, opening the way for a new area of research involving the use of fungal complex in virocontrol at the community level.

The RNA silencing process was investigated in Rosellinia necatrix partitivirus 2 (RnPV2) infecting a non-natural host (*C. parasitica* isolates) (Chiba *et al*., 2013). A wild type fungus and another mutant strain with defective protein processing sRNAs (dicer-like 2) were used. The wild-type *C. parasitica* showed milder symptoms after infection than the defective RNA silencing mutant (called Δ*dcl-2* mutant), suggesting that the antiviral response mechanism detected nonspecific *Partitivirus* sp. as a target. Furthermore, infections involving a defective interfering dsRNA1 (DI-dsRNA1) strain were less effective. By contrast, the natural host (*R. necatrix*) remained asymptomatic after the same treatments. In conclusion, this study suggests the potential for using mycoviruses provided by other fungal species in virocontrol and highlights the need for more detailed knowledge about the RNA silencing process. In a study of transfection of *Partitivirus* sp. (RnPV1) and the *Mycoreovirus* sp. (MyRV3) from *R. necatrix* donor isolates to *Diaporthe* sp., *C. parasitica* and *Valsa ceratosperma* protoplasts, successful horizontal transmission into these fungi was reported (Kanematsu *et al*., 2010). Infection by MyRV3 caused hypovirulence symptoms in all these new hosts. This result suggests a new line in virocontrol techniques.

Sclerotinia sclerotiorum partitivirus 1 (SsPV1), a mycovirus isolated from hypovirulent strains of white mould (*S. sclerotiorum*), has been found to be able to infect *B. cinerea* and also to be transferred via anastomosis among vc types and even overcome incompatibility barriers (Xiao *et al.*, 2014). With regard to the high specificity of this mycovirus in host selection, biosafety in field use is guaranteed (Yu *et al.*, 2013). These noteworthy findings demonstrate the possibility of improving the biological control techniques by using different mycoviruses, even in different pathogenic fungi. This opens up new research lines involving forest pathology biocontrol.

4.3. Future challenges in mycovirus-based biocontrol

Hypovirulence caused by co-infection is an interesting topic in biocontrol. Hypovirulence has been associated with simultaneous infection between MYRV-1 and CHV-1 in *C. parasitica* isolates (Sun *et al.*, 2006). The co-infection produced similar colony changes as single CHV-1 infection, while conidia production and mycelial growth decreased when both viruses were present. Furthermore, accumulation of dsRNA and vertical transmission of MyRV1 increased with co-infection, with no negative effects on CHV-1 genome RNA accumulation. In a more recent study, infection of *B. dothidea* isolates with BdPV1 mycovirus alone did not reduce growth, although the idea of a possible synergistic hypovirulence effect caused by simultaneous infection by BdCV1 and BdPV1 was suggested (Wang *et al.*, 2014). Indeed, co-infection caused by distantly related viruses was recently found to be more stable in isolates of *Heterobasidion* sp. (Vainio *et al.*, 2014). This has important consequences for the distribution of viruses and the co-existence of different viral strains in the same host population. A very recent study showed greater effects of RNA silencing in Rosellinia necatrix victorivirus 1 (RnVV1) hosted by *C. parasitica* than in other mycoviruses naturally hosted by this fungus (CHV1 and MyRV1), suggesting an antagonistic relationship between mycoviruses co-infecting the same isolates (Chiba & Suzuki, 2015). CHV1 and MyRV1 interfered in replication and lateral transmission of RnVV1 and were involved in RNA silencing activation; however, these mycoviruses showed higher resistance of antiviral defence effects and were mainly RnVV1 suppressed, even when the host dicer or Argonaute genes were disrupted. Further studies focusing on the co-infection process are needed. If the combined effects of mycoviruses in its hosts are clarified, new advances in the preventive inoculation of virus complex may be possible.

In depth study of the interactions between mycoviral infections and environmental features is also required. In laboratory assays of *G. abietina* cultures under multiple different growth conditions, mycelial growth was highest in mycovirus free isolates (Romeralo *et al.*, 2012). However, it was not clear whether this phenomenon was mediated by mycoviruses or only by

individual virulence of the strain. New studies focusing on this aspect are required for the development of virocontrol methods.

Research on the persistence of mycoviruses after the use of biocontrol strategies is scarce. In one of the few studies of this aspect, American chestnut plots were evaluated 12 years after biocontrol implementation against chestnut blight (Liu *et al*., 2002). CHV-1 was not detected in any isolate, and biocontrol failure was proposed as a possible reason for this absence. The persistence of CHV-2 and CHV-3 was limited. By contrast, although CHV-4 was common in the study area, attributing its origin to the introduction during biocontrol treatment was regarded as doubtful. Another study reported the disappearance of CHV-1 in European treated plots 24 years after biocontrol application (Robin *et al*., 2010). The authors pointed out that the low diversity of vc is not necessarily related to low persistence, because similar results have been reported in other chestnut forests in Europe, and they concluded that differences in CHV-1 subtype fitness may be the most important factor in the persistence of mycoviruses in field. More research is required to establish the long-term effects of the use of mycoviruses in the field.

5. Conclusions

1. Mycoviruses represent a relatively unknown group in virology and plant pathology sciences. However, the taxonomy of mycoviruses based on genetic sequences and biological characteristics (including antiviral response by hosts) is being improved greatly.

2. Chestnut blight caused by *C. parasitica* is the best known and most successful mycovirus-based biocontrol method in forest pathology. Moreover, it is the only case in which a mycovirus-based biocontrol technique has been satisfactorily implemented. This disease serves as a study model in forestry protection, with particular relevance in the development of new preventive and therapeutic measures centred on several tree species.

3. Mycovirus research focused on diseases caused by the *O. novo-ulmi*, *H. annosum* complex, *G. abietina*, *F. circinatum*, *B. dothidea*, *H. fraxineus* and *R. necatrix* is currently being developed in the forest context. Further studies involving *D. pinea*, *D. scrobiculata*, *V. dahliae* and *V. albo-atrum* pathologies are also needed.

4. Mycovirus-mediated hypovirulence is a current challenge in biocontrol research because of its potential role in the prevention and/or management of plant diseases. It could become an

important tool for maintaining the health of woody species, complementing or totally replacing chemical treatments.

5. Inoculation of fungi with mycoviruses may become a new management tool for forest protection, as used in the treatment of chestnut blight disease.

6. The main targets of study in mycovirus-based biological control are: (i) the mycoviruses that induce hypovirulence in their hosts, (ii) the conditions that affect hypovirulence and the virus silencing process, (iii) the transmission ecology and its biological limitations, (iv) the taxonomical and phylogenetic relationships between mycoviruses and (v) the viability of field biocontrol measures.

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6.2. The fungus as a node in the pathosystem: Original Article II

Anotación *in silico* de cinco genes posiblemente asociados con la patogenicidad en *Fusarium circinatum*

Resumen

La enfermedad del chancro resinoso de los pinos (causada por el hongo *Fusarium circinatum*) es considerada una de las más devastadoras en pinares, plantaciones y viveros forestales en todo el mundo. Pese a la importancia de este fitopatógeno, se desconocen los factores genéticos que desencadenan la virulencia del hongo. En este estudio, cinco genes candidatos (en concreto, *Fcfga1, Fcfgb1, Fcac, Fcrho1* y *FcpacC*) se identificaron *in silico* usando el genoma completo de *F. circinatum* como referencia. Se estudió la similitud de los genes propuestos tomando las secuencias de genes previamente identificados en otras especies de *Fusarium*. La identidad y cobertura entre genes modelo y propuestos superó el 90% tanto para secuencias de nucleótidos como en proteínas. De forma complementaria, se estudió la ontología de los cinco genes candidatos.

Palabras clave: anotación de genes, cAMP-PKA, chancro resinoso de los pinos, MAPK y marchitamiento del cuello de raíz.

In silico annotation of five candidate genes associated with pathogenicity in *Fusarium circinatum*

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Abstract

The pine pitch canker disease caused by the pathogenic fungus *Fusarium circinatum* is one of the most devastating diseases in pine forests, afforestation and nurseries around the world. Despite the importance of this phytopathogen, only a little is known about the genes that drive the infection traits and the virulence factors. In this work, five candidate genes (*i.e. Fcfga1, Fcfgb1, Fcac, Fcrho1* and *FcpacC*) were *in silico* annotated using the whole genome of *F. circinatum* as reference. The similarity of these proposed genes at nucleotide and protein levels with genes previously described in other *Fusarium* species was > 90% of identity and > 90% query coverage in all cases. In addition, the gene ontology of each candidate gene was also investigated.

1. Introduction

Fusarium circinatum Nirenberg & O´Donell is the causal agent of one of the most devastating forest diseases around the world: the pine pitch canker disease. This pathogen infects coniferous species (mainly *Pinus* spp. and *Pseudotsuga menziesii* (Mirb.) Franco), causing wilting and bleeding cankers in mature trees, drastically reducing the value of timber and predisposing the trees to break during windstorms (Wingfield *et al*., 2008). In addition, it causes severe damping-off in seeds and seedlings in nurseries. Despite the high threat that this fungus embodies for native forests and pine plantations, there is limited knowledge about the genes involved in the infection process. On the other hand, the complete genome of *F. circinatum* has been sequenced and made accessible through public databases (Wingfield *et al*., 2012; De Vos *et al*., 2014).

Several virulence-related genes have been annotated in other species of *Fusarium*. Regarding the cell signaling pathways, the guanine nucleotide-binding protein subunits α and β have been reported as related with pathogenesis in fungi. These two subunits play a key role as essential elements upstream of cellular signaling process affecting either the mitogen-activated protein kinase (MAPK) cascade or the cAMP-dependent protein kinase pathway (cAMP-PKA) by triggering virulence factors as in the case of *Fusarium oxysporum* Schltdl. (Guo *et al*., 2016). Likewise, adenylate cyclase has been described as a major element in cAMP-PKA signaling. This pathway is involved with pathogenesis development in several fungi (*e.g. Colletotrichum orbiculare* (Berk.) Arx and *Ustilago maydis* (DC.) Corda), and it is linked to the MAPK pathway in virulence regulation (Kohut *et al*., 2010)

A number of genes known to be involved in metabolic process are also implicated in virulence. More specifically, Martínez-Rocha *et al*. (2008) highlighted the importance of GTP-binding protein as an element implied in the maintenance of a correct cell wall structure in *F. oxysporum*. According to these authors, the loss of the structure could facilitate the recognition of fungal membrane proteins by potential hosts. On the other hand, Caracuel *et al*. (2003) studied the pH signaling transcription factor in the same species and reported that the expression of this gene was reduced when pH becomes acidic (closer to host conditions) favoring the synthesis of virulence factors (*e.g.* cell-wall degrading enzymes).

In this study, we hypothesized that the genome of *F. circinatum* could include pathogenicity genes with high similarity with other species of *Fusarium*. In consequence, the goal of this research was to annotate candidate genes of *F. circinatum* that could be related with virulence.

2. Material and methods

The prediction of five candidate genes was performed using an empiric approach based on homology between annotated genes available in GenBank (https://www.ncbi.nlm.nih.gov/) and the genome of *F. circinatum* (ASM49732v2; genomes of fungal strains FSP34 and GL1327). The following criteria were fulfilled by the genes selected as queries: a) the reference genes were identified in other pathogenic *Fusarium* spp. (*e.g.* genes of *F. oxysporum* reviewed by Michielse & Rep, 2009), b) the complete nucleotide and protein sequences were available in GenBank and c) the biological function of the gene as a pathogenicity trait was previously reported in scientific literature. The accession numbers of genes selected as queries were summarized in Table 1.

The encoding regions of each reference sequence was accessed from GenBank, without removing intron sequences. These reference sequences were compared with the complete genome of *F. circinatum* using MegaBLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (database: whole-genome shotgun contigs (wgs); taxid:48490), to obtain the candidate sequences. The nucleotide sequences for *F. circinatum* were compared with those deposited in GenBank using BLAST and MegaBLAST algorithms in order to ensure the putative homology with target genes (Table 1). The annotation was assessed if the identity between candidate genes and queries was ≥ 90%. In parallel, the similarity at protein level between the candidate sequences and those available in GenBank was also checked by BLASTx using the non-redundant protein sequences (nr) database. The specific databases of Fungi (taxid: 4751) and *Fusarium* sp. (taxid 5506) were selected for more accurate searches (Table 1). Only proteins with a similarity higher than 90% in at least one search were selected for annotation. The software Geneious 6.0.6 (http://www.geneious.com) was used for sequence trimming and alignment (methods: CluscalW for nucleotide sequences; BLOSUM matrix for proteins), while the software AUGUSTUS 2.5.5 (http://augustus.gobics.de/) was required to predict amino acid sequences available in A2. Regarding gene ontology, the 100 best matches of the BLASTx search (*i.e.* BLASTx, nr database, *Fusarium* spp. (taxid 5506) as organism and word size of 3) were used as input data in BLAST2GO 4.1 software (https://www.blast2go.com/; required e-value: 10^{-10}). This software assigned the ontology of each gene using GO database (http://www.geneontology.org/page/go-database).

Table 1. GenBank best matches for five candidate genes related with the pathogenicity of *F. circinatum*.

Gene	Accession number (Reference gene)	Type of sequence	Algorithms	Database	Limit
Fcfga1	GU168785.1	Protein	BLASTx	nr	Fungi (taxid: 4751)
					Fusarium (taxid:5506)
		Nucleotide	BLASTn	nr/nt	Fungi (taxid: 4751)
			MegaBLAST		*Fusarium* (taxid:5506)
Fcfgb1	DQ457053	Protein	BLASTx	nr	Fungi (taxid: 4751)
					Fusarium (taxid:5506)
		Nucleotide	BLASTn	nr/nt	Fungi (taxid: 4751)
			MegaBLAST		*Fusarium* (taxid:5506)
Fcac	HF563555.1	Protein	BLASTx	nr	Fungi (taxid: 4751)
					Fusarium (taxid:5506)
		Nucleotide	BLASTn	nr/nt	Fungi (taxid: 4751)
			MegaBLAST		*Fusarium* (taxid:5506)
Fcrho1	XM_018889260.1	Protein	BLASTx	nr	Fungi (taxid: 4751)
					Fusarium (taxid:5506)
		Nucleotide	BLASTn	nr/nt	Fungi (taxid: 4751)
			MegaBLAST		*Fusarium* (taxid:5506)
FcpacC	XM_018893598.1	Protein	BLASTx	nr	Fungi (taxid: 4751)
					Fusarium (taxid:5506)
		Nucleotide	BLASTn	nr/nt	Fungi (taxid: 4751)
			MegaBLAST		*Fusarium* (taxid:5506)
All	-	Nucleotide	MegaBLAST	wgs	*Fusarium circinatum* (taxid:48490)

Table 1. (Cont.). QC: Query cover; E: E-value; ID: Similarity between sequences; Cd (coordinates): Number of first nucleotide position in the 5´/3´ extreme. nt: nucleotides.

Best match ID	Accession number	QC (%)	E	ID (%)	Cd	Length (nt)
Guanine nucleotide-binding protein subunit alpha [*Hirsutella minnesotensis* 3608]	KJZ72861.1	90%	0	91%		
guanine nucleotide-binding protein [*Fusarium oxysporum* f. *cubense*]	ACF20294.1	93%	0	91%	2594/ 3849	1256
Fusarium oxysporum fga1 gene for guanine nucleotide-binding protein alpha subunit, partial cds	AB072451.1	100%	0	96%		
Fusarium oxysporum fga1 gene for guanine nucleotide-binding protein alpha subunit, partial cds	AB072451.1	100%	0	96%		
Guanine nucleotide-binding protein subunit beta [*Fusarium oxysporum* f. sp. *cubense* race 1]	ENH75085.1	76%	0	98%		
Guanine nucleotide-binding protein subunit beta [*Fusarium oxysporum* f. sp. *cubense* race 1]	ENH75085.1	76%	0	98%	4948 10/49 6220	1411
Fusarium oxysporum fgb1 gene for guanine nucleotide-binding protein beta subunit, partial cds	AB072452.1	100%	0	95%		
Fusarium oxysporum fgb1 gene for guanine nucleotide-binding protein beta subunit, partial cds	AB072452.1	100%	0	95%		
putative adenylate cyclase [*Fusarium fujikuroi*]	KLO87628.1	89%	0	99%		
putative adenylate cyclase [*Fusarium fujikuroi*]	KLO87628.1	89%	0	99%	3308 84/33 8072	7189
Gibberella fujikuroi ac gene for adenylate cyclase, strain IMI58289	HF563555.1	100%	0	95%		
Gibberella fujikuroi ac gene for adenylate cyclase, strain IMI58289	HF563555.1	100%	0	95%		
mitochondrial *Rho* GTPase 1 [*Fusarium oxysporum* f. sp. *cubense* tropical race 4 54006]	EXM09411.1	99%	0	99%		
mitochondrial *Rho* GTPase 1 [*Fusarium oxysporum* f. sp. *cubense* tropical race 4 54006]	EXM09411.1	99%	0	99%	8954 5/914 02	1858
Fusarium oxysporum f. sp. *lycopersici* 4287 mitochondrial *Rho* GTPase 1 partial mRNA	XM_018380 855.1	99%	0	96%		
Fusarium oxysporum f. sp. *lycopersici* 4287 mitochondrial *Rho* GTPase 1 partial mRNA	XM_018380 855.1	99%	0	96%		
pH-response transcription factor *pacC* [*Fusarium oxysporum* f. sp. *vasinfectum* 25433]	EXM37240.1	99%	0	98%		
pH-response transcription factor *pacC* [*Fusarium oxysporum* f. sp. *vasinfectum* 25433]	EXM37240.1	99%	0	98%	1442 18/14 5606	1389
Gibberella fujikuroi pacC gene for transcription factor *PACC*, exons 1-4	AJ514259.1	100%	0	96%		
Fusarium verticillioides 7600 pH-response transcription factor *pacC*/RIM101 mRNA	XM_018893 598.1	100%	0	96%		
-	see text	100%	0	100%	-	-

3. Results and discussion

In this study, five candidate pathogenicity genes were detected in the *F. circinatum* genome using *in silico* methods (*i.e. Fcfga1*, *Fcfgb1*, *Fcac*, *Fcrho1* and *FcpacC*; Table 1, Supplementary files A1, A2 and A3). The role that these gene products plays in cellular signaling pathways has already been addressed in other species of the genus *Fusarium*, but these candidate genes have not been previously described in *F. circinatum*. The proposed annotation was supported by gene ontology and results provided by BLAST2GO agreed with the suggested function of each putative gene (Table 2).

Table 2. Best matches of gene ontology (based on GO database) for five candidate genes of *F. circinatum*.

Candidate gene	Domain	GO term description	GO term ID
Fcfga1	Molecular function	G-protein coupled receptor binding	GO:0001664
	Biological process	regulation of MAPK export from nucleus	GO:0071701
Fcfgb1	Molecular function	signal transducer activity	GO:0004871
	Biological process	heterotrimeric G-protein complex cycle	GO:0031684
Fcac	Molecular function	adenylate cyclase activity	GO:0004016
	Biological process	cAMP biosynthetic process	GO:0006171
Fcrho1	Molecular function	nucleic acid binding	GO:0003676
	Biological process	fungal-type cell wall biogenesis	GO:0009272
FcpacC	Molecular function	nucleic acid binding	GO:0003676
	Biological process	cellular response to alkaline pH	GO:0071469

Minor differences in amino-acid sequence can affect the functionality of the protein. In consequence, high similarities between queries and translated proteins do not guarantee the same biological function. In this study, the similarities between proteins did not reach 100%, nevertheless even in that case the annotation could not be directly assigned (Punta & Ofran, 2008). The results reported here (*i.e.* high homology of sequences and ontology) supported high probability of the same functions between queries and translated proteins. Hence, definitive annotation will require either structural characterization of proteins or biological assays focused on gene expression.

The candidate genes *F. circinatum* putative guanine nucleotide-binding protein subunit alpha (*Fcfga1*) and beta (*Fcfgb1*) were identified in the reference genomic shotguns AYJV02000016.1 (genome of fungal strain FSP34) and JRVE01000002.1 (fungal strain GL1327), respectively (Table 1). Guo *et al*. (2016) studied the disruption effect of *fga2* and

fgb1 genes disruption in *F. oxysporum* f. sp. *cubense* and they found that when the encoding gene of Gα subunit (*fga2*) was silenced, the *in vivo* virulence resulted strongly reduced.

A putative adenylate cyclase (*Fcac*) was found in the genomic region of *F. circinatum* identified as JRVE01000018 (fungal strain GL1327. Table 1). In *Fusarium proliferatum* (Matsush.) Nirenberg either virulence (female fertility and *in planta* pathogenicity) or resistance factors (*i.e.* thermo-tolerance and resistance against oxidative stress) were regulated by a homolog gene of *Fcac* called *Fpacy1* (Kohut *et al*., 2010).

The candidate gene in *F. circinatum* a putative GTP-binding protein (*Fcrho1*) was located in JRVE01000119.1 (fungal strain GL1327. Table 1). The loss of function of the possible orthologue *Rho1* in *F. oxysporum* reduced fungal virulence according to Martínez-Rocha *et al*. (2008). The last candidate gene analyzed was a putative pH signaling transcription factor (*FcpacC*), whose sequence was found in the genomic shotgun JRVE01000056.1 of *F. circinatum* (fungal strain GL1327. Table 1). Caracuel *et al*. (2003) reported that a plausible orthologue of *FcpacC* could repress the expression of some pathogenicity genes in alkaline conditions triggering the virulence factors in acidic environments (plant-fungus interface).

The metabolic pathways that drive the response of *F. circinatum* against external stimuli (*e.g.* host metabolites, nutrients, etc.) have not been deeply investigated. Consequently, the results proposed here could improve the knowledge about the basis of pathogenicity in this phylamentous fungus, contributing to better understand the etiology of pine pitch canker disease.

4. Acknowledgments

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Supplementary material (Supporting information)

A1. Nucleotide sequences of five candidate genes in *Fusarium circinatum*.

A2. Amino acid sequences of five candidate genes in *Fusarium circinatum*.

A3. Alignments of candidate genes and reference ones as nucleotide sequences and protein.

Files available in Wiley-Blackwell´s repository.

https://onlinelibrary.wiley.com/doi/abs/10.1111/efp.12417 [latest accession 15/06/2018]

Use the following QR code to download the supplementary material from Journal´s web page.

6.3. Virus – fungus interactions: Original Article III

Caracterización de cadenas cortas de ARN procedentes de mitovirus infectando el patógeno forestal *Fusarium circinatum*

Resumen

La infección causada por micovirus puede ser diagnosticada mediante el análisis de datos de ARN de cadena corta procedentes de secuenciación masiva. En este estudio se analizó la presencia de micovirus en diez aislados del patógeno forestal *Fusarium circinatum* mediante *high-throughput sequencing* (HTS) de RNA de cadena corta. Las secuencias fueron ensambladas *de novo* y alineadas con los genomas virales. De forma adicional, la presencia de cada cepa de virus en cada aislado se comprobó mediante RT-PCR con cuatro cebadores descritos previamente y siete de nuevo diseño. Los resultados demostraron que la secuenciación HTS posee un gran potencial en el diagnóstico de virus hospedados por *F. circinatum.* Por otra parte, este trabajo supone, hasta donde se tiene conocimiento, el primer estudio sobre la respuesta antiviral por parte de este hongo fitopatógeno habiendo sido posible recuperar fragmentos de RNA derivados de virus y localizado posibles lugares de reconocimiento para la nucleasa dicer, la cual forma parte de la ruta metabólica del silenciamiento de ARN en la mayoría de los eucariotas.

Palabras clave: chancro resinoso de los pinos, dicer, *Mitovirus* sp., *Narnaviridae*, silenciamiento del ARN y vsRNA.

Characterization of small RNAs originating from mitoviruses infecting the conifer pathogen *Fusarium circinatum*

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Abstract

Deep-sequencing of small RNAs has proved effective in the diagnosis of mycoviruses. In this study, the presence of mycoviruses in ten isolates of the phytopathogenic fungus *Fusarium circinatum* was investigated by high-throughput sequencing (HTS) of small RNAs. The contigs resulting from *de novo* assembly of the reads were aligned to viral genomes. The presence of each mycovirus detected in the isolates was confirmed by RT-PCR analysis with four previously described primer pairs and seven new pairs designed on the basis of sequencing data. The findings demonstrate the potential use of HTS for reconstructing previously identified mitoviruses infecting *F. circinatum*.

Keywords: dicer, *Mitovirus* sp., *Narnaviridae*, pine pitch canker, RNA silencing, vsRNA.

1. Introduction

Fusarium circinatum Nirenberg & O´Donell (teleomorph: *Gibberella circinata* Nirenberg & O´Donell) is the causal agent of pine pitch canker (PPC), one of the most devastating diseases in coniferous forests, plantations and nurseries worldwide. This pathogen severely affects *Pinus* species and also *Pseudotsuga menziesii* (Mirb.) Franco, causing pre-, post- and late damping off on seedlings (with mortality rates of up to 90%). In mature trees, it causes wilting, slow growth and bleeding cankers, reducing the economic yield of the affected timber and increasing the risk of trees breaking during windstorms (Wingfield *et al.*, 2008). The fungus is widespread in several parts of America, Europe, Asia and Africa and is mainly spread via soil- and airborne spores, movement of infected material (seedlings, seeds, pruning tools, etc.) and carrier and vector insects (Bezos *et al.*, 2012; Brockerhoff *et al.*, 2016). Although there are no effective means of controlling the disease in the field, biocontrol is currently one of the most promising management options (Bezos *et al.*, 2017).

Fungal viruses (mycoviruses) are frequent in nature, infecting a huge variety of fungi ranging from edible mushrooms to yeasts (Pearson *et al.*, 2009). Mycoviruses are of great interest in plant pathology as some are able to reduce the pathogenicity of their hosts (hypovirulence) (Xie & Jiang, 2014), although many aspects of their biology and ecology remain poorly understood. The best known case of a mycovirus infection affecting fungal virulence is the chestnut blight pathosystem (causal agent *Cryphonectria parasitica* (Murrill) M.E. Barr) (Zamora *et al.*, 2014), in which hypovirulence has been used in practice to control *C. parasitica* infections. This has led to increased interest in the use of mycoviruses as biocontrol agents in infections caused by other phytopathogenic fungi (Peever *et al.*, 2000; Rigling *et al.*, 1989). In relation to the forest pathogen *F. circinatum*, three different members of the genus *Mitovirus* (*Narnaviridae*) have been described: Fusarium circinatum mitovirus 1, 2-1 and 2-2 (FcMV1, FcMV2-1 and FcMV2-2) (Martínez-Álvarez *et al.*, 2014). These viruses are rather prevalent among isolates in northern Spain (Vainio *et al.*, 2015b). Despite the interest surrounding this pathogen in forest health, the effects of FcMV1, FcMV2-1 and FcMV2-2 on *F. circinatum* are not yet fully understood. It has been reported that FcMV1 infection significantly increases fungal virulence in plants (Muñoz-Adalia *et al.*, 2016); however, the effect of mycoviral infection may differ depending on environmental conditions (Botella *et al.*, 2017; Vainio *et al.*, 2010). Hence, the anti-viral response in phytopathogenic fungi deserves further attention.

In recent years, the use of next generation sequencing technology has improved our knowledge of mycovirus diversity and some aspects of mycovirus-host interactions. Thus, mycoviruses have been successfully detected by total RNA (Marzano & Domier, 2016), dsRNA

(Osaki *et al*., 2016) and deep sequencing of small RNAs (Donaire & Ayllón, 2016; Nerva *et al*., 2016; Vainio *et al*., 2015a). The identification of virus-derived small RNA fragments (vsRNA) by high-throughput sequencing (HTS) has revealed cryptic viruses and suggests that both ascomycete and basidiomycete fungi perform a RNA silencing pathway in which vsRNA plays a key role (*e.g.* Donaire and Ayllón (2016); Vainio *et al*. (2015a)).

The RNA silencing process (RNAi) is an important post-transcriptional control pathway described in plants, animals and fungi (Schumann *et al*., 2010). This molecular mechanism is involved in some cellular processes such as gene regulation and defence against selfish nucleic acids. Hence, infection by RNA viruses is thought to trigger the RNA silencing response of the host. The RNAi machinery includes a dicer or dicer-like protein (ribonuclease III-like enzyme) that recognizes viral dsRNA molecules and cleaves them to vsRNA of ~21-25 nucleotides in length (Tauati *et al*., 2014; Tinoco *et al*., 2010). RNA-induced silencing complex (RISC) includes argonaute-like proteins which unwind the paired strands of vsRNA, degrade one of them and use the other to identify the cognate sequences (viral RNA). A ribonuclease H-like enzyme associated with argonaute then degrades the viral target resulting in an anti-viral response (Chen *et al*., 2015; Hammond *et al*., 2008; Zhang *et al*., 2014).

In this study, we hypothesized that *F. circinatum* processes viral RNAs into vsRNA in a similar manner as described for other pathogenic fungi. We investigated whether deep sequencing of small RNAs can reveal new viral strains. The aim of this study was therefore to investigate the practical use of small RNA deep sequencing for the identification of mycoviruses infecting *F. circinatum*.

2. Material and methods

2.1. Fungal isolates, total RNA isolation and high-throughput sequencing of small RNAs

A total of ten *F. circinatum* isolates collected in different regions of Spain were selected for study (Table 1). The fungal isolates were cultured in Petri dishes containing PDA medium (3.90% w/v potato-dextrose-agar, Scharlab S.L., Spain). After culture for a week in darkness at 25 ºC, each isolate was subcultured in cellophane-membrane-covered PDA medium for total RNA extraction.

The total RNA from each isolate was extracted from mycelia ground in liquid nitrogen with the aid of a sterilized pestle. The powdered sample was sequentially treated with TRI Reagent® (Sigma Aldrich Química S.L., Spain) followed by chloroform 99% v/v (PanReac Química, Spain), and the total RNA was then precipitated with isopropanol 70% v/v (Sigma Aldrich). The

pellets were washed with ethanol 75% v/v (PanReac Química) and resuspended in 20 µl of double sterilized Milli-Q water. After RNA extraction, samples were transferred into RNase- and DNase-free tubes (Axygen®, USA) and stored at -80º C. The RNA of each isolate was pooled in two aliquots (20 µl; 2 µl per isolate, final concentration of 1053 ng µl^{-1}). The quality and quantity of RNA in one pooled extract was measured in a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA) and the other aliquot was stored at -80º C to preserve the integrity of RNA. The same pooled extract was run in an 1.20% w/v agarose D1 low EEO (Conda Laboratories, Spain) gel firstly for 10 min at 70 v and then for 65 min at 50 v. After that, the gel was submerged for 25 min in ethidium bromide (Merck, Germany) staining solution 10% v/v and then visualized under UV light. The size of bands was estimated by comparison with DNA Molecular Marker II (Roche Life Science, Spain).

The remaining pool of RNA was sent to Fasteris SA (Switzerland; https://www.fasteris.com) for siRNA library construction and HTS (Illumina HiSeq 2500, Illumina Inc., USA). The sample processing consisted of acrylamide gel purification of small fragments of RNA, single-stranded ligation (3´ and 5´ adapters) and cDNA library generation by reverse transcription and PCR.

2.3. Alignment of Illumina sequence reads and de novo assembly

For the analysis, Illumina adapter sequences were removed from reads with Trimmomatic software v0.32 (Bolger *et al*., 2014). Exploratory analysis of reads was performed with Geneious Pro 6.0.6. (Kearse *et al*., 2012) (Mapping parameters: sensitivity, medium-low; iterations for consensus, two; minimum mapping quality, 99.90%; word length, 18 nt and maximum ambiguity, 4 states), and the complete reads dataset (1-50 nt) was mapped against the following queries: (a) genome of *F. circinatum*, (b) genome of the fungal mitochondria, (c) previously known *F. circinatum* mitoviruses (FcMV1, FcMV2-1 and FcMV2-2) and (d) contigs of interest obtained in this study (see below). The size distribution of reads corresponding to viral genomes (vsRNA) was visualized in a histogram and the mean size was calculated for each strain of virus. All reads between 19-35 nt were mapped against viral genomes by using MISIS v2.7 (Seguin *et al*., 2014) to identify sense and antisense vsRNA. In addition, the hotspots of vsRNAs accumulation throughout viral genomes were analyzed as plausible recognition sites for dicer proteins. The peak values were first identified using "peakPick" package (Weber *et al*., 2014) of software R (R Development Core Team, 2015) (analysis window, ± 10 positions; limit, 12 standard deviations). All peaks that were not covered by > 100 vsRNAs (sense and antisense) were then removed, and the range of mean read size of each hotspot was calculated.

De novo assembly was conducted by computing a total of 33 models in the software Velvet 1.2.10 (Zerbino & Birney, 2008). Each model was defined by three parameters: (a) read size (b) k-mer size and (c) whether or not AssemblyAssembler 1.4 script (Jacob Crawford, Cornell University) was used for the analysis. The complete dataset of reads (read size between 1-50 nt) was used in a total of 12 analyses. In parallel, the complete dataset of reads was mapped against the host genome (*i.e. F. circinatum* genome: Accession ASM49732v2) using Bowtie software 1.2.0 (Langmead *et al.*, 2009). The resulting non-aligned reads (*i.e.* reads that did not match with any region of the fungal genome) were selected for a total of 15 analyses in Velvet. The Bowtie output files showed that most informative reads were ≥14 bp long, and therefore the reads between 14-31 bp were selected for 6 additional models. Regarding the k-mer size, all k-mer between 9-31 bp were used in different analysis (Supplementary material A1). A total of 9 models were run using the post-assembly script Assembly Assembler 1.4., as previously reported for purposes of mycovirus assembly (Vainio *et al.*, 2015a).

The models which simultaneously provided the highest values of N50 and number of contigs generated during Velvet computation were selected for further analysis. The contigs were compared against known sequences in the NCBI GenBank Viruses database (taxid: 10239; https://www.ncbi.nlm.nih.gov/) by using BLASTx and BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify viral sequences. Moreover, the resulting contigs of these models were aligned against FcMV1, FcMV2-1 and FcMV2-2 genomes (Genbank accession numbers: KF803546.1, KF803547.1 and KF803548.1 respectively) in Geneious Pro 6.0.6., in order to estimate the number of effective contigs (contigs that corresponded to the viral genome). Contigs with similarity of less than 90% to reference were removed from the alignment. The percentage sequence coverage was calculated to enable selection of the best model for each mycovirus.

2.4. Analysis of viral prevalence

RNA samples of the isolates included in pool preparation (see above) were used as templates for RT-PCR with selective primers. The cDNA was synthesised using random hexamer primers and PrimeScript™ II Reverse Transcriptase (Takara Bio USA Inc., USA) as previously described (Vainio *et al.*, 2011). The subsequent PCR reactions were carried out using the KAPA Taq PCR kit (Kapa Biosystems, USA) and eleven selective primer pairs (Table 2). The PCR reaction volume was 50 µl and the protocol consisted of denaturation for 10 min at 95º C followed by 37 cycles of 30 s at 95º C, 45 s at the different annealing temperatures summarized in Table 2 and 2 min at 72º C. The final elongation step consisted of 7 min at 72º C (Vainio *et al.*, 2015b). The PCR products were separated for 50 min at 110 v in 1.60% w/v agarose D1

low EEO gel stained with 0.004% v/v 10000 x GelRed^TM (Biotium, USA). The size of the resulting fragments was determined by comparison with a DNA Molecular Marker II (Roche). The products of PCR reactions performed with the primer pairs FcontFOR/FcontREv and FcirCONT/FcirCONTRev were run in 2% w/v agarose D1 low EEO gel under the above-mentioned staining and electrophoretic conditions. In these cases, the amplicon size was estimated by comparison with a 50 bp DNA ladder (Nippon Genetics Europe, Germany). New primer pairs (Table 2) were designed using Primer3Plus software (Untergasser *et al*., 2012) and their homology to query sequences was confirmed using Geneious Pro. 6.0.6. The amplicons were sent to Macrogen Europe Inc. (The Netherlands; www.macrogen.com) for DNA purification and sequencing.

Sequences obtained using selective primers and Sanger sequencing were trimmed using Geneious Pro. 6.0.6 and compared against those deposited in the GenBank (NCBI) database using MegaBLAST (nr/nt database; taxid: 10239). All sequences obtained by direct sequencing of amplicons were aligned against FcMV1, FcMV2-1 and FcMV2-2.

3. Results

3.1. Yield and size distribution of the vsRNA reads

The small RNA deep sequencing analysis included ten *F. circinatum* strains isolated from plant material and bark beetles (Table 1). The quality of the total RNA extracts used for the small RNA library construction was acceptable, based on spectrophotometer (absorbance ratio 260/280: 1.85) and agarose gel analysis (visible bands of 18S and 28S rRNA). The total number of reads produced by the Illumina sequencer was 24415829 (insert size 1-50 bp), corresponding to 1230 megabases. The proportion of clusters that fulfilled the default Illumina quality criteria was 95.73%, and the proportion of bases with a quality score ≥ 30 (Q30) was 96.12%. The numbers of reads per insert size were distributed as follows: 60.26% inserts of 27-50 bp, 38.56% inserts of 18-26 bp and 1.17% inserts with a size ≤ 17 bp (Figure 1).

Regarding the annotation of reads, 86.55% of the obtained reads were assigned to the queries summarized in Figure 2. The sequence coverage was 97.90% for FcMV1 and more than 98.50% for FcMV2-1 and FcMV2-2 (Figure 3). The mean read sizes that mapped with viral genomes were 25.52 ± 0.18 nt for FcMV1, 24.27 ± 0.10 nt for FcMV2-1, 24.99 ± 0.14 nt for Fcmv2-2 (mean and standard error; Figure 4). A total of 11, 21 and 28 hotspots fulfilled the selection criteria for plausible dicer recognition sites in FcMV1, FcMV2-1 and FcMV2-2 respectively. The mean size of reads accumulated in the hotspots ranged between 21.80-31.41 nt in FcMV1, 20.89-29.55 nt in FcMV2-1 and 21.27-30.80 nt in FcMV2-2.

Table 1. Isolates of *Fusarium circinatum* used in this study: geographical origin of the strains and the living organisms from which *F. circinatum* was isolated (source) are provided. The viral infection by Fusarium circinatum mitovirus 1, 2-1, 2-2 (FcMV1, FcMV2-1 and FcMV2-2, respectively), contig 50 and contig 571 (model 310) are shown by the symbol +.

Isolate	Origin	Source	FcMV1	FcMV2-1	FcMV2-2	contig 50	contig 571
FC5	Asturias	*Pinus canariensis* Sweet ex K. Spreng	-	-	-	-	-
FC122	Cantabria	*Tomicus piniperda* L.	-	+	-	-	+
FC179	Cantabria	*T. piniperda*	+	+	+	+	+
FC213	Cantabria	*Pityophthorus pubescens* Marsham	+	+	-	-	+
FC921	Cantabria	*T. piniperda*	-	+	-	-	-
Va70	Cantabria	*Pinus radiata* D. Don	-	-	-	-	-
FC13	Castile and León	*P. radiata*	-	-	-	-	-
FC14	Castile and León	*P. radiata*	-	-	-	-	-
FC20	Galicia	*Pinus nigra* Arnold	-	+	-	-	+
FC24	Basque Country	*P. radiata*	-	-	-	-	-

Table 2. Primer pairs used for FcMV1, FcMV2-1 and FcMV2-2 identification by RT-PCR. *: Sequencing of amplified fragment is required for the identification of each virus strain.

Primer pair	Forward primer	Reverse primer	Annealing temperature (°C)	Amplicon size (bp)	Virus preference	Reference
FMC1F1/ FMC1F1rev1	5´- CGTGGGATTAAAACCCACA AA-3´	5´- TGGTAATCTACCATAGCAATTA YTC-3´	49.5	440	FcMV1	(Vainio et al., 2015b)
FMC3F1/ FMC3Rev1	5´- GAYAGAACTTTACTCAA GATCC -3´	5´- ATTCATCTYTTGGCAAATTCAT A-3´	47.5	461	FCMV2-1* and FCMV2-2*	(Vainio et al., 2015b)
FMC2MFor/ FMC3Rev1	5´- GCATCAAATAGTGCTCTG AC-3´	ATTCATCTYTTGGCAAATTCAT A-3´	49.5	821	FCMV2-1*	(Vainio et al., 2015b)
FMC3MidF/ FMC3Rev1	5´- TCAACCATAAACTGATCCATG T-3´	ATTCATCTYTTGGCAAATTC ATA-3´	48.5	996	FCMV2-2*	(Vainio et al., 2015b)
FMV1BL/ FMV1BLrev	5´- AGGTCAACCTATGGGAG CAT-3´	AGACCACTTATTTCTTTCCCTG A-3´	51	282	FcMV1	This study
FM22for / FM22rev	TGGTTTTGCCAAGGGGT GAC-3´	5´- TACCTCTACTTGGTAGTCCAG TG-3´	53	731	FCMV2-2*	This study
FMNGS1for/ FMNGS1rev	5´- CGTGACAGCCCCCCTCTT ATG-3´	TGACCTTGTAGAATTTCAAGTT GATT-3´	54.5	430	FcMV1	This study
FMNGS21fr/ FMNGS21rv	5´- AAAGAAAACTTCATGATCA GTGCTT-3´	CGTCTGTCAAGACGAAACTTT A-3´	51	970	FCMV2-1*	This study
FMHTS22/ FMHTS22rev	5´- AAACTCTCAAAGAGGTCC AAGG-3´	TCGTATTCAAACATTTCACCAA -3´	51.5	1130	FCMV2-2*	This study
FcontFOR/ FcontREV	5´- GGTCTCGTACGTAATGAA ATTCAAC-3´	5´- CAATGGTTAACAATGTGGCAT A-3´	54.3	163	contig 50* (Model 310)	This study
FcirCONT/ FcirCONTRev	5´- CAGTGACAGACTACTTTA TAG-3´	5´- ATAGCTCAAGATGAATAACAG CCCATCGG-3´	42.4	60	contig 571* (Model 310)	This study

3.2. Contig assembly and mapping

The highest values of N50 and resulting contigs (Supplementary material A1) were obtained for models 204, 310 and 360. The viral contigs provided by these models were identified on the basis of comparison of sequences with those included in the NCBI GenBank Viruses database (taxid: 10239) by using BLASTx and BLASTn. Using this approach, we detected 47, 40 and 40 contigs per model (*i.e.* models 204, 310 and 360 respectively), corresponding to previously known *F. circinatum* mitoviruses and two contigs that were apparently more similar to mitoviruses infecting other fungal species (*i.e.* contig 50 and 571 from model 310. Table 3). The possible origin of these contigs was examined on the basis of RT-PCR with specific primers (see below). However, isolate FC179 was simultaneously infected by the three mitovirus strains and showed positive amplification for the two contigs considered (see below). In addition, only one FcMV2-2 infection was identified among the host isolates, whereas FcMV2-1 was represented by multiple strains (N= 5. Table 1).
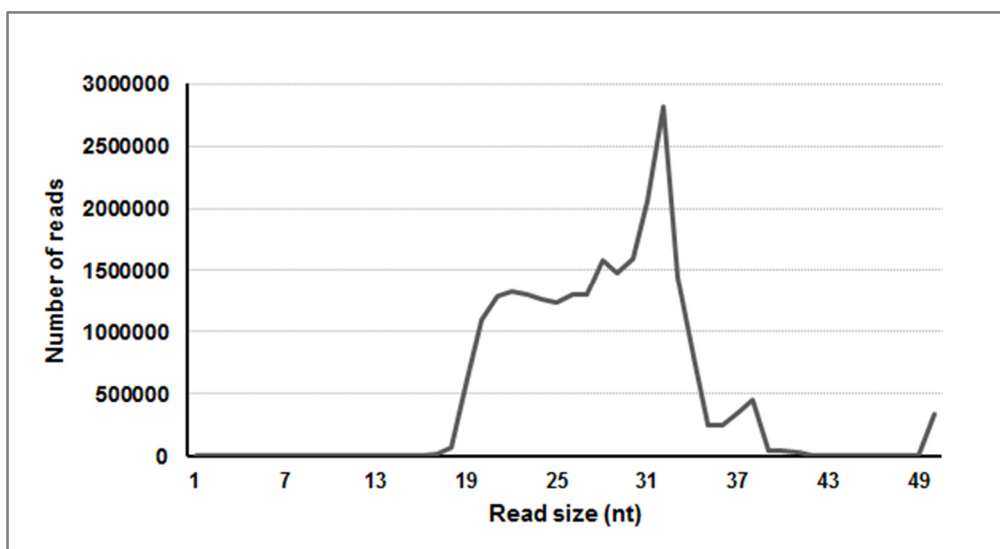
Figure 1. Size distribution of small RNA reads obtained using Illumina HTS.

Based on mapping the contigs against the genomes of known *F. circinatum* mitoviruses, 0.21% of contigs resulting from Model 204 corresponded to FcMV1 genome, 0.22% both with FcMV2-1 and FcMV2-2. Model 310 yielded 0.37% of their contigs as corresponding to FcMV1, 0.37% to FcMV2-1 and 0.40% to FcMV2-2. Model 360 showed that 0.37% of contigs corresponded to the genome of FcMV1, 0.37% with FcMV2-1 and 0.40% with FcMV2-2. The three models showed more than 50% coverage of the viral genome (Table 4), with Model 310 and Model 360 providing the best coverage values.

3.3. Detection of viruses by RT-PCR and mitovirus identification

All the PCR primers used successfully yielded amplification. The primer pair FMC3F1/FMC3Rev1 amplified sequences of FcMV2-1 and FcMV2-2 indistinctly, while the primer pairs FMC1F1/FMC1F1rev1, FMV1BL/FMV1BLrev, FMNGS1for/FMNGS1rev and FcontFOR/FcontREv produced amplicons only in the isolates infected by FCMV1. In the same way, the pairs of primers FMC2MFor/FMC3Rev1 and FMNGS21fr/FMNGS21rv only amplified sequences that corresponded to FcMV2-1, while the primer pairs FMC3MidF/FMC3Rev1, FM22for/FM22rev and FMHTS22/FMHTS22rev amplified fragments of FcMV2-2.
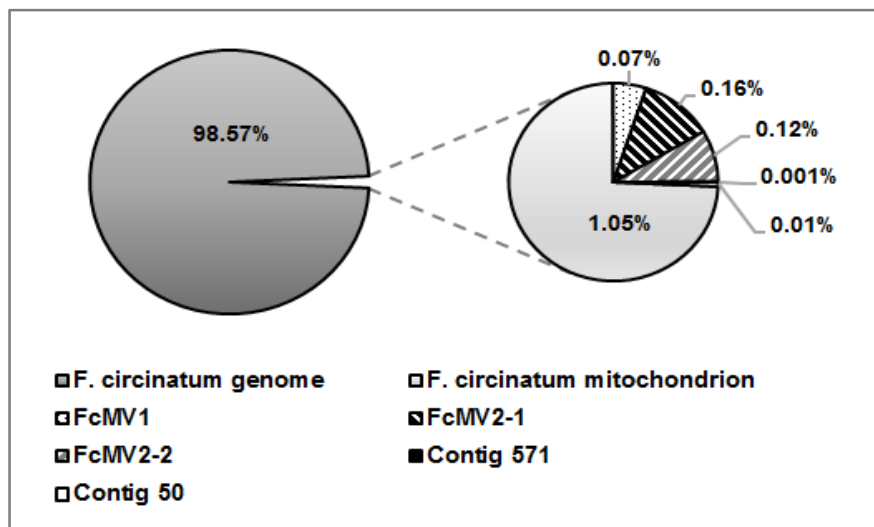

Figure 2. Distribution of annotated reads from HTS analysis by category.

Two new primer pairs were designed based on the contigs 50 and 571 (Table 3). Primer pair FcontFOR/FcontREv amplified a fragment of the expected size (163 nt) in one host isolate (*i.e.* FC179). The genome sequence of FcMV2-2 deposited in GenBank is only partial, and therefore we had to confirm whether contig 50 represents the 3' proximal sequence lacking for this virus. This was able to be verified as contig 50 was aligned with a previously unpublished cloned genome fragment from isolate FcCa070 (Martínez-Álvarez *et al.*, 2014) linking it with the FcMV2-2 sequence end (Supplementary material A2). In addition, the whole dataset was mapped against FcMV2-2 including contig 50 in 3´ extreme (Figure 3d) and the distribution of reads size was also investigated, revealing a mean size of 24.94 ± 0.13 nt in length (Figure 4). The primer pair FcirCONT/FcirCONTRev amplified fragments of similar sample size to that expected for contig 571 in four host isolates (Table 1), irrespective of the presence or absence of previously known Fusarium circinatum mitovirus strains. However, the sequences obtained were not informative, because of the short length of the amplicons (< 50 bp) (Tables 1 and 2). Otherwise, contig 571 shared ~60-65% global nt sequence identity with the three previously known *F. circinatum* mitoviruses.

Table 3. Best matches of contig 50 and contig 571 (Model 310) according to BLAST searches against the GenBank database. L, length of each contig expressed as number of nucleotides; Database, nr (non-redundant protein sequences) and nr/nt (nucleotide collection); QC, Query coverage; E, E-value; ID, Identity.

Model	Contig	L	Algorithms	Database	Limit	Match ID	QC (%)	E	ID (%)	Accession number
310	50	216	BLASTx	nr	Viruses (taxid:10239)	RNA-dependent RNA polymerase [Fusarium poae mitovirus 1]	96%	< 0.01	80%	YP_0092728 98.1
			BLASTn	nr/nt	Viruses (taxid:10239)	Fusarium poae mitovirus 1 genomic RNA, complete genome	97%	< 0.01	81%	LC150564.1
310	571	63	BLASTx	nr	Viruses (taxid:10239)	RNA-dependent RNA polymerase [Sclerotinia sclerotiorum mitovirus 12]	93%	< 0.01	73%	AHF48628.1
			BLASTn	nr/nt	Viruses (taxid:10239)	Rhizoctonia solani mitovirus 11 isolate 42304-9b RNA-dependent RNA polymerase gene, complete cds	73%	< 0.01	80%	KP900906.1

Table 4. Sequence coverage of FcMV1, FcMV2-1 and FcMV2-2 genomes by the resulting effective contigs of the three best models. * , partial genome.

Mycovirus	Model 204	Model 310	Model 360
FcMV1	39.7%	59. %	59.9%
FcMV2-1	52.1%	52.7%	52.7%
FcMV2-2*	35.9%	53.5%	53.5%

A total of 50% of the isolates were virus-free, while 10% had a single infection and 40% of the *F. circinatum* strains were co-infected (Table 1). More specifically, FC921 was infected with FcMV2-1, while FC20 and FC122 were co-infected with FcMV2-1 and the plausible new mitovirus variant (contig 571). In addition, multiple virus infection was observed in two of the isolates: thus isolate FC213 was infected with FcMV1, FcMV2-1 and contig 571, and isolate FC179 was infected with FcMV1, FcMV2-1, FcMV2-2 and contig 571.
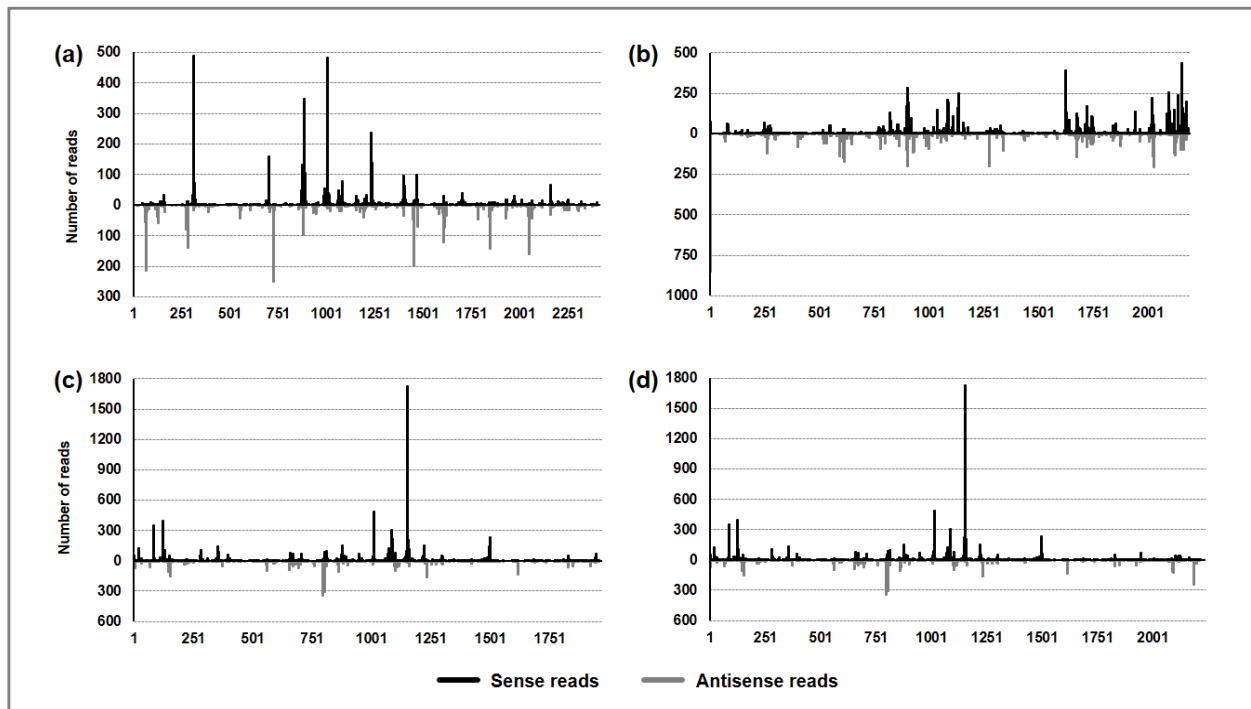

Figure 3. Distribution of reads (19-35 bp) from small RNA sequencing along the (a) FcMV1 (complete genome), (b) FcMV2-1 complete genome, (c) FcMV2-2 partial genome and (d) FcMV2-2 partial genome including contig 50 in 3´ extreme.

4. Discussion

Next generation sequencing represents a powerful tool for the analysis of viral infections (Deakin *et al*., 2017; Nerva *et al*., 2016). In this study, HTS of vsRNA was used for the first time to recover members of *Narnaviridae* infecting *F. circinatum*, demonstrating the suitability of the approach, as previously reported for mycoviruses infecting *Heterobasidion* spp. (Vainio *et al*., 2015a). Vainio *et al.* (2015a) used a similar computing method and reported that 3.70% of resulting contigs corresponded to viruses, while less than 1% did so in this study. Similarly, Donaire and Ayllón (2016) found a higher proportion of reads corresponding to viral genomes. By contrast, similar yields to those found for *F. circinatum* mitoviruses were obtained in other studies focused on plant viruses and mycoviruses (Chen *et al*., 2015; Nerva *et al*., 2016). The rate of viral sequencing may be explained by low quality RNA, although the Q30 values and

the low proportion of reads ≤ 17 nt (Figure 1) did not indicate degradation of the RNA. Possible biological explanations include a low rate of viral replication (latency) at the time when RNA was extracted or reduced activity of the RNAi machinery. In this regard, it is known that mitoviruses replicate inside mitochondria while RNA silencing mainly occurs in the cytoplasm. However, effective RNAi has been reported in other fungi infected by members of *Narnaviridae* including *Mitovirus* spp. (Donaire & Ayllón, 2016). Thus, mitochondrion embodies a key element in signalling pathways which trigger the anti-viral response in humans and other mammals (McBride *et al*., 2006; Moore & Ting, 2008). Recently, Nibert (2017) investigated the relationship between the presence of UGA codons in the genome of mitoviruses and in the genetic code of the host mitochondrion. This triplet implies tryptophan inclusion in the polypeptide chain in the genetic code of mitochondria, although is translated as a stop codon in the standard code. The findings reported by Nibert (2017) suggest that mitoviruses hosted by *F. circinatum* (*i.e.* FcMV1 and FcMV2-1) have a high percentage of UGA codons in their genomes and they could therefore be considered rarely present in cytosol. This finding may explain the low proportion of reads provided by HTS in this study. On the other hand, an signalling pathway involving mitochondria and possible RNA silencing activity inside mitochondria (Donaire & Ayllón, 2016) cannot be ruled out as elements of anti-viral response in this fungus.
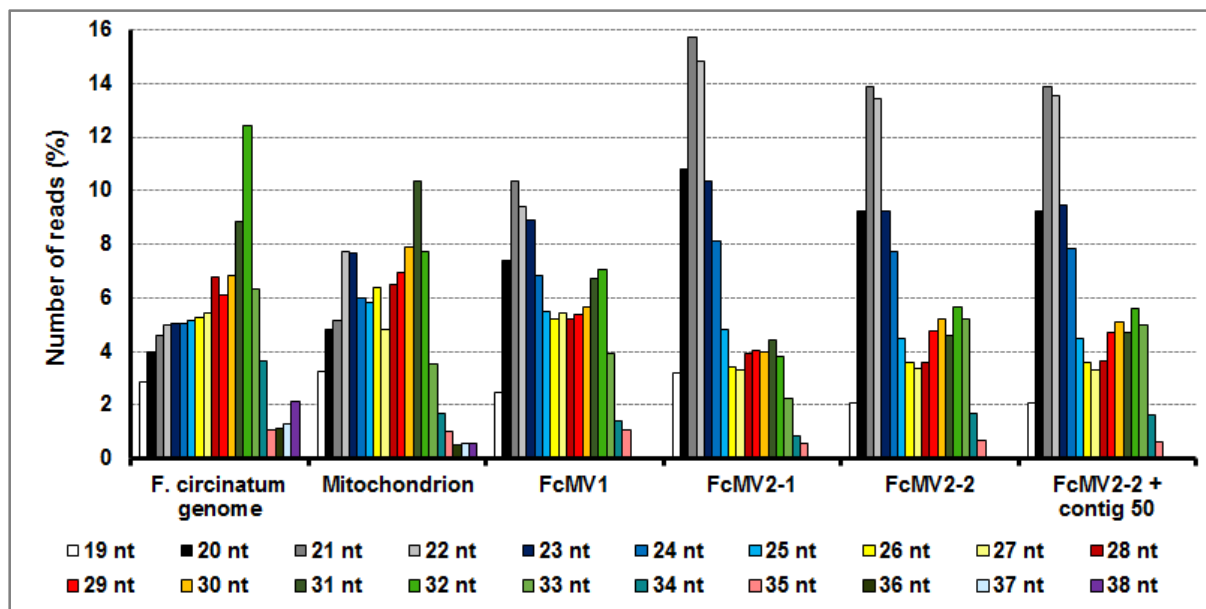

Figure 4. Size distribution of small RNA reads matching with the genome of *F. circinatum*, the genome of fungal mitochondrion and mitoviruses hosted by *F. circinatum* (FcMV1, FcMV2-1, FcMV2-2 and FcMV2-2 including contig 50). Sizes with relative abundance lower than 0.50% are not shown.

To our knowledge, this study represents the first examination of the molecular anti-viral response in *F. circinatum*, and hence vsRNA analysis may provide insights into the process. As shown in Figure 4, the most informative read size ranged between 19-35 nt (*i.e.* > 50% of reads sized between 20-26 nt in each virus strain), which is consistent with the size of vsRNA reported in other filamentous fungi (Dang *et al*., 2011; Vainio *et al*., 2015a; Zhang *et al*., 2008). For *de novo* assembly, either 14-31 nt (Bowtie output) or 1-50 nt reads were used, resulting in a similar contig yield as when k-mer 17 was used. These results show that multiple model computation is required to prevent underestimating reads with biological meaning. Chen *et al*. (2015) also observed multiple vsRNA sizes among HTS reads and suggested the possible participation of multiple dicer-like proteins in RNAi of maize (*Zea mays* L.). In chestnut blight, two dicer proteins (genes *dcl-1-2*) have been identified and their participation in anti-viral response has been reported (Segers *et al*., 2007). Similarly, four argonaute-like genes (*i.e. agl1-4*) have been also characterized in *C. parasitica*, with the *agl2* gene being the only one required for the induction of RNA silencing of viruses (Sun *et al*., 2009). Otherwise, the results reported here confirmed several hotspots along the viral genome, as reported by Donaire *et al*. (2009) and possibly suggesting multiple recognition sites for dicer proteins. Further studies are therefore required to characterize the pathways involving the RNAi machinery (*e.g.* cleavage motifs of dicer) of *F. circinatum*.

In this study, the viral prevalence was moderate to high (*i.e.* 50% of isolates were infected by mycoviruses), while the co-infection rate was moderate, despite the small sample size. These findings are consistent with those of Vainio *et al.* (2015b), who reported that isolates from Cantabria region showed moderate-high rates of single and double infection (> 80% in some locations), while none of the *F. circinatum* strain from Basque Country showed infection. Regarding other geographical locations, isolates from Castile and León and from Asturias did not show any infection; however, 40% of the isolates from Asturias were infected by FcMV1 in the previously mentioned study. Interestingly, the isolate from Galicia (FC20) was co-infected, although this was not detected in an earlier study (Vainio *et al*., 2015b). The most plausible explanation for the new detection is the use of novel primer pairs or the possible low rate of viral replication suggested above. Mycoviruses appear to be more abundant in Cantabria than in other locations. However, the sampling effort was higher than in other parts of the Iberian Peninsula. Schoebel *et al.* (Schoebel *et al*., 2017) studied the genetic diversity of Hymenoscyphus fraxineus mitovirus 1 (HfMV1; *Narnaviridae*) throughout the distribution range of its host, the invasive fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya. The researchers concluded that two phylogenetic groups of viruses are present in Europe, which is consistent with the proposed introduction of two individuals of *H. fraxineus* from Asia. Considering all of these data, we conclude that there is no clear pattern in the

distribution of mycoviruses hosted by *F. circinatum* in Spain. Nonetheless, further studies are required to clarify whether the prevalence of FcMV1, FcMV2-1 and FcMV2-2 is related to a founder effect, as two introductions of *F. circinatum* in Spain have been suggested (Berbegal *et al*., 2013). In this study, small RNA deep sequencing revealed two contigs that showed similarities with mycoviruses not previously reported as being hosted by *F. circinatum*. Regarding the sequencing and read mapping results, we consider that contig 50 represents the 3´ end of the incomplete genome of FcMV2-2. However, the positive RT-PCR amplification using specific primers did not provide enough information to definitively support contig 571 as part of a new viral strain.

In summary, we used deep sequencing to investigate the infection of *Mitovirus* spp. in the forest pathogen *F. circinatum*. The findings show that HTS followed by *de novo* assembly is suitable for detecting previously identified mitoviruses, even for low viral prevalence. By contrast, this method was of limited value for characterizing new viral strains. Finally, we described new primer pairs for use in future research focused on viral infection of *F. circinatum*.

5. Acknowledgements

6. Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Supplementary material (Supporting information)

A1. Modelling parameters and results of models computed for mycoviruses identification. Only models that provided any contig are shown. Best models in bold.

Analysis ID	Insert size (bp)	Script	k-mer	Num. of contigs	N50
MODEL91	Unaligned reads (14-19)	-	11	43	38
MODEL92	Unaligned reads (14-19)	-	13	38	38
MODEL93	Unaligned reads (14-19)	-	15	13	37
MODEL94	Unaligned reads (14-19)	-	17	1	35
MODEL202	1-50	-	13	609	8
MODEL203	1-50	-	15	6911	48
MODEL204	**1-50**	**-**	**17**	**7050**	**54**
MODEL205	1-50	-	19	3700	54
MODEL206	1-50	-	21	1852	52
MODEL207	1-50	-	23	803	53
MODEL208	1-50	-	25	400	54
MODEL209	1-50	-	27	229	57
MODEL210	1-50	-	29	85	172
MODEL211	1-50	-	31	38	521
MODEL300	14-31	Assembly Assembler 1.4.	11-31	2157	67
MODEL310	**14-31**	**Assembly Assembler 1.4.**	**13-27**	**3430**	**71**
MODEL320	14-31	Assembly Assembler 1.4.	15-31	1441	83
MODEL330	14-31	Assembly Assembler 1.4.	9-25	5729	68
MODEL340	14-31	Assembly Assembler 1.4.	11-27	2839	68
MODEL360	**14-31**	**Assembly Assembler 1.4.**	**13-29**	**3431**	**70**
G_MODEL	Unaligned reads (14-19)	Assembly Assembler 1.4.	9-19	73	42
A_MODEL	Unaligned reads (14-19)	Assembly Assembler 1.4.	11-31	14	60
B_MODEL	Unaligned reads (14-19)	Assembly Assembler 1.4.	13-27	6	71

A2. Sequence alignment showing the last 182 nucleotides of FcMV2-2 from isolate FcCa070 (GenBank accession number KF803548), a previously unpublished cloned genome segment from strain FcCa070 (070_M13F), and contig 50 (NODE_50) obtained via small RNA deep sequencing during this study.

```
              1         10        20        30        40        50        60
              |         |         |         |         |         |         |
   KF803548   UUCGCUAGAAAAUUAUAUCUAGAUGAAGAAUAUAAGAUACCAACAACAAUUAAAUCGUUA
   070_M13F   TTCGCTAGAAAATTATATCTAGATGAAGAATATAAGATACCAACAACAATTAAATCGTTA
   NODE_50    ------------------------------------------------------------

   KF803548   AAGACGAUUAUUGGAAUUGGUCUAAAAUCAAAAAUUGUAGAACAAAUUCAAACUCUUGAU
   070_M13F   AAGACGATTATTGGAATTGGTCTAAAATCAAAAATTGTAGAACAAATTCAAACTCTTGAT
   NODE_50    ------------------------------------------------------------

   KF803548   AAUCUUAAAUCUGCCUUUAAUAAGAUGGAUUUCAAACUACCAGAUCCGAUGAGAUCUGCU
   070_M13F   AATCTTAAATCTGCCTTTAATAAGATGGATTTCAAACTACCAGATCCGATGAGATCTGCT
   NODE_50    ------------------------------------------------------------

   KF803548   CA----------------------------------------------------------
   070_M13F   CATATTAAATTACACTTAGTATGACCATTATATAATGGAGTACGTAATGAAATTCAACAT
   NODE_50    ------------------------------------GTACGTAATGAAATTCAACAT

   KF803548   ------------------------------------------------------------
   070_M13F   CTTTATAAAGGATATAATCAGAAAATTGACAAAGATAATATGTCAGTTCACGATTATGTG
   NODE_50    CTTTATAAAGGATATAATCAGAAAATTGACAAAGATAATATGTCAGTTCACGATTATGTG

   KF803548   ------------------------------------------------------------
   070_M13F   TCAGAGGTAAGGATTATCGATT---------------------------------------
   NODE_50    TCAGAGGTAAGAATTATCGATTGACAAGGAATTTATGAGAAACAGCGGCAGAAATATGCC

   KF803548   ------------------------------------------------------------
   070_M13F   ------------------------------------------------------------
   NODE_50    ACATTGTTAACCATTGGATCAACAATGAATAAAGGTTTCTCAGAAGTTAACGAATATATG

   KF803548   ----------------------------
   070_M13F   ----------------------------
   NODE_50    AAAGCTAATAAAGGTAACCCATTAATGGC
```

6.4. Fungus – plant interactions: Original Article IV

E. Jordán Muñoz-Adalia, M. Carmen Cañizares, Mercedes Fernández, Julio J. Diez and M. Dolores García-Pedrajas. The *Fusarium circinatum* gene *Fcrho1*, encoding a putative Rho1 GTPase, is involved in vegetative growth but dispensable for pathogenic development. Manuscript.

El gen *Fcrho1*, que codifica un homólogo putativo de la GTPasa Rho1, participa en el crecimiento vegetativo, pero no en el desarrollo patogénico de *Fusarium circinatum*

Resumen

Fusarium circinatum es el hongo causante del chancro resinoso de los pinos, una de las enfermedades forestales más devastadores a escala mundial. Este hongo causa marchitamiento del cuello de raíz en plántulas de pino, así como reducción del crecimiento, clorosis y aparición de chancros en arbolado adulto (pinares y repoblaciones). La disponibilidad de la secuencia genómica completa de *F. circinatum* hace posible la anotación *in silico* de regiones codificantes por homología con genes identificados en otras especies fúngicas. El gen *Fcrho1* ha sido anotado *in silico* como un homólogo putativo de los genes que codifican la proteína con actividad GTPasa Rho 1. En el presente estudio, se generaron mutantes de deleción carentes de *Fcrho1* en dos cepas silvestres de *F. circinatum* aisladas de árboles afectados por la enfermedad en el norte de España. Para ello, se empleó una versión modificada del método OSCAR que permite la producción de construcciones de deleción en un único paso de clonación, siendo ésta la primera vez que se ha utilizado en la especie *F. circinatum*. Los mutantes obtenidos mostraron una reducción severa en el crecimiento *in vitro*, si bien la producción de microconidios y capacidad germinativa de las esporas no se vio afectada. Se observó además que, a pesar de presentar tasas de crecimiento fuertemente reducidas, los mutantes no mostraban alteraciones en la morfología de las hifas. Por último, un ensayo de virulencia *in vivo* permitió determinar que la reducción del crecimiento observada *in vitro* no estuvo asociada a una menor capacidad de colonizar los tejidos del huésped.

Palabras clave: chancro resinoso de los pinos, mutante de deleción, *Pinus radiata*, protocolo OSCAR, señalización celular y virulencia en planta.

The *Fusarium circinatum* gene *Fcrho1*, encoding a putative Rho1 GTPase, is involved in vegetative growth but dispensable for pathogenic development

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Abstract

Fusarium circinatum is the causal agent of pine pitch canker, one of the most devastating forest diseases worldwide. This fungus causes severe damping-off in pine seedlings and growth reduction, wilting and cankers appearance in pine forests and plantations. A draft of the complete genome sequence of this phytopathogen was recently made available. This information was used to annotate *in silico* the gene *Fcrho1* as a putative homolog of Rho1 GTPase genes. In this study, we generated *Fcrho1* deletion mutants in two *F. circinatum* wildtype strains isolated from damaged trees in northern Spain. For that, we used OSCAR methodology. This approach allows generating deletion constructs in a single cloning step and had not been used before in *F. circinatum*. The resulting deletion mutants showed significantly reduced growth *in vitro* while conidiation and spore germination were not affected. Microscopic observations indicated that hyphal morphology apparently was not altered in the mutants. Finally, an *in vivo* virulence assay showed that the reduced growth rate exhibited by the deletion mutants *in vitro* does not result in any alteration in development of pathogenicity.

Keywords: cell signalling, deletion mutant, *in planta* virulence, OSCAR protocol, *Pinus radiata*, PPC.

1. Introduction

Fusarium circinatum Nirenberg & O'Donell (teleomorph: *Gibberella circinata* Nirenberg & O'Donell) is the causal agent of pine pitch canker disease (PPC). This species is present in fourteen countries throughout Africa, America, Asia and Europe and has become a major threat to coniferous forests, plantations and nurseries worldwide (Wingfield *et al.*, 2008). It causes high rates of mortality in *Pinus* spp. and *Pseudotsuga menziesii* (Mirb.) Franco seedlings as well as reduced growth, the appearance of resin-soaked cankers and intense wilting in mature trees. This disease severely reduces the value of the timber and the yield of the afforestation, leading to important economic losses (Bezos *et al.*, 2017). *F. circinatum* acts as a necrotroph when it infects coniferous plants via the stem; however, the molecular basis of fungal infection is largely unknown. Five candidate genes putatively associated with pathogenicity have recently been annotated *in silico* (Muñoz-Adalia *et al.*, 2018) by using the complete fungal genome as query (Wingfield *et al.*, 2012). However, functional studies are required for definitive characterization of each gene.

The mitogen-activated protein kinase (MAPK) cascades are a crucial signalling pathway in eukaryotes which triggers cellular responses associated with a wide range of external stimuli (*e.g.* environmental chemical conditions, pheromones, etc.). MAPK signalling involves participation of the small Rho-type GTPases proteins, which form a subfamily belonging to the GTPase superfamily. These proteins play an essential role in cell growth polarity by regulating the organization of the actin cytoskeleton as well as participating in the formation of hyphal structures (Araujo-Palomares *et al.*, 2011; Iden & Collard, 2008). These functions make GTPases particularly important in fungal pathogenesis, as abnormal formation of infective structures (*e.g.* appressoria) (Chen *et al.*, 2008) or ineffective mycelial morphogenesis (Zheng *et al.*, 2009) can drastically reduce the fitness of host colonization. In the grass pathogen *Claviceps purpurea* (Fr.) Tul. deletion of the GTPase Rac gene caused excessive branching and absence of polarity in mycelial growth which resulted in avirulence in mutant strains (Rolke & Tudzynski, 2008). Several Rho proteins have been characterized in pathogenic species of *Fusarium*. For instance, in *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) W.C. Snyder & H.N., the *Rho1* gene has been shown to regulate *in planta* virulence as well as the cell wall response to lytic enzymes (Martínez-Rocha *et al.*, 2008). Bluhm *et al.* (2007) studied the function of the GTPase-encoding gene *RAS2* in *Fusarium graminearum* Schwabe, and observed that its deletion resulted in slower mycelial growth and reduction in virulence in wheat (*Triticum* sp.). A later study also reported attenuated virulence in fungal strains of this phytopathogen lacking Rho subfamily members (Zhang *et al.*, 2013). Hence, study of this family of proteins can

contribute to a better understanding of how cellular signalling processes regulate phenotypic responses during plant infection.

A Rho-type GTPase candidate gene, termed *Fcrho1*, has recently been annotated *in silico* in *F. circinatum* (Muñoz-Adalia *et al*., 2018). Lack of knowledge about the genes that drive virulence traits in this phytopathogen demands new research addressing this issue. In consequence, the aims of this study were (i) to generate deletion mutants of the putative Rho1 GTPase gene *Fcrho1* in *F. circinatum* and (ii) to evaluate the phenotypic effects of gene deletion both *in vitro* and *in vivo*.

2. Material and methods

2.1. Fungal material and growth conditions

Two wildtype isolates of *F. circinatum* were selected for study (*i.e.* Fc072 and 011). Both belong to the same mating type (MAT 2) and were isolated from symptomatic Monterey pines (*Pinus radiata* D. Don) in northern Spain (Vainio *et al.*, 2015). The isolates were grown in 100 ml of YEPS (Supplementary material A1) at 25º C and 200 r.p.m shaking for five days. The cultures were filtered using sterilized Miracloth tissues (Sigma Aldrich), and the mycelium of each isolate collected was then ground in liquid nitrogen with a sterile mortar and pestle. The genomic DNA was extracted using Plant DNA kit (Bioline) according to the manufacturer´s instructions.

2.2. Generation of Fcrho1 deletion construct

A modified version of the OSCAR protocol described by Paz *et al*. (2011) and Gold *et al*. (2017) was used for the generation of a construct to delete the entire open reading frame (ORF) of *Fcrho1*. Briefly, specific primers were designed to amplify ~1000 nucleotides (nt) of the 3´ and 5´ regions flanking the *Fcrho1* ORF. Primers were designed on the basis of the sequence reported by Muñoz-Adalia *et al*. (2018) by using primer3 software (http://primer3.ut.ee/) and they included att-tails as described by Paz *et al*. (2011) (Table 1). Flanking regions were then amplified by PCR using genomic DNA from isolate Fc072 as a template. PCR conditions were as follows: 30 s at 98º C followed by 10 cycles of 20 s at 98º C, 20 s at 66º C and 60 s at 72º C (annealing temperature was decreased at a rate of -0.8º C / cycle), and then, a total of 25 cycles of 20 s at 98º C, 20 s at 58º C and 60 s at 72º C with a final elongation step of 5 min at 72º C. To confirm amplification of the right size bands, PCR products were then subjected to electrophoresis on a 1% w/v borate-agarose gel and visualized under UV light by staining with 2×10^{-3}% v/v ethidium bromide (Merck). After that, both PCR fragments were purified from the gel using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) according to

manufacturer´s instructions. A BP clonase reaction was then set up to generate the deletion construct according to Paz *et al.* (2011) and García-Pedrajas *et al.* (2013). The BP clonase reaction contained the purified PCR products, a binary vector compatible with *Agrobacterium tumefaciens* Smith & Townsend - mediated transformation (ATMT), and a second plasmid containing the marker *hygR* conferring resistance to hygromycin B. The binary vector used, pOSCAR-HSVtk, is a variant of pOSCAR (Paz *et al.*, 2011) to which the herpes simplex virus (HSV) thymidine kinase gene HSVtk (Khang *et al.*, 2005) was included to prevent ectopic integration of the transforming T-DNA. The second vector used was pA-Hyg-OSCAR a modified version of pA-Hyg-GFP-OSCAR which contains green fluorescent protein (GFP) cassette for GFP-tagging of deletion mutants (Sarmiento-Villamil *et al.*, 2018). The gene flanks and vectors were incubated with BP clonase (Invitrogen) at 25º C for 16 h. The BP clonase recognizes specific recombination sequences present in the amplified gene flanks and vectors, generating the target construction. Resulting constructs were transformed into *Escherichia coli* Escherich using a standard heat shock method. *Escherichia coli* transformants were selected on LB medium plates amended with 0.06% w/v spectinomycin (Supplementary material A1). Plates were incubated for 12 h at 37º C to allow transformants to grow.

Table 1. Primer pairs used in this study. Bold letters in primers: att-tails included in primers used to amplify *Fcrho1* flanking sequences to generate the deletion construct; nt, nucleotides.

Primer pair	Sequences (5´- 3´)	Size	Region
FcirRHO1j / FcirRHO2q	TATGCGTTGATGCTCTGAGG / TCCAGCTCTCCAAGCTCTTC	855 nt	Exon of *Fcrho1*
FRHOl1j-attB2r / FRHOl2q-attB1r	**GGGGACAGCTTTCTTGTACAAAGTGGAA**AA TTGCCATGGTACGAGAGGCT / **GGGGACTGCTTTTTTGTACAAACTTGT**TTAA GGTTCCATGCCCAAGAGG	940 nt	5´ flanking region of *Fcrho1*
FRHOr1j-attB4 / FRHOr2q-attB3	**GGGGACAACTTTGTATAGAAAAGTTGTT**AA CATGATACCCAGCTCCATCG / **GGGGACAACTTTGTATAATAAAGTTGT**GAT CCCGCTGACGTGAGTATTG	873 nt	3´ flanking region of *Fcrho1*

To analyze plasmid DNA of emerging colonies of *E. coli* and identify those confirmed to harbor the right deletion construct, individual colonies were inoculated in 3 ml of liquid LB medium amended with spectinomycin. Bacterial cells were then mixed with TENS buffer (Supplementary material A1) and 3 M sodium acetate and centrifuged for 5 min at 14000 r.p.m. (rotor radius: 7 cm). The resulting plasmid DNA pellets were washed with 70% v/v ethanol and then dried at 37º C for 20 min. Purified DNA was resuspended in double sterilized water (dsw) and digested with the restriction enzyme Pst1 (FastDigest, Fermentas) for 2 h at 35º C. The digested fragments of DNA were run on an agarose gel as previously described to determine

the pattern of bands (*i.e.* four bands pattern: ~8 kb, 1.65 kb, 1.20 kb and 0.25 kb). A plasmid DNA exhibiting the expected digestion pattern of *Fcrho1* deletion construct was further purified using the NucleoSpin© Plasmid Easy Pure kit (Macherey-Nagel) and transformed into *A. tumefaciens* strain AGL-1 (Hellens *et al.*, 2000). The transformation of *A. tumefaciens* was performed by electroporation in a GenePulser Xcell (BioRad). Cells were incubated in liquid LB medium at 28º C for 2 h prior to selection of transformants on LB plates amended with spectinomycin. These plates were incubated for 48 h at 28º C at which point emergence of colonies resistant to spectinomycin was observed. These bacterial colonies harboring the *Fcrho1* deletion construct were used for ATMT transformation of *F. circinatum* as described below.

2.3. Transformation of F. circinatum *with* Fcrho1 *deletion construct*

Conidial suspensions of *F. circinatum* were prepared to be transformed with the deletion construct using an ATMT method (Paz *et al.*, 2011). For that, *F. circinatum* wildtype strains were grown in YEPS medium (Supplementary material A1) for one week at 24º C and 200 r.p.m. After that, conidia were collected by filtration using sterilized Miracloth and centrifugation at 8000 r.p.m. (rotor radius 10.10 cm) at room temperature for 10 minutes. The resulting pellet was resuspended in 500 µl of dsw and conidia were counted using a haemocytometer under light microscope (25x lens, Zeiss). The final concentration of conidia was adjusted to 10^7 spores / ml.

In parallel, the *A. tumefaciens* AGL-1 cells previously transformed with *Fcrho1* deletion construct, were grown in minimum medium (Supplementary material A1) at 24º C and 200 r.p.m. until the culture reached an optic density of 0.50-0.80 was reached (λ= 600 nm; GeneQuant pro). At that point, 100 µl of each conidial suspension and 100 µl of bacterial culture were mixed in sterile 1.50 ml tubes and then spread on a sterile nitrocellulose film placed on a Petri dish containing induction medium (Supplementary material A1) amended with 200 µM acetosyringone (Sigma Aldrich). Plates containing the mixed cultures were incubated in the dark at 24º C for two days. Then, strips of nitrocellulose were cut using a sterile scalpel and transferred to plates of selection medium amended with 150 µg / ml of hygromycin B (Sigma Aldrich) and three additional antibiotics as shown in Supplementary material A1. Putative transformants of *F. circinatum*, which emerged at the edges of the nitrocellulose strips, were transferred to PDA medium amended with 150 µg / ml of hygromycin B (Supplementary material A1) and then subcultured twice in the same medium to ensure mitotic stability of transforming DNA containing the *hygR* marker.

2.4. Confirmation of deletion mutants

The effective deletion of the target gene in individual transformants was determined by PCR. For that, total DNA was extracted from each transformant according to the method described by Vainio *et al.* (1998) an used for PCR with the primer pair FcirRHO1j / FcirRHO2q which amplify part of the *Fcrho1* ORF (Table 1). PCR was performed under the following conditions: 2 min at 95º C followed by 35 cycles of 35 s at 95º C, 40 s at 55º C and 1 min at 72º C; the final elongation step consisted of 7 min at 72º C. Reagents and electrophoresis conditions were the same as described above. A transformant confirmed to be deleted for *Fcrho1* in each of the two *F. circinatum* strains used, that is Fc072 and 011, was selected for further analysis; these deletion mutants are referred to as Δ*Fcrho1*Fc072 and Δ*Fcrho1*011, according to their genetic background. A transformant with ectopic integration of the deletion construct, and that had therefore an intact *Fcrho1* gene, was also selected for each genetic background. These strains, referred to as Fc072ect2 and 011ect5, were used as controls to discard any potential effect of the transformation procedure on the *F. circinatum* phenotypic characteristics.

2.5. Analysis of hyphal growth and conidiation

We first determined if lack of *Fcrho1* had an effect on the colony growth rate on solid medium. For that, parental wildtype strains Fc072 and 011, deletion mutants Δ*Fcrho1*Fc072 and Δ*Fcrho1*011 and their corresponding ectopic strains, Fc072ect2 and 011ect5, respectively, were grown on PDA (Supplementary material A1). Six plates per strain were prepared and they were maintained under room conditions (23º C ± 2º C; 10-11 hours of light per day) for one week. The size of each colony was calculated daily by measuring two perpendicular axes and calculating the area of the corresponding ellipse (A_c). The mean growth rate (G) was calculated as the mean value of colony size increase between two consecutive observations.

These plates were also used to determine whether lack of *Fcrho1* affects hyphal morphology. For that, 5x5 mm plugs comprising mycelium and PDA medium were cut from four 40 days-old replica plates of each strain. Then, all the samples obtained from each strain were mixed in 50 ml sterile tubes containing 10 ml of dsw. Tubes were incubated at 25º C and 280 r.p.m. for 4 min and then briefly centrifuged at 7000 r.p.m. in a centrifuge with swinging bucket rotor (rotor radius: 7.50 cm). A total amount of 20 µl of water comprising hyphae were collected from the bottom of the tube and transferred with a sterile tip to a haemocytometer. The hyphae structure was inspected under a light microscope (40x lens, Nikon; camera: COOLPIX 4500). Three additional plugs were collected per strain and cultured in 50 ml crystal flasks containing 20 ml of PDB (Supplementary material A1; five replicates per strain). Liquid cultures were incubated at room temperature with shaking (180 r.p.m.) for 48 h. The number of microconidia

(C) and germlings (N) was then counted three times per culture under light microscope as explained for hyphae analysis.

2.6. Pathogenicity assay

An *in vivo* assay was also performed with 75 three-years-old seedlings of *P. radiata* (*i.e.* 10 plants per treatments and 15 plants for control: mean height of plants and standard error: 55.62 ± 2.29 cm). Pine seedlings were placed in growth chambers at 25º C with a 16 h photoperiod for a fortnight for acclimatization prior to inoculation. The plants were watered three times a week with equal amounts of tap water. To inoculate them, an incision was made two centimeters above the root collar with a sterile scalpel and 10 µl of spore suspension (10^6 conidia / ml) were then dropped into the wound. The conidial suspensions of each fungal strain were obtained by liquid culturing in PDB as described above. Control plants were inoculated with the same volume of dsw. The wound was covered with Parafilm® for one week. The inoculated and control seedlings were held in separate plant growth chambers to avoid cross-contamination. The severity of symptoms was monitored after two weeks of infection according to the 0-4 qualitative scale of symptoms described by Correll *et al*. (1991). The intensity of symptoms in each plant was measured every two days from the appearance of symptomatology until 30 days post inoculation (dpi). The data for each seedling were used to calculate the area under the disease progress curve (AUDPC) as previously described (Martínez-Álvarez *et al*., 2014).

One third of seedlings per treatment (*i.e.* dead or dying plants) were selected for re-isolation of *F. circinatum*. Stems were cut into small pieces from two centimeters above the inoculation point and washed by immersion in dsw for 60 s. The samples were then surface sterilized by soaking in 70% v/v ethanol (PanReac Química, Spain) for 60 s and then in 2% v/v sodium hypochlorite (PanReac Química, Spain) for another minute. Finally, the samples were washed again in dsw for 60 s and plated either on PDA or on Spezieller Nährstoffarmer agar medium (Leslie & Summerell, 2006) for morphological identification.

2.7. Statistical analysis

The variations in G, A_c, C, N and AUDPC between treatments were analyzed using Kruskal-Wallis rank sum test in R software (R Development Core Team, 2015). The "DescTools" package (Signorell *et al*., 2015) was used for Dunn´s test computation as post hoc analysis ($P < 0.05$ significance level).

3. Results and discussion

3.1. Production of deletion mutants lacking Fcrho1 *open reading frame.*

To functionally characterize *Fcrho1*, deletion mutants were produced in two different *F. circinatum* strains, Fc072 and 011. In total, 8-10 transformants that exhibited mitotically stable resistance to hygromycin B (*i.e.* correct genomic insertion of the *hygR* fragment) were recorded for each fungal strain after transformation. Genomic DNA was extracted from 3 and 5 transformants obtained in the Fc072 and 011 genetic backgrounds, respectively. A PCR analysis using primers that amplify part of the *Fcrho1* ORF (Table 1) revealed amplicons of the expected size (*i.e.* ectopic strains) in a single transformant of Fc072 while 3 transformants of 011 showed the amplicon corresponding to the exon of *Fcrho1*. Two independent *Fcrho1* deletion mutants were therefore confirmed for each fungal strain. The DNA extractions and PCR analysis were repeated a second time to confirm the correct identification of *Fcrho1* deletion mutant strains. These results represent, as far as we know, the first successful use of the OSCAR protocol to produce deletion mutants in *F. circinatum*. Use of this innovative protocol could be implemented for future gene characterization in PPC fungus as well as in other members of this genus of fungal pathogens.

3.2. Phenotypic characterization of deletion mutants

To characterize the function of the previously *in silico* annotated gene *Fcrho1* (Muñoz-Adalia *et al*., 2018), we started by determining whether the deletion mutants lacking *Fcrho1* were affected in vegetative growth. In *F. graminearum*, deletion of the Rho-type GTPase gene *RHO1* was found to be lethal (Zhang *et al*., 2013). By contrast, in this study it was found that both Δ*Fcrho1*Fc072 and Δ*Fcrho1*011 were able to grow (Figure 1) and also sporulate (Table 2) in different culture media.

The *in vitro* assay showed nevertheless that, although *Fcrho1* is not an essential gene in *F. circinatum*, its absence greatly reduces mycelium growth (Figure 1). Quantification of daily growth rates confirmed that they significantly differed among fungal strains (X^2 = 24.43, d.f. = 5, P < 0.01). More specifically, either Δ*Fcrho1*Fc072 or Δ*Fcrho1*011 showed lower G than their corresponding wildtype and ectopic strains (P < 0.01 in all cases). Mean growth rate varied neither among mutant strains (P = 0.74) nor in the rest of pairwise comparisons (P > 0.39 in all cases) (Table 2). Otherwise, the colony size (A_c) also varied among strains (X^2 = 24.77, d.f. = 5, P < 0.01) being significantly smaller in both mutant strains (P ≤ 0.01 in all cases). No differences were found in A_c among deletion mutants (P = 0.09) (Table 2). The range of growth rates and colony sizes reported here for wildtype and ectopic strains were in line with those provided by Flores-Pacheco *et al.* (2017) for *F. circinatum* strains cultured in PDA and

incubated in darkness for eight days. These observations in ectopic strains confirmed that transformation of *F. circinatum* using the OSCAR protocol caused no deleterious effects.

Reduced mycelial growth was not the only macroscopic change observed in *F. circinatum* upon deletion of *Fcrho1*. Thus, colonies of the loss-of-function strains also exhibited more foamy appearance than their corresponding ectopic and wildtype strains as showed in Figure 1. By contrast, the exploratory observations under light microscope did not reveal noticeably changes in hyphal morphology (*e.g.* hyperbranching; Figure 2) as reported by Zhang *et al.* (2013) in *F. graminearum*. A complex and variable role of GTPase proteins in the regulation of mycelial growth have been reported in fungal species. For example, similarly to our results, in *F. oxysporum* mutant strains lacking *rho1* showed reduced growth on solid media (Martínez-Rocha *et al.*, 2008). Kwon *et al.* (2011) studied the function of five members of the GTPase family in *Aspergillus niger* Tiegh. and found that each protein participates in fungal growth in a different manner. Accordingly, RhoA appears to regulate the formation of the germ tube while other GTPases such as RacA, RhoD and CftA contribute to hyphal tip elongation and mycelial growth. Zhang *et al.* (2013) also reported changes in growth rate in the deletion mutants of four of the five Rho proteins evaluated. These authors found absence of aerial mycelium in *rho4* mutants while deletion of *rho2* resulted in slower growth and severe deformation of spores in minimal medium. Taking together, our results suggest that the *F. circinatum* GTPase *Fchro1* is predominantly involved in the formation of aerial mycelium and hyphal tip elongation.
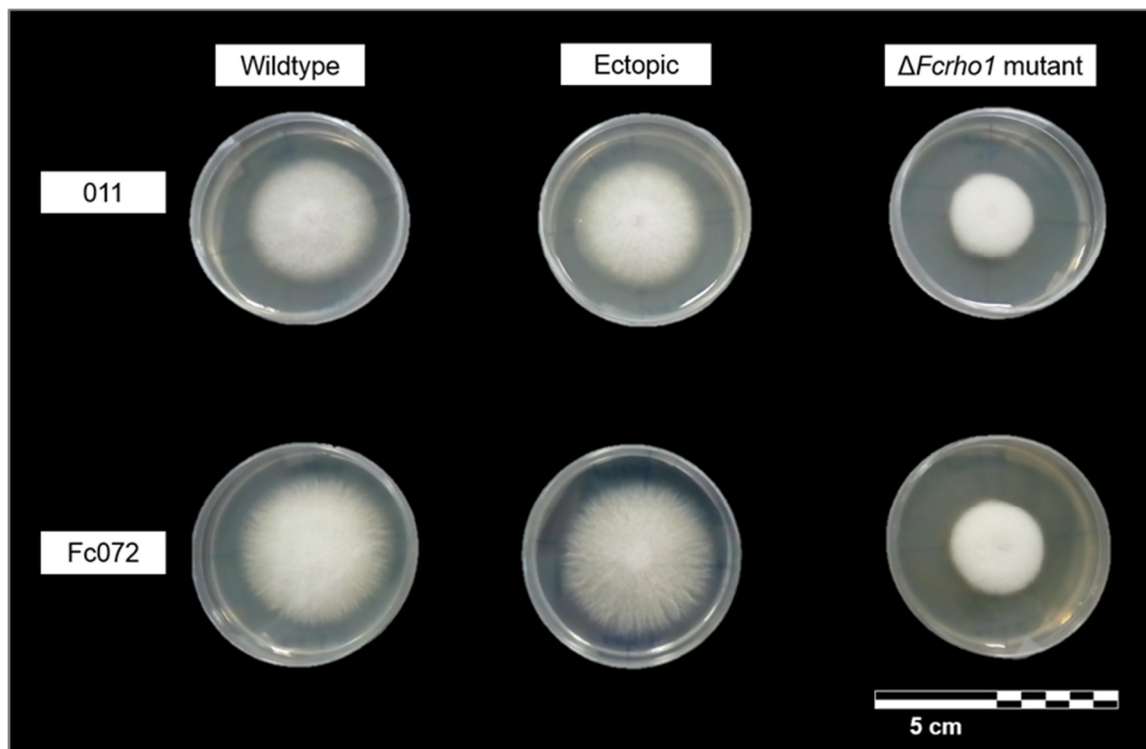

Figure 1. Colonies of *F. circinatum* growing in PDA after one week of culturing.

Table 2. *In vitro* characterization of *F. circinatum* strains. G: average daily growth rate during 7 days of culture; A$_c$: area of fungal colony after culture for 7 days; C: spore density after culture for 48 h of culture; N: germlings density after 48 h of culture. Small letters (a-b) denote significant differences according to Dunn´s test (P < 0.05). Mean values and standard errors are shown.

Isolate	Strain	G (mm² / day)	A$_c$ (mm²)	C (spores / mm²)	N (germlings / mm²)
011	wildtype	764.55 ± 76.04 a	4553.73 ± 251.98 a	525.00 ± 134.49 a	108.33 ± 27.78 a
	011ect5	744.38 ± 41.38 a	4390.89 ± 215.89 a	508.33 ± 83.44 a	83.33 ± 13.28 a
	ΔFcrho1011	331.89 ± 30.72 b	1782.85 ± 35.79 b	393.33 ± 73.49 a	105.00 ± 17.62 a
Fc072	wildtype	793.53 ± 91.55 a	4643.27 ± 184.33 a	353.33 ± 44.17 a	85.00 ± 12.63 a
	Fc072ect2	834.44 ± 63.68 a	4791.97 ± 234.82 a	370.00 ± 61.63 a	71.66 ± 15.01 a
	ΔFcrho1Fc072	302.85 ± 50.27 b	1824.74 ± 89.04 b	451.66 ± 71.10 a	73.33 ± 9.90 a

The molecular basis of virulence traits in PPC fungus are little understood yet. Genes whose deletion affects vegetative growth are often also involved in virulence as they regulate growth processes which are important for the colonization of the host. Functional characterization of candidate genes should therefore include *in vivo* assays that allow identifying virulence factors. Bearing this in mind, in this work we performed *in vivo* virulence assays to determine if the growth defects of *Fcrho1* deletion mutants were associated to a reduced ability to colonize the host. The severity of symptoms 30 dpi did not show significant variations among fungal strains (Figure 3) even though it was significantly lower in control seedlings (X^2 = 35.57, P < 0.01 in all cases), as expected. Our results showed that *Fcrho1* mutants were infective and ready to damage and even kill *P. radiata* seedlings (100% re-isolation rate in each inoculation treatment). These findings show that *Fcrho1* is not an essential regulator of pathogenicity in PPC fungus although they do not rule out the plausible participation of this gene in the disease cycle of this pathogen. It should be noted that the studies performed so far have shown that some Rho proteins play crucial roles in virulence whereas others do not appear to be required for host colonization. For example, in *F. graminearum* a comprehensive analysis of this type of proteins determined that lack of FgCdc42 and FgRho4 activity strongly reduced *in planta* virulence. By contrast, mutant strains lacking the gene encoding FgRho3 maintained a capacity to damage flowering wheat heads to a similar degree as in the wildtype. A broader analysis of Rho GTPase family proteins in *F. circinatum* will be required to determine if this GTPase activity displays overlapped functions in vegetative growth and host colonization in this species. It should be also noted that the four additional genes *in silico* annotated by Muñoz-Adalia *et al.*, (2018) are also good candidates to explore in the search of critical determinant of virulence in the PCC fungus.

Other developmental processes such as asexual sporulation might play a role in the disease cycle of *F. circinatum* and were therefore analyzed in this study. Our results showed no significant effect of *Fcrho1* deletion either on conidiation (X^2 = 2.86, d.f. = 5, P = 0.72) or spore germination (X^2 = 2.50, d.f. = 5, P = 0.77) (Table 2). The role of Rho-family GTPases in spore production is variable and in some cases contrast sharply with our finding for *Fcrho1*. For instance, in *Magnaporthe grisea* (T.T. Hebert) M.E. Barr deletion of the gene econding the Rho-family member Cdc42 strongly reduced conidiation and delayed spore germination (Zheng *et al.*, 2009). In *F. graminearum*, on the other hand, deletion of different Rho protein genes caused dissimilar effects in spore production, effect that also varied in different culture conditions (Zhang *et al.*, 2013). Our *in vitro* results indicate that *Fcrho1* is among the Rho members that do not play a role in regulating asexual reproduction. Mutants lacking this gene in *F. circinatum* are able to complete its asexual cycle and therefore to spread micro- and macroconidia through the vegetal tissues.
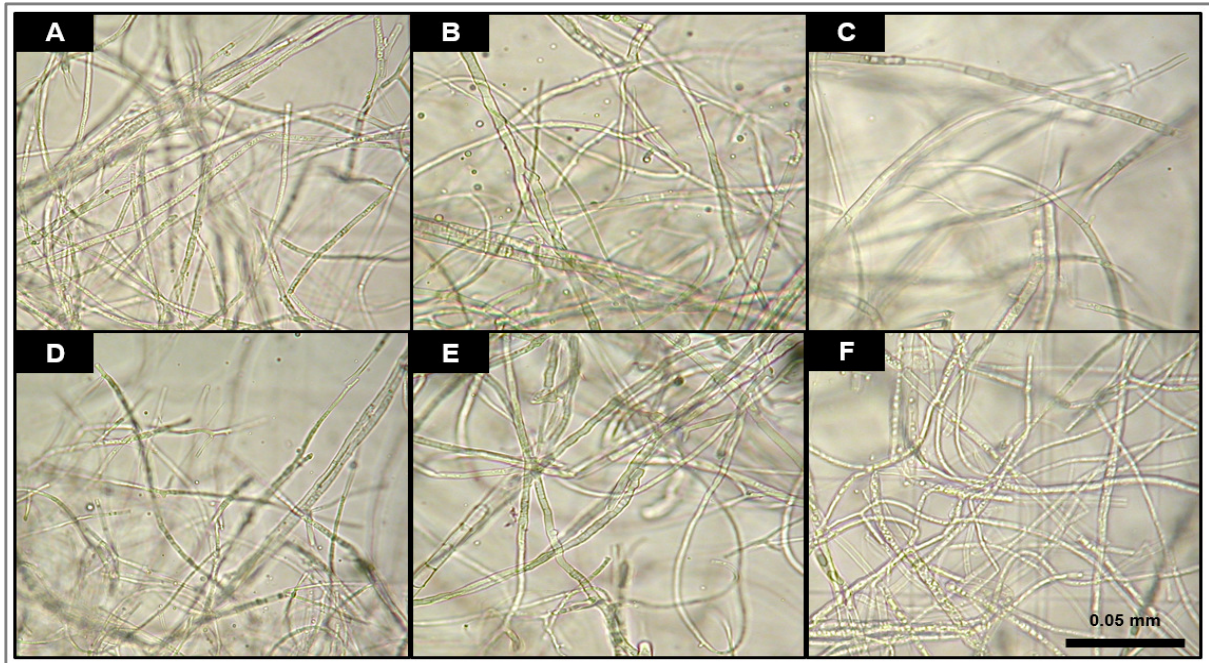
Figure 2. Hyphae of fungal strains 40 days after culture. A: 011 wildtype; B: 011ect5; C: Δ*Fcrho1*011; D: Fc072 wildtype; E: Fc072ect2; F: Δ*Fcrho1*Fc072. Image processing: scaling to common size and +20% brightness.
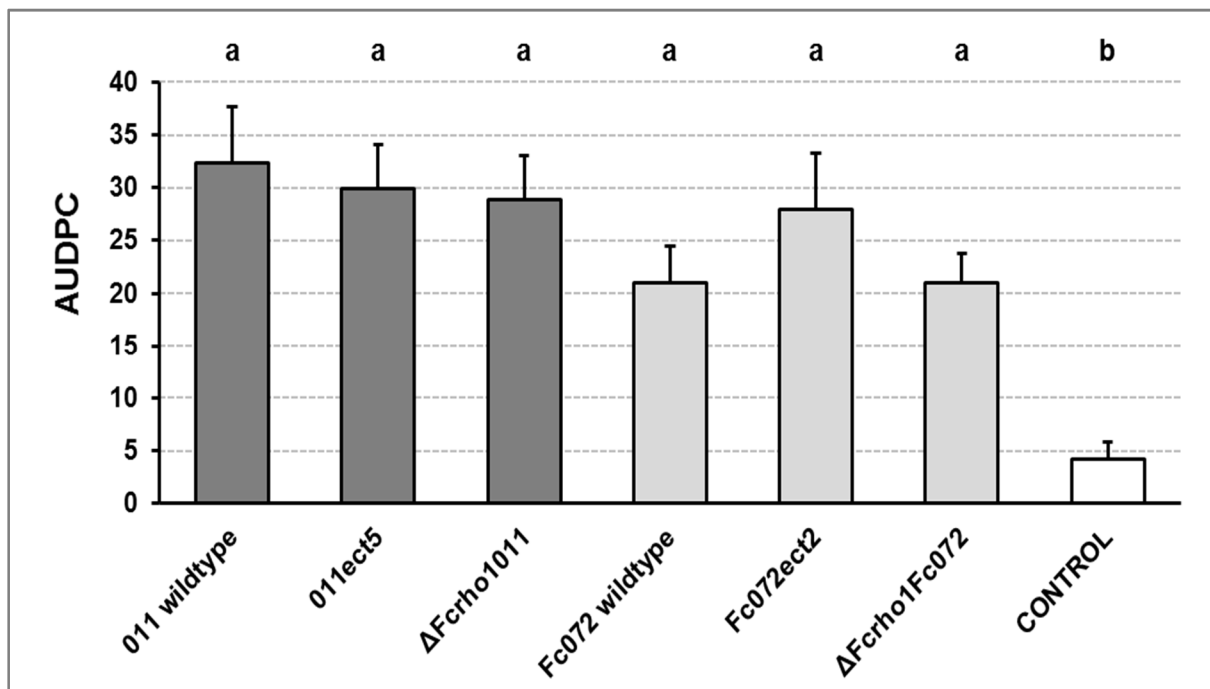

Figure 3. Area under the disease progress curve (AUDPC) of different fungal strains 30 dpi. Small letters (a-b) denote significant differences (Dunn´s test, $P < 0.05$). Comparisons between fungal strains are indicated by colour of plot. Mean values and standard error are shown.

Mutants that show greatly reduced vegetative growth are frequently also affected in their ability to grow during pathogenic development, and thus in turn results in reduced virulence. Yet, we found that *Fcrho1*-lacking strains were as virulent as those having an intact copy of the gene (*i.e.* wildtype strains and ectopic transformants) (Figure 3). It should be noted that plant pathogenic fungi respond to multiple environmental signals, including those coming from the plant, and that the external signals regulating vegetative development and growth in the plant might differ considerably. A possible explanation of the results presented here is, therefore, that *Fcrho1* could be involved only in the pathways that regulate hyphal elongation in response to environmental signals during vegetative growth. Hence, the mutants would respond normally to those signals regulating growth during the host-pathogen interaction, displaying normal growth in that condition. An alternative explanation could be found in the specific host used in our virulence assays. Thus, Monterey pine is known as the most susceptible species to PPC (Martínez-Álvarez *et al*., 2014) and the spatial-temporal analysis of infection provided by Martín-Rodrigues *et al*. (2013) led to think in an inefficient defense of this pine species during trunk colonization by *F. circinatum*. Hence, it is possible that the severe damages observed in our bioassay were due to the low resistance of *P. radiata* that resulted in aggressive infection even for fungal strains with reduced growth rate. The use of alternative host for deletion mutants might clarify whether the observed pattern in pathogenicity is caused by the marked susceptibility of *P. radiata*. In this sense, other members of the genus as *Pinus pinea* L. has shown higher degree of resistance against the pathogen (Iturritxa *et al*., 2013) becoming a complementary model for future inoculation trials.

In summary, in this study we successfully generated *Fcrho1* deletion mutants of *F. circinatum* and use them to perform a preliminar characterization of this candidate gene *in vitro* and *in vivo*. Our results showed that *Fcrho1* is not an essential gene but that its deletion in *F. circinatum* has a great impact on vegetative growth rates. By contrast, asexual reproduction was not affected upon gene deletion. Furthermore, mutant strains were able to effectively damage *P. radiata* seedlings. Additional studies will be required to fully establish the role of *Fcrho1* and other Rho-family GTPases as virulence factors in PPC fungus.

4. Acknowledgments

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Supplementary material (Supporting information)

A1. Culture media and buffers used in this article.

- **Induction medium**: 1% v/v K buffer, 2% v/v M-N solution, 1% v/v $CaCl_2$ (1% w/v), 1% v/v $FeSO_4$ (0.01% w/v), 0.5% v/v spore elements, 0.25% v/v NH_4NO_3 (20% w/v), 1% v/v glycerol (50% v/v), 4% v/v MES (1 M) and 0.5% w/v glucose (2 M). Adjust the medium at pH = 6.

- **LB:** 10% w/v triptone, 5% w/v yeast extract and 10% w/v NaCl (99% w/v).

- **Minimum medium:** 0.1% v/v K buffer (20% w/v K_2HPO_4 and 14.5% w/v KH_2PO_4; pH = 7), 2% M-N solution (3% w/v $MgSO_4·7H_2O$ and 1.5% w/v NaCl), 0.01% v/v $CaCl_2$ (10% w/v), 1% v/v $FeSO_4$ (0.01% w/v), 0.5% v/v spore elements (0.1% w/v of $ZnSO_4·7H_2O$, H_3BO_3, $CuSO_4·5H_2O$ and $MnSO_4·4H_2O$), 2,5% v/v NH_4NO_3 (20% w/v), 0.1% w/v glucose (2 M).

- **PDA:** 3.9% w/v potato dextrose agar.

- **PDB:** 2.8% w/v potato dextrose broth.

- **Selection medium:** 3.9% w/v potato dextrose agar, 0.05% v/v hygromycin B, 0.2% v/v cefotaxim, 0.1% v/v moxalactum and 0.06% w/v streptomycin.

- **TENS:** 1% v/v NaOH (10 N), 5% v/v SDS (10% v/v), 1% Tris base (1 M; pH = 8) and 0.25% v/v EDTA (0.5 M; pH = 8).

- **YEPS:** 1% w/v yeast extract, 2% w/v bacto-peptone and 2% w/v sucrose.

## 6.5. Fungus – plant interactions: Original Article V

Efecto de los micovirus en la virulencia de *Fusarium circinatum* y en la actividad de lacasas

Resumen

Los enzimas lacasas (benzenediol: oxígeno oxidorreductasa, EC 1.10.3.2) desempeñan un importante papel en la degradación de los compuestos fenólicos como la lignina. Estos enzimas son frecuentemente segregados por los hongos y se cree que participan en la colonización del hospedante por parte de los hongos fitopatógenos. Por otra parte, en tiempos recientes se han descrito tres nuevos micovirus infectando al agente causal de la enfermedad del chancro resinoso de los pinos, *Fusarium circinatum*. En este estudio, se analizó el efecto de la infección sencilla y la co-infección por parte de dos de estos micovirus en la actividad de lacasas, en la tasa de crecimiento micelial y en la patogenicidad en planta para catorce cepas del hongo. La actividad extracelular de lacasas se analizó mediante el test Bavendamm junto con el procesamiento de imágenes y métodos espectrofotométricos. Además, se evaluó el crecimiento del micelio, la patogenicidad *in vivo* y la probabilidad de supervivencia de las plántulas de pino insigne (*Pinus radiata* D. Don) infectadas. Los resultados mostraron (i) que el crecimiento micelial de los aislados fue homogéneo, (ii) que la presencia de los virus incrementó la virulencia del hongo, (iii) que la doble infección por parte de los micovirus no causó síntomas en el hospedante y (iv) que los enzimas estudiados podrían participar como herramienta auxiliar en la infección del hospedante.

Palabras clave: análisis de imágenes, chancro resinoso de los pinos, control biológico, oxidasas y ssRNA.

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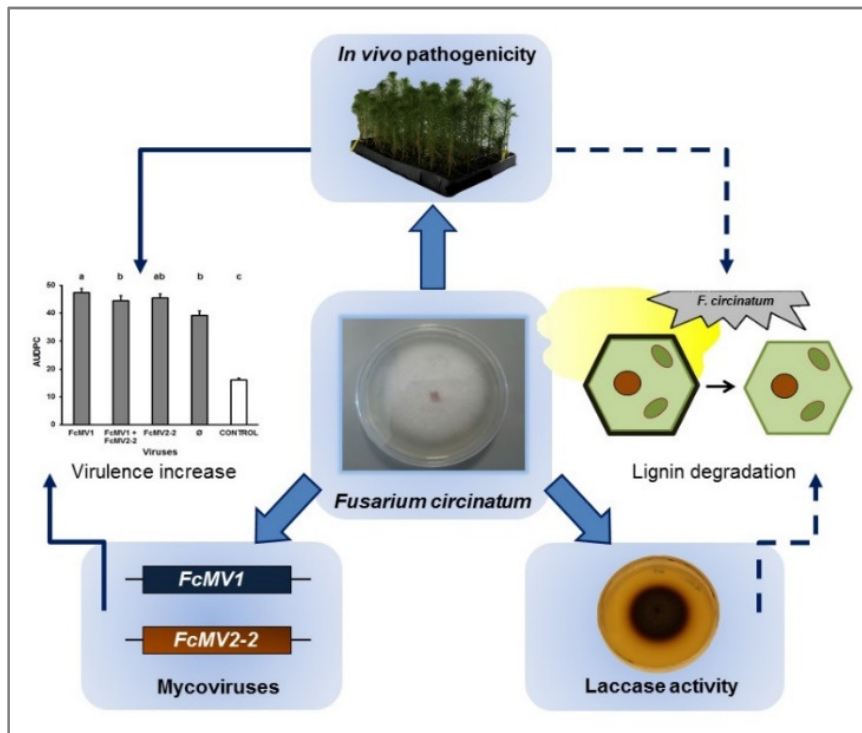
Effect of mycoviruses on the virulence of *Fusarium circinatum* and laccase activity

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Abstract

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

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

1. Introduction

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belong to the multicopper oxidase group of enzymes and are specialized in catalyzing the oxidation of phenolic substrates by reduction of O_2 to H_2O. Laccases are common in eukaryotes, including fungi, and have been widely studied in the phylum Ascomycota (Claus, 2004). These enzymes (molecular weight around 60-70 kDa) are usually extracellular and show a high degree of specificity for degrading polyphenol substrates such as lignin (Baldrian, 2006). They play an essential role in nutrient turnover (mainly nitrogen and carbon) in nature, due to their capacity to degrade lignocelluloses in forest soil and litter, and they are abundant in saprophytic fungi (Criquet *et al.*, 2004). Laccases may also play an important role in host colonization by pathogenic fungi as they can damage host tissues, thus favouring fungal infection (Binz & Canevascini, 1996; Kuo *et al.*, 2015). Additionally, they have important applications in industry (*e.g.* textile and paper industries) as well as in bioremediation and environmental biotechnology (Riva, 2006).

The fungus *Fusarium circinatum* Nirenberg & O'Donnell is the causal agent of pine pitch canker disease. This invasive necrotroph is considered the most important pathogen of pine seedlings in several countries around the world and particularly affects conifers such as Monterey pine (*Pinus radiata* D. Don) and *Pseudotsuga menziesii* (Mirb.) Franco (Martínez-Álvarez *et al.*, 2014; Wingfield *et al.*, 2008). It can infect branches, stems, seeds, cones and roots in host trees of any age, causing pre- and post-emergence damping-off in seedlings (mortality rates up to 90%) and severe damage and reduced growth in adult trees (Aegerter & Gordon, 2006). Pine pitch canker fungus is widespread throughout the world and has been reported in Mexico, USA, Haiti, South Africa, Japan, Korea, Southern Europe and South America (Vainio *et al.*, 2015). The pathogen spreads via the movement of contaminated material (seeds, wood, nursery seedlings, etc.) as well as via air- and soilborne spores and insect vectors (Bezos *et al.*, 2015) and via damage to trees caused by storms or human activities (Bezos *et al.*, 2012). The disease is expected to spread rapidly in the future, and it has been estimated that approximately 10 million hectares of native pine forest and plantations in the EU are potentially endangered (Baker *et al.*, 2010).

Several management measures and treatments for controlling *F. circinatum* have been suggested: application of adaptive silviculture programmes (Gordon *et al.*, 2015), selection of particular species for planting (Martínez-Álvarez *et al.*, 2014), treatment of seeds with hot water (Berbegal *et al.*, 2015), addition of hydrogen peroxide to irrigation water (van Wyk *et al.*, 2012) and biocontrol techniques involving bacteria (Soria *et al.*, 2012) or other fungal species

(Martínez-Álvarez *et al*., 2012). However, although some of these techniques are potentially useful, new methods of biocontrol focused on field and nursery application are required.

Mycoviruses (viruses that infect fungi) are common in many fungal species, including some plant pathogens (Xie & Jiang, 2014). Fifteen families of mycoviruses have been described: these include single-strain RNA viruses which sequence serves as template for RNA-dependent RNA polymerase (RdRp) (ss(+)RNA), viruses that require the intervention of RNA replicase to copy their genome into positive sense (ss(-)RNA) and also viruses with double-strain RNA (dsRNA) and single-strain DNA (ssDNA) (Ghabrial *et al*., 2015; Pearson *et al*., 2009). The effects of mycoviruses on fungi vary from induction of a cryptic state to increase the capacity of host to produce disease (hypervirulence). Although only a few mycoviruses reduce the virulence of their host (hypovirulence), this kind of viruses is of particular interest for biocontrol purposes (Ghabrial & Suzuki, 2009). One of the best known examples of virus-mediated hypovirulence is that involving chestnut blight (causal agent *Cryphonectria parasitica* (Murrill) M. E. Barr). Cryphonectria hypovirus 1 (CHV-1, *Hypoviridae*), which is one of the four *Hypovirus* spp. that infects the fungus, has shown good results in biocontrol treatment and has been shown to reduce fungal virulence (decreased mycelial growth and sporulation rate) (Robin & Heiniger, 2001; Zamora *et al*., 2014). Other mycoviruses hosted by pathogenic fungi have also been identified as promising organisms for biological control (Vainio *et al*., 2012; Zhang *et al*., 2010).

Changes in laccase activity in fungi have been reported in relation to mycoviral infection (Ahn & Lee, 2001; Wang & Nuss, 1998). Laccase activity may also be altered in pathogenic fungi in the presence of mycoviral infection, and reduced enzymatic activity may be associated with lower virulence (Castro *et al*., 2003; Potgieter *et al*., 2013; Rigling *et al*., 1989). Three mycoviruses hosted in mitochondria that infect *F. circinatum* have recently been identified as putative members of *Narnaviridae* (genus *Mitovirus*) and designated Fusarium circinatum mitovirus 1, 2-1 and 2-2 (FcMV1, FcMV2-1 and FcMV2-2) (Martínez-Álvarez *et al*., 2014). Although little is known about the effects of these mycoviruses, any of them that reduce laccase activity could potentially be used to develop a biocontrol technique to treat pine pitch canker disease.

In this study, we hypothesized that *F. circinatum* isolates infected by mycoviruses would show differences in laccase activity relative to isolates not infected by viruses. We also expected to observe a positive correlation between laccase activity and host pathogenicity. To our knowledge, this is the first study focusing on this topic in relation to pine pitch canker disease. The objectives of this study were (i) to analyze the possible effects of mycoviruses FcMV1 and

FcMV2-2 on laccase activity in *F. circinatum*; (ii) to investigate the variations in laccase activity, growth rates and infection development in relation to mycovirus presence, and (iii) to evaluate the relationship between enzyme activity and pathogenicity in Monterey pine seedlings.

2. Material and methods

2.1. Selection of isolates

Seven isolates of *F. circinatum* were obtained from two different locations in northern Spain (Asturias and Cantabria) where wildtypes of this fungus are commonly infected by mycoviruses as previously reported (Vainio *et al*., 2015). Two monosporic cultures for each isolate were selected, and the presence of mycoviruses was confirmed according to Martínez-Álvarez *et al*. (2014). The mating type (MAT) of each isolate was previously investigated (Martínez-Álvarez *et al*., 2014) (Table 1). Briefly, isolates FC104 and FC072 were free of mycovirus and isolates FC104v and FC072v (*i.e.* of the same strains) were infected with FcMV1 ("v" indicates infection with mycovirus). Isolate FC070v was also infected with FcMV1 and isolate FC070w was infected with both FcMV1 and FcMV2-2 ("w" indicates co-infection). Isolates FC020, FC035 and FC042 were free of mycovirus and FC020v, FC035v and FC042v were infected with FcMV2-2. Finally, isolate FC221 was free of mycovirus and isolate FC221w was co-infected with both mycoviruses. FcMV2-1 was not present in the evaluated isolates.

2.2. Bavendamm test

Seven samples of each isolate were cultured in Bavendamm medium to enable estimation of the level of extracellular laccase activity. The fungal isolates were grown in darkness at 25º C in specific media containing 0.50% w/v tannic acid, 1.50% w/v malt extract and 2% w/v agarose. Tannic acid and malt-agarose solutions were prepared with distilled water and autoclaved separately before being mixed together; the pH was adjusted to 4.50 with NaOH 10M (Pointing, 1999; Rigling *et al*., 1989). The global intensity of the enzymatic reaction was evaluated after incubation for five days, and the change in colour of the media (from whitish to dark brown) was assessed according to the following qualitative scale: (-) non appreciable reaction, (+) slight reaction or (++) intense reaction (Figure 1).

2.3. Monitoring for mycelial growth

In parallel to the Bavendamm test, photographs of the Petri dishes containing the fungal isolates were taken every day for five days with a Canon EOS 550D camera (white backlit screen as background and constant light). The photographs were processed using ImageJ 1.48v (Abràmoff *et al*., 2004) in order to quantify the area affected by enzymatic reaction (*i.e.*

brown area over whitish medium) (Barry *et al*., 2009; Lundy *et al*., 2001). The mean area affected by enzymatic reaction (S) and mean growth of the isolate (G; calculated as the mean value of colony size increase between two consecutive observations) were measured daily.

2.4. Laccase activity

The *F. circinatum* isolates were cultured for one week in Bavendamm medium. Three plugs (5x5 mm) comprising mycelia and medium were then removed from the edge of each isolate and transferred to 1.50 ml tubes. Aliquots (1.50 ml) of twice-autoclaved distilled water (4º C) were added to the plug samples to extract crude extracellular laccase. After incubation for thirty minutes at room temperature, the tubes were centrifuged for three minutes at 10^4 g and the supernatant was extracted. The laccase activity was assayed after adding 0.80 ml of 2.50 mM 2,6-dimethoxyphenol (DMP, broad spectrum enzyme substrate) to 0.20 ml of the crude laccase in 100 mM phosphate buffer (pH 6.90) at 37º C (Smit *et al*., 1996). The absorbance of samples was measured at 468 nm and 25º C in a LAN OPTICS (2000-2100) spectrophotometer (Ausec *et al*., 2015). Absorbance was measured immediately and five minutes later. Finally, the increase in absorbance was calculated as an absolute value for the measurement period (ΔA_{0-5}).

2.5. In vivo *pathogenicity*

To test *in vivo* the ability of each strain to cause disease (pathogenicity), the isolates were inoculated into 405 one-year-old nursery seedlings of Monterey pine (*i.e.* 27 replicate seedlings per isolate and 27 control seedlings). A small incision was made two centimeters above the root collar and 10 µl of spore suspension (10^6 spores/ml of distilled water) was inoculated into the wound. In control seedlings, an incision was made in the same way, but distilled water only was inoculated into the wound. The wound was covered with Parafilm® for one week. The treated and control seedlings were held separately in plant growth chambers at 25º C with a 16 h photoperiod. The seedlings were watered three times a week throughout the study period, with equal amounts of distilled water. After one week, the visual severity of symptoms in each plant were assessed every two days during a period of 15 days, according to the following scale: 0 = healthy plant, 1 = necrosis only at the point of inoculation and healthy foliage, 2 = necrosis > 2 cm beyond the point of inoculation, 3 = needles wilting and appreciable dieback and 4 = dead plant (Correll *et al*., 1991) (Figure 1). Finally, the area under the disease progress curve (AUDPC) was calculated as the sum of the area of the corresponding trapezoids as previously described (Martínez-Álvarez *et al*., 2014).

Table 1. Data and results of tests of *Fusarium circinatum* isolates (seven isolates, two monosporic cultures/isolate): origin; host (Pp: *Pinus pinaster* Aiton, Pr: *Pinus radiata*); mating-type (MAT); mycovirus presence (FcMV1/FcMV2-2); intensity of Bavendamm test reaction (B.t.; qualitative scale: -, +, ++); area affected by enzymatic reaction (S); mycelial growth (G); increase of absorbance in five minutes (ΔA_{0-5}) and area under the disease progress curve (AUDPC). Mean values and standard error (SE) are shown. (*) Source of data: Pérez-Sierra et al. (2007).

Isolate	Origin	Host	MAT	FcMV1	FcMV2-2	B.t.	S (mm²) ± SE	G (mm²/day) ± SE	ΔA_{0-5} ± SE	AUDPC ± SE
FC104v	Asturias*	Pp*	1*	✓	-	(++)	524.98 ± 55.15	226.21 ± 35.50	0.09 ± 0.04	44.33 ± 1.75
FC072v	Cantabria	Pr	2	✓	-	(++)	519.32 ± 44.11	252.71 ± 22.62	0.08 ± 0.02	43.35 ± 1.52
FC070v	Cantabria	Pr	2	✓	-	(++)	387.36 ± 45.70	174.91 ± 27.79	0.24 ± 0.06	46.46 ± 1.44
FC070w	Cantabria	Pr	2	✓	✓	(++)	443.03 ± 51.04	224.61 ± 28.30	0.14 ± 0.04	45.40 ± 1.34
FC221w	Cantabria*	Pr*	2*	✓	✓	(++)	542.83 ± 36.29	229.24 ± 26.57	0.15 ± 0.03	43.37 ± 1.36
FC020v	Cantabria	Pr	2	-	✓	(++)	480.60 ± 38.29	220.02 ± 27.82	0.10 ± 0.03	46.29 ± 1.56
FC035v	Cantabria	Pr	2	-	✓	(++)	503.56 ± 43.11	229.89 ± 21.44	0.13 ± 0.04	47.12 ± 1.22
FC042v	Cantabria	Pr	2	-	✓	(++)	447.40 ± 34.71	229.67 ± 22.57	0.32 ± 0.07	49.03 ± 1.63
FC104	Asturias*	Pp*	1*	-	-	(++)	724.22 ± 31.21	332.74 ± 13.01	0.23 ± 0.04	45.14 ± 1.31
FC072	Cantabria	Pr	2	-	-	(++)	504.00 ± 47.13	199.99 ± 34.72	0.10 ± 0.05	41.79 ± 2.14
FC221	Cantabria*	Pr*	2*	-	-	(++)	515.79 ± 46.29	233.29 ± 25.94	0.44 ± 0.15	45.24 ± 1.54
FC020	Cantabria	Pr	2	-	-	(++)	482.57 ± 35.56	231.64 ± 22.50	0.08 ± 0.01	36.92 ± 2.17
FC035	Cantabria	Pr	2	-	-	(++)	417.13 ± 27.38	202.97 ± 14.82	0.14 ± 0.07	45.11 ± 1.32
FC042	Cantabria	Pr	2	-	-	(++)	384.91 ± 15.46	203.73 ± 12.62	0.07 ± 0.02	44.33 ± 1.45

2.6. Statistical analysis

All analyses were performed with R software (R Development Core Team, 2013). The Kruskal-Wallis rank sum tests were carried out with the "Agricolae" package (De Mendiburu, 2009) to analyze the variation in S, G, ΔA_{0-5} and AUDPC values according to two different factors: isolate (14 strains, Table 1) and mycovirus presence (evaluated as follows: not infected (Ø); infected with FcMV1 or FcMV2-2; and co-infected with both mycoviruses). Dunn´s test (Dunn, 1964) was applied for post-hoc analysis of data, with "DescTools" package (Signorell *et al*., 2015). The Pearson's product-moment correlation (Pearson, 1900) was also calculated for (a) G and ΔA_{0-5}, (b) mean values of AUDPC and ΔA_{0-5} for each isolate, and (c) the G and S variables. Survival analysis based on the non-parametric Kaplan-Meier estimator (Kaplan & Meier, 1958) was carried out with "Survival" package (Therneau, 2015). Survival curves were created with the "Survfit" function and the differences between the curves were tested with the "Survdiff" function.
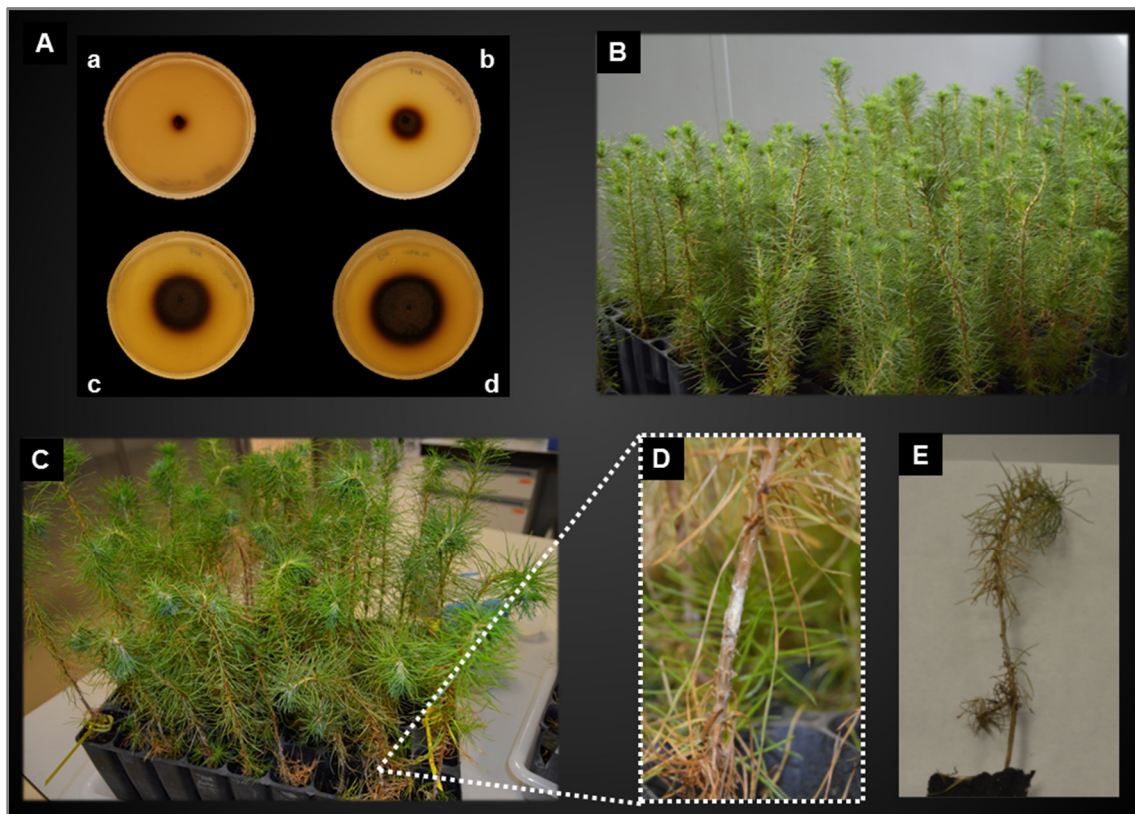

Figure 1. Scheme of the study. A: Bavendamm test progress at four different moments: 24 h (a), 48 h (b), 72 h (c) and 96 h (d) after culture (isolate shown: Fc072). B: Control *Pinus radiata* seedlings on the 13th day of pathogenicity test. C: *Pinus radiata* seedlings inoculated with Fc072v (foreground) and Fc072 (background) on the 13th day of pathogenicity test. D: Detail of resin surrounding the point of inoculation. E: Detail of dead seedling showing the symptomatology of pine pitch canker damping-off.

3. Results

3.1. Bavendamm test and mycelial growth

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

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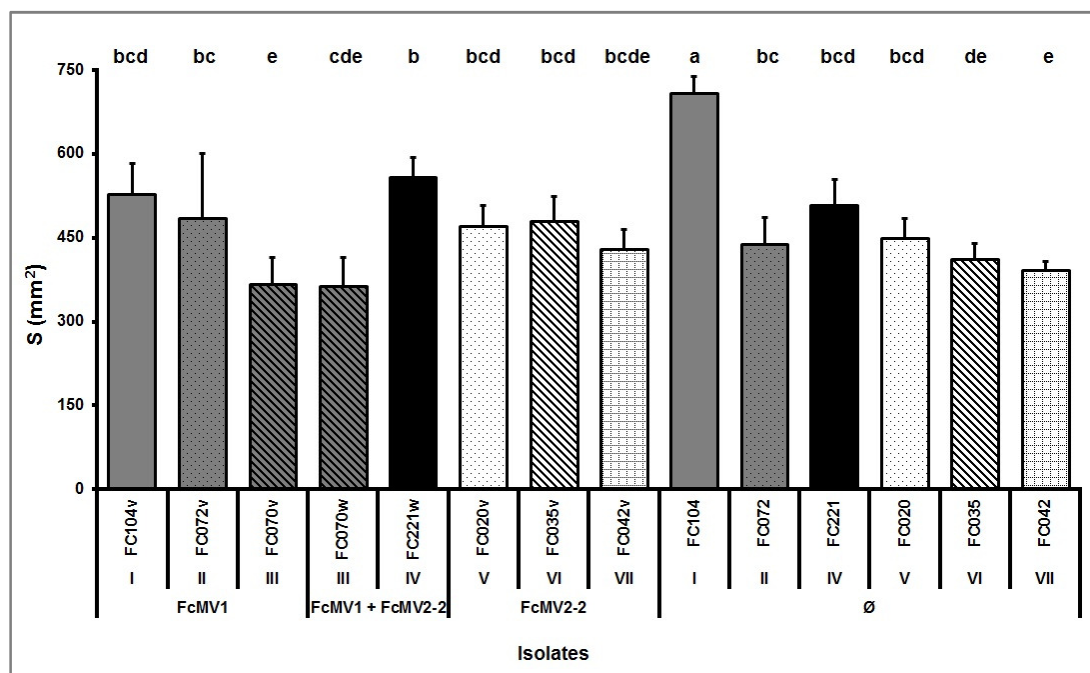

Figure 2. Area affected by enzymatic reaction during the five days of the assay (S) for each fungal isolate. Small letters (a–e) denote significant differences (Dunn´s test, P < 0.05). (Ø): mycovirus-free isolates. Comparisons between pairs of isolates are indicated by colour of plot and roman numbers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

3.2. Laccase activity

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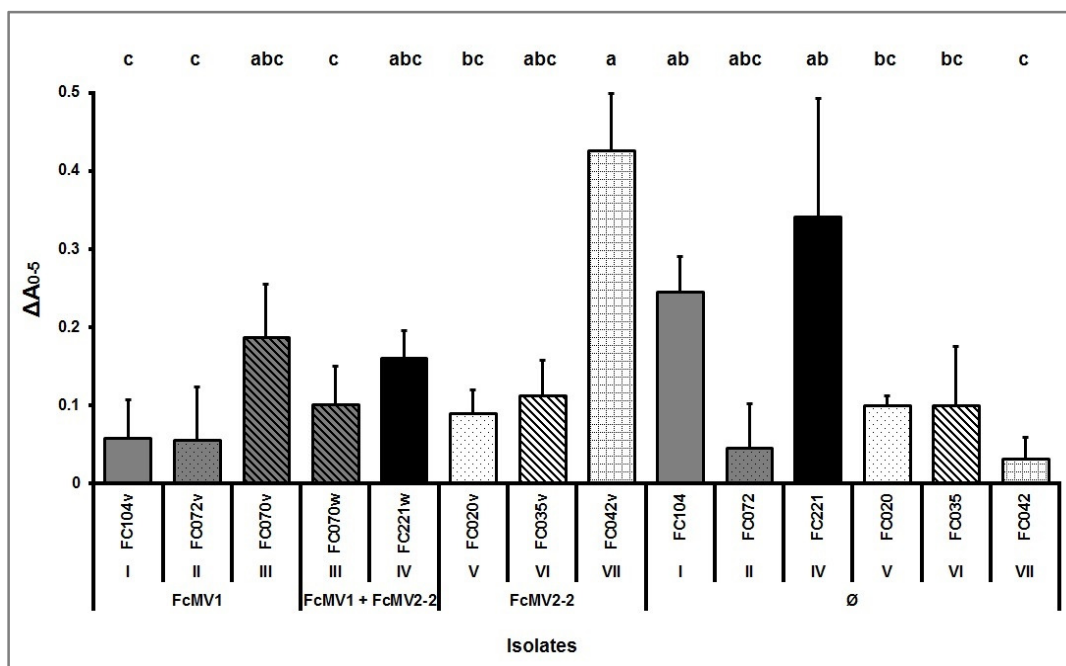

Figure 3. Extracellular laccase activity (ΔA_{0-5}) in the different isolates. Small letters (a–c) denote significant differences (Dunn´s test, P < 0.05). (Ø): virus-free isolates. Comparisons between pairs of isolates are indicated by colour of plot and roman numbers (I-VII). Median values and standard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

3.3. Pathogenicity in vivo

The values of AUDPC obtained in relation to the different treatments varied significantly depending on the isolate ($X^2 = 98.90$; d.f. = 14; P < 0.01). The highest AUDPC value was obtained for FC072v and it was significantly different from that obtained for its pair FC072 (P = 0.02). The lowest value was obtained for seedlings infected with FC042 (mean value 36.92 ± 2.17) and was significant different from the values corresponding to the other isolates (P < 0.03, in all cases) (Figure 4).

The AUDPC also varied significantly in regard to viral infection (X^2 = 25.75; d.f. = 3; P < 0.01). The value was higher in all plants infected by *F. circinatum* isolates than in control seedlings, as expected (< 0.01, in all cases). The AUDPC values were higher in FcMV1-infected fungi than in non-infected (P < 0.01) and co-infected isolates (P = 0.02), but there were no significant differences between FcMV2-2 infected isolates (P = 0.11). There were no significant differences between co-infected isolates and either isolates infected with FcMV2-2 only (P = 0.16) or non-infected isolates (P = 0.40) (Figure 5). The correlation between AUDPC and ΔA_{0-5} as average values for each isolate were almost statistically significant (t = 2.13; d.f. = 13; P = 0.05; ρ = 0.50).
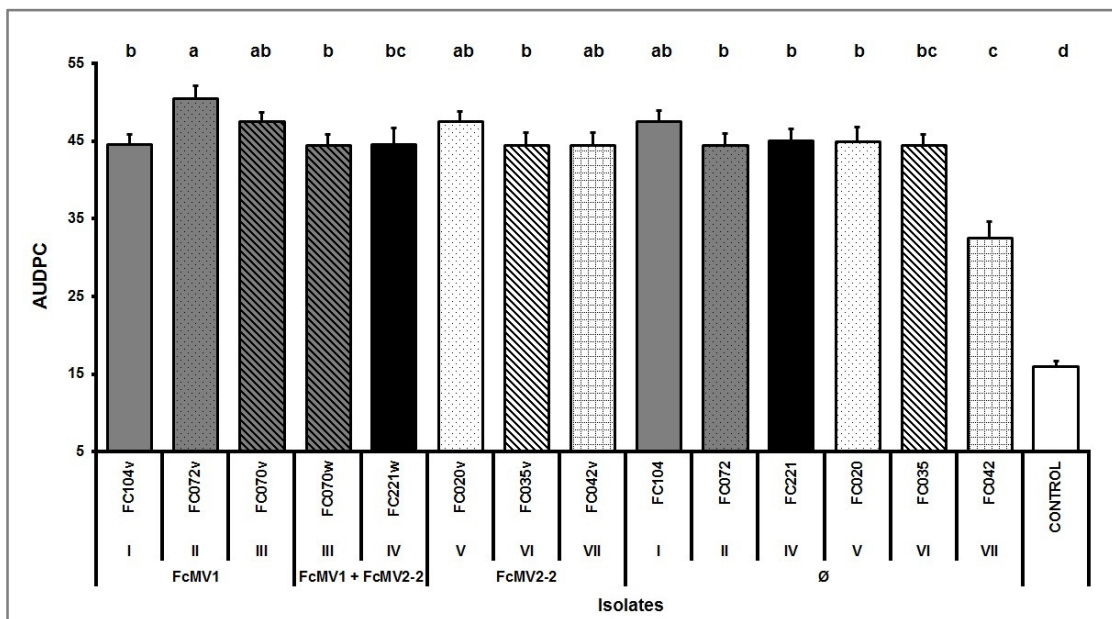

Figure 4. Area under the disease progress curve (AUDPC) for the different fungal isolates. Small letters (a–d) denote significant differences (Dunn´s test, P < 0.05). (Ø): virus-free isolates. Comparisons between pairs of isolates are indicated by colour of plot and roman numbers (I-VII). Median values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

Survival analysis revealed significant differences between treatments (X^2 = 94.50; d.f. = 4; P < 0.01) (Figure 6). The survival probability of seedlings was significantly lower in plants inoculated with isolates infected with FcMV1 than in the virus-free isolates (X^2 = 11.10; d.f. = 1; P < 0.01). FcMV2-2 presence in fungi did not produce any differences in plant host survival relative to non-infected isolates (X^2 = 3.30; d.f. = 1; P = 0.06). No differences were found in seedlings survival probability between isolates infected with FcMV1 or FcMV2-2 (X^2 = 1.50; d.f. = 1; P = 0.22). Likewise, survival probability was not different in plants inoculated with co-infected strains in respect of non-infected isolates (X^2 = 0.40; d.f. = 1; P = 0.52).

4. Discussion

The study findings indicate that laccase activity and the area affected by enzymatic activity were fairly homogeneous in most of the fungal isolates. Only isolate FC104 yielded a higher S value than the other strains. This isolate differed from the others in geographical origin (Asturias region) and in mating type (MAT 1) (Pérez-Sierra *et al*., 2007). The apparent similarity in the S value for other isolates may be related to the low genetic variability among isolates from the Cantabrian population, in which only MAT 2 has been identified (Table 1). The observed differences seem to support the theory that suggests punctual introductions of the fungus in the Iberian Peninsula and a subsequent wide dissemination of the clonal population (Berbegal *et al*., 2013; Iturritxa *et al*., 2011).

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

Infection with a single mycovirus led to higher fungal pathogenicity and lower survival of seedlings infected by *F. circinatum* isolates. FcMV1 infection was associated with higher AUDPC values and lower survival than the other treatments, and FcMV2-2 caused a slight increase in the fungal virulence and a non-significant decrease in the survival relative to the virus-free isolates. In view of these findings, neither of these mycoviruses appear useful for biocontrol purposes (such as with CHV-1 in chestnut blight (Robin & Heiniger, 2001)) because of their lack of capacity to promote hypovirulence in the host. On the other hand, although both FcMV1 and FcMV2-2 were associated with a reduction in survival relative to control seedlings, the AUDPC values increased by < 20% relative to virus-free isolates, and this increment was only significant in FcMV1 (Figure 5). Furthermore, mycelial growth did not vary in relation to mycovirus presence. Taking all this into account, we concluded that neither FcMV1 nor FcMV2-2 induced hypervirulence in their fungal hosts. However further studies are needed to confirm it. Co-infection resulted in similar AUDPC values, plant survival probability and colony growth rates as in the fungal isolates free of mycovirus. This finding contrasts with a previous report of hypovirulence in *C. parasitica* isolates (lower sporulation and mycelial growth) caused by simultaneous infection of CHV-1 and Mycoreovirus 1 (MYRV-1, *Reoviridae*) (Sun *et al*., 2006). In a study involving *Botryosphaeria dothidea* (Moug. ex Fr.) Ces. & De Not., isolates infected with Botryosphaeria dothidea chrysovirus 1 (BdCV1) and Botryosphaeria dothidea partitivirus 1 (BdPV1) showed slower growth rate and lesions were shorter when the fungus was

simultaneously infected by both mycoviruses, suggesting a hypovirulent effect of this multi-viral infection (Wang *et al*., 2014). Simultaneous infection with two putative member of *Partitiviridae* also caused a strong reduction in laccase activity in *Botrytis cinerea* Pers. isolates, and the enzymatic activity was lower than in single infection and wildtype (Potgieter *et al*., 2013). In the present study, co-infection of fungal isolates with FcMV1 and FcMV2-2 did not induce hypovirulence, although further studies focusing on the synergistic effect of mycoviruses within their hosts are required.
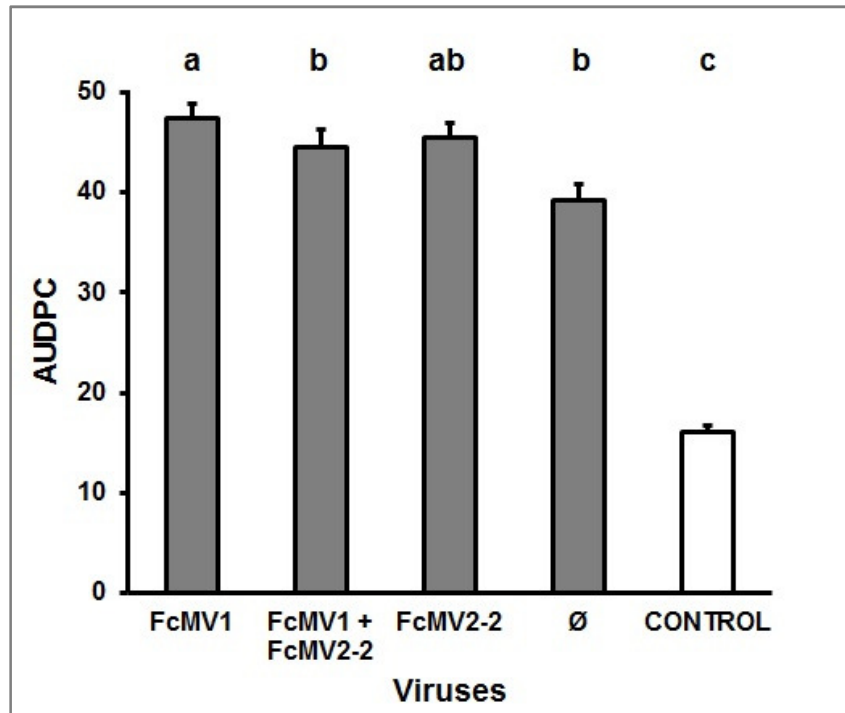

Figure 5. Area under the disease progress curve (AUDPC) in relation to mycovirus presence. Small letters (a–c) denote significant differences (Dunn´s test, P < 0.05). (Ø): virus-free isolates. Median values and standard error are shown.

A previous study reported the presence of mycoviruses in Iberian isolates of *F. circinatum* but not in South African isolates (Vainio *et al*., 2015). It is therefore possible that members of the Iberian population of *F. circinatum* host mycoviruses because the fungi initially introduced in Spain was harbouring viruses at that time (Berbegal *et al*., 2013). On the other hand, this type of mycovirus may play an adaptive role in non-native regions where the fungus has recently been introduced, apparently improving host resilience (Márquez *et al*., 2007). This approach would explain the observed virulence in strains infected with mycovirus and is consistent with the hypothesis supporting ancient co-evolution between mycovirus and fungi, mainly mediated by horizontal transmission of viruses (through mycelial fusion rather than the spread to progeny) (Göker *et al*., 2011; Pearson *et al*., 2009).

Extracellular laccase activity did not vary depending on mycovirus presence. Moreover, mycelial growth rate was not related to enzymatic activity. In contrast, laccase activity varied between isolates and seemed to be related to pathogenicity. Enzymatic activity was only lower in isolates FC221, FC020 and FC104 (significantly lower in the case of FC104) when they were infected, supporting the idea that the mycoviruses do not cause hypovirulence (Ahn & Lee, 2001). By contrast, a strong reduction in laccase activity (indicated by the Bavendamm test reaction and ΔA_{0-5}) was observed in *C. parasitica* isolates infected by dsRNA mycovirus in a study in which isolates that did not produce laccase were identified as hypovirulent strains by a complementary pathogenicity test (Rigling *et al*., 1989). In a study of laccase production in *Ophiostoma ulmi* (Buisman) Nannf. and *Ophiostoma novo-ulmi* Brasier (causal agent of Dutch elm dieback) differences between the two species were observed (Binz & Canevascini, 1996). Thus, the less aggressive *O. ulmi* showed lower or even null laccase activity than the more pathogenic *O. novo-ulmi* (0-0.20 U ml^{-1} *vs* 0.12-0.34 U ml^{-1} respectively). In the aforementioned study comparing two strongly related species with different pathogenicity, the authors proposed laccases as a useful tool for overcoming tree defences. Similarly, higher values of enzymatic activity were obtained for virus-free strains of *Diaporthe ambigua* Nitschke (ΔA_{0-5} for mycovirus-free strains: 0.11-0.17; ΔA_{0-5} of mycovirus-infected strains: 0.01-0.02) and the Bavendamm test was negative in infected and less virulent strains (Smit *et al*., 1996). Similar conclusions have been reached for *B. cinerea* (Castro *et al*., 2003). However, enhanced laccase activity has also been observed in hypovirulent strains of *B. cinerea*, in a study in which the authors concluded that this enzyme was not important in the virulence of the pathogen (Zhang *et al*., 2010). The values obtained in the present study were higher than those reported in previous studies (mean value of ΔA_{0-5} = 0.17 ± 0.03), suggesting intense extracellular laccase activity and ruling out hypovirulence in the isolates.

F. circinatum does not possess specialized infection structures such as apressoria or haustoria. Production of extracellular cell wall-degrading enzymes is therefore expected to be higher in this necrotrophic species (Kikot *et al*., 2009). As *F. circinatum* initially colonizes the host by occupying intercellular spaces, it has been suggested that the fungus would segregate extracellular enzymes in order to degrade the cell wall to obtain nutrients from plant cells (Martín-Rodrigues *et al*., 2013). Other enzymes (*e.g.* cutinases) and mycotoxins (*e.g.* bauvericin) have been identified as important substances in plant infestation by *Fusarium* species (Moretti *et al*., 2007), indicating the involvement of an enzymatic complex in host colonization. Laccase may thus enhance fungal pathogenicity by making cellulose accessible to other enzymes (Bora *et al*., 2005) and probably acts as an initial infection tool enabling *F. circinatum* to overcome tree defenses. This is supported by the findings of a study involving laccase production by *Heterobasidion annosum* (Fr.) Bref. (Kuo *et al*., 2015), in which the

authors also suggested complex uses of laccases during fungal infection. In summary, we conclude that laccases may be important in early host colonization. Nevertheless, complete characterization of these enzymes (chemical structure, molecular weight, suitable thermic and pH range, kinetic constants, etc.) is required (Shin & Lee, 2000) for a better understanding of their metabolism and their participation in the development of pine pitch canker disease.
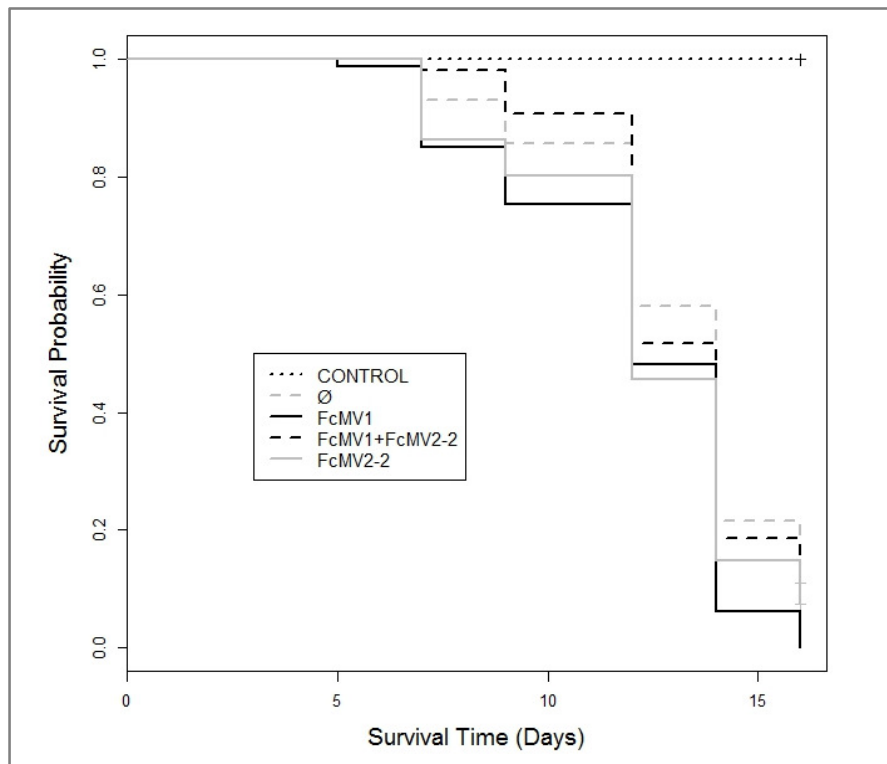

Figure 6. Plot of survival probability determined using the Kaplan-Meier estimate of the survival function for Monterey pine (*Pinus radiata*) seedlings infected with *Fusarium circinatum* in relation to mycovirus presence. (Ø): mycovirus-free isolates.

5. Acknowledgments

6. References

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## 6.6. Fungus – insect – plant interactions: Original Article VI

Sydowia polyspora como especie dominante en la comunidad fúngica transportada por dos especies de *Tomicus* en pinares amenazados por *Fusarium circinatum*

Resumen

Los escolítidos (Coleoptera, Scolytinae), denominados comúnmente barrenillos transportan comunidades de hongos filamentosos actuando como vectores de fitopatópgenos. En este estudio, se investigó la micobiota transportada por dos especies del género *Tomicus* (*Tomicus piniperda* y *Tomicus destruens*) mediante (i) identificación morfólogica y molecular de los taxones, (ii) cálculo de índices de riqueza taxonómica, diversidad, equitatividad, dominancia y foresía, además de llevarse a cabo un (iv) análisis de co-ocurrencia. La micobiota estudiada resultó estar formada por once taxones que aportaron una diversidad moderada y una baja equitatividad. El hongo *Sydowia polyspora* resultó el más abundante y dominó claramente la comunidad. Por otra parte, todas las asociaciones ecológicas entre hongos resultaron aleatorias. Las dos especies de barrenillo (tanto *T. piniperda* como *T. destruens*) fueron capturadas en una plantación de *Pinus radiata* afectada por *Fusarium circinatum* lo que aporta nueva información sobre la distribución simpátrica de ambas especies.

Palabras clave: diversidad fúngica, flora fúngica, foresía, gen *COI*, *Pinus radiata* y *Tomicus destruens.*

Graphical abstract
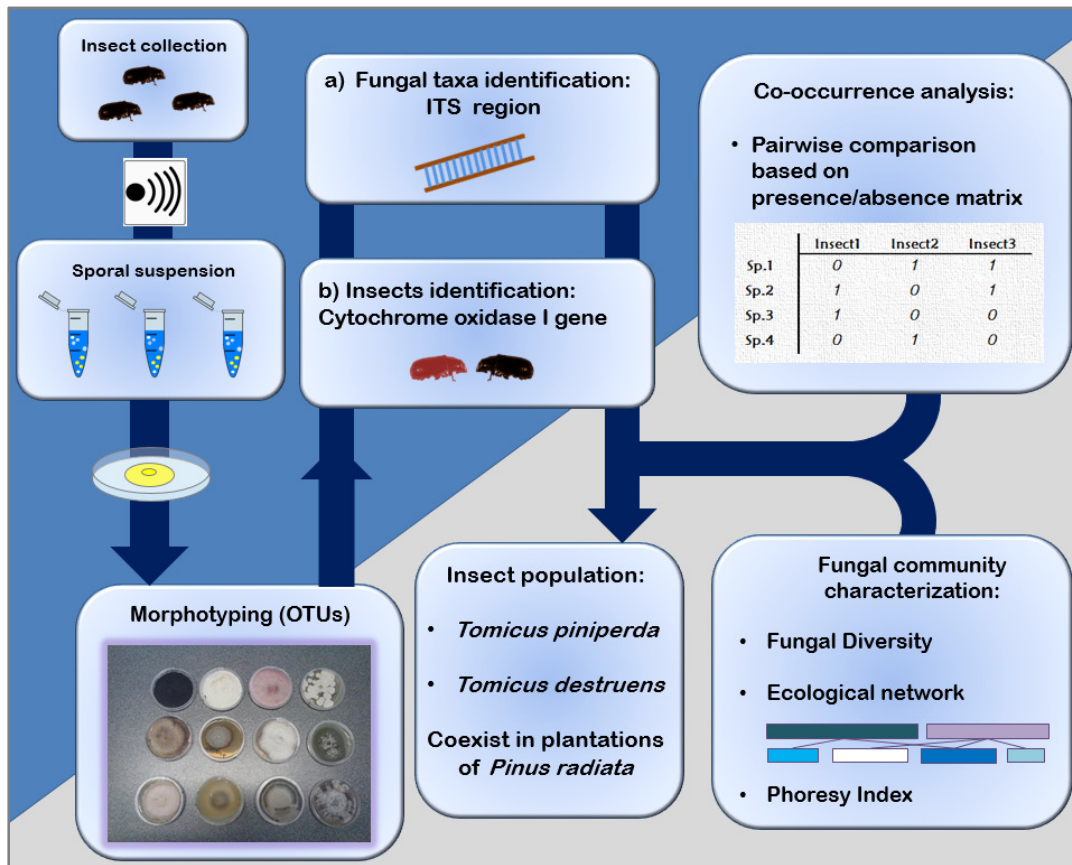
Sydowia polyspora dominates fungal communities carried by two *Tomicus* species in pine plantations threatened by *Fusarium circinatum*

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Abstract

Bark beetles (Coleoptera, Scolytinae) carry a diverse filamentous fungal community sometimes acting as vectors or carriers of phytopathogens. In this study, mycobiota carried by two *Tomicus* species (*Tomicus piniperda* and *Tomicus destruens*) were investigated through (i) morphological and molecular identification of taxa; (ii) taxonomic richness, diversity, evenness, dominance and phoresy indices; (iii) ecological network analysis and (iv) statistical co-occurrence analysis. The studied mycobiota were formed by eleven taxa and showed a moderate fungal diversity with low evenness. The fungus *Sydowia polyspora* was significantly abundant and dominated the community. All the fungal taxa were randomly associated. Both insect species (*T. piniperda* and *T. destruens*) were collected from plantations of *Pinus radiata* infected by *Fusarium circinatum*. The ecological factors that could drive community ecology and phoretic links between fungi and bark beetles are discussed.

1. Introduction

The ecological interactions between fungi and bark beetles (Coleoptera, Scolytinae) are complex and can range in a continuum from symbiosis to parasitism (Six, 2012). The relationship between bark beetles and potentially pathogenic fungi has been broadly studied in forest pathology (Lu & Beer, 2009; Sallé *et al*., 2005; Storer *et al*., 2004), *e.g.*, the Dutch elm disease (DED; causal agents *Ophiostoma ulmi* (Buisman) Melin & Nannf. and *Ophiostoma novo-ulmi* Brasier) and *Scolytus* spp. (Col., Scolytinae) is one of the most well-known pathosystems (Santini & Faccoli, 2014). Despite this interesting association between fungi and insects, the community ecology approach has been scarcely applied to filamentous fungi (Peay *et al*., 2008).

The pine pitch canker disease (PPCD, causal agent *Fusarium circinatum* Nirenberg & O'Donnell; teleomorph *Gibberella circinata* Nirenberg & O'Donnell) is considered the most important disease of pine (*Pinus* spp.) seedlings in several countries, causing high rates of pre-, post-emergence and late damping-off in nurseries. It also causes severe symptoms in adult trees, such as growth reduction, wilting, and the resulting bleeding cankers are dangerous because trees easily break during wind storms (Aegerter *et al*., 2003). This pathogen is widespread around the world, establishing different phoretic relationships with several groups of invertebrates (mainly insects but also others as mollusks and crustaceans (Storer *et al*., 2004)). Regarding insects, thirteen species of Coleoptera and one of Diptera (*Medetera* spp.; Dolichopodidae) have been identified as carriers of PPCD fungus in the USA, as summarized by Brockerhoff *et al*. (2016). In Spain, all identified carriers of *F. circinatum* belong to Curculionidae, including seven species of Scolytinae, one Molytinae and one Entiminae (Iturritxa *et al*., 2011; Romón *et al*., 2007a).

The species that can effectively inoculate a pathogen in a healthy host (vectors) embody a relevant phoretic relationship in forest pathology. In the case of PPCD in the USA, some species of Scolytinae (*i.e.*, *Ips paraconfusus* Lanier, *Ips mexicanus* Hopkins, *Ips plastographus* LeConte, *Pityophthorus setosus* Blackman, *Pityophthorus carmeli* Swaine and *Conophtorus radiatae* Hopkins), as well as *Ernobius punctulatus* (Col., Anobiidae) (Brockerhoff *et al*., 2016) have been proposed as vectors. In Spain, Pine shoot beetle (*Tomicus piniperda* L.; Col. Scolytinae) has recently been identified as a plausible vector of *F. circinatum* (Bezos *et al*., 2015), with a special interest in the North where PPCD infects pine forests and plantations. This phloephagous insect can select different tree species as hosts (mainly *Pinus* sp. but also other genera of conifers such as *Picea* sp. or *Larix* sp.), and it is considered to be a secondary pest in terms of trunk infestation in their native Eurasian range (Annila *et al*., 1999; Fernández

& Costas, 1999). Nevertheless, it is considered an aggressive pest on pine forests and plantations in the USA where it was introduced in 1992 (Haack & Poland, 2001).

In Atlantic habitats, *T. piniperda* sometimes co-occurs with Mediterranean shoot beetle (*Tomicus destruens* Wollaston) (Gallego *et al*., 2004; Vasconcelos *et al*., 2006). Mediterranean shoot beetle causes intense damages in thermophilic woodlands throughout its range, circumscribed to the Mediterranean Basin (Gallego & Galián, 2008; Horn *et al*., 2006, 2012). Despite this, the ecology of this insect in the Atlantic area and its role as a carrier or vector of forest diseases remain understudied. In consequence, more knowledge is needed of the ecology and phoretic communities of these two bark beetles in the areas where they coexist with *F. circinatum*.

We hypothesized that the fungal community carried by the studied bark beetles would be diverse and would include positive and negative interactions among fungal taxa. Additionally, we hypothesized that a few species of fungi would dominate fungal community. Therefore, the aims of this study were (i) to characterise the mycobiota carried by the two *Tomicus* spp. during their hibernation period in pine shoots; (ii) to evaluate the ecological relationships (including dominance) among fungi carried by the bark beetles and (iii) to investigate the possible presence of *Tomicus destruens* in pine stands inhabited by *T. piniperda* and damaged by PPCD.

2. Materials and methods

2.1. Samples collection

During November and December 2015, pine shoots bored by *Tomicus* spp. were directly collected from the ground using circular transects (three hours of sampling per plot during each fieldwork day) in two plots in the Cantabria region (North of Spain): (A) Monterey pine (*Pinus radiata* D. Don) plantations infected by *F. circinatum* (Santibañez, Cabezón de la Sal. UTM 30N, 398813; 4792690. 320 m a.s.l.) and (B) an asymptomatic mixed plantation of European black pine (*Pinus nigra* subsp. *salzmannii* (Dunal) Franco) and Scots pine (*Pinus sylvestris* L.) (San Miguel de Aguayo. UTM 30N, 419587; 4771507. 850 m a.s.l.). Insects inside shoots were removed using sterilised tweezers and stored at 4° C. Each bark beetle was transferred to a single sterilised tube and immersed in 100 µl Tween 80 (PanReac Química, Barcelona, Spain) at 1% v/v. Then, they were sonicated (J. P. Selecta Ultrasons 2.6l; J. P. Selecta, Barcelona, Spain) for 5 s at 40 kHz to obtain spore suspensions (Ambourn *et al*., 2006). The sampling plots showed favorable characteristics for sympatry of *T. piniperda* and *T. destruens*

(Vasconcelos *et al*., 2006); therefore, the body of each insect was reserved for molecular identification.

2.2. Molecular identification of Tomicus *species*

T. piniperda and *T. destruens* share a very similar morphology (Faccoli, 2006). Therefore, molecular identification by PCR was carried out with extracted DNA from all the collected insects according to Vainio *et al*. (Vainio *et al*., 1998). The selected method for species identification was the one described by Kohlmayr *et al*. (2002) based on amplification of cytochrome oxidase I gene (*COI*) using specific primer pairs. Briefly, the primer pair C1-J-2441 (5′-CCTACAGGAATTAAAATTTTTAGATGATTAGC-3′) (Simon *et al*., 1994) and C1-N-2937 (5′-ATATTGGAATCACTCAATTGAG-3′) was used to identify *T. piniperda* samples. Moreover, the primer pair C1-J-2441 and C1-N-2934 (5′-TTCTTGGAATCATTCAATAGAAGTC-3′) was used to ensure the identity of *T. destruens*. PCR was performed with Kapa Taq PCR Kit (Kapa Biosystems, Wilmington, MA, USA) following a protocol of 10 min at 94° C, 36 cycles of 30 s at 94° C, 30 s at 56° C, 1 min at 72° C, and a final extension of 2 min at 72° C. The amplicons were separated in 1.60% w/v agarose gel and stained with 3× GelRed™ (Biotium, Hayward, CA, USA) after running for 18 minutes in an orbital shaker at 85 rpm. The amplicon size was estimated by comparison with a 100 bp ladder (Nippon Genetics Europe, Dueren, Germany).

2.3. Fungal isolation and identification

Fifty microliter aliquots of spore suspensions were initially cultured in a generalist culture medium (*i.e.*, PDA; 3.90% w/v potato-dextrose-agar) amended with streptomycin sulfate salt ($C_{21}H_{39}N_7O_{12}\cdot1.5\ H_2SO_4$; 0.60 mg / l) as a broad-spectrum antibacterial agent. In the cases where bacterial colonies entirely covered the plate, a second 50 µl aliquot of spore suspension was cultured in a nutrient-deficient medium (water-agar; 1.50 mg / l agar) to reduce the number of sterile or uncolonised samples (*i.e.*, insects that did not provide any fungal colony after culturing the spore suspension). Each colony was counted and subcultured onto MEA medium (1.45% w/v agar and 1.93% w/v malt extract) in order to get pure cultures. Fungal colonies were preliminary microscopically characterised (LEITZ DIALUX 22/22 EB; Leitz, Wetzlar, Germany) and visual inspection of colonial characteristics defined operational taxonomical units (OTUs) (Lacap *et al*., 2003). A representative amount of monosporic cultures per OTU from different spore suspensions (*i.e.*, 1–10 pure cultures per OTU depending on the relative abundance; 33 isolates in total) was transferred to cellophane-membrane-covered MOS-agar medium (2.84% w/v orange serum agar, 0.85% w/v agar, 0.76% w/v malt extract and 0.76% w/v dextrose) for DNA extraction and subsequent molecular identification.

DNA extraction was carried out following the protocol described by Vainio *et al*. (1998). OTU homogeneity was verified by using fingerprints amplified with the M13 minisatellite primer (5′-GAGGGTGGCGGTTCT-3′) (Stenlid *et al*., 1994). The PCR reaction was performed in a total volume of 50 µl, and it consisted of the following steps: 10 min at 93° C followed by 45 cycles of 20 s at 93° C, 1 min at 48° C, 20 s at 72° C and a final incubation step of 6 min at 72° C. Furthermore, molecular identification of each OTU was based on rDNA sequencing (*i.e.*, ITS region), which was amplified by using ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as a primer pair (Gardes & Bruns, 1993). The PCR reaction was performed in a total volume of 50 µl, and it consisted of the following steps: 10 min denaturation at 96° C followed by a stepped second phase of 13 cycles of 35 s at 95° C, 55 s at 56° C, 45 s at 72° C; then, 13 cycles of 35 s at 95° C, 55 s at 56° C, 2 min at 72° C and a last step of 8 additional cycles of 35 s at 95° C, 55 s at 56° C, 3 min at 72° C, and finally, an elongation step for 10 min at 72° C (Gardes & Bruns, 1993). The amplification products were separated by electrophoresis in a gel containing 0.90% w/v each of SynerGel™ (Diversified Biotech, Dedham, MA, USA) and 1.60% w/v agarose. DNA fragments were visualized using ethidium bromide staining under UV light.

Amplicons were sent to Macrogen Europe Inc. (www.macrogen.com; Amsterdam, The Netherlands) for DNA sequencing. Sequences were trimmed using Sequence Scanner v1.0 software (Thermo Fisher Scientific, Waltham, MA, USA) and then compared with those deposited in the GenBank (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi) database using BLAST (Altschul *et al*., 1990). Fungal taxa were assigned at species or genus level, always showing a homology equal or higher than 99%. ITS sequences representing each detected molecular variant were submitted to GenBank (Table 1).

The classification of *Fusarium* sp. cultures was done based on morphological traits as well as genetic characterization, by sequencing the ITS regions and studying their phylogenetic relationships with culture collection strains of different *Fusarium* species. The morphological characterization was carried out based on reproductive structures such as microconidia (presence / absence, number of septa, length shape and aggregation in chains, false heads, etc.), macroconidia (size, length, thickness, apical and basal cells shape, number of septa), phialides (mono- and polyphialide presence), and chlamydospores (presence / absence, ornametation, and aggregation) (Leslie & Summerell, 2006). The phylogenetic representation (*i.e.*, dendrogram) was computed among the *Fusarium* isolates from this study and other sequences in GenBank, always including culture collection specimen sequences for each species. The MEGA v6.06 (Tamura *et al*., 2013) software was used to compute a tree using Neighbor-joining as the statistical method, the bootstrap method (1000 replications) as a test

of phylogeny and Kimura2 (Kimura, 1980) as the substitution method. Nevertheless, future studies based on more accurate molecular markers are required to determine the detailed taxonomical status of the *Fusarium* isolates.

2.4. Data analysis

Diversity of fungi was analysed using Shannon (H) and Simpson (D) diversity indices, taxonomic richness (S_{obs}), Sorensen similarity index between plot A and B (I) and Shannon and Simpson taxa evenness indices (J and E, respectively) (Zak & Willing, 2004). Fungal dominance was measured using Camargo's index where dominance was defined if $p_i > 1 / S_{obs}$ (p_i being the number of isolates of taxon i / total number of isolates) (Camargo, 1993). In addition, the EstimateS v9.1.0 (Colwell, 2011) software was used to compute sample-based rarefaction curves (Colwell *et al*., 2012; Gotelli & Colwell, 2001), and 95% confidence intervals (Colwell *et al*., 2004). The same software was used to calculate estimated taxonomic richness through six richness non-parametric estimators: Mao-Tau, Incidence-based Coverage Estimator of species richness (ICE), First and Second order Jackknife richness estimator (Jack 1 and 2), Chao 1 and Chao 2 (Colwell *et al*., 2012).

The phoresy index (PI) based on the pollination probability index previously described by Ne'eman *et al.* (1999) was also calculated for each taxa and carrier *Tomicus* sp. according to Equation 1. This index ranges from 0 where the insects did not yield any fungal isolates (null phoretic association), to 1 where only one taxon was isolated from spore suspensions (intense phoretic association). R software (R Development Core Team, 2015) was used to develop Kruskal-Wallis rank sum tests implemented in the "Agricolae" package (De Mendiburu, 2009) to analyse the PI variation among taxa. Subsequently, Dunn's test was applied for post-hoc analysis, using the "DescTools" package (Signorell *et al*., 2015).

$$PI_{ij} = \left(\frac{n_{ij}}{n_j}\right) \times \left(\frac{N_i}{N}\right) \qquad (1)$$

Where PI_{ij}: phoresy index for fungal taxon i carried by insect j; n_{ij}: number of isolates of taxon i yielded by insect j; n_j: total amount of isolates yielded by insect j; N_i: number of insects that carried taxon i; N: total amount of collected insects.

The relationships between host trees, bark beetles and fungi were analysed using the package "bipartite" (Dormann *et al*., 2008) of R software. This package was used to calculate the connectance (Cn; total number of links in the network / S_{obs}^2) (Dunne *et al*., 2002) and the mean number of links per taxa (L) as well as to represent the sampled mycobiota in a tripartite graph. This statistical software was also used to perform a co-occurrence analysis using the

package "cooccur" (Griffith *et al.*, 2016). The function "co-occur" provides the observed and expected frequencies of co-occurrence by pairs of taxa (F_{obs} and F_{exp} respectively) and two associated probabilities (P_{gt} and P_{lt}) that could be interpreted as p-values (Veech, 2013). These probabilities identify each pairwise association between fungi as follows: a) positive association between taxa if $F_{obs} > F_{exp}$ and $P_{gt} < 0.05$, b) negative association if $F_{obs} < F_{exp}$ and $P_{lt} < 0.05$ or c) random association in the remaining cases.

3. Results

3.1. Shoot collection and insect identification

A total of 499 pine shoots bored by the two *Tomicus* species were collected during the study period. The main collected species were Monterey pine (58.11%; plot A) followed by European black pine and Scots pine (33.27% and 8.62% respectively; plot B). Insect occupation rate was 9.82%, with a total of 49 insects (one insect inside each shoot), while frequencies of insects in each pine species were 5.17% in Monterey pines, 18.07% in European black pines and 9.30% in Scots pines.

Both insect species, *T. destruens* and *T. piniperda*, were present in the study area, but only two individuals captured in the pure Monterey pine stand (plot A) were *T. destruens* (13.33% plot A; 4.08% total amount of insects) while 100% of insects in the mixed stand (plot B) were *T. piniperda*. Two individuals (one from plot A and the other one from plot B) could not be identified at the species level due to the low quality of the extracted DNA. They did not provide any fungal colony information.

3.2. Fungal community characterisation

A total of 113 pure fungal cultures were isolated, belonging to 13 morphological OTUs (2.69 ± 0.10 isolates per insect and 1.38 ± 0.15 OTUs per insect; average values and standard error) (Tables 1 and 2). Fungal cultures were assigned to morphological OTUs based on combined results of morphological investigation and M13 fingerprinting. Species identification was conducted using ITS sequences, which revealed a total of 11 taxa (1.33 ± 0.05 taxa per insect).

In spite of the morphological differences observed in the *Fusarium* sp. isolates in five OTUs (Table 1), ITS sequences were not useful to assign species to OTUs 11, 12 and 13. Dendrogram results (Figure A1) and GenBank best matches supported the clustering of *Fusarium* spp. in three clades (*i.e.*, *Fusarium sporotrichioides* Sherb., *Fusarium lateritium* Nees and *Fusarium* sp.) (Table 1).

Sydowia polyspora (Bref. & Tavel) E. Müll. dominated the whole community (plots A and B), but in plot A, *Pestalotiopisis* sp. was also dominant according to the Camargo's index (Table 2). PI was very low in general, showing low phoretic associations between fungi and *Tomicus* spp. This index varied significantly among fungal taxa (X^2 = 209.74; d.f. = 10; P < 0.01), but the PI of *S. polyspora* was significantly higher than that of other taxa, according to Dunn's test (P < 0.01 in all cases) (Table 2). *F. circinatum* was not isolated from spore suspensions despite the fact that plot A was severely damaged by PPCD.

The sample-based rarefaction curves did not show an asymptotic development (Figure 1). A tripartite graph summarized the observed mycobiota (Figure 2) that showed four shared taxa between bark beetle species (all of them carried by *T. destruens*: *Fusarium* sp., *F. lateritium*, *Pestalotiopsis* sp. and *S. polyspora*). No phoretic fungal taxon was present exclusively in one pine species. All ecological indices calculated for fungal community are shown in Table 3.
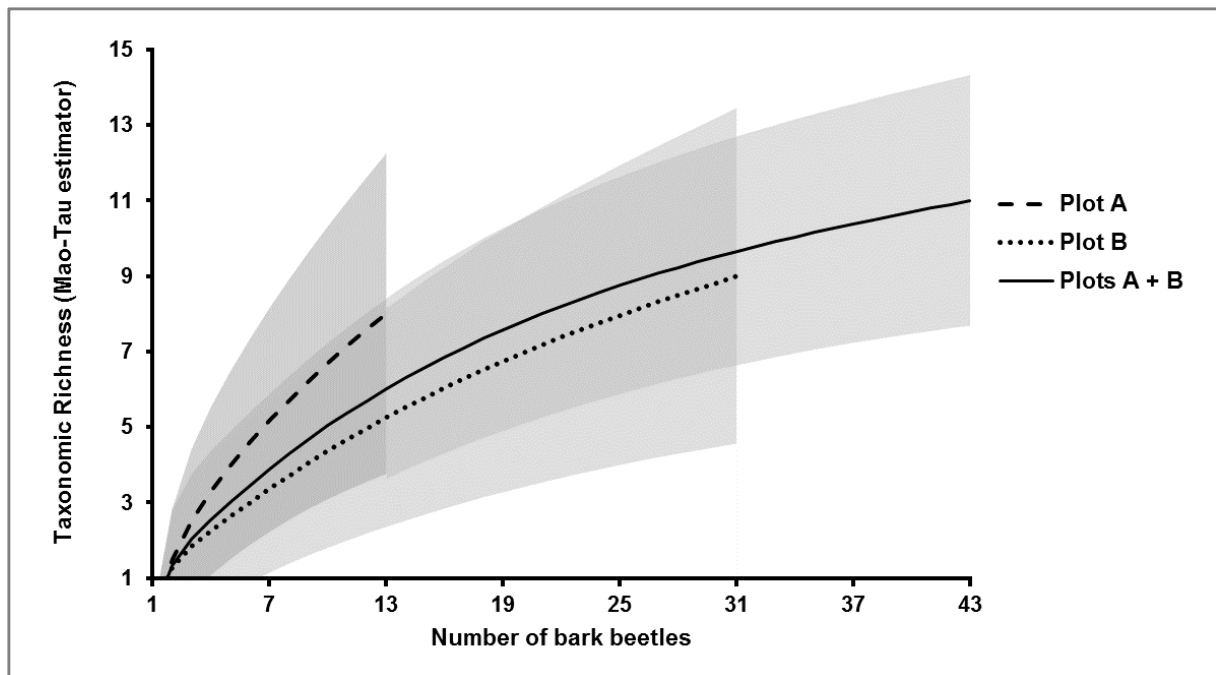

Figure 1. Taxonomic accumulation curves of fungal taxa carried by the two studied *Tomicus* species (shaded areas: 95% confidence intervals). Taxonomic richness computed as expected number of taxa (Mao-Tau estimator).

The analysis of co-occurrence showed 100% random relationships between fungal taxa (P_{gt} and $P_{lt} \geq 0.05$ in all cases). However, the relationship between *S. polyspora* and *Fusarium* sp. showed an F_{obs} lower than the F_{exp}, and the associated probability was slightly higher than the significance level (P_{lt} = 5.72 × 10^{-2}; effect size: −0.45). Results did not vary when an alternative analysis was performed excluding pairs of taxa in which F_{exp} resulted less than one (threshold use; *i.e.*, 89.09% pairs of fungal taxa excluded from the analysis).

Table 1. Data of fungal isolates from *Tomicus* species in the studied plots.

OTU	GenBank Accession Number of Best Matches	Id (%) / Qc (%)	Description of GenBank Best Match	Suggested Name for OTU	Accession Number
1	HG008754.1	100/100	*Sydowia polyspora* (Bref. & Tavel) E. Müll.	*S. polyspora*	KY081694
2	KM199339.1	99/97	*Pestalotiopsis hawaiiensis* Maharachch., K.D. Hyde & Crous	*Pestalotiopsis sp.*	KY081696
3	JX421725.1	100/100	*Phoma herbarum* Westend	*P. herbarum*	KY081697
4	KU182497.1	100/100	*Cladosporium cladosporioides* (Fresen.) G.A. de Vries	*Cladosporium sp.*	KY081699
5	JN617665.1	100/99	*Penicillium westlingii* K.M. Zalessky	*Penicillium sp.*	KY081700
6	JX421733.1	99/100	*Phaeomoniella effusa* Damm & Crous (synonym: *Aequabiliella effusa* (Damm & Crous) Crous)	*P. effusa*	KY081695
7	KU184424.1	99/100	*Ophiostoma canum* (Münch) Syd. & P. Syd.	*O. canum*	KY081698
8	AJ876490.1	99/100	*Mucor hiemalis* Wehmer	*M. hiemalis*	KY081701
9	FJ403224.1	99/100	*Fusarium sporotrichioides* Sherb.	*F. sporotrichioides* *	KY081692
10	AF310978.1	100/100	*Fusarium lateritium* Nees	*F. lateritium* *	KY081690
11	KU516466.1	100/100	*Fusarium avenaceum* (Fr.) Sacc.	*Fusarium sp.* *	KY081689
12	KU516468.1	100/100	*F. avenaceum*	*Fusarium sp.* *	KY081691
13	KU516467.1	100/100	*F. avenaceum*	*Fusarium sp.* *	KY081693

OTU: Operational taxonomical unit; Id: Maximum identity of sequence; Qc: Query coverage of sequence. *: *Fusarium* species were clustered according to GenBank best matches and Neighbor-joining dendrogram results (see Figure A1). Taxonomy according to Robert *et al.* (2005).

Table 2. Relative abundance and phoresy index (PI (%); mean value and standard error (SE)) of fungal taxa carried by the two studied *Tomicus* species in the sampling plots.

Fungal Taxa	Relative Abundance		Total	PI (%) ± SE
	Plot A	Plot B		
Cladosporium sp.	1 (3.23%)	0 (0.00%)	1 (0.88%)	$8.33 \times 10^{-3} \pm 8.33 \times 10^{-3}$a
Fusarium lateritium	1 (3.23%)	2 (2.44%)	3 (2.65%)	0.15 ± 0.08a
Fusarium sporotrichioides	0 (0.00%)	3 (3.66%)	3 (2.65%)	0.04 ± 0.04a
Fusarium sp.	2 (6.45%)	8 (9.76%)	10 (8.85%)	0.74 ± 0.35a
Mucor hiemalis	1 (3.23%)	2 (2.44%)	3 (2.65%)	0.11 ± 0.09a
Ophiostoma canum	0 (0.00%)	2 (2.44%)	2 (1.77%)	0.04 ± 0.04a
Penicillium sp.	3 (9.68%)	0 (0.00%)	3 (2.65%)	0.04 ± 0.04a
Pestalotiopsis sp.	**6 (19.35%)**	1 (1.22%)	7 (6.19%)	0.29 ± 0.16a
Phaeomoniella effusa	2 (6.45%)	3 (3.66%)	5 (4.42%)	0.15 ± 0.10a
Phoma herbarum	0 (0.00%)	4 (4.88%)	4 (3.54%)	0.15 ± 0.08a
Sydowia polyspora	**15 (48.39%)**	**57 (69.51%)**	**72 (63.72%)**	37.84 ± 4.30b
Subtotal	31	82	113	
Total number of bark beetles	49			
Total uncolonised samples	7 (14.28%)			

Plot A: Plantation of *Pinus radiata* infected by *Fusarium circinatum*; Plot B: Asymptomatic plantation of *Pinus nigra* subsp. *salzmanni* and *Pinus sylvestris*. Dominant species according to Camargo's Dominance Index in bold. Small letters (a-b) denote significant differences (Dunn's test, P < 0.05).

4. Discussion

Phoretic fungal communities are driven by complex relationships, and the presence or absence of single involved taxa could have an ecological meaning. The observed richness (S_{obs}) was identical to the value of Chao1; meanwhile, Chao2 and ICE values increased by less than 26%. Jackknife richness estimators 1 and 2 showed values of 35.45% and 53.27% higher, respectively, than S_{obs}, suggesting that a larger sample size would have provided further information. Similarly, accumulation curves computed for the studied mycobiota suggested that more fungal species would have been obtained if more captures had been achieved. The majority of fungal species tended to appear in very low numbers (singletons and doubletons) while only few taxa were really frequent, as phoresy index values suggested (Table 2). This phenomenon seems to be common everywhere in nature, and it has been broadly reported either in fungi-insect associations or fungal endophytes (Arnold & Lutzoni, 2007; Romón *et al*., 2008; Terhonen *et al*., 2011). Although pine host species were different among plots, the

Sorensen index indicated a high-intermediate similarity in the mycobiota among plots (I = 0.70). In addition, the use of an identification method based on culturing in a specific medium implies a detection bias that must be taken into consideration (Botella & Diez, 2011; Giordano *et al*., 2013; Lim *et al*., 2006), even though a generalist medium like PDA is selected. Considering these possible sources of richness underestimation, it is not possible to claim that the whole community was identified. Nevertheless, regarding previously discussed ecological indicators, it is possible to conclude that mycobiota were sufficiently characterised. Results from evaluating fungal community composition by their taxonomical placement agreed with other studies where Dothideomycetes and Sordariomycetes were the main taxonomic groups of fungal communities inhabiting pine species (Botella & Diez, 2011; Sanz-Ros *et al*., 2015).

Table 3. Ecological indices calculated for fungal community.

Ecological Index	Value
Taxonomic richness (S_{obs}) (plot A / plot B / total)	8 / 9 / 11
ICE	13.78
Chao1	11.00
Chao2	12.95
Jack1	14.90
Jack2	16.86
Shannon (H)	1.43
Simpson (D)	0.42
Shannon evenness (J)	0.59
Simpson evenness (E)	0.21
Sorensen index (I)	0.70

ICE: Incidence-based Coverage Estimator of species richness.

The observed fungal diversity carried by scolytids in this study was similar to that reported by Romón *et al.* (2008) for bark beetles *Orthotomicus erosus* Wollaston and *Hylastes attenuatus* Erichson, associated with *F. circinatum* infected plots in the Basque Country (Spain). Meanwhile, fungal evenness was rather low, due to the high presence of a few taxa (dominance) previously commented. Interestingly, this finding accords with studies of fungal endophytes in pine twigs (Sanz-Ros *et al*., 2015). Moreover, lower values of fungal diversity were reported for ophiostomatoid fungi associated with *T. piniperda*, as described by Romón *et al.* (2007b, 2014) in Spain. On the contrary, higher diversity values were observed in northern countries for *Hylurgus ligniperda* Fabricius (Davydenko *et al*., 2014) and even for *T. piniperda* (Jankowiak & Bilański, 2007; Silva *et al*., 2015). An increase in fungal endophytic

diversity in northern latitudes has been suggested (Millberg *et al*., 2015), but how latitudinal gradients can affect phoretic communities composition remains understudied.
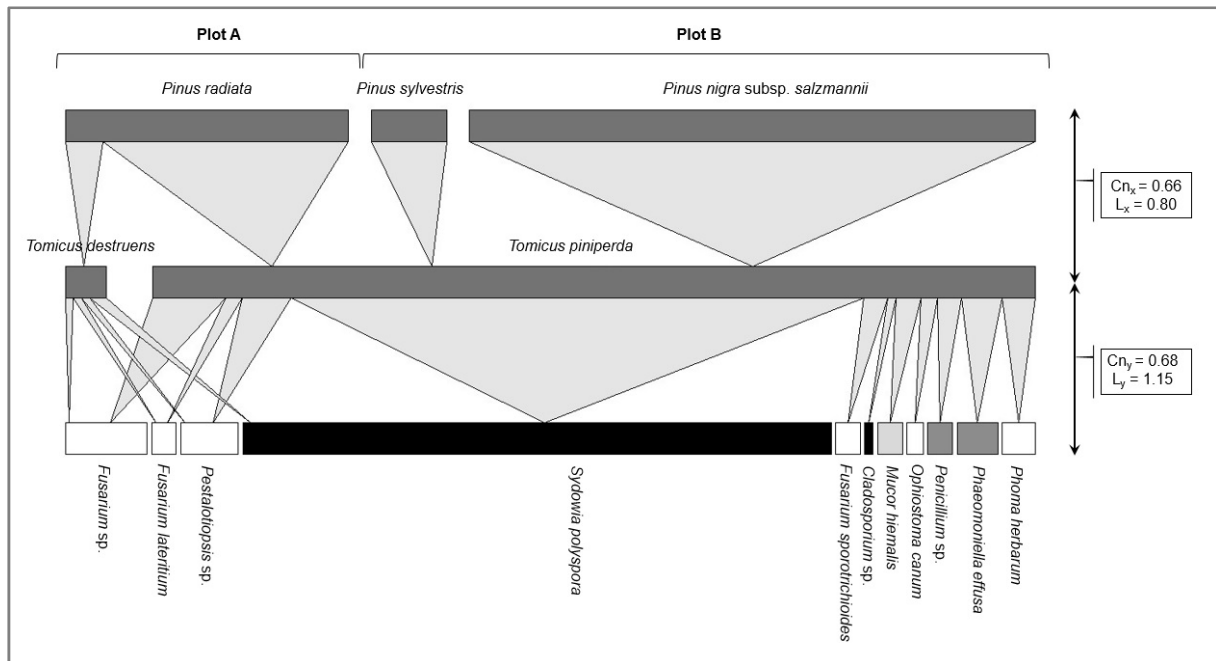

Figure 2. Tripartite graph of sampled mycobiota. Upper level: hosts (*Pinus* spp.); middle level: carrier insects (*Tomicus destruens* and *Tomicus piniperda*); lower level: phoretic fungal taxa. Rectangle width is proportional to the sum of interactions involving each taxon. Fungal classes are represented by colours in lower level boxes: white (Sordariomycetes), black (Dothideomycetes), light grey (Lecanoromycetes) and dark grey (Eurotiomycetes). Cn_x and Cn_y show the connectance for each network level. L_x and L_y show the mean number of links per taxa among levels.

The mycobiota of two studied bark beetles included some *Fusarium* species reported in earlier studies for *Tomicus* genus (Jankowiak, 2005, 2006). The presence of this taxonomical group could be considered noteworthy due to the broad range of hosts and this genus pathogenic potential. Specifically, *F. sporotrichioides* is considered as an opportunistic pathogen while *F. lateritium* is not only considered as nonpathogenic but also as a possible antagonist against *F. circinatum* when *F. lateritium* performs an earlier colonization (Romón *et al*., 2008). In this way, plot A showed evident symptoms of PPCD, and the presence of the pathogen in this area has been proved in previous studies (Bezos *et al*., 2012; Martínez-Álvarez *et al*., 2012). In spite of this, *F. circinatum* was not present in collected insects either from this infected plot or from plot B. The most plausible explanation for this absence could be the previously reported low phoresy rate of *T. piniperda* (Romón *et al*., 2008, 2007a), even in infected pine stands (4% according to Bezos *et al.* (2015)). There is not much information on phoretic capacity of *T. destruens*, although it might be expected to be similar to its sister species. The absence of *F. circinatum* in plot B could suggest that this area was not yet infected by the pathogen. However,

the high dispersion capacity of *F. circinatum* and the presence of plausible vector species indicate that the area cannot be ruled out as potentially threatened by PPCD. According to Bateman *et al.* (2016), the association between *Fusarium* spp. and bark beetles could be explained by the abundant spore production of this genus (phoretic opportunism) and also by the pathogenic behavior of some *Fusarium* species, which could embody an advantage for bark beetles during tree colonization.

The most abundant species *S. polyspora* (anamorph: *Hormonema dematioides* Lagerb. & Melin) requires special attention. This fungus was a dominant species in the studied community and showed the highest and most significant value of PI. *S. polyspora* has been reported as very frequent species carried by insects (Davydenko *et al.*, 2014; Jankowiak & Bilański, 2007) as well as an endophyte and a saprophyte (Lygis *et al.*, 2014; Pirttilä *et al.*, 2003; Terhonen *et al.*, 2011). Therefore, this fungus has been considered a primary coloniser of woodlands litter (Isidorov *et al.*, 2016). In contrast, Talgø *et al.* (2010) identified the possible pathogenic traits of *S. polyspora* that could be involved in current season needle necrosis (CSNN), a disease that affects fir trees (*Abies* spp.) in Europe and the USA. With regard to the role that *S. polyspora* could play in association with bark beetles, Jankowiak and Kurek (2006) proposed that it may embody an advantage during early host colonization. The aforementioned pathogenic behavior related to CSNN could support this hypothesis but, in contrast, Boberg *et al.* (2011) reported that *S. polyspora* mainly consumes soluble compounds in pine needles, suggesting the absence of cellulolytic activity. Hence, the participation of this fungus in host colonization by bark beetles, and specifically with *Tomicus* species, remains unclear. Nevertheless, the observed abundance of *S. polyspora* can be explained by a two-way transmission that may simultaneously take place. On the one hand, the fungus could be abundant in shoots during bark beetle feeding period (endophytic way); on the other hand, insects could be loaded off spores directly from the litter (saprophytic way) where shoots had collected.

In terms of the other fungi observed, some of the genera isolated from spore suspensions have been cited as endophytes of *Pinus* spp, and consequently, could be transient members of the mycobiota. Regarding *Phaeomoniella effusa* Damm & Crous, this species has been previously isolated from old stumps of *Pinus mugo* Turra (Lygis *et al.*, 2014). In the same way, *Pestalotiopsis* sp., which dominated in plot A as reported by Romón *et al.* (2008), has been also considered as an endophyte of *Pinus* spp. (Hu *et al.*, 2007; Zamora *et al.*, 2008). *Phoma* spp. are frequent in the tissues of pines such as sapwood, needles and twigs (Giordano *et al.*, 2009; Zamora *et al.*, 2008) and are also carried by bark beetles (Giordano *et al.*, 2013; Jankowiak & Kurek, 2006; Linnakoski *et al.*, 2016). Sanz-Ros *et al.* (2015) found high rates of

appearance of *Phoma herbarum* Westend in *P. sylvestris* supporting the endophytic role of this fungus.

Associations between ophiostomatoid fungi and bark beetles have been widely reported (Fernández *et al*., 2004; Hausner *et al*., 2005; Jankowiak & Kurek, 2006; Linnakoski *et al*., 2012; Sabbatini-Peverieri *et al*., 2006). In this study the only member of this order was the blue stain fungus *Ophiostoma canum* (Münch) Syd. & P. Syd. This species seems to be more strongly associated with *Tomicus minor* Hartig than *T. piniperda* (Jankowiak, 2005; Solheim *et al*., 2001), which could explain the low frequency observed. However, this fungus has been also isolated from *T. piniperda* (Jankowiak, 2006; Masuya *et al*., 2009), being quite frequent among blue stain fungi carried by this insect species (Silva *et al*., 2015). *Penicillium* spp. have been frequently isolated from several species of bark beetles (Giordano *et al*., 2013; Jankowiak, 2006; Jankowiak & Rossa, 2008). Anemochory is considered the main dispersion method for this genus, being the association between *Penicillium* sp. and bark beetles a consequence of contact during flight. The appearance of *Mucor hiemalis* Wehmer was probably caused by an eventual contact with insects because its spores are frequently distributed in soil and litter (Jankowiak, 2005; Jankowiak & Bilański, 2007). *Cladosposrium* sp. has been also cited as a random phoretic fungus even though the entomopathogenic potencial of this genus has also been reported (Abdel-Baky & Abdel-Salam, 2003; Eken & Hayat, 2009), giving two plausible explanations of its presence in spore suspension.

Antagonism has been broadly documented among fungi (Campanile *et al*., 2007; Jensen *et al*., 2016), driving multiple interactions between species in nature. Curiously, the results provided by co-occurrence analysis only showed random associations among fungal taxa. However, it is noticeable that the relationship between *S. polyspora* and *Fusarium* sp. was close to being negative, according to analysis parameters. In consequence, a larger sample size supported with a confrontation assay could provide more data about the potential antagonistic effects among these fungal taxa (Martínez-Álvarez *et al*., 2016).

In this study, the ecological network analysis was used as an approach to the complexity of the ecosystem starting from the observed mycobiota. The connectance was higher than previously described in other plant-beetle (Meskens *et al*., 2011; Woodcock *et al*., 2013) and fungi-beetle (Schigel, 2011) systems. Our results are circumscribed to the sampled mycobiota; therefore, new network studies in locations along the distribution ranges of these two *Tomicus* spp. could clarify the complexity of the complete ecological network. In addition, *T. piniperda* has been proposed as a predominant species in the study area according to some distribution models (Gallego *et al*., 2004; Horn *et al*., 2012). Nevertheless, its coexistence with *T. destruens*

was observed in plot A, and it has been previously reported in *Pinus pinaster* Aiton stands in other locations of the Iberian Peninsula (Gallego *et al*., 2004; Vasconcelos *et al*., 2006). According to Lieutier *et al*. (2015), the phenology of *T. destruens* can become similar to *T. piniperda* in cold areas. Our sampling areas were located in the North of Spain (Cantabrian Mountains) where annual rainfall is abundant and the climate is not as warm as in the Mediterranean Basin. These climatic aspects could explain why both species feed inside pine shoots during autumn and winter. The number of collected bark beetles was rather low, probably because the population is in an endemic phase in these sampling areas. Therefore, new studies should be performed in order to clarify the ecological interactions between both species in the Atlantic area.

5. Conclusions

1- *Sydowia polyspora* was the most frequent fungus carried by *T. piniperda* and *T. destruens.* This species dominated the sampled mycobiota.

2- The sampled mycobiota showed moderate taxonomic richness and diversity. However, the evenness was rather low.

3- *T. destruens* co-occurs with *T. piniperda* in Monterey pine plantations infected by PPCD in Cantabrian Mountains.

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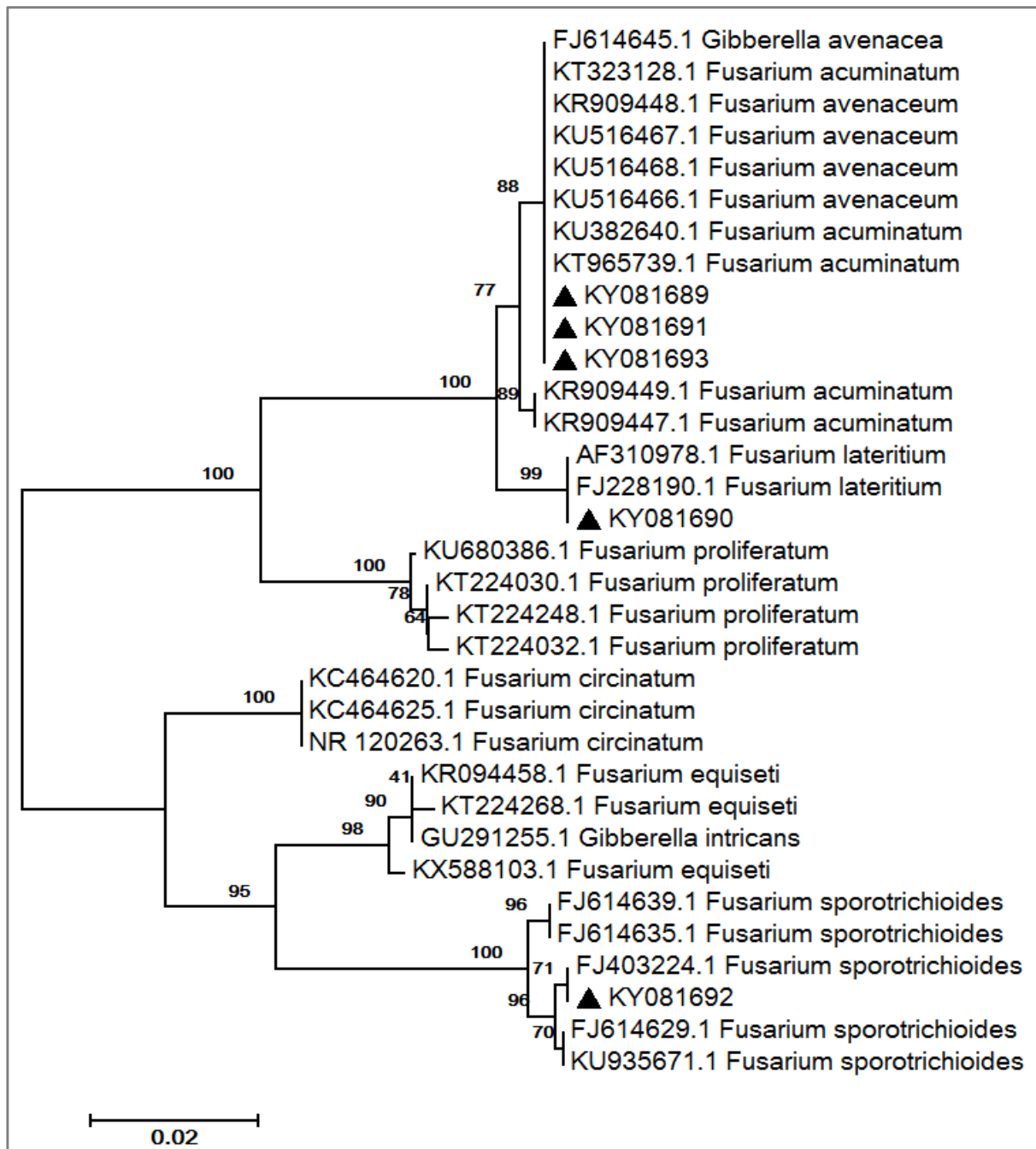
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Supplementary material (Supporting information)

A1. Neighbor-joining dendrogram of ITS showing *Fusarium* spp. clusters. The percentages of replicate trees in which the associated taxa clustered together in the Bootstrap method (1000 replications) are shown next to the branches. This dendrogram is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the dendrogram. Evolutionary distances were computed using the Kimura2 parameter method and are in the units of the number of base substitutions per site. The analysis involved 33 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 435 positions in the final dataset. Black triangles represent sequences of OTUs corresponding to *Fusarium* samples isolated in this study. GenBank accession numbers of each reference sample are provided.

7.1. Fungal viruses as a node in the pathosystem (I)

Current knowledge of fungal pathogen-mycovirus interactions was reviewed with particular emphasis on filamentous fungi that cause diseases in woody plants of interest in agroforestry. Several aspects such as viral taxonomy, molecular description, evolutionary relationships with their hosts and routes of transmission were discussed. Viral diversity hosted by forest pathogens was summarized and the best-known case of virocontrol in forest health (*i.e.* use of CHV-1 to control *C. parasitica*) was also discussed. Advances in virus identification were addressed, along with the reported effects of mycoviruses in native and non-native hosts. In addition, future challenges in mycovirus-based biocontrol were highlighted.

7.2. The fungus as a node in the pathosystem (II)

The complete genome of *F. circinatum* was used as query to identify five candidate genes putatively related to pathogenesis (*i.e. Fcfga1*, *Fcfgb1*, *Fcac*, *Fcrho1* and *FcpacC*). The candidate genes were compared at nucleotide and protein levels with genes included in public databases. The obtained similarities reached up to 90% with annotated pathogenicity-related genes found in other species of *Fusarium*. In addition, the expected biological function of each candidate gene was studied according to the results of the gene ontology analysis.

7.3. Virus – fungus interactions (III)

The anti-viral response of *F. circinatum* to three previously described mitoviruses (*i.e.* FcMV1, FcMV2-1 and FcMV2-2) was studied by high throughput sequencing (HTS). For this purpose, ten Spanish isolates (60% isolated from *Pinus* spp. and 40% from bark beetles) were selected and their total RNA extracted. Next generation sequencing provided more than 24 million reads that were explored, mapped along viral genomes and *de novo* assembled. Most reads belonged to the fungal host genome (98.57% genomic RNA and 1.05% mitochondrial RNA) while 0.07%, 0.12% and 0.16% corresponded to vsRNA belonging to FcMV1, FcMV2-1 and FcMV2-2 viral genomes respectively. *De novo* assembly of reads generated contigs that covered more than 50% of each viral genome. The putative 3´ extreme of FcMV2-2 was also identified (*i.e.* contig 50) correctly aligning with the partial genome deposited in GenBank (NCBI). In addition, another contig with moderate similarity to other *Mitovirus* species was identified (*i.e.* contig 571). Viral prevalence was analyzed in the complete batch of isolates by RT-PCR with four previously reported primer pairs and seven new ones, followed by Sanger sequencing. A total of 50% of isolates were infected by at least one viral strain, and the new

primer pairs proved suitable for detecting mitoviruses in PPC fungus. Mapping of reads together with the subsequent peak analysis revealed several possible recognition sites of the dicer protein, which initiates the RNA silencing process in eukaryotic cells.

7.4. Fungus – plant interactions (IV)

The encoding gene of Rho GTPase previously annotated in *F. circinatum* (*Fcrho1*) was deleted either in Fc072 or in 011 fungal wildtype strains by using a modified version of OSCAR protocol. Transformation rate reached the 66.66% and 40% of analyzed colonies for Fc072 and 011, respectively. Deletion of *Fcrho1* did not promote lethal effect in *F. circinatum*. Nevertheless, suppression of the Rho GTPase encoding gene caused severe growth reduction in a generalist solid medium, whereas the conidiation and the germination of spores in a nutritive liquid medium did not vary after transformation. The hyphal structure was also explored and was found to be similar in wildtype, ectopic and mutant strains. In addition, an inoculation assay was performed with *P. radiata* seedlings to evaluate the *in vivo* virulence. The results showed similar severity of symptoms among fungal strains, and the deletion mutants were able to colonize and even kill the pine seedlings.

7.5. Virus – fungus – plant interactions (V)

The effect of viral infection by FcMV1 and FcMV2-2 was analyzed both *in vitro* and *in vivo* in fourteen monosporic cultures of *F. circinatum* (free of viruses, single infected and co-infected) using homogenic strains for pairwise comparisons. Extracellular laccase activity was rather high in the fourteen monosporic strains. Nevertheless, enzymatic production was highly variable among fungal strains and did not vary in relation to mycovirus infection. In addition, the correlation between laccase production and severity of symptoms in infected *P. radiata* seedlings was almost significant. Statistically significant variation between isolates was observed for *in vivo* virulence in contrast to *in vitro* mycelial growth, which was rather homogeneous across strains. Fungal viruses did not promote any changes in mycelial growth in a specific solid medium containing tannic acid. Otherwise, single infection of the studied mycoviruses produced an increase in the fungal virulence, although only FcMV1 significantly increased the aggressiveness of the host. Co-infection with two mycoviruses did not significantly affect the virulence of the host relative to virus-free stains and double infection was therefore considered cryptic. Survival analysis indicated that survival of pine seedlings inoculated with FcMV1-infected strains was significantly lower than that of the same fungal strain free of viruses.

7.6. Fungus – insect – plant interactions (VI)

The mycobiota of two species of bark beetles (*i.e. T. piniperda* and *T. destruens*) inhabiting pine stands either infected or potentially threatened by *F. circinatum* were studied. The fungal culturable community was formed by thirteen operational taxonomic units (OTUs) defined in relation to micro- and macroscopic morphological features. These OTUs yielded eleven taxa according to minisatellite fingerprinting and ITS amplification by PCR followed by Sanger sequencing. The observed and expected taxonomic richness (six different non-parametric estimators computed) were calculated, and also the sample-based rarefaction curves were computed, showing that a greater sampling effort led to the identification of more taxa. Fungal diversity was moderate, while the evenness was low due to the observed intense dominance by the saprophytic / pathogenic fungus *S. polyspora*. Despite the differences in pine host species, the similarity between plots was moderate and consistent with the observed dominance of a few taxa. Among the members of the mycobiota, the more represented fungal guilds were the endophytes, epiphytes, saprophytes, facultative entomopathogens and plant pathogens. *F. circinatum* was neither phoretically associated with *T. piniperda* nor with *T. destruens*, even though these insects were collected in stands severely damaged by PPC. In the community sampled, only random associations between fungal taxa were found, while the ecological network under study showed a high level of connectance between levels. In addition, a new index termed PI was designed to measure and enable comparison of the intensity of each fungus-insect association. This study highlighted the co-occurrence of *T. piniperda* and *T. destruens* in *P. radiata* stands infected with PPC in northern Spain.

8. General discussion

Plant pathologies have been traditionally studied from the point of view of bidirectional pathogen-host interactions. However, novel approaches are required to better understand the complex associations between participants of a single pathosystem. In this Thesis, a multiple-interaction approach was used to study molecular, cellular and ecological relationships in pine pitch canker disease, one of the most devastating forest pathologies worldwide. Thus, the interactions that take place between mycoviruses, phytopathogenic fungus, plant host, insect vectors *sensu lato* and their associated mycobiota were addressed in six scientific studies (**I-VI**).

Mycoviruses infect diverse clades of fungi inhabiting a wide variety of ecosystems. Interest in these agents has not decreased since their discovery more than 50 years ago. Since then, the classic view of the viruses as simple parasites has evolved, revealing complex host-mycovirus associations (Ahn & Lee, 2001; Márquez *et al.*, 2007). However, several aspects of the evolutionary relationship between fungi and mycoviruses as well as the physiological effects of viral infection in the host remain unclear. The successful use of Cryphonectria hypovirus 1 (CHV-1; *Hypoviridae*), which acts as an hypovirulence-inducer in *C. parasitica*, represents a practical case of mycoviruses being used as effective biocontrol agent in a forest pathology (*i.e.* chestnut blight) (Ding *et al.*, 2007). In other interactions between fungi and mycoviruses deleterious effects have been demonstrated in the hosts (Milgroom & Hillman, 2011), indicating that virology would provide suitable measures for forest and crop protection in the future. As we reviewed (**I**), most forest phytopathogens, ranging from invasive species that severely damage productive plantations to secondary pathogens of understory species, have been described as being infected by members of *Chrysoviridae, Hypoviridae, Megabirnaviridae, Narnaviridae, Partitiviridae, Reoviridae* and *Totiviridae*. Nevertheless, the use of mycoviruses to control plant diseases is mainly limited by the need for exhaustive characterization of symptomatology caused by mycovirus infection. Field trials are also highly desirable in order to evaluate biological control perspectives and viability of mycoviruses-based treatments in the long term (Liu *et al.*, 2002; Yaegashi *et al.*, 2013a).

The transfection of hypovirulence-inducer mycoviruses to non-native pathogenic fungi is attracting interest as a future means of virocontrol. Lee *et al.* (2011) successfully transmitted Fusarium graminearum virus 1-DK21 (FgV1-DK21; unassigned) to three species of the genus *Fusarium* as well as to *C. parasitica* via protoplast fusion. The study findings showed negative effects of viral infection in all artificially infected fungal species. Thus, *in planta* virulence was

significantly reduced not only in *Fusarium* spp. but also in *C. parasitica*, in which the severity of symptoms was lower than in infection by CHV-1. Regarding natural interspecific transmission of mycoviruses, recent research showed that the same viral strains can occasionally be hosted by unrelated fungi in field conditions (*i.e.* viral prevalence < 3% of the fungal material analysed) (Vainio *et al.*, 2017). Replication of mycoviruses in plant cells has also been reported (Nerva *et al.*, 2017) as well as cross-infection of plant virus between the plant host and pathogenic fungi (Andika *et al.*, 2017). These reports of host jumping reveal the complexity of the association between viruses and organisms as taxonomically distant as fungi and plants belonging to a single ecosystem.

On a molecular scale, eukaryotic cells possess a well-conserved pathway called RNA silencing to protect themselves against selfish RNA, including viruses (Figure 5). When viral RNA infects the cell, dicer or dicer-like protein (ribonuclease III-like enzyme) cleaves undesired dsRNA in virus-derived small RNAs (vsRNAs) of around 21-25 nt in length. The vsRNAs are processed by a complex of proteins called RISC (RNA-induced silencing complex) for identifying and subsequently degrading cognate sequences (*i.e.* viral particles), leading to an anti-viral response (Schumann *et al.*, 2010). This molecular route has important implications in the use of mycoviruses for biocontrol purposes as it prevents viral replication inside the cell. Interestingly, the reduction in RNA silencing activity caused by mycoviruses as self-defence has been reported in *C. parasitica* (Nuss, 2011) and *R. necatrix* (Yaegashi *et al.*, 2013b). The study performed in **III** is, as far as we know, the first to focus on the RNA silencing process in *F. circinatum*. Using high throughput sequencing of small RNA, we confirmed infection caused by the mitoviruses FcMv1, FcMv2-1 and FcMv2-2 in ten isolates of PPC fungus from different Spanish regions. *De novo* assembling provided contigs that covered up to 50% of each viral genome, including the lacking 3´ extreme of FcMv2-2, which had not been previously sequenced. Sequencing analysis revealed multiple plausible dicer recognition sites along viral genomes which, together with the distribution size of vsRNA, indicated that *F. circinatum* has a similar anti-viral response to that previously reported in other fungi (Dang *et al.*, 2011). *Mitovirus* spp. are thought to replicate inside the mitochondria since they show high abundance of UGA codons in their genomes (*i.e.* FcMv1 and FcMv2-1 according to Nibert (2017)). The UGA triplet is translated as stop codon in standard genetic code and as a tryptophan codon in the genetic code of mitochondrion (Nibert, 2017), which suggests therefore a low presence and replication rate of these viruses in cytoplasm in which RNA silencing mainly occurs. These considerations may explain the low abundance of recovered viral sequences (less than 1% of complete dataset). Nevertheless, the existence of vsRNA demonstrated that the three mitoviruses are effectively processed by the RNA silencing machinery of the PPC fungus. These findings suggest that viruses may at least occasionally occur in cytosol where the dicer

protein is able to detect and cleave them. Alternatively, it is also possible that signalling processes in the organelle host (McBride *et al*., 2006; Moore & Ting, 2008) may participate in the anti-viral response.
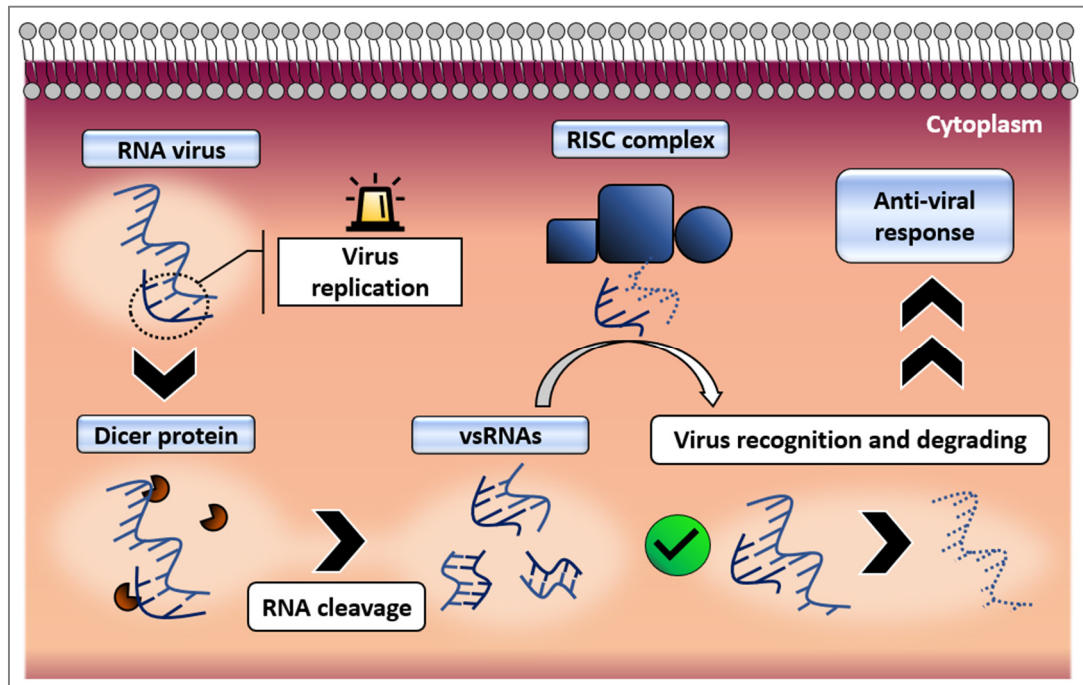

Figure 5. Scheme of RNA silencing process in fungal cell infected by RNA viruses.

Viral infection has a wide range of phenotypical effects in fungi. Some mycoviruses cause a cryptic effect, while others promote changes in colonial morphology and a few reduce any virulence traits such as mycelial growth or enzymatic activity (Pearson *et al*., 2009). Characterization of the effects caused by the single and double infection of FcMV1 and FcMV2-2 was another aim of this doctoral research (**V**). The study findings showed that neither mycelial growth in Bavendamm medium nor extracellular laccase production were affected by viral infection in seven homogenic isolates of *F. circinatum*. These results contrast with those found in other pathosystems in which the presence of mycoviruses strongly altered colony growth (Wang *et al*., 2014) and enzyme production (Potgieter *et al*., 2013). On the other hand, the bioassay performed with *P. radiata* seedlings revealed a significant variation in virulence both among isolates and in relation to mycovirus infection. Accordingly, fungal isolates infected by mycoviruses showed stronger virulence than the corresponding virus-free strains, although only FcMV1 increased the aggressiveness to a significant level. The survival analysis performed in parallel supported these results showing earlier death in seedlings inoculated with virus-infected fungi. In a later study, Flores-Pacheco *et al.* (2017) observed slower growth in mycovirus-infected strains of *F. circinatum* cultured in generalist solid medium (*i.e.* PDA). By contrast, our study (**V**) provided data of fungi growing in a specific medium that simulated

conditions closer to lignified tissues. Changes in culture conditions have been reported to promote different phenotypic responses in fungi infected by mycoviruses (Bryner & Rigling, 2011; Hyder *et al.*, 2013), which may explain the observed discrepancies. Flores-Pacheco *et al.* (2017) also reported the appearance of more sporal germlings in virus-free strains of *F. circinatum*. Notably, this character varied greatly between isolates in that study (*i.e.* sporulation was 5-27 times higher in a single isolate than in the other fungal strains) and may mask phenological patterns attributed to mycovirus infection. This phenomenon, together with sensitivity limitations in viral diagnosis (Papin *et al.*, 2004), demands careful interpretation of the patterns observed in the host during infection by mycoviruses.

The association between mycoviruses and invasive pathogens in non-native areas can reveal data about the origin of the pathogen and the story of introductions. In a recent study, Schoebel *et al.* (2017) observed a high prevalence of two lineages of Hymenoscyphus fraxineus mitovirus 1 infecting the causal agent of ash dieback throughout Europe. Analysis of genetic diversity and viral population structure supported the idea of the introduction of two individuals of *H. fraxineus* from Japan, where viral prevalence was rather low. FcMV1, FcMV2-1 and FcMV2-2 appear to be quite frequent in Spanish isolates of *F. circinatum* either in single infections or co-infections, regardless of the source of isolation (*i.e.* plant material or carrier insects) (Vainio *et al.* (2015b); **III**). Furthermore, a mitovirus-like contig numbered 571 was also present in 40% of studied isolates (**III**) being required further research to clarify whether this may be a fragment of a new virus infecting the phytopathogen. Viral abundance has scarcely been investigated in other countries affected by PPC, although Vainio *et al.* (2015b) analyzed 29 South African isolates all of which were free of viruses. *F. circinatum* was introduced in South Africa from Mexico (Wikler & Gordon, 2000) while it presumably arrived in Spain from the USA and later spread to France and Portugal (Berbegal *et al.*, 2013). A founder effect may explain the high prevalence of mitoviruses in Spain, where *F. circinatum* is thought to have been introduced in two independent episodes (Berbegal *et al.*, 2013). Viral prevalence should thus be studied in the native range of the pathogen as well as in France and Portugal in order to reconstruct the spread of PPC in Spain and neighbouring countries.

The study of viral ecology has proved the advantageous role of viruses in several systems in nature, for example by favouring drought resistance in plants (Xu *et al.*, 2008), conferring thermal-tolerance to endophytes (Márquez *et al.*, 2007) and promoting virulence in phylamentous fungi (Ahn & Lee, 2001). The association between viruses and their host are highly dynamic and can vary from detrimental to beneficial depending on environmental conditions (Hily *et al.*, 2016; Roossinck, 2015b). Our data suggest that infection caused by FcMV1, FcMV2-1 and FcMV2-2 is mainly asymptomatic in *F. circinatum* (**V**), which expends

resources in mounting a protective anti-viral response (**III**). Thus, the major cryptic effect of infection discourages the use of these mitoviruses for biocontrol purposes. However, FcMV1 and FcMV2-2 may become mutualistic under certain conditions; *e.g.* by slightly increasing *in planta* virulence (**V**). This possible beneficial effect could partly compensate in net value the inherent cost to be infected by viruses in cellular terms (Vainio *et al.*, 2018). Considering all data presented through this Thesis, we support the hypothesis that infection of FcMV1, FcMV2-1 and FcMV2-2 in *F. circinatum* represents an example where the relationship between host and virus is mostly defined as commensalism (or not severe parasitism) that would become mutualism when conditions were favorable (*i.e.* mutualism with conditional outcomes *sensu* Bronstein (1994)) (Figure 6).
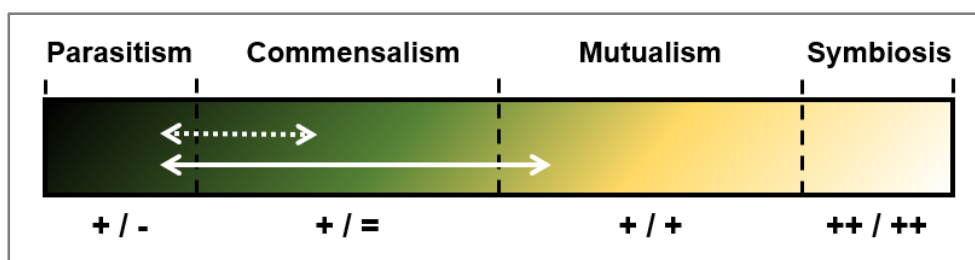

Figure 6. Theoretical representation of the mitoviruses-fungus interaction in PPC. Dashed white line: interaction at cellular level. Solid white line: interaction at colony level. Note that symbols ("+", "-" and "=") correspond to the result of each association for viruses (left symbols) and fungus (right symbols). Design based on Roossinck (2015b).

New generation sequencing technologies and bioinformatics are rapidly improving the knowledge of plant pathogens at genomic level. The complete genome of *F. circinatum* has been sequenced and made available in the public database of the NCBI (Wingfield *et al.*, 2012); nevertheless, the annotation is incipient. Scientific literature has broadly addressed the symptomatology caused by the PPC fungus during host infection (*e.g.* Correll *et al.*, 1991; Martín-García *et al.*, 2017; Roux *et al.*, 2007). By contrast, the genetic mechanisms that trigger virulence factors in this phytopathogen are just beginning to be studied. The results presented in **II** report the *in silico* annotation of five genes of *F. circinatum* putatively related to pathogenicity. The proposed annotation was based on the close homology of sequences at nucleotide and protein levels with previously described genes in other *Fusarium* species. In addition, gene ontology was also described as being consistent with the expected function of each proposed gene.

The mitogen-activated protein kinase (MAPK) cascade is one of the best studied signalling pathways in eukaryotes. This route enables cells to respond correctly to external stimuli such

as nutrient availability, presence of host metabolites or toxins, among others. The heteromeric G protein subunits are key elements upstream of either MAPK or cAMP pathways (Figure 7). One of well-known function of these proteins is their participation to elicit cellular response to pheromones, thus regulating fungal mating. The virulent response of pathogenic fungi are also related to the signalling triggered by these subunits (Deka *et al*., 2016). In the genus *Fusarium*, the genes that encode α and β subunits of G heteromeric proteins (*i.e.* homologous genes to respectively *Fcfga1* and *Fcfgb1* proposed in *F. circinatum*) (**II**) are of particular importance in fungal infection as they regulate multiple physiological traits such as vegetative growth and conidiation. In *F. oxysporum,* Jain *et al*. (2002) reported that disruption of the G_α encoding gene results in lower spore production, altered colony morphology and higher resistance to heat shock. These authors also reported significant *in planta* virulence reduction in gene-lacking mutants. Delgado-Jarana *et al*. (2005) also investigated the contribution of G_β subunit in the pathogenicity of *F. oxysporum* and found severe alteration in hyphae structure and lower virulence after suppression of the corresponding encoding gene. These findings indicate the possible involvement of annotated genes *Fcfga1* and *Fcfgb1* (**II**) in the delivery of virulence factors in the PPC fungus.
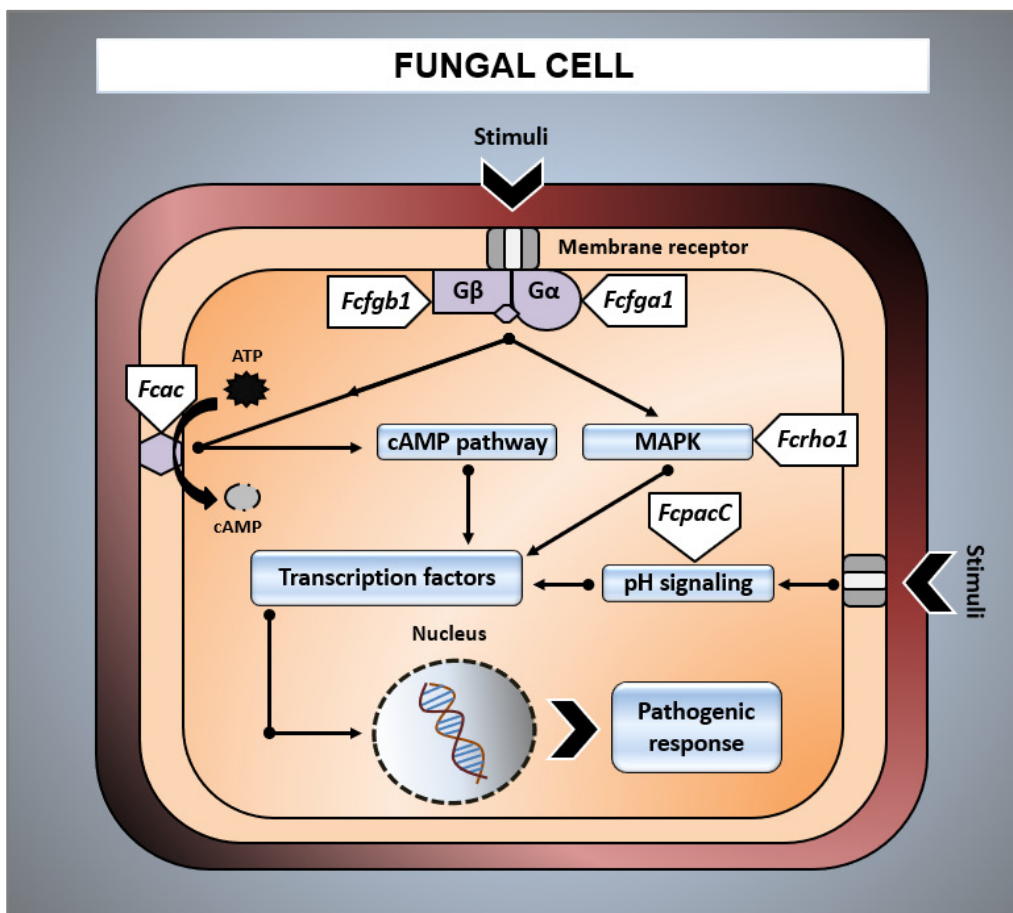

Figure 7. Scheme of metabolic pathways in which the genes *in silico* annotated (white labels) in the Original Article II are thought to be involved.

The participation of putative Rho-type GTPase encoding gene *Fcrho1* was studied in **IV**. The members of this family of small proteins participate as effectors in MAPK signalling pathway by regulating cell wall integrity and growth polarity, among other physiological functions (Levin, 2005). In this respect, our results proved that *Fcrho1* is negligible for life in the PPC fungus as deletion mutants were able to grow, sporulate or colonize living hosts. By contrast, deletion of homologous genes proved lethal to other filamentous fungi (Zhang *et al*., 2013). In *F. circinatum*, suppression of *Fcrho1* severely reduced the growth rate in a generalist solid medium, which is consistent with the data reported in *rho1*-defective strains of *F. oxysporum* (Martínez-Rocha *et al*., 2008). The exploratory examination of mycelium by light microscopy did not reveal notable changes in hyphae architecture, while mutant colonies differed macroscopically relative to the wildtypes. Hence, *Fcrho1* seems to act as an active regulator of hyphal tip elongation and aerial mycelium formation. On the other hand, deletion mutants showed similar virulence to the corresponding untransformed strains, which suggests that this Rho-type GTPase does not regulate pathogenicity *in planta*. Virulence may, therefore, be driven by the participation of multiple genes as suggested for other *Fusarium* species (Guo *et al*., 2016; Zhang *et al*., 2013) as well as by factors related to host physiology (see below).

The cAMP-dependent protein kinase pathway (also termed PKA) has been reported to be an important virulence-related route in some filamentous fungi by regulating hyphal growth, sexual differentiation and the formation of infective structures (Mehrabi *et al*., 2009). This metabolic route acts as a secondary messenger in cell signalling and the deletion of adenylyl cyclase proteins (proposed function of *Fcac* candidate gene; **II**) caused deleterious effects in the conidial formation and production in *Neurospora crassa* Shear & B.O. Dodge (Ivey *et al*., 2002). Furthermore, suppression of a homologous gene negatively affected spore germination and differentiation of infective structures in *Colletotrichum lagenarium* (Pass.) Ellis & Halst. (Yamauchi *et al*., 2004). *F. proliferatum* mutants with disrupted *Fpacy1* gene also showed reduced virulence *in planta*, and tolerance to chemical stress was significantly affected (Kohut *et al*., 2010). Otherwise, pH is a major factor during fungal infection, even driving the production of most suitable metabolites for host colonization (Prusky & Yakoby, 2003). Variations in acidity are thus recognized by membrane receptors and comprise a specific signalling pathway that promotes the corresponding gene expression or silencing (Peñalva *et al*., 2008). Caracuel *et al*. (2003) suggested that the plausible homologous gene of *FcpacC* (**II**) acts as a negative regulator of *F. oxysporum* virulence when conditions are alkaline. In addition, when the environment becomes acidic (more similar than conditions surrounding the host tissues), this gene triggers virulence factors, indicating its importance in fungal virulence. Knowledge about gene functions and the fact that the five proposed genes seem to regulate the expression of virulence factors together support inclusion of these genes in the virulence-associated class

proposed by Wassenaar and Gaastra (2001). The annotation of *Fcfga1*, *Fcfgb1*, *Fcac* and *FcpacC* provides a starting point for the definitive functional characterization of these genes in the PPC fungus. On the other hand, *in vitro* and *in vivo* analyses of *Fcrho1* provided an initial characterization of biological function for this gene in *F. circinatum*.

The ecological behavior of *F. circinatum* has been challenged in recent years. The proven necrotroph role in conifers is currently under discussion as a general profile, as this fungus acts as an endophyte or hemi-biotroph in herbaceous hosts (Swett *et al.*, 2014; Swett & Gordon, 2015) and as a possible saprotroph in forest litter (Serrano *et al.*, 2017). The lack of specialized mycelial structures for damaging host tissues indicates that fungal invasion should be mediated by segregation of cell-degrading enzymes, at least during intercellular colonization, as reported in other species of *Fusarium* (*e.g.* Kikot *et al.* 2009). There is not much information about the production of metabolites by the PPC fungus infecting the tree (*i.e.* mycotoxins and cell-wall degrading enzymes) and, to our knowledge, the study showed in **V** is the first report of extracellular laccase activity in *F. circinatum*. These multicopper oxidases degrade a wide range of phenolic compounds, including lignin, and are usually segregated by phytopathogenic and saprophytic fungi (Baldrian, 2006; Binz & Canevascini, 1996; Criquet *et al.*, 2004). Our results showed intense extracellular laccase activity with an almost significant relationship with the virulence of the PPC fungus infecting *P. radiata* seedlings (**V**). In this regard, Kuo *et al.* (2015) observed overexpression in laccase-encoding genes during initial stages of *H. annosum* infection in *P. sylvestris.* The early infection of *F. circinatum* mainly consisted of intercellular development, concluding with colonization of surrounding areas of cortical parenchyma and xylem resin ducts (Martín-Rodrigues *et al.*, 2013). Hence, it is expected that during the initial colonization stage, the PPC fungus expends resources in segregating cell-wall degrading enzymes such as laccases that allow access to the host´s nutrient reserves. Extracellular laccases can also play a role in fungal protection against defensive metabolites by detoxifying harmful compounds. Molina *et al.* (2008) found a reduction in abietic acid concentration of 95% in the presence of laccases after two hours of treatment. This resin acid has an effective antifungal effect (Kopper *et al*., 2005), comprising a main component in rosin fraction of oleoresin in conifers (Himejima *et al*., 1992; Rezzi *et al*., 2005) including *P. radiata* (Andrews *et al*., 2014; Otto *et al*., 2007). Taking all these data into account, we support the hypothesis of laccases as complementary elements in PPC infection by participating in the degradation of lignified wall of tissues such as xylem and detoxifying defensive compounds triggered by the host as an induced response.

The asexual cycle is considered the predominant reproductive pathway for the PPC fungus in nature (Gordon *et al.*, 2006a), which sporulates over the complete annual cycle (Dvořák *et al*.,

2017). Asexual spores (micro- and macroconidia) carried by wind, rainfall, contaminated pruning tools and vector insects thus represent the main routes of transmission between infected stands and healthy hosts. Spores act as key elements during host colonization; *e.g.* the spores and germlings of *M. grisea* segregate mucilaginous compounds that promote their adhesion on plant, thus facilitating the effective appressorium formation (Hamer *et al.*, 1988; Koga & Nakayachi, 2004). *Phytophthora* sp. produces a wide range of sexual and asexual spores which are guided by chemotaxis and other extracellular stimuli to target the surface of the host (Judelson & Blanco, 2005). The participation of spores during infection has not been studied in *F. circinatum*, although the small size (≤ 10 µm and ≤ 25 µm respectively for microconidia and macroconida, according to Leslie and Summerell (2006)) presumably facilitates transport of the species through water flow, favouring adhesion to and germination in undamaged tissues. Our results also showed that suppression of *Fcrho1* candidate gene did not affect the capacity of *F. circinatum* to sporulate in nutritive liquid medium (**IV**). The observed virulence of mutant strains infecting *P. radiata* seedlings therefore suggests that conidiation may be an important factor in the development of PPC disease cycle. On the other hand, structured vegetative growth is required for effective plant infection in some filamentous fungi, and it is particularly required for the proper formation of infective structures (Chen *et al.*, 2008; Zheng *et al.*, 2009). Enzymatic production appears to be essential during early infection of tree by *F. circinatum* (see above). Nevertheless, the correct formation of mycelium is expected to be a key factor specially in later infection when the xylem collapses because of fungal invasion. By contrast, mutant strains with defective *in vitro* growth caused severe symptoms in pine seedlings (**IV**) suggesting that normal development of hyphae could be recover during plant infection because of specific signalling stimuli. Alternatively, slower growth observed *in vitro* could imply an affordable cost that does not reduce infection fitness in global terms. Further research is required in order to clarify whether these physiological aspects determinate the infection procedure in the PPC causal agent.

The adaptation of pathogens to local hosts, a common phenomenon in nature (Kaltz & Shykoff, 1998), may explain the resistance reported in some *Pinus* spp. (*i.e.* subsection *Oocarpae*) cohabiting with the PPC fungus through their native range (Hodge & Dvorak, 2000). In this regard, natural *P. radiata* forests seem to suffer moderate damage by the disease, whereas plantations outside the native range are frequently affected by epidemic episodes (Gordon *et al.*, 2015). The latter scenario is frequent in northern Spain (**VI**), corresponding to a pattern of encounter between susceptible host and co-evolved pathogen in an exotic environment (Wingfield *et al.*, 2015). In Spain, this co-occurrence between pathogen and host takes place in forest plantations which implies the participation of forest management as a possible predisposing factor, by for instance reducing genetic diversity in plantations (Gordon *et al.*,

2015). All these new environmental conditions are expected to upset the plant-fungus interaction by benefiting the pathogen to the disadvantage of the host or *vice versa* (Parker & Gilbert, 2004). Furthermore, this process will be influenced by selective factors such as the presence / absence of specific nodes in the system (*e.g.* alternative hosts, antagonistic microorganisms or vector insects; see below).

Monterey pine is thus currently considered the most susceptible host of *F. circinatum* (Wingfield *et al.*, 2008). The resistance of *P. radiata* has been demonstrated to be an inheritable trait (Matheson *et al.*, 2006), and the expression profile of some response-regulator genes is lower in susceptible genotypes than in resistant ones (Donoso *et al.*, 2015). Regarding the physiological response to the disease, Martín-Rodrigues *et al.* (2013) proposed that abundant production of resin ducts in this pine species was not only ineffective in hindering advance of the fungus but that it could also facilitate vertical colonization. The high susceptibility in previously reported inoculation trials (Iturritxa *et al.*, 2013; Martínez-Álvarez *et al.*, 2014) was consistent with the severe symptomatology described here (**IV** and **V**). In addition, we found that even *F. circinatum* mutant strains with defective growth *in vitro* caused intense damage to pine seedlings. These observations, together with the possible infection strategy of the fungus (*i.e.* differential importance of enzymatic production and mycelial growth, as discussed above), led us to corroborate the idea of *P. radiata* as the main host in the PPC pathosystem in Spain, with this role probably being caused by the inefficient tree defence during stem colonization.

There are many ways for fungal pathogen spreading in the field such as water flow, wind or animals. The causal agent of pine pitch canker stablishes multiple associations with insects, which are classified as wounding agents, carriers or vectors depending on their capacity to carry fungal propagules or to effectively inoculate the pathogen in a healthy host (Brockerhoff *et al.*, 2016). We studied the participation of the pine shoot beetle in the PPC pathosystem in northern Spain by using a community ecology approach. Thus, *T. piniperda* was found to inhabit an asymptomatic mixed stand of *P. nigra* subsp. *salzmannii* and *P. sylvestris*, while this species co-occurred with *T. destruens* in *P. radiata* plantations severely damaged by *F. circinatum* (**VI**). Both of these insect species have been reported to inhabit *P. pinaster* stands (Gallego *et al.*, 2004), however this is, to our knowledge, the first citation of sympatry in Monterey pine plantations affected by PPC in Spain. The described phenology of *T. destruens* includes a breeding season during the autumn, although we found both species feeding in current shoots prior to stay inside during the hibernation period. This phenomenon suggests that Mediterranean pine shoot beetle undergoes a cycle similar to *T. piniperda* in Cantabrian

Mountains, as already mentioned by Lieutier *et al.* (2015) in colder areas through its geographical range.

Fungal pathogens form part of a community that represents an interesting node complex in the disease system. The mycobiota under study was characterized using several ecological indicators, including the phoresy index (PI) proposed in this Thesis (**VI**). The culturable community of the two studied *Tomicus* species was dominated by *S. polyspora*, a fungus that has been considered a saprophyte of litter as well as an endophyte of coniferous species (Lygis *et al.*, 2014; Pirttilä *et al.*, 2003). By contrast, it has been confirmed as a causal agent of current season needle necrosis (CSNN) in American and European fir trees (*Abies* spp.) (Talgø *et al.*, 2010). A recent study showed that *S. polyspora* can act as a pre-emergent pathogen of *Pinus ponderosa* Douglas ex Lawson & C. Lawson by inhibiting seedling germination (Ridout & Newcombe, 2018). One possible explanation for the observed abundance of this fungus is its frequent endophytic association with *Pinus* spp., as reported in earlier studies (Prihatini *et al.*, 2015; Sanz-Ros *et al.*, 2015). Alternatively, insects may be loaded with fungal propagules when shoots fall to the ground, where *S. polyspora* is expected to be abundant as a saprophyte (Haňáčkova *et al.*, 2015). *F. circinatum* has also been associated with forest litter (Serrano *et al.*, 2017), hence our findings therefore lead to conclude that *F. circinatum* and *S. polyspora* probably co-occur in the soil organic layer of infected plantations. Consequently, the synergetic effect of these two seedling pathogens deserves further research as it may interfere in the restoration of diseased woodland by reducing survival of offspring.

Other endophytic fungi are phoretically associated with both *Tomicus* species (*i.e. Pestalotiopsis* sp., *P. effusa* and *P. herbarum*) as well as saprophytes and epiphytes (*i.e. M. hiemalis* and *Penicillium* sp.) (**VI**). The presence of taxa broadly known as conifer endophytes suggests that these were probably transferred from plant tissues to the exoskeletons of insects while these were feeding on shoots. The case of *P. herbarum* requires special attention as it demonstrates diverse ecological behaviors. Thus, *P. herbarum* has been found associated with pines with high rates of secondary growth (Sanz-Ros *et al.*, 2015), whereas other research supports its potential use as a biocontrol agent against weeds (Stewart-Wade & Boland, 2004; Vikrant *et al.*, 2006). Interestingly, *P. herbarum* has proven able to degrade lignin as a unique source of carbon under laboratory conditions (Bi *et al.*, 2016). This intriguing ability may indicate an unexpected fungus-insect mutualism during trunk infestation and should be addressed in future studies. Although the ophiostomatoid fungi are usually carried by *Tomicus* spp., only *O. canum* was isolated in our study. This species is frequently associated with bark beetles (Romón *et al.*, 2014) and is of high economic importance because it reduces the value of timber acting as blue stain fungus (Solheim *et al.*, 2001). Otherwise, two taxa with

entomopathogenic potential were isolated (*i.e. Pestalotiopsis* sp. and *Cladosporium* sp.). *Pestalotiopsis* sp. has been reported as an entomopathogen in the forest pest *Hemiberlesia pitysophila* Takagi (Homoptera; Diaspididae) (Lv *et al.*, 2011), while the genus *Cladosporium* has been demonstrated to act as a pathogen of mites (Eken & Hayat, 2009).

The interaction between *T. piniperda* and *F. circinatum* in Spain fulfills the criteria described by Wingfield *et al.* (2016) for native-insect and exotic-microorganism associations. Accordingly, this type of relationship is expected to occur when the insect usually transports indigenous microorganisms (*e.g.* other filamentous fungi) and the pathogen is known to be carried by the insect congeners (*i.e.* American bark beetles). Morphological analysis and molecular clustering pointed that *F. circinatum* was not isolated from asymptomatic plantations or from infected *P. radiata* stands, although other species belonging to the same genus were recorded (*i.e. F. sporotrichioides* and *F. lateritium*, among others) (**VI**). Our data were consistent with those of a previous study in which phoretic association between PPC fungus and pine shoot beetle were infrequent (Romón *et al.*, 2008). Notably, Bezos *et al.* (2015) reported phoresy rates of around 4%, also demonstrating the role of *T. piniperda* as a plausible vector of *F. circinatum* according to Leach´s postulates (Leach, 1940). The results of our study confirmed compliance with the first postulate as we detected both *T. piniperda* and *T. destruens* in severely diseased pine plantations. We also provided supporting information about the second postulate, which refers to the contact between healthy host and the plausible vector species (*i.e. T. piniperda*) in conditions suitable for transmission. Both *P. nigra* and *P. sylvestris* have been found to be potential hosts of *F. circinatum*, showing moderate susceptibility to the disease (Iturritxa *et al.*, 2013; Martínez-Álvarez *et al.*, 2014). However, the apparent absence of the pathogen in sampled mycobiota did not rule out its possible latent presence in the asymptomatic area, as recently reported in *P. pinaster* and *P. radiata* stands (Hernández-Escribano *et al.*, 2018a; Swett *et al.*, 2018). The results showed here are consistent with those provided by Bezos *et al.* (2015) regarding *T. piniperda*, whereas the association between the pathogen and *T. destruens* has not previously been analyzed. Accordingly, our results confirmed that the latter species contacts with infected pines in the field, becoming a new possible member of the PPC pathosystem in northern Spain. Altogether, the results reported in **VI** contribute to a better understanding of how bark beetles participate in the pathosystem under study, by connecting different nodes (*i.e.* different pine species and a diverse fungal community formed by endophytes, saprophytes, epiphytes, facultative entomopathogens and plant pathogens) that generate a complex network of interactions.

9. Conclusions / Conclusiones

9.1. Conclusions

[1] Viruses that infect fungi (mycoviruses) are promising candidates for use in managing plant pathologies as some have deleterious effects on their hosts. Mycoviruses that cause a state of weakness (hypovirulence) in phytopathogens have proven useful for biocontrol purposes, with the mycovirus-based control of chestnut blight (causal agent *C. parasitica*) as the best studied case. Several pathogens of woody plants, such as *O. novo-ulmi*, *H. annosum*, *G. abietina*, *F. circinatum*, *B. dothidea*, *H. fraxineus*, *B. cinerea*, *V. dahliae*, *V. albo-atrum*, *D. pinea*, *D. scrobiculata*, *R. necatrix,* as well as *C. parasitica*, have been shown to be infected by members of the families *Chrysoviridae, Hypoviridae, Megabirnaviridae, Narnaviridae, Partitiviridae, Reoviridae* and *Totiviridae*. Nevertheless, some aspects of mycoviruses, such as their role in evolutionary relationships between viruses and fungi, viral transmission and mycovirus-host biological and ecological interactions, require further study to clarify their possible use as biocontrol agents in forest health.

[2] The genome of *F. circinatum* includes at least five genes putatively related to pathogenicity. Annotation of these candidate genes was based on high sequence homology with homologous genes previously described in other species of *Fusarium*. The biological functions obtained by the analysis of gene ontology for each encoding sequence were as follows: *Fcfga1* (G protein α subunit), *Fcfgb1* (G protein β subunit), *Fcac* (adenylate cyclase), *Fcrho1* (Rho-type GTPase) and *FcpacC* (pH transcription factor). These proteins are involved in cellular signalling networks such as the mitogen-activated protein kinase (MAPK) cascade as well as the cAMP-PK and pH transduction pathways. The cellular responses elicited by these routes include triggering of the virulence factors required by fungi to develop infection in their plant hosts.

[3] Study of small RNA reads from high throughput sequencing successfully detected viral infection caused by FcMV1, FcMV2-1 and FcMV2-2 in *F. circinatum*. Sequence analysis revealed that *F. circinatum* performs the RNA silencing pathway (RNAi) in a similar way to that described in other phytopathogenic fungi. The RNA silencing machinery positively targets the three viral strains previously identified as being hosted by PPC fungus, although they are thought to replicate inside mitochondria being infrequent in cytoplasm. It is expected that performance of anti-viral response supposes an energetic cost, hence this virus-fungus interaction could be considered as a kind of commensalism (reversible into parasitism) in cellular terms. Viral prevalence was moderate among fungal strains, which were isolated

either from plants or from bark beetles (Coleoptera, Scolytinae). Furthermore, the lacking 3´ extreme of FcMV2-2 was identified contributing to the complete sequencing of this viral genome. A plausible new viral strain was also detected, showing moderate homology with other members of the genus *Mitovirus*. This RNA particle deserves further study for definitive characterization.

[4] The previously annotated gene *Fcrho1* was deleted in wildtype strains of *F. circinatum*. This gene is negligible for life in PPC fungus. Nevertheless, *Fcrho1* regulates vegetative growth and is mainly involved in the formation of aerial mycelium and hyphal tip elongation. This Rho GTPase encoding gene does not affect the asexual cycle of the fungus. Otherwise, *Fcrho1* does not seem to regulate fungal virulence *in planta* which could be driven by pathogen-dependent (*i.e.* multiple participation of pathogenicity-related genes or physiological traits) and / or host-dependent factors (*i.e.* effectiveness of response during fungal infection). The successful use of a modified version of the OSCAR protocol in PPC fungus represents a promising tool for the genetic manipulation of *F. circinatum* and its application to the functional characterization of genes involved in fungal virulence.

[5] The production of extracellular laccase enzymes in *F. circinatum* was intense and rather variable among isolates. The role of these enzymes during host colonization seems to be particularly important during the first stages of the infection, when laccases may participate in the degradation of lignified tissues as well as in the detoxification of resin acids from the host´s induced response. Single infection and co-infection by FcMV1 and FcMV2-2 did not promote changes in either mycelial growth or in laccase activity under the culture conditions being considered cryptic. By contrast, presence of virus increased the virulence of *F. circinatum* in *P. radiata* seedlings and was significantly higher in single infection with FcMV1. These results suggest that, in terms of colony, the virus-fungus interactions range from commensalism to mutualism depending on the environmental conditions (*in vitro vs in vivo*).

[6] The bark beetle *T. piniperda* was found to inhabit asymptomatic mixed stands of *P. nigra* subsp. *salzmannii* and *P. sylvestris* as well as monospecific plantations of *P. radiata* that have been severely damaged by the PPC disease in northern Spain (Cantabria). In the latter locations, *T. piniperda* co-occurred with *T. destruens*; this represents the first report of this Mediterranean species in Monterey pine stands affected by PPC. Despite the demonstrated contact between diseased trees and both species of bark beetles, *F. circinatum* was not recorded as a member of the phoretic communities under study. However, the sampled mycobiota showed moderate values of taxonomic richness and diversity, and the taxonomic composition was rather similar between plots. The fungal community was formed

by fungi belonging to different guilds, including endophytes, epiphytes, saprophytes, phytopathogens and facultative entomopathogens. The sampled community was dominated by *S. polyspora* (Dothideomycetes). This species has been described as a saprophyte in litter, an endophyte in several plant tissues and also as a conifer pathogen able to infect either mature trees or seedlings at pre-emergence stage. Furthermore, a new indicator of the intensity of fungus-insect associations (termed PI) was designed to characterize the phoretic communities. The study of the mycobiota revealed a complex network of interactions between pines, bark beetles and filamentous fungi.

[7] Study of virus-fungus-insect interactions in PPC pathosystem revealed that the three strains of *Mitovirus* sp. previously identified as hosted by *F. circinatum* embody a relevant node in the disease system in northern Spain. Results of this doctoral research suggest that infection effects caused by these mitoviruses are not static and range in a continuum probably affected by host´s conditions. Future research focused on this virus-fungus interaction is required in order to better understand not only the biology of *F. circinatum* but also the viability of virocontrol of this forest pathology (*i.e.* using mycoviruses naturally infecting *F. circinatum* or other species able to cause hypovirulence in their host).

9.2. Conclusiones

[1] Los virus que infectan de forma natural a los hongos (micovirus) suponen una oportunidad para la patología vegetal puesto que algunos de ellos tienen la capacidad de provocar efectos deletéreos en sus hospedantes. Los micovirus capaces de provocar debilidad (hipovirulencia) en el hospedante han demostrado su potencial para ser empleados en el control biológico de patologías vegetales teniendo su mayor exponente en el control de la enfermedad del chancro del castaño (causada por *C. parasitica*). Así pues, numerosos hongos fitopatógenos presentes en ecosistemas forestales tales como *O. novo-ulmi*, *H. annosum*, *G. abietina*, *F. circinatum*, *B. dothidea*, *H. fraxineus*, *B. cinerea*, *V. dahliae*, *V. albo-atrum*, *D. pinea*, *D. scrobiculata*, *R. necatrix* además de *C. parasitica*, son hospedantes de micovirus pertenecientes a las familias *Chrysoviridae, Hypoviridae, Megabirnaviridae, Narnaviridae, Partitiviridae, Reoviridae* y *Totiviridae*. Sin embargo, se requiere un mayor conocimiento sobre la relación evolutiva micovirus-hongo, así como del proceso de infección, la transmisión de virus y las interacciones virus-hospedante (tanto biológicas como ecológicas). Dichos aspectos requieren de futuras investigaciones que permitan conocer la aplicabilidad de estos micovirus en el control biológico de patologías forestales.

[2] El genoma del hongo fitopatógeno *F. circinatum* incluye al menos cinco genes potencialmente relacionados con la virulencia. Los genes candidatos fueron anotados en base a la alta homología de secuencia con respecto a genes homólogos descritos en otras especies de *Fusarium*. Las funciones biológicas obtenidas mediante ontología génica para las secuencias codificantes descritas fueron: *Fcfga1* (subunidad α de proteína G), *Fcfgb1* (subunidad β de proteína G), *Fcac* (adenil ciclasa), *Fcrho1* (GTPasa tipo Rho) y *FcpacC* (factor de transcripción de pH). Las proteínas mencionadas forman parte de redes de señalización celular tales como la ruta de las MAP-quinasas, la vía de transducción de cAMP-PKA y la ruta de señalización del pH. Entre las respuestas celulares desencadenadas por estas rutas se encuentra el desarrollo de factores de virulencia requeridos por los hongos para acometer la infección de sus hospedantes vegetales.

[3] El estudio de moléculas de ARN de cadena corta obtenidas mediante secuenciación masiva resultó exitoso en el diagnóstico de la infección causada por los mitovirus FcMV1, FcMV2-1 y FcMV2-2 en *F. circinatum*. El análisis de secuencias reveló la existencia de una ruta de silenciamiento de ARN (ARN de interferencia) en *F. circinatum* similar a la descrita en otros hongos fitopatógenos. La maquinaria celular implicada en dicha respuesta antiviral procesa de forma efectiva las tres cepas víricas identificadas en *F. circinatum*, pese a que se considera que estos virus se replican principalmente en la

mitocondria del hospedante siendo infrecuentes en el citoplasma. Cabe esperar que el desarrollo de la respuesta antiviral suponga un coste en términos de recursos energéticos, por tanto, la interacción virus-hongo podría ser considerada como comensalismo (reversible en parasitismo) a nivel celular. La prevalencia vírica resultó moderada entre las cepas fúngicas analizadas, procediendo estas tanto de material vegetal como de insectos escolítidos (Coleoptera, Scolytinae). Además, se obtuvo el extremo 3´ del genoma de FcMV2-2 completando así la secuenciación del mismo. Por otra parte, se detectó una posible nueva molécula viral que mostró homología moderada respecto de otros miembros del género *Mitovirus* previamente identificados. Dicha partícula de ARN requiere de nuevos estudios que permitan su caracterización definitiva.

[4] El gen previamente anotado *Fcrho1* fue delecionado en cepas silvestres de *F. circinatum* resultando prescindible para la vida del hongo. No obstante, *Fcrho1* regula de forma efectiva el crecimiento vegetativo de *F. circinatum*, estando relacionado principalmente con la formación del micelio aéreo y la elongación de las hifas. Se encontró además que este gen codificante de una proteína tipo GTPasa Rho no está relacionado con el desarrollo del ciclo asexual del hongo. Por otra parte, el gen *Fcrho1* no parece ser promotor de la virulencia en planta de *F. circinatum*, la cual podría depender tanto de factores propios del patógeno (*i.e.* patogenicidad regida por múltiples genes o factores fisiológicos) como del hospedante (*i.e.* efectividad de la respuesta frente a la infección). La aplicación exitosa del protocolo OSCAR modificado en el patógeno plantea usos prometedores de esta herramienta en la edición genética de *F. circinatum*, así como en la caracterización funcional de genes relacionados con la patogenicidad del hongo.

[5] La producción de enzimas lacasas extracelulares resultó elevada en *F. circinatum,* aunque se mostró variable entre los distintos aislados del hongo. La participación de estos enzimas en la colonización del hospedante parece ser relevante durante las primeras fases de infección donde podrían participar en la degradación de tejidos lignificados, así como en la detoxificación de ácidos resínicos procedentes de la respuesta inducida del hospedante. Por otra parte, la presencia de los mitovirus FcMV1 y FcMV2-2 en infección sencilla o en coinfección no alteró el crecimiento del hongo ni la actividad de lacasas en las condiciones de cultivo empleadas por lo que se les considera como virus crípticos. Por el contrario, la presencia de estos mitovirus incrementó la virulencia del hongo durante la infección de plántulas de *P. radiata*, siendo este aumento significativo en el caso de infección sencilla por parte de FcMV1. Estos resultados sugieren una interacción dinámica entre el comensalismo y el mutualismo entre hongo y virus a nivel de colonia, que puede ser susceptible de variación en función de determinados factores ambientales (*in vitro vs in vivo*).

[6] El escolítido *T. piniperda* fue encontrado en el norte de España (Cantabria) colonizando tanto repoblaciones asintomáticas mixtas de *P. nigra* subsp. *salzmannii* y *P. sylvestris* como plantaciones monoespecíficas de *P. radiata* infectadas por *F. circinatum*. En estas últimas, se confirmó la presencia simpátrica de *T. piniperda* y de *T. destruens*, lo que supone la primera cita para *T. destruens*, de distribución mayoritariamente mediterránea, en plantaciones infectadas de pino insigne en el norte peninsular. Pese al contacto probado entre árboles afectados por la enfermedad y ambas especies de escolítidos, el patógeno *F. circinatum* no se aisló en la micobiota de los individuos recogidos. Por otra parte, las comunidades de hongos transportadas por ambas especies de insectos mostraron una riqueza de taxones y diversidad moderadas, siendo similar la composición taxonómica entre parcelas. La comunidad fúngica estuvo formada por hongos endófitos, epífitos, saprófitos, fitopatógenos y entomopatógenos facultativos. Por otra parte, la micobiota muestreada estuvo dominada en ambas parcelas por el hongo *S. polyspora* (Clase Dothideomycetes) que ha sido descrito como saprófito en la hojarasca y endófito en numerosos tejidos vegetales, así como patógeno en coníferas adultas y plántulas en estado de preemergencia. Adicionalmente, se diseñó un nuevo indicador de la intensidad de la asociación entre hongo e insecto denominado PI, que puede ser empleado en la caracterización de comunidades foréticas. El estudio de la comunidad fúngica evidenció una compleja red de interacciones entre diferentes especies de pinos, escolítidos y hongos filamentosos.

[7] El estudio de las interacciones virus-hongo-insecto en el patosistema del chancro resinoso de los pinos, evidenció que las tres cepas de *Mitovirus* sp. previamente identificadas en el hongo *F. circinatum*, suponen un nodo de gran relevancia en el mencionado sistema en el norte de España. Los resultados de este trabajo sugieren que los efectos de la infección por parte de los mitovirus estudiados no son estáticos y varían en un continuo probablemente influido por las condiciones del hospedante. Se requiere de nuevos estudios centrados en la caracterización definitiva de esta interacción virus-hongo a fin de conocer en mayor profundidad no solo la biología de *F. circinatum*, sino también, la viabilidad del control biológico de éste patógeno forestal mediante el uso de micovirus (tanto hospedados de forma natural por el hongo, como otras especies que pudieran ser promotoras de hipovirulencia).

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► ii All references cited in the Introduction and Discussion are listed in this section. Specific reference lists are provided in the corresponding Original Articles.

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11. Appendices

11.1. Quick reference of variables

Table Ap1. Main abbreviations used throughout the Thesis. Abbreviations for statistical estimators are not included. Roman numbers denote Original Articles (I-VI).

Abbreviation	Variable	Original article
A_c	Area covered by fungal colony after 7 days of culturing	IV
AUDPC	Area under disease progress curve	IV & V
C	Spore density after 48 h of culturing	IV
Chao1, Chao2	Taxonomical richness (estimated with Chao 1 and Chao 2 estimators, respectively)	VI
Cn_x, Cn_y	Connectance between levels of a network	VI
D	Simpson diversity	VI
ΔA_{0-5}	Increase in absorbance as an absolute value for the 0-5 minutes measurement interval	V
E	Simpson evenness	VI
F_{exp}	Expected number of sites where a pair of taxa co-occurred	VI
F_{obs}	Observed number of sites where a pair of taxa co-occurred	VI
G	Mean diary mycelial growth	IV & V
H	Shannon diversity	VI
I	Sorensen Index	VI
ICE	Taxonomical richness (estimated with the Incidence-based Coverage estimator)	VI
ID	Similarity between sequences	II, III & VI
J	Shannon evenness	VI
Jack1, Jack2	Taxonomical richness (estimated with Jacknife 1 and Jacknife 2 estimators, respectively)	VI
L_x, L_y	Mean number of links per taxa between levels of a network	VI
MAT	Mating type	I, IV & V
N	Sporal germination rate	IV
nt	Nucleotides	I, II & III
OTU	Operational taxonomic unit	VI
P_{gt}, P_{lt}	Co-occurrence probabilities	VI
PI	Phoresy index	VI
p_i	Relative abundance of each taxa	VI
Qc	Query coverage	II, III & VI
S	Mean area affected by enzymatic reaction	V
SE	Standard error	III, IV, V & VI
S_{obs}	Taxonomical richness (observed)	VI
vc	Vegetal compatibility group	I

11.2. List of tables and figures

11.2.1. List of tables

11.2.1.1. Original Article I

► Table 1. Summary of mycoviruses of agroforestry interest. [pp. 44-45]

11.2.1.2 Original Article II

► Table 1. GenBank best matches for five candidate genes related with the pathogenicity of *F. circinatum*. QC: Query cover; E: E-value; ID: Similarity between sequences; Coord.: Number of first nucleotide position in the 5´/3´ extreme. nt: nucleotides. [pp. 72-73]

► Table 2. Best matches of gene ontology (based on GO database) for five candidate genes of *F. circinatum*. [pp. 74]

11.2.1.3. Original Article III

► Table 1. Isolates of *Fusarium circinatum* used in this study: geographical origin of the strains and the living organisms from which *F. circinatum* was isolated (source) are provided. The viral infection by Fusarium circinatum mitovirus 1, 2-1, 2-2 (FcMV1, FcMV2-1 and FcMV2-2, respectively), contig 50 and contig 571 (model 310) are shown by the symbol +. [pp. 87]

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► Table 3. Best matches of contig 50 and contig 571 (Model 310) according to BLAST searches against the GenBank database. L, length of each contig expressed as number of nucleotides; Database, nr (non-redundant protein sequences) and nr/nt (nucleotide collection); QC, Query coverage; E, E-value; ID, Identity. [pp. 91]

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► A1. Modelling parameters and results of models computed for mycoviruses identification. Only models that provided any contig are shown. Best models in bold. [pp. 100]

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11.2.2.2. Original Article I

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► A2. Sequence alignment showing the last 182 nucleotides of FcMV2-2 from isolate FcCa070 (GenBank accession number KF803548), a previously unpublished cloned genome segment from strain FcCa070 (070_M13F), and contig 50 (NODE_50) obtained via small RNA deep sequencing during this study. [pp. 101]

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► Figure 1. Colonies of *F. circinatum* growing in PDA after one week of culture. [pp. 113]

► Figure 2. Hyphae of fungal strains 40 days after culture. A: 011 wildtype; B: 011ect5; C: Δ*Fcrho1*011; D: Fc072 wildtype; E: Fc072ect2; F: Δ*Fcrho1*Fc072. Image processing: scaling to common size and +20% brightness. [pp. 116]

► Figure 3. Area under the disease progress curve (AUDPC) of different fungal strains 30 dpi. Small letters (a-b) denote significant differences (Dunn´s test, $P < 0.05$). Comparisons between fungal strains are indicated by colour of plot. Mean values and standard error are shown. [pp. 116]

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► Figure 1. Scheme of the study. A: Bavendamm test progress at four different moments: 24 h (a), 48 h (b), 72 h (c) and 96 h (d) after culture (isolate shown: Fc072). B: Control *Pinus radiata* seedlings on the 13th day of pathogenicity test. C: *Pinus radiata* seedlings inoculated with Fc072v (foreground) and Fc072 (background) on the 13th day of pathogenicity test. D: Detail of resin surrounding the point of inoculation. E: Detail of dead seedling showing the symptomatology of pine pitch canker damping-off. [pp. 131]

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► Figure 3. Extracellular laccase activity (ΔA_{0-5}) in the different isolates. Small letters (a–c) denote significant differences (Dunn´s test, $P < 0.05$). (Ø): virus-free isolates. Comparisons between pairs of isolates are indicated by colour of plot and roman numbers (I-VII). Median values and standard error are shown. [pp. 133]

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► Figure 1. Taxonomic accumulation curves of fungal taxa carried by the two studied *Tomicus* species (shaded areas: 95% confidence intervals). Taxonomic richness computed as expected number of taxa (Mao-Tau estimator). [pp. 154]

► Figure 2. Tripartite graph of sampled mycobiota. Upper level: hosts (*Pinus* sppp.); middle level: carrier insects (*Tomicus destruens* and *Tomicus piniperda*); lower level: phoretic fungal taxa. Rectangle width is proportional to the sum of interactions involving each taxon. Fungal classes are represented by colours in lower level boxes: white (Sordariomycetes), black (Dothideomycetes), light grey (Lecanoromycetes) and dark grey (Eurotiomycetes). Cn_x and Cn_y show the connectance for each network level. L_x and L_y show the mean number of links per taxa among levels. [pp. 158]

► Figure A1. Neighbor-joining dendrogram of ITS showing *Fusarium* spp. clusters. The percentages of replicate trees in which the associated taxa clustered together in the Bootstrap method (1000 replications) are shown next to the branches. This dendrogram is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the dendrogram. Evolutionary distances were computed using the Kimura2 parameter method and are in the units of the number of base substitutions per site. The analysis involved 33 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 435 positions in the final dataset. Black triangles represent sequences of OTUs corresponding to *Fusarium* samples isolated in this study. GenBank accession numbers of each reference sample are provided. [pp. 170]