Universidad de Valladolid

Instituto Universitario de Investigación iFOR GESTIÓN FORESTAL SOSTENIBLE

UVa iap Escuela Técnica Superior de Ingenierías Agrarias Palencia

DOCTORAL DISSERTATION / TESIS DOCTORAL

Environmentally friendly methods for the integrated management of pine pitch canker (PPC) disease

Métodos ambientalmente respetuosos para el manejo integrado de la enfermedad del chancro resinoso de los pinos

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Agradecimientos

Es una obviedad que una tesis doctoral no la hace una sola persona. Aun así, me parece adecuado recordarlo. Todo lo que aparece en este documento ha sido revisado por mi director de tesis Julio Diez, y como no puede ser de otro modo, se nota la mano de la experiencia tras casi la decena de tesis dirigidas. Aun así, no es este motivo por el cual le estoy más agradecido. Le agradezco aun más haber confiado y creído en mi más que yo mismo, y desde antes incluso que tomara la decisión de realizar el doctorado. Igualmente agradezco el apoyo profesional de Jarkko Hantula y Eeva Vainio, mis tutores durante la estancia de investigación en Finlandia. Gracias a ellos, esos tres meses en el METLA fueron muy productivos y se obtuvieron grandes resultados. Tampoco puedo olvidarme de los coautores de los artículos que presento en este documento. Todos ellos han realizado grandes aportes a la investigación y esta tesis no sería la misma sin su participación. No me olvido tampoco de Milagros de Vallejo y de Juan Blanco por su apoyo logístico en la instalación de los experimentos de campo, y sobre todo por creer en la investigación aunque en ocasiones se encuentre bastante distanciada de la gestión forestal.

Tal vez de lo que me siento más afortunado durante estos años de investigación predoctoral sea del buen ambiente de trabajo existente en el laboratorio. Parte del mérito lo merece de nuevo Julio y su magnifico carácter, pero son sobre todo mis compañeros de laboratorio y oficina los culpables de que ese buen ambiente se haya mantenido tanto en los momentos buenos como en los malos. Todo funciona bien cuando nadie duda en ayudar al resto, y esto ha sido norma durante mis años en el laboratorio.

Para que se puedan llevar a cabo investigaciones como la que se presenta en este documento, es imprescindible contar con fuentes de financiación. De este modo es justo agradecer el apoyo económico del Gobierno de Cantabria, el Ministerio de Medio Ambiente, Medio Rural y Marino, el Ministerio de Economía y Competitividad, el Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), la Acción COST Europea PERMIT FP1002 y el Instituto de Investigación Forestal Finlandés (METLA). Incluyo también en este apartado de agradecimientos a la Universidad de Valladolid por la concesión de la beca de formación de personal investigador (FPI) que he disfrutado durante estos últimos cuatro años.

Quiero dejar el lugar de honor en mis agradecimientos a mi familia. No podría haber llegado al final de este camino sin su apoyo e interés por mi trabajo. Soy como soy por su culpa. Gracias.

Table of contents

Abstract

Fusarium circinatum, the causal agent of pine pitch canker (PPC) disease, has been present in Europe since its detection in Spain in 2004. It causes substantial damage and losses in forest nurseries and pine plantations. Symptoms found in the nurseries include reduced germination of seeds, the wilting of seedlings, shoot and tip dieback, and, finally, the death of seedlings. In adult pines the main symptom is a bleeding canker on the main stem or thick branches. Dieback symptoms in the crown are also common. Economic loss caused by this disease is significant, and there is no known control or way to at least reduce the symptoms caused by this pathogen. Thus, the objective of the work reported in this thesis was to find an environmentally friendly way to minimize the losses caused by the PPC pathogen. The selection of tolerant or resistant hosts is one way to reduce the impact of the disease. Our results confirmed that *F. circinatum* is basically a pine pathogen; the rest of the conifers tested showed resistance to the disease. Among *Pinus* spp., *P. radiata* proved to be the most susceptible host, while *P. pinea* did not develop any symptoms of the disease after being inoculated with the pathogen. Biological control methods have been shown to be a real alternative to the use of fungicides in many plant diseases. More specifically, the antagonism of some fungal endophytes against other fungi can be used to control phytopathogens. In this study, a total of 139 fungal endophytes exhibited antagonism against *F. circinatum* in vitro. Some of these fungi belonged to species that have the potential to be biological control agents (BCAs). A representative sub-set of these endophytes was tested on pine seedlings in the field to confirm the antagonism previously shown in vitro. Two isolates of the species *Chaetomium aureum* and *Alternaria* sp. reduced the symptoms caused by *F. circinatum* in the *P. radiata* seedlings, but no effect was recorded in the other four pine species tested. The hypovirulence caused by mycoviruses is another successful biological control method used for some plant diseases. In this study three novel viral strains were found in a single isolate of *F. circinatum*. These strains were sequenced and described as members of two new species of the *Mitovirus* genus. Finally, another study demonstrated that the Spanish population of *F. circinatum* commonly hosts these strains. In general, this work represents a substantial advance in the study of environmentally friendly alternatives in the management of PPC disease.

Keywords: *Fusarium circinatum*, *Pinus* spp., *Pinus radiata*, conifer, biological control, fungal endophyte, mycovirus, *Mitovirus*, inoculation, invasive species, BCA, susceptibility.

Resumen

Fusarium circinatum, el hongo causante de la enfermedad del chancro resinoso de los pinos (PPC) está presente en Europa desde su detección en España en el año 2004. Este patógeno causa importantes daños y pérdidas tanto en viveros forestales como en plantaciones de pino. Los síntomas que causa en los viveros son reducción de la germinación de las semillas, marchitez de las plántulas, muerte de brotes y finalmente la muerte de las plántulas. En los pinos adultos, el principal síntoma de la enfermedad es la formación de un chancro resinoso en el tronco o sobre las ramas gruesas, aunque también es común la muerte de ramas en la copa. Las pérdidas económicas debidas a la enfermedad son importantes, y hasta el momento no se conoce ninguna manera de controlar o al menos reducir los daños causados por el patógeno. De esta manera, el objetivo de esta tesis es encontrar una manera ambientalmente respetuosa para minimizar los daños causados por el patógeno de la PPC. Una de la alternativas es la selección de hospedantes tolerantes o resistentes para reducir el impacto de la enfermedad. Nuestros resultados confirmaron que *F. circinatum* es básicamente un patógeno de pinos, siendo resistentes a la enfermedad el resto de las especies de coníferas evaluadas. Dentro del género *Pinus*, *P. radiata* fue la especie más susceptible, mientras que por el contrario, *P. pinea* no presentó síntomas de la enfermedad tras su inoculación con el patógeno. Se ha demostrado que ciertos métodos de control biológico son una alternativa real al uso de fungicidas en muchas enfermedades de plantas. En concreto, el antagonismo producido por algunos hongos endófitos frente a otros hongos puede ser utilizado para controlar a algunos fitopatógenos. Un total de 139 hongos endófitos mostraron antagonismo frente a *F. circinatum* in vitro. Algunos de esos hongos pertenecían a especies con potencial como agentes de control biológico (BCAs). Para confirmar que el antagonismo que exhibieron in vitro también ocurría in vivo, una muestra de esos endófitos fue evaluada sobre plántulas de pino en el monte. Dos aislamientos pertenecientes a las especies *Chaetomium aureum* y *Alternaria* sp. redujeron los síntomas causados por *F. circinatum* en las plántulas de *P. radiata*, pero no se observó ningún efecto de estos hongos sobre las otras cuatro especies de pino. Otra técnica de control biológico empleada con éxito frente algunas enfermedades de planta es la hipovirulencia causada por micovirus. En este estudio se encontraron tres nuevas cepas víricas infectando un aislamiento de *F. circinatum*. Estas cepas fueron secuenciadas y descritas como miembros de dos nuevas especies del género *Mitovirus*. Por último se demostró

que esos virus se encontraban comunmente infectando a los aislamientos de la población española de *F. circinatum.* Este trabajo representa un avance sustancial en el estudio de alternativas ambientalmente respetuosas para gestionar la enfermedad de PPC.

Palabras clave: *Fusarium circinatum*, *Pinus* spp., *Pinus radiata*, conífera, control biológico, hongo endófito, micovirus, *Mitovirus*, inoculación, especie invasora, BCA, susceptibilidad.

List of original articles

This thesis is based on the following articles, which will be referred to by their Roman numerals (I-V) in the text.

I. Martínez-Álvarez, P.; Pando, V. & Diez, J.J. 2014. Alternative species to replace Monterey pine plantations affected by pitch canker caused by *Fusarium circinatum* in northern Spain. *Plant Pathology* 63(5): 1086-1094.

II. Martínez-Álvarez, P.; Alves-Santos, F.M. & Diez, J.J. 2012. In vitro and in vivo interactions between *Trichoderma viride* and *Fusarium circinatum*. *Silva Fennica* 46(3): 303-316.

III. Martínez-Álvarez, P.; Fernández-González, R.; Sanz-Ros, A.V.; Pando, V. & Diez, J.J. 2015. Two fungal endophytes reduce the severity of pitch canker disease on *Pinus radiata* seedlings. Submitted to Biological Control.

IV. Martínez-Álvarez, P.; Vainio, E.J.; Botella, L.; Hantula, J. & Diez, J.J. 2014. Three mitovirus strains infecting a single isolate of *Fusarium circinatum* comprise the first putative members of Narnaviridae among species of *Fusarium*. *Archives of Virology* 159(8): 2153-2155.

V. Vainio, E.J.; Martínez-Álvarez, P.; Bezos, D.; Hantula, J. & Diez, J.J. 2015. *Fusarium circinatum* isolates from northern Spain are commonly infected by three distinct types of mitoviruses. *Archives of Virology* 160(8): 2093-2098.

Introduction

Fusarium circinatum, the pine pitch canker pathogen

Fusarium circinatum Nirenberg & O'Donnell (teleomorph = *Gibberella circinata*) is an ascomycete fungus which causes pine pitch canker (PPC), a serious disease present in pines around the world. The taxonomy of the fungus has changed dramatically since its initial discovery. In its first description, written in the mid-twentieth century by Hepting and Roth (1946), the fungus was identified as a species of *Fusarium*, probably belonging the Liseola section. In 1949, the fungus was considered a new form of the species *F. lateritium* (Snyder et al., 1949) due to its similar characteristics. Almost thirty years later, the pathogen was designated *F. moniliforme* var. *subglutinans* (Kuhlman et al., 1978). Afterwards it was named *F. subglutinans* (Nelson et al., 1983) and then *F. subglutinans forma specialis pini* in the 1990s (Correll et al., 1992). The description of the fungus as *F. circinatum*, a member of the *Gibberella fujikuroi* complex, was done seventeen years ago by Nirenberg and O'Donnell (1998), and since then the taxonomy of this species has not undergone any changes. Recently, the genome of *F. circinatum* was sequenced, making it the first eukaryotic organism for which the complete genome has been sequenced in Africa (Wingfield et al., 2012).

When growing in potato-dextrose agar (PDA) media, *F. circinatum* colonies are characterized by white mycelium with purplish tonality. This species epithet is derived from the coiled sterile hyphae, distinctive to this species. They can be found reliably only on Spezieller Nährstoffarmer Agar (SNA) media. *Fusarium circinatum* lacks chlamydospores, although some isolates produce swollen hyphae, similar to and frequently confused with them. The macroconidia are relatively slender and usually have 3 septa. They are formed in the sporodochia, which are an orangish, salmon colour and are sometimes produced on the canker area, although they can be difficult to find. The microconidia are an obovoid, oval or allantoid shape and are produced singly or in small aggregations in mono- and polyphialides (Leslie and Summerell, 2006).

The genetic diversity of *F. circinatum* varies depending on the geographical location. The number of vegetative compatibility (vc) types is high in the southeastern part of the United States (at least 45 vc types) and especially in Mexico, where the pathogen is thought to have originated (Correll et al., 1992; Wikler and Gordon, 2000). On the contrary, the number of vc types is low in the regions were the pathogen was introduced, such as

California, Japan or Spain (Iturritxa et al., 2011). The low genetic diversity of the *F. circinatum* populations in those areas may facilitate the management of the disease using biological control strategies such as the use of mycoviruses that reduce the virulence of the pathogen.

The disease

The PPC pathogen causes substantial damage and loss in pine forests and plantations. The disease is named after its main symptom, the canker. Cankers usually appear on the main stem or on large branches. There, resin formation is abundant and the tissues beneath the canker are soaked in it (Hepting and Roth, 1946). Furthermore, cankers normally cause deformation of the trunk, making it impossible to use in the wood industry. Dieback symptoms are also common on the crown due to the obstruction of water flow caused by the cankers. The wilting and discoloration of needles, which eventually turn red and finally fall off, is a common symptom of the disease as well (Wingfield et al., 2008). The tree finally dies when cankers girdle the trunk or as a result of the loss of structural integrity at the site of the canker formation. In forest nurseries, *F. circinatum* can reduce germination of seeds, cause pre- and post-emergence damping-off, the wilting of seedlings, shoot and tip dieback, and finally lead to the death of the established seedlings (Viljoen et al., 1994) (Figure 1).

The disease was first observed on *Pinus virginiana* Mill. in southeastern United States in the 1940s (Hepting and Roth, 1946). In that part of the States, epidemics occasionally occur and are generally associated with abiotic stress (López-Zamora et al., 2007). Hosts may have co-evolved with the pathogen due to the proximity of this region to the area where the pathogen is said to be endemic. Forty years after its initial detection, the disease was recorded in California affecting landscape pines mainly of the species *P. radiata* D.Don but also *P. muricata* D.Don, *P. pinea* L. and *P. halepensis* Mill. (McCain et al., 1987). Some years later, the hosts and the geographic range of the PPC pathogen increased, affecting native stands of *P. radiata* in the Monterey peninsula and making a transgeneric jump to *Pseudotsuga menziesii* (Mirb.) Franco (Storer et al., 1994). Outside the United States, Hepting and Roth in 1953 reported the disease in Haiti on *P. occidentalis* Sw., and Santos and Tovar (1991) found it in Mexico on several pine species.

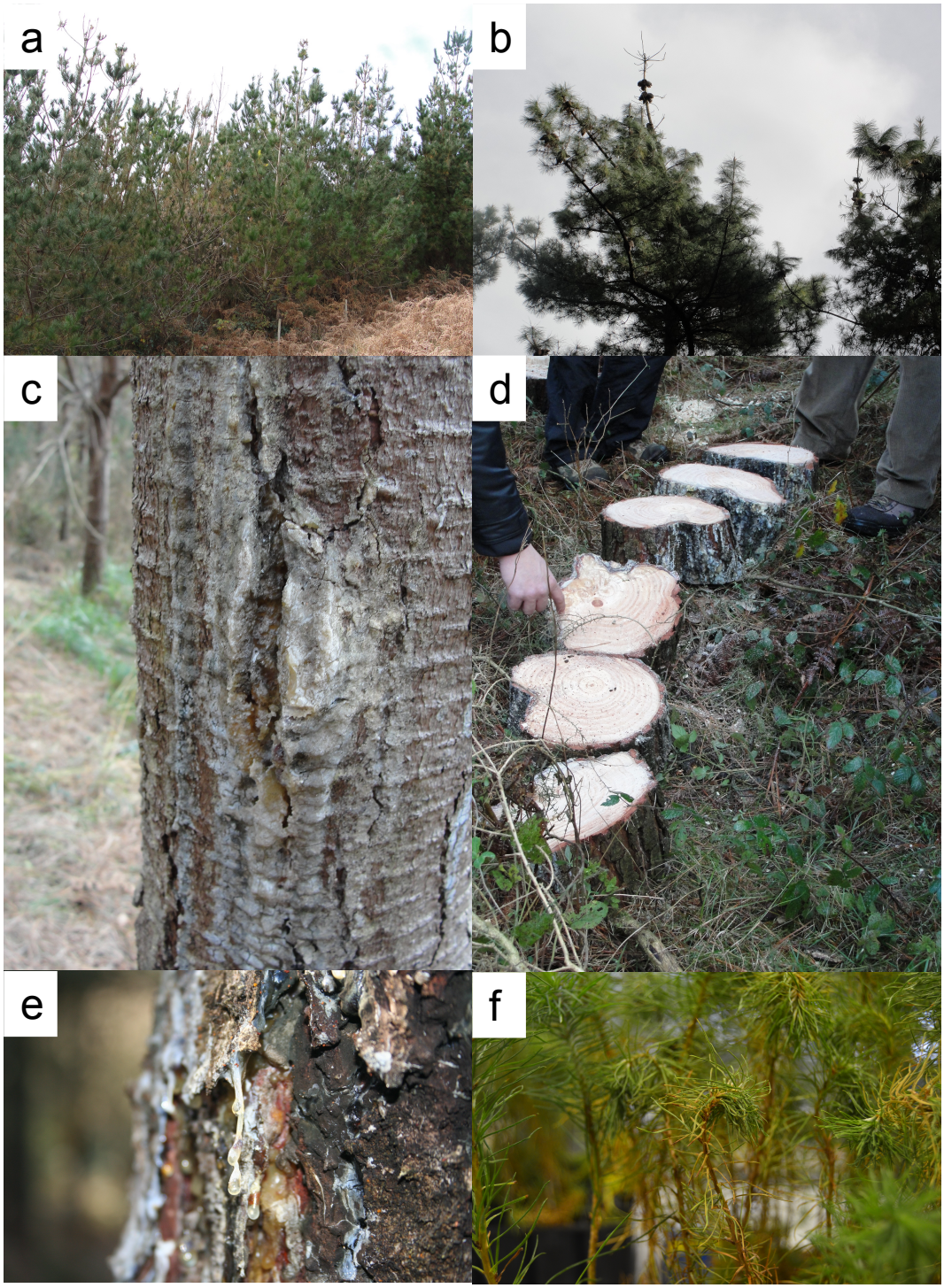

Figure 1: Symptoms of the PPC disease: a) & b) dieback in the crown; c) canker in the main stem of a *P. radiata*; d) deformation in the trunk due to the presence of a canker; e) abundant resin formation in the canker; f) wilting of pine seedlings caused by *F. circinatum*.

PPC disease was first reported outside of North America in Japan in 1989, were it was found to be affecting *P. luchuensis* Mayr. on the islands of Amamiooshima and Okinawa (Kobayashi and Muramoto, 1989). More recently the disease was also reported to be in

another Asian country, South Korea (Cho and Shin, 2004). In 1990 PPC arrived in Africa, particularly South Africa (Viljoen et al., 1994), most likely coming from Mexico (Berbegal et al., 2013; Britz et al., 2001; Wikler and Gordon, 2000). On that occasion, symptoms produced by the pathogen were restricted to root rot in *P. patula* Schiede ex Schltdl. & Cham seedlings in nurseries. *Fusarium circinatum* was considered a nursery pathogen in that country until 2005, when the first outbreak of the disease in a *P. radiata* plantation occurred (Coutinho et al., 2007). The fungus has also spread to Chile (Wingfield et al., 2002), a country in which *P. radiata*, is planted on a wide scale (Fernández and Sarmiento, 2004). There, the pathogen is limited to forest nurseries and plantations between 1 and 4 years old, and it is believed that the diseased trees were probably infected at the nursery stage (Moraga-Suazo et al., 2014).

PPC disease was reported to have arrived to Europe in 2004 when the pathogen was found to be present in pine nurseries in Asturias, and was affecting both *P. radiata* and *P. pinaster*. Later the same year it was found in a 20-year-old *P. radiata* plantation in Cantabria (both regions are in northern Spain) (Landeras et al., 2005). However, there is some evidence to support the fact that *F. circinatum* has been present in the country since the 1990s (Dwinell et al., 1998; Laucirica and Muguruza, 1997; MAPA, 1996). Recently, (Berbegal et al., 2013) concluded that the United States is probably the source of the pathogen causing the infections in Spain. France confirmed the detection of *F. circinatum* in 2005 on a *P. menziesii* tree (approximately 20 years old), in a private garden at Perpignan (EPPO, 2006). It was found in Italy on *P. pinea* and *P. halepensis* in urban parks and gardens (Carlucci et al., 2007). Portugal was the last country in Europe to report the disease. There, *F. circinatum* was detected in 2007 in a nursery and caused damage to *P. radiata* and *P. pinaster* Aiton seedlings (Bragança et al., 2009). Recently, Uruguay (Alonso and Bettucci, 2009), Colombia (Steenkamp et al., 2012) and Brazil (Pfenning et al., 2014) were added to the list of countries in which the PPC disease is present. As one can see, PPC is widely distributed around the world. Nevertheless, some countries where *P. radiata* is widely planted, such as Australia and New Zealand, still remain free of the pathogen. There, the risk of infection is very high and strict measures to avoid the introduction of *F. circinatum* are taken.

Currently, PPC disease in Spain is restricted to *P. radiata* and *P. pinaster* plantations in the north of the country. However, this pathogen has been occasionally detected and eradicated from some forest nurseries in central Spain, where it was affecting different

pine species. Galicia, Asturias, Cantabria, País Vasco, Navarra and Castilla y León are the regions in which *F. circinatum* is currently present (Figure 2). The susceptibility of the species *P. radiata* and *P. pinaster,* together with the climatic conditions (mild temperatures and moisture), may determine the distribution of the pathogen. In Galicia, the fungus mainly infects *P. pinaster*, while in Cantabria, País Vasco and Navarra the disease is exclusive to *P. radiata*. In Asturias both hosts have been found to be affected by the pathogen, and in Castilla y León the pathogen has been detected in *P. radiata* plantations in the north of León and Burgos provinces, together with forest nurseries in Valladolid.

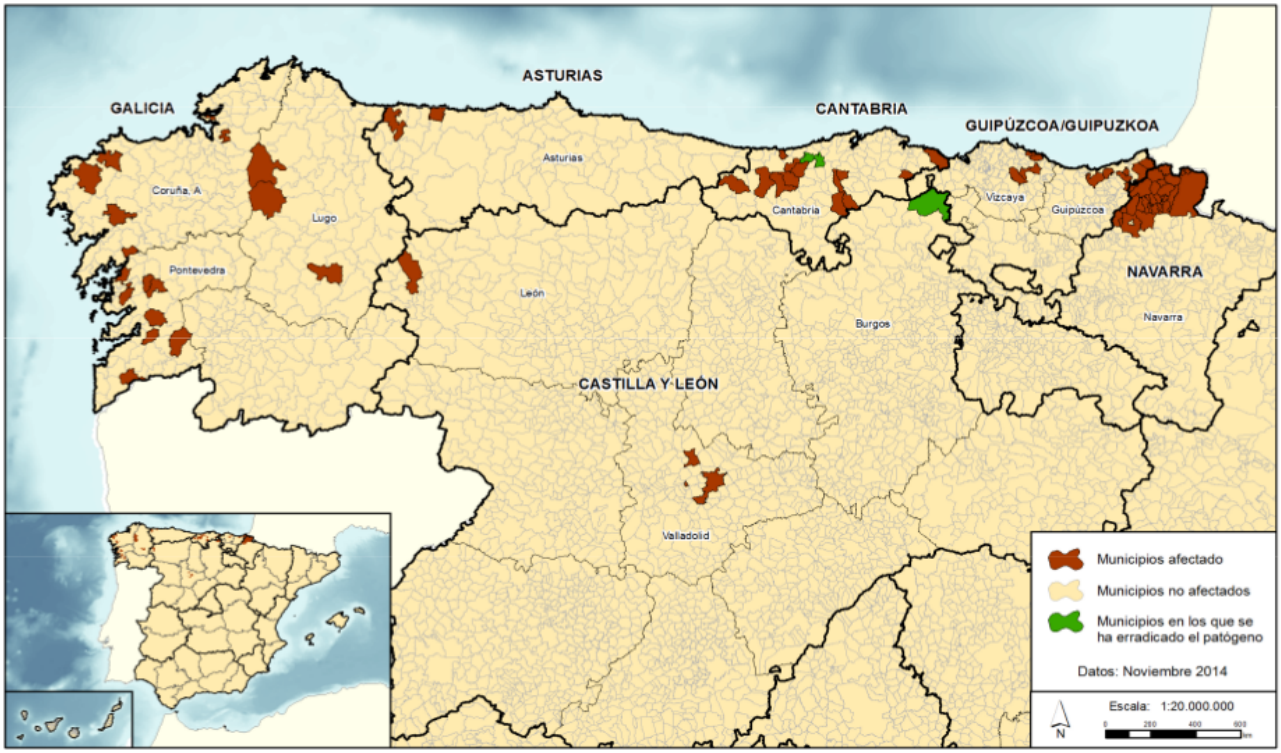

Figure 2: Distribution of the PPC disease in Spain. In brown, municipalities in which *F. circinatum* is present; in green, municipalities in which *F. circinatum* has been eradicated. Source: Ministerio de Agricultura, Alimentación y Medio Ambiente, November 2014.

As previously mentioned, the diversity of the population of this pathogen in Spain is very reduced. Only two different groups of multilocus genotypes were found in the study carried out by Berbegal et al. (2013), and therefore it is thought that *F. circinatum* was introduced into Spain at least twice. According to the results of this study, the *F. circinatum* isolates detected in the first infected nursery in País Vasco in 1997 (Dwinell et al., 1998) and deposited in the Fusarium Research Center collection (Pennsylvania State University)

belong to the most common multilocus genotypes of the two groups, MLG32 (Berbegal et al., 2013). Therefore it is probable that one of those introductions occurred in the País Vasco region, and it spread from there to the rest of the regions in northern Spain. The other group of multilocus genotypes is represented by MLG59, which is restricted to the northwest of the country (Berbegal et al., 2013). Galicia may be the second place of origin of *F. circinatum*, taking into account that the disease was reported to be in that region in 1996 (MAPA, 1996).

It is difficult to know how the pathogen first got into Spain, although it is likely that the original source of inoculum consisted of imported seeds used in nurseries (Berbegal et al., 2013). The fact that some seedlings look healthy despite having latent infections may have facilitated the dispersion of the fungus to the forests (Wingfield et al., 2008). Once in the field, the pathogen could have been transported via asexual spores (microconidia or macroconidia) by wind (Correll et al., 1991), insects (Storer et al., 2004) or pruning tools (Bezos et al., 2012) for new pine stands.

The hosts

Until now *F. circinatum* is exclusively a pine species pathogen, although some trees of the species *P. menziesii* were found susceptible to the disease (Gordon et al., 2006; Storer et al., 1994). At least 57 pine species have been reported as being susceptible to the PPC pathogen after observing symptoms of the disease on seedlings or adult trees or performing inoculation experiments (reviewed by Wingfield et al., 2008). However, susceptibility varies among pine species. For example, while species like *P. pinea* or *P. canariensis* seem to be resistant to the pathogen (Gordon et al., 1998; Iturritxa et al., 2013), there is evidence that *P. radiata* is the most susceptible species to the disease (Wingfield et al., 2008). It is also the most widely-planted pine in the world (Critchfield and Elbert, 1966), Chile being the country with the most surface planted with this species (more than one and a half million hectares). Other countries with large areas of *P. radiata* plantations are Argentina, Uruguay, South Africa, Australia and New Zealand (Fernández and Sarmiento, 2004). *Fusarium circinatum* is still absent from the last two countries, and due to the importance of this pine species there, the occurrence of the disease would have serious economic, ecological, and social impacts. In Spain, around 275 000 hectares are planted with this pine (Fernández and Sarmiento, 2004), a relatively small area in comparison with native pines (3.6% of the total area covered by coniferous species). However, due to its fast growth and short rotation time, it provides 25% of the conifer

timber in Spain (Hermoso et al., 2007).

Since the detection of *F. circinatum* (Landeras et al., 2005), some regions in Spain have stopped planting *P. radiata* due to the ban on using *Pinus* spp. and *P. menziesii* for the reforestation of the affected areas (MAPA, 2006). The production of *P. radiata* seedlings in forest nurseries was also reduced due to the risk of contamination from the pathogen and the consequent loss in crop and yield.

Apart from pines and *P. menziesii*, the susceptibility of a variety of other plant species including trees and herbaceous plants has been tested, with the pathogen failing to infect them (McCain et al., 1987; Wingfield et al., 2008). On the other hand, *F. circinatum* has been found to infect grasses as a symptomless endophyte (Swett and Gordon, 2012). This fact makes the eradication of the disease even more difficult in most parts of the world where the pathogen is well-established; grasses may serve as a reservoir of inoculum which, in turn, influences the occurrence of the disease in pine nurseries and plantations (Swett et al., 2014). An intensive sampling must be done to detect the presence of the pathogen in many other plant species in order to improve the management of the disease. Furthermore, *s*usceptibility to PPC disease in other conifer species must be evaluated to find alternatives to *P. radiata* in the areas where the pathogen is already present.

Measures for the control of the disease. Biological control.

At present there are no means of controlling PPC disease in adult trees in forest or plantations. However, given the seedborne character of *F. circinatum,* some encouraging results have been obtained by the use of different strategies to reduce the presence of the pathogen in pine seeds. For example hot water treatments (51–52ºC for 30 min) were found effective in reducing *F. circinatum* contamination in seeds (Agustí-Brisach et al., 2012; Berbegal et al., 2015). Furthermore, several studies found that hydrogen peroxide is a good disinfectant of the contaminated seeds (Berbegal et al., 2015; Dwinell and Fraedrich, 1999). Unfortunately, these methods do not prevent the arrival of infected seeds to the forest nurseries and seedlings to the forest. To reduce the impact of the disease, an integrated management approach is necessary, and, at the same time, the role of biological control is crucial due to the advantages it has over the use of chemicals.

Cook and Baker (1983) defined biological control as the reduction of the amount of inoculum or disease-producing activity of a pathogen produced by or through one or more organisms other than man. The organisms mentioned in the definition are (1) avirulent or

hypovirulent strains of the pathogen, (2) the host plant manipulated either genetically, by cultural practices, or with microorganisms toward greater or more effective resistance to the pathogen, and (3) antagonists of the pathogen, i.e. microorganisms that interfere with the survival or disease-producing activities of the pathogen.

Biological control of plant diseases has many advantages when compared to the use of chemicals. For instance, fungicides and bactericides have promoted the development of pathogen strains resistant to chemicals, which has been a problem for at least the last thirty years (Dekker and Georgopoulus, 1982). Besides, most chemicals are nonspecific and broadspectrum in their effect, and they can produce undesirable effects on non-targeted organisms, such as the fungal or bacterial endophytes, which can play an important role as antagonists of the pathogens (Spokes et al., 1981). Humans may be harmed as well, specifically when pesticides are employed in agriculture and potentially enter into the food chain (Kniewald, 2003). Another advantage is that longer-term control is achieved because biological control agents (BCAs) act as a host-specific control method continually present and constantly impacting the target pathogen. Thus, although it may be expensive to introduce due to research costs, it can be quite economical in the long term (Cook and Baker, 1983). The importance of biological control methods is even higher in forests where, day after day, chemical use is more and more restricted (EU, 2009a).

As explained before, there are several strategies classified as biocontrols in the fight against pathogens. However, here we will focus our attention only on the control of plant pathogens through the use of fungal endophytes and mycoviruses.

Biological control using fungal endophytes

Many definitions for the term fungal endophytes can be found in scientific literature. One of the most accepted definitions says that fungal endophytes are fungi that are able to infect their hosts without causing visible symptoms of disease (Petrini, 1991). Typically, they may be divided into three types: (1) pathogens of another host that are non-pathogenic in their endophytic relationship; (2) non-pathogenic fungi; and (3) pathogens that have been rendered non-pathogenic yet are still capable of colonization by selection methods or genetic alteration (Backman and Sikora, 2008). Endophytes can produce many benefits for the host plants. For instance, various studies have demonstrated that plants infected with endophytes obtain growth promotion (Barka et al., 2002), resistance to drought stress (Swarthout et al, 2009), tolerance to unsuitable soil conditions (Malinowski et al., 2004),

greater access to nutrients (White et al., 1997), and improved defense against herbivorous animals (Carroll, 1988) and pathogens (Arnold et al., 2003). Regarding the latter benefit of endophytes, the mechanisms implemented to protect the plant against the infection of plant pathogens can be grouped into: direct effects, indirect effects and ecological effects (Gao et al., 2010). In the case of direct effects, endophytes directly suppress pathogens by producing antibiotics (Richardson et al., 2014) or secreting lytic enzymes (Tripathi et al., 2008). Induction of plant resistance, stimulation of the plant's secondary metabolites and promotion of plant growth and physiology all are indirect effects that the endophytes have on the plant to help it reduce damage caused by pathogens. Finally, examples of ecological effects are the occupation of an ecological niche, as well as hyperparasitism and predation (Gao et al., 2010).

One group of fungi stands out among the endophytes because of their potential as a biological control agent of plant diseases: genus *Trichoderma* (Howell, 2003). This group is well known and worldwide in occurrence. One of the most salient characteristics of the group is their ability to parasitize other fungi (Weindling, 1932), but they also produce antibiotic substances that are inhibitory to many plant pathogens (Howell and Stipanovic, 1983). However, the principal mechanism in the biocontrol process of *Trichoderma* spp. is the competition for space and nutrients in the rhizosphere (Howell, 2003). The growth of these fungi is not restricted to the soil and plant roots, but rather they are also able to colonize the phloem and even the sapwood of trees (Jankowiak, 2006). To understand the potential of this group of fungi as BCAs of phytopathogenic fungi, it is important to point out that 90% of the applications performed to control plant diseases have been carried out with different strains of the genus *Trichoderma* (Benítez et al., 2004). There are many examples of *Trichoderma* spp. employed in the successful control of different plant diseases (Abdullah et al., 2008; Latunde-Dada, 1993; Ruano-Rosa et al., 2010) which, in some cases, are caused by pathogens of the genus *Fusarium* (Basak and Basak, 2011; Bernal-Vicente et al., 2009; Sivan et al., 1987). *Trichoderma* spp. have potential not only to control fungal pathogens but also bacteria (Phupiewkham et al., 2015).

Regarding tree diseases, the most well-known example of biocontrol is the one carried out by *Phlebiopsis gigantea* (Fr.) Jülich against *Heterobasidion annosum* (Fr.) Bref, considered the most harmful forest pathogen in economic terms in the Northern Hemisphere (Woodward et al., 1998). However, the importance of endophytes as BCAs is not restricted to forests but also extends to nurseries (Capieau et al., 2004). The production of seedlings

carrying antagonistic endophytes to the most aggressive pathogens may be the future of biocontrol in forest pathology.

Regarding PPC disease, very little has been done to reduce the impact of the pathogen using fungal endophytes. Antagonistic interactions between *F. circinatum* and the fungal species *Penicillium chrysogenum* Link. and *Fusarium lateritium* Ness. were observed in an experiment performed by Romón et al. (2008). Similarly, Soria et al. (2012) showed that two endophytic bacteria (*Bacillus subtilis* Cohn and *Burkholderia* sp.) were antagonists to the pitch canker pathogen. Finally, the potential use of *Trichoderma* spp. and *Clonostachys* spp. strains to control *F. circinatum* on *P. radiata* seedlings was evaluated by (Moraga-Suazo et al. 2011). One of the strains of *Clonostachys* sp. tested significantly increased the survival of *P. radiata* seedlings, but no effect was observed with the *Trichoderma* strains. The same conclusions were reached in previous studies in which *Trichoderma* spp. were tested as BCAs of the disease (Dumroese et al., 1988; Mitchell et al., 2004).

Biological control using mycoviruses

Mycoviruses or fungal viruses are widespread in all major taxa of fungi. Most of them have dsRNA (double-stranded RNA) genomes, although an increasing number of positive or negative ssRNA (single-stranded RNA) and ssDNA (single-stranded DNA) viruses have been isolated and characterized in recent years (Ghabrial et al., 2015). Mycoviruses are, in general, associated with latent infections in their hosts (Ghabrial and Suzuki, 2009). However, in some cases they induce different symptoms such as changes in growth, colour, sporulation, and sometimes enhancement (hypervirulence) or attenuation of fungal virulence (hypovirulence) (Ghabrial and Suzuki, 2009; Pearson et al., 2009). Hypovirulence in the host's physiology is the most important reason why plant pathologists are interested in mycoviruses and why mycoviruses can be used as BCAs.

The mycovirus that dominates in the context of plant pathology is the Cryphonectria hypovirus 1 (CHV1) (Heiniger and Rigling, 1994), which has been successfully used as a BCA for the chestnut blight pathogen throughout Europe (Robin and Heiniger, 2001; Turchetti et al., 2008; Zamora et al., 2014). This hypovirus reduces mycelial growth and sporulation of *Cryphonectria parasitica*. It also produces changes in the morphology and colour of the fungus colony (Peever et al., 2000; Rigling et al., 1989). After being infected, the pathogen is only capable of forming superficial healing cankers on stems, allowing

trees to survive after the attack (Nuss, 1992).

Among mycoviruses, one genus only found in fungi stands out due to its importance in the biological control of plant diseases. This is the genus, *Mitovirus*, which belongs to the family Narnaviridae, a group in which the members have the simplest genomes of any autonomous RNA virus (Ghabrial et al., 2015). Putative members of the genus *Mitovirus* are located and translated in the mitochondria (Polashock and Hillman, 1994), where they mostly occur as dsRNA replicative forms (Ghabrial, 1998). In some cases they exhibit phenotypic changes and cause hypovirulence in major plant pathogens, such as *Botrytis cinerea* (Wu et al., 2010), *Ophiostoma novo-ulmi* (Rogers et al., 1987) or *Sclerotinia homoeocarpa* (Deng et al., 2003).

The use of mycoviruses to control fungal diseases of plants could be a promising method when the genetic diversity of the populations of the pathogen is low, for example, when the introduction of the fungus in a region is recent or when sexual reproduction is absent. This is the case with the pitch canker pathogen in Spain (Berbegal et al., 2013). However there is still a long way to go in the development of a biological control tool using mycoviruses, and it requires first finding a virus producing hypovirulence then finding out the limitations in its transmission.

Aims of the study

Fusarium circinatum is a threat to the productivity of pine plantations and forests around the world. Forest owners, nurseries, as well as wood and pulp industries from the countries in which the disease is present, are already suffering the consequences. Together with its economic impact, the presence of the PPC pathogen has also had serious social and ecological implications. Currently there is no way of controlling PPC disease in seedlings in nurseries or in adult trees in plantations. Furthermore, the application of pesticides in the forest is becoming more and more restricted because of the subsequent impact on the rest of the ecosystem. Therefore, the overall aim of the work described in this thesis was to find an environmentally friendly way to minimize the losses caused by the PPC pathogen.

More specifically the objectives were:

1. To identify species that are susceptible and resistant to the PPC pathogen among the conifers commonly planted in northern Spain and to search for an alternative to *P. radiata* in the diseased areas (I & III).

2. To assess the effectiveness of different fungal endophytes against *F. circinatum* in laboratory conditions (II & III).

3. To evaluate the effect of several fungal endophytes on *F. circinatum* in five pine species seedlings in the field (III).

4. To detect the presence of mycoviruses hosted by the *F. circinatum* isolates, and to obtain their sequences (IV).

5. To examine the population structure of *Mitovirus* spp. hosted by the isolates of *F. circinatum* (V).

Material and methods

Sampling and experimentation sites

- Eleven *P. radiata* plantations in Cantabria (Spain), in which the presence of *F. circinatum* had been previously confirmed, were sampled to isolate the pathogen (Figure 3) (II).

- Four plots were used to establish the susceptibility to *F. circinatum* experiment, three of them located next to plantations affected by the PPC disease (San Sebastian de Garabandal, Santibañez and Villafufre) and the fourth one in a disease-free area to be used as a control (Hermosa) (Figure 4) (I).

- One plot in which the experiment of antagonism with fungal endophytes was performed (Puentenansa) (Figure 5) (III).

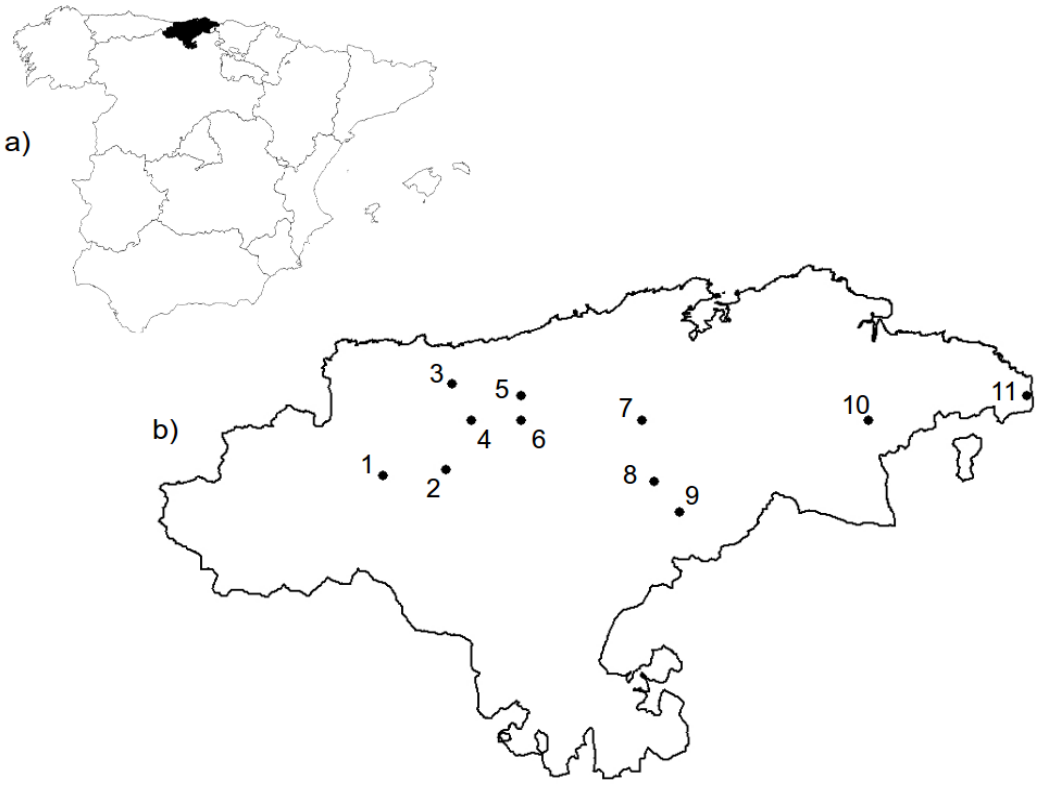

Figure 3: Location of the eleven *P. radiata* plantations in Cantabria (Spain) were sampled to isolate the pathogen; a) in Spain, b) in the Autonomous Region of Cantabria. The numbers refer to municipalities: 1 = Rionansa, 2 = Cabuérniga, 3 = Comillas, 4 = Cabezón de la Sal, 5 & 6 = Mazcuerras, 7 = Villafufre, 8 = Santiurde de Toranzo, 9 = San Pedro del Romeral, 10 = Ramales de la Victoria and 11 = Castro Urdiales.

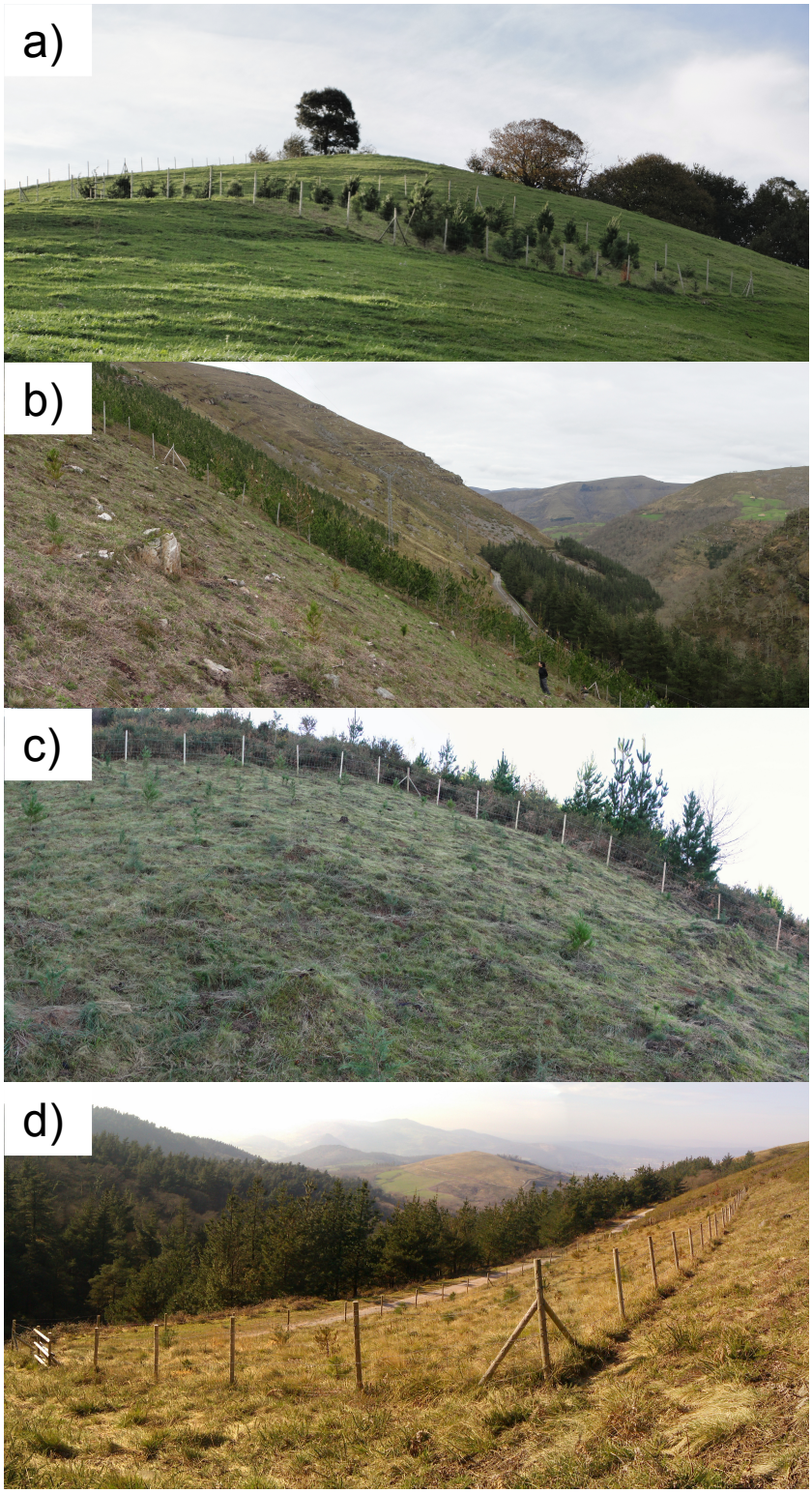

Figure 4: Pictures of the four plots established to test the susceptibility to *F. circinatum* of different conifers. One of them was located in a disease-free area to be used as a control; a) Hermosa. The other three were located next to plantations affected by the PPC disease; b) San Sebastian de Garabandal, c) Santibañez and d) Villafufre.

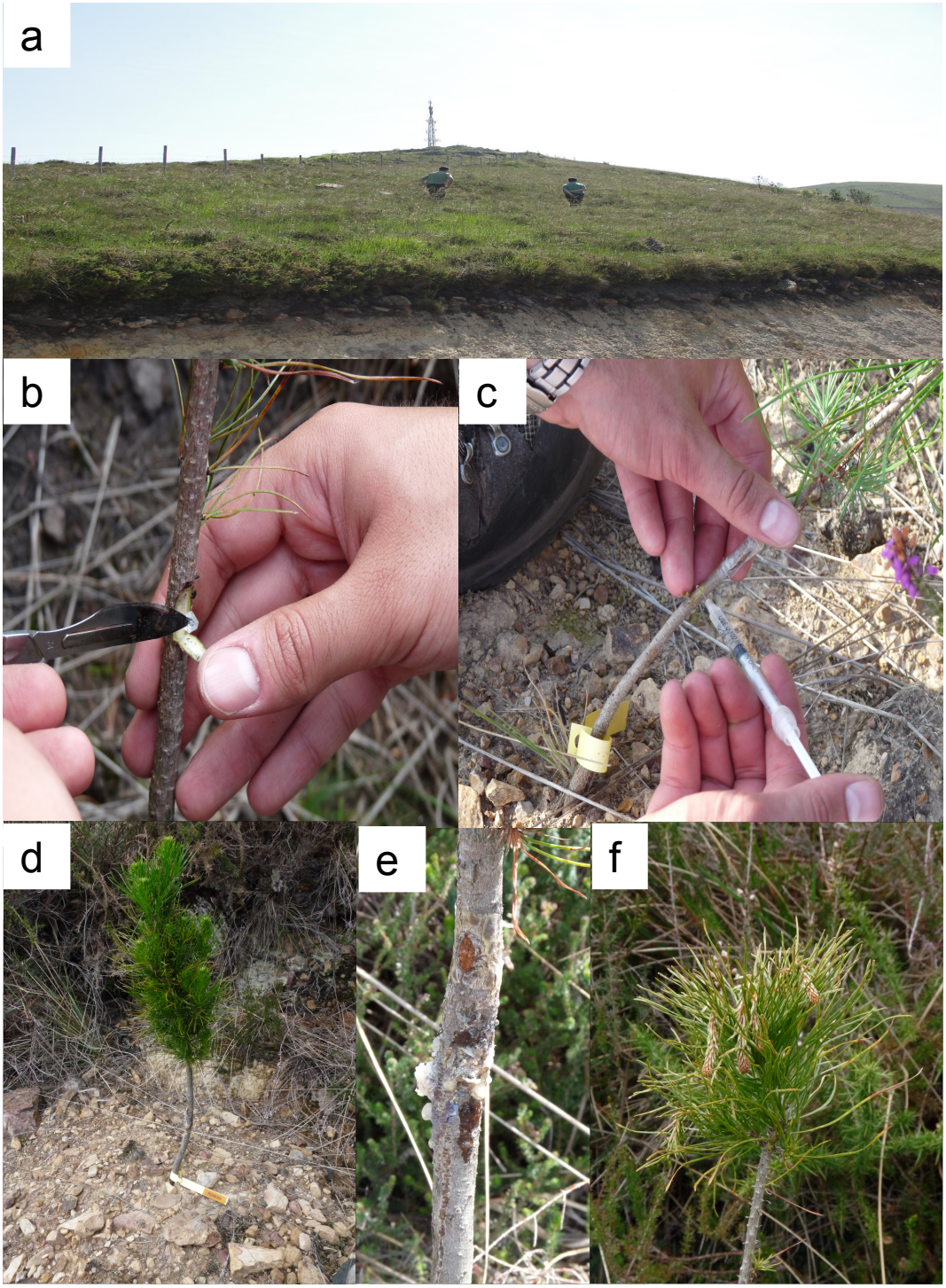

Figure 5: Pictures of the experiment of antagonism with fungal endophytes in the field; a) general view of the plot, b) inoculation of the endophytes, c) inoculation of *F. circinatum*, d) healthy *P. radiata* seedling, e) necrosis and resin formation at the inoculation point, f) wilting of a inoculated *P. radiata* seedling.

Fungal isolates

- Cantabrian *F. circinatum* collection (101 isolates) (I, II, III, IV & V).

- Spanish *F. circinatum* collection (25 isolates), provided by the Instituto Agroforestal Mediterráneo (Universidad Politécnica de Valencia) (III & V).

- South African *F. circinatum* collection (29 isolates), provided by the Forestry and Agricultural Biotechnology Institute (University of Pretoria) (V).

- Fungal endophytes collection (547 isolates), obtained in the Calabazanos Forest Health Center (Junta de Castilla y León) and in the Forest Entomology and Pathology Laboratory (University of Valladolid) (II & III).

Methods

Isolation of Fusarium circinatum and fungal endophytes

- Samples: Needles, twigs, stem bark, stem xylem, cones and seeds were collected in order to isolate *F. circinatum* (I, II & III) and other fungi (II) associated with PPC disease.

- Surface sterilization: The samples were washed in running tap water for one minute, soaked in 70% alcohol for two minutes, and soaked twice in 3% sodium hypochlorite solution, for two minutes each time. The samples were finally immersed twice in sterile distilled water, for two minutes each time (I, II & III).

- Plating: Samples were plated onto potato-dextrose agar (PDA) media supplemented with 0.5 g/l of streptomycin sulphate (I, II & III).

Morphological identification of fungal species

- *Fusarium circinatum*: Colonies were transferred to Spezieller Nährstoffarmer Agar (SNA) media, observed under microscope and identified with the help of the taxonomic key written by Leslie and Summerell, (2006) (I, II).

- Other fungal species: Identification was performed on PDA media, with the help of different taxonomic keys (Hanlin, 1990; Kiffer and Morelet, 1997; Leslie and Summerell, 2006; Sutton, 1980; Watanabe, 1993) (II).

DNA isolation

- Protocol modified from Vainio et al. (1998) (I, II & III).

DsRNA extraction

- Protocol modified from Morris and Dodds (1979) (IV & V).

Polymerase Chain Reaction (PCR) amplification

- A fragment of the IGS rDNA region (ca 360 bp), with specific primers for *F. circinatum* CIRC1A and CIRC4A (I, II & III).

- Two fragments of the genomic DNA with specific primers for *F. circinatum* mating type determination: MAT1p2 and MAT1p3 for MAT-1 (ca 380 bp); GcHMG1 and GcHMG2 for MAT-2 (ca 190 bp) (I).

- Internal Transcribed Spacer (ITS) of the ribosomal DNA (rDNA) (ca 600 bp), with primers 1F and 4 (II & III).

- The Elongation Factor 1 alpha EF (ca 700 bp) with primers EF1 and EF2 (II & III).

- Large Subunit (LSU) rDNA with primers LR0R and LR16 (III).

- Complementary DNA (cDNA) with T4 RNA primer (IV &V).

- Complementary DNA (cDNA) with selective primer pairs: FMC1F1/FMC1Rev1 and FMC3F1/FMC3Rev1 (V).

Electrophoresis

- Agarose gels for amplified DNA fragments (I, II & III).

- Agarose gels for RNA molecules (IV & V)

DNA and RNA purification

- PCR product purification (II, III, V)

- DsRNA purification (IV, V)

Reverse transcription and cloning

- Reverse transcription performed with RevertAid H minus M-MuLV reverse transcriptase (IV & V).

- Cloning using the pCR2.1-TOPO cloning vector (IV).

Bioinformatics and phylogenetics analysis

- Nucleotide search using nucleotide sequence as query (Blastn) in the NCBI GenBank database (II, III, IV & V).

- Primer design (V).

- Dendrogram construction by Neighbor-Joining clustering with MEGA 5 software (IV & V).

- Recombination detection methods: RDP, GeneConv, Chimaera, MaxChi, SiScan and 3Seq, with RDP4 (v.4.16) software (V).

Statistical analyses

- Analysis of variance (ANOVA) (II).

- Kruskal-Wallis test (II).

- Fisher Least Significant Difference (LSD) test (I, II, III).

- Scheffe test (II).

- Tukey–Kramer test (I).

- T-test (III)

- Linear mixed model analysis of variance (I & III).

- Linear mixed model analysis of variance with repeated measurements (III).

Results

1. *Susceptibility of conifers commonly planted in northern Spain to the PPC pathogen* (I & III).

Pinus radiata was confirmed to be highly susceptible to *F. circinatum*. Although the emergence rate of this species was slightly affected by the pathogen, mortality of the seedlings reached 98%, 67% and 74% in the different experiments performed. The susceptibility of the rest of the pine species tested differed depending on the experimental conditions (age of the seedlings and environmental conditions). All pine species were susceptible to the pathogen in the assay performed in the laboratory with newly germinated-seedlings. However, in the experiments performed in the field with two-year-old seedlings, *P. pinea* was found to be resistant to *F. circinatum,* as well as the rest of the conifers not in the genus *Pinus.* Susceptibility of *Pinus nigra* Arnold*, P. pinaster, P. sylvestris* L.*, P. uncinata* Mill. was not clear after the evaluation due to the fact that significant differences among the seedlings inoculated with *F. circinatum* and the control treatment were found in some plots but not in others.

2. *Effectiveness of different fungal endophytes against F. circinatum in laboratory conditions* (II & III).

One hundred thirty nine fungal endophytes among a total of 547 assessed produced antagonism against *F. circinatum* in vitro. The number of antagonists detected by the indicators was always higher in the confrontations against isolate Fc221 (mating type 2) of the pathogen than against Fc70 (mating type 1), and these differences were found to be significant according to the linear mixed model. Significant differences were also found between the four different indicators used to detect the antagonism, with the indicator which detected a higher number of antagonists (82 against Fc70 and 99 against Fc221) being the percentage inhibition of the radial growth and the most restrictive indicator being the percentage of inhibition zone (42 and 62 respectively). Among the antagonistic endophytes, a total of 41 were identified as species of the genus *Trichoderma*, a widely studied group of fungi because of its potential as a BCA.

3. *Reduction of the symptoms caused by F. circinatum on pine seedlings due to fungal endophytes* (III).

Two isolates among the six tested, *Chaetomium aureum* Chivers and *Alternaria* sp.,

significantly reduced the symptoms produced by *F. circinatum* on the *P. radiata* seedlings (27.8% and 22.2% respectively). However, they were not able to stop the reduction in growth caused by the pathogen. No effect of these two endophytes was recorded in the seedlings from the rest of pine species tested. Similarly, the other four endophytes evaluated did not succeed in controlling the pathogen in any of the five pine species planted except for a questionable reduction on *P. sylvestris* by the endophyte *Trichoderma spirale* Bissett.

4. *Detection of mitoviruses infecting F. circinatum* (IV).

Three putative viral RdRp sequences were detected in a single isolate of *F. circinatum* (FcCa070), the first time that mycoviruses have been recorded as having infected the PPC pathogen. After the phylogenetic analysis was conducted, they clustered with the 'clade I' mitoviruses, and they seem to belong to at least two phylogenetically distinct groups. They were named Fusarium circinatum mitovirus 1 (FcMV1), FcMV2-1 and FcMV2-2. The three described strains are the first members of the family Narnaviridae detected in a fungus of the genus *Fusarium*. The length of the genomes was 2419 bp, 2193 bp and 1973 bp respectively, although the sequences of FcMV2-1 and FcMV2-2 were partial.

5. *Genetic structure of the population of Mitovirus spp. hosted by F. circinatum* (V).

In 43 of the 135 *F. circinatum* isolates (31.9%) mitoviruses were detected by PCR amplification using the mitovirus primers designed. The South African isolates as well as the isolates of other *Fusarium* spp. analyzed in the study did not exhibit dsRNA elements, and, therefore, viruses were only detected in the Spanish collection of the pathogen. Each sequence obtained was highly similar to one of the three mitovirus strains from *F. circinatum* characterized previously (FcMV1, FcMV2-1 and FcMV2-2), which suggests that they were separate genetic entities (i.e., mitovirus types), each showing highly similar sequence variants (with more than 95% similarity). The fact that the same haplotypes were detected at more than one collection site, in some cases located in different provinces, suggests that highly similar viral sequence variants occur throughout northern Spain. Besides, the same sequence variants were found to be hosted by isolates of the pathogen belonging to both mating types, thus the occurrence of the mitoviruses is apparently not restricted by the mating type compatibility.

Discussion

The economic loss caused by PPC disease has been substantial since *F. circinatum* was first detected on *P. radiata* and *P. pinaster* seedlings in a forest nursery in Asturias and on a *P. radiata* plantation in Cantabria in 2004 (both regions being located in northern Spain) (Landeras et al., 2005). Currently the disease has spread throughout northern Spain affecting mainly pine plantations of these two species. The risk of dispersion of the disease to other disease-free countries or other tree species is high. Thus, other European countries have reported the presence of the pathogen in other pine species (Bragança et al., 2009; Carlucci et al., 2007; EPPO, 2006). Therefore, there is a need to know the susceptibility of the different conifers commonly planted in Europe to PPC disease in order to be aware of new possible hosts of the pathogen and to find an alternative to *P. radiata*, the most susceptible species to *F. circinatum* (Wingfield et al., 2008). On the other hand, no effective methods exist at the present time to control or reduce the losses caused by this pathogen, so it is urgent to examine methods to control or at least minimize the damages caused by the PPC pathogen. We must focus on environmentally friendly methods due to the existing concern about the use of pesticides in the forest and the European recommendations of avoiding the use of chemicals (EU, 2009b).

One of the keys to reducing the impact of phytopathogens lies in the search for tolerant or resistant host species, provenances, varieties and specimens (Agrios, 1997). With this goal in mind, we conducted several experiments in which the effects of *F. circinatum* were assessed on several conifer species. Our results confirmed that *P. radiata* is the most susceptible species to PPC disease, as other authors have stated before (McCain et al., 1987; Gordon et al., 1998, 2001; Hodge and Dvorak, 2000; Iturritxa et al., 2012). Furthermore, the susceptibility of some other important pine species, such as *P. nigra*, *P. pinaster*, *P. sylvestris* and *P. uncinata,* to *F. circinatum* varied among experiments. The age of the inoculated seedlings together with their provenance and the different environmental conditions of the experimental plots may be the reasons behind this variation in susceptibility. Other studies showed different results on this subject. For instance, the susceptibility of *P. sylvestris* was confirmed by Mccain et al. (1987), Enebak and Stanosz (2003) and Pérez-Sierra et al. (2007); but in the experiment performed by Carlucci et al. (2007) *P. sylvestris* proved resistant to the pathogen. Regarding *P. pinaster*, most authors agree on defining the species as susceptible to *F. circinatum* (Carlucci et al., 2007; Pérez-Sierra et al., 2007), although the provenance may play an important role in

the susceptibility of this pine species (Iturritxa et al., 2012). Variation in the susceptibility of *P. nigra* has been also reported. While in the experiments carried out by Carlucci et al. (2007) and Iturritxa et al. (2012, 2013) *P. nigra* was found to be resistant to *F. circinatum*, Pérez-Sierra et al. (2007) found damage on the seedlings inoculated with the pathogen.

Pinus pinea was the only pine clearly resistant to PPC disease among the species tested in our experiments. This result is consistent with those obtained by Iturritxa et al. (2012, 2013) and Gordon et al. (1998) but differed from the findings of McCain et al. (1987) and Carlucci et al. (2007). Again we consider the provenance of the seedlings and the experimental conditions responsible for these different results. On the other hand, some other conifers were inoculated to test their susceptibility to *F. circinatum*. None of them showed symptoms caused by the pathogen during the trials except in the experiment performed to test the effect on germination; in this test, damage was recorded in the soft tissues of the newly germinated seedlings. Our results confirm the fact that *F. circinatum* is mainly a pine pathogen (Wingfield et al., 2008), but it has been demonstrated that it can cause damping off on conifers of other genera. To our knowledge our work is the first to assess the effect of the pitch canker pathogen on the following species: *Abies alba* Mill*., Chamaecyparis lawsoniana* (A. Murray) Parl., *Larix decidua* Mill., *Picea abies* Karst., *Cedrus atlantica* (Endl.) Manetti ex Carrière, *Cupressocyparis leylandii* (A.B. Jacks. & Dallim.) Dallim. & A.B. Jacks. and *Thuja plicata* Donn ex D.Don.

In Spain, planting pines in the areas in which *F. circinatum* has been detected is forbidden (MAPA, 2006). Thus, an alternative to the previously highly productive *P. radiata* is needed. Many forest owners in northern Spain have changed to *Eucalyptus* spp. (mainly *E. globulus* Labill. and *E. nitens* Deane & Maiden) to reforest their land, particularly in Cantabria, but these tree species have a serious pest, *Gonipterus scutellatus* Gyll., and disease, *Mycosphaerella* spp., both of which reduce the crop yield as well. Some of the conifers tested in our experiments are real alternatives to the utilization of *P. radiata* in those areas affected by the disease. For instance, there are stands of *C. lawsoniana* close to *P. radiata* diseased plantations that show promising growth and phytosanitary conditions. Similarly, pines such as *P. sylvestris* and *P. pinaster* are well-adapted to the climatic conditions of northern Spain and are less susceptible to *F. circinatum* than *P. radiata,* could be an alternative in the disease-free areas. Despite their slower growth, the symptoms caused by the pathogen on these pine species would be smaller and the economic loss lesser.

Integrated management is needed to control PPC disease, and thereby, the selection of a resistant or tolerant host must be accompanied by other methods. Detection and eradication of the pathogen in the forest nurseries is key to preventing the arrival of the pathogen to the forest. And although methods to eliminate the pathogen from the seeds such as the use of disinfectants (Berbegal et al., 2015) or thermotherapy (Agustí-Brisach et al., 2012) have been studied and have obtained promising results, they may not be enough. The use of fungal endophytes as BCAs has been confirmed as a real alternative to using fungicides in other plant disease situations (Abdullah et al., 2008; Arnold et al., 2003), making the study of fungal endophytes against *F. circinatum* a priority.

The antagonistic effect of a total of 139 isolates of fungal endophytes against *F. circinatum* was observed in vitro. Significant differences were found between the four different indicators used to assess the interactions between the pathogen and the endophytes.

The strategies employed by the endophytes to inhibit the growth of the *F. circinatum* colony were diverse, and, therefore, the use of several complementary methods was crucial in order to select the endophytes, regardless of the strategies they used to control the pathogen. The use of more than one method in the antagonism experiments to identify the best BCA candidates is highly recommended. On the other hand, differences were found between the MAT-1 and MAT-2 isolates of the pathogen. More endophytes were antagonistic against the MAT-2 isolate of *F. circinatum* than against the MAT-1 isolate. Differences between mating types of this fungus have been previously reported, for example in pathogenicity (Pérez-Sierra et al., 2007), sensitivity to thermotherapy (Agustí-Brisach et al., 2012), and even in some morphological characteristics (Pérez-Sierra et al., 2007). However, all these findings have been reported in the Spanish population of the pathogen, and, taking into account the low diversity level of this population (Berbegal et al., 2013), it is possible that the differences are due to the genotypes rather than to the mating type.

Most of the endophytes with the best results in the in vitro antagonism experiment belonged to the genus *Trichoderma*. The fast growth of these fungi in the soil and the plant roots is well known (Howell, 2003) and also occurs on artificial culture medium. In our experiments, the *Trichoderma* isolates reduced the space and nutrients available for the pathogen very fast, which is the reason why we found many isolates of this genus to be antagonistic. But competition is not the only mechanism this group of fungi has to exert biocontrol against fungal phytopathogens. They have been reported as being able to

modify environmental conditions, promote plant growth and plant defensive mechanisms produce antibiosis and parasitize other fungi (Howell, 2003). As a result, there are many examples of plant diseases successfully controlled by species of *Trichoderma*, and, in fact, some strains of these species are registered for commercial use (Harman, 2000).

The antagonism that some fungi show against the pathogens on artificial culture media is not always maintained on the host plant. Although many endophytes resulted antagonistic to *F. circinatum* after the assays performed in vitro, evaluating their effect in vivo is essential. Therefore, the trial was carried out in the field in order to assess the antagonism in real environmental conditions of the diseased areas. Two endophytes of the species *Chaetomium aureum* and *Alternaria* sp. significantly reduced the damages caused by *F. circinatum* in the *P. radiata* seedlings. *Chaetomium aureum* was previously reported as a BCA of both *Magnaporthe grisea* (T.T. Hebert) M.E. Barr and *Rhizoctonia solani* J.G. Kühn, causal agents of rice blast and sheath blight respectively (Wang et al., 2013). On the other hand, although *Alternaria* is a fungal genus that includes saprophytic, endophytic and pathogenic species (Thomma, 2003), some of them have been used as antagonists of plant pathogens. For instance, Campanile et al. (2007) found *A. alternata* to be antagonistic to *Diplodia corticola* A.J.L. Phillips, A. Alves & J. Luque, pathogen of *Quercus* spp. Nevertheless, the progression of the disease in the pine seedlings was not affected by the *Trichoderma* spp. tested, even though the best endophyte isolates in the in vitro experiments belonged to this genus. As mentioned before, the in planta tests are essential to finding BCAs of plant diseases, and our work has confirmed this statement.

To confirm that *C. aureum* and *Alternaria* sp. can be used as effective BCAs of PPC disease and if they can live inside the tissues of the host tree in the long term, further studies are needed. If it becomes clear that they reduce the damages caused by this pathogen, production of pine seedlings in the nurseries infected with these endophytes would be the best way to implement this advance in the prevention and control of the disease.

The use of mycoviruses could be another option for the biological control of the disease (Nuss, 2005). The first step in this case is to search for viral molecules in the host, indicating an infection in the fungus. Therefore, we conducted a screening to detect dsRNA particles in the Spanish population of *F. circinatum*. The occurrence of a band (ca 2.5 kb) in the electrophoresis gel denoted the presence of at least one virus in one of the isolates of the pathogen. After sequencing and performing a phylogenetic tree, we

concluded that there were three viral strains belonging to two new different species of the genus *Mitovirus* infecting the fungal isolate. They were named Fusarium circinatum mitovirus 1 (FcMV1), FcMV2-1 and FcMV2-2. Although studies to test the effect of these viruses on *F. circinatum* have not been done yet, the fact that these viral strains belong to the genus *Mitovirus* is encouraging, since some species of this virus group have been reported to reduce the virulence of some destructive plant pathogens (Deng et al., 2003; Rogers et al., 1987; Wu et al., 2010).

Specific primers were designed for each one of the newfound viruses in order to find out their occurrence in different collections of *F. circinatum* isolates. Viruses were common in the Spanish population of the pathogen with almost 32% of the isolates infected by at least one of the three viral strains. Same sequence variants were found at different locations throughout northern Spain, some of them many kilometers apart from one another. This suggests that the diversity of the pathogen is low in northern Spain as some authors have stated before (Berbegal et al., 2013; Iturritxa et al., 2011), and it supports the hypothesis that the pathogen was introduced twice into Spain (Berbegal et al., 2013). Similarly, the occurrence of the mitoviruses is apparently not restricted by the mating type compatibility, contrary to the observations of other authors (Coppin et al., 1997; Milgroom and Hillman, 2011), since same sequence variants were found in both MAT-1 and MAT-2 isolates.

No dsRNA particles were found in the South African isolates provided by the FABI (the Forestry and Agricultural Biotechnology Institute of the University of Pretoria) nor in other *Fusarium* spp. included in the study. The Spanish population of the pathogen presumably originates from the United States of America (Berbegal et al., 2013), while the pathogen in South Africa was probably introduced via Mexico (Wikler and Gordon, 2000). This difference, together with the low diversity of the Spanish population of *F. circinatum* (Berbegal et al., 2013; Iturritxa et al., 2011), may be the explanation for the different infection rates of the viruses in the isolates of the fungus from the two countries. However, we should not discard other hypothesis such us the transmission of the viruses from the host plant, other fungi or even from insect vectors (Ghabrial, 1998), and therefore this issue must be studied more thoroughly.

The *modus operandi* if hypovirulence caused by any of the mitovirus is detected would be the same as that being carried out in the case of the chestnut blight caused by *Cryphonectria parasitica* (Zamora et al., 2014), although many important studies regarding, for example, vertical and horizontal transmission of the viruses are still missing.

The low genetic diversity of the pathogen in northern Spain where only two vc types were found (Iturritxa et al., 2011), is promising for the future of this technique as a biological control.

Pitch canker disease is widely spread in northern Spain so in our opinion the eradication of the pathogen is not feasible. To reduce the damages caused by *F. circinatum* and avoid infecting new areas or tree hosts, an integrated management of the disease is imperative, as reported in other cases of plant diseases (Köhl, 2006; Wingfield and Swart, 1994). In this integrated management, environmentally friendly methods such as the selection of species that are tolerant or resistant to the pathogen and the use of biological control techniques using endophytes or mycoviruses must be among the most important tools implemented in the near future.

Conclusions

1. The species *P. radiata* was highly susceptible to PPC disease. Other pine species tested (*P. pinaster*, *P. sylvestris*, *P. nigra* and *P. uncinata*) showed symptoms caused by the pathogen, although their susceptibility varied among experiments due to the different provenance of the seedlings and environmental conditions of the experimental plots. *Pinus pinea* as well as the rest of the conifers tested were found to be resistant to the PPC pathogen. To reduce the symptoms and loss caused by *F. circinatum,* the substitution of *P. radiata* plantations in the areas where the pathogen is present is essential.

2. A total of 139 endophytes among 547 tested, were effective in controlling the growth of *F. circinatum* in vitro by way of at least one of the four indicators of antagonism used. A high percentage of the effective endophyte isolates belonged to *Trichoderma* spp., a genus recognized for its potential use in biocontrol.

3. Two fungal endophyte isolates belonging to the species *C. aureum* and *Alternaria* sp. substantially reduced the symptoms produced by *F. circinatum* in the *P. radiata* seedlings. They have the potential to be used as BCAs of the disease, but they still need to be studied in more depth. These fungi along with the others tested did not cause any effect on the rest of the pine species assayed.

4. Three novel members of the genus *Mitovirus,* Fusarium circinatum mitovirus 1 (FcMV1), FcMV2-1 and FcMV2-2, were detected in an isolate of *F. circinatum* and their genome sequenced. They are the first viruses reported as being hosted by *F. circinatum*, and it is the first time that mitovirus genome sequences of *Fusarium* sp. have been described.

5. Viruses FcMV1, FcMV2-1 and FcMV2-2, as well as their closely related sequence variants were relatively common in Spain. No dsRNA particles were detected in the South African collection of isolates of *F. circinatum* nor in the isolates of other *Fusarium* spp. Different origins of the Spanish and the South African populations of the pathogen may be the reason for these differences in presence of the virus.

6. According to the findings of this study, the use of environmentally friendly methods to manage PPC disease is plausible. However more studies are necessary to minimize the symptoms caused by the PPC pathogen in nurseries and in forests using these techniques.

Conclusiones

1. La especie *P. radiata* fue altamente susceptible a la enfermedad del PPC. Otras especies evaluadas (*P. pinaster*, *P. sylvestris*, *P. nigra* y *P. uncinata*) presentaron daños causados por el patógeno, aunque su susceptibilidad varió entre experimentos debido a la diferente procedencia de las plántulas y a las diferentes condiciones ambientales de las parcelas de ensayo. La especie *P. pinea* resultó ser resistente al patógeno del PPC así como el resto de las coníferas evaluadas. La sustitución de las plantaciones de *P. radiata* en las áreas donde el patógeno está presente es básica para reducir los daños y pérdidas causados por *F. circinatum*.

2. Un total de 139 endófitos de entre los 547 estudiados, controlaron el crecimiento in vitro de *F. circinatum* según al menos uno de los indicadores de antagonismo utilizados. Un alto porcentaje de estos aislamientos de endófitos pertenecieron a especies de *Trichoderma*, un género reconocido por su potencial en biocontrol.

3. Dos aislamientos de hongos endófitos pertenecientes a las especies *C. aureum* y *Alternaria* sp. redujeron sustancialmente los síntomas causados por *F. circinatum* sobre las plantas de *P. radiata*. Estos aislamientos tienen potencial para ser utilizados como BCAs de la enfermedad, pero todavía necesitan ser estudiados más a fondo. Por el contrario, estos hongos así como los otros evaluados no causaron efecto alguno sobre el resto de las especies ensayadas.

4. Tres nuevas cepas del género *Mitovirus*, Fusarium circinatum mitovirus 1 (FcMV1), FcMV2-1 y FcMV2-2, fueron detectadas en un aislamiento de *F. circinatum* y su genoma secuenciado. Estos son los primeros virus encontrados en *F. circinatum*, y es también la primera vez que se describen secuencias de mitovirus en una especie del género *Fusarium*.

5. Los virus FcMV1, FcMV2-1 y FcMV2-2, así como sus variantes, fueron relativamente comunes en España. Por el contrario no se detectaron partículas de dsRNA en la colección de aislamientos de *F. circinatum* sudafricana ni en los aislamientos de otras especies de *Fusarium*. El hecho de que las poblaciones española y sudafricana tengan diferente origen podría ser la razón de las diferencias encontradas en la presencia de los virus.

6. De acuerdo con los resultados de este estudio, el uso de métodos ambientalmente respetuosos para gestionar la enfermedad del PPC es factible. Sin embargo, son necesarios más estudios para minimizar los daños causados por el patógeno del PPC en los viveros así como en los árboles adultos en el monte mediante estas técnicas.

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Original article I

Martínez-Álvarez, P.; Pando, V. & Diez, J.J. 2014. Alternative species to replace Monterey pine plantations affected by pitch canker caused by *Fusarium circinatum* in northern Spain. *Plant Pathology* 63(5): 1086-1094.

Resumen

Especies alternativas para reemplazar las plantaciones de pino radiata afectadas por la enfermedad del chancro resinoso del pino causada por *Fusarium circinatum* en el norte de España.

El patógeno causante de la enfermedad del chancro resinoso del pino, *Fusarium circinatum*, fue encontrado por primera vez en 2004 en el norte de España causando daños en viveros forestales y en plantaciones de pino. Desde entonces se ha reducido el número de plantaciones establecidas en la región como resultado de la prohibición de plantar *Pinus* spp. y *Pseudotsuga menziesii* en las áreas afectadas por la enfermedad. Sin embargo, aunque la mayoría de las especies de pino son susceptibles al patógeno en la fase de vivero, aun no se tiene mucha información sobre el efecto del hongo en los árboles adultos en el monte. Además no se conoce la susceptibilidad a *F. circinatum* de algunas de las especies, tanto nativas como exóticas, plantadas en la zona. El objetivo del estudio fue evaluar la susceptibilidad de varias coníferas comunmente plantadas en el norte de España al patógeno del chancro resinoso del pino. Para ello se llevaron a cabo dos experimentos diferentes, uno de ellos en condiciones de laboratorio y otro en el campo. Aunque la mayor parte de las coníferas se vieron afectadas por el patógeno en el ensayo de laboratorio, solamente *Pinus radiata*, *Pinus nigra*, *Pinus pinaster* y *Pinus uncinata* fueron susceptibles al patógeno en el campo.

Palabras clave: conífera, emergencia, ensayo de campo, *Pinus radiata*, supervivencia, susceptibilidad.

Plant Pathology (2014) **63**, 1086–1094 Doi: 10.1111/ppa.12187

Alternative species to replace Monterey pine plantations affected by pitch canker caused by *Fusarium circinatum* in northern Spain

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The pitch canker pathogen *Fusarium circinatum* was first found to cause damage in nurseries and pine plantations in northern Spain in 2004. Since then, establishment of pine plantations in the region has decreased as a result of the prohibitions placed on planting *Pinus* spp. and *Pseudotsuga menziesii* in areas affected by the disease. However, although most pine species have been found to be susceptible to the pathogen under nursery conditions, little is known about how the fungus affects the trees in the field. Furthermore, it is not known whether some of the native or exotic species commonly planted in the area are also susceptible to *F. circinatum*. The aim of this study was to evaluate the susceptibility of several conifer species commonly planted in northern Spain to the pitch canker pathogen. For this purpose, two different trials were carried out, one under controlled laboratory conditions and the other in the field. Although most of the conifers were affected by the pathogen in the laboratory tests, only *Pinus radiata*, *Pinus nigra*, *Pinus pinaster* and *Pinus uncinata* were susceptible to the pathogen in the field.

Keywords: conifer, emergence, field test, *Pinus radiata*, survival, susceptibility

Introduction

Monterey pine (*Pinus radiata*) is widely distributed throughout the world. In Spain, it occupies an area of 215 000 ha, almost entirely in the northern coastal region (Fernández & Sarmiento, 2004). It is highly productive in this region because the climate is conducive to fast growth of the trees. However, the species is particularly susceptible to pitch canker disease (Correll *et al.*, 1991), caused by *Fusarium circinatum* (Nirenberg & O'Donnell, 1998). This ascomycete fungus causes damping off, shoot and tip die-back and also death of the seedlings in nurseries (Viljoen *et al.*, 1994). In adult trees, the most important symptom is a bleeding resinous canker on the main stem, terminals and large branches (Hepting & Roth, 1946), although the pathogen also causes shoot die-back (Correll *et al.*, 1991). Some trees eventually die, often as a result of stem breakage as a result of loss of structural integrity at the site of canker formation.

Pitch canker is one of the most important diseases of pine species worldwide. Apart from Spain, it is already present in the USA (Hepting & Roth, 1946), Mexico (Guerra-Santos, 1998), Haiti (Hepting & Roth, 1953), South Africa (Viljoen *et al.*, 1994; Coutinho *et al.*, 2007), Japan (Muramoto & Dwinell, 1990), Chile (Wingfield *et al.*, 2002), Korea (Cho & Shin, 2004), France (EPPO, 2004), Italy (Carlucci *et al.*, 2007), Portugal (Bragança *et al.*, 2009) and Uruguay (Alonso & Bettucci, 2009). In the European and Mediterranean Plant Protection Organization (EPPO) region, it appears on the list of pests (A2) recommended for regulation as quarantine pests (EPPO, 2005). The first report of the disease in Spain was made in 2005 by Landeras *et al.* (2005), although the pathogen is thought to have caused damage in Spain since 1997 (Dwinell *et al.*, 1998). A Royal Decree, which appeared after the official detection of the pathogen, forbids, among other activities, planting *Pinus* spp. and *Pseudotsuga menziesii* in the affected areas (Ministerio de Agricultura Pesca y Alimentación, 2006). Production of timber in some of the infected provinces has decreased significantly since implementation of these measures. Nursery losses have also been substantial, as all susceptible material must be destroyed when an infected seedling is detected. A few nurseries in Spain continue to produce Monterey pine seedlings despite the high risk of infection by *F. circinatum* and its devastating consequences. However, forest owners are now asking for an alternative crop to plant on their land.

Several conifer species have been tested for their susceptibility to *F. circinatum*. However, the effect of the pitch canker pathogen on some of the native or introduced species planted in Spain has not yet been studied. Moreover, some species tested for susceptibility to *F. circinatum* in other countries have not yet been tested in the environmental conditions of northern Spain. Although many authors have focused their efforts on

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Published online 20 January 2014

Plant Pathology

Pinus spp., further research is needed to detect other possible resistant genera. The aims of this study were therefore: (i) to examine the effect of *F. circinatum* on different conifer species to determine whether the pathogen may threaten other hosts present in the country, and (ii) to identify possible alternatives to Monterey pine for planting in northern Spain.

Materials and methods

Laboratory experiment

Fifteen species of conifers were tested for susceptibility to *F. circinatum* in a laboratory assay (Table 1). In the summer of 2009, 224 seeds per species were sown in nursery trays filled with peat and vermiculite (1:1, v:v), which was previously autoclaved twice for 1 h at 120°C. Prior to sowing, the seeds were washed repeatedly with sterile distilled water (SDW) and then soaked in SDW for 12 h to promote germination. The seeds were then soaked in hydrogen peroxide (3%) for 30 min and finally washed twice with SDW to remove the remaining hydrogen peroxide.

Half of the seeds were sown in planting medium inoculated with *F. circinatum* isolate FcCa6 (as described below), collected 1 year before from a *P. radiata* plantation in Cantabria (Spain). The virulence of this isolate has already been demonstrated (Martínez-Álvarez *et al.*, 2012). This isolate belongs to mating type 2 (MAT-2), which is the most frequent in the area. The other half of the seeds were sown in medium that was not inoculated (treatment control).

Table 1 Species used in the laboratory and field trials and their respective provenances

	Provenance of the seedlings	
Species	Laboratory assay	Field assay
Abies alba	ES02 Pirineo Central	ES02 Pirineo Central
Cedrus atlantica	–	Unidentified
Chamaecyparis lawsoniana	Navarra	Unidentified
Cupressocyparis leylandii	–	Unidentified
Juniperus thurifera	ES26 Serranía de Cuenca	–
Larix decidua	Germany 83702	–
Libocedrus decurrens	Segovia	–
Picea abies	East Europe	East Europe
Pinus pinaster	ES11 Rodenales de Molina	ES08 Meseta castellana
Pinus nigra corsicana	Corsica	902 Sud-ouest (France)
Pinus radiata	03 Litoral astur-cantabro-Galicia	Unidentified
Pinus strobus	USA	–
Pinus sylvestris	Sierra de Guadarrama	ES10 Sierra de Guadarrama
Pinus uncinata	Pirineo Central	ESC Sierra de Gúdar
Pseudotsuga menziesii	USA 430 Washington	430 Washington, Randle
Sequoia sempervirens	Navarra	–
Sequoiadendron giganteum	USA	Unidentified
Thuja plicata lobbii	–	Unidentified

For inoculation, 1 mL of a spore suspension (10^6 spores mL^{-1}) was added to the substrate in each pot. To prepare the inoculum, four pieces of mycelium grown in potato dextrose agar (PDA) amended with 0·5 g L^{-1} streptomycin sulphate (to prevent bacterial growth) were placed in an Erlenmeyer flask containing 50 mL malt extract medium (20 g L^{-1}). Spore production was induced in an orbital shaker and spores were recovered from the culture by filtration through cheesecloth to remove mycelium from the suspension. The concentration of the spores was determined with a haemocytometer, and was adjusted to 10^6 spores mL^{-1} with SDW.

Seed emergence and the number of healthy seedlings were enumerated weekly. At the end of the experiment, 90 days after inoculation, *F. circinatum* was reisolated from the seedlings (10% per treatment were checked) to verify its presence in the necrotic lesions.

Field trial

Four plots in northern Spain were selected for the study. Three of the plots were located beside *P. radiata* plantations severely affected by pitch canker disease; the fourth plot was established more than 10 km from plantations of susceptible species affected by the disease, for use as controls. The characteristics of the plots are shown in Table 2. The susceptibility of 13 conifer species (Table 1) was tested. Twelve seedlings per species in each location were planted manually (2 × 2 m spacing) in June 2009. Each plot was fenced off to prevent the trees being damaged by wild and domestic herbivores.

In November 2010, seedlings were inoculated with *F. circinatum*. Different isolates of the pathogen were used in each of the three plots. To prevent the introduction of new fungal material to the area, each isolate was obtained from the nearest diseased pine growing outside the plot in which seedlings were being tested. Xylem next to the canker was extracted with the aid of an axe and isolated in the laboratory, as described by Martínez-Álvarez *et al.* (2012). To identify the isolates, morphological characteristics such as size, shape and colour of spores and other reproductive structures such as polyphialides and sterile coiled hyphae were used. Genomic DNA was extracted following the protocol described by Vainio *et al.* (1998) and subjected to PCR using the specific primers for *F. circinatum* CIRC1A and CIRC4A, described by Schweigkofler *et al.* (2004), and Dynazyme II DNA polymerase, according to the manufacturer's recommendations (Finnzymes Ltd.). The mating type of each isolate was also determined by PCR with Dynazyme II DNA polymerase and the primers GcHMG1, GcHMG2, MAT1p2 and MAT1p3 as described by Wallace & Covert (2000) and Schweigkofler *et al.* (2004).

The spore suspension used in the inoculation was prepared in the same way as for the laboratory experiment, and the concentration of the spores was likewise adjusted to 10^6 spores mL^{-1}. The trees were inoculated in the field by making a wound with a scalpel in the stem of the seedling and pouring 10 μL of the spore solution into the wound. The wound was then covered with a strip of Parafilm to prevent desiccation.

Plots were visited every 2 months to measure collar diameter, height and diameter of the crown of the seedlings. The seedlings were also scored for disease symptoms following the method proposed by Correll *et al.* (1991), in which each inoculation was rated on a scale of 0 (healthy) to 4 (branch girdled and foliage dead distal to the point of inoculation). Disease progress

Table 2 Characteristics of the sites where the plots for the field trial were installed

	HER	SAN	SSG	VIL
Location	Hermosa	Santibañez	San Sebastian de Garabandal	Villafufre
Municipality	Medio Cudeyo	Cabezón de la Sal	Rionansa	Villafufre
Altitude (m a.s.l.)	190	340	500	510
Orientation	Northeast	North	Southeast	Southwest
Slope (%)	20	35	70	30
Annual precipitation (mm)	1581	1133	1160	1650
Mean temperature (°C)	14	13	13	12
Minimum mean temperature (°C)	9	8	7	7
Maximum mean temperature (°C)	19	18	19	17
UTM coordinate X	439743	398672	384887	427396
UTM coordinate Y	4800821	4792705	4784978	4793017

Climate data according to Atlas Climático Digital de la Península Ibérica (Ninyerola *et al.*, 2005). UTM coordinates in European Terrestrial Reference System 1989 (ETRS89) spindle 30.

curves for each plant were constructed by plotting the scores against time. The area under the disease progress curve (AUDPC) was calculated as the sum of the area of the corresponding trapezoids.

Data analysis

Laboratory experiment

A mixed-model ANOVA with two between-subjects factors (treatments and species) and one within-subjects factor of repeated measures (time of measurement) was used to evaluate the emergence and survival of seedlings. Multiple comparisons were made with the data of the last measure using the Tukey–Kramer test. All analyses were carried out using SAS v. 9.1 software.

Field trial

A mixed-model ANOVA with two between-subjects factors (plot and species) and one within-subjects factor of repeated measures (time of measurement) was used to evaluate the dependent variables collar diameter, height and diameter of the crown of the seedlings. A mixed-model ANOVA with a factorial design was used to study the variable AUDPC, considering the plots (four levels) and species (seven levels, the species which showed symptoms in any of the plots or visits) as factors. Multiple comparisons were made using Fisher's LSD test. All analyses were carried out using SAS v. 9.1 software.

Results

Laboratory experiment

The analysis revealed statistically significant differences among species, treatment and time of measurement in seedling emergence, as well as in the interaction between species and the time of measurement (Table 3). The emergence rate 90 days after sowing was highest for *Pinus sylvestris* (94·6%) and *P. radiata* (92·9%) in the control treatment. In contrast, emergence of *Sequoia sempervirens*, *P. menziesii*, *Libocedrus decurrens* and *Juniperus thurifera* was very low (<10%) in both treatments, and therefore these species were not included in the statistical analysis.

Inoculation of *F. circinatum* had different effects on seedling emergence, depending on the species. In *Abies alba*, *Larix decidua*, *Pinus nigra*, *Pinus pinaster*, *P. radiata*, *Pinus strobus*, *Pinus sylvestris* and *Pinus uncinata*, the emergence rate was lower when the pathogen spore suspension was added to the substrate than in the controls ($P < 0{\cdot}0001$ in each case; Fig. 1). In contrast, seedling emergence was not reduced in *Chamaecyparis lawsoniana*, *Picea abies* or *Sequoiadendron giganteum*. The analysis also revealed differences in seedling survival between species, treatments and the interaction, but not between the measurement times (Table 3). Ninety days after the beginning of the experiment, differences between treatments were evident in most species. The only species with no significant difference in survival rate of inoculated and control (uninoculated) seedlings was *C. lawsoniana* (Fig. 2). In the other species, between 1·35% (*P. radiata*) and 70·0% (*S. giganteum*) of the inoculated seedlings were still alive at the end of the experiment.

Field trial

The dependent variables total height, collar diameter and crown diameter of seedlings were significantly affected by site, species, time of measurement and the interactions between these factors (Table 4). The relative increases in the variables total height, collar diameter and crown diameter of seedlings during the 3 years of the experiment are shown in Table 5. Increases in total height and collar diameter during the assay were greatest in *P. pinaster* and *P. radiata* at the control site.

Although *F. circinatum* was present in the area, natural infection did not occur and none of the seedlings displayed symptoms of the disease 17 months after the plantations were established. At this point, it was decided to inoculate the conifers with a local strain of *F. circinatum* at each site, to further test their susceptibility.

Table 3 Three-way ANOVA table for emergence and survival of seedlings in the laboratory trial

Dependent variable	Source	d.f.	*F*-value	*P*-value
Emergence	Treatment	1	332·29	<0·0001
	Species	10	1060·47	<0·0001
	Time of measurement	4	4·12	0·0029
	Treatment*Species	10	50·91	<0·0001
	Treatment*Time of measurement	4	0·36	0·8373
	Species*Time of measurement	40	1·69	0·0077
	Treatment*Species*Time of measurement	40	0·40	0·9996
Survival	Treatment	1	2590·90	<0·0001
	Species	10	128·86	<0·0001
	Time of measurement	4	1·50	0·2009
	Treatment*Species	10	139·09	<0·0001
	Treatment*Time of measurement	4	0·52	0·7224
	Species*Time of measurement	40	0·74	0·8337
	Treatment*Species*Time of measurement	40	0·90	0·6429

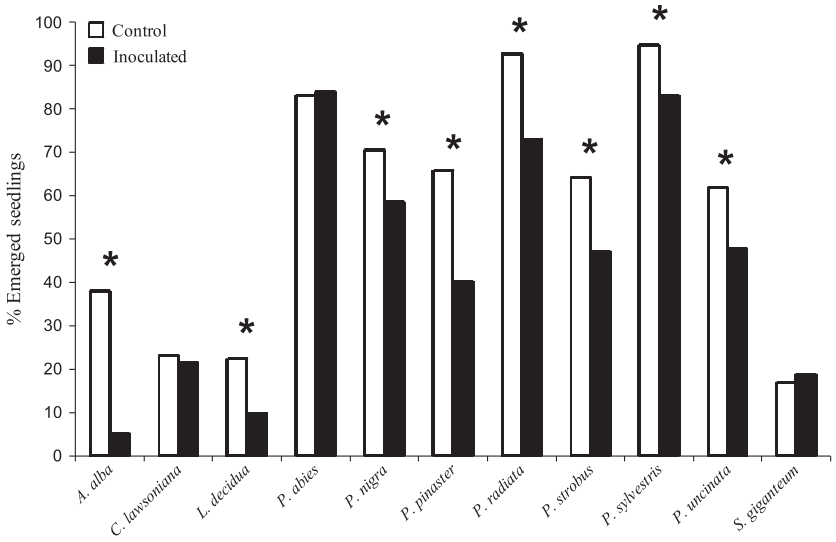

Figure 1 Percentage of emerged seedlings of 11 species grown in substrate inoculated with *Fusarium circinatum*. Asterisk denotes significant difference ($P < 0·0001$) between plants grown in inoculated substrate and control plants in uninoculated substrate (Tukey–Kramer multiple range test).

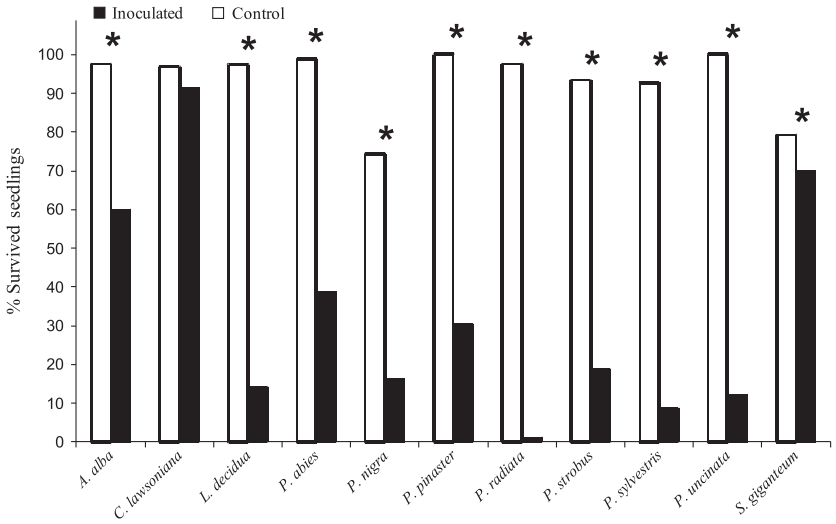

Figure 2 Percentage seedling survival of 11 species grown in substrate inoculated with *Fusarium circinatum*. Asterisk denotes significant difference ($P < 0·05$) between plants grown in inoculated substrate and control plants in uninoculated substrate (Tukey–Kramer multiple range test).

Of the species tested, *P. radiata* was the most susceptible to the pitch canker disease pathogen. The AUDPC for this species, considering all plots, was about five times larger than for *P. nigra* and *P. pinaster*, the second and third species in the ranking ($P < 0{\cdot}0001$). In contrast, the AUDPC for *A. alba*, *Cedrus atlantica*, *C. lawsoniana*, *Cupressocyparis leylandii*, *S. giganteum* and *Thuja plicata* was zero because none of the seedlings presented symptoms of the disease in any of the plots or visits. The AUDPC values for each species in the four plots established are shown in Figure 3.

In general, trees in the Santibañez (SAN) and San Sebastian de Garabandal (SSG) plots displayed more symptoms of disease than trees in the Villafufre (VIL) plot ($P = 0{\cdot}019$ and $0{\cdot}021$ respectively). On the other hand, damage was insignificant in the seedlings in the control plot in Hermosa (HER). For *P. radiata*, the AUDPC was largest in the SSG plot and was significantly larger than those in the VIL and control (HER) plots ($P = 0{\cdot}001$ and $<0{\cdot}0001$, respectively), but not significantly different from that of plot SAN ($P = 0{\cdot}080$). *Pinus nigra* and *P. radiata* were the only species found to be susceptible to pitch canker in the three inoculated plots. However, while 67% of the *P. radiata* seedlings died as a result of infection by the pathogen by the end of the assay, no seedlings of *P. nigra* died. For *P. pinaster* and *P. uncinata*, the AUDPC was only different from corresponding control AUDPC in plots VIL and SAN, respectively. The AUDPC values for *P. sylvestris*, *P. abies* and *P. menziesii* were not different in the inoculated plots and control plot.

Discussion

Pitch canker disease of pines caused by *F. circinatum* was first officially recognized in Spain in 2005 (Landeras *et al.*, 2005). In areas where the pathogen has been detected, planting species that are susceptible to the disease is forbidden (Ministerio de Agricultura Pesca y Alimentación, 2006). In the national programme designed to eradicate and control the pathogen (Ministerio de Agricultura Pesca y Alimentación, 2006), only *Pinus* spp. and *P. menziesii* are considered susceptible to the fungus. However, many species used in plantations in northern Spain have not been tested for their susceptibility to the pathogen. Moreover, most studies testing the effect of the pathogen on different hosts have been carried out with seedlings under controlled nursery conditions (Viljoen *et al.*, 1995; Pérez-Sierra *et al.*, 2007; Kim *et al.*, 2008). As the environment plays an important role in the interaction between the pathogen and the plant, the current study tested the susceptibility of different species using seedlings in controlled conditions in both the laboratory and the field, in forest areas where the pathogen occurs naturally.

The results of both laboratory and field trials confirmed that Monterey pine is highly susceptible to pitch canker disease, as previously reported (McCain *et al.*, 1987; Gordon *et al.*, 1998; Landeras *et al.*, 2005; Martínez-Álvarez *et al.*, 2012). In Spain, *P. radiata* is also the species most affected by the fungus. *Pinus pinaster* plantations are also affected (Pintos *et al.*, 2006; Vivas *et al.*, 2011), although the symptoms and yield losses are much lower than in *P. radiata*. Susceptibility of some other species such as *P. menziesii*, *C. lawsoniana* or *P. abies*, occasionally planted in northern Spain to produce timber, is not known. Thus, to the authors' knowledge, this is the first time a trial has been carried out to test the effect of *F. circinatum* on *A. alba*, *C. lawsoniana*, *L. decidua*, *P. abies*, *P. uncinata*, *C. atlantica*, *C. leylandii* and *T. plicata*.

The results of the laboratory and field trials were quite different. In the laboratory experiment, all species, except *C. lawsoniana*, were affected by the pathogen,

Table 4 Three-way ANOVA table for the total height, collar diameter and crown diameter of seedlings in the field trial

Dependent variable	Source	d.f.	*F*-value	*P*-value
Total height	Site	3	429·11	<0·0001
	Species	12	776·77	<0·0001
	Time of measurement	10	1357·4	<0·0001
	Site*Species	36	30·15	<0·0001
	Site*Time of measurement	30	151·87	<0·0001
	Species*Time of measurement	120	57·04	<0·0001
	Site*Species*Time of measurement	360	8·71	<0·0001
Collar diameter	Site	3	639·6	<0·0001
	Species	12	256·5	<0·0001
	Time of measurement	10	1008	<0·0001
	Site*Species	36	38·56	<0·0001
	Site*Time of measurement	27	188·88	<0·0001
	Species*Time of measurement	120	30·33	<0·0001
	Site*Species*Time of measurement	324	8·26	<0·0001
Crown diameter	Site	3	588·67	<0·0001
	Species	12	272·79	<0·0001
	Time of measurement	10	923·73	<0·0001
	Site*Species	36	28·21	<0·0001
	Site*Time of measurement	30	127·93	<0·0001
	Species*Time of measurement	120	24·43	<0·0001
	Site*Species*Time of measurement	360	6·51	<0·0001

Table 5 Relative increase in collar diameter, total height and crown diameter in 13 coniferous species at four locations over a period of 3 years

	HER (control)		SAN		SSG		VIL	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Collar diameter								
A. alba	2·34	0·58	0·75	0·28	0·83	0·21	0·92	0·39
C. atlantica	5·44	1·59	0·86	0·28	0·68	0·28	1·15	0·46
C. lawsoniana	15·07	4·77	3·32	2·53	3·41	3·22	3·13	1·11
C. leylandii	9·51	1·98	1·53	0·56	1·49	0·50	1·83	0·37
P. abies	3·43	1·68	0·94	0·94	0·51	0·31	1·11	0·79
P. nigra	8·14	2·53	3·34	0·73	3·19	1·19	3·64	0·94
P. pinaster	15·5	2·63	5·83	1·99	5·32	1·61	6·76	2·80
P. radiata	19·78	3·51	2·43	0·26	5·69	–	3·33	1·77
P. sylvestris	7·42	1·86	3·68	1·33	3·69	1·47	4·15	1·37
P. uncinata	2·95	0·79	1·24	0·38	1·26	0·70	1·66	0·36
P. menziesii	6·53	3·07	1·44	1·46	1·19	0·36	1·73	0·74
S. giganteum	2·94	0·72	0·92	0·19	0·58	0·29	0·93	0·19
T. plicata	4·79	0·95	0·84	0·29	0·76	0·28	0·71	0·27
Total height								
A. alba	2·59	0·99	1·09	0·50	1·29	0·65	0·92	0·26
C. atlantica	5·50	1·48	0·97	0·37	0·55	0·24	1·14	0·47
C. lawsoniana	7·75	1·96	3·18	1·81	2·05	0·46	2·79	0·82
C. leylandii	3·21	0·55	1·33	2·32	0·29	0·14	0·79	0·21
P. abies	3·23	1·09	0·61	0·49	0·58	0·40	0·96	0·39
P. nigra	6·59	1·87	4·17	0·84	4·05	1·17	3·83	1·37
P. pinaster	8·92	1·62	5·80	1·47	5·48	1·46	6·00	2·12
P. radiata	14·89	4·39	4·18	1·05	6·25	–	3·93	1·50
P. sylvestris	4·07	1·18	2·14	0·75	2·49	1·34	2·29	0·61
P. uncinata	0·91	0·61	0·45	0·18	0·38	0·29	0·48	0·19
P. menziesii	4·48	4·53	0·96	0·84	0·90	0·38	1·46	0·67
S. giganteum	2·58	4·88	0·86	0·26	0·26	0·13	0·56	0·19
T. plicata	2·22	0·29	1·00	0·27	0·76	0·15	1·12	1·20
Crown diameter								
A. alba	8·55	2·48	3·37	1·49	3·22	1·25	2·49	0·99
C. atlantica	8·20	2·44	1·89	0·35	1·36	0·71	2·48	0·53
C. lawsoniana	8·60	2·98	3·95	2·39	2·78	1·00	3·58	2·01
C. leylandii	10·88	2·48	2·18	0·70	1·14	0·36	3·28	1·42
P. abies	5·59	1·79	1·62	1·12	1·33	0·61	3·09	2·24
P. nigra	5·24	1·34	2·79	0·88	2·17	0·69	3·13	1·08
P. pinaster	15·34	4·01	7·85	1·70	8·13	1·62	7·99	1·95
P. radiata	16·84	3·94	3·29	1·26	5·27	–	2·42	0·94
P. sylvestris	6·74	3·52	4·84	2·56	3·29	1·62	2·92	1·51
P. uncinata	1·84	0·53	1·51	0·59	1·76	0·60	1·80	0·88
P. menziesii	2·49	0·63	1·21	0·91	1·33	0·84	1·65	0·63
S. giganteum	2·62	1·00	1·57	0·65	0·95	0·47	4·48	9·66
T. plicata	3·82	1·22	0·83	0·37	0·46	0·29	0·84	0·46

whereas in the field trial only *P. radiata* (in every plot), *P. nigra* (in every plot), *P. pinaster* (in VIL) and *P. uncinata* (in SAN) showed any effects relative to the control plot. This may be as a result of the age of the seedling, the environment and the amount of inoculum. The tissue of the newly germinated seedlings in the laboratory trial was soft and easy for the pathogen to invade. However, the tissue of seedlings planted in the field was more lignified and the defences of the seedlings were better developed, so that it would be more difficult for the pathogen to infect the tissues. In addition, the temperature in the field was not always optimal for growth of the fungus, unlike in the laboratory where it was controlled. The lack of correspondence between susceptibility at emergence and in older trees has been reported previously (Aegerter & Gordon, 2006). High mortality rates were also observed in newly germinated *P. radiata* seedlings (Martínez-Álvarez *et al.*, 2012) and in 10-month-old *Pinus patula* seedlings (Porter *et al.*, 2009).

Although the inoculation was made in the same way in all seedlings, and despite the high susceptibility of *P. radiata*, in the field trial some of the seedlings of this species (30·6%) did not display important symptoms of the disease or they recovered from initial resin bleeding. Resistance to pitch canker has been observed in *P. radiata* (Gordon *et al.*, 1998; Storer *et al.*, 1999; Matheson *et al.*, 2006). Resistance levels of 30% in natural stands,

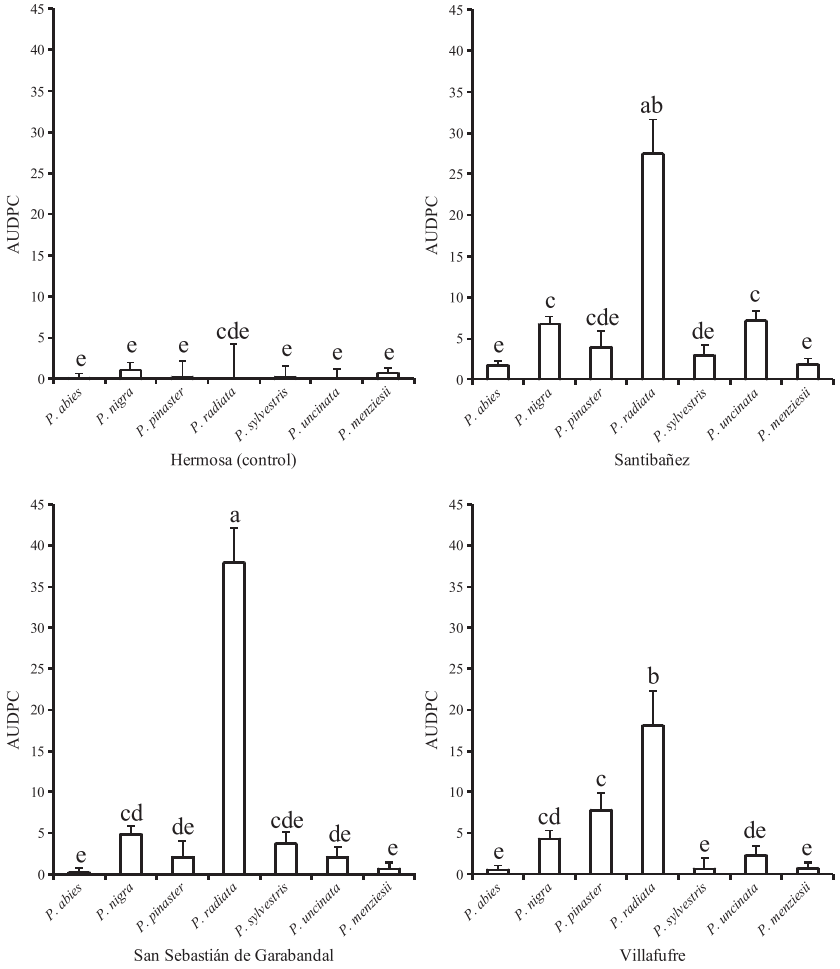

Figure 3 Area under the disease progress curve (AUDPC) for seven species at four locations. Only those species that displayed symptoms of disease in any of the plots or visits are shown in the figure. Different letters (a–e) denote significant differences ($P < 0{\cdot}05$) among all columns (Tukey–Kramer multiple range test).

and 5% in plantations, have been reported (Storer *et al.*, 1999).

In the present field trial, neither *P. sylvestris* nor *P. menziesii* was susceptible to pitch canker disease, although mortality was observed in the laboratory trial. These species have been described as susceptible in many other studies (McCain *et al.*, 1987; Enebak & Stanosz, 2003; Gordon *et al.*, 2006; Pérez-Sierra *et al.*, 2007). However, susceptibility sometimes differs more among provenances than among species (Hansson, 1998). The findings of the current study explain why no symptoms of the disease have been reported in *P. sylvestris* in nature, although many plantations of this species are located beside severely affected *P. radiata* plantations, for example in Cantabria. However, these species are strongly affected by the pathogen in nurseries, as shown in the laboratory trial and in some other studies (McCain *et al.*, 1987; Enebak & Stanosz, 2003; Gordon *et al.*, 2006; Pérez-Sierra *et al.*, 2007), and therefore strict phytosanitary measures are needed.

Although the experimental plots were established next to severely diseased *P. radiata* plantations (except the control plot), none of the seedlings displayed symptoms of the disease in the first year and a half after planting. Therefore, natural infection does not occur as readily as was expected, at least at the young stage of the host. It is possible that in most of the diseased plantations in northern Spain either (i) the seedlings were infected in nurseries in the years preceding the first report of the disease, as seedlings can be infected without showing any symptoms (Storer *et al.*, 1998), or (ii) the trees were infected at a later stage through the wounds caused by pruning (Bezos *et al.*, 2012). However, transmission can also occur via insects, as demonstrated for *Pythiopthorus* spp. in California (Storer *et al.*, 2004). In fact, further experiments are currently in progress to test the role of *Tomicus piniperda* as a vector of the disease in this region (D. Bezos, University of Valladolid, Spain, personal communication).

Abies alba, *C. atlantica*, *C. lawsoniana*, *C. leylandii*, *S. giganteum* and *T. plicata* did not display any symptoms of the disease, and therefore the AUDPC for all of these species was zero. Among these, only *S. giganteum* has been tested previously for its susceptibility to *F. circinatum* (McCain *et al.*, 1987), and the present findings are consistent with the previous study. All of these species can be used in plantations in the areas affected by the disease because there is no risk of infection by the pathogen and

planting is not prohibited (Ministerio de Agricultura Pesca y Alimentación, 2006). In particular, some plantations of *C. lawsoniana* have been established in the Cantabria region and their growth is promising. Although no information is available about the susceptibility of *S. sempervirens* to the pathogen in the field, trees in a single stand established in Cantabria around 70 years ago are now more than 40 m tall, with large volumes of timber being produced and no symptoms of the disease. Because of the uniqueness and size of the trees, this stand was declared a Natural Monument in 2003 and included in the Network of Protected Natural Areas in Cantabria.

Further studies are required to fully establish the susceptibility of the most important European conifer species to *F. circinatum*. In the case of the widespread species *P. sylvestris*, such studies are essential to prevent the rapid spread of the disease from southern to northern Europe.

Acknowledgements

This research was financially supported by the Government of Cantabria, the Ministry of the Environment and Rural and Marine Affairs, Ministry of Agriculture and INIA. The authors also thank Milagros de Vallejo, Juan Blanco and the technical staff at the 'Dirección General de Montes y Conservación de la Naturaleza (Government of Cantabria)' for help in establishing the plots used in the field trial.

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Original article II

Martínez-Álvarez, P.; Alves-Santos, F.M. & Diez, J.J. 2012. *In vitro* and *in vivo* interactions between *Trichoderma viride* and *Fusarium circinatum*. *Silva Fennica* 46(3): 303-316.

Resumen

Interacciones *in vitro* e *in vivo* entre *Trichoderma viride* y *Fusarium circinatum*

Fusarium circinatum, el hongo causante de la enfermedad del chancro resinoso de los pinos, está presente en Europa desde al menos el año 2003, cuando fue detectado en el norte de España causando daños severos en viveros forestales y plantaciones de pino. En este estudio se evaluó un método de control biológico de la enfermedad con *Trichoderma viride*, un hongo empleado de manera exitosa contra muchos otros patógenos. Se llevaron a cabo ensayos tanto *in vitro* como *in vivo* para evaluar la eficacia del antagonismo producido por esta especie fúngica frente a *F. circinatum*. El aislamiento de *T. viride* redujo a la mitad y de manera significativa la longitud de la colonia de *F. circinatum* en el ensayo *in vitro*. Sin embargo, aunque se probaron tres concentraciones diferentes de inoculo de *T. viride*, no pudieron obtenerse conclusiones claras con respecto al efecto producido sobre las plantas de *Pinus radiata*. A nuestro conocimiento, este es el primer estudio llevado a cabo con el objetivo de utilizar *Trichoderma* spp. para controlar la enfermedad del chancro resinoso del pino.

Palabras clave: chancro resinoso, antagonismo, biocontrol, agentes de control biológico, endofitos, *Pinus radiata*, España.

SILVA FENNICA

Silva Fennica 46(3) research articles

www.metla.fi/silvafennica · ISSN 0037-5330
The Finnish Society of Forest Science · The Finnish Forest Research Institute

In Vitro and In Vivo Interactions between *Trichoderma viride* and *Fusarium circinatum*

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Martínez-Álvarez, P., Alves-Santos, F.M & Diez, J.J. 2012. In vitro and in vivo interactions between *Trichoderma viride* and *Fusarium circinatum*. Silva Fennica 46(3): 303–316.

Fusarium circinatum, a fungus that causes pitch canker disease, has been present in Europe since at least 2003, when it was detected in northern Spain and found to be producing severe damage in tree nurseries and pine plantations. In this study, we tested a method of biological control of the disease with *Trichoderma viride*, a fungal species successfully used against many other pathogens. In vitro and in vivo assays were carried out to test the efficacy of this antagonist in controlling *F. circinatum*. The *T. viride* isolate exerted a significant effect on the growth of *F. circinatum* in the in vitro assay, reducing the length of the pathogen colony by half. However, although we tested three different concentrations of the *T. viride* spore solution, no clear conclusions were obtained with regard to the effects on the *Pinus radiata* seedlings. To our knowledge, this is the first study carried out with the aim of using *Trichoderma* spp. to control pitch canker disease.

Keywords pitch canker, antagonism, biocontrol, biological control agents, endophytes, *Pinus radiata*, Spain
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Received 18 November 2011 **Revised** 3 May 2012 **Accepted** 20 June 2012
Available at http://www.metla.fi/silvafennica/full/sf46/sf463303.pdf

1 Introduction

Fusarium circinatum Nirenberg & O'Donnell (teleomorph = *Gibberella circinata*) is a highly virulent pathogenic fungus in species of the genus *Pinus*, causing a disease called pitch canker. It was first detected in 1945 in the southeastern United States (Hepting and Roth 1946) and was hypothesized to be endemic there and in Mexico (Gordon et al. 1996, Guerra-Santos 1998). Since then, *F. circinatum* has also been found in Haiti (Hepting and Roth 1953), South Africa (Viljoen et al. 1994, Coutinho et al. 2007), Japan (Kobayashi 2007), Chile (Wingfield et al. 2002), Korea (Cho and Shin 2004), France (EPPO 2004), Italy (Carlucci et al. 2007), Portugal (Bragança et al. 2009), Uruguay (Alonso and Bettucci 2009) and Spain (Landeras et al. 2005).

The most common symptom of the disease is a bleeding, resinous canker on the trunk, terminals or large branches (Hepting and Roth 1946). The canker is usually sunken and the bark is retained, whereas the wood beneath the canker is deeply pitch-soaked (Dwinell et al. 1985). The pathogen also causes shoot die-back in adult trees (Correll et al. 1991), and in seedlings, it causes damping off, shoot and tip die-back and death (Viljoen et al. 1994). Nowadays, *F. circinatum* is the most important pathogen of *Pinus* seedlings in several countries around the world (Coutinho et al. 2007, Jacobs et al. 2007, Pérez-Sierra et al. 2007). In Spain, the presence of the pathogen in nurseries and plantations has resulted in crop and yield losses, loss of revenue due to the high costs invested in monitoring and control, and an exportation ban (Pérez-Sierra et al. 2007). Taking into account the high risk of spread of the pathogen to other European countries, it is essential to advance rapidly in understanding and controlling the disease.

Monterey pine (*Pinus radiata* D. Don) is the exotic conifer most commonly used for reforestation in northern Spain, covering an area of approximately 200 000 ha (Hermoso et al. 2007). Plantations are located from sea level to an altitude of 500 meters in areas with mild temperatures and minimum annual precipitation of 900 mm (Ceballos and Ruiz de la Torre 1979). Although *P. radiata* occupies a relatively small area in comparison with native pines (3.6% of the total area covered by coniferous species), it provides 25% of the conifer timber in Spain (Hermoso et al. 2007). However, timber production may be in jeopardy because *P. radiata* and other members of the Attenuata Group (subsection Oocarpa) are the species considered most susceptible to pitch-canker (Gordon et al. 2001). In fact, it is currently difficult to obtain *P. radiata* seedlings, or even seeds, from forest nurseries in Spain because culture of this species has been abandoned in some regions. In response to these problems, the Spanish Government has developed a national programme aimed at evaluating the distribution of the disease, preventing its spread and developing control measures (Ministerio de Agricultura 2006).

Fungal endophytes, which colonize living plant tissues without causing any immediate negative effects (Hirsch and Braun 1992), are currently considered important because of their potential use in the biological control of plant diseases (Zabalgogeazcoa 2008). The use of endophytes may have some advantages over the use of chemicals, to which many organisms become resistant. *Trichoderma* is a frequent endophyte in conifers, and it is one of the most commonly used genera of fungi in the biological control of plant diseases (Rosa and Herrera 2009). *Trichoderma viride* Pers. is one of the species most widely known for its capacity to control plant diseases (Kolombet et al. 2001, Eslaminejad Parizi et al. 2012), in some cases caused by *Fusarium* spp. (John et al. 2010, Basak and Basak 2011). However, studies investigating the biological control of pitch canker disease are scarce (Romón et al. 2008), and to our knowledge the effect of *T. viride* on the pathogen *F. circinatum* has not yet been tested.

The aims of the present study were as follows: a) to analyze the possible antagonistic effects of *T. viride* on the in vitro growth of the pathogen *F. circinatum*, and b) to evaluate the potential use of *T. viride* for biocontrol of the pathogen in seeds and seedlings of Monterey pine.

2 Materials and Methods

2.1 Fungal Isolates

Eleven *P. radiata* plantations (in northern Spain), in which pitch canker disease was previously

Table 1. List of the sites sampled and their characteristics. UTM Coordinates in European Terrestrial Reference System 1989 (ETRS89) spindle 30. m.a.s.l. = meters above sea level.

Site number	Site code	Site name	Age of the plantation (years)	UTM Coordinates (x, y)	Altitude (m.a.s.l.)
1	SPR	San Pedro del Romeral	17–37	432325, 4778396	517
2	VIL	Villafufre	35	425804, 4792808	413
3	SSG	San Sebastian de Garabandal	5–18	383982, 4784771	510
4	COM	Comillas	9–36	395568, 4798793	265
5	VEJ	Vejorís	12–30	427391, 4783206	410
6	RVI	Ramales de la Victoria	15–25	462157, 4793728	406
7	SIB	Sierra de Ibio	15–25	407147, 4796973	161
8	IBI	Ibio	30	406643, 4792301	435
9	SAN	Santibañez	20	398930, 4792735	342
10	RCA	Renedo de Cabuérniga	30	395678, 4785428	485
11	ONT	Ontón	12	487282, 4797283	295

detected, were sampled in summer and autumn 2008 for isolation of *F. circinatum* and *Trichoderma* spp. The site characteristics are listed in Table 1. Needles, twigs, stem bark, stem xylem, cones and seeds were collected from four trees at each site. Stem xylem was extracted by two different procedures, with a Pressler borer and with an axe. The sampled material was selected and processed within 24 hours. The samples were surface sterilized before isolation of the endophytic fungi. Samples were washed in running tap water for one minute, soaked in 70% alcohol for two minutes, and soaked twice in 3% sodium hypochlorite solution, for two minutes each time. Finally, the samples were immersed twice in sterile distilled water, for two minutes each time, to remove any possible remains of the hypochlorite.

The sterilized fragments were placed on potato dextrose agar (PDA), enriched with 0.5 g/l of streptomycin sulphate (to prevent bacterial growth), in Petri plates. The plates were then incubated at room temperature. Three days later, growing mycelia were subcultured in fresh plates containing the same medium. The fungal cultures were stored at 25 °C in growth chambers in the dark for seven days, and then under normal laboratory conditions for another seven days. Fungal isolates were then classified into morphotypes. One colony of each fungal morphotype was conserved in Petri plates containing PDA at 4 °C until morphological identification was confirmed. Cultures were identified according to morphological characteristics such as size, shape and colour of spores and other reproductive structures. Different taxonomic keys were used for fungal identification (Sutton 1980, Hanlin 1990, Watanabe 1993, Kiffer and Morelet 1997, Leslie and Summerell 2006).

Molecular identification was performed to confirm *F. circinatum* and *T. viride* isolates. Genomic DNA was isolated following the protocol described by Vainio et al. (1998). Polymerase Chain Reaction (PCR) was then carried out with Dynazyme II DNA-polymerase, according to the conditions recommended by the manufacturer (Finnzymes Ltd, Espoo, Finland). The concentration of DNA used was 2 µM. A fragment of the IGS rDNA region (ca 360 bp) was amplified with specific primers for *F. circinatum* CIRC1A (5′-CTTGGCTCGAGAAGGG-3′) and CIRC4A (5′-ACCTACCCTACACCTCTCACT-3′), as described by Schweigkofler et al. (2004). The PCR programme consisted of an initial step of 3 min at 94 °C followed by 45 denaturation cycles at 94 °C for 35 s, annealing at 64 °C for 55 s, and an elongation at 72 °C for 50 s. The final extension was performed at 72 °C for 12 min. For *T. viride*, the ITS rDNA region (ca 600 bp) was amplified with primers 1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and 4 (5′-TCCTCCGCTTATTGATATGC-3′) (Vilgalys and Hester 1990). Samples were denaturized by incubation for 10 min at 95 °C, after which 34 cycles of amplification were carried out as follows: 13 times: 35 s at 95 °C, 55 s at 55 °C and 45 s at 72 °C; 13 times: 35 s at 95 °C, 55 s at 55 °C and 2 min at 72 °C and finally, 9 times: 35 s at 95° C, 55 s at 55 °C and 3 min at 72 °C. On completion

of these cycles, the reaction was followed by 7 min of extension at 72 °C. To ensure the identity of the *T. viride* isolate, the elongation factor 1 alpha (ca 700 bp) was also amplified with EF1 (5′-ATGGGTAAGGA(A/G)GACAAGAC-3′) and EF2 (5′-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3′) primers (O'Donnell et al. 1998). The samples were denaturized by incubation for 5 min at 95 °C, and were then subjected to 35 denaturation cycles at 94 °C for 50 s, annealing at 60 °C for 50 s, and elongation at 72 °C for 1 min.

DNA amplification products were checked under UV light after runs of 1 hour and 30 minutes in 1% TAE-buffer at 3 V/cm in 1% agarose gels (FMC BioProducts, Rockland, ME, USA) containing 1× TAE (40 mM Tris-acetate pH 8.0, 1 mM EDTA) and 10 µl of GelRedTM Nucleic Gel Acid Gel Stain (Biotium). The marker used to estimate the size of the amplification products was λ-DNA Hind III – ΦX174Hae III (DyNAzymeTM DNA Polymerase Kit).

In the case of the *T. viride* isolate, PCR products were purified with NucleoSpin® Extract II 10/2007 Rev. 06 (Macherey-Nagel Gmbh and Co.KG), and one µl of each purified PCR product was then pipetted into 9 µl of 1× tracking dye. The samples were then loaded on a 1% agarose gel containing 10 µl of GelRedTM and 1× TAE buffer. The runs were performed in 1× TAE buffer for 10–15 min at 90 V/30 cm, and the gels were then observed under UV light and photographed. The concentration was determined by visual comparison between each sample and a series of known standard concentrations of λ-DNA (5, 10, 20, 40, 80 and 160 ng/µl). The ITS rDNA and the elongation factor 1 alpha were sequenced by Secugen (Madrid, Spain). Sequences were obtained to determine preliminary identification at a higher taxonomy level by BLAST search. The sequences were submitted in the EMBL/GenBank database (accession number for ITS: HE802170; and for elongation factor 1 alpha: HE802169).

2.2 In Vitro Antagonism

Antagonism between *F. circinatum* and *T. viride* was studied on PDA. Seven representative isolates of the pathogen and one isolate of *T. viride* were tested. A square plug (4 mm of side) of growing mycelium taken from the pathogen was placed 10 mm from the edge of the plate. A similar plug of endophyte mycelium was placed in front of the pathogen and 10 mm from the opposite edge of the plate. Five replicates were prepared per treatment (pathogen × endophyte). The plates were maintained under laboratory conditions for six days and the increase in the mycelial length of *F. circinatum* colonies was measured along three axes from the middle of the plug, one joining both fungal plugs and the other two forming an angle of 45° with it, as described by Santamaría et al. (2007). The difference between the mean length of the lateral axes and the length of the middle axis was used as an indicator of the shape of the colony and therefore of the effect of *T. viride* on the growth of *F. circinatum*. When the value of the index was greater than one, the endophyte was considered to have reduced the growth of the pathogenic colony.

2.3 In Vivo Experiments

The same isolates used in the in vitro assay were also used in the in vivo experiments. Erlenmeyer flasks containing 50 ml of malt extract agar (MEA, 20 g/l) were inoculated with each *F. circinatum* isolate to achieve the spore suspension. The inoculum consisted of four pieces of fungal mycelium grown in PDA with streptomycin sulphate. Spore production was induced in an orbital shaker and the spores were recovered from culture by filtration through cheesecloth, to prevent the presence of mycelium in the solution. In the case of *T. viride*, spores were recovered by rinsing the Petri plates with sterile distilled water and filtering the resulting suspension. A haemocytometer was used to determine the concentration of the spores (10^6 spores/ml in the case of *F. circinatum* and 10^7 (T1), 10^6 (T2) and 10^5 (T3) spores/ml for *T. viride*).

A total of 2688 seeds of provenance "03 litoral astur-cántabro-Galicia", provided by the Consellería do Medio Rural (Xunta de Galicia, Spain), were sown to observe the effect of the fungi on the plant material. The seeds were first washed repeatedly with sterile distilled water and submerged in water for twelve hours, to improve germination. They were then maintained in hydrogen

peroxide (3%) for 30 minutes and finally washed twice with sterile distilled water to remove the remaining hydrogen peroxide.

The seeds were then sown in nursery seed trays with cells of volume 250 ml. The substrate used in the experiment consisted of a mixture of peat and vermiculite (1:1), which was autoclaved twice for one hour at 120 °C. After the seeds were sown, one ml of the spore suspension of the *F. circinatum* isolate and another one ml of the *T. viride* suspension were added to the substrate. In the case of the control treatments, one ml of sterile distilled water was used in place of the spore suspension. The trays were then maintained under controlled conditions of temperature (20 °C) and photoperiod (light/darkness 16/8 hours) inside a growth chamber. The seedlings were watered once a week, with twenty millilitres of sterile distilled water, and the progress of the assay was checked. The assay consisted of 32 different treatments resulting from pairing the seven isolates of *F. circinatum* and the *F. circinatum*-free control, with the three concentrations of *T. viride* and the *T. viride*-free control. Seed germination (emergence) was measured once a week, and the number of dead seedlings was counted ten weeks after sowing. At the end of the experiment, *F. circinatum* was re-isolated from the seedlings (10% were checked) to verify its presence in the necrotic lesions.

2.4 Statistical Analyses

The Relative Isolation Frequency (R.I.F.) was calculated as:

$$\text{R.I.F.} = n_{ij} / N_{ij} \times 100$$

where n_{ij} is the number of the isolates of the fungus found in site i and in tissue j, and N_{ij} is the number of samples examined in site i and in tissue j (Santamaría and Diez 2005). Analysis of variance (ANOVA) was performed to check for significant differences among the R.I.F. values, and a Least Significance Difference (LSD) test for multiple comparisons was used when significant differences were observed.

ANOVA was also performed in order to detect differences between treatments with respect to germination and mortality rate as well as in vitro antagonism. Multiple comparisons were made with the Scheffe test. Assumption of normality was checked on the residuals with the Shapiro-Wilks test. When this assumption and equal variance hypothesis for parametric testing failed, a Kruskal-Wallis test was performed. All statistical analyses were done with Statgraphics Plus 5.1 software (Statistical Graphics, Rockville, MD, USA).

3 Results

3.1 Fungal Isolates

A total of 96 isolates of *F. circinatum* were obtained from 9 of the 11 sampled plantations. No isolates were found at sites 1 (San Pedro del Romeral) or 6 (Ramales de la Victoria), although the disease was reported to be present at these sites. Significant differences in the occurrence of the pathogen in the plant material were recorded. Most of the isolates (91.7%) came from stem xylem extracted with the aid of an axe. Pitch-soaked and dark tissue was indicative of *F. circinatum* infection. The pathogen was even isolated from the midpoint of the trunk. The fungus was also obtained from twigs (4.2%), stem bark (2.1%) and from cores of stem xylem extracted with a Pressler borer (2.1%). The pathogen did not appear on needles, cones or seeds (Fig. 1). On the other hand, only one isolate of *T. viride* was obtained. This was isolated from cone samples of a tree in which *F. circinatum* was absent (site 5, Vejorís).

Another 20 fungal species were also found, thirteen of which were identified. Excluding *F. circinatum*, the most frequently isolated fungi in the plant material were *Pestalotiopsis funerea* (Desm.) Steyaert and *Sphaeropsis sapinea* (Fr.) Dyco et Sutton. The values of the R.I.F. of each endophyte (in relation to the plant material in which fungal species appeared) are shown in Table 2.

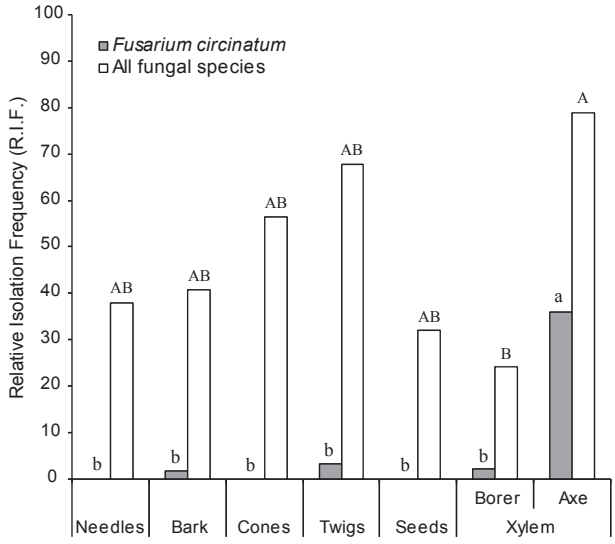

Fig. 1. Relative Isolation Frequency (R.I.F.) of *Fusarium circinatum* (black bars) and all fungal species detected (white bars), in relation to the tissue sampled and their respective homogeneous groups. Columns indicated by the same letter (a–b to denote significant differences in the abundance of *F. circinatum*, and A–B in the abundance of all fungi isolated) are not significantly different at $p = 0.05$ (LSD multiple range test).

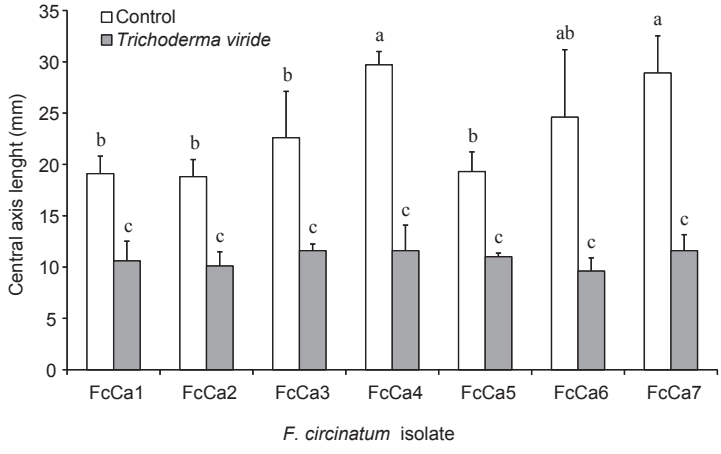

Fig. 2. Distribution of the variable length of the central axis of the *F. circinatum* colony in the control treatment (white bars) and in dual culture with *T. viride* (black bars). Error bars are standard deviations. Letters (a–c) denote significant differences ($p < 0.05$) among all columns (Scheffe multiple range test).

Table 2. Relative Isolation Frequency (R.I.F.) of the fungal species detected, in relation to the tissue sampled.

Fungal endophytes	Plant material							
	Needles	Stem bark	Cones	Twigs	Seeds	Stem xylem		Total
						Borer	Axe	
Aureobasidium pullulans (de Bary) Arnaud		0.83		3.20				0.72
Botrytis sp. Micheli ex Pers.					6.67		0.41	0.29
Fusarium circinatum Niremberg & O'Donnell		1.67		3.20		2.11	35.92	13.81
Gliocladium roseum (Link) Bainier	3.33			0.80				0.29
Nigrospora sacchari (Speg.) Mason		0.83		3.20				0.72
Paecilomyces sp. Bain.		0.83					0.41	0.29
Penicillium sp. Link		1.67					17.55	6.47
Pestalotiopsis funerea (Desm.) Steyaert	33.33	19.17	16.92	16.80		21.05	16.33	17.99
Phialophora cinerescens (Wollenw.) van Beyma		1.67		0.80			0.41	0.58
Phoma sp. Sacc.		0.83						0.14
Phomopsis sp. (Sacc.) Bubak			4.62	3.20				1.01
Pseudeurotium sp. Beyma			3.08			1.05	1.63	1.01
Sordaria fimicola (Rob. ex Desm.) Ces & De Not.				2.40				0.43
Sphaeropsis sapinea (Fr .) Dyco et Sutton	23.33	17.50	40.00	42.40	6.67		2.45	16.40
Trichoderma viride Pers.			1.54					0.14
Unidentified fungus 1			4.62	0.80				0.58
Unidentified fungus 2	3.33			1.60	26.67		2.86	2.01
Unidentified fungus 3			3.08	2.40	13.33			1.01
Unidentified fungus 4				0.80				0.14
Unidentified fungus 5				1.60				0.29
Unidentified fungus 6		0.83		0.80				0.29
Unidentified fungus 7							0.82	0.29
Total	38.00	40.74	56.47	67.74	32.00	24.21	78.78	64.89

3.2 In Vitro Antagonism

T. viride exerted a significant antagonistic effect on *F. circinatum* after dual culture on PDA for six days, and the decrease in the linear growth of all isolates ranged from 60% to 43% (Fig. 2). Although the increase in mycelial length differed significantly between the isolates of *F. circinatum* (the FcCa1, FcCa2, FcCa3 and FcCa5 isolates grew less than FcCa4 and FcCa7 isolates), the inhibition caused by *T. viride* reduced the increase in mycelial length of all isolates to a similar level (Fig. 2). Furthermore, the shape of the *F. circinatum* colony indicated that the presence of the endophyte significantly affected growth of the pathogen (Table 3).

3.3 In Vivo Experiments

3.3.1 Germination

The pathogen *F. circinatum* reduced the germination of *P. radiata* seeds by 14% ($p<0.01$). This reduction was reflected by the percentage of seed germination, which decreased from 92% in the absence of pathogen, to 78% when spores of *F. circinatum* were added to the substrate. The lowest rate of germination was 73%, obtained for FcCa6 isolate, whereas 82% of the seeds germinated in the case of isolate FcCa3, which appeared to be the least aggressive isolate. Despite the apparently different percentages of germination, no significant differences were found among the

Table 3. Two-way ANOVA table for the colony shape indicator in the antagonism in vitro assay. Below, data of the shape indicator for incubations of the seven isolates of the pathogen with and without *T. viride*. Letters (a–b) denote significant differences between treatments (control and *T. viride*).

Source	Degrees of freedom	Mean squares	F-value	p-value
Fusarium circinatum strain	6	388929.00	2.19	0.0577
Trichoderma viride	1	116036.00	6.53	0.0134
F. circinatum strain × *T. viride*	6	17869.00	1.00	0.4314

	FcCa1	FcCa2	FcCa3	FcCa4	FcCa5	FcCa6	FcCa7
Control	0.15 a	–0.05 b	0.15 a	–1.00 b	–0.05 a	2.30 a	0.80 a
T. viride	0.95 a	1.00 a	1.55 a	0.85 a	0.80 a	1.45 a	1.40 a

Table 4. Homogeneous groups of all the treatments tested in the germination assay ten weeks after inoculation, and the germination rate of each (T0 = 0, T1 = 10^7, T2 = 10^6, T3 = 10^5 *T. viride* spores/ml). Treatments indicated by the same lower case letter (a–g) are not significantly different at p = 0.05 (Scheffe multiple range test). Letters denote differences within the whole table.

Treatment	Germination (%)			
	T0	T1	T2	T3
Control	91.67 ab	80.95 abcdefg	94.05 a	90.48 abc
FcCa1	79.76 bcdef	86.90 abcd	77.38 cdef	59.52 g
FcCa2	78.57 bcdef	59.52 g	78.57 bcdef	72.62 efg
FcCa3	82.14 abcdef	85.71 abcde	84.52 abcdef	79.76 bcdef
FcCa4	77.38 cdef	82.14 abcdef	82.14 abcdef	78.57 bcdef
FcCa5	79.76 bcdef	71.43 fg	73.81 def	76.19 def
FcCa6	72.62 efg	76.19 def	79.76 bcdef	72.62 efg
FcCa7	77.38 cdef	79.76 bcdef	75.00 def	85.71 abcde

Table 5. Homogeneous groups of all the treatments tested in the survival assay ten weeks after inoculation, and the mortality rate of each (T0 = 0, T1 = 10^7, T2 = 10^6, T3 = 10^5 *T. viride* spores/ml). Treatments indicated by the same lower case letter (a–c) are not significantly different at p = 0.05 (Scheffe multiple range test). Letters denote differences within the whole table.

Treatment	Dead seedlings (%)			
	T0	T1	T2	T3
Control	17.86 c	3.17 c	2.78 c	2.38 c
FcCa1	93.65 a	87.30 a	93.65 a	59.13 b
FcCa2	83.73 ab	74.60 ab	92.86 a	88.89 a
FcCa3	97.22 a	96.83 a	83.73 ab	96.03 a
FcCa4	98.81 a	93.25 a	75.79 ab	92.86 a
FcCa5	90.87 a	87.30 a	86.51 a	87.70 a
FcCa6	92.06 a	79.37 ab	92.86 a	91.27 a
FcCa7	100.00 a	98.81 a	96.03 a	96.83 a

seven *F. circinatum* isolates (Table 4, treatment T0).

The rate of germination of the seeds treated exclusively with *T. viride* differed depending on the concentration of spores used. When the highest concentration of spores (T1, 10^7 spores/ml) was added to the substrate, the rate of germination fell to 81%. The values for the lower concentrations of spores or the control without the fungus were slightly higher, ranging from 94 to 90% respectively.

The effects of *T. viride* on germination of *F. circinatum* differed, and no correlation with spore concentration or *F. circinatum* isolate was established. The percentage of seeds germinated per *F. circinatum* isolate in relation to the concentration of *T. viride* spores varied widely (Table 4).

3.3.2 Survival

The survival of Monterey pine seedlings was severely affected by the pitch-canker pathogen, which caused significant mortality ($p<0.001$). The damage and losses caused by the pathogen were much higher than the effect observed in the germination assay (rates of mortality ranged between 83.7 and 100%). However, no differences among the seven isolates of *F. circinatum* were found. Although some deaths were observed in the control treatment (29.8%), no isolates of the pathogen were recovered from the non-inoculated control seedlings. On the contrary, *F. circinatum* was re-isolated from 100% of the checked seedlings.

With one exception, *T. viride* failed to protect *P. radiata* seedlings against mortality caused by *F. circinatum* (Table 5). The antagonist only caused a significant reduction in the growth of isolate FcCa1 (mortality rate decreased from 93.7 to 59.1%) when 10^5 spores/ml of the fungus were added to the substrate.

4 Discussion

Global trade and tourism are increasing the movement of tree pathogens to new environments, where they infect new hosts with which they never co-evolve. Pitch canker disease in Spain is a good example of this global threat. The pathogen is supposed to have arrived from California in legally imported Monterrey pine seed (Laucirica and Muguruza 1997). Since the pathogen was detected in 2003 it has spread rapidly, colonizing many forest nurseries and pine stands in Northern Spain. Despite the actions taken by the Spanish Government (Ministerio de Agricultura 2006), Monterrey pine plantations are currently heavily affected and native pine species are threatened by this pathogen. Despite great research efforts during the last decade, few studies related to the disease have been carried out in Spain (Pérez-Sierra et al. 2007, Romón et al. 2008, Iturritxa et al. 2011).

The first step in working with a new pathogen is to optimize the method of isolation. Although *Fusarium* spp. isolates are often not as recalcitrant as e.g. *Phytophthora* spp. isolates (Streito et al. 2002), and theoretically all symptomatic tissues are suitable for isolation of the pathogen (Coutinho et al. 2007), most of the isolates were obtained from stem xylem. We found that the best way to isolate *F. circinatum* from adult trees was to use large samples of stem xylem (extracted with an axe), from which a smaller piece was obtained after surface sterilization. Although all plant material was obtained from symptomatic trees, no isolates were obtained from seeds, in contrast with Storer et al. (1998) who obtained the pathogen from up to 83% of the seeds collected from cones on recently infected branches. The absence of isolates from seeds may be due to subtle differences in sterilization protocols that are not reported in the literature, but may affect the final result.

The low occurrence of *T. viride* in the present study may be inversely correlated with the high presence of *F. circinatum* in the plantations surveyed. Thus, the antagonistic effect of this fungus may appear on cones from which *F. circinatum* was not isolated but *T. viride* was obtained. A similar relationship between *T. viride* Pers. Ex Fr. and *F. oxysporum* Schlecht. and *F. verticillioides*. (Sacc.) Nirenberg was also found in a study of the seasonal effect of the soil-borne fungi in forest nurseries (low levels of *T. viride* and high levels of *Fusarium* spp. in spring, and the opposite in autumn), by Martín-Pinto et al. (2006), who sug-

gested that *Trichoderma* may exert antagonistic effects on nursery diseases caused by *Fusarium* spp. Different *Trichoderma* spp. have repeatedly been described as antagonistic to many fungal pathogens diseases (Capieau et al. 2004, Perazzolli et al. 2011) and even sold as biological fungicides (Liñán 2010).

Some of the fungal species that appeared in the plant material, such as *P. funerea*, *Phialophora* sp. Medlar, and *Phomopsis* sp. (Sacc.) Bubak (Table 2), have been described as common fungi associated with conifers (Hoff et al. 2004, Zamora et al. 2008, Botella et al. 2010). Other fungi identified, such as *Aureobasidium pullulans* (de Bary) Arnaud, *Penicillium* sp. Link, *Phoma* sp. Sacc. and *Sordaria fimicola* (Rob. Ex Desm.) Ces & De Not., are ubiquitous taxa that are often isolated from very different host genera (Collado et al. 2000, Martín-Pinto et al. 2004, Santamaría and Diez 2005, Zamora et al. 2008, Botella et al. 2010, Botella and Diez 2011, Martín-García et al. 2011).

The presence of *F. circinatum* had a devastating effect on seedlings, even killing 100% of them in one of the treatments tested. Inoculation with fungal isolates FcCa3, FcCa4, FcCa6 and FcCa7 resulted in all of the seedlings presenting symptoms ten weeks later. In the case of isolate FcCa2, only 6% of the seedlings survived, whereas in the control treatment, more than 70% of the seedlings survived. Similar results were obtained by Porter et al. (2009) with *P. patula* Schiede & Deppe seedlings, all of which died within twelve weeks of inoculation. On the contrary, lower mortalities were reported by Enebak and Stanosz (2003) in *P. banksiana* Lamb. (18.9% of seedlings died), *P. resinosa* Sol. Ex Ait. (0%), *P. strobus* L. (14.8%), *P. sylvestris* L. (0%) and *P. nigra* Arnold (4.4%). The pine species, age of the seedlings (3 years) and the lower concentration of the spores (100 000 spores/ml) may have caused the different mortality rates recorded in this assay. Aegerter and Gordon (2006) obtained mortality rates ranging from 3.5 to 52% in *P. radiata* seedlings, although the method of inoculation was different from that used in the present study (the latter authors immersed and vortexed the seeds for ten seconds in a suspension of 10^3 spores of *G. circinata* per ml of water). *F. circinatum* is clearly a devastating fungus in forest nurseries in terms of the mortality that it causes. By contrast, in studies using exactly the same methodology, the percentage mortality caused by other pathogenic species such as *F. oxysporum* and *F. verticillioides* was very low (Machón et al. 2006, Machón et al. 2009).

Although isolates were collected from different geographical areas, no significant differences were observed in either the germination or survival of the seedlings among the seven isolates of *F. circinatum*. The recent introduction of the pathogen (first reported in northern Spain by Landeras et al. 2005) may explain the low phenotypic variability of the isolates used in this assay. A study of vegetative compatibility groups (VCGs) carried out in the nearby Basque Country, found only two VCGs of the same mating type, demonstrating the low level diversity of the population in the area (Iturritxa et al. 2011), in comparison with the 45 VCGs found in Florida, where the disease was established many years ago (Correll et al. 1992). Furthermore, sexual reproduction in *F. circinatum* has not been observed in Spain, although mating types 1 and 2 have been detected (Pérez-Sierra et al. 2007). Although eradication of the pathogen in Spain is technically impossible, taking into account the vast area affected by the fungi, the homogeneity of *F. circinatum* populations may facilitate future management of this disease.

Biological control in forest diseases is increasingly important. *Trichoderma* is one of the fungal genera most commonly used as a biological control agent (BCA). Ninety percent of the antagonists used to control plant diseases belong to this genus (Benitez et al. 2004), which is why we chose *T. viride* (from among all endophytes that appeared on Monterrey pine) to perform this trial. *T. viride* exerted an antagonistic effect on the in vitro growth of *F. circinatum*.

Although *T. viride* has been observed to be a good BCA with an important effect on some diseases caused by *Fusarium* (John et al. 2010, Basak and Basak 2011), and it had an effect on *F. circinatum* in the in vitro assay, no significant reduction in mortality rates was observed in the present study, with the exception of the slight effect on isolate FcCa1. The biocontrol capacity of *Trichoderma* species may decrease under real conditions of inoculation (Bernal-Vicente et al. 2009). Better results may have been obtained by

adding *T. viride* to the substrate some days before the pathogen. Nonetheless, good results in the in vitro assay are not always good indicators of positive antagonistic effects in vivo (Campanile et al. 2007). Furthermore, the wide intraspecific diversity in *T. viride* species may explain the low in vivo effect. On the other hand, this low effect enabled us to test the low variability in the pathogenicity of the *F. circinatum* isolates. It would be interesting to test other strains of *T. viride*, as well as some other naturally occurring fungi, as potential BCAs. It should be borne in mind that the use of chemicals to control forest diseases is often not allowed or is expected to be banned in the future, and that biocontrol may be one of the best options for controlling pitch-canker in *P. radiata* and other tree diseases, e.g. as successfully achieved by the use of *Phlebiopsis gigantea* to control *Heterobasidion annosum* (Sun et al. 2009), in order to protect the health of forests and forest nurseries. Further assays with more isolates and endophyte species are underway in an attempt to discover a fungus that is able to reduce the effects of the pitch canker disease pathogen.

Acknowledgements

This research was supported by the Government of Cantabria, the Ministry of the Environment and Rural and Marine Affairs, Ministry of Agriculture, INIA and by grants provided by the University of Valladolid. We thank Milagros de Vallejo and Juan Blanco for their help in the sampling, and Leticia Botella for her useful comments of the manuscript.

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Total of 66 references

Original article III

Martínez-Álvarez, P.; Fernández-González, R.; Sanz-Ros, A.V.; Pando, V. & Diez, J.J. 2015. Two fungal endophytes reduce the severity of pitch canker disease on *Pinus radiata* seedlings. Submitted to Biological Control.

Resumen

Dos hongos endofitos reducen la severidad de la enfermedad del chancro resinoso del pino en plántulas de *Pinus radiata*.

La enfermedad del chancro resinoso de los pinos, causada por el hongo *Fusarium circinatum*, no tiene hasta el momento métodos de control conocidos. Las restricciones en el uso de productos químicos y fungicidas que existen en la actualidad en el monte hacen necesaria la investigación hacia métodos alternativos para reducir los daños causados por el patógeno. El control biológico con hongos endófitos es una estrategia prometedora y respetuosa con el medio ambiente. En este estudio, 154 aislados de hongos endófitos fueron seleccionados de una colección de 546 tras un ensayo de enfrentamientos preliminares. Los aislamientos fueron después probados frente a *F. circinatum* en un experimento de antagonismo *in vitro*. Para detectar y cuantificar el antagonismo producido por los endófitos frente al patógeno, se utilizaron cinco indicadores diferentes. Seis aislamientos con resultados prometedores *in vitro*, fueron inoculados junto al patógeno en plantas de *Pinus radiata*, *P. sylvestris*, *P. pinaster*, *P. nigra* y *P. pinea* en el monte, para saber si eran capaces de reducir los daños causados por *F. circinatum*. Un total de 138 endófitos mostraron actividad antagonista frente a *F. circinatum* en el experimento *in vitro*. En el ensayo de campo, los endófitos *Chaetomium aureum y Alternaria sp.* redujeron la variable AUDPC en las plántulas de *P. radiata*, indicando que pueden ser buenos candidatos para su uso como agentes de control biológico (BCAs) de la enfermedad.

Palabras clave: *Fusarium circinatum*, ensayo de campo, inoculación, cultivos duales, agentes de control biológico.

Two fungal endophytes reduce the severity of pitch canker disease in *Pinus radiata* seedlings

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Abstract

Pitch canker disease, which affects pines and is caused by the fungus *Fusarium circinatum*, cannot be effectively controlled at present. Current restrictions on the use of chemicals and fungicides in forests are driving research into alternative methods of reducing the damage caused by the pathogen. Biological control with fungal endophytes is a promising and environmentally friendly strategy. In this study, 154 endophyte isolates were selected from a collection of 546 fungi tested in a preliminary confrontation assay. These isolates were then tested against *F. circinatum* in an in vitro antagonism experiment. Five different indicators were used to detect and quantify the antagonistic activity directed towards the pathogen by the endophytes. The six isolates that showed the most promising results were inoculated in the field, together with the pathogen, into seedlings of *Pinus radiata*, *P. sylvestris*, *P. pinaster*, *P. nigra* and *P. pinea,* to test whether they could reduce the damage caused by *F. circinatum*. In total, 138 endophytes displayed antagonistic activity towards *F. circinatum* in the dual cultures of the in vitro experiment. In the field test, the endophytes *Chaetomium aureum* and *Alternaria sp.* reduced the area under disease

progress curve (AUDPC) for the *P. radiata* seedlings, indicating that they may therefore be suitable for use as biological control agents (BCAs) of the disease.

Keywords: *Fusarium circinatum*, field test, inoculation, dual cultures, biological control agents.

1. Introduction

Pitch canker is a virulent disease of pines that is caused by the fungus *Fusarium circinatum* Nirenberg & O'Donnell (teleomorph = *Gibberella circinata*). The pathogen is characterized by microconidia in false heads that are formed on mono- and polyphialides, the absence of chlamydospores, the presence of coiled sterile hyphae and the formation of conidiophores on erect aerial mycelium (Nirenberg and O'Donnell, 1998). *Fusarium circinatum* causes damage to seedlings in nurseries as well as to adult trees in forests. In seedlings, symptoms include damping-off and wilting (Viljoen et al., 1994). In mature trees, the main symptom is a bleeding resinous canker on the stem or thick branches, although branch die-back also occurs (Dwinell et al., 1985). Mortality in adult pines is estimated to range between 5 and 25% in susceptible species (EFSA, 2010).

The pitch canker pathogen was first recorded in 1946 as causing damage to *Pinus virginiana* Mill. in the south-eastern United States of America (Hepting and Roth, 1946). It was later found in Haiti (Hepting and Roth, 1953), California (McCain et al., 1987) and Mexico (Guerra-Santos, 1998). More recently, globalization has led to the pathogen reaching other countries far from its origin, and the disease is now found in South Africa (Viljoen et al., 1994), Japan (Kobayashi, 2007), Chile (Wingfield et al., 2002), Korea (Cho and Shin, 2004), France (EPPO, 2006), Spain (Landeras et al., 2005), Italy (Carlucci et al., 2007), Portugal (Bragança et al., 2009), Uruguay (Alonso and Bettucci, 2009), Colombia (Steenkamp et al., 2012) and Brazil (Pfenning et al., 2014).

At present there is no effective means of controlling pitch canker disease in nursery seedlings or in adult trees in forest plantations. Some studies have been conducted to analyze the effect of different fungicides on *F. circinatum* (Mitchell et al., 2004; Runion et al., 1993). However, the discouraging results -together with the increasing awareness of the detrimental effects that fungicides may have on the environment, particularly in forests- are driving the search for alternative methods of control. For example, a study of the effect of hot water on the survival of *F. circinatum* on pine seeds, Agustí-Brisach, et al, (2012)

concluded that hot water treatments (51–52ºC for 30 min) can be used to reduce *F. circinatum* contamination on *P. radiata* seeds. Similarly, hydrogen peroxide has also been found to be effective for disinfecting seeds (Dwinell and Fraedrich, 1999). Unfortunately, although these methods reduce the numbers of infected seeds, they do not prevent seedlings that carry the pathogen reaching the forest. An integrated management approach is needed to reduce the impact of the disease. The use of biological control methods, which have shown good results in the control of other fungal diseases, is imperative because of the need to eliminate the use of chemicals in forests and nurseries. Chestnut blight is a good example of the successful use of biological control. The hypovirulence caused by certain viruses found in some fungal isolates is associated with decline of the disease in some European regions (Heiniger and Rigling, 1994). Although viruses hosted by *F. circinatum* have been recently detected (Martínez-Álvarez et al., 2014b), further research is needed to evaluate whether these viruses are able to produce hypovirulence in the fungus. Only two different vegetative compatibility (vc) types have been found in the Spanish population of *F. circinatum* (Iturritxa et al., 2011). The very low genetic diversity, in comparison with the 45 vc types present in Florida (USA), may be due to the limited and recent introduction of the pathogen in Spain (Berbegal et al., 2013) and is encouraging in relation to controlling the disease in the country, or at least minimizing the damage caused by the pathogen in nurseries and plantations.

Fungal endophytes, i.e. fungi that can infect their hosts without causing visible symptoms of disease (Petrini, 1991) are used successfully as biological control agents (BCAs) in the fight against some fungal diseases (Arnold et al., 2003). The mechanisms that fungal endophytes use against pathogens can be classified in three groups: direct effects (interaction between endophytes and pathogens), indirect effects (enhanced plant defense) and ecological effects (occupation of ecological niche) (Gao et al., 2010). Endophytes can be extracted from the same ecosystem in which they will be used as BCAs, so that there will be no impact on the environment. Another advantage over the use of chemicals is that organisms generally do not become resistant to endophytes. Furthermore, endophytes may have other beneficial effects on plants, such as enhanced growth (Barka et al., 2002), resistance to drought stress (Swarthout et al., 2009), tolerance to unsuitable soil conditions (Malinowski et al., 2004) and protection against herbivores (Carroll, 1988). These advantages, together with the good results obtained with other fungal diseases, have encouraged us to study the effectiveness of fungal endophytes in controlling the pitch canker pathogen. Thus, the aims of the present study were (1) to

detect and identify in vitro antagonists to *F. circinatum* from among a collection of fungal endophytes obtained from pines, and (2) to test the in vivo effect of the endophytes on the incidence of the disease in five pine species inoculated with the pitch canker pathogen.

2. Material and Methods

2.1 Fungal material and preliminary confrontation assays

Most of the endophytes tested in the assay were isolated in the Forest Health Centre of Calabazanos (Junta de Castilla y León), and a few were obtained from the Forest Entomology and Pathology Laboratory at the University of Valladolid. Most of the fungi were isolated from different tissues of *Pinus* spp. Once in the laboratory, the plant material was washed in tap water and surface sterilized by immersion in 70% ethanol (for 1 min), in 3% sodium hypochlorite (1 min), and finally in sterile distilled water (1 min) to remove any remaining traces of disinfectants. The samples were immediately dried, by placing them on sterile filter paper, before being cut in small pieces for plating on potato-dextrose-agar (PDA) with 0.5 g/l of streptomycin sulphate (to prevent bacterial growth). The plates were placed in growth chambers in the dark at 25ºC and frequently examined to detect all fungi that appeared in fresh plates (which were then subcultured). Several species were isolated from tissue cultures when antagonism of a fungal species was observed. A total of 546 fungal isolates were obtained in this way.

Two different isolates of *F. circinatum* were used in the study. Isolate Fc70 belongs to mating type 1 and was obtained from *P. radiata* in Asturias (Spain). Isolate Fc221 corresponds to mating type 2 and was collected from the same host species, in this case in Cantabria (Spain). Both isolates were provided by the Instituto Agroforestal Mediterráneo in Valencia (Spain), and their pathogenicity has been confirmed previously (Pérez-Sierra et al., 2007).

In order to reduce the number of endophytes included in the in vitro antagonism study, a preliminary trial was performed with all 546 endophytes, in which the pathogen was tested against four endophytes in the same PDA plate. The four endophytes were placed (at the edge of the plate) at the ends of two perpendicular axes crossing the centre of the plate where the plug of *F. circinatum* was cultured. The control treatment consisted of plates in which the five positions were occupied by *F. circinatum*. The plates were incubated in the dark at 25ºC and examined every two days to detect any interactions between fungi. When

growth of the pathogenic fungal colony decreased or the shape of the colony was modified, the causative endophyte was selected for the in vitro antagonism experiment. The selected isolates were stored on filter paper at -20ºC and in 15% glycerol at -80ºC.

The selected endophytes were identified by molecular techniques. Genomic DNA was isolated from fungal cultures following the protocol described by Vainio et al. (1998). The Polymerase Chain Reaction (PCR) was then used to amplify the internal transcribed spacer (ITS) region of rDNA (ca 600 bp) with primers 1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes and Bruns, 1993) and 4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990). The PCR mixture (50 µl) included one unit of KAPA Taq DNA-polymerase (Kapa Biosystems, Boston), Kapa Taq buffer 1X, 200 µM of dNTPs, 0.5 µM of each primer and 1 µl of DNA. The PCR involved initial denaturation for 2 min at 95°C, followed by 35 cycles of 40 s at 95°C, 55 s at 55°C and 1 min at 72°C. On completion of these cycles, the reaction was followed by extension of 10 min at 72°C. To ensure identification of the isolates, elongation factor 1 alpha (ca 700 bp) was also amplified by PCR with EF1 (5′-ATGGGTAAGGA(A/G)GACAAGAC-3′) and EF2 (5′-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3′) primer pair (O'Donnell et al., 1998). PCR was carried out using an initial denaturation step of 2 min at 94 °C, followed by 36 cycles of 94°C for 30 s (denaturation), at 62 °C for 55 s (annealing), and at 72°C for 1 min (elongation), and a final elongation step at 72 ºC for 10 min. Finally, LSU rDNA was amplified using the LR0R and LR16 primer pair (Vilgalys and Hester, 1990). PCR involved initial denaturation at 94ºC for 3 min, followed by 38 cycles at 94ºC for 35 s, 54ºC for 40 s and 72ºC for 1 min and a final elongation step at 72ºC for 10 min. The PCR mixtures used for elongation of factor 1 alpha and LSU were the same as used for ITS 1F-4, as previously described. PCR products were amended with 1 µl of 6X loading dye solution (50 mM EDTA, 30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol), and they were separated by electrophoresis in runs of 1 hour in 1X TAE buffer (40 mM Tris base, 0.114% glacial acetic acid and 1 mM EDTA (pH=8)) at 5 V/cm in 1.8 % agarose gels (type I standard PB, Panreac Química S.A.) and posterior staining with 3x GelRed™ solution (Biotium), following the manufacturer's instructions. A 100 bp ladder was used to estimate the size of the amplification products (Exact Gene, Fisher Scientific International Inc.). The electrophoresis results were examined under UV light and processed with GenSnap Software (Synoptics Ltd., Cambridge, UK). PCR products were purified with NucleoSpin® Extract II kit 10/2007 Rev. 06 (Macherey-Nagel Gmbh and Co.KG) and retested by electrophoresis. The DNA concentration was automatically determined with GenTools

software (Synoptics Ltd., Cambridge, UK) and by comparison with size markers. Samples were sent to CENIT Support Systems (Madrid, Spain) where the different DNA fragments were sequenced. Geneious v.6 software was used to process the sequences and the BLAST tool to compare the fragments with those deposited in GenBank (NCBI) database (Benson et al., 2002). The fungal isolates were named according to the best GenBank match if at least 98% matched with a sequence consisting of at least 450 bp.

2.2 In vitro antagonism experiment

To evaluate the antagonistic effect of the selected endophytes on the *F. circinatum* colony, dual cultures were grown on PDA plates. A 4 mm side plug of the pathogen was placed 5 mm from the edge of the plate and the endophyte under test was placed at the opposite edge. The central axis (axis C) and two axes at 45º (lateral axes) were drawn on the bottom of the plate before the fungi were cultured (Figure 1). Five replicates were prepared per endophyte-pathogen confrontation. In the control treatment, the respective isolate of *F. circinatum* was placed in both positions. Measurements were made ten days after the fungi were plated.

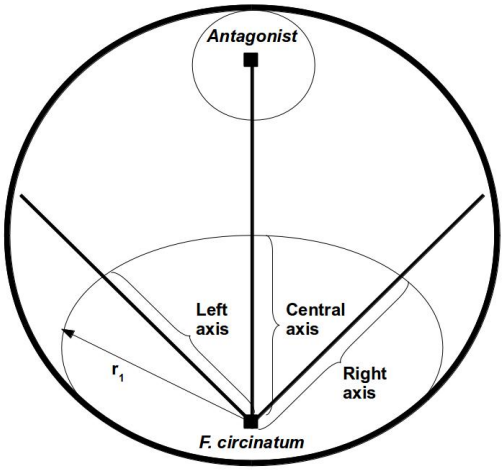

Figure 1. Diagram showing the mode of inoculation of the plates with *F. circinatum* and the potential antagonist in the in vitro antagonism experiment. r_1 = maximum radius of the colony of the fungal pathogen.

Four different indicators were used to test the antagonism exercised by the endophyte: (1) length of the central axis (axis C) of the colony of the pathogen; (2) the shape coefficient

(Santamaría et al., 2007), calculated as the difference between the average of the lateral axis and the central axis; (3) percentage inhibition of radial growth (Royse and Ries, 1978), calculated by the equation [100 x (r_1 - axis C) / r_1], where r_1 is the maximum radius of the colony of the pathogen in any direction; and (4) percentage inhibition zone (Orole and Adejumo, 2009), calculated by the equation [(A - B) / A] x 100, where A is the radius of pathogen in the control plate and B is the radius of the pathogen in the dual culture plate.

2.3 Field test

A total of 1500 two-year-old seedlings were planted in June 2012 in Puentenansa, Cantabria (Spain) in an area where the pathogen has previously been detected. The plot characteristics are summarized in Table 1. The seedlings belonged to five different pine species: *P. radiata*, *P. sylvestris* L., *P. nigra* Arnold, *P. pinaster* Aiton and *P. pinea* L. Each species was represented by 300 seedlings distributed in three blocks. Collar diameter, total height and crown base height of the seedlings were measured at the beginning (April 2013) and at the end of the trial (July 2014).

Table 1. Characteristics of the plot where the field trial was established. UTM coordinates in ETRS 89 spindle 30. m.a.s.l. = metres above sea level. Climate data according to "Atlas Climático Digital de la Península Ibérica" (Ninyerola et al., 2005).

UTM coordinate X	386979
UTM coordinate Y	4788566
Location	Puentenansa
Municipality	Rionansa
Altitude (m.a.s.l.)	525 – 615
Orientation	southwest
Slope **(%)**	50
Anual precipitation (mm)	1092
Mean temperature (ºC)	11
Minimum mean temperature (ºC)	5
Maximum mean temperature (ºC)	17

In July 2013, seedlings were inoculated with the candidate antagonistic endophytes. Six endophytes were selected from among the isolates tested in the in vitro experiment. These were selected because they belong to fungal species of interest as BCAs and the good results they yielded in the in vitro antagonism experiment. The seedlings were inoculated with the pathogen in September 2013, 42 days after the inoculation with the six endophytes. Although the genetic diversity of *F. circinatum* in northern Spain is very low (Berbegal et al., 2013; Iturritxa et al., 2011), the pathogen was isolated, as described in Martínez-Álvarez et al. (2012), from a canker of a Monterey pine close to the plot where the study was conducted, thus avoiding the introduction of new genetic material of the pathogen in the area. To inoculate the fungi, a cut was made in the stem of the seedling to enable insertion of the plug (16 mm^2) with mycelium of the endophyte or addition of one drop (20 µl) of a suspension of the pathogen spores (10^6 spores/ml). A plug of sterile agar or one drop of sterile distilled water was used in the control seedlings. The injury was immediately covered with Parafilm® to prevent desiccation and contamination. The pathogen was inoculated two cm below the site of inoculation of the endophyte, according to previous inoculation experiments (Romeralo et al., 2015). The treatments tested in the field experiment are described in Table 2.

Table 2. Treatments tested in the field experiment.

Code	Inoculation July 2013	Inoculation September 2013
Control	Sterile potato dextrose agar plug	Sterile distilled water
CF	Sterile potato dextrose agar plug	*F. circinatum* spore solution
031W	Endophyte isolate HP031	Sterile distilled water
047W	Endophyte isolate HP047	Sterile distilled water
066W	Endophyte isolate HP066	Sterile distilled water
143W	Endophyte isolate HP143	Sterile distilled water
151W	Endophyte isolate HP151	Sterile distilled water
155W	Endophyte isolate HP155	Sterile distilled water
031F	Endophyte isolate HP031	*F. circinatum* spore solution
047F	Endophyte isolate HP047	*F. circinatum* spore solution
066F	Endophyte isolate HP066	*F. circinatum* spore solution
143F	Endophyte isolate HP143	*F. circinatum* spore solution
151F	Endophyte isolate HP151	*F. circinatum* spore solution
155F	Endophyte isolate HP155	*F. circinatum* spore solution

The plot was visited four times (December 2013, March 2014, May 2014 and July 2014) to evaluate any damage that the pathogen had caused to the seedlings. For this purpose, the seedlings were visually scored for disease symptoms following the method proposed by Correll et al. (1991), in which each inoculation was rated on a scale of 0 (healthy) to 4 (girdled branch and dead foliage distal to the point of inoculation). Disease progress curves were constructed for each plant by plotting the scores against time elapsed since inoculation. The area under the disease progress curve (AUDPC) was calculated as the sum of the area of the corresponding trapezoids.

2.4 Statistical analysis

In vitro_antagonism experiment: A linear mixed model analysis of variance with repeated measures was used. A factorial design was applied in which two between-subjects factors (isolate of *F. circinatum* with two levels and endophyte with one hundred and fifty five levels) and a within-subjects factor (time), with two levels, were considered. In order to solve the problem of high heterogeneity of variance due to the endophyte factor, the levels were divided into seven groups with different random variances for each. One tailed t tests were used to compare Ls-means between each endophyte and the control level within each isolate of *F. circinatum* and each time. The mathematical formulation of the model is expressed as follows:

$$Y_{ij(l)k;t} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \gamma_t + \alpha\gamma_{it} + \beta\gamma_{jt} + \alpha\beta\gamma_{ijt} + \varepsilon_{ij(l)k;t}$$

where i = 1, 2 for the isolates, j = 1,…,155 for the antagonists, l = 1,…,7 for the groups of antagonists, k = 1, …, 5 for the replicates and t = 1, 2 for the two repeated measurements, and where $Y_{ij(l)k;t}$ = the observed value of the dependent variable for the replication k of isolate i with endophyte j into group l at time t; μ = general mean effect; α_i = main effect of time on the *F. circinatum* isolate i; β_j = main effect of endophyte j; $\alpha\beta_{ij}$ = interaction effect between isolate i and endophyte j; γ_t = main effect of time t; $\alpha\gamma_{it}$ = interaction effect between isolate i and time t; $\beta\gamma_{jt}$ = interaction effect between antagonist j and time t; $\alpha\beta\gamma_{ijt}$ = triple interaction effect between isolate i , antagonist j and time t; and $\varepsilon_{ij(l)k;t}$ = random error in the dependent variable for replication k of isolate i with antagonist j into group l at time t. The following assumptions were applied: $\varepsilon_{ij(l)k;t} \sim N(0, \sigma^2_{ilt})$, with σ^2_{ilt} = random variance for the errors in isolate i and the group of endophytes l at time t; and $\mathrm{Cov}(\varepsilon_{ij(l)k;t}, \varepsilon_{i'j'(l')k';t'}) = \omega_{il}$ if i = i', j = j', l = l', k = k', for t ≠ t', or $\mathrm{Cov}(\varepsilon_{ij(l)k;t}, \varepsilon_{i'j'(l')k';t'}) = 0$ in any other case,

with ω_{il} covariance between errors at different time for isolate *i* and the group of endophytes *l.* The model thus included forty-two variance parameters (twenty eight variance parameters and fourteen covariance parameters), which were estimated using the Minimum Variance Quadratic Unbiased Estimators method (MIVQUE0).

Field test: A linear mixed model analysis of variance with three factors in a split-plot design was used. The pine species factor with five levels was used for the whole plots in a randomized block design with three blocks. The factor *F. circinatum* (yes or no) (two levels) and the factor endophyte (seven levels) were used for the split-plots in a factorial design within each whole-plot. In order to solve the problem of high heterogeneity of variance due to the factors pine species and *F. circinatum*, different random variances were used for each of the ten combinations of these two factors. Fisher's LSD test was used to compare the Ls-means. The mathematical formulation of the model was expressed as follows:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \omega_{ij} + \gamma_k + \delta_l + \gamma\delta_{kl} + \alpha\gamma_{ik} + \alpha\delta_{il} + \alpha\gamma\delta_{ikl} + \varepsilon_{ijkl}$$

where i = 1,…,5 for the pine species, j = 1, 2, 3 for the blocks, k = 1, 2 for the levels of the *F. circinatum* factor and l = 1,…,7 for the level of the endophyte factor, and where Y_{ijkl} = the observed value of the dependent variable for the split-plot with the k level of F. circinatum and l level of endophyte in the whole-plot with pine species i in block j; μ = general mean effect; α_i = main effect of the pine species i; β_j = main effect of the block j; ω_{ij} = random error for the whole-plot of the pine species i in block j; γ_k = main effect of the k-level of factor F. circinatum; δ_l = main effect of the l-level of factor endophyte; $\gamma\delta_{kl}$ = interaction effect between the k-level of F. circinatum factor and the l-level of endophyte factor; $\alpha\gamma_{ik}$ = interaction effect between pine species i and the k-level of F. circinatum factor; $\alpha\delta_{il}$ = interaction effect between pine species i and the l-level of endophyte factor; $\alpha\gamma\delta_{ikl}$ = triple interaction effect for pine species I, the k-level of F. circinatum factor and the l-level of endophyte factor; and ε_{ijkl} = random error in the dependent variable for the split-plot with the k level of F. circinatum and l level of endophyte in the whole-plot with pine species i in block j. The following assumptions were used: $\omega_{ij} \sim N(0, \theta^2)$, with θ^2 = random variance for the whole-plots error; $\varepsilon_{ijkl} \sim N(0, \sigma^2_{il})$, with σ^2_{il} = random variance for the split-plot error for pine species i and endophyte l; and that all variables ω_{ij} and ε_{ijkl} are independent for any values of i, j, k and l. The model thus included eleven variance parameters, which were estimated using the Restricted Maximum Likelihood method (REML).

3. Results

3.1 Preliminary confrontation assays

A total of 546 isolates of *Pinus* spp. endophytes were tested against *F. circinatum* in the preliminary confrontation assays. Of these, 154 were selected for inclusion in the in vitro antagonism study because of the effect they produced on the growth or shape of the colonies of the pathogen. Occupation of ecological niche (ecological effects) was the strategy most commonly exhibited by the endophytes, although antibiosis (direct effects) was also observed in some cases.

3.2 In vitro antagonism experiment

The linear mixed model revealed significant differences in the four indicators of antagonism used for the 154 endophytes tested. For all indicators (except for the second, i.e. shape coefficient) statistically significant differences were also found between the two isolates of *F. circinatum* plated in the dual cultures (Fc70 and Fc221) (Table 3).

Table 3. Summary of the results of the two-way ANOVA of the four indicators used in the in vitro antagonism experiment.

Dependent variable	Source	d.f.	F-value	P-value
Lenght of the central axis of the colony	Endophyte	154	108.08	<0.001
	F. circinatum	1	39.49	<0.001
	Endophyte * *F. circinatum*	154	19.82	<0.001
Shape coefficient	Endophyte	154	12.76	<0.001
	F. circinatum	1	3.19	0.074
	Endophyte * *F. circinatum*	154	5.56	<0.001
Percentage inhibition of the radial growth	Endophyte	154	9.83	<0.001
	F. circinatum	1	31.00	<0.001
	Endophyte * *F. circinatum*	154	3.18	<0.001
Percentage of inhibition zone	Endophyte	154	96.23	<0.001
	F. circinatum	1	4111.64	<0.001
	Endophyte * *F. circinatum*	154	29.37	<0.001

In total, 138 of the 154 endophyte isolates tested showed an antagonistic effect towards at least one of the two isolates of *F. circinatum* and with at least one of the four indicators

used. The identity of the endophytes (at least to the genus level) is shown in Table 4. Percentage inhibition of radial growth was the indicator that detected the greatest number of antagonists (82 against Fc70 and 99 against Fc221), while length of the central axis (41 and 64 respectively) and percentage of inhibition zone (42 and 62 respectively) were the most restrictive indicators. Growth of isolate Fc221 of *F. circinatum* (MAT-2) was more easily controlled by the endophytes than that of isolate Fc70 (MAT-1), as the number of antagonists detected by the four indicators was always higher in the confrontations against Fc221 than against Fc70. Thus, nine endophytes showed antagonism (using the four indicators) towards the MAT-2 isolate of *F. circinatum*, but none in the case of the MAT-1 isolate. Thirty nine of the endophytes with antagonistic effect towards *F. circinatum* belonged to the genus *Trichoderma*, which is the most widely represented genus in the list.

3.3 Field test

The amount of damage caused by *F. circinatum* on the seedlings differed between the pine species inoculated (p-value<0.001). *Pinus radiata* was the most susceptible of the five species tested, and the AUDPC for the seedlings, in which only the pathogen was inoculated, was almost five times higher than the same variable in *P. sylvestris,* the second species in the ranking. These two species, together with *P. pinaster*, should be considered as susceptible to the pathogen, as the value of the variable AUDPC was significantly higher in the CF treatment (inoculation of the pathogen but not the endophyte) than in the control (neither the pathogen nor the endophyte were inoculated). On the contrary, the AUDPC was not significantly different between those two treatments for the species *P. nigra* and *P. pinea*, which cannot therefore be considered as susceptible to *F. circinatum* (Figure 2).

Fusarium circinatum also yielded a reduction in growth of the pine seedlings. In the case of *P. radiata*, the growth in collar diameter was reduced by 50.6% ($t=8.84$, $p<0.001$) and 48.6% in height ($t=8.30$, $p<0.001$). Similarly, the growth of the live crown length of the *P. radiata* seedlings inoculated with the pathogen was 58.0% less than in the control seedlings ($t=6.54$, $p<0.001$). In the other pine species, the reduction was smaller and not statistically significant (Table 5).

Table 4. Identity of the endophytes after comparison in the GenBank database of the ITS, EF or LSU sequences, as well as the significance (*) for each endophyte of the five indicators evaluated in the in vitro antagonism assay. Accession numbers of the EF sequences in bold.

Code	Significative indicators								Identity	Accession number	
	MAT-1				MAT-2					ITS / EF	LSU
	1	2	3	4	1	2	3	4			
HP001	*			*	*		*	*	*Trichoderma virens*	KT323108	KT323213
HP002	*			*		*	*		*Fusarium sp.*	KT323109	KT323214
HP003						*	*		*Neopestalotiopsis clavispora*		KT323215
HP004	*		*	*	*		*	*	*Trichoderma virens*	KT323110	KT323216
HP005	*		*	*	*		*	*	*Trichoderma virens*	KT323111	KT323217
HP006						*	*		*Fusarium sp.*	KT323112	KT323218
HP007						*	*		*Diaporthe sp.*	KT323113	KT323219
HP008			*			*	*		*Bionectria ochroleuca*	KT323114	KT323220
HP009			*			*	*		*Phomopsis sp.*	KT323115	KT323221
HP010	*			*	*		*	*	*Trichoderma harzianum*	KT323116	KT323222
HP011	*			*	*		*	*	*Trichoderma harzianum*	KT323117	KT323223
HP012	*			*	*			*	*Trichoderma sp.*	KT323118	KT323224
HP013	*			*	*				*Biscogniauxia mediterranea*		KT323225
HP014	*		*	*	*		*		*Trichoderma harzianum*	KT323119	KT323226
HP015		*	*			*	*		*Diaporthe leucospermi*	KT323120	KT323227
HP016						*	*		*Diaporthe sp.*	KT323121	KT323228
HP017		*	*			*	*		*Truncatella angustata*	KT323122	KT323229
HP018		*	*			*	*		*Macrophomina phaseolina*		KT323230
HP019		*							*Nectria balsamea*	KT323123	KT323231
HP020						*	*		*Penicillium glabrum*	KT323124	KT323232
HP021					*		*	*	*Fusarium sp.*	KT323125	
HP022		*	*			*	*		*Fusarium sp.*	KT323126	KT323233
HP023		*	*			*	*		*Microdochium sp.*	KT323127	KT323234
HP024						*	*		*Fusarium acuminatum*	KT323128	KT323235
HP025		*	*			*	*		*Daldinia childiae*	KT323129	KT323236
HP026		*	*	*	*		*	*	*Trichoderma atroviride*	KT323130	KT323237
HP027	*		*	*	*			*	*Trichoderma atroviride*	KT323131	KT323238
HP028	*			*	*		*	*	*Trichoderma atroviride*	KT323132	KT323239
HP032		*	*		*	*	*	*	*Trichoderma spirale*	KT323134	KT323243
HP033		*	*		*			*	*Trichoderma spirale*	KT323135	KT323244
HP034		*	*		*	*	*	*	*Trichoderma spirale*	KT323136	KT323245
HP035		*	*			*	*		*Biscogniauxia mediterranea*		KT323246
HP036						*	*		*Alternaria sp.*	KT323137	KT323247
HP037						*	*		*Phaecytostroma ambiguum*	KT323138	KT323248
HP038					*	*	*	*	*Aspergillus leporis*	KT323139	

Code	Significative indicators								Identity	Accession number	
	MAT-1				MAT-2					ITS / EF	LSU
	1	2	3	4	1	2	3	4			
HP039		*	*			*	*		*Biscogniauxia mediterranea*	KT323140	KT323249
HP040			*		*	*	*	*	*Macrophomina phaseolina*		KT323250
HP041						*	*		*Biscogniauxia mediterranea*		KT323251
HP042							*		*Biscogniauxia mediterranea*		KT323252
HP043					*			*	*Penicillium glabrum*		KT323253
HP046						*	*		*Trichoderma harzianum*		KT323256
HP048	*			*	*			*	*Trichoderma atroviride*		KT323258
HP049					*		*	*	*Trichoderma atroviride*		KT323259
HP050		*							*Pezicula sp.*	KT323143	KT323260
HP052		*							*Bionectria ochroleuca*	KT323145	KT323262
HP054						*	*		*Schizophyllum commune*	KT323145	KT323264
HP056		*	*			*	*		*Diaporthe viticola*	KT323149	KT323266
HP057						*	*		*Alternaria sp.*	KT323150	KT323267
HP060						*			*Neonectria radicicola*		KT323270
HP062		*							*Penicillium sp.*	KT323154	KT323272
HP065		*	*			*	*		*Penicillium sp.*	KT323155	KT323274
HP066		*	*			*	*		*Alternaria sp.*	KT323156	KT323275
HP067		*	*			*	*		*Neonectria radicicola*	KT323157	KT323276
HP068		*	*			*	*		*Penicillium decaturense*	KT323158	KT323277
HP069		*	*			*	*		*Fusarium oxysporum*	**KT323357**	KT323278
HP070						*	*		*Penicillium biourgeianum*	KT323159	KT323279
HP071		*							*Penicillium biourgeianum*	KT323160	KT323280
HP072		*							*Chaetomium sp.*	KT323161	KT323281
HP073					*			*	*Biscogniauxia mediterranea*		KT323282
HP075			*		*			*	*Sordaria sp.*	KT323162	KT323284
HP076		*	*			*			*Phoma herbarum*	KT323163	KT323285
HP077		*	*			*			*Sydowia polyspora*	KT323164	KT323286
HP078		*	*			*	*		*Alternaria sp.*	KT323165	KT323287
HP079						*	*		*Fusarium equiseti*	**KT356155**	KT323288
HP080		*							*Preussia sp.*	KT323166	KT323289
HP081		*	*			*	*		*Epicoccum nigrum*	KT323167	KT323290
HP082	*		*	*	*		*	*	*Trichoderma viride*	KT323168	KT323291
HP083			*		*			*	*Biscogniauxia mediterranea*		KT323292
HP084						*	*		*Coniophora puteana*	KT323169	KT323293
HP085		*	*						*Fusarium proliferatum*	**KT356156**	KT323294

Code	Significative indicators								Identity	Accession number	
	MAT-1				MAT-2					ITS / EF	LSU
	1	2	3	4	1	2	3	4			
HP086		*	*			*	*		*Fusarium proliferatum*	**KT356157**	KT323295
HP087		*							*Neonectria radicicola*	KT323170	KT323296
HP088		*	*			*	*		*Fusarium sp.*	**KT356158**	KT323297
HP089						*			*Phialocephala sp.*	KT323171	KT323298
HP090	*		*	*	*		*	*	*Trichoderma viride*		KT323299
HP091		*							*Neonectria sp.*		KT323300
HP092	*			*	*			*	*Trichoderma atroviride*		KT323301
HP093	*			*	*			*	*Trichoderma viride*		KT323302
HP094		*							*Phialocephala sp.*	KT323172	KT323303
HP095		*	*			*	*		*Fusarium sp.*	**KT356159**	KT323304
HP096						*	*		*Phomopsis sp.*	KT323173	
HP097	*			*	*		*	*	-		
HP098	*		*	*	*		*	*	*Trichoderma viride*		KT323305
HP099			*		*		*	*	*Trichoderma harzianum*	KT323174	KT323306
HP100	*		*	*	*		*	*	*Trichoderma harzianum*	KT323175	KT323307
HP101			*						*Mucor moelleri*	KT323176	KT323308
HP102		*	*			*	*		*Ceratobasidium sp.*		KT323309
HP103		*	*			*	*		*Ceratobasidium sp.*		KT323310
HP104			*		*	*	*	*	-		
HP105	*			*	*	*	*	*	*Trichoderma sp.*	KT323177	
HP106	*		*	*	*	*	*	*	*Trichoderma sp.*		KT323311
HP107	*			*	*		*	*	*Trichoderma harzianum*	KT323178	KT323312
HP108		*	*			*	*		*Phomopsis sp.*	KT323179	KT323313
HP109	*		*	*	*	*	*	*	*Trichoderma sp.*		KT323314
HP110	*		*	*	*	*	*	*	*Trichoderma sp.*		KT323315
HP112		*				*			*Nemania diffusa*	KT323181	KT323317
HP113						*	*		*Bionectria ochroleuca*	KT323182	
HP114			*			*	*		*Daldinia sp.*	KT323183	KT323318
HP115			*		*	*	*	*	*Daldinia sp.*	KT323184	KT323319
HP116			*						*Biscogniauxia mediterranea*	KT323185	KT323320
HP117	*		*	*	*	*	*	*	*Trichoderma atroviride*		KT323321
HP118					*	*	*	*	*Biscogniauxia mediterranea*	KT323186	KT323322
HP119	*		*	*	*	*	*	*	*Trichoderma atroviride*		KT323323
HP120			*		*		*	*	*Daldinia sp.*	KT323187	KT323324

Code	Significative indicators								Identity	Accession number	
	MAT-1				MAT-2					ITS / EF	LSU
	1	2	3	4	1	2	3	4			
HP121			*			*	*		*Biscogniauxia mediterranea*	KT323188	KT323325
HP122			*		*			*	-		
HP124			*		*			*	*Daldinia sp.*	KT323190	KT323327
HP125			*		*	*	*	*	-		
HP126	*	*	*		*	*	*		*Trichoderma atroviride*		KT323328
HP127		*	*			*	*		*Diaporthe viticola*	KT323191	KT323329
HP128		*	*		*	*	*	*	*Botryotinia fuckeliana*	KT323192	KT323330
HP129		*	*			*			*Nectria sp.*	KT323193	KT323331
HP130		*				*			*Epicoccum nigrum*	KT323194	KT323332
HP131							*		*Daldinia sp.*	KT323195	KT323333
HP132					*		*	*	*Biscogniauxia mediterranea*	KT323196	KT323334
HP133		*	*			*	*		*Nectria sp.*	KT323197	KT323335
HP134		*	*		*			*	*Trichoderma atroviride*		KT323336
HP135	*		*	*	*			*	*Absidia coerulea*		KT323337
HP136	*		*	*	*		*	*	*Trichoderma atroviride*		KT323338
HP137	*		*	*	*	*	*	*	*Trichoderma sp.*	KT323198	KT323339
HP138	*			*	*	*	*	*	*Mucor hiemalis*	KT323199	
HP139	*		*	*	*	*	*	*	*Mucor hiemalis*	KT323200	KT323340
HP140	*		*	*	*		*	*	*Trichoderma asperellum*	KT323201	KT323341
HP141					*	*	*	*	*Absidia coerulea*		KT323342
HP142	*		*	*	*	*	*	*	*Mucor hiemalis*	KT323202	KT323343
HP143	*		*	*	*		*	*	*Trichoderma asperellum*	KT323203	KT323344
HP144	*		*	*	*		*	*	*Nigrospora sp.*	KT323204	KT323345
HP145			*						*Diplodia pinea*	KT323205	KT323346
HP146			*			*			*Diplodia pinea*	KT323206	KT323347
HP147		*	*			*	*		*Schizophyllum commune*	KT323207	KT323348
HP148		*	*		*		*	*	*Chaetomium globosum*	KT323208	KT323349
HP149	*		*	*	*		*	*	*Aspergillus flavus*	KT323209	KT323350
HP150	*		*	*	*		*	*	*Trichoderma atroviride*		KT323351
HP151	*		*	*	*	*	*	*	*Trichoderma atroviride*		KT323352
HP152						*	*		*Epicoccum nigrum*	KT323210	KT323353
HP153			*						*Sordaria fimicola*	KT323211	KT323354
HP154			*						*Diplodia pinea*	KT323212	KT323355
HP155	*		*	*	*		*	*	*Trichoderma viride*		KT323356

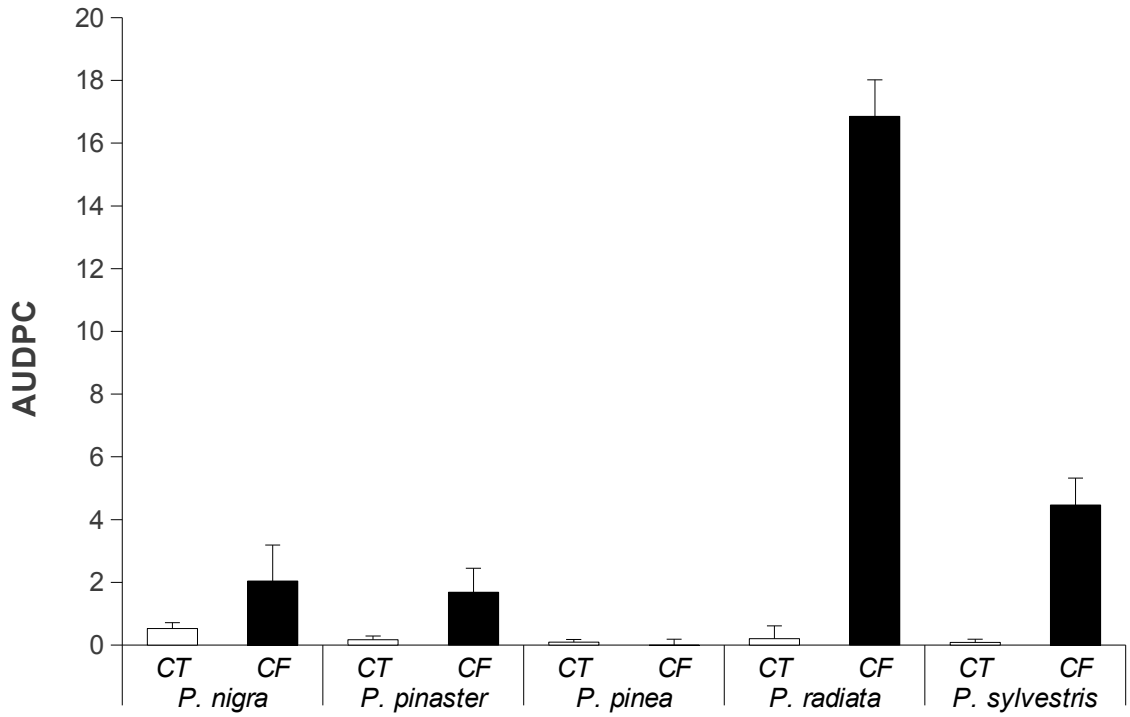

Figure 2: AUDPC (area under disease progress curve) for the seedlings of the five pine species inoculated with *F. circinatum* (CF) and the control treatment (CT). In both cases, the seedlings were not inoculated with endophytes. Error bars are standard errors. Different letters (a–e) denote significant differences (P < 0.05) between all columns (Fisher's LSD test).

Table 5. Data for growth in collar diameter, total height and live crown length of the pine seedlings in the treatments CF and CT (control). The percentage reduction relative to the control was only calculated for the species with significant differences between both treatments. Different letters (a–d) denote significant differences (P < 0.05) within the same column (Fisher's LSD test).

Species	Treatment	Growth in collar diameter		Growth in height		Growth in live crown lenght	
		Value	Reduction (%)	Value	Reduction (%)	Value	Reduction (%)
P. nigra	CT	3.24 BC		16.15 B		7.43 BC	
	CF	3.37 B		15.91 B		7.80 BC	
P. pinaster	CT	4.62 A		23.10 A		11.43 AB	
	CF	4.39 A		21.89 A		8.41 BC	
P. pinea	CT	2.73 D		11.99 C		6.72 BC	
	CF	2.85 CD		11.36 C		4.77 CD	
P. radiata	CT	4.62 A		19.76 A		16.27 A	
	CF	2.28 D	50.6	10.16 C	48.6	6.83 BC	58.0
P. sylvestris	CT	3.43 B		11.91 C		1.45 D	
	CF	4.07 AB		13.24 BC		1.16 D	

The following six endophytes were tested in the in vivo experiment because of their importance as BCAs and the good results obtained in the in vitro assay: HP031 (*Trichoderma spirale* Bissett), HP047 (*Chaetomium aureum* Chivers), HP066 (*Alternaria* sp.), HP143 (*Trichoderma asperellum* Samuels, Lieckf. & Nirenberg), HP151 (*Trichoderma atroviride* P. Karst.) and HP155 (*Trichoderma viride* Pers.). We confirmed that these did not cause any damage to the seedlings when inoculated alone, as the differences between AUDPC for CT and the treatments on which only the respective endophyte was applied were not significant for all pine species and endophyte. The endophytes HP047 and HP066 significantly reduced the damage (indicated by the AUDPC) caused by *F. circinatum* in *P. radiata* by respectively 27.8% (p=0.005) and 22.2% (p=0.025) (Figure 3). However, they were not able to prevent the decrease in growth caused by the pitch canker pathogen. For *P. sylvestris,* the seedlings inoculated with the endophyte HP031 and *F. circinatum* yielded a value of 47.8% for the AUDPC, which is apparently lower than the value obtained for the seedlings only inoculated with the pathogen (p=0.081). In the case of the three remaining species, no reduction was detected with any of the six endophytes.

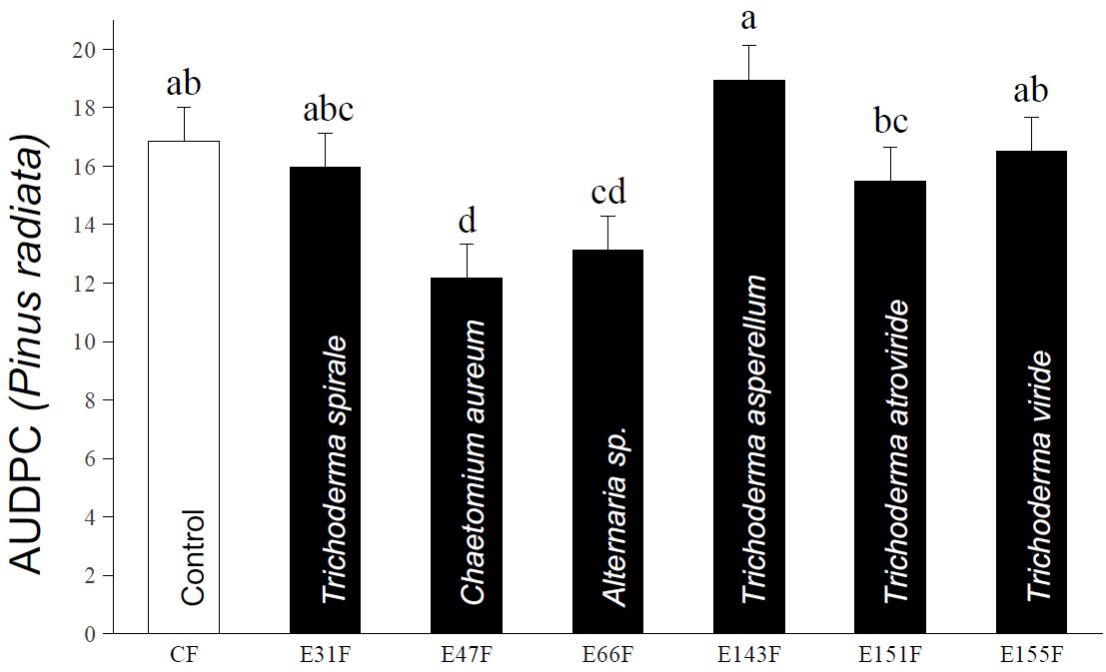

Figure 3: AUDPC (area under disease progress curve) for the species *P. radiata* when the seedlings were inoculated with *F. circinatum* (CF) only or with the different endophytes together with *F. circinatum*. Error bars are standard errors. Different letters (a–d) denote significant differences (P < 0.05) between all columns (Fisher's LSD test).

4. Discussion

The pitch canker pathogen causes substantial economic losses in pine plantations and nurseries around the world. Countries where *F. circinatum* is present are making a great effort to search for measures to control the disease or at least to minimize the damage caused by the pathogen. However, very few studies have considered biological control as a means of fighting against *F. circinatum*. In this study, we sought candidate BCAs for pitch canker disease from among a collection of 546 pine fungal endophytes. Of these, 154 showed antagonistic effect towards the pitch canker pathogen in a preliminary test. These isolates were therefore studied more exhaustively in the in vitro antagonism experiment. The vast majority of the endophytes tested showed fast growth on culture media, and occupation of ecological niche (ecological effects) was therefore the mechanism most frequently observed in the in vitro antagonism assay. Moreover, some of the fungi produced antibiosis (direct effects), thus inhibiting growth of the pathogen in the culture medium. According to at least one of the indicators used, 138 of the fungi tested in the in vitro experiment were found to be antagonists of either the MAT-1 or the MAT-2 isolates of *F. circinatum*. Of these, 39 were identified as *Trichoderma* spp., a genus first reported to show potential as a BCA in the 1930s (Howell, 2003). The genus has previously been studied as a means of controlling the pitch canker disease pathogen. Thus, two strains of *Trichoderma* spp. reduced post-emergence mortality of *P. radiata* seedlings caused by *F. circinatum* in an experiment performed by Moraga-Suazo et al. (2011). Similarly, Mitchell et al. (2005) observed that a strain of *T. harzianum* restricted growth of *F. circinatum* on culture media and even caused the collapse of the hyphae after 7 days. Apart from the *Trichoderma* spp., some of the 138 isolates were found some to belong to genera of interest in the biological control of different plant diseases. This is the case with *Diaporthe* spp. (Prada et al., 2009) or *Microdochium* spp. (Foxroberts and Deacon, 1988).

The use of different indicators of antagonism helped us to find endophytes with antagonistic activity towards *F. circinatum* that probably would have not been detected if only one indicator had been used. The endophytes show diverse types of ability to control the growth of the pathogenic colonies, and therefore several indicators must be used to enable detection of the antagonism produced via different mechanisms. However, more endophytes were antagonistic towards the *F. circinatum* VA221 (MAT-2) isolate than towards VA70 (MAT-1). Differences between mating types of *F. circinatum* have previously been reported in relation to other aspects such as pathogenicity and sensitivity to hot

water treatment. Thus, isolates of MAT-2 seem to be less virulent (Pérez-Sierra et al., 2007), more sensitive to hot water treatment (Agusti-Brisach et al., 2012) and, according to the present results, more easily controlled by fungal endophytes in artificial medium.

The antagonism that some fungi showed towards the pathogens on artificial culture media was not always maintained on the host plant. Therefore, despite obtaining good results in the in vitro experiment, we believe that an in vivo experiment must be conducted in the field. All pine species tested in this assay are among those 57 considered susceptible to *F. circinatum* (reviewed by Wingfield et al., 2008)). However, we found that damage caused by the pathogen on *P. pinea* and *P. nigra* was not significantly higher than in the control treatments. Resistance of these two pine species to the pitch canker pathogen was also recorded by Iturritxa et al. (2012). Similarly, Iturritxa et al. (2013) found that *P. pinea* was resistant to *F. circinatum* and showed an intermediate level of response to the pathogenic *P. nigra*. Similarly, in the trial performed by Carlucci et al. (2007), the authors did not observe any symptoms on *P. nigra*, but they found that *P. pinea* was susceptible to the pitch canker pathogen. On the other hand, in a study performed by Martínez-Álvarez et al. (2014a), *P. nigra* was the second most susceptible species to the pitch canker pathogen, just after *P. radiata*, but *P. sylvestris* did not produce symptoms of the disease after the inoculation with the pathogen. The different results obtained in these studies show that the susceptibility of different pine species may depend on many factors, such as the provenance of the seedlings, the age at which the seedlings were inoculated, the method of inoculation and the environmental conditions. Clearly, *P. radiata* is one of the pine species most susceptible to pitch canker disease, as shown in previous studies and confirmed here. The mortality rate calculated for seedlings of this species (74%) is very similar to that obtained by other authors (Hodge and Dvorak, 2000; Martínez-Álvarez et al., 2014a).

The endophyte isolates HP047 and HP066 significantly reduced the damage caused by the pathogen *F. circinatum* on *P. radiata* seedlings. These were isolated from *Chaetomium aureum* and *Alternaria* sp. respectively, and both have previously been reported as BCAs. Thus, *C. aureum* was recently found to be an effective biocontroller of the rice blast pathogen *Magnaporthe grisea* (T.T. Hebert) M.E. Barr and sheath blight pathogen *Rhizoctonia solani* J.G. Kühn both in in vitro and in vivo (Wang et al., 2013). On the other hand, *Alternaria* is a fungal genus that includes saprophytic, endophytic and pathogenic species (Thomma, 2003). Some of these have been used as antagonists of other plant

pathogens, because some metabolites of *Alternaria* species display a variety of types of biological activity such as phytotoxic, cytotoxic and antimicrobial activity (Lou et al., 2013). This is the case of the study performed by Campanile et al. (2007), in which *Alternaria alternata* (Fr.) Keissl. showed considerable antagonistic activity towards *Diplodia corticola* A.J.L. Phillips, A. Alves & J. Luque, a fungus that causes dieback and canker disease of the apical twigs and branches of *Quercus* spp. Similarly, Feng and Ma (2010) extracted four compounds from a culture of *Alternaria* sp. that significantly inhibited the growth of several phytopathogenic fungi, including some *Fusarium* species.

Although the *Trichoderma* isolates tested in the study produced good results in the in vitro assay, the same level of effectiveness was not observed under in vivo conditions. The different behaviour of the isolates may be explained by the method of inoculation. Although they are able to colonize the phloem and even the sapwood of the trees (Jankowiak, 2006), *Trichoderma* spp. are typically soil fungi and are usually present in the rhizosphere (Harman et al., 2004). It is possible that the time of 42 days was not long enough for the fungi to become established in the host and colonize the tissues that the pathogen will then infect. Producing pine seedlings on a substrate amended with the *Trichoderma* isolates to be tested may be a better way of using this specific group of endophytes as BCAs.

This study reports promising findings in relation to the use of fungal endophytes for biological control of pitch canker disease, although further research is needed to confirm the findings. The isolates HP047 (*C. aureum*) and HP066 (*Alternaria* sp.), which reduced the damage caused by the pathogen, should be studied in greater detail with the aim of increasing their positive effect on diseased seedlings. Different methods of applying the antagonists should be tested along with many other endophytes to find an effective BCA for the disease.

Acknowledgements

This research was financially supported by the Government of Cantabria, the Ministry of the Environment and Rural and Marine Affairs, Ministry of Agriculture, INIA and by grants provided by the University of Valladolid. The Instituto Agroforestal Mediterráneo provided the *F. circinatum* isolates for the in vitro antagonism experiment. The authors also thank Milagros de Vallejo, Juan Blanco and the technical staff at the 'Dirección General de Montes y Conservación de la Naturaleza (Government of Cantabria)' for their help in the

establishment of the plot used in the field experiment, and África Miravalles for laboratory assistance.

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Original article IV

Martínez-Álvarez, P.; Vainio, E.J.; Botella, L.; Hantula, J. & Diez, J.J. 2014. Three mitovirus strains infecting a single isolate of *Fusarium circinatum* comprise the first putative members of Narnaviridae among species of *Fusarium*. *Archives of Virology* 159(8): 2153-2155.

Resumen

Tres cepas de mitovirus que infectan un solo aislamiento de *Fusarium circinatum* constituyen los primeros miembros putativos de la familia Narnaviridae en *Fusarium* spp.

Fusarium circinatum Nirenberg & O'Donnell (teleomorfo = *Gibberella circinata*) es el causante de la enfermedad del chancro resinoso de los pinos. Está presente en Europa desde el año 2004, y particularmente en España afectando a las especies *Pinus radiata* y *P. pinaster* tanto en viveros forestales como en plantaciones en el monte. La enfermedad se ha extendido a otros países europeos, tales como Francia, Italia y Portugal. En este artículo, se describen tres nuevos miembros del género Mitovirus obtenidos de un aislamiento español de *F. circinatum:* Fusarium circinatum mitovirus 1 (FcMV1), FcMV2-1 and FcMV2-2. Usando la tabla de traducción mitocondrial, el genoma completo de FcMV1 (2419 bp) codifica para una ARN polimerasa dependiente de ARN (RdRp) de 731 aminoácidos (contenido GC del 30% aproximadamente). Los genomas parciales de FcMV2-1 y FcMV2-2 (2193 y 1973 bp, respectivamente) comparten aproximadamente el 48% de la secuencia de la RdRp a nivel de aminoácidos y podrían considerarse pertenecientes a la misma especie. FcMV1 es claramente diferente, presentando un 32-35% de similitud con las otras cepas. Sin embargo, FcMV1 mostró un 46% de similitud con Thielaviopsis basicola mitovirus. Este es el primer estudio que detecta virus en *F. circinatum*, así como el primero en que se describen secuencias de mitovirus obtenidas en *Fusarium* spp.

Palabras clave: chancro resinoso del pino, virus, RdRp, virología.

Arch Virol (2014) 159:2153–2155
DOI 10.1007/s00705-014-2012-8

ANNOTATED SEQUENCE RECORD

Three mitovirus strains infecting a single isolate of *Fusarium circinatum* are the first putative members of the family *Narnaviridae* detected in a fungus of the genus *Fusarium*

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Received: 12 November 2013 / Accepted: 27 January 2014 / Published online: 12 February 2014

Abstract *Fusarium circinatum* Nirenberg & O'Donnell (teleomorph = *Gibberella circinata*) is the causal agent of pitch canker disease of pines. Since 2004 it has been present in Europe, particularly in northern Spain, affecting *P. radiata* and *P. pinaster* in plantations and nurseries. The disease has now also spread to other European countries, including France, Italy and Portugal. In this report, we describe three novel members of the genus *Mitovirus* from a Spanish isolate of *F. circinatum*: *Fusarium circinatum* mitovirus 1 (FcMV1), FcMV2-1 and FcMV2-2. Using a mitochondrial translation table, the complete 2419-bp genome of FcMV1 encodes an RNA-dependent RNA polymerase of 731 amino acids (GC-content *ca* 30 %). The partial genomes of FcMV2-1 and FcMV2-2 (2193 and 1973 bp, respectively) share *ca* 48 % RdRp sequence similarity at the aa level and might be regarded as conspecific, while FcMV1 is clearly distinct, showing 32-35 % polymerase similarity to the other strains. However, FcMV1 shared 46 % protein-level similarity with *Thielaviopsis basicola* mitovirus. This is the first study to report viruses in *F. circinatum*, as well as the first time that mitovirus genome sequences are described from *Fusarium* spp.

Nucleotide sequence data reported in this study are available in the GenBank under the accession numbers KF803546-48.

Electronic supplementary material The online version of this article (doi:10.1007/s00705-014-2012-8) contains supplementary material, which is available to authorized users.

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Introduction

Pitch canker is a severe disease of conifers caused by the fungus *Fusarium circinatum* Nirenberg & O'Donnell (teleomorph = *Gibberella circinata*). The main symptom caused by this pathogen is a resinous bleeding canker in the trunk or large branches of the tree [9]. Infected trees eventually die due to girdling of the stem or as a result of stem breakage at the site of the canker. The fungus has a global distribution, and Monterey pine (*Pinus radiata* D. Don), the most commonly used exotic pine in forest plantations around the world, is also one of the species most susceptible to the disease [5]. There is no effective treatment to control the disease so far.

Fungal viruses of the genus *Mitovirus* belong to the family *Narnaviridae*, the members of which are the simplest viruses known. Although they have a (+)ssRNA linear genome of approximately 2.5 kb, they usually occur as dsRNA replicative forms in the mitochondria, where they are located and translated. Mitoviruses have been found in many phytopathogenic fungi, including both ascomycetes and basidiomycetes [6]. Fungi of the genus *Fusarium* host diverse viruses, including members of the families *Chrysoviridae*, *Hypoviridae*, *Partitiviridae* and *Totiviridae* [2], as well as the yet unassigned virus FgV1 (*Fusarium graminearum* virus 1), which causes

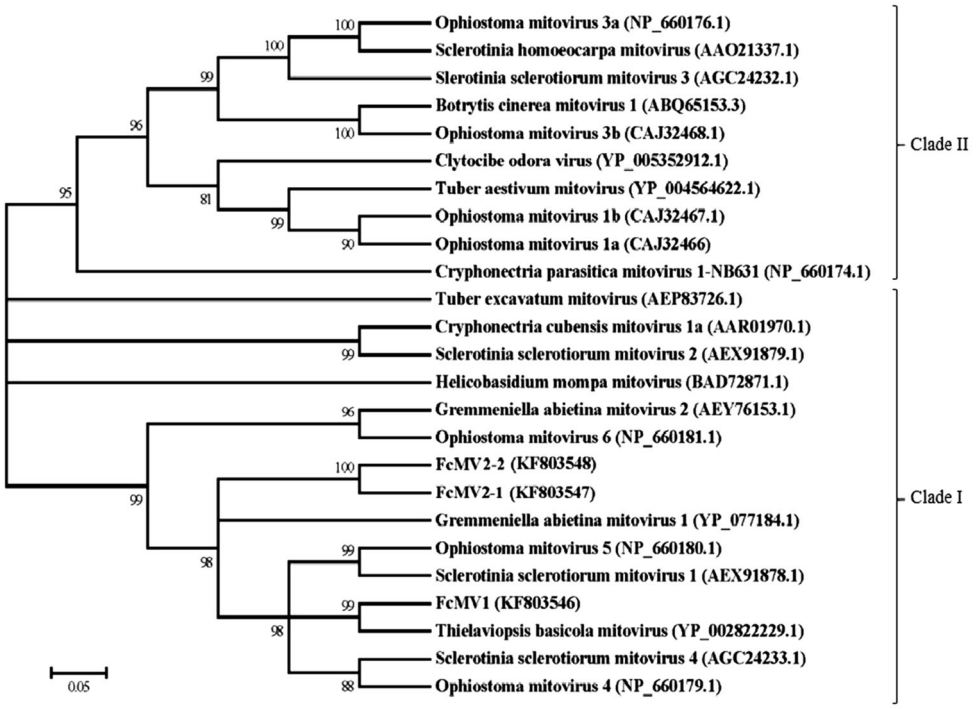

Fig. 1 Neighbor-joining phylogenetic tree based on the amino acid sequences of the RdRp of FcMV1, FcMV2-1, FcMV2-2 and the most similar mitoviruses available in GenBank. The bootstrap consensus tree was inferred from 1000 replicates. Branches corresponding to partitions reproduced in fewer than 80 % of the bootstrap replicates were collapsed. The percentage of replicate trees in which the associated sequences clustered together in the bootstrap test is shown next to each branch. Evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. All positions containing gaps and missing data were eliminated. There were a total of 420 positions in the final dataset

hypovirulence in its host [3]. Although dsRNAs have been reported to be associated with *Fusarium proliferatum* mitochondria [8], no mitovirus genome sequences have been described so far from members of the genus *Fusarium*.

The complete genome described here as belonging to *Fusarium circinatum* mitovirus 1 (FcMV1) and the partial genomes of FcMV2-1 and FcMV2-2 represent the first of occurrences a virus in *F. circinatum*, and these viruses are the first members of the family *Narnaviridae* found to infect members of the genus *Fusarium*.

Provenance of the virus material

Fusarium circinatum strain FcCa070 was isolated from the xylem of a *P. radiata* tree collected on October 6, 2008. The location was Monte Corona, Cantabria (Spain), which is a plantation severely affected by the disease. Fungal cultures were established from plant material as described previously [11]. CF11 cellulose affinity chromatography was used to extract putative viral double-stranded RNA (dsRNA) molecules from lyophilized cells as described earlier [12, 14, 15].

Complementary DNA was generated using random priming, and the sequences of the distal ends of the viral genome were determined by adapter ligation, followed by PCR amplification as described earlier [10].

PCR-amplified cDNA products were cloned into the pCR2.1-TOPO cloning vector (Invitrogen, Carlsbad, USA) and sequenced at Macrogen Inc. (www.macrogen.com). Sequence positions with insufficient coverage were determined using specific primers.

Phylogenetic analysis was conducted using Geneious Pro 5.1.6 and MEGA 5 [13].

Sequence properties

Three putative viral RdRp sequences were detected in one isolate of *F. circinatum*.

The total length of the FcMV1 genome was 2419 bp. Using a mitochondrial translation table, a single ORF of 2196 bp was found. This ORF codes for a RdRp of 731 amino acids (aa), with a molecular weight of 85.4 kDa and G + C content of 29.7 %. The partial genome of FcMV2-1 had a length of 2193 bp and 28.8 % G + C. The sequence included the complete RdRp coding region but not the ends of the genome. The length of the ORF was 2175 bp, and the RdRp protein was predicted to be 724 aa, with a molecular weight of 84.7 kDa. In the case

of FcMV2-2, we obtained 1973 bp of sequence (596 aa), but the protein coding region was incomplete at the 3′ end.

All three putative virus genomes described in this study clustered with the 'clade I' mitoviruses [6] (Fig. 1). Nevertheless, there seem to be at least two phylogenetically distinct groups (i.e., FcMV1 versus FcMV2-1 and FcMV2-2) that share a low level of RdRp similarity (*ca* 32-36 %). Species delimitation criteria for mitoviruses have not yet been precisely defined. Current ICTV criteria define mitovirus variants with less than 40 % of RdRp protein similarity as members of different species, and those with more than 90 % similarity as strains of the same species [7]. FcMV2-1 and FcMV2-2 share *ca* 48 % RdRp sequence similarity at the aa level, which is higher than the current 40 % threshold for species delimitation but lower than the 90% identity required for conspecific strains. However, as these strains occur in the same host species, we suggest regarding them as strains of the same species. FcMV1 and *Thielaviopsis basicola* mitovirus share 46 % aa-level sequence similarity (Table S1). Due to the fact that their host species represent different orders of the fungal kingdom, this cannot be interpreted as a clear case of horizontal virus transfer, as in the case of *Ophiostoma novo-ulmi* mitovirus 3a-Ld [4] or *Gremmeniella abietina* mitovirus [1]. Therefore, we suggest considering FcMV1 and *T. basicola* mitovirus members of different species. Our observations may suggest a need for changes in the species delimitation criteria for mitoviruses (i.e., raising the threshold of 40 % for species delimitation).

Acknowledgments We are grateful to D. de Vega, M. Kashif, M.-L. Santanen, and S. Sarsila for their help in the laboratory work. This research was supported by the Ministry of Economy and Competitiveness (Project: AGL2012-39912), a short-term scientific mission of the EU COST Action FP1002 (PERMIT), and by grants provided by the University of Valladolid, the Academy of Finland (decision number 251193), and the Finnish Forest Research Institute.

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Original article V

Vainio, E.J.; Martínez-Álvarez, P.; Bezos, D.; Hantula, J. & Diez, J.J. 2015. *Fusarium circinatum* isolates from northern Spain are commonly infected by three distinct types of mitoviruses. *Archives of Virology* 160(8):2093-2098.

Resumen

Los aislamientos de *Fusarium circinatum* del norte de España están comúnmente infectados por tres tipos diferentes de mitovirus.

El chancro resinoso es una importante enfermedad de los pinos causada por el hongo ascomicete *Gibberella circinata* (anamorfo = *Fusarium circinatum*). En este hongo se describieron con anterioridad tres cepas de mitovirus distintas: Fusarium circinatum mitovirus 1 (FcMV1), FcMV2-1 and FcMV2-2. En este estudio se investiga la frecuencia de infección y las variaciones de la población de estos virus y sus variantes relacionados en el norte de España mediante RT-PCR y secuenciación. Cada cepa vírica y sus variantes de secuencia relacionados mostraron un 95% de similitud y fueron designados "virus types". Todos los "virus types" fueron comunes en España con una frecuencia estimada del 18.5%, 8.9% y 16.3% para FcMV1, FcMV2-1 y FcMV2-2, respectivamente.

Palabras clave: *Gibberella circinata*, chancro resinoso, ssRNA, Narnaviridae, población vírica.

Arch Virol (2015) 160:2093–2098
DOI 10.1007/s00705-015-2462-7

BRIEF REPORT

Fusarium circinatum isolates from northern Spain are commonly infected by three distinct mitoviruses

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Received: 4 March 2015 / Accepted: 20 May 2015 / Published online: 31 May 2015

Abstract Pitch canker is a serious disease of pines caused by the ascomycete fungus *Gibberella circinata* (anamorph = *Fusarium circinatum*). Three distinct mitovirus strains have been described in this fungus: Fusarium circinatum mitovirus 1 (FcMV1), FcMV2-1 and FcMV2-2. Here, we investigated the frequency and population variation of these viruses and closely related sequence variants in northern Spain using RT-PCR and sequencing. Each virus strain and similar sequence variants shared >95 % sequence identity and were collectively designated as virus types. All virus types were relatively common in Spain, with estimated prevalence of 18.5 %, 8.9 % and 16.3 % for FcMV1, FcMV2-1 and FcMV2-2, respectively.

Keywords *Gibberella circinata* · Pitch canker · ssRNA · *Narnaviridae* · Virus population

Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number(s) KP726378–KP726394.

Electronic supplementary material The online version of this article (doi:10.1007/s00705-015-2462-7) contains supplementary material, which is available to authorized users.

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The ascomycete fungus *Gibberella circinata* Nirenberg & O'Donnell (anamorph = *Fusarium circinatum*) causes a serious disease known as pitch canker on *Pinus* spp. The pathogen initiates resinous bleeding cankers on the tree trunks or large branches, and infected trees can eventually die due to girdling or stem breakage. The fungus was first reported in North Carolina [6] and Mexico, and has since spread into Haiti, South Africa, Japan, Chile, Korea, Southern Europe, Uruguay [reviewed in reference 12], Colombia [17] and Brazil [15]. *F. circinatum* has moved between plantations areas via contaminated seed and seedlings, and the fungus spreads further through airborne spores, insect vectors and water splash. Mechanical damage that provides infection courts increases the risks of infection [1]. The fungus is considered to be native in Mexico [24], the Caribbean and the southeastern USA.

Fungi of the genus *Fusarium* are hosts to diverse viruses, including members of the families *Chrysoviridae*, *Hypoviridae*, *Partitiviridae* and *Totiviridae* [3], as well as the yet unassigned Fusarium graminearum virus 1, which reduces the growth rate and virulence of its host [4]. We recently characterized three distinct strains of mitoviruses co-infecting *F. circinatum* isolate FcCa070: Fusarium circinatum mitovirus 1 (FcMV1) and two strains of Fusarium circinatum mitovirus 2 (FcMV2-1 and FcMV2-2) [13]. Fungal viruses in the genus *Mitovirus* belong to the family *Narnaviridae*, which are the simplest known viruses. They have a positive-sense ssRNA genome of ~2.5-4.4 kb, are located in mitochondria, and utilize the mitochondrial translation table [7, 22]. Mitoviruses have been found in many phytopathogenic fungi, including both ascomycetes and basidiomycetes. Some mitoviruses appear to mediate reduced virulence (hypovirulence) in important plant pathogens, including *Botrytis cinerea*, *Chalara elegans*, *Ophiostoma novo-ulmi*, *Rhizoctonia solani* and *Sclerotinia*

homeocarpa [reviewed in reference 7]. In this study, we examined the occurrence of mitoviruses in *F. circinatum* isolates collected from northern Spain, as well as 29 isolates of *F. circinatum* from South Africa, and five isolates of other species of *Fusarium* from Cantabria (northern Spain). Each new virus sequence determined in this study closely resembled that of one of the previously determined strains (FcMV1, FcMV2-1 or FcMV2-2), and we classified mitovirus sequence variants sharing >95 % similarity with one of these strains as mitovirus 'types'.

Fungal cultures were established from infected plant or insect tissues as described previously [11], and collection data for the *Fusarium* isolates used for virus screening are listed in Tables S1 and S2. Two different approaches were used for virus screening: CF11 cellulose affinity chromatography was used to detect viral dsRNA elements, and specific mitovirus types were detected by RT-PCR with selective primers. CF11 cellulose affinity chromatography was conducted using lyophilized cells as described previously [13, 19]. Complementary DNA for the RT-PCR was generated by random priming using previously described techniques [9, 20]. The following PCR primers were designed for virus screening and sequence determination: FMC3F1 (5′-GAY AGA ACT TTT ACT CAA GAT CC-3′), FMC3Rev1 (5′-ATT CAT CTY TTG GCA AAT TCA TA-3′), FMC1F1 (5′-CGT GGA TTA AAA CCC ACA AA-3′) and FMC1Rev1 (5′-TGG TAA TCT ACC ATA GCA ATT AYT C-3′). Primer pair FMC1F1/FMC1Rev1 is specific for FcMV1, whereas primer pair FMC3F1/FMC3Rev1 preferably detects virus types FcMV1, FcMV2-1 or FcMV2-2, depending on the DNA polymerase used (fusion-type versus conventional) and annealing temperature. The amplification products cover two different genome locations: the product obtained with primer pair FMC1F1/FMC1Rev1 covers nt 511-935 in the FcMV1 genome, whereas the product obtained with FMC3F1/FMC3Rev1 covers nt 1000-1478. PCRs were conducted in volumes of 50 µl, including 1-2 µl of the cDNA product, 25 pmol of each primer, 10 nmol of dNTPs and 1-1.25 units of a DNA polymerase as recommended by the manufacturer. The amplification conditions used for primer pair FMC1F1/FMC1Rev1 were as follows: 10 min at 95 °C, followed by 37 cycles of 30 s at 95 °C, 45 s at 53 °C, 2 min at 72 °C; and a final extension of 7 min at 72 °C. The same PCR program was used for primer pair FMC3F1/FMC3Rev1, but the annealing temperature was adjusted according to the polymerase enzyme used. The amplification of FcMV2-1 from isolates with FcMV2 co-infections was carried out using a conventional DNA polymerase – Dynazyme II (Thermo Scientific), DreamTaq (Thermo Scientific) or Biotools Native DNA polymerase (Biotools B & M Labs) – and an annealing temperature of 53 °C, whereas FcMV2-2 was amplified using a fusion domain containing thermostable DNA polymerase – Phusion (Thermo Scientific), PhireII (Thermo Scientific), or Q5 (New England Biolabs) – and an annealing temperature of 59 °C.

The amplicons were sequenced at Macrogen Inc. (www.macrogen.com). Regardless of the polymerase used, the sequencing results were constant for single viral sequence variants. Sequence variation was examined and phylogenetic analysis was performed using Geneious Pro 5.5.8 (Biomatters Ltd.) and MEGA 5 [18]. The frequency of sequence variants (='haplotypes') at each collection site was compared to an estimated random distribution using AMOVA, implemented in Arlequin 3.5 [5], in order to examine the level of sequence differentiation among the collection sites (i.e., 'population differentiation'). Putative recombination events were identified with RDP4 (v.4.16) [10] using the recombination detection methods RDP, GeneConv, Chimaera, MaxChi, SiScan and 3Seq (highest probability was set as 0.1).

Using CF11 chromatography, we detected dsRNA elements in 14 of the 135 *F. circinatum* isolates analyzed (Table S1). No dsRNA elements were detected among the 29 South African isolates of *F. circinatum* or the five isolates of other species of *Fusarium* (Table S2). RT-PCR using the mitovirus primers yielded amplification products from 43 *F. circinatum* isolates (31.9 %). Therefore, most of the mitovirus infections were not detectable as dsRNA. This is a common phenomenon, likely resulting from the fact that mitoviruses have ssRNA genomes, and the replicative dsRNA intermediates may have a low titer [e.g., 14, 16, 22]. Notably, co-infections by two or three mitovirus types were common, and resulted in a total of 59 virus incidences among the Spanish host isolates. Co-infections with FcMV1 and FcMV2-2 were found in nine isolates, and five isolates hosted both FcMV2-1 and FcMV2-2. One isolate hosted all three virus types.

Genetic variation within the mitovirus population was assessed by sequence analysis. The amplicon length was 425 nt for FMC1F1/FMC1Rev1 and 479 nt for FMC3F1/FMC3Rev1. The partial sequences were deposited in the GenBank database with accession numbers KP726378–KP726394 (Table 1). Based on BlastN and phylogenetic analysis including 312–351 nt sites (Fig. 1), each sequence was highly similar to one of the three mitovirus strains from *F. circinatum* FcCa070 characterized previously. This suggests that FcMV1, FcMV2-1 and FcMV2-2 are separate genetic entities (i.e., virus 'types'), each of which consists of highly similar sequence variants. Sequence identity among variants representing single virus types was >95 % (Table 1). At the protein level, the sequences were highly conserved. All three variants of FcMV2-2 were 100 % identical at the protein level, whereas three different variants of FcMV2-1 shared identical protein sequences, and

Table 1 Viral sequence characteristics

Virus strain	Variant (haplotype)[a]	Host isolate	GenBank accession no.	Primer pair	Identity (% nt) with FcMV-strains from isolate 070[b]	Sequence length	Host isolates harboring the same sequence variant (sequence length in bp)
FcMV1	a	070	KF803546	FMC1F1/Rev1	100 %	2419	**047**(337), **072**(351), **VA104**(325)
	a	070	KF803546	FMC3F1/Rev1	100 %	2419	**042**(146)[c], **045**(386), **048**(433)
	b	60_2010	KP726378	FMC1F1/Rev1	98.5 %	332	-
	c	Saint1.7	KP726379	FMC1F1/Rev1	98.4 %	322	-
	d	069	KP726380	FMC1F1/Rev1	98.3 %	354	**067**(322)
	d	069	-	FMC3F1/Rev1	100 %	392[d]	-
	e	Saint1.189	KP726381	FMC1F1/Rev1	98.0 %	322[d]	-
	f	22_2010	KP726382	FMC1F1/Rev1	97.9 %	327	-
	g	VA221	KP726383	FMC1F1/Rev1	97.8 %	348[d]	-
	h	100	KP726384	FMC1F1/Rev1	97.6 %	327	**099**(322)
	i	001	KP726385	FMC1F1/Rev1	97.2 %	326	-
	j	VA215	KP726386	FMC1F1/Rev1	95.4 %	327	-
	j	VA215	KP726387	FMC3F1/Rev1	98.3 %	372[d]	**043**(56)[cd], **050**(285)[e]
	k	052	KP726388	FMC3F1/Rev1	99.8 %	405	**051**(415)[e]
	l	041	KP726389	FMC3F1/Rev1	99.2 %	392	**068**(390), **073**(376)
FcMV2-1	a	070	KF803547	FMC3F1/Rev1	100 %	2193	**053**(132)[c]
	b	009	KP726390	FMC3F1/Rev1	99.7 %	369	**005**(390)[cd], **007**(356), **010**(354)[cd], **020**(364)
	c	018	KP726391	FMC3F1/Rev1	99.5 %	388	**019**(395)[c], **026**(371)[c], **032**(399)
	d	046	KP726392	FMC3F1/Rev1	99.5 %	389	-
FcMV2-2	a	070	KF803548	FMC3F1/Rev1	100 %	1973	**010**(359), **020**(363), **026**(363), **033**(340), **035**(360), **042**(117)[c], **054**(359)[c], **073**(321), **099**(358), **100**(372), **VA221**(394), **VA253**(333), **Saint1.7**(60)[c]
	b	004	KP726393	FMC3F1/Rev1	99.5 %	398	**001**(424), **002**(364)[d], **003**(405), **005**(394), **007**(402)[e], **22_2010**(224)
	c	60_2010	KP726394	FMC3F1/Rev1	99.3 %	355[d]	-

[a] FcMV1 sequence variants obtained using the two different primer pairs are considered distinct unless they were obtained from the same host isolate (variants FcMV1-a, FcMV1-j)

[b] Variants of FcMV1 are compared to FcMV1-a; variants of FcMV2-1, to FcMV2-1-a; and variants of FcMV2-2, to FcMV2-2-a

[c] Sequence is in one direction only. All other sequence variants have sequence coverage of at least two times. Sequences determined in one direction only (N = 10) were assigned to haplotypes according to the most reliable sequence region (56-395 nt, depending on sequence quality)

[d] The sequence included one ambiguous nucleotide site (double base calls), and the haplotype assignment was made according to the dominant nucleotide character

[e] The sequence included 2-3 ambiguous nucleotide sites (double base calls), and the haplotype assignment was made according to the dominant nucleotide character

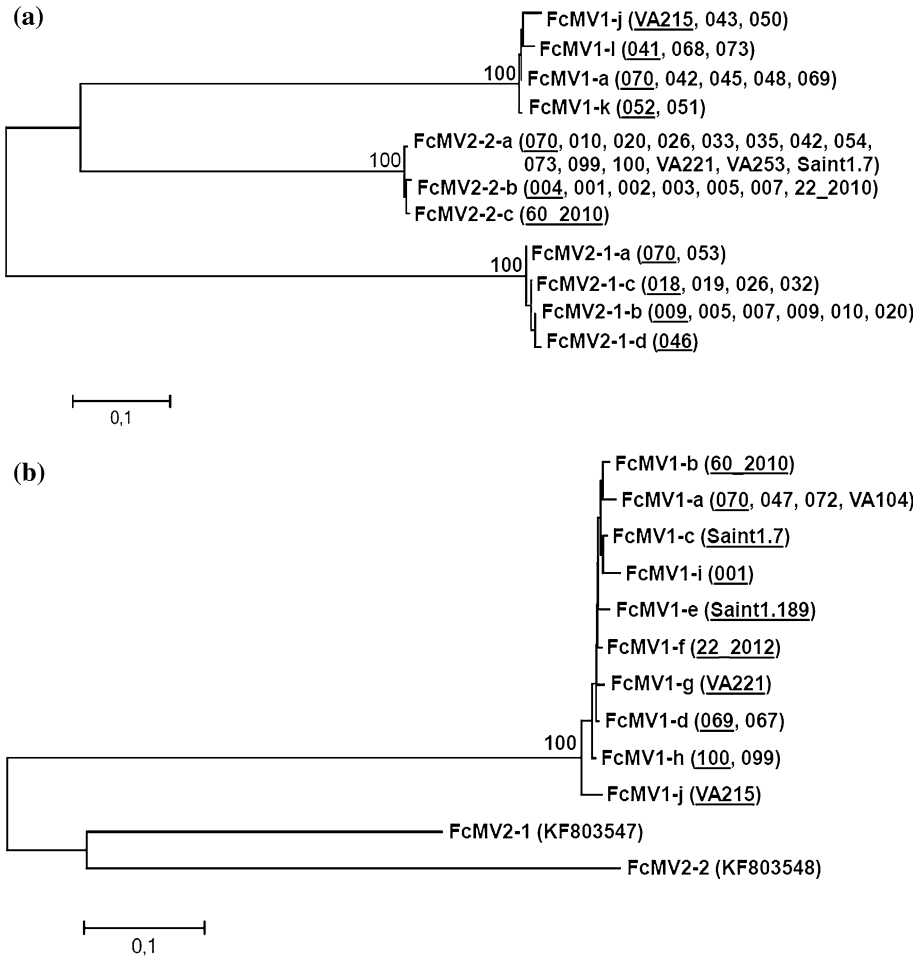

Fig. 1 Neighbor-joining dendrograms for sequence variants obtained using primer pair FMC3F1/FMC3Rev1 (**a**) or FMC1F1/FMC1Rev1 (**b**). *F. circinatum* isolates harboring the same virus haplotype (identical sequences) are shown in parentheses (see Table 1), and the representative sequence selected for dendrogram construction is underlined. Bootstrap probabilities of >90 % (1000 replicates) are shown next to branches. Distances are shown as the number of substitutions per site. Positions containing gaps or missing data were eliminated, and the number of positions used was 351 for FMC3F1/FMC3Rev1 (**a**) and 312 for FMC1F1/FMC1Rev1 (**b**)

variant FcMV2-1-c differed from them by one amino acid residue. In the case of FcMV1, different variants shared 96.9-99.3 % identity in the sequence region flanked by FMC3F1/FMC3Rev1 (1-3 aa mismatches) and 95.4-100 % in the region flanked by FMC1F1/FMC1Rev1.

The majority of the *F. circinatum* isolates investigated were from Cantabria (Fig. 2). Viruses occurred in 26 (39.4 %) of the 66 isolates from Cabezón de la Sal in Cantabria, and co-infections were found in seven isolates, resulting in a total of 33 viral infections (Table S1). In Rionansa, five (83.3 %) of the six isolates were co-infected, and the number of viral infections was 10. In Comillas, six (85.7 %) of the seven host isolates were virus-infected, and there was one double and one triple co-infection. The two isolates from Castro-Urdiales were both infected by FcMV1. However, isolates from some locations were devoid of viral infections: 17 isolates from Villafufre, 11 isolates from Mazcuerras and one isolate from Cabuérniga in Cantabria were all virus-free. Moreover, in Santiurde de Toranzo, we observed unknown dsRNA elements of ca. 2.5 kb in four *F. circinatum* isolates (Table S1). Whether they represent additional mitoviruses or members of other virus species is still unknown, but they remained undetected using our set of primers. Similarly, two unknown dsRNA virus infections were observed in Cabezón de la Sal. Outside the Cantabria region, we found two FcMV1 infections in five isolates from Asturias and one FcMV2-2 infection in seven isolates from Galicia. No viruses were detected in the four *F. circinatum* isolates from País Vasco, and none were found in the South African isolates or members of other *Fusarium* species (Table S2). It should be noted that many FcMV1 sequence variants were only detected with one of the primer pairs used, which suggests that the mitovirus frequencies reported here are conservative estimates.

Many sequence variants (i.e., haplotypes; see Table 1) were found at more than one collection site: FcMV1-a from Cabezón de la Sal, Comillas and Asturias; FMC1-j from Cabezón de la Sal and Asturias; FcMV1-l from Cabezón de la Sal and Comillas; FcMV2-2-a from Cabezón de la Sal, Comillas, Rionansa, and Galicia; and FcMV2-2-b from Rionansa, Cabezón de la Sal and Castro-Urdiales. Even though the sequences are partial, this suggests that highly similar viral sequence variants occur throughout northern

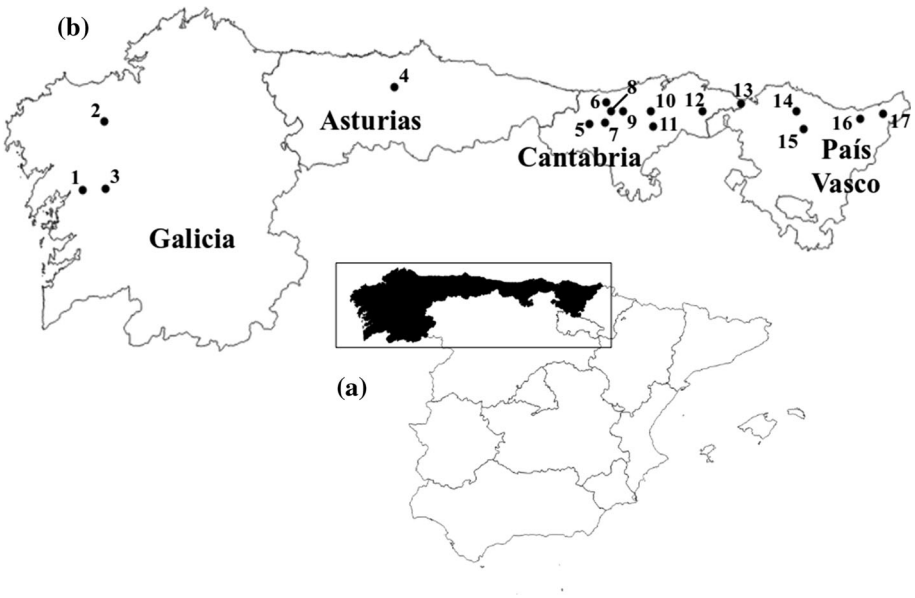

Fig. 2 Collection sites of *F. circinatum* isolates in Spain (**a**) and in the autonomous communities of Galicia, Asturias, Cantabria and País Vasco (**b**). The numbers refer to municipalities: 1 = Valga, 2 = Ordes, 3 = A Estrada, 4 = Grado, 5 = Rionansa, 6 = Comillas, 7 = Cabuérniga, 8 = Cabezón de la Sal, 9 = Mazcuerras, 10 = Villafufre, 11 = Santiurde de Toranzo, 12 = Ramales de la Victoria, 13 = Castro Urdiales, 14 = Muxika, 15 = Iurreta, 16 = Aia and 17 = Hernani

Spain. Moreover, host mating type compatibility did not seem to restrict the occurrence of mitoviruses. While the vast majority of *F. circinatum* isolates analyzed encompassed mating type idiomorph *MAT1-2* [23], which is the only mating type found in Cantabria [2], there were a total of eight host isolates with the *MAT1-1* mating type in Asturias or Galicia (Table S1). FcMV1 or FcMV2-2 was detected in two of the *MAT1-1* isolates, namely *F. circinatum* VA104 and VA253, respectively. Notably, the latter isolate originated from Galicia and hosted a sequence variant that was identical over 333 nt to FcMV2-2-a, hosted by isolate 070 (=FcCa070) from Comillas, Cantabria. These collection sites are located more than 250 kilometers apart.

Single collection sites typically harbored more than one virus sequence variant, and all three virus types occurred in Cabezón de la Sal and Comillas. Moreover, based on AMOVA analysis, there seemed to be a low-level geographical differentiation between the locations in terms of viral polymorphism. Considering all haplotypes obtained using primer pair FMC3F1/FMC3Rev1, 85.6 % of the variation was attributable to within-population variation, and only 14.4 % was attributable to between-population variation ($P = 0.03$). Individual analysis of haplotypes representing each virus type showed similar results: the level of within-population variation was 79.8-91.3 % in FcMV1 (sequence regions flanked by FMC3F1/FMC3Rev1 and FMC1F1/FMC1Rev1, respectively), 64.0 % in FcMV2-1, and 85.2 % in FcMV2-2, but these results were only tentative ($0.27 \geq P \geq 0.10$).

The number of sequence variants was higher for the sequence region flanked by primer pair FMC1F1/FMC1Rev1 than for that flanked by primer pair FMC3F1/FMC3Rev1. This is due to differences in the level of sequence polymorphism in the sequence regions analyzed: the FcMV1, FcMV2-1 and FcMV2-2 genomes share ~52.2-58.2 % identity at the sequence region flanked by primer pair FMC1F1/FMC1Rev1, whereas sequence identity in the sequence region flanked by primer pair FMC3F1/FMC3Rev1 is higher, and the three virus types share 62.4-67.1 % identity. The sequence region covered by FMC3F1/FMC3Rev1 spans the conserved amino acid sequence 'motif IV' in the RdRp-like proteins encoded by mitochondrial viruses, as determined by Hong et al. [8], whereas the sequence region covered by FMC1F1/FMC1Rev1 is more variable, with only the last 5-14 amino acids overlapping with conserved motif I.

Two putative recombination events were detected using the RDP program among the FcMV1 sequences obtained using primer pair FMC1F1/FMC1Rev1. One putative breakpoint (recombination event) was located at nucleotide site 645 in the FcMV1 genome and was identified by MaxChi, Geneconv and SiScan to have occurred among sequence variants FcMV1-a, FcMV1-g and FcMV1-j ($P = 8.2^{-03}$). Another putative breakpoint detected by 3Seq was located at site 625 in the FcMV1 genome and seemed to have occurred among sequence variants FcMV1-j, FcMV1-b and FcMV1-f ($P = 2.4^{-02}$). No recombination events were detected in the conserved sequence region amplified using primer pair FMC3F1/FMC3Rev1.

North America (USA) is considered to be the area of origin of the Spanish population of *F. circinatum*, and there seem to have been at least two introductions of the pathogen into Spain, followed by clonal spread [2]. As a result, only two to three *F. circinatum* clones occur in Spain. The South African population is considerably more

diverse than the Spanish population and probably originates from Mexico [24]. The *F. circinatum* mycovirus diversity in the United States remains to be investigated and might shed more light on the dispersal pattern of this pathogen, i.e., finding identical virus strains at these geographically distant locations might confirm speculations about the origin of the fungal host. In this study, we observed vast differences in the incidence of viral infection of *F. circinatum* between nearby collection sites. For example, viruses were absent in eleven isolates in Mazcuerras, whereas viruses were common in the nearby ($\sim$8 km distance) Cabezón de la Sal. In contrast, single virus types in Spain appeared to have a wide geographical distribution. Because the presence or absence of viruses in sexual and asexual spores may have a profound effect on the dispersal patterns of mycoviruses [21], future studies are needed to investigate whether viruses are present in the conidia or ascospores of *F. circinatum*.

F. circinatum mitoviruses seem to be common and polymorphic in northern Spain. The occurrence of several viral types and variants at single collection sites is consistent with the occurrence of single clonal genotypes of *F. circinatum* throughout a wide geographical region in northern Spain.

Acknowledgements This study was financially supported by the Academy of Finland (decision numbers 251193 and 258520), the Spanish Ministry of Economy and Competitiveness (Project: AGL2012-39912), the Government of Cantabria, EU COST Actions FP1002 and FA1103 and the University of Valladolid. Instituto Agroforestal Mediterráneo and the Forestry and Agricultural Biotechnology Institute of the University of Pretoria provided strains for mitovirus screening. We are grateful to M.-L. Santanen, S. Sarsila, E. Simón Vicente, M. de Vallejo and J. Blanco for technical assistance, and Prof. M. Wingfield for commenting on the manuscript.

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