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

Pablo Martínez-Álvarez, Fernando Manuel Alves-Santos and Julio Javier Diez

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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
Addresses Sustainable Forest Management Research Institute, University of Valladolid – INIA, Avenida de Madrid 44, 34071 Palencia, Spain **E-mail** (Martínez-Álvarez) pmtnez@pvs.uva.es
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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.

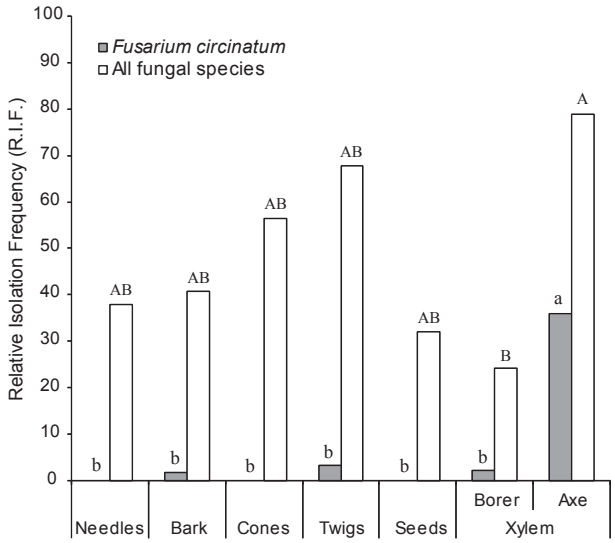

Fig. 1. Relative Isolation Frequency (R.I.F.) of *Fusarium circinatum* (black bars) and all fungal species detected (white bars), in relation to the tissue sampled and their respective homogeneous groups. Columns indicated by the same letter (a–b to denote significant differences in the abundance of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

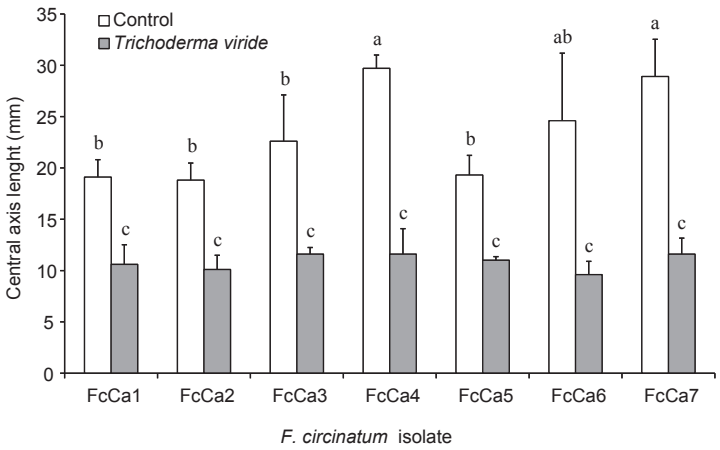

Fig. 2. Distribution of the variable length of the central axis of the *F. circinatum* colony in the control treatment (white bars) and in dual culture with *T. viride* (black bars). Error bars are standard deviations. Letters (a–c) denote significant differences ($p < 0.05$) among all columns (Scheffe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

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