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

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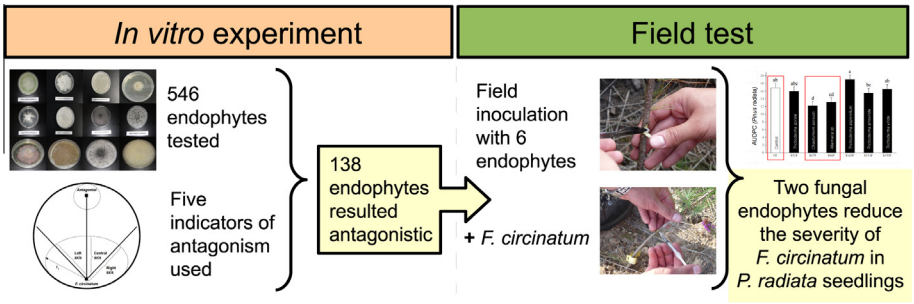
HIGHLIGHTS

- Five hundred forty-six endophytes were tested against *F. circinatum* *in vitro*.
- The antagonistic activity of the endophytes was quantify using five indicators.
- The six endophytes that showed the most promising results were tested in the field.
- *Chaetomium aureum* and *Alternaria* sp. reduced the damages caused by the pathogen.

GRAPHICAL ABSTRACT



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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. Four different types of indicators (length of the central axis of the colony of the pathogen, the shape coefficient, percentage inhibition of radial growth and percentage inhibition zone) 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.

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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 reported in 1946, 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). 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 (van der Werf, 1996), particularly in forests) are driving the search for alternative methods of control. For example, in 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 (51–52 °C 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 biological impact since new species will be not introduced in 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), protection against herbivores (Carroll, 1988) and against important pests (Vega et al., 2008). 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 at the Forest Health Centre of Calabazanos (Junta de Castilla y León), and a few were obtained from the collection of the Forest Entomology and Pathology Laboratory at the University of Valladolid. Most of the fungi were isolated from different tissues of *Pinus* spp. (needles, twigs, bark, cones and seedlings). Firstly, 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 25 °C 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 used 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 25 °C 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 −20 °C and in 15% glycerol at −80 °C.

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 1×, 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 belonging to the genus *Fusarium*, elongation factor 1 alpha (ca 700 bp) was also amplified by PCR with EF1 (5′-ATGGGTAAGGA(A/G)GACAA GAC-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 °C for 10 min. Finally, LSU rDNA was amplified using the LR0R and LR16 primer pair (Vilgalys and Hester, 1990). PCR involved initial denaturation at 94 °C for 3 min, followed by 38 cycles at 94 °C for 35 s, 54 °C for 40 s and 72 °C for 1 min and a final elongation step at 72 °C 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 6× 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 h in 1× 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 3× 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 45° (lateral axes) were drawn on the bottom of the plate before the fungi were cultured (Fig. 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.

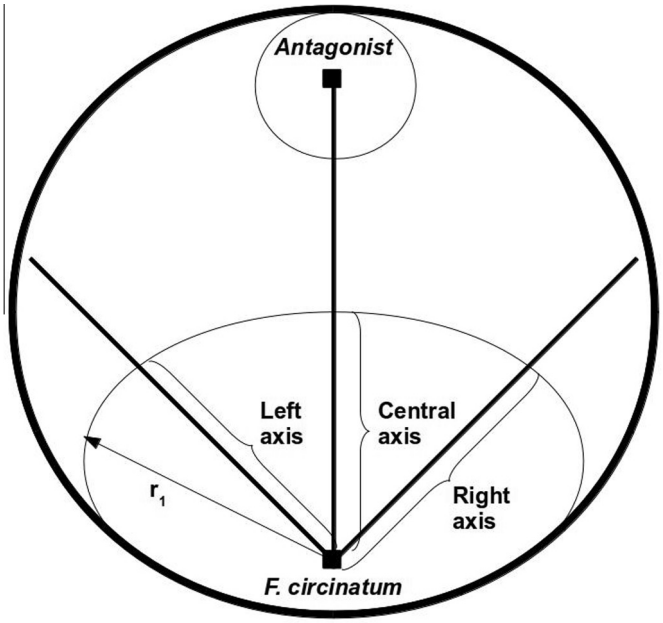

Fig. 1. Diagram showing the mode of inoculation of the plates with *F. circinatum* and the potential antagonist in the *in vitro* antagonism experiment. r_1 = maximum radius of the colony of the fungal pathogen.

Four different type of 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 $[100 \times (r_1 - \text{axis } C)/r_1]$, 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 $[(A - B)/A] \times 100$, 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*	386,979
UTM coordinate *Y*	4,788,566
Location	Puentenansa
Municipality	Rionansa
Altitude (m.a.s.l.)	525–615
Orientation	Southwest
Slope (%)	50
Annual precipitation (mm)	1092
Mean temperature (°C)	11
Minimum mean temperature (°C)	5
Maximum mean temperature (°C)	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 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 for the *in vitro* antagonism experiment. 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:

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

where $i = 1, 2$ for the isolates, $j = 1, \ldots, 155$ for the antagonists, $l = 1, \ldots, 7$ for the groups of antagonists, $k = 1, \ldots, 5$ 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 $t \neq t'$, 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:

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

where $i = 1, \ldots, 5$ for the pine species, $j = 1, 2, 3$ for the blocks, $k = 1, 2$ for the levels of the *F. circinatum* factor and $l = 1, \ldots, 7$ 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

Table 2
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
Length 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

two isolates of *F. circinatum* plated in the dual cultures (Fc70 and Fc221) (Table 2).

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 3. 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 symptoms 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* (Fig. 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 4).

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 species did not cause any damage to the seedlings when inoculated alone, as the differences between AUDPC for CT (control treatment) 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% ($p = 0.025$) (Fig. 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.

4. Discussion

The vast majority of the endophytes tested against *F. circinatum* 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 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

Table 3
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 four indicators evaluated in the *in vitro* antagonism assay (length of the central axis of the colony of the pathogen, the shape coefficient, percentage inhibition of radial growth and percentage inhibition zone). Only the endophytes with signficant antagonism against the pathogen are included in the list. 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	
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

Table 3 (*continued*)

Code	Significative indicators								Identity	Accession number	
	MAT-1				MAT-2					ITS/EF	LSU
	1	2	3	4	1	2	3	4			
HP085		*	*						*Fusarium proliferatum*	**KT356156**	KT323294
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
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

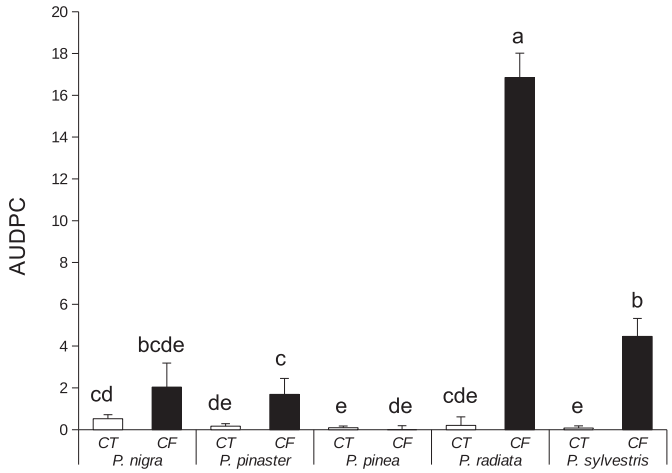

Fig. 2. AUDPC (area under disease progress curve) for the seedlings of the five pine species inoculated with *F. circinatum* (CF) and the control treatment (CT). In both cases, the seedlings were not inoculated with endophytes. Error bars are standard errors. Different letters (a–e) denote significant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

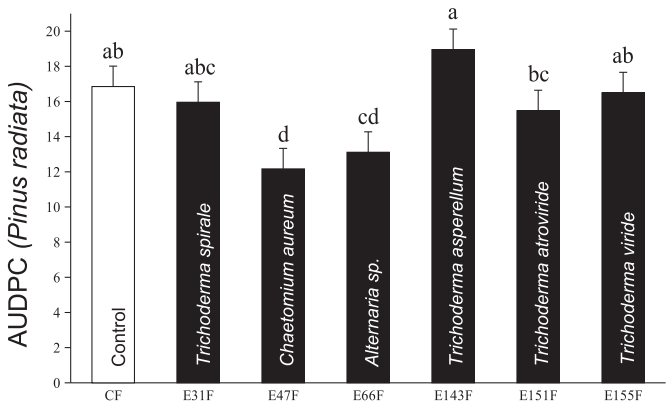

Fig. 3. AUDPC (area under disease progress curve) for the species *P. radiata* when the seedlings were inoculated with *F. circinatum* (CF) only or with the different endophytes together with *F. circinatum*. Error bars are standard errors. Different letters (a–d) denote significant differences ($p 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

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 (Agustí-Brisach et al., 2012) and, according to the present results, more easily controlled by fungal endophytes in artificial medium. However, since just one isolate of each one of the mating types was used, we cannot assure that the differences are due to the mating type or to a genetic difference among the isolates.

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 *P. nigra* showed an intermediate level of response to the pathogen. 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 identified as *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

Table 4
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 length		
		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	

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

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