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Antagonistic activity of fungal endophyte filtrates against *Gremmeniella abietina* infections on Aleppo pine seedlings

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Abstract Biological control agents (BCAs), and among them some species of fungal endophytes, are potential substitutes for chemical pesticides in the control of plant diseases due to their non-toxicity to human beings and their surrounding environment. One mode of action of fungal BCAs is through their bioactive, extracellular products, which can inhibit the growth of pathogens. In this study, the effect of fungal filtrates from four endophyte isolates (*Trichoderma viride*, *Aureobasidium pullulans*, *Aureobasidium* sp. and the unknown endophyte 20.1) on the advance of the pathogen *Gremmeniella abietina* on 2-year *Pinus halepensis* seedlings was evaluated. Both preventive and therapeutic treatments of the filtrates were studied by applying the filtrates either before or after the pathogen inoculation, respectively. Since *G. abietina* is a necrotrophic fungus, the length of the necrosis produced by the pathogen was used as response variable in our experiment. In order to explore the chemical composition of the fungal filtrates, a simple HPLC screening of UV-absorbing components was conducted. The results of the study showed that all fungal filtrates were able to reduce the advance of *G. abietina* when compared to the control seedlings, regardless of the time of inoculation and the treatment. Low-molecular weight phenolic compounds could be detected in some but not all filtrates, warranting further studies on the possible role of these compounds in fungal filtrates.

Keywords Biological control agents · Antibiosis · Secondary metabolites · Forest pathogens · *Trichoderma* · *Aureobasidium*

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Introduction

The Aleppo pine (*Pinus halepensis* Mill.) is one of the most common species in the Mediterranean, its forest area spanning more than 3 million ha and more than 800.000 ha in Spain (Gil et al. 1996). This species can

withstand a wide variety of environmental conditions and soil features, and it presents a high resistance to drought. Because of its ecological plasticity, it has been used for reforestation in degraded areas and for plantations with commercial purposes in Spain (Gil et al. 1996). However, over the last few years, environmental conditions have been unfavourable for *P. halepensis*, especially in the north western part of the Iberian Peninsula where it grows outside its optimum natural habitat (Abelló 1998). In 1999, the fungal pathogen *Gremmeniella abietina* (Lagerberg) Morelet (anamorph *Brunchorstia pinea* (P. Karsten) Höhnel) was detected and isolated from *P. halepensis* plantations in northern Spain causing defoliation, discoloration, terminal twig distortion and cankers (Santamaría et al. 2003). The fungus infects the trees during the spring, but the external symptoms appear after a latent period of the host (Ylimartimo et al. 1997). Ascomycetous fungi belonging to the genus *Gremmeniella* are all pathogens; they have been found all over the Northern Hemisphere spreading diseases on several conifer species. The most important damages have been recorded on *Pinus*. Both seedlings and adult trees may be affected, and, on several occasions, epidemic outbreaks have led to the destruction of natural forests and restored stands (Yokota 1975; Dorworth 1979; Laflamme and Lachance 1987; Kaitera and Jalkanen 1992; Kaitera et al. 1998; Wulff et al. 2006).

The control of *G. abietina* has varied from silvicultural to chemical practices. Some of the silvicultural techniques performed in the forests, like pruning and removing dead trees, may decrease the source of inoculum and thus slow the spread of the pathogen (Laflamme 1999). In some nurseries, the applications of synthetic fungicides such as chlorothalonil have been used to reduce *G. abietina* infections although mainly as an emergency measure (Skilling and Waddell 1970; Smerlis 1980). Nevertheless, there is currently an increasing interest in finding effective biological control methods, e.g., recent EU legislation (Council DIRECTIVE 2009) recommended sustainable forest management and protecting forests and their biodiversity giving priority to non-chemical methods of plant protection.

With the use of synthetic fungicides in forestry progressively more restricted by the strengthening of regulatory limitations and the risks of detrimental effects on the environment (Brimner and Boland 2003) more and more apparent, finding biological solutions is becoming an increasingly attractive control strategy against plant pathogens (Cook et al. 1996; Pal and McSpadden Gardener 2006). Biological control is the use of living organisms to fight against a disease and is based on the antagonism of pathogens by the presence or the activities of other microorganisms. However, other authors broaden the definition and include not only the use of antagonistic microorganisms, but also the application of naturally derived bioactive compounds (Talibi et al. 2014). These microbial antagonists are known as biological control agents (BCAs). The interaction of a BCA and a pathogen include: (i) mycoparasitism, the pathogen is directly attacked by a BCA that kills it or its propagules; (ii) antibiosis and metabolite production, i.e., the BCAs produce substances that are toxic to the pathogen; (iii) competition for nutrients, i.e., the BCAs occupy the same ecological niche of the pathogen and therefore deplete the nutrients necessary for its establishment; (iv) induction of the plant defence system, i.e., the stimulation of the host plant defences by the presence of the BCAs; and (v) the barrier effect, caused by the presence of mycorrhizal fungi (Schoeman et al. 1999; Alabouvette et al. 2006; Ownley and Windham 2007; Heydari and Pessarakli 2010; Diez and Alves-Santos 2011). Among the potential BCAs there are several fungal endophytes, i.e., fungi that live inside the plant tissue and maintain either a neutral, detrimental or beneficial relationship with the host plant (Sieber 2007; Backman and Sikora 2008). In other studies previously conducted, several species of fungal endophytes were able to reduce the growth of *G. abietina*. For example, *Phaeotheca dimorphospora* Desrochers and Ouellette inhibited the mycelial growth of the colonies, the germination of the spores and the spread of the pathogen on seedlings of red pines (Yang et al. 1995). Santamaría et al. (2007) observed a reduction or even an inhibition of the growth of Spanish isolates of *G. abietina* on Petri dishes it was confronted with some endophytes such as *Trichoderma, Aureobasidion, Cladosporium* and some unknown fungus called 20.1. Lastly, Romeralo et al. (2015) observed that *Trichoderma viride*, *Aureobasidion pullulans*, the endophyte 20.1 and a *Leotiomycete* reduced the progression of *G. abietina* when inoculating both with mycelia on plants.

To protect themselves from the attack of the pathogens, plants have several defence mechanisms known as constitutive, if they already exist in the plant before the infection, or induced if they are produced as a

consequence of it. The induced response leads to the production of some hormones to extend the communication within the plant preparing it to prevent future infections which is called systemic acquired resistance (Agrios 1997; Franceschi et al. 2005). The presence of some BCAs has shown to activate this defence system effectively against other fungal pathogens (Muñoz et al. 2008; Regliński et al. 2012).

Antibiotics, which are involved in the mechanisms employed by the BCAs, are microbial extracellular toxins that may eradicate other microbial cells. Most microbes produce and secrete one or more compounds with antibiotic activity. In some instances, antibiotics produced by microorganisms have been shown to be particularly effective at suppressing plant pathogens (Pal and McSpadden Gardener 2006). They include not only antibiotics sensu stricto but also bactericides, cell wall degrading enzymes, and volatile compounds with antifungal activity (Alabouvette et al. 2006). The role of antibiotics in biocontrol has been studied with genetic analyses by using mutants that do not produce antibiotics (Lo 1998). Apparently, antibiotic production is not specific to certain species. Different species may produce the same antibiotics or secondary metabolites, while products of different strains of the same species may turn out to be quite distinct (Lo 1998). Even different secondary metabolites produced by a single strain of a BCA might be responsible for the antagonistic activity towards different pathogens (Alabouvette et al. 2006). Examples of antifungal metabolites produced by either fungi or bacteria are: phenazine, produced by *Pseudomonas fluorescens* Migula; cladosporin produced by *Cladosporium cladosporioides* (Fresen.) G.A. de Vries; gliovirin and gliotoxin produced by *Trichoderma virens* (J.H. Mill., Giddens & A.A. Foster) Arx, and alkylpirones and peptaibol produced by *T. harzianum* Rifai (Lo 1998; Alabouvette et al. 2006; Wang et al. 2013).

Although biologically-based methods are desirable, there are only a few cases when they are applied in practice when managing forest diseases. One example is the control of the root and butt rot pathogen *Heterobasidion annosum* (Fr.) Bref. with the fungus *Phlebiopsis gigantea* (Fr.) Jülich. In Scandinavia, *Phlebiopsis* stump treatment is commonly applied, as it may reduce *H. annosum* colonization on stump surfaces by 89–99 % compared to untreated stumps (Thor and Stenlid 2005). Another example of biological control of forest disease in Europe is the control of the Chestnut blight fungus (*Cryphonectria parasitica* (Murr.) Barr.) using hypovirulent pathogen strains. The infection of the fungus produces cankers on stems and branches. Hypovirulent strains host viruses from the genus *Hypovirus* that reduce the virulence of these strains and are also transmissible by hyphal anastomosis (Anagnostakis and Day 1979; Polashock et al. 1997).

Since some endophytes had such good results in reducing the growth of the pathogen both in vitro (Santamaría et al. 2007) and in vivo (Romeralo et al. 2015) our hypothesis was that these endophyte's filtrates would be able to reduce or stop the progression of *G. abietina* once in the seedlings. Consequently, in the present study, the suitability of selected fungal endophytes filtrates in the control of the *G. abietina* is described. The specific goals of the present work were: (i) to test if endophyte filtrates can provide preventive or therapeutic protection against *G. abietina* in *P. halepensis* seedlings, and (ii) to screen the filtrates for UV-absorbing compounds to characterize the chemical composition of the fungal filtrates. The results are discussed with special emphasis on the potential use of the tested fungal filtrates as a novel, bio-based tool in the control of *G. abietina* in *P. halepensis* seedlings.

Materials and methods

Plant material, fungal isolates and filtrates

The experiment was conducted in December 2011 (mean T°=4.4 °C) and January 2012 (mean T°= 3.4 °C) in the shade cloth greenhouse of the College of Agricultural Engineering at the University of Valladolid, in Palencia, Spain. Two-year-old containerized Aleppo pine seedlings were used to perform this experiment obtained from the Central Nursery of the Castilla y León regional government. The seedlings (n=840) had a mean root collar diameter and height of 3.03 mm± 0.73 and 17.13 cm±2.64 respectively (mean±standard deviation). Six months prior to the inoculations, all standard nursery treatments against pests and fungi were stopped. Once in the greenhouse, the seedlings were watered regularly.

All the *G. abietina* and the endophyte's isolates (Table 1) came from a collection at the University of Valladolid Forest Pathology Lab. The *G. abietina* isolates were selected randomly whereas the endophytes were the same used in previous experiments with

Table 1 Characteristics of the *G. abietina* isolates and the endophytes, host, species, place of origin in Spain and year of isolation

Behaviour	Isolate	Name	Species	Origin	Province	Year of isolation
Pathogen	G1	Z0-10-01	*G. abietina*	Valle de Cerrato	Palencia	2010
	G2	Z0-10-02	*G. abietina*	Valle de Cerrato	Palencia	2010
	G3	P1-8	*G. abietina*	Valle de Cerrato	Palencia	2007
	G4	P1-12	*G. abietina*	Valle de Cerrato	Palencia	2007
	G5	VAI-13	*G. abietina*	Villalba de los Alcores	Valladolid	2003
	G6	00P-7	*G. abietina*	Valle de Cerrato	Palencia	2001
Endophytes	E1	1778 AB	*Trichoderma viride*	Tordehumos	Valladolid	2009
	E2	1077 4A	*Aureobasidium pullulans*	Valle de Cerrato	Palencia	2009
	E3	1812 RA 1-b	*Aureobasidium sp.*	Valle de Cerrato	Palencia	2009
	E4	20.1	Unknown Deuteromycete	Quintanilla de Onésimo	Valladolid	2004

success in reducing *G. abietina* mycelial growth in vitro (Santamaría et al. 2007) and in vivo (Romeralo et al. 2015). The endophytes *Trichoderma viride* Pers. *Aureobasidium* sp., *A. pullulans* (de Bary & Löwenthal) G. Arnaud and endophyte 20.1 (which did not match with any known fungus in the BLAST database) were grown on PDA (potato, dextrose, agar) at room temperature (25±2 °C) for 2 weeks while the *G. abietina* isolates were cultured on MOS-agar (modified orange, serum-agar) at 15 °C (Müller et al. 1994). To obtain the fungal filtrates from the endophytes, several pieces of mycelial agar plugs were placed into Erlenmeyer flasks containing 250 ml of PDB (potato, dextrose, broth) and incubated at room temperature in the orbital shaker with constant movement for 3 months. After this period, the broth culture was filtered twice with Whatman® qualitative filter paper, Grade 1 (Whatman International Ltd, Maidstone, UK), in order to separate the broth and mycelia. The filtrates were preserved in refrigerators at 4 °C until the time of inoculation.

Experimental design, *G. abietina* inoculations and application of fungal filtrates

In order to know if the presence of the endophytic filtrate was able to either prevent *G. abietina* infections or reduce its growth, two treatments were performed: (i) preventive, a primary treatment with the endophyte filtrates followed by a challenge inoculation with the pathogen 1 week later; and (ii) therapeutic, primary inoculation of the pathogen followed by treatment with the endophyte filtrates 1 week later. To perform the inoculation with the pathogen, we used mycelium to ensure that the infection would take place; conidial suspension was found to be less effective in previous results obtained with Spanish isolates in our lab (unpublished data). Therefore, a small wound was made with a sterile scalpel at 10 cm from the shoot apex, and a small piece of 0.25 cm^2 of mycelial agar of *G. abietina* cultures was placed in the wound and covered with Parafilm® to avoid desiccation. Treatments with the fungal filtrates were done with a sterile syringe at 8 cm from the top after making a small wound with a sterile scalpel. Afterwards, four drops of the endophyte filtrates were placed into the wound that was covered with Parafilm. Control treatments were made with sterile agar and broth filtrates. The inoculations were performed in December and January in accordance with descriptions that the pathogen colonizes the living host tissues only during the dormant season (Ranta et al. 2000). Three weeks after all inoculations were finished, the whole experiment was repeated. The experiment had a completely randomized factorial design with six repetitions per combination and four factors: (i) pathogen (six *G. abietina* isolates + water-inoculated control), (ii) endophytes' filtrate (filtrate from four endophyte isolates + sterile broth filtrate as a control), (iii) time of inoculation (December or January), and (iv) treatment (preventive or therapeutic). Thus, every combination consisted of the artificial inoculation of one of the 70 possibilities of "pathogen / endophyte filtrate / treatment". In order to avoid uncontrolled infections among adjacent seedlings, the plants were placed 5 cm from each other.

Evaluation and measurement of the seedlings and re-isolation of the pathogen

Seedlings were kept under the shade cloth greenhouse at ambient temperature until symptoms of the disease

started to appear. In June, the seedlings were cut and brought to the laboratory in order to quantify the damages. Several parameters of the seedlings were measured and evaluated: (i) total length of the plant (cm), (ii) diameter at root collar (mm), (iii) presence of cankers (presence/absence), and (iv) length of the necrosis (cm). In order to measure the necrosis produced by the advance of the pathogen, the seedlings were cut lengthwise. Since *G. abietina* is a necrotroph, the necrosis length was considered to be an appropriate indicator of the progression of the disease (Adomas and Asiegbu 2007). The response variable of our experiment was the relative necrosis length and was defined as the relationship between the necrosis length vs. the total length of the plant (Santamaría et al. 2006).

To confirm Koch's postulates, (i.e., that the necroses were indeed produced by *G. abietina*) we proceeded to re-isolate the pathogen from four seedlings of every combination of pathogen / endophyte filtrate / treatment (280 seedlings in total). From every sample, a portion of 6 cm was cut and submerged into 100 ml of sterilized distilled water for 1 min; followed by 2 min in 2 % NaClO and 2 min in 96 % etanol then placed into MOS-agar plates, incubated at 15 °C for 15 days and revised daily for the emergence of any *G. abietina* colonies.

Qualitative analysis of organic compounds of fungal filtrates by extraction

Given the expected low concentration of organic compounds in raw extracts (Pal and McSpadden Gardener 2006) the samples were subject to a concentration step prior to analysis. Concentration was determined in a total volume of 360 ml of *T. viride,* 90 ml of *Aureobasidium sp.,* 90 ml of *A. pullulans,* 360 ml of endophyte 20.1 and 90 ml of control broth. For the isolation of metabolites, multiple batches were needed. In each batch 45 ml of fungal filtrates were extracted with 25 ml of ethyl acetate (EtOAc). For that aim, a stirrer Vibromatic 680–750 U/min (10 min ×6) was used. The interphases were also preserved and extracted with brine (40 ml). Later, the combined organic phases were filtered with a C18 solid phase extraction cartridge (Sigma-Aldrich) at vacuum pressure. Afterwards, 5 ml of acetonitrile was used as elution buffer, and the samples were stored at 4 °C until needed for the chromatography analysis.

Screening of UV-absorbing phenolic compounds in the extracts

To elucidate the chemical characters of the EtOAc extracts, the samples were subjected to liquid chromatographic analysis, targeting the UV-absorbing phenolic compounds. The filtrates were first filtered through disposable filters (0.45 μ pore size) before their injection into HPLC. The HPLC system was a Merck Hitachi LaChrom device consisting of a D-7100 pump, D-7200 autosampler, D-7300 column oven at 40 °C, and a D-7455 DAD detector scanning the absorbance between 220 and 400 nm. Separation was achieved on a HyPurity C18 (Thermo Scientific, Waltham, MA, USA) column using the gradient of water (acidified with *o*-phosphoric acid to pH3; A) and methanol (B) as follows: 10 % B (0–1 min); 10–70 % B (1–20 min); 70 % B (20–23 min); 70–100 % B (23–30 min), followed by flushing and equilibration to initial conditions. The flow rate was 0.8 ml/min and the injection volume was 40 μl. The UV-spectra, collected at 200 to 400 nm, was compared to the spectral data of a standard compound library.

Statistical analysis

To evaluate the effect of time of inoculation, treatment, *G. abietina* isolate, endophyte filtrate and their interactions on the relative necrosis length we performed a linear mixed model (SAS Institute Inc. SAS/STAT® 2004) because of the high heterogeneity of variances in some levels of our factors (Levene Test). In a linear model all levels of the factors should have the same variance (homoscedasticity) thus; we used a linear mixed model that allows using different variances for any of the levels of the factors. By grouping our factors in pairs we obtain different combinations of variance parameters which produced different models. The best model was chosen according to the lowest values of the Bayesian Information Criterion (BIC) and in compliance to the normality, linearity and homoscedasticity of the residuals, checked by graphical procedures and the Kolmogorov-Smirnov test. Furthermore, in order to explore if the effect of the filtrates was different whether the pathogen was isolated or not, we divided the data into two subsets: samples with success in re-isolating *G. abietina* (Ga positive), and data without success (Ga negative). For every subset we performed a linear mixed model (because of the heteroscedasticity of the data) with the relative necrosis length as the response variable

and *G. abietina* isolate, endophyte filtrate and their interaction as the explanatory variables.

The random errors of all models were supposed to be independent and with normal distribution for the relative necrosis length. In all the statistical analyses a 5 % level of significance was used. When significant differences were found in the test type III table of the model, a Tukey-Kramer HSD test was applied to compare the means.

Lastly, a non parametric Kruskal-Wallis test was used to observe the effect of the extracts, time of inoculation, treatment and isolates on the visual severity (using the following scale: 0 symptomless; 1 chlorosis; 2 dieback; 3 dry needles; 4 dead plant) after it was found that the data did not follow a normal distribution in a Shapiro-Wilk test. Then, the same test was applied to compare the means of the factors that presented significant p-values. These analyses were performed with R software (R Development Core Team 2008, version 3.1.2 Vienna, Austria, http://www.r-project.org).

Results

Symptoms of *G. abietina* infections and reisolation of the pathogen

Four months after the artificial inoculations of *G. abietina*, a total of 740 (100 were symptomless) seedlings started to show symptoms of the disease such as chlorosis (61 %) (Fig. 1a), dieback (29 %) (Fig. 1b), dry needles (3 %) and cankers (1 %). No dead plants were found. Tissues around the inoculation site turned a brown colour (Fig. 1c). The pathogen grew upwards in most of the seedlings; growth both upwards and downwards was found in two seedlings. The symptoms were attributed to *G. abietina* infections given that fruiting bodies were observed in 38 % of the seedlings (Fig. 1d) while no fruiting bodies were observed in the control inoculations. Fruiting bodies were found in 49 % of the seedlings inoculated in December and in 28 % inoculated in January. Moreover, *G. abietina* could be re-isolated in 20 % of the samples; 22 % of the seedlings that were inoculated in December and 18 % of those from January and no *G.abietina* was isolated from the controls.

Effects of the factors on necrosis and visual severity

The effect of the four factors on necrosis length was explored by a linear mixed model, which was selected according to the lowest BIC value (Table 2). The best model had no random effects and 4-variance parameters, one variance for every time of inoculation-treatment combination. Three factors, time of inoculation, endophyte filtrate and *G. abietina* isolate, had a statistically significant effect on the relative necrosis length as well as the interaction time of inoculation*isolate (Table 3). The presence of the endophyte filtrates reduced the advance of the pathogen in the seedlings regardless of the endophyte isolate, time of inoculation, treatment and *G. abietina* isolate (Table 3). The control seedlings (with no endophyte filtrate) presented a relative necrosis length greater than the seedlings which were inoculated with the filtrates of *T. viride*, *A. pullulans*, *Aureobasidum* sp. and the Endophyte 20.1 (Fig. 2).

The inoculation with any isolate of *G. abietina* resulted in more extensive necrosis, as compared to the control seedlings (not *G. abietina* isolate inoculated) despite the time of inoculation, the treatment and the type of inoculated endophyte filtrate (Table 4). Nevertheless, some differences were found between the *G. abietina* isolates as indicated by the significant isolate effect (Table 3). Furthermore, the necrosis produced by isolates showed temporal variation, as indicated by the significant interaction between time of inoculation and isolate (Table 3). In December the *G. abietina* isolates G2, G3 and G5 resulted in more extensive necrosis than the rest of the isolates (G1, G4) whereas in January only G3 and G5 produced more necrosis than the rest; G2 was not as effective as in the first round.

The average relative necrosis length was significantly higher ($p<0.001$) in seedlings inoculated in December (0.112 ± 0.003) (mean value±standard error) than in January (0.098 ± 0.003). Nevertheless, no difference in necrosis length was found ($p=0.80$) between the preventive and therapeutic treatments, (0.106 ± 0.003, and 0.104 ± 0.003, respectively).

The results were very similar when analyzing the Ga-positive and the Ga-negative seedlings. There was a significant effect of the filtrates ($p=0.001$), the isolates ($p<0.001$) and their interaction ($p=0.01$) on the relative necrosis length of the Ga-positive seedlings. Furthermore, we also observed a significant effect of the filtrates ($p<0.001$), the *G. abietina* isolates ($p<0.001$) and their interaction ($p=0.001$) on the relative necrosis length on the seedlings without success in isolating the pathogen. The Tukey Kramer test revealed that in both models, the seedlings inoculated with any of the filtrates

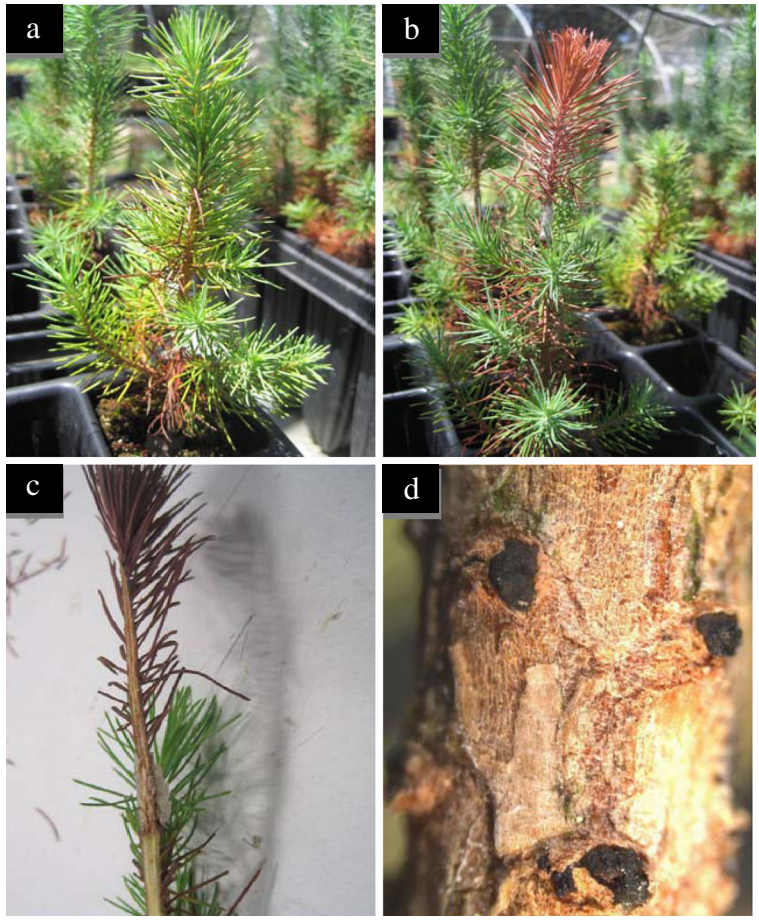

Fig. 1 Symptoms of (**a**) chlorosis and (**b**) dieback; (**c**) brownish tissues in a endophyte-control plant; and (**d**) *G. abietina* fruiting bodies (10×)

presented significantly lower necrosis than the controls although the efficacy depended on the isolate of *G.abietina* that was co-inoculated. In the Ga-positive seedlings the controls presented a higher necrosis length compared to one or more filtrate in seedlings inoculated with isolates G1, G2, G3, or G6. Furthermore, Ga-negative seedlings presented differences among control seedlings and the ones inoculated with any filtrate in isolates G1, G2, G4, G5, G6 and G7.

Table 2 Description and comparison of the models according to the Bayesian Information Criterion (BIC)

Factors	Group	Covariance parameters	Model selection criteria BIC
4	Time of inoculation_Isolate	14	−1780.8
4	Time of inoculation_Endophyte	10	−1785.9
4	Time of Inoculation_Treatment [a]	4	−1798.2
4	Treatment_Endophyte	10	−1795.3
4	Treatment_Isolate	14	−1778.6
4	Endophyte_Isolate	35	−1685.7

[a] Selected model

The Kruskal-Wallis test revealed that there were significant differences in the severity of the seedlings inoculated with different filtrates ($H=31.83$; df.=4; $p<0.001$) and *G. abietina* isolates ($H=96.36$; df.=6; $p<0.001$). Nevertheless, there were no significant differences between the two time of inoculations, December and January ($H=0.70$; df.=1; $p=0.40$) or the treatments, preventive or therapeutic ($H=0.003$; df.=1; $p=0.96$). The seedlings that were inoculated with the filtrate of *T. viride* had less mean visual severity than the ones inoculated with *Aureobasidum* sp. ($p=0.03$) or the Endophyte 20.1 ($p=0.02$). Furthermore, seedlings inoculated with the Endophyte 20.1 had less mean visual severity than the ones inoculated with the rest of the filtrates except the control ones. No differences were

Table 3 Test type 3 fixed effects for Relative Necrosis Length

Dependent variable	Effect	DF	F-value	Pr>F
All seedlings (n=840)				
Relative Necrosis Length	Endophyte	4	13.4	<0.0001
	Isolate	6	52.2	<0.0001
	Time of inoculation (TI)	1	14.94	0.0001
	Treatment (T)	1	0.07	0.80
	Endophyte*Isolate	24	1.39	0.10
	Endophyte*TI	4	0.73	0.57
	Endophyte*T	4	2.23	0.63
	Isolate*TI	6	9.52	<0.0001
	Isolate*T	6	1.62	0.14
	TI*T	1	0.24	0.62
	Endophye*Isolate*TI	24	0.56	0.96
	Endophye*Isolate*T	24	1.09	0.35
	Endophyte*TI*T	4	1.12	0.35
	Isolate*TI*T	6	2.01	0.06
	Endophyte*Isolate*TI*T	24	1.16	0.28

found between the controls and the seedlings inoculated with the rest of the filtrates. Regarding the *G. abietina* isolates, the control seedlings presented lower mean severity than the ones inoculated with the isolates G3 (p=0.02) and G5 (p=0.05).

UV-absorbing compounds of the filtrates

The identification of phenolic compounds was performed through the comparison of chromatographic retention times and UV spectra with those of commercial standards, when available. The HPLC analysis indicated that the EtOAc fractions of the fungal filtrates contained some phenolic compounds. In the filtrate from *T. viride* two peaks were found in the UV region (detection at 254 nm) (Fig. 3), these were identified on basis of the UV-spectrum as hydroxybenzoic acids. In the filtrate from endophyte 20.1, three additional distinct peaks were found showing identical spectra but without a match in library records. We did not detect any phenolic peaks in the filtrates of the two *Aureobasidion* endophytes or in the control broth.

Discussion

In this study, we explored the possibility of controlling the pathogenic fungus *G. abietina* with fungal filtrates

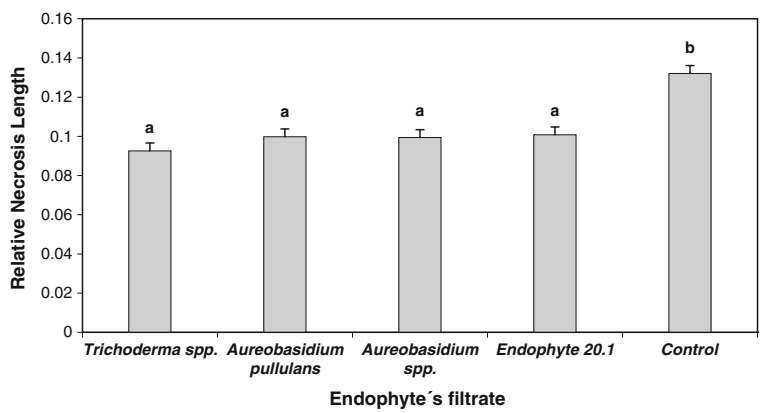

Fig. 2 Average relative necrosis length found in *Pinus halepensis* seedlings when inoculating both *G.abietina* isolates with the different endophyte filtrates. Control seedlings had no endophyte but *G. abietina* isolate. Means with a different letter were significantly different from p<0.05 (Tukey's HSD Test). Bars represent standard error (n=70)

Table 4 Relative Necrosis Length caused by six *G. abietina* isolates in two repeated experiments. Shown are the mean values±standard errors (n=70)

Isolate	Time of inoculation		Total [1]
	T1	T2	
G1	0.097±0.008 b[2] A[3]	0.107±0.007 b B	0.102±0.005 b
G2	0.173±0.008 d B	0.099±0.007 b A	0.136±0.005 c
G3	0.145±0.008 cd B	0.109±0.007 bc A	0.127±0.005 c
G4	0.095±0.008 b A	0.099±0.007 b B	0.097±0.005 b
G5	0.134±0.008 c A	0.138±0.007 c B	0.136±0.005 c
G6	0.113±0.008 b B	0.096±0.007 b A	0.104±0.005 b
G-Control	0.028±0.008 a A	0.035±0.007 a B	0.032±0.005 a
TOTAL [4]	0.112±0.003 B	0.098±0.003 A	

[1] Average necrosis when combinig all the times of inoculation together

[2] Means without a common small letter in the same column show values significantly different from $p<0.05$ (ANOVA Tukey's HSD Test)

[3] Means without a common capital letter in the same row values significantly different from $p<0.05$ (ANOVA Tukey's HSD Test)

[4] Average necrosis when combining all the *G.abietina* isolates together

from selected BCAs. According to the symptoms observed (i.e., cholorosis, dieback, cankers and death of the plants), the presence of fruiting bodies and absence of fungal signs in the controls, we concluded that it was likely that the infections were produced by *G. abietina*. The re-isolation of the fungus was lower than the percentage obtained in a previous study by Santamaría et al. (2007) who obtained 66 %. Nevertheless, isolating *G. abietina* from vegetal material is especially challenging even when the sample material has fruiting bodies as previously observed in our lab (Romeralo et al. 2015). Besides the slow growth of the pathogen, another hypothesis explaining why it was so difficult to isolate it could be because the fungus was not alive until the end of the experiment. A successful activation of the plant defence mechanism after the dormancy period (around March) could be responsible for excluding the pathogen in some of the seedlings. This plant defence mechanism would include the formation of ligno-suberized boundaries followed by the restoration of cambial activity, tissue regeneration and the production of fungal degrading enzymes by the host previously described as being key factors in the resistance of Pine species to the pathogen (Simard et al. 2001, 2013). Isolates from this fungus grow very slowly in media, even if it is specific media like MOS-agar and the pathogen is growing in its optimal temperature of 15 °C. Even so, necrosis was apparently produced by the pathogen because the seedlings inoculated with *G. abietina* isolates had significantly greater necrosis length than the controls, which were not infected by the pathogen. The small necroses observed in the controls were probably the result of the wound made by the scalpel as also seen previously (Doğmuş-Lehtijärvi et al. 2012). The different isolates of *G. abietina* also varied in their ability to cause necrosis in the tested plants. This concurs with previous reports (Terho and Uotila 1999; Santamaría et al. 2006, 2007), which have shown that virulence can vary within isolates.

Other factors influenced the extent of necrosis in our study. Temporal variation was found in necrosis length: the seedlings inoculated in December exhibited longer necrosis and more fruiting bodies than the seedlings that were inoculated in January. This result coincides with those obtained by Doğmuş-Lehtijärvi et al. (2012) who found that from several inoculations made with Turkish isolates of *G. abietina* on several periods of the year (September, November, December and January), the ones made in December (mean T°=4.1 °C) presented the highest necrosis. In our experiment, the colder weather in January (mean T°=3.1 °C) than in December (mean T°=4.4 °C) seems unlikely to be a limitation for the development of the fungus since it has been reported to grow at temperatures as low as −6 °C (Marosy et al. 1989). Therefore, the highest necrosis in December could be explained by the fact that the fungus had 3 weeks more to grow inside the plant until March when

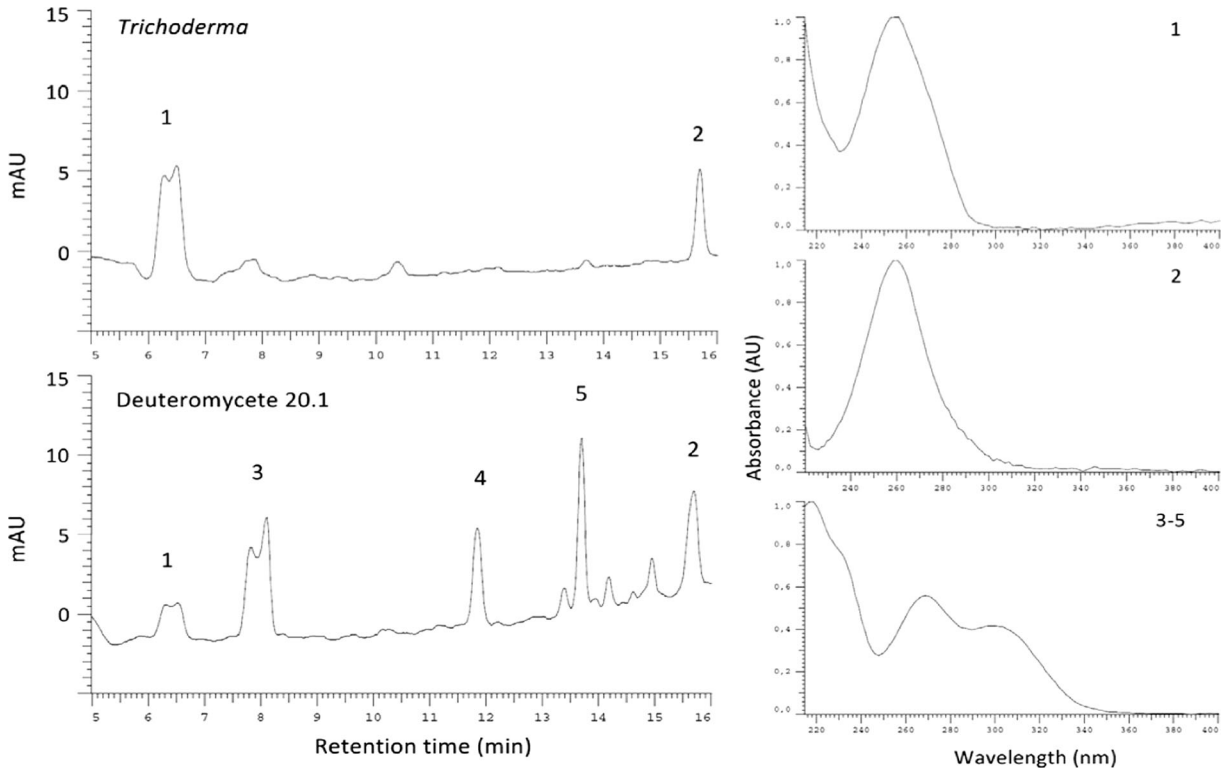

Fig. 3 Chromatographic retention times and UV spectra of *T. viride* and Endophyte 20.1. Peaks 1 and 2 best matched with p-hydroxybenzoic acid in the library (over 98 % of the spectrum form). Peaks 3–5 had a spectrum that did not match any of the library compounds

the temperatures started to increase and the defence system of the plant would be activated again.

In our experiment, there was not a significant effect of the treatment (preventive or therapeutic) on the necrosis length produced by the pathogen or the visual severity of the disease. Due to the short time between treatments (1 week) and due to the fact that the plants were submerged in the dormancy period by the time of the inoculations, it is likely that there was no activation of the defence mechanism of the plants. Nevertheless, although pine dormancy is described as the absence of growth (and in the case of Aleppo pine the growth in height is known to stop at temperatures below 10 °C) some activities have been reported to happen during dormancy in this species as opposed to other conifers. Puertolas Simon et al. (2005) found that Aleppo pine seedlings maintain their photosynthetic ability during cold hardening. Furthermore increases in shoot dry weight (which indicates some cambial activities) and in starch reserves have also been reported during this period (Tinus et al. 2000; Fernández Martínez et al. 2003). Therefore, although some activity or activation of the defence system of the plants will remain during the dormancy period, it was not enough to lead to a different response among the treatments in our experiment.

The results of our study indicate that the filtrates of all the tested endophytes reduced the necrosis produced by *G. abietina* in the seedlings. The filtrates had a similar effect whether the pathogen was isolated from the seedlings or not, suggesting that the pathogen could be alive until the end of the experiment, but it was difficult to isolate because of the features of this fungus. Another explanation could be that the pathogen was not alive until the end of the experiment, and that the effects of the filtrates were produced during the first months after the inoculations. The biological control agents (BCAs) may antagonize the pathogens through several modes of action and revealing them is useful for easier registration procedures at the commercialization stage (Castoria et al. 2001). Our results show that the mechanisms of the studied BCAs were likely linked to production of extracellular metabolites, since the filtrates alone resulted in necrosis reduction whereas the competition for

nutrients or the microbial antagonism would involve the presence of the BCAs themselves. Similar results were reported in other studies where the presence of fungal filtrates was able to decrease the mycelial growth of several pathogens like *Diplodia corticola* A.J.L. Phillips, A. Alves & J. Luque (Campanile et al. 2007) or *Sclerotinia sclerotiorum* (Lib.) de Bary (Zhang et al. 2014). An induction of the resistance in plants has been reported as well as a consequence of the presence of fungal filtrates (Viecelli et al. 2009).

The visual severity was not a good indicator in our experiment, as most of the seedlings presented symptoms of chlorosis, and this was not enough to pinpoint a difference of effectivity of the filtrates or the damage produced by the different *G. abietina* isolates. A more accurate scale and the examination along a longer period of time (throughout the whole experiment) would be recommended to improve these results in future experiments.

The inoculation of the filtrates of *T. viride* in the seedlings was able to reduce the necrosis produced by *G. abietina* as compared to the controls. The success of *Trichoderma* filtrates was previously reported in reducing the spore germination or the mycelial growth of other plant pathogens such as *Claviceps africana* Freder., Mantle & De Milliano (Bhuiyan et al. 2003) or *Ophiostoma novo-ulmi* Brasier (Díaz et al. 2013). According to our results, the filtrates of *Trichoderma* spp. were found to have some phenolic compounds. Although these phenols might contribute to the observed antagonism, results from previous studies have pointed out the presence in the fungus' filtrates of other potential chemical agents. Indeed, a wide range of non-volatile and volatile antifungal substances produced by *Trichoderma* spp. have been identified (Reino et al. 2008; Howell 2003), such as gliotoxin, viridin, harzianopyridone, harziandione and peptaibols (Vinale et al. 2008) as well as hydrolytic enzymes such as chitinase and glucanase (Aziz et al. 1993; Schirmböck et al. 1994).

Our results showed that inoculation with *Aureobasidium* (both *A. pullulans* and *Aureobasidium* sp.) filtrates also resulted in a reduction of the necrosis length, as compared to the controls. In previous studies, an antagonistic behaviour of different isolates of this genus through different mechanisms has been reported, including the presence of volatile compounds (Mari et al. 2012), competition for nutrients (Bencheqroun et al. 2007; Zhang et al. 2010), and induction of phytoalexins (Rühmann et al. 2013). The results from the Castoria et al. (2001) study showed that *A. pullulans* was an effective BCA against postharvest fungal pathogens, most likely due to the production of enzymes such as β-1,3-glucanase(s) and nagase(s) that were acting against fungal walls. Nevertheless, the same authors reported that neither antibacterial nor antifungal compounds were present in ethylacetate filtrates obtained from the culture filtrate of the fungus; which coincided with our results that we could not detect any UV-absorbing metabolites in the *Aureobasidium* filtrates.

The seedlings that were inoculated with the filtrate of the endophyte 20.1 exhibited reduced necrosis length compared to the controls. A previous study performed in vitro by Santamaría et al. (2007) showed a complete inhibition of Spanish isolates of *G. abietina* on cultures when the filtrate of this fungus was present, suggesting that there was some antifungal compound in the filtrate. Furthermore, Romeralo et al. (2015) observed that the presence of the mycelia of this fungus resulted in a reduction of necrosis produced by *G. abietina* on *P. halepensis* seedlings. We found that the filtrates of this fungus did contain a few phenolic compounds. Therefore it is probable that antioxidant activity and toxicity of these compounds might have contributed to the apparent antagonistic activity of this fungus against the pathogen. Thus, their potential involvement in restriction of necrosis length should be studied further along with a more comprehensive chemical profiling of the filtrates.

In conclusion, both the preventive and therapeutic treatments of *P. halepensis* seedlings with filtrates of four endophyte isolates (*Trichoderma viride*, *Aureobasidium pullulans*, *Aureobasidium* sp. and Endophtyte 20.1) were effective against necrosis development caused by *G. abietina* infection. However, there was some temporal variability in responses, indicating the complexity of the system. Not all fungal filtrates contained phenolics in amounts that were detectable with our HPLC method, suggesting that such compounds were not a general factor behind the preventive or therapeutic effect or that they were in such low concentrations that we could not detect them. Further studies, including more inoculation intervals and shorter incubation periods, could provide more accurate results about the efficacy of the filtrates and timing of activation of the defence mechanisms. A more comprehensive chemical profiling of the filtrates is recommended in the future.

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