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Fungal endophytes reduce necrosis length produced by *Gremmeniella abietina* in *Pinus halepensis* seedlings

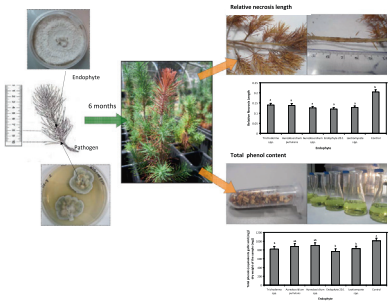
C. Romeralo [a,b,*], O. Santamaría [c], V. Pando [a,d], J.J. Diez [a,b]

[a] Sustainable Forest Management Research Institute, University of Valladolid-INIA, Avda. Madrid 44, Building E, 34004 Palencia, Spain
[b] Department of Vegetal Production and Natural Resources, Higher Technical School of Agrarian Engineering, University of Valladolid, Avda. Madrid, s/n, 34004 Palencia, Spain
[c] Department of Engineering of Agronomic and Forest Environment, School of Agrarian Engineering, University of Extremadura, Avda. Adolfo Suárez, s/n, 06007 Badajoz, Spain
[d] Department of Statistics and Operative Research, Higher Technical School of Agrarian Engineering, University of Valladolid, Avda. Madrid, s/n, 34004 Palencia, Spain

HIGHLIGHTS

- Aleppo pine seedlings were inoculated with endophytes and *Gremmeniella abietina*.
- When symptoms of decline were observed in the seedlings they were cut and brought to the lab.
- Necrosis and content of total phenols as activation of response system were measured.
- The presence of endophytes reduced the necrosis length produced by *G. abietina*.
- The inoculation of endophytes did not promote a higher production of phenolic compounds.

GRAPHICAL ABSTRACT



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ABSTRACT

Gremmeniella abietina (Lagerberg) Morelet is a pathogenic fungus that causes severe damage in coniferous forests, causing the death of the trees, in Central and Northern Europe, North America and Japan. Biological control (i.e. the use of biologically antagonistic organisms) is being considered as an alternative and an eco-friendly method to deal with plant diseases. Among such organisms several fungal endophytes have been successfully used to reduce or inhibit the growth of pathogens. Thus, the aim of this study was to evaluate the ability of several fungal endophytes to reduce the *G. abietina* spreading on pine seedlings, with the goal of exploring the mechanisms involved in that reduction. The experiment was carried out on two-year old *Pinus halepensis* seedlings under greenhouse conditions. Five fungal endophytes (*Trichoderma* spp., *Aureobasidium pullulans*, *Aureobasidium* spp., endophyte 20.1 and *Leotiomycete* spp.) obtained from healthy *P. halepensis* trees were used to evaluate their effect on six *G. abietina* isolates. The pathogen and the endophyte were both inoculated in every seedling. At the end of the experiment seedlings were cut and brought to the laboratory where the necrosis length and total phenol content of the plant were measured. The pathogen presence was determined by a nested PCR with specific primers of *G. abietina*. The presence of all endophytes significantly reduced the necrosis length caused by *G. abietina* in most of the cases. However, the phenolic content of the plant, which is an indicator of the activation of the plant's defence mechanisms, had not increased with the endophyte inoculation. Therefore, the reduction in the necrosis observed could

* Corresponding author at: Sustainable Forest Management Research Institute, University of Valladolid-INIA, Avda. Madrid 44, Building E, 34004 Palencia, Spain.
E-mail address: carmen.romeralo@pvs.uva.es (C. Romeralo).

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be attributed to a direct effect of the endophyte on the *Gremmeniella* spreading. These results suggest that the use of fungal endophytes could be an effective way to protect against *G. abietina* infections.

1. Introduction

The ascomycete fungus *Gremmeniella abietina* (Lagerberg) Morelet (anamorph *Brunchorstia pinea* (P. Karsten) Höhnel) is a pathogen whose infection produces cankers on stems and trunks, dieback and causes the death of the trees (Donaubauer, 1972). The fungus, which is native to Europe, has extended to most European countries, the east of North America and Japan (Yokota, 1975; Dorworth, 1979; Kaitera and Jalkanen, 1992). In some of the countries where it occurs severe damage has been registered in both natural forests and plantations, sometimes causing devastation of huge areas (Kaitera et al., 1998; Wulff et al., 2006). Trees from genera *Picea* and *Pinus* are their principal hosts although some damages have been also found in *Abies*, *Larix* and *Pseudotsuga* trees. In Spain, *G. abietina* was detected for first time in 1929 causing damage to *Pinus pinaster* (Martínez, 1933), and to *Pinus halepensis* in 1999 on forest plantations (Santamaría et al., 2003). Although no epidemic outbreaks have been registered yet in Spain it causes serious damages to trees affected by the disease. The main symptoms of the infection in Spanish stands are crown defoliation, dieback and distortion of terminal twigs, which occasionally leads to the death of the tree (Santamaría et al., 2003). Control measures of the disease might include silvicultural practices, like pruning lower branches or removing dead trees, to avoid the expansion of the pathogen and to reduce the source of inoculum (Laflamme, 1999). The application of fungicides has been also used but mostly as an emergency measure on nurseries (Hopkin and McKenney, 1995) although currently the application of chemical products is not recommended in the forests of the European Union (COM 659/2013, 20th of September).

Biological control is considered an alternative method in plant disease control. Since the use of chemical products has been reduced due to their harmful effect on the environment, the biological antagonisms are expected to become an important part of the control methods against plant pathogens (Cook, 1993). Fungi are being used more and more as commercial biological control agents, providing alternatives to chemical pesticides for combating insect pests, nematodes, and plant-pathogenic fungi (Deacon, 2006). Among them, fungal endophytes could be also used with this purpose. Fungal endophytes have been described by several authors as organisms that live inside the plant tissue and maintain a neutral, beneficial or detrimental relationship with the plant (Backman and Sikora, 2008). Other authors describe endophytes as fungi that live inside plant parts that produce no symptoms or signs of infection (Arnold et al., 2003; Deacon, 2006; Sieber, 2007). They have been previously used as biological control agents because they can inhibit or reduce the pathogen growth by micoparasitism, antibiosis, metabolites production, competition for the nutrients and induced resistance of the plant (Heydari and Pessarakli, 2010). Biological control by means of fungal endophytes has already demonstrated its suitability against *G. abietina* infections. The fungal endophyte *Phaeotheca dimorphospora* Desrochers & Ouellette was previously described to have inhibited *in vitro* the growth of *G. abietina* colonies, the germination of the spores and the spread of the pathogen on seedlings of red pine (*Pinus resinosa* Ait.) (Yang et al., 1995). Furthermore, several endophytes produced a reduction or an inhibition of the mycelial growth of some Spanish isolates of *G. abietina* which belonged to the genus *Trichoderma*, *Aureobasidion* and some unknown genus (Santamaría et al., 2007).

The defence mechanisms of conifers against pathogens can be classified as constitutive which are already in the tree before the contact with the pathogen, and induced, that are activated as a consequence of the interaction with the pathogen. If the induced defences serve as prevention against future infections then is called acquired resistance. The activation of the systemic induced resistance (SIR) in the host can be due to the presence of fungal endophytes and this mechanism has been already found to be effective against other fungal pathogens like *Diplodia pinea* (Desmaz.) J. Kickx fil. (Muñoz et al., 2008; Regliński et al., 2012). This activation produces either new compounds or an increase of the concentration of others that already existed in the plant (Franceschi et al., 2005). As a result of the activation of the plant's defence system an increase of the phenolic compounds has been frequently observed in lesion margins and transition zones in living sapwood of many trees, (Pearce, 1996). Other frequent responses include: an increase of the peroxidase and chitinase activity, a higher lignin content, a higher amount of glucanases that can degrade components of living organisms, the presence of toxic proteins and inhibitors of enzymes (Takahama and Oniki, 2000; Howell, 2003; Franceschi et al., 2005; Adomas and Asiegbu, 2006). In infections caused by *G. abietina* the defence mechanisms of conifers include an increase in the peroxidase activity and in lignin content with the accumulation of phenolic compounds in some cell walls (Cvikrova et al., 2006). Furthermore, the formation of a lignosuberized barrier has been shown to be a limiting component of the progression of *G. abietina* (Ylimartimo et al., 1997).

The reduction of *G. abietina* growth because of the endophyte antagonism has been previously studied in *in vitro* experiments (Santamaría et al., 2007) nevertheless no inoculation tests have been carried out yet. Thus, the main objectives of this study were (i) to evaluate the potential of several fungal endophytes to be used as biocontrol agents by testing the effect of their presence on the necrosis produced by *G. abietina* on the seedlings and (ii) to observe if the inoculation of the endophytes was able to activate the defence system of the plant by means of the measurement of the concentration of the total phenolic compounds.

2. Materials and methods

2.1. Plant and fungal material

To test the antagonistic effect of the endophytes and the pathogen *in vivo*, we performed artificial inoculations in healthy *P. halepensis* seedlings. The experiment was carried out in the greenhouse located at the University of Valladolid [Universal Transverse Mercator (UTM) coordinates: 4607558, 353022]. Containerized two-year old seedlings of *P. halepensis* (height: 16.77 ± 2.49 mm (mean ± standard error); diameter: 3.16 ± 0.74 mm) provided by "El Serranillo" Nursery from the Ministry of Agriculture and Environment and the Central Nursery from the regional government of Castilla y León were used for the experiment. Seedlings had been grown according to nursery practice and 6 months before inoculation, any fungicides, pesticides or herbicides were not applied to them. The seedlings in the experimental greenhouse were watered to field capacity every 2 to 3 days.

The fungal material (Table 1) consisted of six Spanish isolates of *G. abietina* isolated from adult trees showing the typical symptoms

Table 1
Characteristics of the isolates.

Behavior	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 sp.*	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 1	Quintanilla de Onésimo	Valladolid	2004
	E5	638 AB 2-b	*Leotiomycete sp.*	Tordehumos	Valladolid	2009

of *Gremmeniella* disease and five fungal endophytes previously isolated from symptomless *P. halepensis* trees and identified by Santamaría et al. (2007), Botella and Diez (2011) and Botella et al. (2010). The fungal endophytes corresponded to *Trichoderma* spp., endophyte 20.1 (not identified), *Aureobasidium* spp., *Aureobasidium pullulans* (de Bary) G. Arnaud and *Leotiomycete* spp. species. Some of these species had previously been shown reduction of mycelial growth of Spanish *G. abietina* in *in vitro* experiments (Santamaría et al., 2007). Isolates were sub cultivated in culture media MOS-agar (modified orange serum-agar, Müller et al., 1994) and PDA (potato, dextrose, and agar, Scharlau) some weeks before the inoculations in order to have enough mycelium.

2.2. Experimental design and inoculations

The experiment was arranged in a completely randomized factorial design with eight repetitions per treatment and two factors: 'pathogen' (six isolates + control), and 'endophyte species' (five endophytes species + control). Every treatment consisted of the artificial inoculation of one of the 42 combination of "pathogen ∗ endophyte species". Seedlings were placed 5 cm apart, without any direct contact between them, to avoid secondary infections. For inoculations, two wounds were made on the same side of the shoot axis; *Gremmeniella* isolate was placed at 10 cm below the shoot apex and the endophyte at 8 cm below the shoot apex. Therefore both fungal organisms were separated by 2 cm, which is considered an appropriate distance to test interaction between them. Each wound was made with a sterile scalpel. After, a small piece of culture medium from the margin of an actively growing colony was placed in the wound and finally the wounds were covered with Parafilm®. Control treatments were performed with sterile culture media. Inoculations were made in December in order to mimic the natural behavior of the fungus (Ranta et al., 2000). Three weeks after the first inoculations, the experiment was repeated. Therefore, two times of inoculation were performed. Thus, a total of 672 seedlings were inoculated twice in the two rounds of this experiment.

2.3. Necrosis length and other plant-related measurements

Six months after the inoculations the plants were cut and brought to the laboratory. The total length of the seedlings, the diameter at root collar and the necrosis length were measured in all the seedlings. In order to get a more accurate observation of the necrosis length, shoots were halved lengthwise before taking the measurements. The necrosis produced by the fungus was considered a quality indicator of the disease's advance because *G. abietina* is an organ-specialized pathogen with a necrotrophic behavior that kills stem tissue during colonization (Adomas and Asiegbu, 2006). The relationship among necrosis and total length of the seedling was defined as relative necrosis length and was used as response variable as previously described (Santamaría et al., 2006).

2.4. Total phenols extraction and quantification

Changes in the concentration of total phenols were used to measure the activation of the defence mechanisms of the plants to the inoculation of *G. abietina* and the endophytes. Six seedlings of every combination (a total of 252 samples) were selected and were analyzed for phenol content. The preparation of the samples and the total phenols extraction was done following the protocol described by Peñuelas et al. (1996) and Robles et al. (2003) slightly modified. Firstly the samples were dried in the oven at 40 °C for a week and then ground into powder. To extract the total phenols, 20 ml 70% (v/v) methanol solution (acidified with some drops of 1 M HCl) were added to every sample. The samples were left in the orbital shaker at continuous movement for 1.5 h and then filtered. To quantify the phenolic compounds we used the protocol described by Singleton and Rossi (1965) with some modifications. The reagents Folin–Ciocalteu (Sigma–Aldrich) and Na_2CO_3 (20% w/v) were used to produce the colorimetric reaction in the samples. After 1.5 h in darkness at room temperature the absorbance at 760 nm was measured 4 times for each sample with a spectrophotometer (Spectrum SP-2000UV, LAN Optics). To quantify the results, Gallic acid was used as the standard.

2.5. Re-isolation of G. abietina: DNA extraction and nested PCR

In order to confirm that *G. abietina* was the fungus responsible for the damage in the seedlings two methods were performed: (i) observation of the fruiting bodies and (ii) DNA extraction and amplification of the fungus. The observation of fruiting bodies was done in all the seedlings of the experiment. The fruiting bodies produced in the seedlings were taken and observed under the microscope to confirm morphologically that they belonged to *G. abietina*. The DNA extraction was made directly from the necrotic tissue from 168 seedlings. A 10 cm piece of the seedling was cut, freeze-dried for 24 h and ground into a fine powder with tungsten beads. Then, DNA was extracted following the Hamelin et al. (2000) protocol. To heighten sensitivity of detection, a nested PCR was used to amplify the 18S region (840 bp) of the rDNA. Amplifications were performed as described in Zeng et al. (2005) protocol but instead of using DNA products to perform the first round of PCR, we used dilutions of 1:100 and/or 1:1000 from DNA extracts. Nested PCR was carried out with specific primers for *G. abietina* (Zeng et al., 2005): NS. Grem 3 (5′-AACCTTGA ACTTGGTTGGTT-3′), NS. Grem 4 (5′-TGGTGGAGTGTTGCCACT-3′) in the first round followed by a second round of PCR with the primers NS. Grem 5 (5′-CACTGATCCGACCGGGT-3′) and NS. Grem

6 (5′-CCTTTCGGACAAGGAAGG-3′). PCR products (5 μl) were analyzed by electrophoresis on 1.2% agarose gels in 1× TAE buffer. The gels were stained with GelRed™ and visualized under UV light.

2.6. Statistical analysis

All statistical analyses were performed with SAS 9.2 Inc (2004) program. Due to the high variance heterogeneity of the data, the effect of time of inoculation, isolates, endophytes and their interactions on necrosis length and phenols content was evaluated by a linear mixed model with the MIXED procedure in SAS. The mathematical formulation of the model was:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + \alpha\beta_{ij} + \alpha\gamma_{ik} + \beta\gamma_{jk} + \alpha\beta\gamma_{ijk} + \varepsilon_{ijkl}$$

with $i = 1, 2$ for the time of inoculation, $j = 1,\ldots,6$ for the type of endophyte and $k = 1,..,7$ for the isolates, and Y_{ijkl} = the observed value of the dependent variable for the replication l of the time of inoculation i, the type of endophyte j and the isolates k; μ = general mean effect; α_i = main effect of the time of inoculation i; β_j = main effect of the type of endophyte j; γ_k = main effect of the isolate k; $\alpha\beta_{ij}$ = interaction effect of the time of inoculation i and the type of endophyte j; $\alpha\gamma_{ik}$ = interaction effect of the time of inoculation i with the isolate k; $\beta\gamma_{jk}$ = interaction effect of the type of endophyte j and the isolate k; $\alpha\beta\gamma_{jk}$ = triple interaction effect of the time of inoculation i, the type of endophyte j and the isolate k; ε_{ijkl} = random error in the dependent variable for the replication l of the time of inoculation i, the type of endophyte j and the isolate k.

We suppose that the random errors ε_{ijkl} are independent, with normal distribution $\varepsilon_{ijkl} \sim N(0, \sigma^2_{ij})$ for the relative necrosis length and $\varepsilon_{ijkl} \sim N(0, \sigma^2_{ik})$ for the total phenolic content. Therefore the mixed linear models have 12 parameters of variance for the relative necrosis and 14 parameters of variance for the total phenolic content.

To choose the best model among the possibilities we used the lowest Bayesian (BIC) and the Akaike information criterion (AIC). Normality, linearity and homoscedasticity of the residuals were checked by the Kolmogorov–Smirnov test and graphical procedures. A 5% level of significance was used in the statistical analyses. A Tukey–Kramer HSD test was applied for the comparisons of means when significant differences were found in the ANOVA table. To explore a possible relationship among necrosis length and total phenols content a non-parametric Spearmańs correlation test ($p = 0.05$) was performed.

3. Results

3.1. Effectiveness of the Gremmeniella infections

The first symptoms caused by the *G. abietina* infection were observed a few weeks after the inoculations. Symptoms observed consisted of dried needles, discoloration, necrosis, cankers and the death of some plants. We found fruiting bodies of *G. abietina* in the 42% of the seedlings inoculated in time of inoculation 1 while only the 3% of seedlings had pycnidia in the time of inoculation 2. There were not fruiting bodies on control seedlings. A first attempt of re-isolating the fungus by means of traditional subculture on PDA was performed but nested PCR had to be used based on the lower percentage of re-isolation obtained. The results from nested PCR showed that 53.52 % of the samples with *G. abietina* inoculations showed the presence of the specific band of approx. 840 bp (Fig. 1) in the gel electrophoresis. We found some differences based on the time of inoculations; in round one, the 58.33% of the samples were successfully amplified as compared to 47.22% of the samples from round two.

3.2. Relative necrosis length

The best linear mixed model was selected for relative necrosis length (RLN) according to the lowest AIC and BIC values. A linear mixed model with 12 variance parameters (one variance for every time of inoculation-isolate) and no random effects was used to test the effect of the time of inoculation, the *Gremmeniella* isolate, the endophyte species and their interactions on the necrosis length. The ANOVA table showed that all the main effects: time of inoculation, endophyte and isolate as well as the interactions time of inoculation * isolate and endophyte * isolate were all statistically significant variables (Table 2). The Tukey–Kramer test revealed that, on average, seedlings inoculated on time of inoculation 1 had significantly higher relative necrosis length measurement than those inoculated in time of inoculation 2 (Table 3). Regarding the *Gremmeniella* isolates, seedlings inoculated with any of them, regardless which endophyte was also inoculated, showed relative necroses length higher than that observed in the controls (G-control) (Table 3). In both times of inoculation, G-control seedlings had less RLN than the seedlings inoculated with the *G. abietina* isolates (Table 3). Among the *G. abietina* isolates we also found some differences; G1 had lower RLN than G2, G3, G4, G5 and G6. Among them, G2 produced the significantly highest necrosis length in the seedlings, regardless the time of inoculation (Table 3). Differences in necrosis were also observed among the other isolates, although it was dependent on the time of inoculation (Table 3).

The presence of all endophytes reduced significantly the necrosis produced by *G. abietina* (Table 4) but no differences were found among the various endophyte species (Fig. 2). When analyzed separately, the data showed that the effect of each endophyte was different for each *Gremmeniella* isolate. In the case of isolate G1, only the endophyte E5 (*Leotiomycete* spp.) was significantly effective reducing the RLN caused by the pathogen. In the case of G2, two of the endophytes (E3 (*Aureobasidium* spp.) and E4 (endophyte 20.1)) reduced significantly the RLN caused by the pathogen. For G3, none of the endophytes was able to reduce significantly the necrosis length; whereas for G4, G5, and G6, the five endophytes were able to reduce the necrosis caused by the pathogen (Table 4)

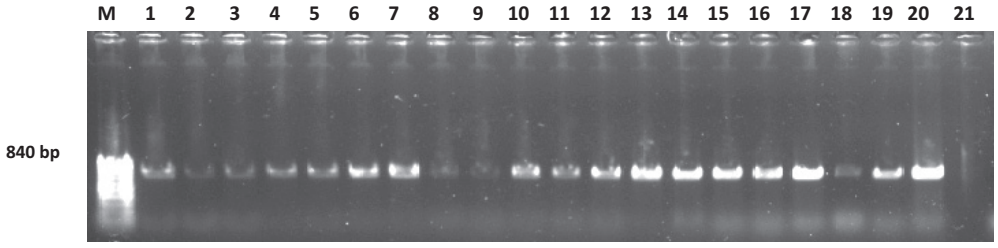

Fig. 1. Detection of *G. abietina* in *P. halepensis* seedlings. PCR products of nested PCR. Lanes 1–19: samples from infected seedlings. Lane 20: positive control using *G. abietina* DNA. Lane 21: negative control. M: marker 100 bp DNA Ladder.

Table 2
ANOVA table for relative necrosis length and total phenols content.

Dependent variable	Effect	DF	*F*-value	Pr > *F*
Relative necrosis length	Time of inoculation (TI)	1	98.95	<0.0001
	Endophyte	5	11.31	<0.0001
	Isolate	6	64.16	<0.0001
	TI * endophyte	5	1.32	0.2547
	TI * isolate	6	14.13	<0.0001
	Endophyte * isolate	30	1.72	0.0108
	TI * endophye * isolate	30	1.04	0.4072
Total phenols content	Time of inoculation (TI)	1	44.78	<0.0001
	Endophyte	5	2.1	0.0683
	Isolate	6	3.52	0.0049
	TI * endophyte	5	11.91	<0.0001
	TI * isolate	6	9.01	<0.0001
	Endophyte * isolate	30	0.97	0.5218
	TI * endophye * isolate	30	0.97	0.5116

Table 3
Relative necrosis length. Mean value ± standard error (SE).

Isolate	Time of inoculation		Total[a]
	T1	T2	
G1	0.0544 ± 0.015 b[b] A[c]	0.091 ± 0.0087 b B	0.0727 ± 0.0087 b
G2	0.2676 ± 0.015 d B	0.1445 ± 0.0087 d A	0.206 ± 0.0087 d
G3	0.2383 ± 0.015 cd B	0.1288 ± 0.0087 cd A	0.1835 ± 0.0087 cd
G4	0.236 ± 0.015 d B	0.1184 ± 0.0087 c A	0.1772 ± 0.0087 c
G5	0.2239 ± 0.015 c B	0.1313 ± 0.0087 cd A	0.1776 ± 0.0087 c
G6	0.1985 ± 0.015 c B	0.1329 ± 0.0087 cd A	0.1657 ± 0.0087 c
G-control	0.0108 ± 0.015 a A	0.0255 ± 0.0087 a B	0.0182 ± 0.0087 a
Total[d]	0.1757 ± 0.0057 B	0.1104 ± 0.0033 A	

[a] Average necrosis when combining all the times of inoculation together.
[b] Means without a common small letter in the same column show values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test).
[c] Means without a common capital letter in the same row values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test).
[d] Average necrosis when combining all the *G. abietina* isolates together.

compared to the control seedlings. The susceptibility of the different *Gremmeniella* isolates to each endophyte can be also analyzed in Table 4. The isolate G1 was the most susceptible to the influence of all the endophytes. Even for three cases (for E2 (*A. pullulans*), E4 (endophyte 20.1), and E5 (*Leotiomycete* spp.) the endophyte reduced the necrosis length of this isolate to that observed in the controls (Table 4). In contrast, the isolate G2 seemed to be the most resistant to the influence of any endophyte antagonism, especially for E2 (*A. pullulans*) and E5 (*Leotiomycete* spp.). The rest of the *Gremmeniella* isolates showed a susceptibility to the endophyte influence in between those two extremes. None of the endophytes were pathogenic for *P. halepensis* seedlings as no significant differences were found in the RLN between the E-control and the G-control of either endophyte.

3.3. Total phenols content

A linear mixed model with 14 variance parameters (one variance for every combination isolate * endophyte) and no random effects was selected according to AIC and BIC criteria to test the effect of the time of inoculation, the *Gremmeniella* isolate, the endophytes species and their interactions on the total phenols content. The ANOVA table (Table 2) for the linear mixed model showed that all the following variables to be significant: time of inoculation, isolate, time of inoculation * endophyte and time of inoculation * isolate. On the contrary, the endophyte species and the rest of interactions did not show significant influence on the total phenol concentration. On average, plants inoculated at the time of inoculation 2 had higher total phenolic content than those

Table 4
Relative necrosis length. Mean value ± standard error (SE).

Isolate	Endophyte						Total[a]
	E1	E2	E3	E4	E5	E-control	
G1	0.082 ± 0.0213 b[b] AB[c]	0.0609 ± 0.0225 a AB	0.0807 ± 0.0173 b AB	0.0669 ± 0.0201 a AB	0.0408 ± 0.0191 a A	0.105 ± 0.0263 b B	0.0727 ± 0.0087 b
G2	0.1645 ± 0.0213 c A	0.2343 ± 0.0225 c BC	0.1476 ± 0.0173 c A	0.1798 ± 0.0201 b AB	0.2238 ± 0.0191 c BC	0.2863 ± 0.0263 cd C	0.206 ± 0.0087 d
G3	0.194 ± 0.0213 c A	0.1773 ± 0.0225 bc A	0.1863 ± 0.0173 c A	0.1565 ± 0.0201 b A	0.171 ± 0.0191 bc A	0.216 ± 0.0263 c A	0.1835 ± 0.0087 cd
G4	0.1742 ± 0.0213 c A	0.1594 ± 0.0225 b A	0.148 ± 0.0173 c A	0.1322 ± 0.0201 b A	0.1519 ± 0.0191 b A	0.2974 ± 0.0263 d B	0.1772 ± 0.0087 c
G5	0.2021 ± 0.0213 c B	0.1465 ± 0.0225 b AB	0.1715 ± 0.0173 c AB	0.1396 ± 0.0201 b A	0.1369 ± 0.0191 b A	0.2693 ± 0.0263 cd C	0.1776 ± 0.0087 c
G6	0.1446 ± 0.0213 c A	0.1574 ± 0.0225 b A	0.139 ± 0.0173 c A	0.1345 ± 0.0201 b A	0.1605 ± 0.0191 b A	0.2584 ± 0.0263 cd B	0.1657 ± 0.0087 c
G-control	0.0193 ± 0.0213 a A	0.0307 ± 0.0225 a A	0.0137 ± 0.0173 a A	0.0307 ± 0.0201 a A	0.0103 ± 0.0191 a A	0.0044 ± 0.0263 a A	0.0182 ± 0.0087 a
Total[d]	0.1401 ± 0.008 A	0.1381 ± 0.0085 A	0.1267 ± 0.0065 A	0.12 ± 0.0076 A	0.1279 ± 0.0072 A	0.2052 ± 0.0099 B	

[a] Average growth when combining all the endophytes together.
[b] Means without a common small letter in the same column show values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test).
[c] Means without a common capital letter in the same row values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test).
[d] Average necrosis when combining all the *G. abietina* isolates together.

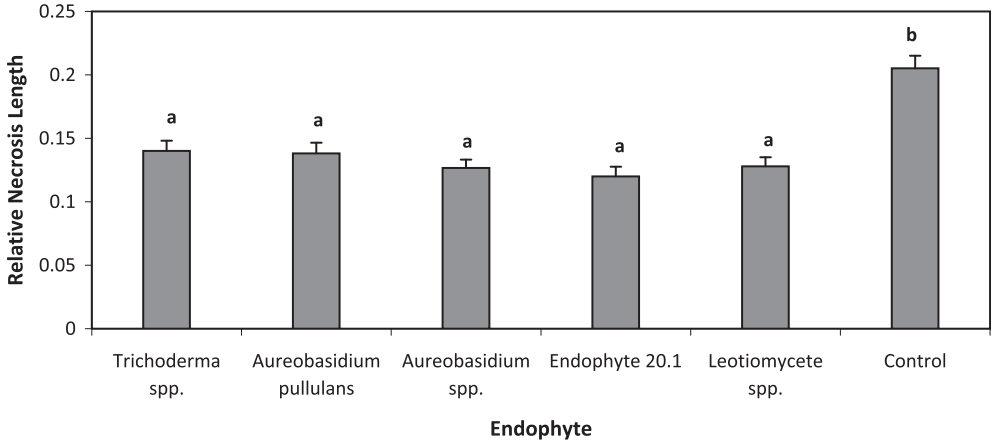

Fig. 2. Relative necrosis length found in every seedling when inoculating both *G. abietina* isolates with the different endophytes. Control seedlings had no endophyte but *G. abietina* isolate. Means with a different letter were significantly different from $p < 0.05$ (Turkey's HSD Test). Bars represent standard error.

Table 5
Total phenols (equivalents Gallic acid (mg)/ dry weight of the sample (g)). Mean value ± standard error (SE).

Isolate	Time of inoculation		Total[a]
	T1	T2	
G1	1282.44 ± 98.78 a[b] A[c]	1043.03 ± 63.93 ab B	1162.73 ± 58.83 a
G2	349.81 ± 45.99 c B	1038.47 ± 89.56 ab A	694.14 ± 50.34 bc
G3	515.87 ± 115.74 bc B	888.19 ± 78.43 b A	702.03 ± 69.9 bc
G4	486.79 ± 119.93 c B	1087.66 ± 79.81 ab A	787.22 ± 72.03 bc
G5	385.57 ± 110.47 c B	983.55 ± 74.11ab A	684.56 ± 66.51 c
G6	694.67 ± 74.34 b B	1049.55 ± 81.83 ab A	872.11 ± 55.28 b
G-control	1239.87 ± 112.54 a A	1122.11 ± 83.42 a A	1180.99 ± 70.04 a
Total[d]	707.86 ± 37.81 B	1030.37 ± 29.89 A	

[a] Average growth when combining both times of inoculation.
[b] Means without a common small letter in the same column show values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test).
[c] Means without a common capital letter in the same row values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test).
[d] Average phenols content when combining all the *G. abietina* isolates together.

Table 6
Total phenols (equivalents Gallic acid (mg)/ dry weight of the sample (g)). Mean value ± standard error (SE).

Endophyte	Time of inoculation		Total[a]
	T1	T2	
E1	585.01 ± 93.28 b[b] B[c]	1060.75 ± 72.76 a A	822.88 ± 59.15 b
E2	621.42 ± 104.29 b B	1137.07 ± 72.76 a A	879.24 ± 63.58 ab
E3	924.38 ± 94.36 a A	884.68 ± 72.76 b A	904.53 ± 59.58 ab
E4	695.9 ± 89.71 ab A	841.69 ± 72.76 b A	768.8 ± 57.76 b
E5	589.7 ± 85.62 b B	1068.47 ± 72.76 a A	829.08 ± 56.18 b
E-control	830.75 ± 87.18 ab B	1189.54 ± 75.42 a A	1010.14 ± 57.64 a
Total[d]	707.86 ± 37.81 B	1030.37 ± 29.89 A	

[a] Average growth when combining both times of inoculation.
[b] Means without a common small letter in the same column show values significantly different from $p < 0.05$ (ANOVA Tukeýs HSD Test).
[c] Means without a common capital letter in the same row values significantly different from $p < 0.05$ (ANOVA Tukey's HSD Test).
[d] Average phenols when combining all the endophytes together.

inoculated on time of inoculation 1 (Table 5). Among the isolates, G-control seedlings and the ones inoculated with isolate G1 presented the highest concentrations of total phenols (Table 5). At the time of inoculation 1 the G-control seedlings (no *Gremmeniella* isolate) and the ones inoculated with the isolate G1 had a significantly higher content of total phenols than the seedlings inoculated with the rest of *G. abietina* isolates. There were differences also among the other isolates. Seedlings inoculated with G6 had higher phenolic content than the seedlings inoculated with G2, G4 and G5. In time of inoculation 2 G-control seedlings had higher phenolic content than seedlings with G3, but there were no differences between G-control and the rest of the *Gremmeniella* isolates.

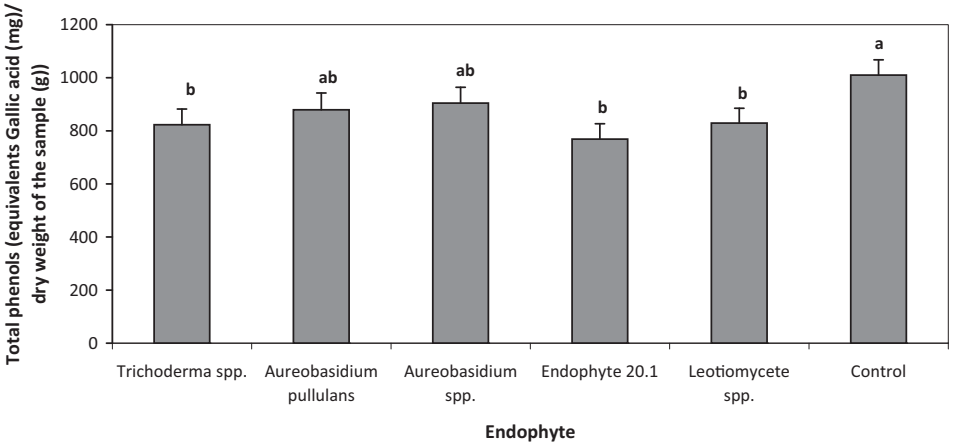

Fig. 3. Total phenols content, (equivalents Gallic acid (mg)/dry weight of the samples (g)) found in every seedling when inoculating both *G. abietina* isolates with the different endophytes. Control seedlings had no endophyte but *G. abietina* isolate. Means without a common letter were not significantly different from $p < 0.05$ (Turkey's HSD Test). Bars represent standard error.

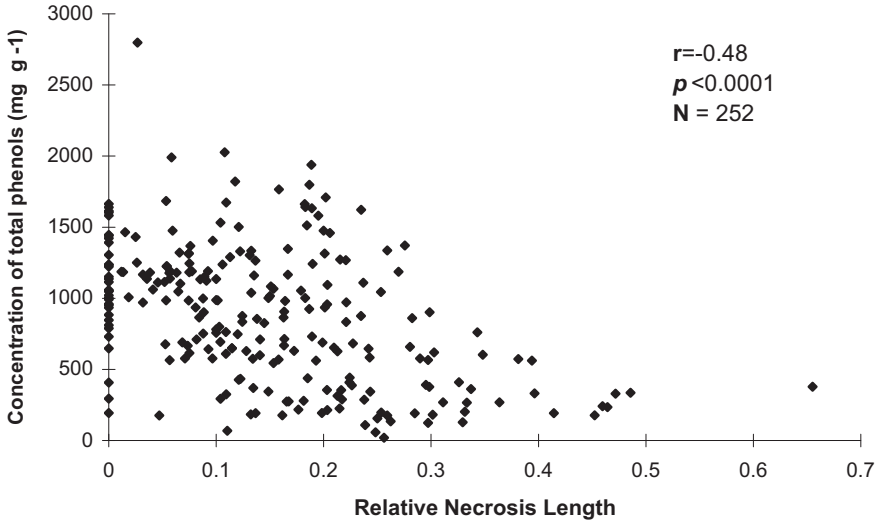

Fig. 4. Correlation among concentration of total phenols (milligrams equivalents of Gallic acid per gram of dried sample) and relative necrosis length.

Among endophytes, at both times of inoculation, none of the endophytes were able to promote a higher phenolic production in the plants compared to E-controls (Fig. 3 and Table 6). Nevertheless, some differences among endophytes were found. At the time of inoculation 1, seedlings inoculated with E3 (*Aureobasidium* spp.) produced a higher total phenol content than seedlings inoculated with E1 (*Trichoderma* spp.), E2 (*A. pullulans*) and E5 (*Leotiomycete* spp.) (Table 6). In the second inoculation time, seedlings inoculated with E1 (*Trichoderma* spp.), E2 (*A. pullulans*), E5 (*Leotiomycete* spp.) and the E-control seedlings presented a significantly higher amount of total phenols than seedlings inoculated with E3 (*Aureobasidium* spp.) and E4 (endophyte 20.1) (Table 6). Relative necrosis length showed a negative correlation ($r = -0.48002$; $p < 0.0001$) with the total phenolic content (Fig. 4).

4. Discussion

This study explores the effectiveness of biological control against *G. abietina* in Aleppo pine seedlings. Our results showed that the inoculation of *G. abietina* produced symptoms of chlorosis, dieback, necrosis, cankers and the death of some seedlings. These symptoms were similar to those previously reported in others studies (Uotila, 1993; Santamaría et al., 2003, 2006). The results also showed that at the time of inoculation 1 there were fruiting bodies in 42% of the seedlings inoculated with *G. abietina* isolates while at the time of inoculation 2 this percentage was only 3%. Furthermore, the necrosis length was also statistically higher at the time of inoculation 1 than at the time of inoculation 2. Both facts confirm that in our study the time of infection was very important in terms of damage and control. The results of DNA amplification were consistent with this statement: 58.33% of the samples from seedlings inoculated in the first round were successfully amplified whereas in round 2 the percentage was 47.22% of the samples. The longer the necrosis length, the greater the fruiting bodies production and the higher percentage of re-isolation by means of DNA amplification observed at the time of inoculation 1 could be explained by the fact that *G. abietina* is more active during the dormant season and its activity is influenced by the resource allocation process (Ranta et al., 2000). Therefore, when inoculations were carried out at the time of inoculation 1, the fungal pathogen was growing during a longer period in the optimal conditions for this fungus development.

The attempt of re-isolating the fungus by means of a traditional subculture on PDA media was a failure in our experiment as opposed to Santamaría et al. (2007) who obtained a 66% of re-isolation of *G. abietina* with this method. Because of that, the nested PCR was performed and 53% of the samples from the seedlings inoculated were successfully amplified with the specific *G. abietina* primers of the 18S region. Although the ITS region has been recently described as the international barcode for fungi and the marker of choice for studying the fungal diversity (Schoch et al., 2012; Kõljalg et al., 2013) the use of the 18S region to amplify the DNA is less likely to produce false negative detections (Zeng et al., 2005). Furthermore, the use of specific primers for *G. abietina* simplified the process as no-sequencing was needed, and the results could be visualized directly in the electrophoresis' gel.

Results from our experiment showed that generally speaking the presence of all the endophytes reduced the necrosis length produced by *G. abietina* although the efficacy of the control depended also on the time of inoculation. Several mechanisms could be responsible for that reduction. It has been reported that biological control agents may produce substances that directly attacks the pathogens or that induce the systemic resistance which, in turn, reduce the pathogen incidence in the plant host (Paul and Sharma, 2002; Gao et al., 2010; Akila et al., 2011). For example, in seedlings of *Theobroma cacao* L., inoculation with endophytes significantly reduced leaf necrosis and mortality caused by a major foliar pathogen. On the pathogen-infected leaves that did survive, necrotic lesions were significantly larger on leaves without endophytes than on leaves with endophytes (Arnold et al., 2003). Biocontrol agents employ an assortment of mechanisms to control plant diseases that vary with the host, the pathogen and also with the biocontrol agent involved in the interaction. In addition, these mechanisms are also influenced by the environmental conditions where the interaction is produced: leaf chemistry, soil type, temperature, pH, moisture of the plant and the possible occurrence of other endophytic species (Arnold et al., 2003; Howell, 2003; Miles et al., 2012).

In our study, the isolate from genera *Trichoderma* spp. was able to successfully control part of the spreading of *G. abietina*, as it caused a reduction of the necrosis produced by the pathogen in the seedlings compared to the control ones. Good results on *in vitro* experiments were previously shown in Santamaría et al. (2007) in the reduction of mycelial growth of *G. abietina* on dual culture tests. This genus has been reported as an effective tool

against pathogens before. For instance, it was effective against *Fusarium circinatum* Nirenberg & O'Donnell on *in vitro* experiments (Martínez-Álvarez et al., 2012), against *Cytospora chrysosperma* (Pers.) Fr. both on *in vitro* and on *in vivo* tests (Yi and Chi, 2011) against *Botrytis cinerea* Pers. in Scots pine seedlings (Capieau et al., 2004) against *D. pinea* on *in vivo* experiments in *Pinus banksiana* Lamb. (Santamaría et al., 2012) and against the causal agent of Dutch Elm disease in laboratory tests conducted under *in vitro* conditions (Díaz et al., 2013). It is especially effective against pathogens that colonize the rhizosphere, because it has been shown to increase the root growth of the plant as well as its systemic resistance (Inbar et al., 1994; Regliński et al., 2012). The efficiency of *Trichoderma* as a biological control agent depends on the pathogen species they are confronted with. But in general terms, *Trichoderma* can be considered a good candidate to be used as biocontrol agent due to its high capacity and rate of reproduction; they are able to survive when the environmental conditions are not favorable, they can modify the rhizosphere, they present the ability to transport glucose rapidly which gives them an advantage in nutrient competition and they can promote plant growth and better defence mechanisms (Benítez et al., 2004). In some cases *Trichoderma* strains combine several of those mechanisms to successfully fight against the phytopathogenic fungi.

In addition to *Trichoderma*, our results showed that the inoculation with isolates of the *Aureobasidium* genus (both *A. pullulans* E2 and *Aureobasidion* spp. E3) also produced a reduction in the necrosis length caused by *G. abietina*. Previous studies have also pointed out the antagonistic activity of the species *A. pullulans* against several fungal pathogens such as *Penicillium expansum* Link (Mounir et al., 2007) and *Aspergillus carbonarius* (Bainier) Thom (Dimakopoulou et al., 2008). Miles et al. (2012) observed that *A. pullulans* was the most successful biocontrol agent based on the evaluation of its efficacy on *in vivo* test against *Rhizoctonia solani* J.G. Kühn, bacteria and oomycetes on tomato plants. This genus is especially effective on postharvest pathogens of several fruits and its mechanisms of success include the induction of plant defence responses and the competition of nutrients (Banani et al., 2014). The endophyte named 20.1 also reduced the necrosis length produced by *G. abietina* in the seedlings compared to the control plants. Although no identification of the species has been made yet, a previous study performed *in vitro* by Santamaría et al. (2007), showed that in Petri Dishes, this endophyte completely inhibited *G. abietina*'s growth. Furthermore, this endophyte produced a brownish pigment around the colony on culture media so that *G. abietina* colonies became more compact and dense. These results suggested that the endophyte could produce some antifungal compounds which slow down the growth of *G. abietina*. Lastly, the isolate from *Leotiomycete* genus was also able to reduce the necrosis produced by *G. abietina* in the seedlings. Very scarce literature was found about *Leotiomycetes* fungus as a biocontrol agent. Nevertheless, Miles et al. (2012) tested several endophytes, including one member of *Leotiomycete* class, *Botrytis fabae* Sardiña which can acts as a plant pathogen as well, and concluded that it reduced the growth of other pathogens such as *B. cinerea*, *Fusarium oxysporum* E.F. Sm. & Swingle, *Phytophthora infestans* (Mont.) de Bary, and *R. solani*.

Among the mechanisms which explain the reduction in the necrosis caused by the pathogen when an endophyte was also inoculated, several authors (Muñoz et al., 2008; Regliński et al., 2012) have proposed that endophytes might activate a systemic induced resistance (SIR) mechanism in the host plant which might contribute to reduce the incidence of the disease. An increase in the concentration of phenolic compounds has been previously related to the activation of the induced defence mechanisms of the plant. For instance, *Trichoderma* spp. strains produce compounds that induce the synthesis and accumulation of phytoalexins, flavonoids, terpenoids, phenolic derivatives, aglycones and other antimicrobial compounds (Benítez et al., 2004). Nevertheless, the results of the present study showed that the presence of the endophytes, did not produce an increase of the total phenols. Therefore, although some phenolic production would have been instigated by the endophytes, it would not have been enough to retain the pathogen development. Consequently, in our case the reduction in the necrosis length caused by *G. abietina* observed when the endophyte was also inoculated could be likely caused, rather than by a systemic induced resistance mechanism, by the direct effect of the endophyte on the pathogen. In this case, competition and/or antibioses could be the mechanisms more likely involved in the observed antagonism.

On the other hand, in our study, the infection of the seedlings by *G. abietina* did not produce an increase in the total contents of phenolic acids compared to the control seedlings. This was inconsistent with the fact that an increase in phenolic compounds would be expected since they have been previously described as a part of conifer defence against *G. abietina* (Cvikrova et al., 2006). In addition, in most of woody plants phenols have been studied as markers for pathogen resistance (Witzell and Martín, 2008). It is known that wounding or an invasion of the bark has been shown to activate polyphenolic parenchyma cells, which includes cell expansion and accumulation of increased amounts of phenols. Thus, phenolic compounds can act as antifungal agents and can bind hydrolytic enzymes secreted by pathogens, thus inhibiting their spread into tissues (Franceschi et al., 2005). Nevertheless, relationships among such substances composition and resistance to pathogens are unclear and sometimes contradictory and some authors report finding no such correlations. For instance, there were no correlations between monoterpene composition and resistance to *Heterobasidion annosum (Fr.) Bref., (1888)* (Pearce, 1996). Varying relationships have also been found between lesion length produced by other pathogens and secondary metabolites (Wallis et al., 2008; Witzell and Martín, 2008). The lack of response in the plant's phenols production by plants as a consequence of the pathogen inoculation could be explained by the fact that the seedlings used in the experiments were young (2-year old) and still developing. Therefore, the suberization of their cell walls may not have been complete. Furthermore, *G. abietina* has reported to have some phenoloxidase activity because it could grow trough the bract cells surrounded by lignified and suberized cell walls and filled with phenols (Ylimartimo et al., 1997). In addition, Simard et al. (2013) observed that lignin, suberin and other phenolic compounds could be degraded by *G. abietina* in the transition zones of the infection. Thus, the low suberization of the cells linked to the ability to degrade phenolic compounds of *G. abietina* could explain the lack in response of the plants regarding phenolic production.

In the current study, the control seedlings without *G. abietina* inoculations showed the highest concentration of phenolic compounds. In our case, the higher phenolic content found in the not inoculated seedlings could be explained by the fact that the analyses were made 6 months after inoculation when plants were very affected by the pathogen. Under this condition it is supposed that the capacity of the plants to produce these defensive compounds could be reduced and if the analyses had been performed only some weeks after inoculation, the phenols contained in the plant tissues would have been higher. Therefore, it could be hypothesized that the induced resistance mechanisms of the plant are activated when the infection is produced. Nevertheless, further experiments including several samplings along the infection process should be carried out to confirm this hypothesis.

This study provides additional knowledge about the effects of the inoculation of *G. abietina* and fungal endophytes in Aleppo pine seedlings. It can be concluded that the use of fungal endophytes

could be a suitable strategy to reduce the incidence of plant pathogens like *G. abietina* in pine seedlings. This statement is made based on the results obtained in which the inoculation of an endophyte into a plant reduced the advance of the pathogen; although the efficiency of the control depended on the moment of inoculation. The inoculation with an endophyte did not promote a higher production of phenolic compounds, which are considered a good indicator of such induced resistance mechanism. However, it seemed that competition and/or antibiosis were the mechanisms responsible for that reduction, rather than a systemic induced resistance mechanism. Nevertheless, further studies of biological antagonisms are recommended, particularly about the mechanisms they employ to interact with the pathogens.

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