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Native rhizobacteria as biocontrol agents of *Heterobasidion annosum* s.s. and *Armillaria mellea* infection of *Pinus radiata*

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HIGHLIGHTS

- Native *Pinus radiata* rhizobacteria inhibit growth of fungal pathogens *H. annosum* and *A. mellea*.
- *P. fluorescens* S32R2 and *E. billingiae* S31R1 were strong antagonists of both fungi in vitro.
- *P. fluorescens* S32R2 and *B. simplex* S11R41 best protected *P. radiata* seedlings against both fungi.

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ABSTRACT

Biocontrol bacteria *Pseudomonas fluorescens*, *Bacillus simplex* and two different strains of *Erwinia billingiae* were isolated from the rhizosphere of a healthy tree located in a *Pinus radiata* plantation with high presence of fungal pathogens. The bacteria were selected based on a high level of antagonism in vitro against *P. radiata* pathogens *Heterobasidion annosum* s.s. (68.6–99.3% area inhibition percent (AIP)) and *Armillaria mellea* (64.8%–94.2% AIP). None of the bacteria were pathogenic for two-month-old seedlings of *P. radiata*. *P. fluorescens* and *B. simplex* reduced the incidence of *H. annosum* and *A. mellea* infection on *P. radiata*. While *H. annosum* was detected in 90% of seedlings that were not inoculated with bacteria, detection was reduced to 40% and 55% of seedlings treated with *P. fluorescens* and *B. simplex*, respectively. Following infection with *A. mellea*, 54% of the seedlings that were not treated with bacteria died, whereas for those treated with *P. fluorescens*, *B. simplex* and the two strains of *E. billingiae*, the proportion of plants that died was 13.1%, 7.1%, 3.6% and 11.6% respectively.

1. Introduction

Root and butt rot diseases of trees are often caused by several fungal species within *Heterobasidion* Bref. and *Armillaria* (Fr.) Staude genera and are characterized by chlorotic leaves, progressive thinning of the crown, slower leader growth, and rapid tree death (Edmonds et al., 2000). *Armillaria* is distributed worldwide (Wingfield et al., 2010) with a broad host range such as conifers, hardwoods, shrubs and some herbaceous plants (Williams et al., 1986). In contrast, *Heterobasidion* distribution is mainly limited to coniferous forests and plantations in the northern hemisphere (Korhonen et al., 1998).

Three percent of the total tree plantations worldwide consist of *Pinus radiata* D. Don (Monterey pine), covering over four million hectares, mainly in New Zealand, Chile, Australia, Spain and South Africa, where they are an important part of the economy. *P. radiata* is the most extensively planted exotic conifer, most productive (Mead, 2013), and susceptible to infection by both *Heterobasidion* and *Armillaria*. Studies conducted in New Zealand showed that species of *Armillaria* caused mortality rates between 20 and 50% in the first six years of *P. radiata* stands (Hood and Sandberg, 1993), and 6–13% losses of the potential volume in a 28-year-old *P. radiata* plantation (MacKenzie, 1987). *Heterobasidion annosum* sensu stricto (s.s.) (Niemela and Korhonen, 1998) caused high levels of disease in three-year-old *P. radiata* seedlings (Doğmuş-Lehtijärvi et al., 2016), and gaps in plantations in northern Spain (Mesanza and Iturritxa, 2012). Timber volume losses caused by *Heterobasidion* infection are due to tree decay, diameter growth reduction, wind throw, and stand susceptibility to storm damages (Garbelotto and Gonthier, 2013). The infected trees are also more susceptible to other factors such as bark beetle infestations (Goheen and Otrosina, 1998).

Both fungi can be transmitted to new hosts by direct contact between hyphae and roots, or by basidiospores (Redfern and

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Filip, 1991; Rishbeth, 1959). *Armillaria* also forms rhizomorphs, root-like structures that can spread through the ground (Redfern and Filip, 1991), and *Heterobasidion* produces conidiospores (Hughes, 1953). The ability of these fungal structures to mediate host infection is variable and depends on many factors, such as fungal species, host resistance, and environmental conditions.

Currently, *Heterobasidion* infections are managed using silvicultural, chemical and biological methods. Silvicultural practices include planting less susceptible tree species, stump removal, using proper planting and mixture schemes, and thinning when the spores are not dispersing. Chemical treatments are based on urea and borate (Pratt and Lloyd, 1996; Johansson et al., 2002), and biological control requires inoculating stumps with the fungus *Phlebiopsis gigantea* (Fr.) Jülich (Asiegbu et al., 2005). *Armillaria* treatment includes silvicultural methods (e.g., root collar excavation, stump and residual root removal, and planting less susceptible tree species), soil fumigants such as methyl bromide and carbon disulphide, and the application of the soil-borne fungus *Trichoderma* Pers. after fumigation (Baumgartner et al., 2011). However, the application of these treatments is limited and often ineffective due to factors such as level of infection, environmental conditions and risks, cost, and legislation, among others (Asiegbu et al., 2005; Gonthier and Thor, 2013; Baumgartner et al., 2011; Shaw and Roth, 1978). The best defence against these fungal infections is prevention.

Biological control with bacteria has proven effective against several fungal pathogens of agronomic crops (Mark et al., 2006) and in fewer cases against forest fungal pathogens (Singh et al., 2008). Antagonism by bacteria is achieved by different mechanisms including antibiosis, competition for nutrients, parasitism, and induced resistance in the host (Whipps, 2001). Other factors that influence the efficacy of biocontrol bacteria are their capacity to colonize the rhizosphere or the host seeds, and to adapt to soil conditions (Mark et al., 2006). The probability of isolating microorganisms from the environment that demonstrate an antibiosis effect in vitro is relatively high, but many of these are not effective when applied *in planta* where plant host responses to and impact on microbial activity are also important. Crop studies have shown that strains isolated from native soils have the best chance of protecting plants as they are adapted to the soil conditions and therefore can compete effectively with other indigenous microbes.

The objective of this study was to isolate and characterize the ability of some bacteria native to the *P. radiata* rhizosphere to inhibit the growth of *A. mellea* and *H. annosum* in vitro and reduce their pathogenic effects in *P. radiata* seedlings. Effective bacterial inoculants offer a prophylactic nursery treatment to complement the current integrated management strategies.

2. Materials and methods

2.1. Microorganisms

The *H. annosum* s.s. and *A. mellea* strains used in this study were isolated from basidiocarps present in a *Pinus sylvestrys* plantation in Alava, Spain and on an *Acer* spp. located in Biscay, Spain, respectively. Both fungal strains proved to have high virulence against different tree species including *P. radiata*. Identification, characterization and efficacy of both pathogens were described in previous studies (Mesanza and Iturritxa, 2012; Mesanza and Iturritxa, 2013a, 2013b). The fungi were routinely grown at 20 °C in the dark on malt extract agar (MEA).

Bacterial strains were isolated from the rhizosphere of a healthy tree located in a *P. radiata* plantation (Latitude: 43°06′46″N; Longitude: 2°38′35″W, Abadiano, Biscay, Basque Country, Spain) with high presence of fungal pathogens. Samples containing tree roots and surrounding soil were collected and stored at 4 °C. To extract ectorhizosphere bacteria, 5 g of root samples were suspended in 45 ml sterile 0.85% NaCl, shaken for 3 min, and the supernatant was decanted into sterile tubes. To obtain endorhizosphere bacteria, 5 g of roots were washed with sterile 0.85% NaCl and then homogenized with an adapted drill (Optimun Maschinen, Germany) in 50 ml of the same solution and the supernatant was collected. Serial dilutions of the supernatants were plated on Luria Bertani (LB) agar (Miller, Fisher Scientific) and grown overnight at room temperature.

2.2. In vitro fungal antagonism assay

Two hundred isolated rhizobacteria were initially screened, in triplicate, for antagonistic effects against *H. annosum* and *A. mellea*. Approximately 2 mm^2 of fungal mycelium was transfer into wells of a six-well plate (Nunc) containing ISP2 agar (4 g/L yeast extract, 10 g/L malt extract, 4 g/L glucose and 20 g/L agar, pH 7.3; Shirling and Gottlieb, 1966), determined to be suitable for both fungal and bacterial growth, and was grown for two days before applying the bacterial suspension. Bacterial cultures, prepared in triplicate from independent colonies grown for two days into LB broth, were applied in a thin line using a sterile inoculation loop 0.5 cm from the fungi. *Escherichia coli* TOP10 (Invitrogen) served as a negative control. After 60 days of growth at room temperature (20–25 °C), inhibition of fungal growth by the bacteria was visually assessed.

The effect of antagonistic bacterial strains was confirmed in a second in vitro antagonism assay. The bacterial cultures were washed twice with 0.03 M $MgSO_4$ and the final concentration adjusted to an OD_{600nm} of 0.5 before applying to wells containing fungi as described above. Bacterial antagonism was defined as Area Inhibition Percentage (AIP): AIP (%) = (A − B)/A * 100, where A and B are the surface area covered by the fungus in control (no bacteria) and treated (with bacteria) plates, respectively.

2.3. Bacterial identification and pathogenicity determination

Single colonies of effective fungal antagonistic bacteria were grown overnight in 3 ml LB broth at 30 °C for DNA extraction with Wizard Genomic DNA Purification Kit (Promega, USA). The 16S rRNA gene was amplified using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Pickering, ON) and the primer pairs 46f and 536r (Mummey and Stahl, 2004), E334f and E939r (Baker et al., 2003), and E786f (Baker et al., 2003) and E1491r (Smit et al., 1997) (Table 1). The PCR conditions were as follows: 5 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at the appropriate annealing temperature, and 1 min at 72 °C, and a final 1 min at 72 °C. The purified PCR products were sequenced by Robarts Research Institute (London, ON). The sequences were manually assembled using Mega 4.0 software, and then analyzed using the Ribosomal Database Project (Cole et al., 2014), Greengenes (DeSantis et al., 2006) and GenBank (Benson et al., 2007) databases.

The possible phytopathogenicity of the bacterial strains was assessed by watering two-month-old *P. radiata* seedlings with 5 ml of bacterial suspension. *P. radiata* seedlings were grown from seeds (Sheffield's Seed Co. Inc., NY) that were surface sterilized by placing them in 2.1% sodium hypochlorite solution for 10 min with shaking, and then rinsing them thoroughly with water (Wenny and Dumroese, 1987). Seeds were soaked in water for 24 h and then stratified for 15 days at 4 °C. Bacterial cultures were grown for two days at room temperature in LB broth, washed twice with 0.03 M $MgSO_4$, and the final concentration adjusted to an OD_{600nm} of 0.5. Seedlings were also treated with a control solution of 0.03 M $MgSO_4$. A total of 40 plants per treatment were grown in sand Turface (69% silica sand, 29% Turface, 2% $MgCO_3$) in 12 cm × 3 cm pots under constant temperature (16 h photoperiod, day/night

Table 1
Primers used in this study.

Organism, gene	Name	Primer sequences (5′ → 3′)	Reference
P. radiata, actin gene	AprF	AGCAACTGGGATGACATGGA	This study
	AprR	TGCCTTGGGATTCAGAGGAG	This study
H. annosum s.s., rRNA gene (primary primers)	HaPF	TCCTTGACCCTTAGGCATTG	This study
	HaPR	TCCATGCGAAGAACTTCAGG	This study
H. annosum s.s., ITS region (nested primers)	MJF	GGTCCTGTCTGGCTTTGC	Hantula and Vainio (2003)
	MJR	CTGAAGCACACCTTGCCA	Hantula and Vainio (2003)
Bacteria, 16S rRNA gene	46f	GCCTAACACATGCAAGTCGA	Mummey and Stahl (2004)
	536r	GTATTACCGCGGCTGCTGG	Mummey and Stahl (2004)
	E334f	CCAGACTCCTACGGGAGGCAGC	Baker et al. (2003)
	E939r	TTGTGCGGGCCCCCGTCAATTC	Baker et al. (2003)
	E786f	GATTAGATACCCTGGTAG	Baker et al. (2003)
	E1491r	GGTTACCTTGTTACGACTT	Smit et al. (1997)

temperature of 23/17 °C) (Chanway et al., 1991). After one month the seedlings were examined for disease symptoms.

2.4. In vivo biocontrol assay

The *A. mellea* inoculum was prepared following the procedure of Beckman and Pusey (2001). Briefly, pieces of fungal mycelium were placed on a preparation of benomyl-dichloran-streptomycin agar (Worrall, 1991) with autoclaved *Quercus* spp. acorns and incubated for approximately one month at room temperature in the dark. *H. annosum* was grown in MEA for one month. Bacterial suspensions (OD_{600nm} of 1) were prepared in 0.03 M $MgSO_4$ as described above. An *E. coli* suspension and 0.03 M $MgSO_4$ were included as negative controls.

A total of 100 one-year-old *P. radiata* seedlings (Explotaciones Forestales Jiménez Araba s.l. Nursery, Vitoria, Spain) were inoculated with each bacterial strain by immersing root balls in the bacterial suspension for one hour. The inoculation was repeated after one week. After seven days, 65 of the seedlings treated with each bacterial strain were inoculated with *A. mellea* by placing acorns colonized by the fungus in contact with tree roots, one acorn per seedling (Beckman and Pusey, 2001). The remaining seedlings were inoculated with 1 cm^2 of *H. annosum* mycelial fragments. Experiments were laid out in a completely randomized design with two factors (pathogens and bacterial treatments). *P. radiata* seedlings were grown in 11 × 11 × 22 cm pots (1600 cc volume), using a mix of peat moss (2/3 peat, 1/3 perlite and fertilizer NPK; N = 200–450 mg/l, P_2O_5 = 200–500 mg/l, K_2O = 300–550 mg/l). They were maintained for 110 days in a biosafety level 2 greenhouse at a mean temperature of 18 ± 5 °C, with a relative humidity of 55–60% and without supplemental light. Upon completion of the experiment, roots were carefully cleaned by rinsing with water, and stem length, collar diameter and, in the case of the plants inoculated with *A. mellea*, dry root weight were measured. To obtain the dry weight, roots were dried in an oven (Selecta) at 60 °C for 72 h and then weighed on an analytical balance (OHaus). Stem length and collar diameter measurements were analyzed together as slenderness index (SI) using the formula: SI = stem diameter (mm)/((stem length (cm)/10) + 2) (Schmidt-Vogt, 1980). The size of the lesions caused by *A. mellea* was determined by measuring the length of mycelial colonization under the seedlings' bark.

2.5. Heterobasidion detection by nested PCR

Nested PCR was used to detect the presence of *H. annosum* in *P. radiata* seedlings. Ten plants per treatment were randomly chosen and the roots were cleaned by rinsing with water. Three centimeters of pine roots were cut (one cm above and two cm below the first secondary root) and the DNA extracted using DNeasy Plant Maxi Kit according to the manufacturers protocol (QIAGEN). Because PCR inhibitors were present in root extracts, 360 µl of each sample were repurified by ethanol precipitation and the DNA resuspended in 36 µl of 0.1X TE buffer.

In the first PCR, primers HaPF and HaPR that flank the internal transcribed spacer (ITS) were used to amplify a fragment of the rRNA gene (Table 1). The reaction mixture was composed of 2.5 µl of 10X Buffer (NEB), 50 nM of each primer, 200 µM dNTPs (NEB), 1.25 U of Taq DNA polymerase (NEB) and 1.3 µl of sample DNA (1:10, 1:25 and 1:50 dilutions) in a final volume of 25 µl. The cycling conditions were as follows: 10 min at 95 °C, 15 cycles of 30 s at 95 °C, 45 s of annealing at 59.5 °C, and 2 min at 72 °C, and a final 1 min at 72 °C. PCR controls included a water blank, *H. annosum* DNA only, and *P. radiata* DNA only.

The second PCR was performed using 300 nM of each specific primer for *H. annosum* s.s. ITS region MJF and MJR (Table 1), 2 µl of 10X Buffer (NEB), 200 nM dNTPs (NEB), 1 U of Taq DNA polymerase (NEB) and 5 µl of the products of the first PCR, in a final volume of 20 µl. Cycling conditions were: 3 min at 95 °C, 40 cycles of 30 s at 95 °C, 30 s of annealing at 58 °C and 15 s at 72 °C, and a final 1 min at 72 °C. PCR controls included all the controls from the first PCR reaction, a water blank, *H. annosum* DNA only, and *P. radiata* DNA only. All the PCR products were visualized in 1.5% (w/v) agarose gels.

The *P. radiata* actin gene was used as a reference gene to ensure that PCR inhibitors did not contribute to false negative results from *H. annosum* ITS amplification. PCR reactions contained 2.5 µl of 10X Buffer (NEB), 300 nM of each primer AprF and AprR (Table 1), 200 nM dNTPs (NEB), 1.25 U of Taq DNA polymerase (NEB) and 1.3 µl of the DNA sample (1:10, 1:25 and 1:50 dilutions). PCR conditions were as follows: 3 min at 95 °C, 40 cycles of 30 s at 95 °C, 30 s of annealing at 58 °C and 15 s at 72 °C, and a final 1 min at 72 °C.

All the amplification reactions for all the samples were replicated once.

2.6. Statistical analysis

Differences in the ability of the bacterial strains to inhibit fungal growth in vitro were analyzed by one way ANOVA with Waller-Duncan post-hoc test using SPSS software (SPSS Inc.). Values of $p < 0.05$ were considered significant. The differences in *Armillaria* disease severity among bacterial treatments in vivo were determined by Pearson's chi-square test. Plants were scored as healthy (without symptoms of infection), dead or with lesions. The strength of association between categorical variables was measured with Cramer's V; adjusted standardized residuals were checked in order to determine the significant differences between categories. The rest of the measurements such as lesion size, stem diameter, height, SI, and dry root weight were analyzed by one way ANOVA, using Hochberg's GT2 and Games-Howell post hoc comparisons for the data from the plants inoculated with *A. mellea* and *H. annosum*, respectively. Data for dead plants were removed from the ANOVA analysis for diameter, height, SI and dry root weight and values of $p < 0.05$ were considered significant.

3. Results

3.1. Bacterial inhibition of fungal growth in vitro

From 200 bacterial strains isolated from the rhizosphere of a healthy *P. radiata* tree, seven were selected as potential biocontrol agents based on their ability to inhibit *H. annosum* and *A. mellea* growth in vitro. Four strains were isolated from the endorhizosphere and three from the ectorhizosphere. The bacterial treatments had a significant inhibitory effect on *H. annosum* ($F(8, 18) = 73.9$, $p < 0.05$) and *A. mellea* ($F(8, 18) = 111.4$, $p < 0.05$). All seven isolates reduced the area covered by *A. mellea* mycelia by 58.6–94.2% and rhizomorph formation (Figs. 1 and 2). In contrast, *E. coli* increased mycelial growth of *A. mellea* by an average of 21% (Fig. 2). All isolates except S22L11 reduced *H. annosum* mycelial growth (Figs. 1 and 2). The most effective bacterial treatments against both fungi were S32R2 and S31R1 with inhibition values of 99% for *H. annosum*, and 94.2% and 83.4% respectively for *A. mellea* (Fig. 2).

3.2. Identification and pathogenicity determination of bacterial strains

Based on their 16S rRNA gene sequence identity to known bacteria, the antagonistic bacterial strains were identified as follows: strain S32R2 as *Pseudomonas fluorescens*; S22L11 as *Bacillus weihenstephanensis* or *B. mycoides*; S11R41 as *Bacillus*

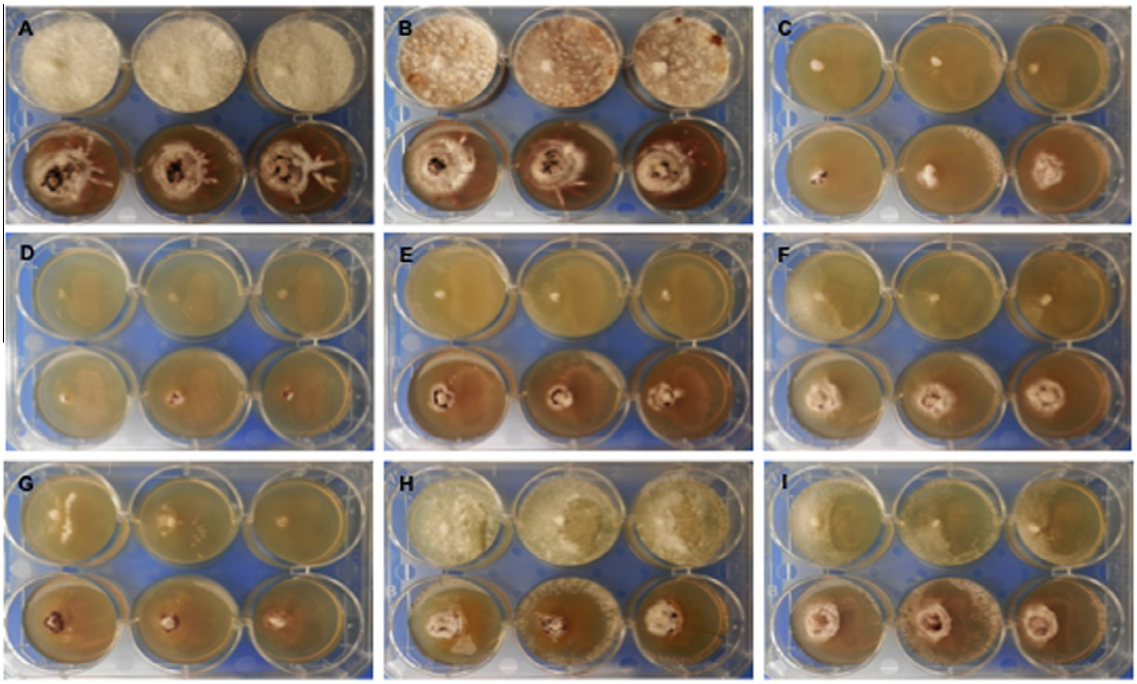

Fig. 1. In vitro fungal antagonism assay. Plates were inoculated with *E. coli* (A), no bacteria (B), S31R1 (C), S32R2 (D), S11R41 (E), S23L3 (F), S11R21 (G), S22L11 (H), or S31L1 (I). The three upper wells of each plate were inoculated with *H. annosum* s.s., and the bottom wells with *A. mellea*.

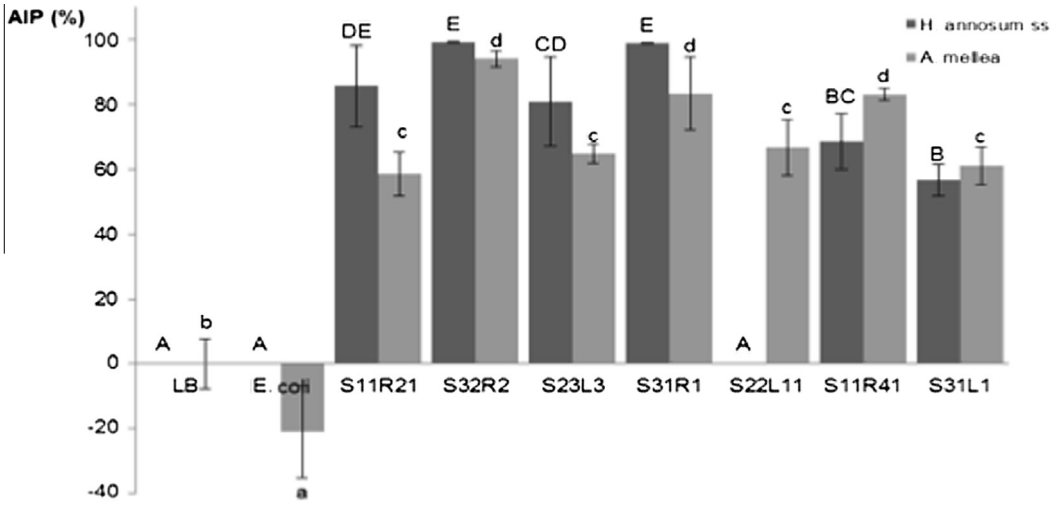

Fig. 2. Area inhibition percent (AIP) of the tested bacterial strains against *H. annosum* s.s. (dark grey) and *A. mellea* (light grey). Error bars show the standard deviation of the 3 independent replicates. Statistically significant differences of $p < 0.05$ between treatments for *A. mellea* and *H. annosum* are presented with different lowercase or capital letters, respectively.

Table 2
Bacterial strains and their closest homologues.

Bacterial strain	Closest homologue	S-ab score[a], Identity[b] or Similarity[c]	Accession number
S32R2	*P. fluorescens* str. B67	99.79%[a]	EU169164.1
S22L11	*B. weihenstephanensis* str. WSBC 10204	100%[a]	AM747230.1
	B. mycoides CIP 103472	1.0[b]	AM747229
S11R41	*B. simplex* str. LMG 21002	1.0[b]	AJ628745.1
	Brevibacterium frigoritolerans; type strain DSM 8801	1.0[b]	AM747813
S31L1	*Rahnella* sp. str. CDC 21234	99.79%[a]	U88435.1
	R. aquatilis str. 334	99.04%[a]	X79940.1
S11R21	*P. fluorescens* 2312	0.993[b]	EU360313
	P. costantinii	99%[c]	AF374472
	P. poae zol-15	0.993[b]	JQ782898
S23L3	*E. billingiae* str. Eb661	99.93%[a]	FP236843
S31R1	*E. billingiae* str. Eb661	100%[a]	FP236843

[a] Ribosomal Database Project.
[b] GenBank.
[c] Greengenes.

simplex or *Brevibacterium frigoritolerans*; S31L1 as *Rahnella* sp., possibly *Rahnella aquatilis*; S11R21 as possibly *Pseudomonas poae*, *P. costantinii* or *P. trivialis*; and S23L3 and S31R1 as *Erwinia billingiae* (Table 2). Three were not tested further, S22L11 and S31L1 due to the possibility of being human or animal pathogens (Stenfors et al., 2002; Goodwin et al., 1994; Chang et al., 1999), and S11R21 because *P. costantinii* is considered a pathogen for cultivated mushrooms (Munsch et al., 2002). Two-month-old pine seedlings inoculated with the remaining four bacterial strains did not exhibit any visible symptoms of disease, such as necrosis of any part of the plants, spots on the needles, needle distortion, cankers, or general decline.

3.3. In vivo biocontrol assay

A significant difference was observed among the bacterial treatments in the health status of the plants inoculated with *A. mellea* (χ^2 (10) = 44.2, $p < 0.001$), and a Cramer's V of 0.323 indicated a medium-large effect size. Of the plants that were not treated with bacteria, 54% were not healthy (i.e., they were dead or had a lesion), and contributed 40.4% of the total number of dead plants in all treatments (Fig. 3). In contrast, treatment with the biocontrol bacteria resulted in fewer dead plants. The proportion of dead plants within a bacterial treatment ranged from 3.6 to 13.1%. *E. billingiae* S23L3 and *B. simplex* S11R41 contributed the least (3.5% and 8.8%, respectively) to the total number of dead plants in all treatments. When the adjusted standardized residuals were examined, the group of plants that were not treated with bacteria had significant positive values in the dead category ($z = 5.2$; $p < 0.001$), meaning that more plants than expected by chance were dead and, significant negative values in the healthy category ($z = -5.2$; $p < 0.001$), meaning that fewer plants than expected were healthy. The plants treated with *B. simplex* S11R41 had significant positive values in the healthy category ($z = 2.6$; $p < 0.01$) and significant negative values in the dead category ($z = -2.0$; $p < 0.05$). Plants treated with *E. billingiae* S23L3 had significant negative values in the dead category ($z = -2.6$; $p < 0.01$) but significant positive values in the lesion category ($z = 2.1$; $p < 0.05$) (Fig. 3). When present, lesion sizes were not significantly different among the treatments (data not shown).

Among the live plants infected with *A. mellea* significant differences in height ($F(5, 308) = 5.64$, $p < 0.05$) (Fig. 4A), diameter ($F(5, 312) = 2.58$, $p < 0.05$) (Fig. 4B) and SI ($F(5, 308) = 3.19$, $p < 0.05$) (Fig. 4C) were found among the treatments. No significant differences were found among treatments for dry root weight ($F(5, 286) = 0.53$) (data not shown). Plants with no bacterial treatment had the lowest average height (47.9 cm ± 10.4) and

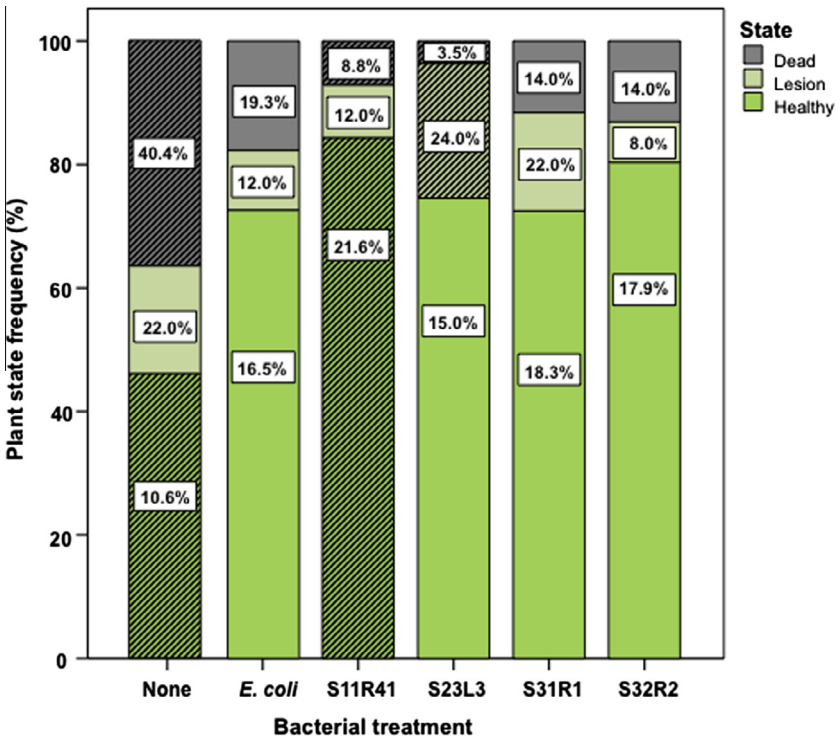

Fig. 3. Dead, healthy or fungal lesion-containing pine seedlings inoculated with *A. mellea*. Counts are represented as percentage of the total number of plants within each bacterial treatment. The percentages shown in the boxes in each segment represent the contribution to the total number of plants in each state among all of the bacterial treatments. Darker segments indicate significant *z* scores ($p < 0.05$).

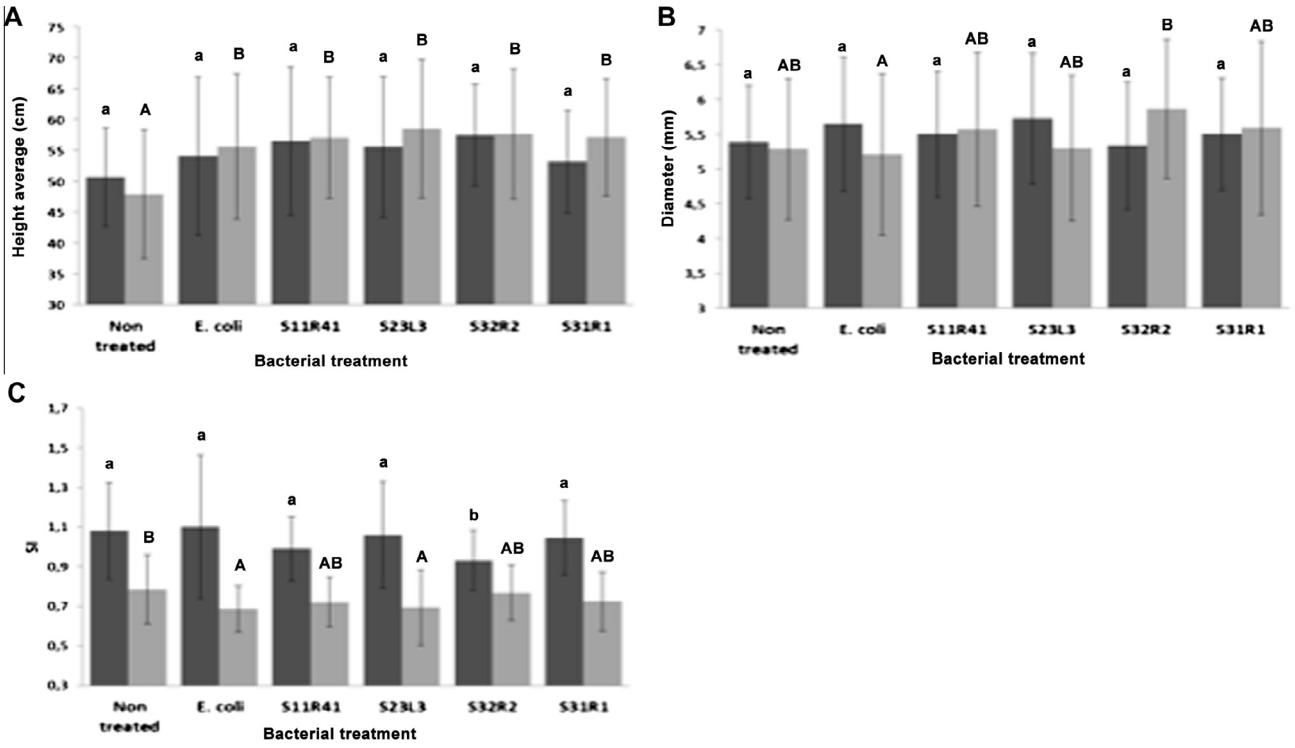

Fig. 4. Height (A), diameter (B) and slenderness index (C) for seedlings inoculated with *H. annosum* (dark grey) or *A. mellea* (light grey) and treated with bacteria. Error bars show the standard deviation of the means. Statistically significant differences of $p < 0.05$ between treatments for *H. annosum* and *A. mellea* are presented with different lowercase or capital letters, respectively.

treatment with any of the bacterial strains increased height by 19% on average (Fig. 4A). Plants treated with *E. coli* had the smallest diameter average (5.2 mm ± 1.2), and plants treated with *P. fluorescens* S32R2 had the highest (5.9 mm ± 1) (Fig. 4B). The lowest average SI, thus the least robust plants, was for plants treated with *E. coli* (0.69 ± 0.12) and *E. billingiae* S23L3 (0.69 ± 0.19) (Fig. 4C), and the highest for untreated plants (0.78 ± 0.17).

The plants infected with *H. annosum* presented significant differences in SI values ($F(5, 200) = 2.28$, $p < 0.05$) among the treatments (Fig. 4C). Plants treated with *P. fluorescens* S32R2 had the lowest SI average (0.93 ± 0.15). Bacterial treatments did not result in significant differences in height ($F(5, 200) = 1.99$) (Fig. 4A) or in diameter ($F(5, 200) = 0.99$) (Fig. 4B).

3.4. Detection of *H. annosum* infection

Symptoms of *H. annosum* infection were not apparent in any of the inoculated seedlings. To determine if the fungus was present in the seedlings, and if any of the bacterial treatments influenced this, DNA was extracted from root fragments and used as a template to amplify the *H. annosum* ITS region and the *P. radiata* actin gene. Products from PCR amplification of the *P radiata* actin gene were obtained in 57 of the 60 samples tested using different concentrations of DNA (Fig. 5). This indicates that PCR was not inhibited by substances in the DNA extracted from plant roots. The samples and DNA concentrations with a positive result for the actin gene were tested for the presence of *H. annosum*. In this case, 43 samples

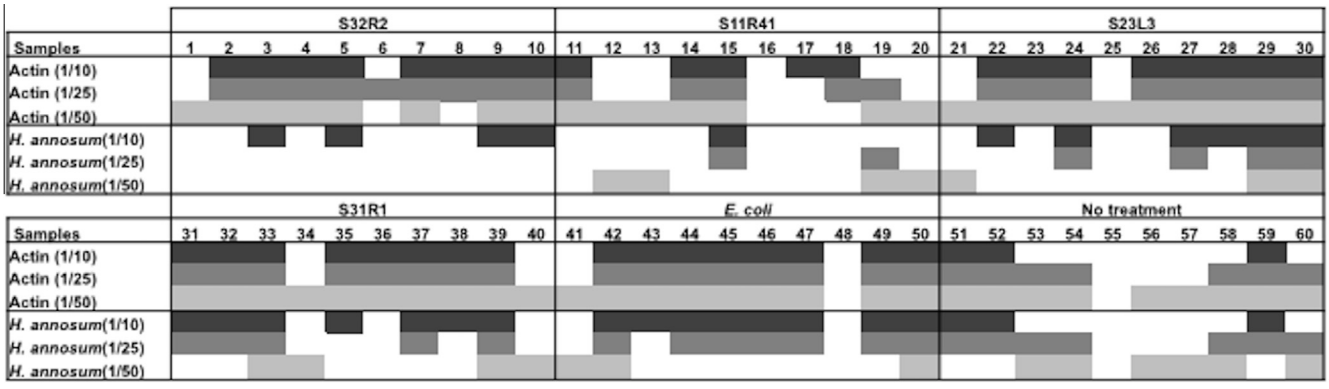

Fig. 5. Detection of *H. annosum* in DNA extracted from infected *P. radiata* roots treated with biocontrol bacteria. A positive result for the *P. radiata* actin gene or the *H. annosum* ITS region is indicated as a shaded box (DNA dilution shown).

yielded a specific amplification product (Fig. 5). The percentage of positives in the groups treated with *P. fluorescens* S32R2, *B. simplex* S11R41, *E. billingiae* S23L3, and *E. billingiae* S31R1 were 40%, 55.5%, 70% and 90%, respectively. All of the actin-positive samples with *E. coli* or without bacterial treatment were positive for a *H. annosum*-specific ITS sequence. The same result was obtained in both experimental replications.

4. Discussion

In the present study, we report the isolation of native bacteria from a healthy tree in a *P. radiata* stand with high levels of fungal infection and the ability of some of these bacterial strains to inhibit the growth in vitro and the pathogenic effects *in planta* of two forest fungal pathogens, *H. annosum* s.s. and *A. mellea*. A few studies have described the antagonistic effects of biocontrol bacteria against forest fungal pathogens in vitro, although their ability to reduce fungal growth in trees was not always demonstrated. Dumas (1992) isolated bacteria from soils of the boreal mixed wood forest of Ontario that inhibited *A. mellea* in vitro, however, inhibition of infection in trees was not reported. Singh et al. (2008) reported the antagonistic effect of *Bacillus subtilis* against *Macrophomina phaseolina* in *Pinus roxburghii* (Sarg.), and *Phytophthora cinnamomi* infection was reduced in *Fagus sylvatica* and *Quercus* sp. treated with *Bacillus amyloliquefaciens* (Lefort et al., 2013). Murray and Woodward (2003) concluded that weight losses caused by *Heterobasidion* in spruce wood cubes were lower when they were simultaneously inoculated with *H. annosum* and biocontrol bacteria.

In our case, native biocontrol bacteria were isolated in anticipation of their greater potential for adaptation to the host and conditions under which they will be applied. The selected bacteria were not pathogenic for seedlings of *P. radiata* nor are *P. fluorescens*, *E. billingiae*, and *B. simplex* (*B. frigoritolerans*) known to be human or animal pathogens. Some strains of *P. fluorescens* are commercially available and exhibit effective rhizosphere colonization, plant growth promoting activity (Mark et al., 2006), and antifungal activities against crop pathogens due to the production of secondary metabolites such as 2,4-diacetylphloroglucinol, phenazine and pyoverdin (Boruah and Kumar, 2002). *E. billingiae* is epiphytic and has shown biocontrol activity against fire blight caused by *E. amylovora* (Jakovljevic et al., 2008). *B. simplex*, an endospore forming bacteria (Heyrman et al., 2005), promoted the growth of tomato and wheat (Hassen and Labuschagne, 2010) and showed antifungal effects against *Fusarium oxysporum* in vitro (Schwartz et al., 2013).

P. fluorescens S32R2 and *B. simplex* S11R41 were strong antagonists of *H. annosum* and *A. mellea* in vitro, and performed best against both fungal strains in vivo. *P. fluorescens* S32R2 reduced *H. annosum* growth by 99% in vitro and fungal presence by 60% in *P. radiata* seedlings. *B. simplex* reduced *H. annosum* growth to a lesser extent (68%) in vitro, but reduced the presence of the fungus in pine seedlings by 45%. On the other hand, *E. billingiae* strains S31R1 and S23L3 performed strongly against *H. annosum* in vitro (99% and 81% AIP, respectively), but reduced the presence of the fungus by only 10% and 30%, respectively, in vivo.

In the in vitro antagonism assay against *A. mellea*, *P. fluorescens* S32R2 had the highest antifungal activity (94.2% AIP). As a plant treatment, this bacterial strain showed a similar antagonistic effect as *B. simplex* S11R41, (80.3% and 84.3% of the plants, respectively, were healthy following fungal infection compared with 46% of untreated plants), although *B. simplex* S11R41 was less inhibitory in vitro (83.2% AIP). When compared with plants that were not treated with bacteria, antagonistic effects were detected for *E. coli* in the pines inoculated with *A. mellea*; 73% of the plants were healthy following fungal infection. This was surprising because *E. coli* did not inhibit *A. mellea* in vitro (−21% AIP). The consistent (Singh et al., 2008) or inconsistent (Coombs et al., 2004; Inderiati and Franco, 2008) relationship between the antagonistic effect of biocontrol bacteria in vivo and in vitro has been previously reported and, in general, is related to plant characteristics and environment (Tolba and Soliman, 2013).

The number of plants with *A. mellea* lesions was smaller in the seedlings treated with *B. simplex* S11R41 (8.6%) and *P. fluorescens* S32R2 (6.6%) compared with 17.5% of plants with lesions for the untreated controls, although the size of fungal lesions, when present, was not different among the treatments. Fewer plants treated with bacteria died. Bacterial treatment may have a systemic protective effect, but not a strong antagonistic effect once the fungal infection is established.

When plant growth in the presence of *A. mellea* was analyzed, a significant difference was detected in the height, diameter and SI among the treatments. Plants treated with *P. fluorescens* S32R2 had the highest values in height and diameter. On the other hand, untreated plants and plants treated with *E. coli* had the lowest values in height and diameter, respectively, and SI values were higher for the untreated plants (0.78) and smaller for the plants treated with *E. coli* (0.69). A significant effect on height or diameter was not detected among bacterial treatments for plants treated with *H. annosum*, but *P. fluorescens* treatment had the highest value for plant height and the smallest for diameter, resulting in a significant decrease of SI. The mean values of SI were consistently different between the seedlings inoculated with *H. annosum* (1.04 ± 0.25) and those inoculated with *A. mellea* (0.73 ± 0.15). SI values of 1 are considered normal for *P. ponderosa* seedlings (Olivo and Buduba, 2006), which is similar to the SI values of *P. radiata* seedlings treated with *H. annosum* in this study. This suggests that the severity of infection was greater for *A. mellea*, and although *H. annosum* was present, the infection was not sufficient to cause disease symptoms in any of the treatments at the time of the assessment. The time to manifestation of disease following *H. annosum* infection is known to be variable (months to years) and is dependent on the levels of inoculum and inoculation method, and accelerated by plant stress (Zaluma et al., 2016; Doğmuş-Lehtijärvi et al., 2016; Prieto-Recio et al., 2014).

In conclusion, the isolated bacterial strains selected on the basis of an antibiosis effect on the fungal pathogens *H. annosum* s.s. and *A. mellea* in vitro reduced the pathogenic effects of *A. mellea* and the presence of *H. annosum* in *P. radiata* seedlings. We propose, as part of an integrated management strategy, an early application of antagonistic bacteria in the nursery. Early application is advantageous for several reasons, the volume of bacteria needed is lower and can be applied under controlled conditions, the bacteria have time to colonize and adapt to the rhizosphere conditions, and consequently, the seedlings are protected before they are in contact with the fungi. Future studies are needed to understand the mechanisms of antibiosis and bacterial-fungal interactions in other tree species. In addition, the effects of the bacteria on other beneficial rhizobacteria and mycorrhizal fungi in the rhizosphere community, and the ability of the biocontrol bacteria to compete effectively with indigenous microorganisms must be assessed, although they are native to *P. radiata* roots and therefore expected to be well-adapted to the conditions under which they will be applied. Nonetheless, the biocontrol bacteria characterized here show promise as a treatment to mitigate the damage by devastating pine pathogens against which there are few options available.

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