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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.

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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

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