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Biocontrol of *Fusarium circinatum* Infection of Young *Pinus radiata* Trees

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Academic Editors: Robert A. Blanchette and Timothy A. Martin
Received: 28 October 2016; Accepted: 18 January 2017; Published: 26 January 2017

Abstract: Pitch canker, caused by the fungus *Fusarium circinatum*, is a major disease of *Pinus radiata* currently controlled to some extent in nurseries by good hygiene and application of synthetic fungicides. The aim of this study was to evaluate alternative strategies to control fungal infections in nurseries and young pine plantations. The antagonistic effects of biocontrol bacteria and essential oils against *F. circinatum* in vitro and in young *P. radiata* trees were assessed. *Pseudomonas fluorescens*, *Erwinia billingiae*, and *Bacillus simplex* reduced the growth of the fungus in vitro by 17%–29%, and decreased the density of the mycelial mat. In young *P. radiata* trees, the length of *F. circinatum* lesions was reduced by 22%–25% by the same bacterial strains. Direct application of cinnamon and/or clove essential oils to wounds in stems of two-year-old *P. radiata* trees also limited the damage caused by *F. circinatum*. Lesion length was reduced by 51% following treatment with cinnamon oil (10% *v*/*v*), and by 45% following treatment with clove oil (15% *v*/*v*) or a combination of both oils. However, the oils were toxic to younger trees. The biocontrol bacteria and essential oils show promise as prophylactic treatments to reduce the devastating effects of *F. circinatum* on *P. radiata*.

Keywords: *Fusarium circinatum*; *Pinus radiata*; biocontrol bacteria; essential oils

1. Introduction

The fungus *Fusarium circinatum* Nirenberg & O'Donnell is the causative agent of pitch canker, a major disease of *Pinus radiata* D. Don or Monterey pine [1]. The disease is characterized by resinous lesions at the site of infection on the trunk and branches, and branch dieback (necrosis from the branch tip to the site of infection) as a result of vascular tissue obstruction [2]. Infection reduces growth of the tree and may result in death, often from increased susceptibility to biotic and abiotic stresses [2]. Infected seedlings have a high mortality rate, especially in nurseries, where root infections can be extensive [3,4]. The pathogen spreads through contaminated seeds, and through dissemination of spores via wind, rain, or insect vectors; however, disease results only when wounds in the protective bark are infected.

F. circinatum outbreaks and epidemics are common in nurseries and plantations in many regions of the world and are responsible for high mortality and reduced yields, causing substantial economic losses. The fungus is believed to have originated in Mexico and spread north into the native stands of *P. radiata* in the southeastern US [5]. It is now a major threat in the coastal southwestern US [6],

Chile [7], northern Spain [8], Portugal [9], South Africa [3], and potentially in regions of Australia, New Zealand, China, and Brazil, where *P. radiata* is widely planted and climatic conditions are highly suited for establishment of the pathogen [10]. Although *F. circinatum* is primarily a pathogen of *P. radiata* [11], an important commercial species, other *Pinus* spp. such as *P. muricata* D. Don (bishop pine), *P. taeda* L. (loblolly pine), *P. elliotii* Engelm. *var. elliotii* (slash pine), and *Pseudotsuga menziesii* (Mirb) Franco (Douglas fir) are also susceptible to the pathogen [12,13].

At present, there are few effective options to treat or prevent infections of this fungal pathogen. Good forest management such as planting less susceptible varieties of pine, rapid removal of infected trees, and quarantine measures, including restrictions on importation of wood products, are the only mechanisms that have proven effective in reducing the spread of *F. circinatum*. Measures to prevent seed contamination such as hot water treatment can reduce infections in nurseries [14]. Although effective, widespread application of fungicides such as benomyl may promote resistance and are no longer allowed in some countries (e.g., EU directive 91/414/CEE) [15].

Biological control agents have been used in agriculture over the last century as an alternative to chemical pesticides and have proven effective in protecting crops against many pathogens, including fungi [16–18]. In contrast, less has been done to investigate the potential of biocontrol agents, such as beneficial microorganisms, to protect pine trees from fungal pathogens. Commercial and native strains of the biocontrol fungi *Trichoderma harzianum* Rifai and *T. viride* Pers. were shown to be effective against *F. circinatum* in vitro, however, they did not inhibit growth of the pathogen on *P. radiata* seeds [15] or seedlings [19]. Application of methyl jasmonate to induce systemic resistance also failed to protect pine seedlings from disease caused by *F. circinatum* and other fungi [20]. Essential oils extracted from plants are also known to have fungicidal properties [21–23]. Several essential oils, including cinnamon, oregano, thyme, lavender, tea tree, Japanese mint, clove, rose geranium, and lemongrass were shown to inhibit growth of *F. circinatum* in vitro [15,24], although the high concentrations required to inhibit the fungus may also inhibit seed germination [15]. To address the need for more effective strategies to mitigate the damage caused by *F. circinatum*, we evaluated the efficacy of pine rhizobacterial isolates and essential oils (cinnamon and clove oil) to inhibit fungal growth in vitro and reduce symptoms of fungal infection in young *P. radiata* trees.

2. Materials and Methods

2.1. Microorganisms

The *F. circinatum* strain (CECT20759) used in this study was isolated from a *P. radiata* plantation in Gipuzkoa, Spain, and is associated with vegetative compatibility group (VCG) A and Mating type 2 (Mat-2), the only mating type present in the Basque Country [15]. Isolate CECT20759 does not show significant differences in virulence in relation to Mating type 1 [15]. To assess the specificity of the biocontrol agents in pathogen control, two other fungal pathogens of forest tree species that spread through infection of seeds and seedlings in nurseries were also tested. *Diplodia sapinea* (Fr.) Fuckel 1870 strain DPV24 was isolated from an adult *P. radiata* tree in Bizkaia, Spain [25], and *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips 2006, CMW37773, was isolated from an adult *Eucalyptus globulus* Labill tree in Bizkaia, Spain [26]. The fungi were cultured on potato dextrose agar (PDA; Cultimed) at 20 °C in the dark.

Bacterial strains *Pseudomonas fluorescens* S32R2, *Bacillus simplex* S11R41, and *Erwinia billingiae* S31R1 were isolated from the endorhizosphere and *E. billingiae* S23L3 from the ectorhizosphere of a healthy pine tree (*P. radiata*) in a plantation in Abadiano, Bizkaia, Spain, with a high density of diseased trees, and were shown to inhibit the growth of fungal pathogens *Heterobasidion annosum* and *Armillaria mellea* in vitro and pathogenic effects in *P. radiata* [27]. For in vitro and in planta fungal antagonism assays, single bacterial colonies were inoculated into Luria Bertani broth and grown for two days at room temperature (20–25 °C). Cells were washed twice with 0.03 M $MgSO_4$ and the final suspension adjusted to an optical density measured at 600 nm of 0.5. Laboratory strain *Escherichia coli* TOP10

(Invitrogen Life Technologies, Carlsbad, CA, USA) and grass rhizosphere isolate *P. fluorescens* J11 (C. Patten culture collection), used as controls, were prepared in the same manner.

2.2. In Vitro Bacterial-Fungal Antagonism Assay

Bacteria and fungi were prepared as described above and inoculated into wells of a six-well plate (Nunc, Sigma-Aldrich Co. LLC., St. Louis, MO, USA) containing International Streptomyces Project 2 (ISP2) agar (4 g/L yeast extract, 10 g/L malt extract, 4 g/L glucose, and 20 g/L agar, pH 7.3; [28]). In preliminary experiments, this medium was determined to be suitable for growth of the bacterial and fungal strains. Bacterial cultures, prepared in triplicate from independent colonies, were applied in a thin line using a sterile inoculation loop. A sterile toothpick was used to transfer a small amount (1 mm^3) of the fungal mycelium to the agar plates. The plates were incubated for one week at 23 °C and 53% humidity. Inhibition of fungal growth by the bacteria was determined by calculating the Area Inhibition Percentage (AIP): AIP (%) = $(A - B)/A \times 100$, where A and B are the surface area covered by the fungus in control (no bacteria) and treated (with bacteria) plates, respectively. Differences in the AIP among the treatments were analyzed by one-way analysis of variance (ANOVA) with Waller-Duncan post-hoc test using SPSS software (SPSS Inc., Chicago, IL, USA). Values of $p < 0.05$ were considered significant.

2.3. In Vivo Bacterial Biocontrol Assay in P. radiata

Two-year-old *P. radiata* trees (Deba Seed Orchard, HS-Q-20/002, Basque Country, Spain) were grown at the Explotaciones Forestales Jiménez Araba nursery (Vitoria, Spain) before transfer to a biosafety level 2 greenhouse for cultivation at 18 ± 5 °C, with a relative humidity of 55%–60% and without supplemental light throughout the experimental period (May–August). The trees were contained in 300 mL pots with a mix of peat moss (2/3 volume), perlite (1/3 volume), and NPK fertilizer (N = 200–450 mg/L, P_2O_5 = 200–500 mg/L, K_2O = 300–550 mg/L). After four weeks of acclimatization to greenhouse conditions, approximately 2 cm were removed from the top of the trees to expose the pith, and 5 µL of bacterial culture, prepared as described above, or 0.03 M $MgSO_4$ as a negative control were added to the wound. After one week, trees were reinoculated with the bacterial suspensions. The trees were not watered for three days following each inoculation to allow the bacteria to infect the tree tissue. At least 30 trees were inoculated with each bacterial strain and the pots containing the treated trees were arranged in trays in a randomized block design.

After the second inoculation, the trees were left for one week to allow the bacteria to proliferate and the trees to recover from removal of their tops, and then were infected with an aqueous fungal spore suspension prepared as follows. Sterile water (1 mL) was added to plates containing seven-day-old cultures of *F. circinatum*. A sterile microscope slide was used to suspend the mycelium and spores. The suspension was then filtered through two layers of sterile gauze to remove the mycelia and collect the spores. The density of spores was quantified using a Neubauer chamber and sterile water was added to obtain a final concentration of 25 spores per µL. Small wounds, deep enough to reach the sapwood, were made on the main stem using a drill bit (1.6 mm diameter), and 2 µL of the aqueous spore suspension were deposited into each wound. Control trees were treated in the same manner but were inoculated with 2 µL of sterile water. Six weeks after inoculation, bark and phloem were removed with pruning scissors and the length of the lesions around inoculation points was measured using an electronic caliper [29]. The experiment was repeated once in the same manner.

Data was analyzed by a two-way ANOVA using the statistical package JMP (v7, SAS Institute Inc., Cary, NC, USA) with block (tray) and bacterial treatment as predictor variables and lesion size as the response variable. Significant differences ($p < 0.05$) among the treatments were determined using a post-hoc Tukey honest significant difference (HSD) test.

2.4. In Vitro Essential Oils Inhibition Assay

Cinnamon, fennel, and clove oils (Sanoflore Laboratory, Gigors et Lozeron, France) were tested at several concentrations (5%, 10%, 15%, 50%, and 100% v/v) for the ability to inhibit fungal growth in vitro. Sweet almond oil (Guinama Laboratory, Valencia, Spain) was used as a diluent as it did not affect the growth of the fungus or the development of spores in preliminary tests. Each well of a 12-well plate (Nunc, Sigma-Aldrich Co. LLC., St. Louis, MO, USA) containing PDA was inoculated with *F. circinatum* mycelium as described above, and approximately 100 µL of the prepared oils were applied to cover the surface of the well. To determine if the vapors from the oils impacted fungal growth, six outer wells in each plate were treated with the oils, while six inner wells were left untreated. Six replicates of each oil concentration were applied.

After one week, the coverage of the mycelial mat was measured using electronic calipers and AIP was calculated as described above. The oil concentrations that completely inhibited the growth of the fungus were assessed for fungicidal or fungistatic activity by transferring a small amount of mycelia from the oil-treated plates, in triplicate, to fresh medium without oil. A treatment was deemed fungicidal if no new mycelial growth was detected and fungistatic if growth was initiated on the oil-free medium.

The impact of essential oil application on the biocontrol bacterial strains was analyzed by inoculating a bacterial suspension (prepared as described above) and, after one day, essential oils that showed fungicidal activity into wells of a six-well plate (Nunc, Sigma-Aldrich Co. LLC., St. Louis, MO, USA) containing ISP2 agar. The plates were incubated for one week at 32 °C. Inhibition of bacterial growth was assessed by comparing growth of bacteria treated with the oils to that for the control treatment without oils.

2.5. In Vivo Essential oils Inhibition Assay in P. radiata

To assess the phytoxicity of the essential oils, the lowest concentration of cinnamon and/or clove oil (5 µL) determined to have a fungicidal effect in vitro was applied to the excised tops of one-year-old *P. radiata* trees in the same manner as the bacterial treatments, and to small wounds made in the stem of two-year-old *P. radiata* trees using a drill bit as described above (12 trees/treatment). The oils were prepared in almond oil as the diluent and an almond oil treatment was included as a control. Lesions were measured four weeks after application of oil treatments. Data was analyzed by a two-way ANOVA with tissue age and oil treatment as predictor variables and lesion size as the response variable. Significant differences ($p < 0.05$) among the treatments were determined using a post-hoc Tukey HSD test.

To assess the antifungal effect of the essential oils in vivo, 5 µL of a *F. circinatum* spore suspension, prepared as described above, were applied to the stem wounds one day after treatment with each oil or combination of oils (30 trees/treatment). Almond oil, the diluent for the essential oils, was applied as a control treatment. The trees were maintained in the greenhouse as described above. After six weeks the size of the lesions was measured using a digital caliper. Data was analyzed by a one-way ANOVA with oil treatment as the predictor variable and lesion size as the response variable. Significant differences ($p < 0.05$) among the treatments were determined using a post-hoc Tukey HSD test.

3. Results

3.1. Antagonism of F. circinatum by Bacteria In Vitro

The rhizobacterial strains significantly inhibited the growth of *F. circinatum* on ISP2 agar plates ($F_{5,12} = 27.46$, $p < 0.001$). The AIP was greater for *P. fluorescens* S32R2, *E. billingiae* S31R1, and *E. billingiae* S23L3 treatments than for *B. simplex* S11R41 (Figure 1). Fungal growth was arrested as it approached the boundary of bacteria and microscopic examination of the interface between the mycelia and the bacteria revealed a decrease in the density of the mycelial mat. *E. coli* also inhibited fungal growth with an AIP value similar to that of four biocontrol strains. Treatment with the grass root isolate

P. fluorescens J11 resulted in a low AIP value and the fungal mycelium grew over most of the surface of the agar, therefore this strain was not tested further. Neither *D. pinea* nor *N. parvum* growth were inhibited by any of the bacterial strains (AIP = 0).

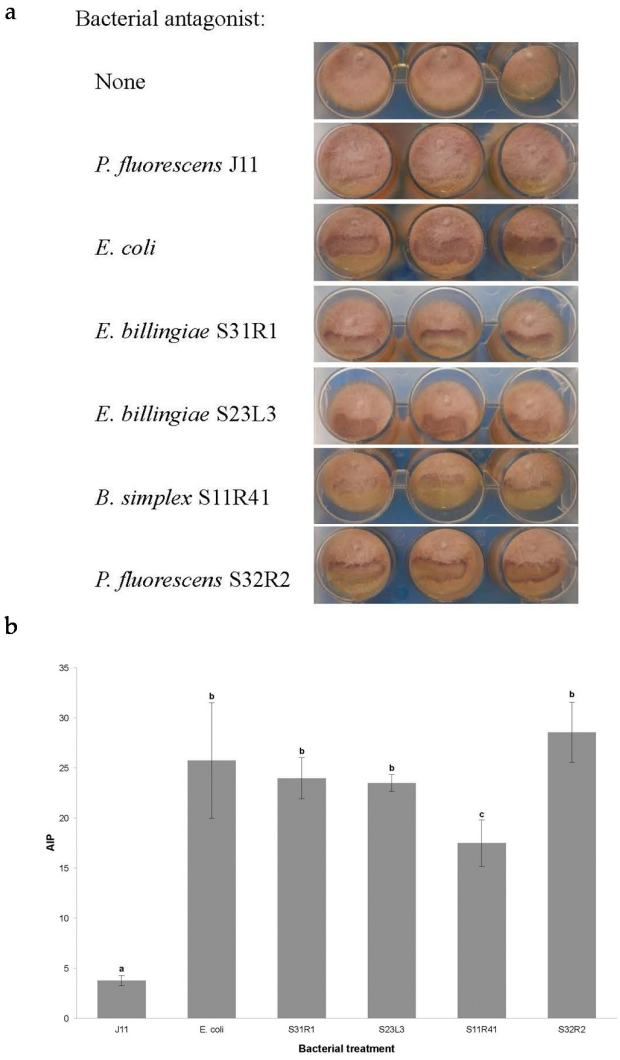

Figure 1. Antagonism of *F. circinatum* by bacteria in dual culture plate assays on International Streptomyces Project 2 (ISP2) agar (**a**); Area inhibition percent (AIP) of the tested bacterial strains against *F. circinatum* (**b**). Error bars show the standard deviation of the three independent replicates. Statistically significant differences of $p < 0.05$ between treatments are indicated with different lowercase letters. S31R1: *E. billingiae* S31R1; S23L3: *E. billingiae* S23L3; S11R41: *B. simplex* S11R41; S32R2: *P. fluorescens* S32R2.

3.2. *Inhibition of* F. circinatum *Lesions by Bacteria in Young* P. radiata *Trees*

Treatment of *P. radiata* with the pine rhizobacterial isolates significantly reduced the size of the lesions caused by *F. circinatum* infection (Experiment (Exp.) 1, $F_{6,211} = 13.52$, $p < 0.0001$; Exp. 2, $F_{6,189} = 17.13$, $p < 0.0001$). The reduction in the length of the lesions was similar among the bacterial strains, about 78% of the length of untreated *F. circinatum* lesions six weeks after introduction of the fungus, although in the first experiment the effect of *B. simplex* S11R41 was not significantly different from the control treatment without bacteria (Figure 2). Neither treatment with *E. coli* nor $MgSO_4$ reduced the size of the fungal lesions. While tray position (block) influenced lesion size (Exp. 2, $F_{7,189} = 4.20$, $p = 0.0002$) due to one of the eight trays of trees having a smaller mean lesion length

compared to the others, there was no significant interaction between tray position and bacterial treatment (Exp. 2, $F_{42,189} = 0.99$, $p = 0.49$).

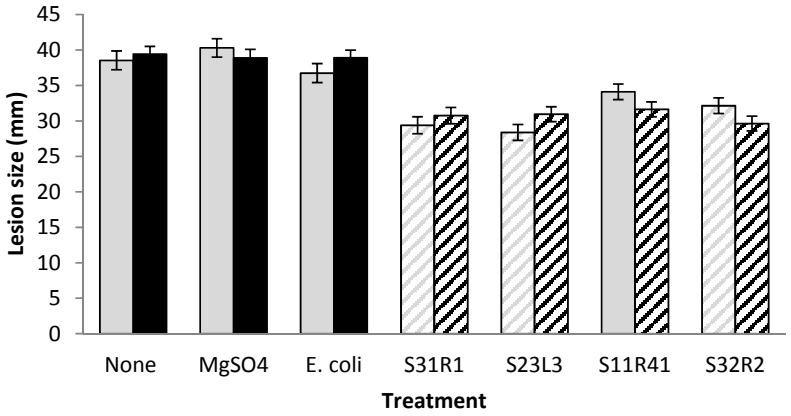

Figure 2. Size of *F. circinatum* lesions on two-year-old *P. radiata* trees treated with pine rhizobacteria. Values shown are the means of at least 30 lesions ± standard error in two separate experiments (Exp. 1, solid and hatched grey bars; Exp. 2, solid and hatched black bars); hatched bars indicate lesions that are significantly different from those on untreated (*F. circinatum* only) trees ($p < 0.05$).

3.3. Effect of Essential Oils In Vitro

Application of undiluted fennel, cinnamon, or clove oil directly to the wells containing *F. circinatum* completely inhibited the growth of the fungus (Table 1), and the vapors emitted by the undiluted oils were sufficient to limit the growth of the untreated fungus in the central wells of the plate. The 50% dilution of each of the oils produced similar inhibitory results. Cinnamon oil diluted to 10% and clove oil to 15% also completely inhibited fungal growth and were fungicidal. However, the inhibitory effect was reduced at lower oil concentrations to 82.7 AIP in the case of 5% cinnamon oil, and 87.7 and 69.0 AIP for 10% and 5% clove oil, respectively. Fennel oil, even when undiluted, had a fungistatic activity and there was no inhibition of growth at concentrations below 50%. Mycelia grew throughout the area covered by 5%–15% fennel oil, and therefore it was excluded from further testing. All of the essential oils at all of the concentrations tested inhibited the growth of the bacterial strains.

Table 1. Antifungal activities of essential cinnamon, clove, and fennel oils diluted with sweet almond oil. Area inhibition percent (AIP) was assessed after one week of exposure to the oil. The oils were determined to be fungicidal if no growth occurred after an inoculum was transferred to fresh media without oil or fungistatic if growth resumed after transfer. ND, not determined.

Oil	Concentration (% *v*/*v*)	AIP	Effect on *F. circinatum*
Cinnamon	5	82.7 ± 1.0	ND
	10	100	Fungicidal
	15	100	Fungicidal
	50	100	Fungicidal
	100	100	Fungicidal
Clove	5	69.0 ± 2.1	ND
	10	87.7 ± 1.8	ND
	15	100	Fungicidal
	50	100	Fungicidal
	100	100	Fungicidal
Fennel	5	0	ND
	10	0	ND
	15	0	ND
	50	100	Fungistatic
	100	100	Fungistatic

3.4. Inhibition of F. circinatum *Lesions by Essential Oils in Young* P. radiata *Trees*

The essential oils had a significant toxic effect on *P. radiata* trees ($F_{3,88}$ = 31.76, p = 0.0001) although phytotoxicity was dependent on the age of the tree tissue ($F_{3,88}$ = 30.32, p = 0.0001). One-year-old seedlings exhibited signs of phytotoxicity within three days of treatment with the cinnamon and clove oils. Symptoms were similar to those produced by herbicide treatments, including epinasty or curling and twisting of needles and stems followed by necrosis [30]. Chlorotic tissue extended to 14.18 and 18.21 mm from the site of cinnamon and clove oil application, respectively, compared to 1.74 mm for the control almond oil treatment, and was especially pronounced for the combined cinnamon and clove oil treatment (24.16 mm) (Table 2). In contrast, the essential oils were not toxic to older *P. radiata* tissues. The average lesion size was 3.31 mm and none of the oil treatments damaged two-year-old pine trees to a greater extent than the control treatment (Table 2). Due to the toxicity of the oils to one-year-old pine seedlings, their anti-fungal effect was tested only in older trees.

Table 2. Phytotoxicity of essential oils in one- and two-year-old *P. radiata* tissue. Cinnamon and clove oils were diluted to the indicated concentration in sweet almond oil. Values shown are the means of 12 lesions ± standard error. Statistically significant ($p < 0.05$) differences in lesion sizes among the treatments are indicated with a different lowercase letter.

Essential Oil Treatment	Concentration (% *v*/*v*)	Lesion Size (mm)	
		1-Year Old Tissue	2-Year-Old Tissue
Almond (diluent)	100	1.74 ± 0.18 ^{c}	3.27 ± 0.11 ^{c}
Cinnamon	10	18.22 ± 1.38 ^{b}	3.19 ± 0.14 ^{c}
Clove	15	14.18 ± 0.97 ^{b}	3.16 ± 0.12 ^{c}
Cinnamon + clove	10, 15	24.16 ± 2.94 ^{a}	3.62 ± 0.13 ^{c}

Treatment of wounds in stems of two-year-old *P. radiata* trees with clove and/or cinnamon oils prior to *F. circinatum* infection reduced the size of the lesions caused by the fungus ($F_{3,116}$ = 33.74, $p < 0.0001$). Six weeks after fungal infection, the length of the lesions treated with 10% cinnamon oil was reduced by 51% compared to those treated with the diluent sweet almond oil (Table 3). Clove oil (15%) or a combination of the two oils reduced lesion length by 45%.

Table 3. Efficacy of essential oils in reducing the size of *F. circinatum* lesions in *P. radiata*. Essential oils were applied one day prior to infection with *F. circinatum*, and lesions were measured six weeks after the infection. Cinnamon and clove oils were diluted to the indicated concentration with sweet almond oil. Values shown are the means of 30 lesions ± standard error. Statistically significant ($p < 0.05$) differences in lesion sizes among the treatments are indicated with a different lowercase letter.

Essential Oil Treatment	Concentration (% *v*/*v*)	Lesion Size (mm)
Almond (diluent)	100	38.08 ± 1.98 ^{a}
Cinnamon	10	18.60 ± 1.38 ^{b}
Clove	15	20.83 ± 1.29 ^{b}
Cinnamon + clove	10, 15	20.79 ± 1.50 ^{b}

4. Discussion

4.1. Biocontrol Bacteria

The biocontrol bacteria *P. fluorescens* S32R2, *B. simplex* S11R41, *E. billingiae* S31R1, and *E. billingiae* S23L3, isolated from the roots of a healthy pine tree, inhibited *F. circinatum* growth in vitro and reduced the symptoms of pitch canker disease on young *P. radiata* trees. In a previous study, Mesanza et al. [27] reported the antagonist effect of these bacterial strains against the pine pathogens *H. annosum* and *A. mellea*. In that study, *P. fluorescens* S32R2 and *B. simplex* S11R41 had the greatest antagonistic effect

in vitro and in vivo. In the present study, *P. fluorescens* S32R2 and the two strains of *E. billingiae* reduced *F. circinatum* lesion length to the greatest extent in young *P. radiata* trees. In contrast, the pine pathogens *D. sapinea* and *N. parvum* were not inhibited by any of the bacterial strains. This may suggest that the bacteria can outcompete some fungi, but not others, for nutrients and/or that they produce anti-fungal compounds that are effective against some fungi.

Pseudomonads are well known as biocontrol agents that are effective against several plant pathogens including pathogenic fungi such as *Rhizoctonia solani*, *R. oryzae*, *Pythium ultimum*, and *P. irregulare* [31]. *P. fluorescens* reduced the severity of *Fusarium* (mainly *F. oxysporum*)-induced wilt on a variety of crops [32–36]. Pseudomonads produce several antifungal metabolites including cyclic lipopeptides, phloroglucinols, phenazines, pyoluteorin, and pyrrolnitrin [37] or may induce local or systemic resistance in the plant against the pathogen [38]. Fungal inhibition is not characteristic of all pseudomonads, as indicated by the inability of *P. fluorescens* J11 to inhibit growth of *F. circinatum* in vitro.

Erwinia spp. are mainly plant pathogens, however, *E. billingiae* is part of the normal commensal microbiota of pome fruit trees and is well adapted to plant colonization including resistance to environmental stresses [39–41]. It is an antagonist of the closely related species *E. amylovora* that causes fire blight on apples and pears [42]. Currently, the mechanism by which *E. billingiae* inhibits fungal growth is not known.

B. simplex is a plant growth-promoting bacterium that can enhance the biomass of tomato and wheat [43,44], and induce rooting and root growth of kiwifruit stem cuttings [45] In addition, this bacterium showed antifungal effects against *F. oxysporum* in vitro [46] and *Phytophthora erythroseptica*, a pathogen of potato [47].

Although the biocontrol bacteria were isolated from the pine rhizosphere, they were able to inhibit *F. circinatum* growth on aerial tissues, suggesting that the bacterial strains are well adapted to grow in both plant environments. Plant epiphytic strains of *P. fluorescens* are common (e.g., [48]) and *E. billingae* strains have been isolated from the phyllosphere of apple, hawthorn, and pear [39]. The ability to colonize both the rhizo- and phyllosphere of a plant is a desirable characteristic of a bacterial treatment to control *F. circinatum*, which can also infect pine roots [49,50].

The bacteria were introduced through the excised tops of the trees to ensure that they and/or their antifungal products were able to reach the vascular tissue for possible systemic dissemination. This was done to optimize the potential for interactions between the fungus and bacteria/bacterial metabolites. While this is an aggressive treatment, control plants that were wounded in a similar manner (but not inoculated with fungus or bacteria), did not exhibit any signs of additional damage and recovered well. Cutting the tops of pine trees (typically 4–6 cm) is used in practice to obtain cuttings and to reinforce the root system before planting [51].

While *E. coli* inhibited *F. circinatum* in vitro, it was ineffective against the fungus in vivo. The difference in efficacy may reflect the mechanism by which *E. coli* inhibits *F. circinatum*, perhaps by outcompetition for nutrients. The *E. coli* strain used as a control in this study is a common laboratory strain selected for rapid growth under laboratory conditions. While the ISP2 agar used to test antifungal activity in vitro was determined to be a suitable growth substrate for both the fungal and bacterial isolates, in vivo growth factors such as nutrients, pH, and osmolarity are determined by the tree and may not be suitable for proliferation of *E. coli*, which is not a plant-associated bacterium. On the other hand, the biocontrol strains are native to pine trees and are adapted to growth under those conditions.

4.2. Essential Oils

Essential oils extracted from a variety of plants have been proposed as safe, natural alternatives to synthetic fungicides. Many in vitro studies have reported the efficacy of essential oils against fungi including sapstain fungi (*Ophiostoma piceae*, *Aureobasidum pullulans*, *Alternaria alternata*, *Gliocladium viride*) [52], *Fusarium* spp. [15,24,53], and *D. pinea* [15]. However, plant conditions may change the efficiency of essential oils, therefore fungal inhibition must be assessed in situ. Soylu et al. [54] reported the efficacy of oregano essential oil on tomato plants against *Botrytis cinerea*. Clove oil applied on guava

seedlings roots completely inhibited *F. oxysporum*, *Botryodiplodia theobromae*, and *R. solani* [55], and cumin, geranium, and basil essential oils were effective against six species of *Fusarium* both in vitro and on cumin plants when applied directly to soil during planting in concentrations ranging from 2% to 8% (*v*/*v*) [56]. In this study, the direct application of cinnamon and/or clove oils to wounds in the stems of two-year-old *P. radiata* trees limited the damage to the trees by *F. circinatum*. The size of the lesions was reduced to the same extent by the individual and combined oils treatments. However, essential oils may be phytotoxic depending on their composition and concentration, the age of the plant, and the method of application [57,58]. In our study, cinnamon (10%) and clove (15%) oils had a toxic effect when applied to one-year-old *P. radiata* seedlings, especially when the two oils were combined, but were not toxic to older trees.

The antifungal activity of essential oils may be attributed to reactive aldehydes, flavonoids, or terpenes that disrupt cell membranes [59,60]. Cinnamaldehyde, a major component of cinnamon and some other essential oils, is a potent antifungal agent. When applied to tobacco, cinnamaldehyde reduced symptoms of tobacco black shank disease caused by *Phytophthora parasitica* var. *nicotianae* [61]. It also inhibited the growth of the tree pathogenic fungi *R. solani*, *Ganoderma australe*, *Collectotrichum gloeosporioides*, and to a lesser extent, *Fusarium solani* [62], and was active against *Phytophthora cactorum* and *F. circinatum* [23]. Eugenol, a phenylpropanoid, is a major component of clove oil and is known to have antifungal properties [63], including toxicity to several species of *Fusarium* [64]. Cinnamaldehyde and eugenol may be responsible for the inhibitory effect of cinnamon and clove oils, respectively, against *F. circinatum* demonstrated in this study.

5. Conclusions

The results presented herein demonstrate the ability of four pine rhizobacterial isolates, and cinnamon and clove essential oils, to inhibit the growth of *F. circinatum* in vitro and in young *P. radiata* trees. These biocontrol agents show promise as early, prophylactic treatments in nurseries to supplement existing control measures such as seed sanitization and/or cultivation of more resistant tree species to reduce fungal infections. However, the biocontrol treatments cannot be combined as the essential oils are toxic to the antagonistic bacteria. The oils are also toxic to *P. radiata* seedlings and therefore their application is restricted to trees older than one year. The use of natural compounds and indigenous antagonistic organisms may represent an alternative to the use of synthetic fungicides to control *F. circinatum* infections in nurseries and young pine plantations; however, additional studies are needed to optimize the method of application, including treatments to protect against root infections. The future of control strategies in forest ecosystems should be directed to an integrated system in which biocontrol agents and natural products play a crucial role.

Acknowledgments: This work was supported by Spanish Project RTA 2013-00048-C03-03 INIA and the European Union Healthy Forest Project LIFE14 ENV/ES/000179 to E.I., a grant from the New Brunswick Innovation Foundation to C.L.P., and a grant from the European Union 7th Framework Programme (Marie Curie Action) to E.I. and C.L.P.

Author Contributions: E.I., M.E.-R., and R.R. conceived and designed the experiments; E.I., M.E.-R., R.R., and T.T. performed the experiments; E.I., M.N., T.T., and C.L.P. analyzed the data; E.I., M.N., and C.L.P. contributed reagents/materials/analysis tools; C.L.P. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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