Compost Tea Reduces the Susceptibility of *Pinus radiata* to *Fusarium circinatum* in Nursery Production

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ABSTRACT

Nutrition is one of the factors that most limits forestry plant growth; thus, current production in nurseries is based on conventional fertilization focused on enhancing vigor. However, an excessive intake of mineral nitrogen can cause morphological imbalances and the formation of more succulent tissues which, consequently, increase susceptibility to plant pathogens. *Fusarium circinatum* is the causal agent of pitch canker in plants of the *Pinus* genus, with *Pinus radiata* being the species most susceptible to this disease. This study compares the response of *P. radiata* seedlings to infection by *F. circinatum* as influenced by two fertilizers—conventional and aerated compost tea (ACT)—applied during the nursery phase. The potential of ACT against *F. circinatum* was first tested in vitro, where it was found to inhibit the pathogen's mycelial growth and conidial germination. In the greenhouse, infected plants

fertilized with ACT exhibited less severe internal and external symptoms of pitch canker and lower levels of pathogen colonization of both stems and needles than with conventional fertilizer. An analysis of the hormone content and defense-related gene expression shows greater salicylic acid production and phenylalanine ammonium-lyase and chalcone synthase expression in ACT-fertilized pine. All of the parameters assessed are consistent in showing that biofertilization with ACT reduces the susceptibility of pine seedlings to the disease compared with conventional fertilization.

Keywords: bacteriology, biofertilizer, biological control, defense-related genes, disease control and pest management, hormones, mycology, symptomatology, systemic resistance

In 1946, a pathogenic fungus of several pine species was detected in the southeastern United States. In the following decades it spread to populations of *Pinus radiata* D. Don in California (Britz et al. 2001; Gordon 2006). It was identified as the phytopathogenic ascomycete fungus *Fusarium circinatum* (Nirenberg and O'Donnell 1998). Since becoming distributed throughout the United States, the pathogen has spread to nurseries and plantations in Australia, Chile, Colombia, Italy, Japan, South Africa, Spain, and New Zealand, probably due to the ability of the fungus to lie dormant in seed or wood of asymptomatic trees (Britz et al. 2001; Schweigkofler et al. 2004; Steenkamp et al. 2012; Wingfield et al. 2008). The disease was named pine pitch canker (Hepting and Roth 1946) and mainly affects conifers of the *Pinus* genus. However, it has been detected in more than 40 species (Viljoen et al. 1994) in closely related genera. *P. radiata* is the most susceptible *Pinus* sp. because it has comparatively lower genetic diversity and is cultivated in monocultures over vast areas (Schweigkofler et al. 2004). In Spain, *F. circinatum* has been detected infecting *P. pinaster*, *P. radiata*, and *P. sylvestris* in both nurseries and forest plantations (Landeras et al. 2005) and has caused widespread alarm due to its potential economic, ecological, and social impact (Wingfield et al. 2001). It is one of the most serious diseases that conifers can suffer anywhere because it progresses rapidly, is efficiently disseminated, and is highly lethal (O'Donnell et al.

1998). Conventional disease control practices are not effective against this species (Reglinski et al. 2004) and *F. circinatum* is currently on the European and Mediterranean Plant Protection Organization (EPPO) A2 List of pests recommended for regulation as quarantine pests (EPPO code GIBBCI).

F. circinatum is capable of causing damage at any time in the pine's life cycle—flowering, seed, seedling, or mature tree (Pérez-Sierra et al. 2007; Wingfield et al. 2008)—and may even be present asymptomatically. In winter, the pycnidia of this fungus are produced on infested needles, branches, and cones. When spring arrives, the conidia infect the tree buds before the needles begin their development and cause the first symptoms of wilting in late spring or early summer. In the nursery, the fungus is typically found on the seed and, when the latter is sown in *Sphagnum* peat, the pathogen begins to develop, causing the plant to die before or after germinating (Wingfield et al. 2008). Conventionally, this pathogen has been considered airborne, requiring entry through mechanical wounds. However, recently it has been shown that it is able to behave as a soilborne pathogen and infect plants via root penetration, taking advantage of the small wounds produced by formation of new ramifications of secondary roots (Martín-Rodríguez et al. 2015). Moreover, the structure of the *Sphagnum* peat acts as a reservoir for *F. circinatum*, due to its porous cavities and the amount of organic matter that it contains (Martín-Rodríguez et al. 2015).

The standard practices for pine production in Basque Country involve conventional fertilizers applied on a slow-release basis to promote vigorous growth, reduce stress, and improve overall tree health (Blodgett et al. 2005). This type of fertilizer alters the original edaphic communities and, due to the large amount of macronutrients applied, can be conducive to the emergence and expansion of pests and increase the susceptibility of plants to phytopathogenic fungal infection (Bengtsson et al. 2005; Konishi et al. 2001). This means that chemical pesticides must be used. The application of such pesticides is increasingly being called into question because of their negative effects on the environment and on human beings.

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Thus, there is growing interest in finding other types of fertilization that maintain the yield and reduce losses due to pests and pathogens.

Compost teas (CTs) are produced by macerating composted remains that contain large populations of microorganisms, many of them beneficial. There are two types of CT, depending on whether the organic matter oxidizes in aerobic conditions or not. Although the most widely studied are nonaerated CTs, aerated compost teas (ACTs) have recently begun to attract more attention. Several of the most abundant microorganisms present in ACTs have been highlighted by different authors as having growth-promoting properties in horticultural plants, among them species of the genera *Pseudomonas* (Cheng et al. 2007; Patten and Glick 2002; Shaharoon et al. 2008), *Serratia* (Hameeda et al. 2008; Selvakumar et al. 2008), and *Bacillus* (Gutiérrez-Mañero et al. 2001; Iqbal et al. 2018; Lateef et al. 2015). Several authors have reported that *Bacillus* is the most abundant genera followed by *Pseudomonas*, *Serratia*, *Penicillium*, and *Trichoderma* (Droffner et al. 1995; Kim et al. 2015). The presence of these microorganisms makes CTs a valuable choice because they show an effective ability to suppress phytopathogenic microorganisms (Scheuerell and Mahaffee 2004; Siddiqui et al. 2009; Weltzien 1991), improve soil fertility (Naidu et al. 2010), and stimulate plant enzymes involved in defense against pathogens (Siddiqui et al. 2009). However, to date, there have been no studies of the effect of ACT application on the growth or susceptibility to pathogen infection of trees used in forestry production.

In this study, our aim was to determine whether fertilization with an ACT reduces the susceptibility of *P. radiata* to infection by *F. circinatum*. To that end, the susceptibility to *F. circinatum* of 2-year-old plants produced in a nursery with ACT, as an alternative to conventional fertilization, was tested. In addition, the potential fungicide effect of ACT was also tested in vitro, taking into account the demonstrated infective potential of *F. circinatum* as a soilborne agent in nurseries (Martín-Rodríguez et al. 2015). The value of this work mainly lies in the fact that the effect of ACT on forestry plant health has not previously been described and the results presented here can lay the basis for redirecting current methods of fertilization toward a more sustainable model.

TABLE 1. Physicochemical properties of aerated compost tea

Parameter	Value ^z
Moisture (wt/wt)	99.1%
Dry matter (wt/wt)	0.91%
Density at 20°C (g/ml)	1.011
pH	7.84
Electrical conductivity at 25°C (mS/cm)	14.3
Oxidation-reduction potential (mV)	-339.25
Ashes (wt/wt)	0.981%
Total organic matter	Ø
Total humic extract	Ø
Nitrogen (total) (wt/wt)	0.1449%
Nitrogen (ammoniacal) (wt/wt)	0.0183%
Nitrogen (organic) (wt/wt)	0.1266%
Total phosphorus (P ₂ O ₅)	Ø
Total potassium (K ₂ O) (wt/wt)	0.463%
Total calcium (CaO) (wt/wt)	0.0131%
Total magnesium (MgO) (wt/wt)	0.0508%
Total sulfur (SO ₃) (wt/wt)	0.0634%
Total sodium (Na ₂ O) (wt/wt)	2.037%
Total boron (B) (mg/kg)	2.56
Total iron (Fe)	Ø
Total manganese (Mn)	Ø
Total zinc (Zn) (mg/kg)	0.92
Vitamin B1(mg/kg)	1.21
Vitamin B2, B3, C, E, and B9	Ø
Gibberellins (GA), cytokinins (CK), and auxins	Ø

^z Symbol Ø indicates value below the detection threshold.

MATERIALS AND METHODS

Physicochemical and microbiological characteristics of ACT. The compost used was produced by GARBIKER AB, S.A. and consisted of 90% agricultural waste (mainly cabbage, kale, red cabbage, pumpkin, and tomato) from large producers and 10% garden waste. The production of ACT is detailed in the Materials and Methods section of Otero et al. (2019) but is briefly described here. To produce the aerated tea, this compost was mixed with water (1:10 compost/water [wt/vol]) in a brewing tank and fermented at room temperature (22 ± 2°C) for at least 4 days with continuous aeration supplied by an aquarium pump (Naidu et al. 2010). In addition, based on the results obtained by Naidu et al. (2010), yeast extract (7 g per 100 g of compost) was added to increase the microbial populations in the ACT. Once fermentation was completed, the solid fraction was removed, and the extract was stored in polyethylene barrels in darkness at room temperature until use.

The physicochemical properties of the fermented extract were determined by Fitosoil Laboratorios S.L. using inductively coupled plasma atomic emission spectroscopy and ionic chromatography in accordance with the relevant UNE-EN ISO standards and Regulation (CE) number 2003/2003 of the European Parliament and are detailed in Table 1. The microbial DNA was extracted by a modified version of the cetyltrimethylammonium bromide (CTAB) procedure described by Möller et al. (1992), detailed in the “Quantification of plant hormones, defense-related gene expression and pathogen present in pine tissues” section. Metabarcoding analysis was performed by the Genomics and Proteomics Service of SGIKER (UPV/EHU) following the protocol “16S metagenomic Sequencing Library Preparations” by Illumina, available on its website (https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf), using an MiSeq sequencer. The sequences from the metagenomic analysis were contrasted in the Silva database (<https://www.arb-silva.de/>). The sequences were analyzed within Quantitative Insights Into Microbial Ecology (QIIME 1.9) (Caporaso et al. 2010). The taxonomy was assigned to each representative sequence of bacteria and archaea using SILVA 119. Sequences were clustered into operational taxonomic units with UCLUST (Edgar 2010), using reference approaches with a 97% identity threshold. The cultivable microorganisms in the CT were analyzed by using Sanger sequencing following the protocol of Murray and Thompson (1980). The sequences generated after Sanger sequencing were compared with the sequences available in the NCBI.

Plant and fungal growth. The Fc-GFP1 strain, an *F. circinatum* isolate transformed with a green fluorescent protein (GFP) (Martín-Rodríguez et al. 2013), was used to assess the effect of ACT on pathogen development and the susceptibility of *P. radiata*. Fungal cultures were established from colonies resulting from the subculture of a single conidium (stored in 25% glycerol [vol/vol] at -80°C) and were routinely grown at 22 to 28°C on potato dextrose agar (PDA) for up to 2 weeks. *P. radiata* seed were obtained from an orchard belonging to the Basque Country's Genetic Improvement Program.

In vitro experiment. The direct fungistatic effects of ACT on pathogen growth, mycelial biomass, mycelial radial growth, and conidia germination were assessed using in vitro studies on amended media. The preparation of the culture media was carried out as follows. To obtain 0, 25, 50, 75, and 100% (vol/vol) ACT concentrations in each flask, the same amount of PDA agar (39 g liter⁻¹) was added to each one and then 1,000, 750, 500, 250 ml, and 0 ml of distilled water, respectively, was poured into the flask. Because ACT contains live microorganisms and thermosensitive molecules, it was decided to add only 100 ml of ACT to the flask which was assigned the 100% concentration to dissolve the agar and then pour the rest when the mix had been tempered and not

completely solidified. The same procedure was followed for the rest of the flasks, carefully adding the remaining 0, 250, 500, 750, and 900 ml of ACT while gently shaking the flask containing the sterilized, molten PDA medium prior to pouring 10 ml into 90-mm-diameter Petri dishes. After that, a mycelial disk (5 mm in diameter) obtained from the edge of a 14-day-old *F. circinatum* colony was placed at the center of the PDA agar plate and sealed with parafilm. Plates were incubated for 14 days in darkness at 25°C. Then, colony growth was measured as the area of media covered by mycelium and mycelium biomass according to the protocol described by Eng et al. (2003).

To assess the effect of ACT on germination, conidia were extracted from 14-day-old colonies grown on PDA by adding 10 ml of sterile KCl at 0.5% (wt/vol) and scraping with a scalpel from the surface of the colony. The concentration of the suspension was adjusted to 10^3 conidia ml⁻¹ using a hemocytometer. Petri dishes containing the amended medium were inoculated by spreading a 0.1 ml aliquot of the suspension on each amended plate. Plates were incubated for 7 days in darkness at 25°C. After the incubation period, the germinated colonies were visually counted and, knowing the inoculated initial concentration, the percentage of inhibition was calculated.

Greenhouse experiment. The standard production process of *P. radiata* begins with staggered sowing in containers, which are maintained outdoors for a minimum of 9 months and later removed. For this work, the experiment was conducted in the forest nursery of BASALAN S.A. in Bizkaia, Spain. Two batches of pine seed were sown in December and March over a mixture of white and black *Sphagnum* peat (70:30 [vol/vol]) in Arnabat trays of 35 cells (200 cm³). Each tray was an experimental unit, and trays were arranged in a randomized block design, dividing the experiment into three blocks. During their time in the nursery, seedlings were maintained under regular irrigation and received three different types of fertilization: (i) control, in which plants received only water during their growth; (ii) conventional, in which the slow-release fertilizer Plantacote Plus 14:18:15 NPK + 2 Mg (Aglukon, Düsseldorf, Germany) was mixed with peat substrate at the recommended dose of 3 g/liter prior to filling the trays (Ortega et al. 2006); and (iii) ACT, in which the CT was applied once a month by directly watering the substrate with 50 ml of an aqueous dilution of 1:5 (vol/vol). At the end of this period in the nursery, the conventional fertilized plants had received 0.84, 1.08, and 0.9 g of nitrogen, phosphorus, and potassium respectively, by fertilization. The plants fertilized with ACT received a total of 0.1, 0.0, and 0.41 g of nitrogen, phosphorus, and potassium respectively, by fertilization.

After 9 months, arbitrarily selected plants were transplanted into QuickPot trays of 6 cells (1,600 cm³), using the same substrate mixture. For each batch, 54 trays were prepared, 18 for each of the three fertilization treatments (water, conventional, and ACT). These plants continued growing in the nursery up to the age of 17 months. Then, they were moved into a high-security glasshouse under controlled conditions (85% relative humidity, with a 12-h photoperiod, providing 300 μ mol m⁻² s⁻¹ photosynthetic active radiation) and, after a 4-week acclimatization period, the inoculation was carried out.

The 54 trays of plants were divided into three different homogeneous sets for different purposes. Set 1 was inoculated with *F. circinatum* and used for most of the data in the study. In total, 27 plants were destructively sampled 2 days after inoculation, 18 of which were used for hormone quantification and 9 of which were used for quantifying gene expression. The remaining plants were rated for whole-plant disease symptoms once weekly for 14 weeks. During this period, 36 of the remaining plants showing wilting symptoms were processed to determine canker length under the bark and necrotic area of cross sections, and 12 of the remaining plants were processed to quantify the amount of pathogen present in host tissue. Set 2 received a 1- μ l droplet of distilled sterile water (mock inoculation) and, from here, 18 plants were processed for

noninfected plant hormone quantification. Set 3 was left unmanipulated as a negative environmental control. Thirty plants from this last set were biometrically characterized by measuring their height, root collar diameter, and shoot or root dry biomass after drying at 80°C for 48 h. The inoculation process consisted of removing one needle fascicle from the middle of the stem and adding a 1- μ l droplet of the conidia suspension (2.5×10^5 conidia ml⁻¹) or sterile water directly at the wound site (Martín-Rodríguez et al. 2013).

Two ratings of disease severity, one based on aerial symptoms of the plant and the other on the amount of resin exuded, were obtained weekly through 14 weeks postinoculation. The rating scale for aerial disease symptoms was as follows: 1 = healthy plant with no necrosis, 2 = healthy plant with necrosis only at the inoculation point, 3 = necrosis 2 cm above and below the point of inoculation, 4 = wilting with crown dieback, and 5 = reddening foliage above the point of inoculation (Correll et al. 1991). The rating scale for the amount of resin exuded was as follows: 0 = low resin exudation, 1 = moderate exudation, 2 = ≤ 0.5 cm from the wound, 3 = > 0.5 and < 4 cm from the wound, and 4 = > 4 cm from the wound (Bonello and Blodgett 2003). With these data, the disease progression rate (DPR) was calculated using the formula $DPR = \sum X_i / n$, where X_i = average of severity degrees and n = weeks after inoculation.

In order to verify the real extent of the symptomatology, the bark was removed from the stems of some of those plants. In all, 18 plants showing irreversible signs of wilting (severity scale of 5) and 18 plants apparently asymptomatic (severity scale of 1) were chosen at the end of the experiment. The procedure consisted of making longitudinal sections so as to measure with a ruler the canker length and transversal sections to determine the necrotic area using ImageJ software.

Quantification of plant hormones. Hormone content was analyzed at 2 days postinfection by sending a pool of pulverized and lyophilized stems and needles from infected and uninfected pine trees to the CETEMAS Research Institute (Technological and Forestry Center for Wood) in Asturias, Spain. Hormone detection and quantification was carried out by liquid chromatography (LC) (ultrahigh-performance LC, Agilent 1290 Infinity; Agilent) with a triple quadrupole mass detector (Agilent 6460 Triple series Quad LC mass spectrometry; Agilent).

The plant material used in the experiments that followed consisted of 2-cm sections of the stems (1 cm above and below the inoculation point) and the needles around them, which were then pulverized using liquid nitrogen and a mortar and stored at -80°C until use.

Defense-related gene expression. To assess the influence of fertilizer treatment on the plant's defense response, we quantified the expression of genes within the pathogenesis-related (PR) protein family (chitinase [CHI] and glucanase [GLU]) or those that are involved in the synthesis of secondary metabolites of proven antimicrobial action (phenylalanine ammonium-lyase [PAL], chalcone synthase [CHS], and hydroxymethylglutaryl-CoA reductase 2 [HMG-CoA2]) in needles and stems of plants 2 days after inoculation. To determine gene expression, a modified version of the method described by Chang et al. (1993) was used to extract RNA. This modification consisted of adding two volumes of cold isopropanol to the sample with LiCl, letting the RNA precipitate at -20°C for 2 h before centrifuging, and, finally, adding 100% ethanol to wash the sample. To remove the DNA, the Recombinant DNase I (RNase-free) kit from Takara (Clontech Laboratories Inc.) was used following the manufacturer's instructions. For enzyme elimination, phenol-chloroform extraction was chosen. Finally, the pellet was resuspended in 50 μ l of sterile ultrapure water and kept at -20°C until use. Subsequently, sample concentration was standardized and the retrotranscription reaction was carried out using the PrimeScript RT reagent kit (Perfect Real Time) from Takara (Clontech Laboratories Inc.). The reaction and the PCR program were prepared according to the manufacturer's instructions.

Once the complementary DNA was obtained, the quantitative PCR was prepared with the following final concentrations: 1× SYBR, 0.2 µM each primer, 1× ROX, and 2 µl of cDNA in a final volume of 15 µl. The sequences of the primers used are described in Table 2. These genes were selected because they are within the PR protein family (QUI and GLU) or because they are involved in the synthesis of secondary metabolites of proven antimicrobial action.

The PCR program was implemented in a StepOne Plus thermocycler (Applied Biosystems) and consisted of an initial denaturation at 94°C for 10 min; followed by 45 cycles of denaturation at 94°C for 15 s and annealing or elongation at 60°C for 1 min; and ending with an automatic final cycle which consisted of continuously measuring the fluorescence emitted in the range of 60 to 95°C, increasing the temperature 0.2°C/s. This step enables the dissociation kinetics of the amplified fragments to be monitored and the melt curve to be determined. The calculation of the expression was carried out by relative quantification applying the formula $2^{-\Delta C_T}$, with ΔC_T being the difference between the cycle threshold (C_T) of the incognita gene and the C_T of the housekeeping gene. The housekeeping gene used in this experiment was *NLY* (Martín-Rodrigues et al. 2013).

Quantification of the pathogen present in pine tissues.

After disease severity was assessed in the greenhouse experiment, the fungus present in the plant tissues was quantified. DNA was extracted using a modified version of the CTAB procedure described by Möller et al. (1992). This modification consisted of adding 1,250 µl of cold isopropanol after adding LiCl and allowing DNA to precipitate for 24 h at -20°C before centrifuging. The SYBR Premix Ex Taq™ (TliRNaseH Plus) kit from Takara (Clontech Laboratories Inc.) was used to prepare the reaction. The primers used and the PCR procedure followed were as described by Martín-Rodrigues et al. (2013), with the following final concentrations: 1× SYBR, 0.2 µM each primer, 1× ROX, and DNA at 1 ng/µl in a final reaction volume of 15 µl. The calculation of the expression was made by absolute quantification, having previously made a regression line with different concentrations of DNA and their different C_T values. Once the DNA concentration of the sample was obtained, in order to normalize the samples, the nanograms per microliter of the *FCIR* gene were divided between the *NLY* gene (housekeeping) concentration values (Martín-Rodrigues et al. 2013).

Statistical analyses. The data obtained from the two rounds carried out over time were merged for the statistical analyses, which were carried out using SPSS Statistics software (v.18; IBM). In order to identify differences between treatments in the case of the in vitro experiment, defense gene induction, and pathogen concentration assays, one-way analysis of variance (ANOVA) and the *t* test were used. As post hoc tests, S-N-K and Tukey were used if the homogeneity of variances was met, and Dunnett's C if it was not. Due to the lack of independence of the data, in the disease progression experiment, the data were treated using a repeated-measures ANOVA. In addition, to check the dissimilarity between

the different blocks and treatment groups in canker dimensions, pine plant growth, and defense hormone concentration experiments, a linear mixed model was used, stipulating that the fixed effects were the different nutritional treatments and the random effects were the blocks. In all cases, it was determined that there was statistical significance for values of $\alpha \leq 0.05$.

RESULTS

Physicochemical and microbiological characteristics of ACT. The results for both the analysis of bacterial metagenome and the count of cultivable species are shown in Table 3. The predominant phyla detected in the CT were, in descending order, Bacteroidetes, Firmicutes, and Proteobacteria. The main cultivable species were *Ochrobactrum tritici*, *Pseudomonas aeruginosa*, *Gordonia terrae*, *Bacillus subtilis*, and *B. licheniformis*.

In vitro experiment. In all of the parameters studied in vitro to test the ACT effect on *Fusarium* development, an inhibitory trend was noted even at low ACT concentrations (25%). However, significant differences were observed in the development of the

TABLE 3. Most abundant bacterial phyla detected by metagenomic analysis and representativeness of the main microbial species detected after the count of colony forming units in Petri dishes

Phylum or species	Percentage (%)
Metabarcoding	
Bacteroidetes	15.6
Firmicutes	15.5
Proteobacteria (β-proteobacteria)	14.5
Proteobacteria (α-proteobacteria)	13.7
Proteobacteria (δ-proteobacteria)	11.6
Others	6.1
Spirochaetes	4.6
Synergistetes	4.5
Proteobacteria (γ-proteobacteria)	4.4
Tenericutes	3.6
Proteobacteria (ε-proteobacteria)	2.8
Actinobacteria	1.7
Euryarchaeota	1.4
Cultivable microorganisms	
<i>Ochrobactrum tritici</i>	24.53
<i>Pseudomonas aeruginosa</i>	17.41
<i>Gordonia terrae</i>	15.83
<i>Bacillus subtilis</i>	14.24
<i>B. licheniformis</i>	11.87
<i>B. pumilus</i>	5.54
<i>B. safensis</i>	4.75
<i>B. velezensis</i>	3.96
<i>Serratia marcescens</i>	1.58
<i>P. fluorescens</i>	0.14
<i>P. putida</i>	0.12
Fungal species	0.04

TABLE 2. Sequences of the primers used to detect the induction of plant defense-related genes

Gene name	GenBank access	Primer name ^z	Primer sequence (5'–3')	Citation
Chitinase class III	4327172	QUI-F	AAATCTTCAGCATCGCCA	Boava et al. 2010
	...	QUI-R	CCCAATAGACAGCAATTTCTC	...
Glucanase	103498635	GLU-1	TTYGATGCMYTKKTGGAT	Rivera et al. 2002
	...	GLU-2	TCRTCARACATRGCAA	...
Phenylalanine ammonium-lyase	JQ765748.1	PAL-F	AGAACACGGTGAGCC	Jin et al. 2013
	...	PAL-R	CATAGGCAAGCACATA	...
Chalcone synthase	AJ413277	CHS-F	GGGAATCAACGGTTTGGAA	De Keyser et al. 2013
	...	CHS-R	CTCGGGCTTAAGGCTCAACTT	...
Hydroxymethylglutaryl-CoA reductase 2	AF110383	HMG2-F	ATTACCTTCAGATGAATACGCT	Villar-Luna et al. 2015
	...	HMG2-R	CTCTCTATGTTTGTGCTGGGT	...

^z Chitinase (QUI), glucanase (GLU), phenylalanine ammonium-lyase (PAL), chalcone synthase (CHS), and hydroxymethylglutaryl-CoA reductase 2 (HMG2).

pathogen at different concentrations: the greatest biomass inhibition occurred at concentrations of 75 and 100%, where growth in biomass was reduced by 30 and 38%, respectively; the highest area reduction was observed at a concentration of 75%, where growth in area was reduced by 45%; and the greatest germination inhibition occurred at a concentration of 100%, showing a reduction in conidia germination of 79% (Fig. 1). All of these percentages have been normalized with respect to the control values (0%).

Plant growth. Biometric values for plants fertilized with the conventional treatment were 8, 31, and 50% higher for height and grams of dry weight of needles and stem, respectively, when compared with ACT-fertilized plants. The roots of the ACT-fertilized pine showed the highest biomass but it was not significantly different from conventionally fertilized roots (Table 4).

Greenhouse experiment. For disease symptoms, the DPR showed significant differences between plants fertilized with ACT and the rest of the treatments; in this case a lower index was observed in ACT-fertilized plants at 98 days (14 weeks) post-inoculation. Disease severity DPR values on the final rating date were 33 and 22% higher in water and conventional treatments, respectively, when compared with ACT-treated plants. This pattern contrasted with that of resin, in which the degree of resinification in water presented significant differences compared with the conventional treatment. However, statistical analysis did not detect differences between conventional and ACT treatments (Fig. 2).

Canker length was significantly smaller (67%) in the pine trees fertilized with ACT than in those fertilized conventionally (Fig. 3). The same trend was observed in the percentage of necrotic area, which was 26% lower. As for the presumed asymptomatic pine trees, it was found that, although they did not show external signs of disease, on the inside they displayed tissue necrosis. Those asymptomatic pine trees fertilized with ACT also had the lowest percentage of affected tissue (Fig. 3).

Quantification of plant hormones. In relation to hormone content, infected pine trees with conventional fertilizer showed significant increases of 19, 31, and 62% in indolacetic acid,

Fig. 1. Effect of increases in the concentration of aerated compost tea (25, 50, 75, and 100%) on colony area, biomass, and conidia germination rate inhibition of *Fusarium circinatum* compared with control treatment (0%). Sample size = 10. Means with different letters within each variable are statistically different ($\alpha \leq 0.05$) based on the P values obtained using the analysis of variance test. Error bars indicate the standard error.

gibberellins, and brassinosteroids, respectively, compared with those irrigated with water. The ACT-fertilized infected pine showed a significant increase in concentration of 208% in salicylic acid and 33% in brassinosteroids compared with conventional-fertilized infected plants. In the case of brassinosteroids, the increase was especially notable for castasterone and brassinolides. In the latter treatment, the concentration of salicylic acid increased to the detriment of all other stress hormones but especially gibberellins (Table 5).

Defense-related gene expression. The change in relative expression in response to *F. circinatum* inoculation was significantly higher in ACT-fertilized plants compared with conventional or water for QUI in stems, PAL in both needles and stems, and HMG-CoA2 in stems (Fig. 4). No differences were found in the expression of the HMG-CoA2 gene in the needles of any of the treatments.

Quantification of the pathogen present in pine tissues. The amount of fungus found in the stem of symptomatic plants was significantly higher (17.73%) in conventionally fertilized pine trees than in those grown with ACT. The same trend was observed in the case of asymptomatic pine, where the amount of the pathogen was significantly higher (99.94%) in stems of conventionally fertilized pine. This trend was also observed in the amount of fungus present

Fig. 2. Disease progression rate (DPR) based on degree of plant severity and degree of resin exudation observed at 98 days after inoculation with *Fusarium circinatum* of *Pinus radiata* plants grown for 18 months in a nursery with water, conventional fertilizer, and aerated compost tea (ACT). Sample size = 18. Means with different letters within each variable are statistically different ($\alpha \leq 0.05$) based on the P values obtained using the repeated measures analysis of variance test. Error bars indicate the standard error.

TABLE 4. Growth of 18-month-old noninoculated pine trees as influenced by fertilizer treatment^a

Treatment	Height (cm)	Diameter (mm)	Needles (gDW)	Stem (gDW)	Root (gDW)
Water	22.9 ± 1.1 b	3.5 ± 0.21 b	1.1 ± 0.17 c	0.5 ± 0.05 c	1.3 ± 0.14 b
Conventional	66.6 ± 2.1 a	7.7 ± 0.46 a	7.4 ± 0.58 a	6.8 ± 0.95 a	9.4 ± 0.36 ab
Aerated compost tea	61.4 ± 1.3 ab	7.2 ± 0.19 a	5.1 ± 0.78 b	3.4 ± 0.26 b	10.7 ± 0.39 a

^a Means within a measurement column followed by different letters are statistically different ($\alpha \leq 0.05$) based on the P values obtained using the linear mixed model. gDW = grams dry weight and symbol ± indicates mean standard error above and below average.

in needles: under conventional fertilization, it was 96.59% higher than in ACT in the needles of symptomatic plants and 99.88% higher for asymptomatic plants (Fig. 5).

DISCUSSION

This research found that ACT reduced the severity of pitch canker of *Pinus radiata* caused by *F. circinatum*. This was due to limitation in the development and progression of the pathogen compared with conventional fertilization.

The ACT assessed here is a liquid organic matrix which contains a low concentration of macro- and micronutrients and vitamins, but whose main importance lies in its concentration of microorganisms from different genera, which coexist and act synergistically, increasing the benefits for the plants to which it is applied. The most abundant microorganisms in the ACT used in this research were species of, in descending order, *Bacillus* (40.4%), *Ochrobactrum* (24.5%), *Pseudomonas* (17.7%), *Gordonia* (15.8%), and *Serratia* (1.6%). Similarly, Kim et al. (2015) found that *Bacillus* (63%) and *Ochrobactrum* (13%) were the two most abundant bacteria in a tea obtained from composting of rice straw, vermicompost, and cypress bark.

ACTs are considered effective biofertilizers because they contain a rich microbial community composed of nitrogen-fixing bacteria and phosphorus and potassium solubilizers, many of which are described as plant-growth-promoting rhizobacteria. Fungi account for only 1% but include species of the genera *Aspergillus* and *Penicillium* that add value to the product by being plant-growth-promoting fungi.

The ACT used in this study was found to be directly antagonistic to *F. circinatum* in vitro. In these fertilizers, predominate bacterial

species of the genera *Bacillus* and *Pseudomonas*, *Actinomyces* and acid-lactic bacteria, and fungi in *Trichoderma* and other genera (Naidu et al. 2010; Siddiqui et al. 2009; Welke 2005) are found. These have been shown over the years to have an antagonistic effect on pathogens (Allona et al. 1996; Cheah and Page 1997; Domracheva et al. 2010; Veluthakkal and Dasgupta 2010). That effect was found in our work via the assay in which *F. circinatum* was cultured in plates with increasing concentrations of ACT. It was observed that, with a concentration of just 25%, there was significant inhibition of growth and viability of conidia compared with the control (0%), which helped to reduce their presence in the substrate (in this case peat) where ACT was applied. The use of these CTs as fertilizers would thus also be desirable in the case of infections of *P. radiata* roots that are initiated by inoculum in peat, because this fungus is able to survive in *Sphagnum* peat and infect *P. radiata* roots, as Martín-Rodriguez et al. (2015) demonstrate in their article.

It has also been proven that the microorganisms present in CTs are capable of both stimulating the induced resistance of plants and inhibiting pathogenic microorganisms by antibiosis mechanisms, parasitism, and competition for resources (Kai et al. 1990; Kim et al. 2015; Palmer et al. 2010; Scheuerell and Mahaffee 2002; Siddiqui et al. 2009; Weltzien 1991). In their review, Scheuerell and Mahaffee (2002) emphasized the importance of preinfection application and frequency of fertilization as a preventive treatment, which would depend mainly on the pathogen reproductive rates, propagation methods, and environmental conditions. Due to this phenomenon of displacement by competition, CT can be applied as an undiluted foliar spray to attempt to cover at least 60 to 70% of the surface area. The natural niche of most of the bacteria and fungi

Fig. 3. A, Canker length (in centimeters) and B, percentage of necrotic surface in a cross section of the stem of symptomatic and asymptomatic *Pinus radiata* plants, grown for 18 months with water, conventional fertilizer, and aerated compost tea (ACT). Sample size = 12. Means with different letters within each variable are statistically different ($\alpha \leq 0.05$) based on the *P* values obtained using the linear mixed model. Error bars indicate the standard error.

TABLE 5. Concentration of stress, growth, and defense hormones in 18-month-old pine trees without infection (–Fc) and 2 days after inoculation with *Fusarium circinatum* (+Fc) for different types of fertilization^x

Fertil. ^y	Hormones (µg/g of tissue)								
	Abscisic acid	Jasmonic acid	Salicylic acid	Indolacetic acid	Cytokinins	Gibberellins	Brassinosteroids	Castasterone ^z	Brassinolides ^z
–Fc									
Water	0.11 ± 0.00 NS	0.00 ± 0.00 NS	0.04 ± 0.01 NS	0.11 ± 0.01 b	0.31 ± 0.01 b	0.05 ± 0.00 b	10.74 ± 0.46 NS	8.64 ± 1.37 NS	1.38 ± 0.19 a
Conv	0.14 ± 0.01 NS	0.00 ± 0.00 NS	0.03 ± 0.01 NS	0.31 ± 0.02 a	0.30 ± 0.01 b	0.08 ± 0.01 a	9.47 ± 0.76 NS	6.98 ± 1.16 NS	0.87 ± 0.12 b
ACT	0.16 ± 0.01 NS	0.00 ± 0.00 NS	0.04 ± 0.03 NS	0.19 ± 0.01 b	0.43 ± 0.02 a	0.08 ± 0.01 a	9.39 ± 0.96 NS	6.68 ± 1.13 NS	1.35 ± 0.18 a
+Fc									
Water	0.15 ± 0.01 NS	0.09 ± 0.02 a	0.10 ± 0.01 b	0.14 ± 0.01 b	0.11 ± 0.02 NS	0.06 ± 0.03 b	5.73 ± 0.72 c	4.57 ± 0.48 b	1.11 ± 0.09 b
Conv	0.15 ± 0.01 NS	0.00 ± 0.00 b	0.06 ± 0.00 c	0.16 ± 0.01 a	0.11 ± 0.01 NS	0.07 ± 0.00 a	9.27 ± 0.42 b	4.92 ± 0.93 b	0.86 ± 0.07 c
ACT	0.13 ± 0.01 NS	0.00 ± 0.00 b	0.18 ± 0.03 a	0.16 ± 0.01 a	0.10 ± 0.02 NS	0.05 ± 0.01 b	12.34 ± 0.56 a	10.72 ± 0.84 a	1.50 ± 0.11 a

^x Sample size *n* = 12. Means within a measurement column followed by different letters are statistically different ($\alpha \leq 0.05$) based on the *P* values obtained using the linear mixed model. NS = not significant and symbol ± indicates standard error above and below average.

^y Fertilization: water control, conventional (Conv), and aerated compost tea (ACT).

^z Castasterone and brassinolides are part of the brassinosteroids family, along with homobrassinolides and 24-Epibrassinolides.

present in ACTs is the plant phyllosphere and, when they return to their optimum environment, they are able to survive in the aerial parts of plants and become great competitors against airborne pathogens (Ingham 2005). This form of application could be of interest in future experiments to prevent the entry of *F. circinatum* via stem wounds.

During the pine inoculation assay, two facts were observed. The first was that the symptomatology took longer to appear in the pine fertilized with ACT than with the other two types of fertilization; thus, the severity of the infection depended on something more than plant biometrics. Second, the length and area of the canker of infected plants already showed differences from one treatment to another, with plants fertilized with ACT displaying smaller lesions. The effectiveness of ACT lies not only in the delay and reduction of disease symptoms but also in the increase of the number of asymptomatic plants with respect to conventional fertilization. Not all the asymptomatic plants showed evident tissue damage when the bark was removed; however, those that did could have eliminated or contained the pathogen in some way, which would explain why, after 98 days of duration of the experiment, they did not present

greater symptoms. Because of this effect, ACT could be considered as a preventive treatment of the disease.

The amount of fungus found in the pine trees after 98 days postinoculation was noteworthy. The pathogen concentration in the different organs of both symptomatic and nonsymptomatic plants was significantly lower in plants fertilized with ACT, possibly due, in part, to a decrease in inorganic nitrogen content, which would restrict growth and sporulation of fungi (Ingham 2005; Paustian and Schnürer 1987; Treseder and Allen 2002). Surprisingly, the pathogen was detected in the needles surrounding the infected area. That would indicate that it is able to propagate to the vascular tissue once it has colonized the stem, creating a new dispersion vector that needs to be considered when the phytopathogen is detected in nurseries or in the field, although its concentration in needles was found to be lower than in stems.

In addition, it can be seen that the type of fertilization applied influenced the hormonal content of the plant. The higher production of salicylic acid and brassinosteroids observed in inoculated plants fertilized with ACT could have influenced plant defense. O'Donnell et al. (2001) showed that salicylic acid was an important molecule in

Fig. 4. Fold change in relative expression within *Pinus radiata* inoculated with *Fusarium circinatum* over noninoculated plants at 2 days after inoculation. Genes were chitinase (QUI), phenylalanine ammonium-lyase (PAL), chalcone synthase (CHS), and hydroxymethylglutaryl-CoA reductase 2 (HMG-CoA2). Sample size = 6. ACT = aerated compost tea. Means with different letters within each variable are statistically different ($\alpha \leq 0.05$) based on the *P* values obtained using the analysis of variance test. Error bars indicate the standard error.

Fig. 5. Absolute quantification of the DNA of *Fusarium circinatum* in stems and needles of symptomatic and asymptomatic *Pinus radiata* plants, grown for 18 months with water, conventional fertilizer, and aerated compost tea (ACT). *FCIR* is the reference gene for detecting *F. circinatum* and *NLY* is the gene used as housekeeping. Asterisks indicate significant differences according to the *t* test. Sample size = 6. Error bars indicate the standard error.

disease response, because they observed a correlation between salicylic acid accumulation in infected tissues and necrosis, which would prevent the spread of the pathogen and decrease its concentration. In addition, De Torres Zabala et al. (2009) pointed out that jasmonic acid is unlikely to contribute to early stages of infection and that, if it appears, it is probably because of a wound, as happened in our study in plants irrigated with water at 2 days postinoculation, where jasmonic acid was detected.

The increase in brassinolides in ACT-fertilized plants may have contributed to the decrease in pitch canker. It has been discovered that brassinolides, which have traditionally been related with the plant growth hormone regulation, also have the ability to induce resistance to infection in tobacco and rice plants against viruses, bacteria, and fungi (Nakashita et al. 2003; Wang 2012). According to these authors, the resistance mediated by brassinolides does not require the biosynthesis of salicylic acid and does not lead protein PR expression; therefore, it seems not to be related to systemic acquired resistance.

Although disease severity was lower in ACT-treated plants, more resin was produced by conventionally fertilized plants. Resin is one of the main defenses of *Pinus* spp. to seal wounds and expel or trap invasive organisms (Morse et al. 2004; Phillips and Croteau 1999). Resin also contains effective compounds for inhibiting phytopathogens such as monoterpenes, diterpenes, and sesquiterpenes (Mumm and Hilker 2006; Nagy et al. 2006), which might be found in greater concentration in the resin of ACT-fertilized plants. Determining qualitatively and quantitatively the presence of these organic compounds could be an interesting analysis for future studies.

Our finding that selected defense genes were upregulated to a higher degree in ACT-fertilized plants when compared with conventionally fertilized plants agrees with previous studies on the influence of CTs on plant defense. These studies have proven that crop fertilization with CTs improves the hosts' defenses by reducing susceptibility to phytopathogenic infection through the induction of synthesis of various enzymes with oxidase activity such as peroxidase and polyphenol oxidase, or involved in the synthesis of phenolic compounds such as PAL (Chittoor et al. 1990; Siddiqui et al. 2009; Weltzien 1991). In our study, both PAL and CHS genes, which codify for enzymes involved in production of compounds with proven antimicrobial activity such as flavonoids, phenylpropanoids, and phytoalexins (Azcón-Bieto and Talón 2008; Donoso et al. 2015), are expressed to a high degree in the plants fertilized with ACT. QUI or HMG-COA2 codifying genes, involved in isoprenoid synthesis, are also overexpressed in ACT-fertilized plants. In particular, isoprenoids play an important role because many of them are involved in defense, given that essential oils or resins have antimicrobial activity (Azcón-Bieto and Talón 2008). A greater expression of these genes inoculated in ACT-fertilized pine could explain the differences in concentration of *F. circinatum* found in comparison with the other two types of fertilization, because an increase in defenses in the short term would reduce the amount of fungus that could colonize the xylem through the cortex.

Conclusions. Applying ACT during the growth phase of *P. radiata* in a nursery reduces the mortality rate and the severity of both external and internal symptoms caused by infection by the pathogen *F. circinatum*. In addition, this type of fertilization favors the defensive capabilities of the plant by stimulating its defense mechanisms and fostering a hostile environment that hinders the advancement of the infection, thus helping to reduce susceptibility to the pathogen.

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