Fungal communities influence root exudation rates in pine seedlings

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Abstract

Root exudates are hypothesized to play a central role in belowground food webs, nutrient turnover, and soil C dynamics in forests, but little is known about the extent to which root-associated microbial communities influence exudation rates in trees. We used a novel experimental technique to inoculate loblolly pine (Pinus taeda L.) seedlings with indigenous forest fungi to examine how diverse fungal communities influence exudation. Surface-sterilized seeds were sown in intact, unsieved soil cores for 14 weeks to promote root colonization by fungi. After 14 weeks, we transferred seedlings and root-associated fungi into cuvettes and measured exudate accumulation in trap solutions. Both the abundance and identity of root-associated fungi influenced exudation. Exudation rates were greatest in root systems least colonized by ectomycorrhizal (ECM) fungi and most colonized by putative pathogenic and saprotrophic fungi. However, the ECM community composition was not a strong determinant of exudation rates. These results suggest that environmental conditions that influence the degree to which tree roots are colonized by pathogenic and saprotrophic vs. mutualistic fungi are likely to mediate fluxes of labile C in forest soils, with consequences for soil biogeochemistry and ecosystem processes.

Introduction

Root exudates are soluble organic compounds that provide a semi-continuous input of labile C to soil in contrast to the more transient inputs of recalcitrant C from root and hyphal litter and debris. These compounds enhance nutrient availability in the zone of soil adjacent to roots by mobilizing poorly soluble mineral nutrients (e.g., by the release of chelating agents and alterations in the pH milieu; Jones & Darrah, 1994; Hartmann et al., 2009; Marschner et al., 2011) and provisioning substrates for fast-turnover rhizosphere microorganisms (Phillips et al., 2012). In forest ecosystems, root exudates represent 1–5% of net primary production (Grayston et al., 1997) and may increase by up to 50% in response to atmospheric CO2 enrichment (Phillips et al., 2011). Hence, root exudates are likely to play an increasingly important role in influencing ecosystem C and nutrient dynamics under global climate change by enhancing nutrient release and promoting feedbacks to plant productivity (Drake et al., 2011). Despite recent recognition of the ecosystem consequences of changing exudation patterns (Frank & Groffman, 2009; Gärdenäs et al., 2011), substantial knowledge gaps remain owing to challenges associated with identifying mechanistic linkages between roots, soils, and microbial communities.

Previous investigations have mostly considered exudation as a plant physiological process mediated by abiotic factors such as light, elevated CO2, soil fertility, and temperature (Neumann & Römheld, 2007). And while it has long been known that exudates play a central role in structuring soil microbial communities (Rovira, 1965; Graham et al., 1981; Garbaye, 1991; Broeckling et al., 2008), most exudation studies have been conducted in sterile hydroponic systems, with limited consideration of how soil microorganisms influence this flux (Jones et al.,
In the few studies of exudation in nonsterile systems, roots are generally colonized by single fungal taxa or simple 'communities' comprised of a few easily cultured taxa (Leyval & Berthelin, 1993; van Schöll et al., 2006; Fransson & Johansson, 2010). Ectomycorrhizal (ECM) fungi are strong sinks for C (Jones et al., 2004), and differences in ECM taxa can have differential effects on the amount and chemical composition of exudates released (Ingham & Molina, 1991; Rosling et al., 2004; Fransson & Johansson, 2010). However, most studies of the effects of ECM fungi on exudation have used fungal taxa that are easy to isolate and culture in vitro (e.g. Hebeloma, Laccaria, Paxillus, Suillus). Thus, we have an incomplete view of how indigenous ECM fungi influence exudation rates in forest soils. This knowledge gap has hindered our ability to predict whether changes in environmental drivers will indirectly affect exudation owing to shifts in mycorrhizal communities (Avis et al., 2007; Högberg et al., 2007; Lagomarsino et al., 2007).

In addition to ECM fungi, most roots are colonized by a diverse assemblage of other fungi, including pathogens and saprotrophs (Kent & Triplett, 2002). Plants respond to pathogen attack, for example, by releasing defense compounds (oxalic acids, phytoalexins, proteins, and other yet unknown substances) as exudates. While it has long been known that exudates provide substrates for root pathogen germination and activity in the rhizosphere of agronomic plants (Hiltner, 1904; Schroth & Hildebrand, 1964; Lim & Lockwood, 1988; Lynch & Whipp, 1990; Nelson, 1990; Steinkellner et al., 2007), few studies have examined the role of pathogens on exudation in tree roots and even fewer have considered the degree to which pathogens interact with ECM fungal communities to influence exudation.

In a recent in situ investigation of exudation in a loblolly pine (Pinus taeda L.) plantation in North Carolina, USA, Phillips et al. (2011) reported variation in mass-specific exudation rates from pine roots ranging three orders of magnitude (Phillips et al., 2008, 2011). Because the vast majority of roots at this site are colonized by ECM fungi – and single root systems are often colonized by a diverse suite of fungi (Hersh et al., 2012) – much of the variation in exudation rates may arise from differences in root-associated fungal communities. The goal of this experiment is to investigate the degree to which diverse assemblage of indigenous fungi influences exudation rates in loblolly pine. We used a novel experimental technique to promote the colonization of seedlings by similar fungal taxa as mature trees in the forest and measured exudation rates from roots growing in glass beads. We hypothesized that seedlings colonized by ECM taxa, which dominate in nutrient poor soils, would have high exudation rates and that seedlings colonized by ECM species found in fertile soils would have low exudation rates as a consequence of a lower necessity to acquire poorly soluble nutrients from these soils. Further, we hypothesized that seedlings colonized by the most diverse suite of fungal species would have the lowest exudation rates (niche complementarity hypothesis; sensu Tilman et al., 1997) owing to a more complete utilization of exudates by the root-associated microorganisms, that is, there is a higher probability of the occurrence of species that have elevated mycelial production and act as sinks for root carbon. This is the first study, to our knowledge, to examine exudation rates in tree roots colonized by diverse indigenous soil microorganisms.

Materials and methods

Seedling inoculation

Soils were collected from the Duke Forest free-air carbon enrichment (FACE) experiment, North Carolina, USA, in October 2009. At this site, a loblolly pine (P. taeda L.) plantation was established in 1983 following clear cutting and burning. Pines occupy > 80% of the basal area, and nearly all pine roots are colonized by ECM fungi or nonmycorrhizal fungal associates (Parrent et al., 2006). Most of the hardwood tree species that have formed in the understory form associations with arbuscular mycorrhizal fungi (e.g. Liquidambar styraciflua, Ulmus alata, Cornus florida, Acer rubrum, Cercis canadensis). The Duke FACE experiment consisted of eight randomly selected 30-m-diameter plots, with four control plots and four treatment plots that received elevated atmospheric CO2 concentrations for 14 years (August 1996 to October 2010, c. 200 μmol mol−1 in excess of ambient). Since 2005, nitrogen fertilizer (NH4NO3) was added to half of each plot in a single dose in the spring of every year at the rate of 11.2 g N m−2 a−1.

We collected intact, that is, undisturbed soil cores (5.1 cm in diameter) from the upper 15 cm of surface soils at random locations in each subplot at the site (2 CO2 × 2 N × 4 replicates; n = 16 subplots). Five replicate cores were collected from each subplot. Soil cores were kept intact in Plexiglas tubes, sealed in plastic bags and transported on ice to the greenhouse. Five loblolly pine seeds (surface sterilized in 1% bleach solution and cold stratified at 4 °C for 3 weeks) from a first generation orchard mix from the North Carolina Piedmont (NCSU seed lot #SOM8) were sown into each intact core. This facilitated the colonization of pine roots with diverse root-associated fungi from the forest soil (cf. Avis & Charvat, 2005). Soil cores in covered Plexiglas tubes were set up in a randomized block design in a climate chamber. A 14-h diurnal photoperiod was maintained in each chamber with cool-white fluorescent lamps.
(c. 120 μmol m⁻² s⁻¹). The chamber temperature and relative humidity averaged 23/17 °C day/night and 75%, respectively. All cores were irrigated periodically and rotated within the growth chamber. After 4 weeks, pine seedlings were moved to a greenhouse until the end of this experiment in February 2010 (14 weeks after pine seedling germination). The photoperiod in the greenhouse chamber was maintained at 14 h with high-pressure sodium lights (c. 500 μmol m⁻² s⁻¹ PAR). We selected one healthy pine seedling per soil core for later analyses (n = 80 total).

**Root exudate collection**

Root exudates were collected after transferring seedlings from pots into cuvettes filled with 750-μm-diameter glass beads (Phillips et al., 2008). A single pine seedling was removed from each core, and all soil adhering to the root system was carefully removed with deionized water and fine forceps to maintain the integrity of the mycorrhizal root tips. We took extreme caution to ensure that the fungal mantle and hyphal strands were maintained during this process although some hyphal severing inevitably occurred. Seedlings were then placed into root cuvettes filled with sterile, acid-washed glass beads moistened with C-free nutrient solution (0.5 mM NH₄NO₃, 0.1 mM KH₂PO₄, 0.2 mM K₂SO₄, 0.15 mM MgSO₄, 0.3 mM CaCl₂). In this solution culture system, the glass beads provided the mechanical impedance and porosity of soils but in a matrix free of C. Sterile cuvettes with beads and nutrient solutions (i.e. no plants) were included as controls. Seedlings were allowed to equilibrate in the cuvette environment for 24–48 h before being flushed with dilute nutrient solution using a low-pressure vacuum pump. Trap solutions containing exudates were collected from each cuvette, filtered through sterile syringe filters (Whatman Glass Microfiber Filters, Grade GF/F) and frozen at −20 °C. We added new nutrient solution, equilibrated for 24 h and repeated the trap solution collection two more times. Trap solutions were analyzed for dissolved organic C and total N on a nonpurgeable organic carbon and total nitrogen analyzer (Shimadzu TOC-VCPN and TNM-1; Shimadzu Scientific Instruments, Columbia). Net mass-specific exudation rates (gross root and hyphal exudation minus reabsorption and microbial consumption) were calculated as the total amount of C flushed from each root system over the incubation period divided by the total root mass (μg C g⁻¹ h⁻¹).

**Root vitality test**

To examine whether placement of roots into cuvettes influenced root vitality and exudation rates, we measured triphenyltetrazolium chloride (TTC) in root tissue at the beginning and end of the 7 day incubation period. For the TTC test, we added 1.5 mL of TTC buffer solution (0.1 M potassium phosphate buffer, pH 7.0, with 0.6% TTC and 0.05% Tween 20) to c. 45 mg of fresh root tissue (cf. Ruf & Brunner, 2003). Samples were put in a vacuum for 45 min to support the infiltration of the TTC buffer and were subsequently incubated for 15 h at 30 °C in the dark. During this time, colorless TTC was reduced to red triphenyl-formazan depending on the activity of the mitochondrial respiratory chain. After incubation, the buffer solution was removed and the roots carefully rinsed with DDI water. Samples were centrifuged for 2 min at 10 000 g, and the remaining supernatant water was removed. The root tissue was ground with 1 mL of 95% ethanol, centrifuged (10 000 g, 2 min), and the supernatant analyzed at 520 nm on a microplate reader (SpectraMax 190 Absorbance Microplate Reader; Molecular Devices, Sunnyvale). We used boiled roots as a control. The reactivity of the samples with TTC was calculated from the absorption of triphenyl-formazan per g dry mass (A₅₂₀ g⁻¹).

**ECM colonization and molecular identification of root-associated fungi**

After the exudate collection, we quantified the degree of ECM colonization on 50 root tips per seedling by examining the size, color, and morphology of fungal structures (mantle type and extra-radical hyphae) found on root surfaces (Agerer, 1991; Goodman et al., 1996). Additionally, from a randomly selected subset of the plants (n = 37), 16 ECM root tips per plant were randomly collected under a dissecting microscope, by dividing the total root system into eight even sections and picking the first two ECM tips from each section. Each tip was individually placed into a well of a 96-well plate for DNA analysis. DNA was extracted from each sample with REDExtract-N-Amp Plant PCR Kit (Sigma, St. Louis). We used the primers ITS1-F and ITS4 (Gardes & Bruns, 1993) to amplify the ribosomal internal transcribed spacers (ITS1 and ITS2) and 5.8S ribosomal RNA gene by PCR following Avis et al. (2008). Positive amplicons were excised and purified by GELase Agarose Gel-Digesting Preparation (Epicentre Biotechnologies, Madison), then cycle sequenced using Big Dye version 3.1 and screened on a 3730 DNA Analyzer (Applied Biosystems, Carlsbad) at the Pritzker Laboratory for Molecular Evolution and Systematics at the Field Museum of Natural History in Chicago, Illinois, USA. Sequence length and quality were then examined using SEQUENCHER 3.0 (GeneCodes, Ann Arbor). Sequences were compared to those in GenBank by BLAST analysis to check for matches to fungi found in...
four previous studies conducted at Duke Forest (O’Brien et al., 2005; Parrent et al., 2006; Parrent & Vilgalys, 2007; Hersh et al., 2012).

Fungal identification based on these sequences was made in two ways. First, the BLAST comparisons performed to find matches to previously identified Duke Forest fungi were also used to identify a sequence to genus or family in most cases. This approach was called ‘Max OTU’ as the operational taxonomic units (OTU) were generally inclusive for a given lineage. Because this approach could use nearly all of the sequences generated (even short, sometimes poor quality sequences) but was conservative, it lumped closely related but different taxa (e.g. species of the same genus). To determine how many additional taxa were in the MaxOTU, we also used a more exclusive approach on a subset of sequences which excluded shorter, poorer quality sequences. To do this, we used CLUSTALW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to align sequences within the same genus (or family if a genus-level distinction was not made) and then conducted phylogenetic analyses on these alignments using maximum likelihood employed by RAXML-HPC2 7.2.8 (Stamatakis, 2006; Stamatakis et al., 2008) via the CIPRES portal (www.phylo.org/sub_sections/portal/). Operational taxon units (OTU) determined as terminal clades with > 70 fast bootstrap support. This approach resulted in more exclusive groups and was referred to as ‘Min OTU’. The trophic status or nutritional mode for each OTU (e.g. ECM, pathogen, and saprotroph) was assigned based on previous studies (Parrent & Vilgalys, 2007; Hersh et al., 2012) and known phylogenetic relationships.

**Biomass production and root and shoot morphology**

Tree seedlings were divided into aboveground and belowground biomass. Roots were sorted by diameter (fine roots < 2 mm, coarse roots > 2 mm). Fine root and leaf area measurements were conducted by optical surface measurement with a camera and scale and the computer programs WINRHIZO (Régent Instrument, Canada) and IMAGEJ 1.43 (Wayne Rasband), respectively. After analysis, leaves, stems, coarse roots, and fine roots were dried (48 h, 70 °C) and weighed.

**Statistical analyses**

Statistical analyses were conducted with the SAS, version 9.2 (Statistical Analyses System; SAS Institute Inc., Cary), Canoco for WINDOWS 4.5 (Biometrics – Plant Research International, Wageningen, the Netherlands) and r (http://www.R-project.org). ECM colonization was calculated as percentage of root tips colonized per 50 root tips examined and multiplied by the number of root tips per plant and normalized by arc sine square root transformation prior to statistical analyses. Probability of fit to normal distribution was tested by a Shapiro–Wilk test. In the case of Gaussian distribution, means were compared with a one-factorial ANOVA followed by a Scheffe test to analyze differences (in case of unbalanced data, the PROC GLM procedure was used). The datasets deviating from normal distribution were compared by one-way Kruskal–Wallis single-factor analyses of variance and nonparametric multiple comparison tests after Wilcoxon to locate the differences. To analyze for covariance, a generalized linear model was applied to root exudation rates using the frequency of root infection with pathogen fungal species as the main effect and the number of root tips as a covariate. Additionally, the influences of treatment at soil origin (CO₂ treatment at soil origin; N treatment at soil origin) or spatial location (Duke FACE ring; quadrant of Duke FACE ring) on root exudation were assessed with a four-factorial analysis of variance. The composition of the root-associated fungal community was analyzed by principal component analysis (PCA), with root parameters as environmental variables. We applied linear regression analyses to quantify the influence of the most important PCA axes on various root characteristics. For analyses of fungal response to treatments, we used the ‘lm’ then ‘anova’ function in R to see whether fungal OTU richness was affected by treatment. A chi-squared analysis was performed to check for any responses to treatment by individual OTU. We refer to a P value of 0.05 as statistically significant.

**Results**

A total of 608 root tips from 37 seedlings were examined in this study. Most of the fungi we found were ECM basidiomycetes (e.g. Atheliaceae, Tomentella, Thelephoraceae) followed by pathogens (e.g. Cylindrocarpon) and ascomycetes (e.g. Wilcoxia; Supporting Information, Table S1, S2). We found ECM fungi on the roots of all seedlings but pathogens or saprotrophs on less than half (Table S2). Seventy-two percent (315/438) of the recovered sequences and about 50% of the OTU matched those from previous field-based studies in the Duke Forest (Table S2). Many of the pathogens have previously been found at this site, with species of Cylindrocarpon being abundant (Hersh et al., 2012). Almost 40% of the seedlings of our study were infected with pathogens. Although we found some absolute (totals) differences in the number of OTU per CO₂ or N treatment (Table S2), these differences were not statistically significant (Table S3a). We did not detect significant responses by any individual OTU to the treatments.
Placement of roots into cuvettes had no negative effects on the vitality of loblolly pine roots as measured by TTC reactivity. Dead roots by contrast had significantly decreased reactivity ($P < 0.0001$). Moreover, there was no carry-over effect of the CO2 or N treatment at soil origin on root exudation of loblolly pine seedlings (Table S3b). Hence, all samples were treated as individual samples independent from treatment at origin. Overall, mass-specific exudation rates varied by several orders of magnitude and were associated with several aboveground and belowground variables (Table 1). Mean exudation rate was $32 \mu g C g^{-1} h^{-1}$, which is slightly higher than in other investigations (Johansson et al., 2008; Phillips et al., 2009, 2011; Fransson & Johansson, 2010). In general, pine seedlings with the highest exudation rates had the least root mass and root areas, although leaf mass and leaf areas were not affected (Table 1).

Root-associated fungal identity had strong effects on net exudation rates. Exudation rates increased by two times when loblolly pine seedlings were infected by putative pathogen, saprotroph, and ECM taxa, as opposed to pine seedlings that were colonized by ECM taxa only (Fig. 1a). Loblolly pine seedlings with high pathogen or saprotroph colonization had low ECM colonization (Fig. 1b). Root exudation decreased exponentially with the number of fungal root tips (Fig. 1c). An analysis of covariance applied to the exudation data, using the percentage of pathogen infection as the categorical variable and number of root tips as a continuous covariate, demonstrated that the effect of root pathogen infection was highly significant, even when controlled for the number of root tips. The number of root tips was a significant covariate, but with a negative coefficient: the higher the frequency of pathogen infection, the lower the number of root tips and the more C was released by exudation. Accordingly, a regression analysis revealed an exponential relationship between the numbers of fungal root tips and fine root exudation rate of loblolly pine seedlings infected with either ECM fungi (•) or with ectomycorrhizas and root-associated fungal pathogens and saprotrophs (RF (○); root exudation $= 19 + \exp \left( -0.01 \times \text{(root tips} - 393) \right)$; $n = 37$).

**Table 1.** Pearson correlation coefficients for the relationship between mass-specific root exudation ($\mu mol C g^{-1} h^{-1}$) and several leaf and root characteristics of loblolly pine (Pinus taeda L.) seedlings inoculated with different root-associated fungal communities ($n = 80$)

<table>
<thead>
<tr>
<th>Root exudation</th>
<th>$r$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant mass (g)</td>
<td>$-0.27$</td>
<td>*</td>
</tr>
<tr>
<td>Leaf mass (g)</td>
<td>$-0.18$</td>
<td>ns</td>
</tr>
<tr>
<td>Leaf area (cm$^2$)</td>
<td>$-0.17$</td>
<td>ns</td>
</tr>
<tr>
<td>Root mass (g)</td>
<td>$-0.37$</td>
<td>**</td>
</tr>
<tr>
<td>Root area (cm$^2$)</td>
<td>$-0.36$</td>
<td>**</td>
</tr>
<tr>
<td>Number of root tips (n)</td>
<td>$-0.34$</td>
<td>**</td>
</tr>
<tr>
<td>Root/shoot ratio (g g$^{-1}$)</td>
<td>$-0.40$</td>
<td>**</td>
</tr>
</tbody>
</table>

Significant differences at $P \leq 0.01$ and 0.05 are marked with ** and *, respectively.

Fig. 1. (a) Mean and standard error of NPOC root exudation rate of loblolly pine (Pinus taeda L.) seedlings infected with either ECM fungi or with ectomycorrhizas and root-associated fungal pathogens and saprotrophs (RF; $F = 5.2$, $n = 37$). The associated table shows the F-value and associated probability of error $P$ from an analysis of covariance for root exudation rate, using frequency of root infection with pathogen fungal species as the main effect and number of root tips as a covariate. (b) Relationship between the percentage of fungal root tips and percentage of pathogen and saprotroph root tips of loblolly pine seedlings inoculated with soil from different origins of the Duke FACE experiment (RF $F = 49.0$, ECM; pathogens: $r = 0.81$, $P < 0.001$; saprotrophs: $r = 0.82$, $P < 0.001$). (c) Relationship between the numbers of fungal root tips and fine root exudation rate of loblolly pine seedlings infected with either ECM fungi (•) or with ectomycorrhizas and root-associated fungal pathogens and saprotrophs (RF (○); root exudation $= 19 + \exp \left( -0.01 \times \text{(root tips} - 393) \right)$; $n = 37$).
negative relationship between exudation and the number of ECM root tips ($r = 0.40, P = 0.01$).

Using a PCA, we identified three main gradients in the composition of the fungal community (Fig. 2): on the first PCA axis, potentially pathogen and saprotrophic fungal taxa had high loadings (Cochliobulus, Trichoderma, Oidiodendron, Mortierella, and Cylindrocarpon), which explained 42% of the total variance. This first axis was highly significantly correlated with root mass–based exudation rate ($r = 0.44, P < 0.01$; Table 2). Root/shoot ratio and root mass were opposite to root exudation on the first PCA axis. Accordingly, root mass ($r = -0.37, P = 0.001$) and root/shoot ratio ($r = -0.40, P = 0.001$) decreased as exudation rate increased (Table 1). The second PCA axis explained another 23% of the total variance of the community composition (taxa with high loading: Piloderma, Inocybe, and uncultured Ascomycete; Fig. 2). The fungal community composition explained by the second PCA axis was only marginally significantly correlated with exudation ($r = 0.29, P = 0.08$; Table 2) and did not influence other root characteristics. The third PCA axis explained 12% of the variance in the fungal community composition (total explained variance by the first three PCA axes 76%) and was mainly influenced by the pathogen fungus Chytridiomycota and the ECM Rhizopogon (Fig. 2). This portion of the fungal community influenced the specific root area of pine seedlings but not root exudation.

### Table 2. Pearson correlation coefficients for the relationship between the PCA axes (cf. Fig. 2) and root mass–based root exudation of loblolly pine (Pinus taeda L.) seedlings inoculated with different root-associated fungal communities ($n = 37$)

<table>
<thead>
<tr>
<th>Root exudation</th>
<th>PCA axis 1: Pathogens</th>
<th>PCA axis 2:</th>
<th>PCA axis 3:</th>
</tr>
</thead>
<tbody>
<tr>
<td>$r$</td>
<td>0.44</td>
<td>0.29</td>
<td>-0.06</td>
</tr>
<tr>
<td>$P$</td>
<td>0.01</td>
<td>0.08</td>
<td>ns</td>
</tr>
</tbody>
</table>

### Discussion

Investigations on the influence of indigenous forest fungal communities on tree root exudation rates are exceedingly rare. Here, we report a strong relationship between root-associated fungi and exudation rates, as root systems with the greatest percentage of ECM tips had the lowest net mass-specific exudation rates, and roots with putative pathogenic and saprotrophic taxa had exudation rates twofold greater than those not containing these taxa. Collectively, these results suggest that root-associated fungi differ in their effects on or response to exudation rates, depending on whether they are mutualists or pathogens.

### Methodological considerations

The method we used to culture fungi on seedlings appeared to simulate field conditions to a reasonable extent. We recovered a large number of fungi that had previously been found in field studies at this site, and the most abundant taxa found in our study corresponded to nearly the same abundant taxa as those found by Parrent et al. (2006) and Parrent & Vilgalys (2007) (i.e. Russula, Tylospora, which is in the Athelicaeae as we have treated it and the thelephoroid clade that we have treated as Thelephoraceae). In addition, many of these fungi (e.g. Russula, Inocybe) are considered ‘late-stage’ (Deacon & Fleming, 1992), typical of mature habitats and hard to culture by other commonly used culture methods. We also regularly found Cylindrocarpon/Neonectria, the most impactful pine seedling pathogen at this site (Hersh et al., 2012).

Root exudates are frequently collected in aerated trap solutions or by percolating solution through a C-free matrix such as sand or glass beads (Neumann & Römheld, 2007). While these methods reduce the likelihood that exudates will be reacquired by roots (common for some amino acids; cf. Phillips et al., 2004) or metabolized by rhizoplane microorganisms, there is a trade-off in that vital interactions between roots, soil particles, and microorganisms are absent. For example, the lack of mechanical impedance to root growth in liquid media may decrease...
exudation (Boeuf-Tremblay et al., 1995; Grolleau-Renaud et al., 1998), and the use of axenic culture systems may underestimate exudation given microbial-induced stimulation of exudation (Meharg & Kilham, 1991, 1995; Phillips et al., 2004). Further, exudate collection from soil by percolation or by the use of adsorption media often leads to incomplete leaching of certain exudate compounds (Neumann & Römheld, 2007).

We chose a combination of the two approaches by inoculating pine seedlings in forest soil and transferring them to sterile glass beads for the collection of trap solutions. This method has the advantage of recovering indigenous fungal communities while growing seedlings with natural mechanical forces in soils. Although we cannot rule out that some root cells and mycelia may be damaged during the transfer – a process that would likely increase net C flux from roots – such artifacts would not be biased toward a specific group of fungal taxa and thus should have little impact on our general findings. Under these conditions, collection time is also an important factor for the recovery of root exudates as affected by microbial consumption and reuptake of exudates by roots (Jones et al., 2004; Personeni et al., 2007). In previous experimental tests, an incubation period of 24 h has proven to result in an optimum carbon accumulation with the associated advantage of spanning a diurnal cycle (Phillips et al., 2008). As such, we are confident that our exudation patterns reflect differences related to the root-associated fungal communities and not experimental artifacts.

**Effects of CO₂ and N on fungi**

We found no effects of CO₂ and N on fungal richness or the frequency at which we found individual fungal OTU. In part, this is similar to what was found in the field by Parrent et al. (2006) and Parrent & Vilgalys (2007) where the primary responses to these treatments were by individual taxon response and only four (of 72 total) of the most abundant taxa responded to N fertilization. Although we did not see any significant shifts in taxa response, the trend of more frequent *Russula* in fertilized plots was consistent with *Russula* G in Parrent et al. (2006) and an increasing appreciation of certain nitrophilic *Russula* taxa (Avis, 2012). The reasons we may not have seen responses to N especially given how often N is a major factor that structures soil fungal communities (Lilleskov et al., 2011) may be a seasonal effect or that our sample sizes were small.

**Influence of mycorrhizal fungi on exudation**

ECM fungi have long been hypothesized to reduce root exudation rates (Marx, 1972; Graham & Menge, 1982; Harley & Smith, 1983; Graham & Egel, 1988) but there have been few investigations of tree roots colonized by diverse fungal communities. ECM fungi form a fungal sheath around root tips, which retards apoplastic movement of ions into the root cortex (Behrmann & Heys, 1992; Bücking et al., 2002) depending on the conditions of the ECM tip (Vesk et al., 2000). Likewise, the exudation of low molecular weight organic compounds from the elongation zone directly behind root tips is likely to be hindered by ECM sheath formation (Neumann & Römheld, 2007). However, because ECM fungi also exude compounds such as oxalic acid from hyphal tips (Ahoenen-Jonnarth et al., 2000; Sandnes et al., 2005), the total amount of C exuded to soils from colonized roots may be equal to or greater than in uncolonized roots. In our study, seedlings with a greater percentage of ECM root tips had lower mass-specific exudation rates, consistent with the response of other *Pinus* seedlings (Leyval & Berthelin, 1993; Fransson & Johansson, 2010). Importantly, these effects were unrelated to ECM community composition (Fig. 2) indicating that the identity of the mycorrhizal fungi had little effect on exudation. This result is surprising given previous investigations that have used artificially inoculated trees and have reported that individual, early successional (i.e. easy to culture) ECM taxa strongly influence both the quantity and chemical composition of exudates in several tree species (Krupa et al., 2013, 2012 Federation of European Microbiological Societies Published by Blackwell Publishing Ltd. All rights reserved)

**Relationship between exudation and root pathogens and saprotrophs**

We found that pine seedlings colonized by putative pathogens and saprotrophs exuded twice as much C as uninfected pines. We suggest three possible reasons for high exudation rates in pathogen-infected roots. First, the presence of pathogens and saprotrophs on the root surface may induce exudation, as plants infected by pathogens increase exudation to communicate with rhizosphere bacteria or stimulate ECM fungi to produce defense substances. Barley has been found to communicate with root-associated bacteria by exuding diffusible molecules, which increase the antifungal activity of bacteria (Jousset et al., 2011). Likewise, ECM roots can suppress root pathogens by producing oxalic acids (Duchesne et al., 1989; Kope et al., 1991) and extracellular enzymes such as chitinases and glucanases as defenses against pathogens.
Thus, the enhanced exudation rates may be an induced response to the presence of the pathogen on the root surface.

Second, it is also possible that seedlings that have low ECM colonization rates leak more C, and as such, indirectly increase pathogen activity on the root surface. In this way, roots with low degrees of ECM colonization would use less carbon to build up fungal structures, that is, the fungal sheath and release more of the volatile or water-soluble molecules (e.g. glucose, Ca\(^{2+}\), ethanol, amino acids, organic acids, and lipids) that attract pathogens (Lim & Lockwood, 1988; Nelson, 1990). After recognition, fungi can direct their zoospore movement or hyphal growth into the direction of the exudation source (Deacon & Donaldson, 1993; Ruttledge & Nelson, 1997; Tyler, 2002; van West et al., 2002). Thus, high exudation rates can affect pathogens directly by raising their germination, nutritional status before penetration or their saprotrophic and pathogenic activities (Schroth & Hildebrand, 1964).

A third possibility is that the enhanced exudation rates were caused by pathogen attack of the root cortical tissue, degradation of plant cell walls and destruction of the root phloem (necrotrophic pathogen invasion). In our investigation, the presence of root pathogens and saprotrophs did not affect shoot biomass, but did reduce C allocation to root production in favor of root exudation. Exudation rates were positively correlated with potential root respiration (as measured by a TTC test of root vitality), that is, roots infected by pathogen and saprotroph fungi released more carbon from their more vital root system \((r = 0.63, P = 0.02)\). Decreased root tissue density and increased specific root area (SRA) and root respiration of these plants are typical for lower root orders of shorter longevity and faster turnover (Eissenstat et al., 2000; Valenzuela-Estrada et al., 2008). Likely, the described nonmycorrhizal fungi of our study were biotrophs, which did not deconstruct the root tissue, but grew between host root cells and invaded only a few with nutrient-absorbing haustoria.

Some ECM taxa (Cantharellaceae, Russula and Thelephoraceae) tolerated and benefitted from the presence of these pathogen and saprotroph species as their percentage root inoculation increased when nonmycorrhizal species were present. Others (Tomentella, Atheliacae, Wilcoxina) were impacted by the presence of putative pathogen and saprotroph species or were absent (Laccaria, Inocybe).

We conclude that exudation studies conducted in sterile hydroponic solutions or in nonsterile systems using only single fungal taxa or simple communities of easily cultured species give an incomplete view of the magnitude of this flux in field soils. In contrast to our hypothesis, the diversity of fungi had little effect on the amount of carbon exuded from roots. Rather, exudation rates were related to the level of mycorrhization and the presence of putative pathogens. As such, global change factors (e.g. N deposition, drought, etc.) that influence mycorrhizal colonization and the proliferation of pathogenic fungi will likely have consequences for soil biogeochemistry through their indirect effects on root exudation patterns.

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References


Fungal communities influence tree root exudation


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Taxonomic identification and trophic status of the identified fungal species colonizing loblolly pine (*Pinus taeda* L.) seedlings grown in soil from different origins of the Duke FACE experiment.

**Table S2.** Summary of operational taxonomic units (OTU) at two binning levels.

**Table S3.** (a) Significance of the effect of CO2 treatment at soil origin (CO2), N treatment at soil origin (N), quadrant of Duke FACE ring (LOC), and their interactions on operational taxonomic units (OTU) at two binning levels according to three-factorial analyses of variance (*n* = 37). (b) Significance of the effect of CO2 treatment at soil origin (CO2), N treatment at soil origin (N), Duke FACE ring (RING), quadrant of Duke FACE ring (LOC), and their interactions on the variance of root exudation according to four-factorial analyses of variance (*n* = 80).