Phenolic root exudate and tissue compounds vary widely among temperate forest tree species and have contrasting effects on soil microbial respiration

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Summary

- Root-soil interactions fundamentally affect the terrestrial carbon (C) cycle and thereby ecosystem feedbacks to climate change. This study addressed the question whether the secondary metabolism of different temperate forest tree species can affect soil microbial respiration. We hypothesized that phenolics can both increase and decrease respiration depending on their function as food source, mobilizer of other soil resources, signaling compound, or toxin.
- We analyzed the phenolic compounds from root exudates and root tissue extracts of six tree species grown in a greenhouse using high-performance liquid chromatography (HPLC). We then tested the effect of individual phenolic compounds, representing the major identified phenylpropanoid compound classes, on microbial respiration through a five-day soil incubation.
- Phenolic root profiles were highly species-specific. Of the eight classes identified, flavonoids were the most abundant with flavanols being the predominating sub-class. Phenolic effects on microbial respiration ranged from a 26% decrease to a 46% increase, with reduced respiration occurring in the presence of compounds possessing a catechol ring.
- Tree species variation in root phenolic composition influences the magnitude and direction of root effects on microbial respiration. Our data support the hypothesis that functional group rather than biosynthetic class determines the root phenolic effect on soil C cycling.

Keywords: phenolics, root priming, root exudates, rhizosphere, secondary metabolism, temperate forest tree species

Introduction

Plant roots create hotspots of biological activity in soil interacting with a diverse community of microorganisms, invertebrates and neighboring plants (Badri *et al.*, 2009; Philippot *et al.*, 2013). Recent evidence highlights that these root-soil interactions are responsible for up to one-third of the total carbon (C) mineralized in temperate forest soils and are crucial to understanding terrestrial biogeochemical cycles (Finzi *et al.*, 2015; Kuzyakov & Blagodatskaya, 2015). Because soil respiration is the primary means by which C fixed by plants returns to the atmosphere (Schlesinger & Andrews, 2000), studying the mechanisms by which roots alter soil CO_2 emissions is particularly important in order to improve global C models and estimate ecosystem feedbacks to climate change (Norby & Jackson, 2000; Drigo *et al.*, 2008; Phillips *et al.*, 2012; Warren *et al.*, 2015). Of particular interest is the question how plant roots within hours to days can turn bulk soil into a rhizosphere with distinctly different biological, chemical and physical characteristics (Hinsinger *et al.*, 2005). Yet, our knowledge of the mechanisms driving differences in rhizosphere biogeochemical processes commonly observed among different plant species, environmental conditions and seasons remains limited (Wang *et al.*, 2001; Chen *et al.*, 2002; Berg & Smalla, 2009; Suseela & Dukes, 2013).

Rhizodeposits, such as root exudates and decomposing tissue, are some of the key drivers in shaping the rhizosphere environment (McCully, 1999) and have been suggested as explanation for the observed species differences in rhizosphere C cycling (Cheng *et al.*, 2003; Dijkstra & Cheng, 2007; Bengtson *et al.*, 2012; de Graaff *et al.*, 2014). Because root products are comprised of an extremely diverse group of compounds (Badri & Vivanco, 2009; Haichar *et al.*, 2014), their chemical composition and associated effects on rhizosphere biochemistry and metabolism are challenging to study. To date, soil incubation experiments using simulated rhizosphere setups with bulk soil samples receiving artificial root exudate applications have mainly focused on primary metabolites like sugars and organic acids (Landi *et al.*, 2006; Kuzyakov *et al.*, 2007). These studies have demonstrated that roots, through different biotic and abiotic mechanisms, indeed influence the rate of soil organic matter (SOM) decomposition (Kuzyakov, 2010; Keiluweit *et al.*, 2015), a process termed “root or rhizosphere priming” (Kuzyakov, 2002; Dijkstra *et al.*, 2013). Yet, it is unclear whether root secondary metabolites act through similar mechanisms. While studies have analyzed relationships between root exudate quantity and soil biogeochemical processes (Dijkstra & Cheng, 2007; Drake *et al.*, 2011; Phillips *et al.*, 2011;

Cheng *et al.*, 2014), the role of root chemical composition in shaping the microbial and biochemical environment of the rhizosphere is poorly understood.

Reported priming effects range from a 79% reduction to a 500% increase in SOM decomposition rate (Huo *et al.*, 2017). While different priming mechanisms have been proposed and may simultaneously be at play (Blagodatskaya & Kuzyakov, 2008; Mason-Jones & Kuzyakov, 2017), most of these hypotheses have a common assumption: namely, that root substrates increase microbial respiration through an increased supply of available C and energy (Horvath, 1972; Fontaine *et al.*, 2004, 2011; Hamer & Marschner, 2005). However, the assumption that root exudates offer a readily available food source to microbes, thus increasing respiration, does not necessarily hold for secondary metabolites. In particular, compounds synthesized in the phenylpropanoid pathway have different modes of action in the rhizosphere. Depending on their chemical structure, concentration and environment, phenolic compounds can function as food source or toxin (Shaw *et al.*, 2006), influence enzyme activity (Salminen & Karonen, 2011), control nitrogen availability (Northup *et al.*, 1995; Schmidt-Rohr *et al.*, 2004), and act as signaling molecules (Haichar *et al.*, 2014). Moreover, recent evidence suggests that phenolic root exudates are responsible for shifting microbial community composition (Badri *et al.*, 2013). While these findings support the hypothesis that phenolics contribute to the wide range of root effects on soil C cycling reported in the scientific literature, studies have not tested root phenolic effects on microbial respiration.

Secondary metabolites are more species-specific than primary metabolites (Salminen & Karonen, 2011), which make them a more likely candidate to drive species variation in rhizosphere C cycling. Unfortunately, the challenge associated with root exudate collection and analysis has limited the number of studies on variation in root phenolic composition. Detecting low phenolic concentrations, minimizing damage to roots during sample collection, and limiting contamination from soil are only some of difficulties in root exudate analysis (Shi *et al.*, 2013). One of the few studies measuring tree root exudates reported qualitative and quantitative species differences in the sugars, organic acids and amino acids exuded by 18-day-old seedlings (Smith, 1969). Recent work identified different organic acids dominating the soil around silver birch versus Norway spruce roots (Sandnes *et al.*, 2005). Moreover, tree species associating with ectomycorrhizal fungi were found to exude higher amounts of total organic C than species colonized by arbuscular mycorrhizae, which also had an impact on rhizosphere biogeochemistry

(Yin *et al.*, 2014). While these findings confirm species differences in the amount and composition of root exudates, the role of secondary metabolites remains unclear. We know little about which phenylpropanoid classes and compounds dominate root exudate profiles (Cesco *et al.*, 2010), how diverse phenolic root exudates are, and to what degree we can detect tree species-specific profiles.

Studies focusing on root exudate characterization rarely link their results to the chemical composition of the fine root tissue, even though comparing root tissue and exudate compounds could enhance our understanding of root exudate transport. Moreover, research on how fine root traits influence soil biogeochemistry could enhance our general understanding of soil ecosystem functioning (McCormack *et al.*, 2017). Root turnover constitutes relatively large pulses of plant tissue into the soil (Nadelhoffer & Raich, 1992; Jackson *et al.*, 1997) and may act as an unexplored mechanism of root priming. Although studies have acknowledged fine root phenolics as an important factor influencing their own decomposition (Berg *et al.*, 1993; Silver & Miya, 2001), these characteristics have not been linked to external effects on SOM dynamics.

Overall, these remaining unknowns about tree species variation in root phenolics and the effect of secondary metabolites on microbial respiration limit the conclusions we can draw about the drivers of rhizosphere development and the mechanisms of root-facilitated C cycling in forest soils. Without more information about secondary metabolite diversity of root products, soil incubation experiments focusing on root priming are constrained to the few verified primary metabolites. Therefore, we examined the effect of root phenolic compounds released by two coniferous and four deciduous temperate forest tree species on microbial respiration using high-performance liquid chromatography (HPLC) and a five-day soil incubation with “plant-verified” phenolic compounds. We hypothesized that tree phenolic compounds have contrasting effects on soil microbial respiration, which is primarily driven by the phenylpropanoid class of the compound. While we expected phenolics functioning as food source, signaling compound or mobilizer of other soil resources to increase respiration, compounds with toxicity or anti-nutritive properties should suppress microbial activity. The results of this study demonstrate that root phenolic profiles are tree species-specific and that individual phenolic compounds can have divergent effects on soil microbial respiration.

Materials and Methods

Tree growing conditions

Two and three-year-old bare root seedlings (*N*=12) of *Acer saccharum* L. (sugar maple), *Fagus grandifolia* Ehrh. (American beech), *Pinus strobus* L. (eastern white pine), *Picea abies* L. (Norway spruce), and *Quercus rubra* L. *(*red oak), and transplants of *Alnus rugosa* L. (speckled alder) were planted in 4 L pots with turface (Profile Products LLC, Buffalo Grove, IL, USA) in a greenhouse with 16 hours of light at the Guterman Bioclimate Laboratory (Cornell University, Ithaca, NY, USA) from 23rd of March until the beginning of June. The temperature fluctuated from 20°C during the day to 17°C at night. Trees were watered to full water holding capacity twice a day and fertilized once a week with a 300 ppm nutrient solution (15N : 5P : 15K : 4Ca : 2Mg).

Prior to root exudate collection, root systems were thoroughly rinsed. As recovery from washing, trees were transferred to a hydroponic growing system for four days. This was done in two batches on the 3rd and 17th of June. Each batch included six replicates of each tree species. Each 53 L hydroponic tank (Centrex Plastics, LCC Rugged Tote, Lowe's, USA) was covered with a foam board (Kingspan Insulation Unfaced Polysterene Foam Board Insulation, Lowe's, USA) with six holes to hold trees of the same species. An airstone was placed in each tank and connected to a pump. Hydroponic growing medium consisted of a modified Hoagland solution containing 0.6 mM NH_4NO_3, 0.1 mM KH_2PO_4, 0.25 mM KNO_3, 0.2 mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.3 µM EDTA-Fe, 0.005 mM H_3BO_4, 0.001 mM $MnSO_4$, 0.1 µM $ZnSO_4$, 0.1 µM $CuSO_4$, and 0.015 µM $(NH_4)_6MO_7O_{24}$ (Benton Jones, 1997).

Root exudate collection and root tissue sampling

To collect phenolic root exudates, we used three 1 x 5 cm acetate cellulose strips (Sterlitech Corporation, Kent, WA, USA) that specifically adsorb phenolic compounds out of solution (Bolaños-Vásquez & Werner, 1997). After four days in hydroponics, trees were taken from their hydroponic tanks and placed into individual 950 mL mason jars containing the acetate cellulose strips and 1 mM $CaCl_2$ and 5 mM MES buffer solution. To test whether microbial degradation of the compounds occurs during exudate collection, we also added 0.01 g L^{-1} Micropur (Katadyn®, Switzerland) to sterilize the collection solution for half of the samples (Oburger *et al.*, 2014; Schindlegger *et al.*, 2014). After 24 hours, acetate cellulose membranes were removed, rinsed

with water and extracted twice with 3 mL 100% methanol and once with 3 mL 90% methanol. For each extraction, strips were incubated with methanol solution in a 15 ml Falcon tube, vortexed for 10 seconds, left in solution for 10 minutes, and then vortexed for another 10 seconds. The resulting methanol extracts were evaporated to dryness at 32°C under vacuum (CentriVap Concentrator and Cold Trap, Labconco, Kansas City, MO, USA), re-dissolved in 100 µL 90% methanol, and stored in a -80°C freezer until HPLC analysis.

After root exudate collection, 0.5-1 g of fresh absorptive roots were randomly selected, rinsed and frozen for each tree replicate. Roots were freeze-dried and milled with a Thomas Wiley Mill to mesh size 60 (Thomas Scientific, Swedesboro, NJ, USA). Phenolic compounds were extracted from 20-30 mg of the root tissue samples using a FastPrep tissue homogenizer (MP Biomedicals® LLC, Santa Ana, CA, USA) at 6.5 m s^{-1} for 90 seconds with 0.9 g grinding beads (Zirconia/Silica 2.3 mm, Biospec® Products Inc., Bartlesville, OK, USA) and 1 mL of 90% methanol. Subsequently, samples were centrifuged at 4°C for 15 min at 20,931 g force (Centrifuge 5804 R, Eppendorf, Hauppauge, NY, USA). The supernatants were transferred to crimp vials with PTFE/rubber septa and analyzed for phenolic compound composition.

Phenolic compound analysis and biosynthetic class assignment

15 µL aliquots of root exudate and root tissue extracts were analyzed for phenolic composition using HPLC (Hewlett Packard1100 Series, Agilent Technologies, Santa Clara, CA, USA) on a XDB-C18 column (4.6 mm x 250 mm, ZORBAX Eclipse XDB, Agilent Technologies, Santa Clara, CA, USA). We modified the analysis from a method for identifying alkaloids, phenolics and diterpene glycosides (Keinänen *et al.*, 2001) with the solvents (A) 0.25% H_3PO_4 in water (pH 2.2) and (B) acetonitrile. Elution system was as follows: 0-4 min, 5-5% of B, 4-24 min, 5-60 % of B, and 24-34 min, 60-95% of B with a flow rate of 0.7 mL min^{-1}. Absorption spectra were measured at 210, 254, 280, 320, and 360 nm.

Retention times and UV-vis spectra of peaks found in the chromatograms of the six tree species were compared to 36 different standards from ten phenylpropanoid and diterpene classes and seven flavonoid sub-classes (Table S1). For each chromatogram, we used the total number of peaks and total peak area in absorption units (AU) as measures of root exudate diversity and abundance. In addition, we determined the phenylpropanoid class of each identified chemical peak by fitting the spectra of the standards to the sample spectra using Bio-Rad KnowItAll®

2017 Spectroscopy Software (Bio-Rad Laboratories, Philadelphia, PA, USA). A phenylpropanoid class or flavonoid sub-class was assigned (1) if the highest fit had a Hit Quality Index (HQI) above 95 out of 100, (2) if the HQI values of the three first hits were above 85 and from the same class or (3) if the highest fit had a HQI value above 90 and passed visual inspection. The chemicals that did not pass this test were compared to each other. If the unknown spectra fit to others with a HQI value of 95 or higher and formed a group of more than three members, we assigned them to a separate group. A total of three groups with similar absorption spectra were formed (Fig. S1). All other chemicals were assigned to the unknown group without matching criteria. We used the number of classes per root exudate or tissue sample as a measure of plant biosynthetic diversity.

Soil microbial respiration

To analyze phenolic effects on microbial respiration, we added individual compounds from the identified biosynthetic classes to a forest soil and measured soil CO_2 emissions over a five-day period. This approach is similar to the experimental setup of other soil incubation studies that created an artificial and simplified rhizosphere to study the effect of single root exudates on soil microbial activity and SOM decomposition (Renella *et al.*, 2006; Keiluweit *et al.* 2015), except that we added the chemicals as solids to an air-dried soil at the start of the experiment due to their insolubility in water. Soil was collected from the first 0-15 cm mineral soil in the Arnot Forest (Central NY, USA). The specific area of our collection was dominated by *A. saccharum, F. grandifolia* and *P. strobus*. Total organic C was 4.6 g kg^{-1} soil and total organic matter measured with loss on ignition was 10.5%. Total soil nitrogen and phosphorus were 0.34 g kg^{-1} soil and 12.6 mg kg^{-1} respectively. The cation exchange capacity was 16.47 mol_c kg^{-1} with exchangeable cations at levels of 211 mg Ca kg^{-1}, 767 K mg kg^{-1}, 279 mg kg^{-1} and 589 mg Na kg^{-1} soil. Soil pH was 4.5 in water. Prior to the respiration test, the soil was air-dried and sieved (< 2mm).

We tested a total of nine compounds from seven different biosynthetic classes (*N*=8) representing the biochemical spectrum of compounds in our samples, including benzoic acid (benzoic acid-derivative), caffeic acid (cinnamic acid-derivative), arbutin (chalcone), catechol (simple phenol), resveratrol (stillbene), and abietic acid (diterpene). Since flavonoids were the predominating class identified, we included catechin (flavanol), naringenin (flavanone) and

taxifolin (flavanonol) to represent the three flavonoid sub-classes exuded by the tree seedlings' roots. In addition to the root compounds, glucose as a positive control and "no addition" as a baseline were included as treatments.

The phenolic, diterpene and sugar compounds were added as dry solids to the air-dried forest soil. The compound application rate of 1 mg-C g^{-1} soil was based on the fine root concentration of catechin, which ranged from 0.05 to 2.8 mg-C g^{-1} tissue. First, we prepared one large single sample by adding the soil and respective treatment compound in a jar, which was thoroughly shaken and horizontally rolled back and forth on a lab bench for thirty times. The control was prepared similarly but did not receive any compounds. Secondly, 2 grams of the soil-compound mixtures were individually weighed out as replicates into 20 mL glass scintillation vials. At the start of the experiment, water was added to 60% water holding capacity and the scintillation vials were placed in 237 mL mason jars (Newell Brands, Hoboken, NJ, USA) together with a vial containing a 10 mL 0.09 M potassium hydroxide (KOH) solution. Jars were sealed and put in a dark incubation chamber with a constant temperature of 20°C. We started measuring soil respiration exactly one day after water addition and continued measurements every 24 hours for five days.

The KOH solution functioned as a trap absorbing the respired CO_2 through an acid-base neutralization reaction lowering the electrical conductivity of the trapping solution (Zibilske, 1994; Whitman *et al.* 2014). To estimate soil CO_2 flux from the different treatments, we measured the decrease in electrical conductivity of the KOH trap with a benchtop conductivity meter (Model 860032, Sper Scientific Direct, Scottsdale, AZ, USA). After each measurement, the KOH solution was renewed to ensure a large excess of OH^- to keep the C in solution as carbonate. We used jars injected with 2.5, 5, 10 or 20 mg-C of 100% CO_2 gas (N=3) as calibration standards to calculate CO_2 concentration. KOH traps in blank jars corrected for the amount of atmospheric CO_2.

Statistical analysis

All statistical analyses were performed in R (R Core Team, 2017). We used the packages lsmeans (Lenth, 2016), vegan (Oksanen, 2017) and ade4 (Dray & Dufour, 2007) for performing Tukey honest significant difference (HSD) tests with significance level (α) set at 0.05 and non-metric multidimensional scaling (NMDS). In addition, we performed Pearson's Chi-squared tests

to compare class chemical composition between root exudate and tissue samples. Moreover, we ran multiple linear regression models to estimate the effect of species, class and sterility of the sampling solution on root exudate abundance and to test the effect of time and compound identity on respiration. Lastly, we used Kruskal-Wallis test to compare compound diversity between root exudate and tissue samples.

The sum of relative compound abundances was standardized by dividing the total number of absorption units (AU) by belowground biomass for root exudates and by fine root mass for the tissue samples. Compound and class diversity were defined as number of compounds per sample and number of compounds belonging to one class per sample respectively. As an additional measure of diversity, we calculated the Shannon index (Shannon, 1948) for each sample using the number of compounds per class. To comply with normality and equal variance of residuals assumptions, the sum of relative abundance, class abundance, relative class abundance, number of compounds per sample, root exudate catechin concentration, and cumulative respiration were log-transformed. Daily respiration rates were transformed using a square root.

NMDS analyses were performed on the relative abundance of root exudate and tissue compounds using Bray-Curtis distances (Kruskal & Wish, 1978). All root compounds with determined and undetermined identity were included. Stress values were calculated to estimate how well the ordination plots represented the higher-dimensional relationships. Stress values < 0.2 and preferably closer to 0.1 yield usable ordination plots (Clarke, 1993). We drew *a priori* clusters in the ordination plots based on the species in each sample. To test for differences in compound composition between species, we performed a permutational multivariate analysis of variance (PERMANOVA) using the adonis function from the vegan package with permutations set to 9999 (Anderson, 2001). A Mantel test determined the correlation between species distances in the exudate and tissue plots. Additionally, we extracted the distances from the individual sample points to the centroid of their species cluster to get a measure of intraspecific variation. Using ANOVA, we tested the effect of sample origin (root exudate or tissue) on cluster tightness.

Results

Phenolic root exudate and tissue compounds

Out of the 1044 root exudate and 1215 root tissue peaks, we found 286 individual compounds belonging to eight different phenylpropanoid classes, one diterpene class, three unidentified chemical classes with similar UV-vis absorption characteristics (group A, B, C) and a set of unknowns (Table 1). Out of the 181 unique root exudates, we determined the identity of five compounds through matching spectra and retention times with one of the standards (Table S2). These included three flavonoids (catechin, naringenin, taxifolin), one stilbene (resveratrol) and one benzoic acid derivative (syringic acid). In the case of the 137 unique tissue compounds, we determined the identity of five compounds (Table S2) including one cinnamic acid derivative (caffeic acid), two benzoic acid derivatives (syringic acid, gallic acid) and two flavonoids (catechin, taxifolin). Catechin was the most frequently detected compound in both the phenolic root exudate and tissue analysis.

The sum of relative compound abundances varied among biosynthetic classes. Root exudates showed the highest abundance in flavonoids, chalcones and diterpenes, while tissue flavonoids had the highest abundance than any other tissue biosynthetic class ($p < 0.05$, Fig. S2). Within the flavonoid class, flavanols had higher abundance than any of the other sub-classes in both root exudate and tissue samples (Fig. S3, S4). For each tree species, sterilizing the collection solution using Micropur tablets significantly increased the total abundance of root exudates detected per sample ($p = 0.002$), when species was also included as independent variable in the linear model. Adding batch number as factor to this same model also affected the sum of relative compound abundances ($p = 0.04$) with the root exudates collected on the earlier sampling date showing a higher relative amount.

The biosynthetic class composition of the root exudates and root tissue extracts was different (Pearson's Chi-square test, $p < 0.001$,) across the tested tree species. Overall, the number of classes found in the root tissue was higher than those detected in root exudate samples for all tree species ($p < 0.05$). The fine root tissue also contained a higher number of compounds than the root exudate samples ($p < 0.001$), except for *P. abies* that showed no difference and *A. rugosa* that had a higher number of root exudates. While the proportion of flavonoids, chalcones and diterpenes was significantly higher in root exudate samples than in the root tissue extracts, the

reverse was true for the simple phenols, benzoic acid, cinnamic acid and tannic acid derivatives ($p < 0.05$, Fig. S5).

Species variation in phenolic root exudates and fine root tissue compounds

The phenolic composition of root exudates and tissue extracts differed among species (PERMANOVA, $p < 0.001$, Fig. 1). NMDS analysis showed that the species-specific pool of root exudate profiles did not overlap with those from other species except for *F. grandifolia* and *Q. rubra*, (Fig. 1a). All species had their unique phenolic profiles in root tissue (Fig. 1b). The Mantel test demonstrated that the species distances in the exudate and tissue plot were strongly correlated ($r = 0.72$, $p < 0.001$). Comparing the distances of the species data points to the centroids of their respective clusters demonstrated that the intraspecific variation in fine root tissue composition was lower than root exudate composition for all species ($p < 0.001$, Fig, S6).

Root exudate compound and class diversity were different among tree species ($p < 0.001$, Table 2). *Alnus rugosa, P. abies* and *P. strobus* exuded the highest number of compounds, followed by *A. saccharum* and *Q. rubra*, and lastly by *F. grandifolia*. In terms of number of classes and the Shannon index of root exudates, *A. rugosa* scored higher than all others. *Acer saccharum, F. grandifolia* and *Q. rubra* had the lowest Shannon index and class count ($p < 0.05$). On the other hand, the number of root tissue compounds was similar for all species except for *P. strobus* that had a higher relative amount of compounds than the others. Class diversity and Shannon index of root tissue secondary metabolism followed similar patterns as those in root exudate profiles. *Alnus rugosa* and *P. abies* had the highest class diversity and Shannon index followed by *P. strobus*. Finally, *A. saccharum*, *F. grandifolia* and *Q. rubra* scored the lowest with *A. saccharum* having the bottommost value ($p < 0.05$).

The sum of relative root exudate abundances was also different among the tree species ($p < 0.001$, Table 2). *Pinus strobus* exuded by far the largest concentration of compounds followed by *A. rugosa* and *P. abies* and then *A. saccharum* and *F. grandifolia.* We detected the lowest quantity of root exudates for *Q. rubra* ($p < 0.05$). In comparison to the species effects on root exudate abundance, root tissue concentration showed a similar trend. Again, *P. strobus* contained the highest and *Q. rubra* the lowest tissue concentration of phenolic compounds ($p < 0.05$). Yet, *A. rugosa, A. saccharum, F. grandifolia* and *P. abies* all had the same tissue concentration of phenolics.

The amount of catechin in root exudate and tissue samples was different across tree species ($p < 0.001$, Table 2) with *P. strobus* having the highest and *Q. rubra* and *F. grandifolia* the lowest concentrations. In the root tissue extracts, we did not detect catechin for *P. strobus* and *F. grandifolia*. *Picea abies* showed the highest tissue concentration of catechin, while *A. rugosa* and *Q. rubra* samples contained the lowest amounts ($p < 0.05$).

Phenolic effects on soil microbial respiration

Root phenolic compounds had divergent, compound-specific effects on cumulative respiration ($p < 0.001$, Fig. 2) ranging from a 26% decrease to a 46% increase (Table 3). Benzoic acid and naringenin increased respiration in comparison to the untreated control ($p < 0.05$) utilizing 40% and 31% of the total added C (Table 3). In contrast, the application of taxifolin, caffeic acid, catechol and catechin reduced microbial respiration ($p < 0.05$). The soil replicates receiving an addition of abietic acid, arbutin or resveratrol had similar levels of respiration as the untreated control. These results indicate that even if compounds come from the same biosynthetic class, for instance the flavonoids naringenin, taxifolin and catechin, they can both suppress and stimulate respiration. Interestingly, the biosynthetically diverse compounds that reduced respiration had the same catechol ring functional group in common. Functional groups of the other compounds included carboxyl and phenol groups. However, these commonalities were not exclusively shared among the compounds with similar effects on respiration.

The compound identity, day and their interaction had a significant effect on daily respiration ($R^2 = 0.88$, $p < 0.001$, Fig. 3). The compounds increasing cumulative respiration after five days showed varying patterns over time. While benzoic acid caused lower microbial respiration than all chemicals except for catechol and arbutin on the first day ($p < 0.05$), this was the reverse on the second day where it was among the treatments with the highest level of respiration. On the third through fifth day, benzoic acid induced the highest respiration surpassing glucose ($p < 0.05$). Similarly, the soils with naringenin additions respired a similar amount of C as the control on the first day, but had consistently higher respiration than the control on the following days ($p < 0.05$). With added glucose, soil microbial respiration was higher than in all other treatments on the first day ($p < 0.05$), but this rate dropped back to same level as the control by day five.

Except for the first day where the catechol treatment had the lowest respiration rate ($p < 0.05$), the phenolic compounds reducing cumulative respiration had similar respiration rates over

time ($p > 0.05$). Yet, these same compounds decreased respiration in comparison to the control on different days. Soil replicates with catechol and taxifolin additions respired less than the control on the first day ($p < 0.05$) but had similar levels of respiration for the following days. In contrast, the catechin and caffeic acid treatments had similar levels of respiration as the control on day one. In the catechin treatment, respiration dropped on the second day and remained lower than the control until the fifth day ($p < 0.05$). For the caffeic acid treatment, respiration was only lower than the control on day five ($p < 0.05$).

The abietic acid treatment maintained similar levels of soil respiration as the control for the entire duration of the incubation. While resveratrol and arbutin had no effect on cumulative respiration, they both suppressed respiration on the first day ($p < 0.05$) with arbutin decreasing respiration to a higher degree than resveratrol ($p < 0.05$).

Discussion

Suppression of soil microbial respiration

In contrast to our initial expectations, these data support the hypothesis that functional group rather than biosynthetic class is responsible for determining root phenolic effects on microbial respiration. Different flavonoids were found to both increase and decrease soil respiration. Moreover, phenolics reducing respiration did not have the same biosynthetic class but instead possessed a catechol ring. These soil treatments with catechol-containing compounds also resulted in similar daily respiration trends over the five-day incubation period suggesting analogous modes of action (Fig. 3c).

Other studies have demonstrated that catechol can be toxic to microbes (Capasso *et al.*, 1995; Boyd *et al.*, 1997) and decrease the rates of lignin and SOM decomposition (Hamer & Marschner, 2002, 2005). The most common toxicity mechanisms of catechol-containing compounds include phenoxyl radicals formation, generation of reactive oxygen species (ROS), DNA damage, protein inactivation and disruption of membrane function (Schweigert *et al.*, 2001; Galati *et al.*, 2002). In addition, catechol-derivatives act as chelators altering iron availability, which can decrease microbial activity and growth (Loper & Buyer, 1991). In medical biology, studies have found that phenolic mode of action is linked to functional groups and particularly point at the difference between compounds that have a phenol or catechol ring (Galati *et al.*, 2002; Galati & O'Brien, 2004). Research on plant-herbivore interactions also

concludes that phenolic mode of action depends on functional groups like the number of hydroxyl groups (Salminen & Karonen, 2011). While these studies and our findings suggest that functional group determines the specific effect of different root phenolic compounds on soil microbial respiration, further experiments using a larger set of compounds are necessary to test this hypothesis and to unravel the specific mechanism by which catechol-containing compounds may reduce soil microbial respiration under different rhizosphere conditions.

Even though arbutin and benzoic acid did not decrease cumulative respiration, the decrease in respiration in comparison the control on the first day (Fig. 3a,b) could also indicate toxicity (Thiele-Bruhn & Beck, 2005). Unlike the catechol-containing compounds, this effect was temporary suggesting that toxic-tolerant microbial groups increased their abundance or activity on later days stimulating respiration. Microbial population shifts after compound application can indeed occur within a few hours to days (Blum & Shafer, 1988; Cleveland *et al.*, 2007). Yet, the timeline of phenolic effects on microbial respiration warrants further investigation.

Stimulatory phenolic effects on soil microbial respiration

Two different C sources could have fueled the increase in cumulative respiration (Fig. 2): namely, endogenous soil C or added C from compound application. Because the increase in respiration of the soils treated with glucose, benzoic acid and naringenin was 68%, 40% and 30% of the total added C respectively (Table 3), we conclude that not all C from the compound application was metabolized. Therefore, it is likely that any increase in soil CO_2 emissions in comparison to the control came predominantly from the added substrate. This process called substrate-induced respiration (Anderson & Domsch, 1978; Lin & Brookes, 1999) has been proposed as mechanism of phenolic effects on soil respiration in the field and laboratory (Blum & Shafer, 1988; Qu & Wang, 2008). The difference in timing of peak respiration between glucose, benzoic acid and naringenin (Fig. 3a) can be explained by the time needed to ramp up biosynthesis of enzymes for breaking down more complex substrates than sugars (Stemmer *et al.*, 1998; Allison & Vitousek, 2005; Poll *et al.*, 2008). It is possible that the abietic, arbutin and resveratrol treatments with similar respiration levels as the control (Fig. 2), required more time to be broken down and would have increased cumulative respiration if the incubation was continued for a longer duration.

Besides substrate-induced respiration, other stimulatory processes may have occurred. Naringenin could have enhanced microbial activity as signaling compound attracting specific bacteria or inducing fungal hyphal growth and spore germination (Chabot *et al.*, 1992; Cooper, 2007; Perry *et al.*, 2007). Triggering of microbial metabolism may have played an additional role (Mason-Jones & Kuzyakov, 2017). Lastly, a recent study showed that oxalic acid released mineral-bound endogenous soil C contributing to the root priming effect (Keiluweit *et al.*, 2015). This mechanism could explain part of the stimulatory effect of benzoic acid that, like oxalic acid, possesses a carboxyl functional group.

Interestingly, the majority of phenolic compounds tested in this study did not have a stimulatory effect on microbial respiration (Fig. 2). This is in contrast with other soil incubation studies simulating root priming with the addition of primary metabolites (Landi *et al.*, 2006; Keiluweit *et al.*, 2015). Hence, our results suggest that phenolic compounds are more resistant to degradation by soil microbes than other root exudates and have other functions than primary metabolites in the rhizosphere. Yet, this hypothesis needs further testing because we did not identify many of the 286 unique root chemicals detected with HPLC analysis (Table 1, Fig. 1) and we only tested nine compounds from seven biosynthetic classes for their effects on microbial respiration.

Potential implications of tree phenolics for rhizosphere dynamics

This study shows that trees have species-specific phenolic profiles (Fig. 1) and that root compound diversity varies across tree species (Table 2). While root exudate composition has been proposed as driver of plant species differences in rhizosphere characteristics, few studies have measured and confirmed this chemical variation. The higher intraspecific variation in compound composition among root exudates in comparison to root tissue (Fig. S6) is most likely due to lower concentrations in root exudate samples, indicating that better detection techniques would make the root exudate profiles even more species-specific. Previous studies have shown that root exudates can drive shifts in rhizosphere microbial community composition (Broeckling *et al.*, 2008; Haichar *et al.*, 2008) and that species-specific and genotype-specific chemical root profiles may translate into a unique rhizosphere microbial community (Micallef *et al.*, 2009). Our findings together with these previous studies should motivate further testing of the

hypothesis that distinct root phenolic profiles cause shifts in microbial communities with different metabolic traits, which indirectly influences rhizosphere C and nutrient cycling.

All tree species contained the flavanol catechin in their root exudates, tissue extracts or both (Table 2). Moreover, flavanols dominated the chemical profiles of most of the studied tree species (Fig. S3, Fig. S4). Root exudation of catechin has been observed in other studies, with suggested functionality including metal-chelation and allelopathy (Hughes *et al.*, 1999; Kidd *et al.*, 2001; He *et al.*, 2009). In this study, catechin reduced microbial respiration (Fig. 2) and most likely functioned as a toxin. Therefore, besides using the quantity of root products released into the rhizosphere to predict root priming (Cheng *et al.*, 2014), the concentration of respiration-suppressing root compounds should be considered as factor explaining species variation in soil CO_2 emissions. This study indeed showed that catechin concentration in root substrates significantly varied across tree species (Table 2). Future studies should estimate the proportion of phenolics and respiration-suppressing compounds of total C exuded. This information could help determine to what extent phenolics may influence rhizosphere respiration when exuded with other primary metabolites.

We emphasize that our findings require further testing under more natural rhizosphere conditions. Measuring root exudate effects on rhizosphere processes is extremely challenging because of the diversity of chemicals exuded and limited rhizosphere access. Therefore, previous studies produced simulated rhizospheres, where bulk soil samples received a single-compound application through an artificial root or membrane filter mimicking the transformation of bulk to rhizosphere soil driven by roots (Renella *et al.*, 2006; Kuzyakov *et al.*, 2007). We took a similar approach but, unlike other studies, we focused on secondary metabolites and analyzed the root exudates of different tree species to inform the compound selection for the incubation study. Applying a single root chemical to a soil and measuring microbial respiration over time has the benefit of isolating the mechanistic effects of individual compounds. However, the limitation of this method is that the “rhizosphere” is simplified and other root effects like water and nutrient uptake or root respiration are excluded. Moreover, determining the quantity of exudate that should be added is tricky. The amount of phenolics added in this study is within the range of total carbon added to soil by other studies yet it is likely a higher amount than what a thin layer of rhizosphere soil receives. Overall, our findings provide support that secondary metabolites are

485 an important factor to consider in rhizosphere dynamics. Yet, additional studies are essential to
486 test root phenolic function in natural rhizospheres and at the forest ecosystem-scale.
487
488 *Phenolic release into soil*
489 The higher proportions of flavonoids, chalcones and diterpenes in root exudates in comparison to
490 the root tissue (Fig. S5) and overall higher tissue class diversity suggest that roots can partly
491 control which chemicals and phenylpropanoid classes are exuded. Several forms of membrane
492 transport have been proposed for flavonoids including active secretion (Buer *et al.*, 2007;
493 Sugiyama *et al.*, 2007; Cesco *et al.*, 2010). Selective compound exudation hints that these
494 chemicals perform a function for the tree such as chemo-attractants, toxins or inhibitors of
495 particular microbial processes (Cesco *et al.*, 2012; Sugiyama & Yazaki, 2014; Venturi & Keel,
496 2016). Through actively changing the secretion of root exudates, plants may also influence
497 microbial respiration. This raises the question to what extent individual plants can alter exudation
498 as a function of their environment. While this has been shown in plant-symbiont interactions and
499 during nutrient deficiency (van Scholl *et al.*, 2006; Badri *et al.*, 2009), future studies should
500 examine how plants may alter secondary metabolite production in response to their soil
501 environment to control the microbial metabolism of their rhizospheres.
502 The difference in phenolic composition between root exudate and fine root tissue samples
503 (Fig. S5) suggests that the compounds released during root exudation and root turnover vary
504 greatly. A recent meta-analysis by Huo *et al.* (2017) listed chemical variation as one of the
505 reasons for why root exudates and plant litter may influence SOM decomposition differently.
506 Besides differences in chemical composition, we expect that amount of phenolic compounds
507 released during root exudation and turnover varies. However, quantifying root exudates is
508 extremely challenging. Because we used a hydroponic setup, the low concentration of phenolics
509 in the collection solution may have drawn a higher amount of root exudates from the root than
510 under natural settings (Vranova *et al.*, 2013). This would be a more significant limitation for
511 compounds that reach the rhizosphere through passive transport than for compounds transported
512 by active secretion. Moreover, transferring the root systems from a solid medium to hydroponics
513 may have caused disturbance and changed exudation rates. While we should be cautious about
514 quantitative statements regarding root exudate concentrations, we can still conclude that root
515 exudates and fine root tissue within one species have distinct phenolic fingerprints. Furthermore,

our results are relative across species. The conclusion that root phenolic profiles are species-specific is a new contribution to the field of root-soil interactions.

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Author Contribution

MJZ and TLB designed the experiments. MJZ executed experiments and AK helped with HPLC analysis. MJZ, AK and TLB discussed results and conclusions of study. MJZ wrote manuscript. TLB and AK edited manuscript drafts.

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Supporting Information

Fig. S1 UV-vis spectra of groups of unidentified compounds with similar absorption characteristics.

Fig. S2 Biosynthetic class relative abundance in root exudates and tissue extracts.

Fig. S3 Relative abundance of flavonoid sub-classes in root exudates by tree species.

Fig. S4 Relative abundance of flavonoid sub-classes in root tissue by tree species.

Fig. S5 Proportions of biosynthetic classes in the sum of relative abundances of root exudate and tissue samples by tree species.

Fig. S6 Distance of species points in NMDS ordination plot to centroid of their respective clusters in the root exudate and tissue samples.

Table S1 Chemical standards used for compound identification and class assignment

Table S2 The number of root exudate and tissue samples that showed an exact match with a phenolic reference during HPLC analysis.

Figures and Tables

Table 1. Total number of root exudates and tissue compounds identified by biosynthetic class from the root exudate and tissue samples of *A. saccharum, A. rugosa, F. grandifolia, P. abies, P. strobus* and *Q. rubra* (*N*=12 except for *F. grandifolia* and *Q. rubra* with N=8, hence 64 tree replicates in total).

Class	Root exudate	Root tissue
Benzoic acid-derivatives	5	116
Chalcones	73	27
Cinnamic acid-derivatives	26	86
Diterpenes*	61	49
Flavonoids	608	505
Flavanols	436	424
Flavanones	43	0
Flavanonols	29	1
Flavonols	0	12
Unknowns	100	68
Phenols	15	22
Stillbenes	8	1
Tannins	0	13
Class A	27	193
Class B	52	41
Class C	67	57
Unknowns	102	105
Total	**1044**	**1215**

*Diterpenes are not phenolic compounds.

Table 2. Tree species variation in phenolic root exudate and tissue diversity and abundance (means and standard errors; *N*=12, except for *F. grandifolia* and *Q. rubra* with *N*=8).

Species	Sample	Number of compounds	Number of classes	Shannon index	Sum of relative compound abundances (10^{-3} AU^2 g^{-1} root)	Catechin concentration (µg g^{-1} root)
Acer saccharum	**Exudate**	6.6 ± 0.7^{b}	1.3 ± 0.1^{d}	0.05 ± 0.02^{d}	2.1 ± 0.5^{c}	19.19 ± 5.72^{b}
Alnus rugosa	**Exudate**	28.9 ± 1.6^{a}	7.9 ± 0.3^{a}	0.85 ± 0.01^{a}	9.3 ± 2.8^{b}	4.06 ± 1.24^{c}
Fagus grandifolia	**Exudate**	2.9 ± 0.4^{c}	1.6 ± 0.2^{d}	0.17 ± 0.05^{cd}	0.8 ± 0.2^{c}	2.60 ± 2.39^{c}
Picea abies	**Exudate**	20.0 ± 2.0^{a}	3.2 ± 0.3^{c}	0.26 ± 0.04^{bc}	7.6 ± 1.1^{b}	23.54 ± 5.15^{b}
Pinus strobus	**Exudate**	26.8 ± 1.5^{a}	4.5 ± 0.2^{b}	0.34 ± 0.2^{b}	28.6 ± 3.2^{a}	116.06 ± 15.65^{a}
Quercus rubra	**Exudate**	4.12 ± 0.5^{bc}	1.3 ± 0.2^{d}	0.06 ± 0.04^{d}	0.2 ± 0.0^{d}	1.00 ± 0.53^{c}
Acer saccharum	**Tissue**	15.3 ± 0.5^{b}	4.3 ± 0.3^{c}	0.41 ± 0.03^{d}	1111 ± 85^{b}	1450 ± 250^{b}
Alnus rugosa	**Tissue**	18.4 ± 1.7^{b}	8.0 ± 0.1^{a}	0.85 ± 0.01^{a}	992 ± 107^{bc}	400 ± 120^{c}
Fagus grandifolia	**Tissue**	15.8 ± 0.3^{b}	4.3 ± 0.2^{c}	0.52 ± 0.01^{c}	1127 ± 64^{b}	
Picea abies	**Tissue**	16.5 ± 0.3^{b}	8.5 ± 0.2^{a}	0.82 ± 0.01^{a}	1127 ± 105^{b}	3010 ± 300^{a}
Pinus strobus	**Tissue**	22.8 ± 0.7^{a}	7.0 ± 0.0^{b}	0.64 ± 0.01^{b}	1956 ± 59^{a}	
Quercus rubra	**Tissue**	15.5 ± 0.6^{b}	4.7 ± 0.2^{c}	0.53 ± 0.03^{c}	685 ± 68^{c}	700 ± 130^{bc}

Contrasting letters indicate significant differences ($p < 0.05$) among tree species within root exudate or tissue samples as determined by Tukey HSD test. Note that the sum of relative compound abundances and catechin concentration for root exudates and tissue compounds cannot be compared. While the sum of relative compound abundances was standardized by belowground biomass for the root exudate samples, this was done by dividing by the fine root mass extracted for the tissue samples.

Table 3. Phenolic and diterpene compound-induced changes in soil microbial respiration and proportion of carbon respired from compound applications (means and standard errors, *N* = 8).

Compound	Change in soil microbial respiration relative to control (%)	Proportion of added carbon respired (%)
Abietic acid	1 ± 2	1 ± 2
Arbutin	-14 ± 1	0 (-12 ± 1)*
Benzoic acid	46 ± 8	40 ± 7
Caffeic acid	-19 ± 2	0 (-17 ± 2)*
Catechin	-26 ± 2	0 (-22 ± 2)*
Catechol	-25 ± 2	0 (-21 ± 2)*
Naringenin	35 ± 4	30 ± 4
Quercetin	-4 ± 3	0 (-3 ± 2)*
Resveratrol	-15 ± 3	0 (-13 ± 2)*
Taxifolin	-16 ± 2	0 (-14 ± 3)*
Glucose	59 ± 5	68 ± 6

The change in respiration relative to the control was calculated by dividing the difference of carbon respired between the control and treatment soils by the amount of carbon respired in the control. We calculated the proportion of added carbon respired by dividing the difference of carbon respired between the control and treatment soils by the total amount of carbon added through the phenolic or diterpene application. Glucose is included as comparison.
*Because the amount of added carbon cannot be negative, we changed the negative values to zero. The true value from the calculation is shown in parentheses.

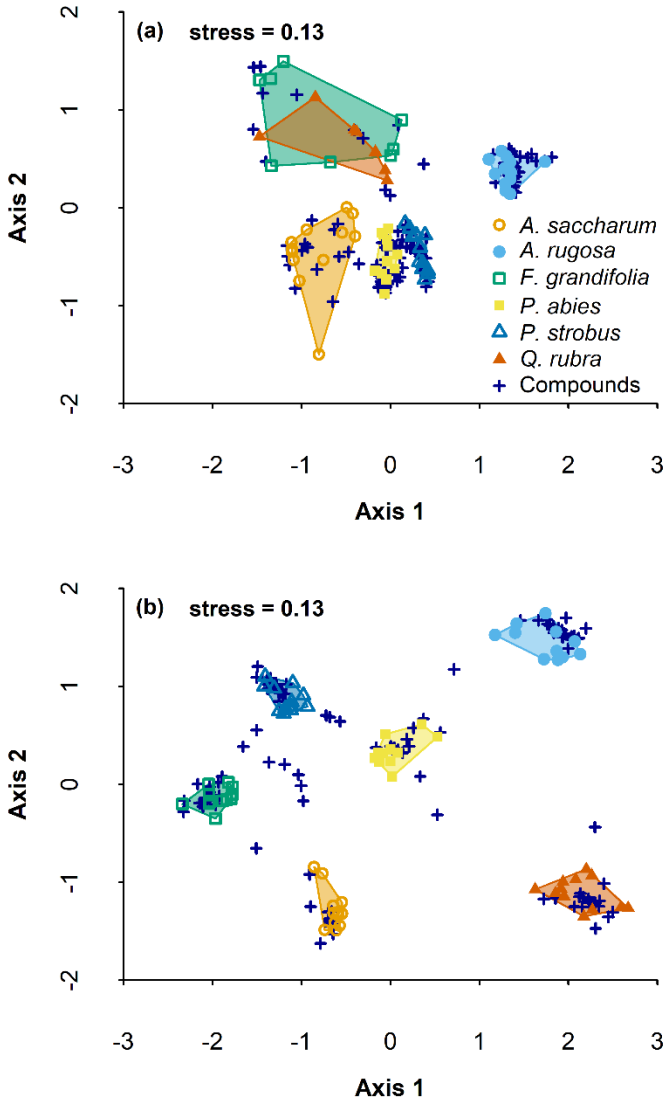

Fig. 1 NMDS ordination of phenolic and diterpene composition of (a) root exudates and (b) fine root tissue for temperate forest tree species *A. saccharum* (orange), *A. rugosa* (light blue), *F. grandifolia* (green), *P. abies* (yellow), *P. strobus* (dark blue) and *Q. rubra* (dark red) (*N*=12 except for *F. grandifolia* and *Q. rubra* with *N*=8). Root compounds are indicated by a blue cross. Both compounds with determined and undetermined identity from HPLC analysis are included.

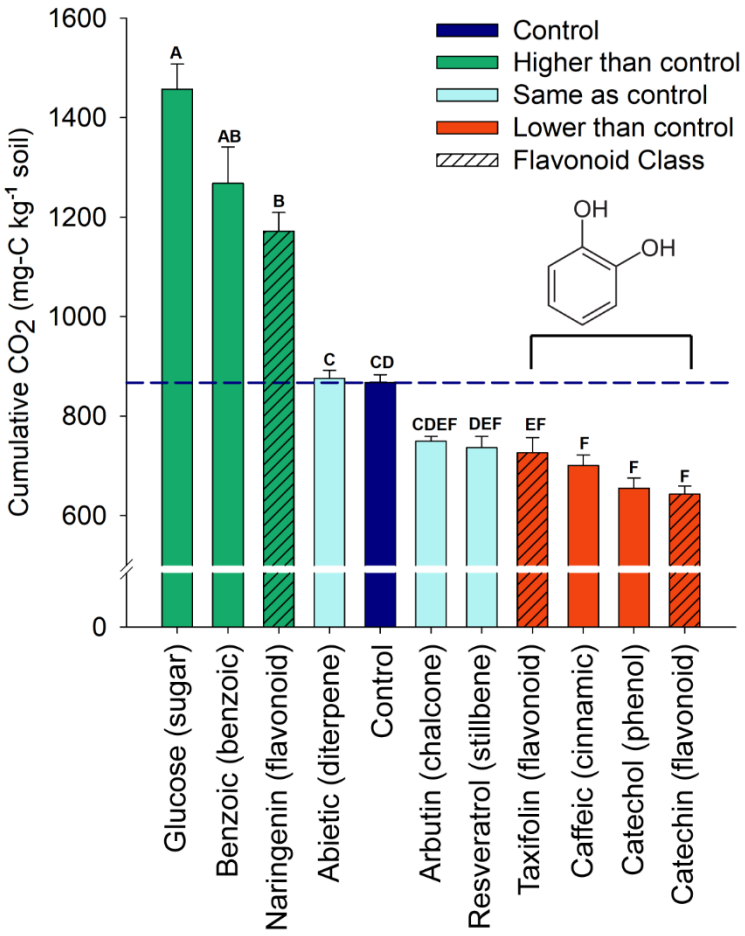

Fig. 2 Cumulative respiration measured over a five-day period as influenced by phenolic and diterpene compounds belonging to different biosynthetic classes (means and standard errors, *N*=8). Biosynthetic classes are in parentheses. Different capital letters indicate significant differences ($p < 0.05$) as determined by Tukey HSD test. Colors indicate difference in cumulative respiration in comparison to control (dark blue): higher (green), no difference (light blue) and lower (red).

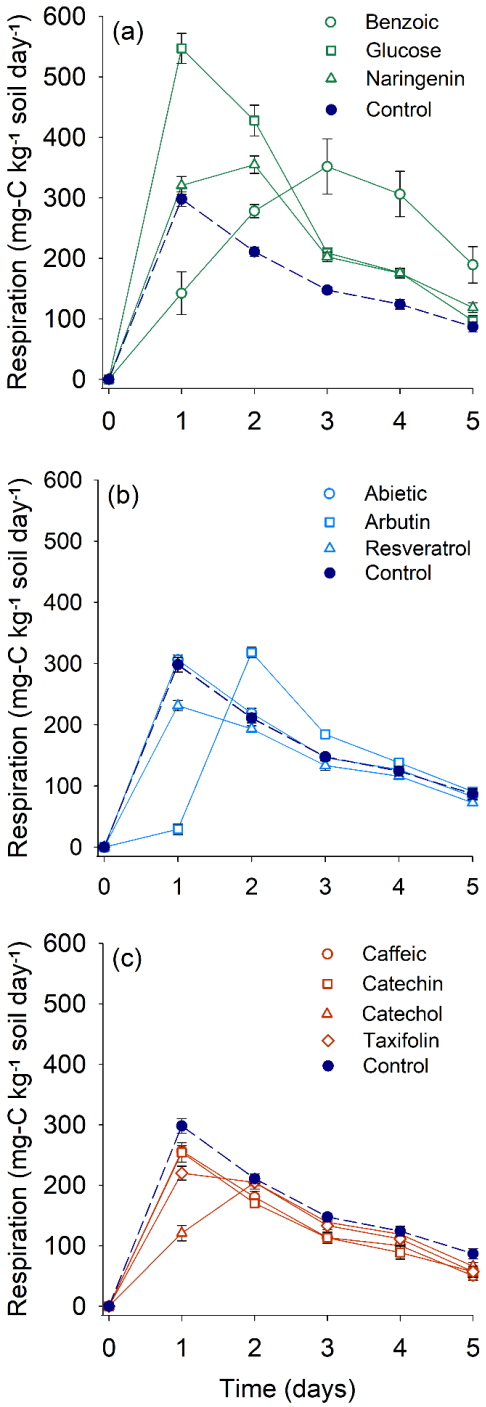

Fig. 3 Daily respiration of soil-phenolic and -diterpene compound mixtures that (a) increased cumulative respiration relative to the control, (b) did not affect cumulative respiration relative to the control, and (c) decreased cumulative respiration relative to control (means and standard errors, *N*=8). The control indicated by the dark blue closed circle and dashed line is included in each panel as a reference. For the biosynthetic class of each compound, see Fig. 2.