# ROOT AND RHIZOSPHERE INTERACTIONS OF TEMPERATE FOREST TREE SPECIES IN A CHANGING CLIMATE

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Root-soil interactions are crucial parts of the terrestrial carbon (C) cycle. To get a better understanding of their role in forest ecosystem feedbacks to climate change, this dissertation explored (1) how tree species variation in root phenolic profiles influence soil C cycling and (2) to what extent seasonal drought affects fine root growth dynamics in a mixed-species forests of European beech (*Fagus sylvatica* [L.]) and Norway spruce (*Picea abies* [L.] Karst).

For the first question, I modified a root exudate collection system in order to measure root phenolic composition of two coniferous and four deciduous forest tree species using high-performance liquid chromatography (HPLC). I tested the effects of nine of these phenolic compounds on soil microbial respiration. These findings helped inform the next incubation experiment, which made use of $^{13}C$-labeling techniques and different microbial assays to analyze the mechanistic effects of phenolic-glucose interactions on soil organic matter (SOM) decomposition and associated soil microbial dynamics. Overall, my experimental results showed that root phenolic composition is highly species-specific and can have both stimulatory and inhibitory effects on soil microbial respiration. Moreover, I found that root phenolic effects on soil

biogeochemistry are more persistent than glucose, influencing SOM decomposition rates and associated enzyme activities as well as inducing shifts in bacterial community composition.

To answer the second question, I studied root growth dynamics in a mature mixed beech-spruce forest in southern Germany under throughfall-exclusion over a four-year period by means of minirhizotron imaging. While fine root production of both *F. sylvatica* and *P. abies* decreased under throughfall-exclusion, I found an increase in fine root lifespan in response to seasonal water limitation. Yet, in the interspecific stands of *F. sylvatica* and *P. abies,* a decline in root production was not observed, suggesting that the belowground productivity of mixed-species forests is less affected by seasonal drought than monospecific forest stands.

In conclusion, these findings show the varying magnitude and direction of root phenolic effects on SOM decomposition and the responsiveness of fine roots to drought, emphasizing the importance of studying root-facilitated C cycling in a changing climate.

# BIOGRAPHICAL SKETCH

Marie Jasmijn Zwetsloot was born in Westernieland in the Netherlands on July 29th in 1989. She grew up with her younger sister and brother, playing outside in her parents' fruit and vegetable garden and dreaming about exploring the natural world of other countries. In 2005, she received a scholarship from the Dutch branch of the United World Colleges (UWC) organization to complete the last two years of her secondary education at Atlantic College in St. Donat's in Wales. She returned to the Netherlands in 2007 to start a Bachelor of Arts at University College Maastricht (UCM) where she wrote a research thesis evaluating the sustainable development practices of a Dutch NGO with cacao farmers in Nicaragua. As part of her undergraduate degree, she studied at Universidad de San Francisco de Quito in Ecuador for one semester and did a research internship at the Center for Human values at Princeton University in the United States. She also took additional classes in plant and soil science at Wageningen University. Funded by a Fulbright fellowship and the Dutch Huygens Talent Scholarship Program, she came to Cornell University in 2011 to obtain a research master's in soil science. Under the supervision of Johannes Lehmann, she published two research articles on plant phosphorus (P) availability from bone char in soils. After graduating in 2013, she started a PhD program with Taryn Bauerle in the School of Integrative Plant Science at Cornell University.

To my Ithaca friends

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# CHAPTER 1: INTRODUCTION

## *Root and rhizosphere interactions in forest ecosystems under climate change*

Forests store approximately 50% of all terrestrial carbon (Bonan 2008) with a significant proportion of assimilated carbon being directly allocated to roots. Because fine roots and root-associated microorganism are the primary supply of plant carbon to soil (Rasse *et al.*, 2005; Clemmensen *et al.*, 2013), the spatio-temporal patterns of fine root growth dynamics and turnover have important consequences for soil carbon storage and biogeochemical cycles (Gill & Jackson, 2000; Norby & Jackson, 2000).Yet, fine roots do not solely function as a channel of soil carbon inputs. Roots interact with a diverse community of microorganisms, invertebrates and neighboring plants creating hotspots of biological activity (Badri *et al.*, 2009; Philippot *et al.*, 2013). Previous research demonstrated that rhizosphere interactions can control up to one-third of the total carbon mineralized in temperate forests soils (Finzi *et al.*, 2015). Hence, while fine roots function as a soil organic matter (SOM) source, they potentially also accelerate soil carbon and nutrient cycling (Phillips *et al.*, 2012). These two contrasting effects complicate our understanding of the role of fine roots in terrestrial biogeochemistry. Recent studies have indeed emphasized the need for research on root-soil interactions to estimate forest ecosystem feedbacks to climate change (Drigo *et al.*, 2008; Warren *et al.*, 2015; Pausch & Kuzyakov, 2018).

Fine root production and soil respiration depend on aboveground carbon assimilation for resources (Matamala & Schlesinger, 2000; Ekblad & Högberg, 2001) and can respond to changes in soil resource distribution and availability (Hodge,

2005). Climate change will affect plant growth conditions and resource availability in forest ecosystems (Allen *et al.*, 2010; Lindner *et al.*, 2010; Williams *et al.*, 2013). Therefore, these changing environmental factors should also have a profound impact on root growth dynamics and rhizosphere interactions. However, before one can answer such a broad-scale question as the effect of climate change on roots and root-facilitated biogeochemical processes, it is important to get a better understanding of the mechanisms driving root-soil interactions. Because of the challenges associated with measuring root exudates and rhizosphere processes (Shi *et al.*, 2013), simple questions about root exudate chemical composition, tree species differences in root exudate profiles and the consequential effects of this chemical diversity on rhizosphere dynamics remain unanswered. Moreover, in order to estimate the combined effect of the predicted changes in temperature, precipitation patterns and atmospheric $CO_2$ concentration (Pearson & Palmer, 2000; Dai, 2013; Seneviratne *et al.*, 2014; Gosling & Arnell, 2016), these processes require individual study. While there are a considerable amount of studies on the effect of elevated $CO_2$ on root-mediated soil biogeochemical cycles (Pregitzer *et al.*, 1995; Pendall *et al.*, 2004; Heath *et al.*, 2005; Pritchard *et al.*, 2008), results from the few studies investigating forest root growth dynamics under changing water availability are inconclusive. Hence, the aim of this dissertation is to provide answers to these questions ranging from the rhizosphere to ecosystem scale. Specifically, the objectives are 1) to investigate the significance of tree root exudate chemistry to rhizosphere dynamics and 2) to analyze patterns of fine root production and lifespan in temperate forest ecosystems under seasonal drought.

***Root exudates and rhizosphere dynamics***

Root exudation – the process through which roots release organic and inorganic compounds into the soil (Hawes *et al.*, 2000; Pinton *et al.*, 2009) – has received little scientific attention yet may be the key to understanding root-facilitated carbon cycling in forest ecosystems. Through the supply of labile carbon, root exudation provides energy for microbial growth and production of extracellular enzymes involved in the breakdown of root litter and SOM (Kuzyakov, 2010). These root-associated changes in decomposition rate have been termed "root priming" or "rhizosphere priming" (Dijkstra *et al.*, 2013). Studies have reported both negative and positive priming in response to labile carbon additions (Hamer & Marschner, 2005; Renella *et al.*, 2007; Guenet *et al.*, 2012), but have not been able to fully explain these opposite trends.

Surprisingly, root exudate chemistry has not been tested as a mechanism controlling the magnitude and direction of rhizosphere priming. Root exudates comprise a highly diverse group of chemicals (Pinton *et al.*, 2009), which may differentially affect the rhizosphere environment (Keiluweit *et al.*, 2015). While the chemical composition of root exudates are expected to vary greatly among tree species (van Hees *et al.*, 2000; Sandnes *et al.*, 2005), direct root exudate samples of trees have not been collected and analyzed for chemical characteristics. Variation in secondary metabolites among plant species is often higher than in primary metabolites (Salminen & Karonen, 2011). The secondary metabolites of particular interest to this dissertation are phenolic compounds. Depending on the chemical structure and concentration, phenolic effects on microbes can range from energy source to toxicity, influence soil extracellular enzyme activity, and precipitate nitrogen out of solution (Shaw *et al.*,

2006; Dijkstra *et al.*, 2013). These diverse functions of different phenolic root exudates may partly explain the varying degrees of root priming reported in the scientific literature and help us understand species-specific effects of roots on soil carbon cycling in forests.

Recent evidence also suggests that phenolic root exudates play an important role in driving shifts in microbial communities (Badri *et al.*, 2013). However, most studies investigating root exudation effects on rhizosphere microbial communities use annual plants at particular developmental stages (Badri *et al.*, 2009; Chaparro *et al.*, 2013, 2014) or labile organic acids and sugars (Shi *et al.*, 2011), thus ignoring the diversity of compounds that may have a more significant impact in forest soils. So far no one has traced how phenolics may have compound-specific impacts on rhizosphere microbial communities and how changes in community composition may translate to biological function. Unraveling these interactions between plant roots, micro-organisms and soil chemical processes is crucial in order to enhance our understanding of rhizosphere development and its role in the terrestrial carbon cycle.

A common experimental setup to study root exudate effects on SOM decomposition is to create a simplified rhizosphere using membrane filters or artificial roots through which a continuous or one-time application of a single compound is delivered to the soil (Kuzyakov *et al.*, 2007; Renella *et al.*, 2007; Keiluweit *et al.*, 2015). Most of these studies used glucose or other primary metabolites to simulate root exudation (Hamer & Marschner, 2005; Landi *et al.*, 2006; Kuzyakov *et al.*, 2007). This approach does not only ignore the chemical diversity of root products released by plants into the soil, but also overlooks the interactions between different root exudates

in the rhizosphere. While these incubation studies were the first and important steps towards a better understanding of how root exudates shape the biochemical environment of the rhizosphere, it is essential to take these experiments to the next level by including questions about the interactive effects of primary and secondary metabolites on rhizosphere biogeochemistry.

***Drought-induced changes in fine root production and lifespan***

Unlike studies of root exudation, many studies have focused on analyzing fine root production and turnover patterns (Jackson *et al.*, 1997; Gill & Jackson, 2000; Norby & Jackson, 2000; Norby *et al.*, 2001). However, in the context of water limitation, results are inconclusive. Limitation in belowground resources is expected to lead to increased belowground carbon allocation (Bloom *et al.*, 1985; Coomes & Grubb, 2000) for root production and maintenance; however, this may not be the case during drought, which can reduce aboveground assimilation of carbon (McDowell & Sevanto, 2010).

Both increases and decreases in fine root production have been reported in response to drought (Mainiero & Kazda, 2006; Gaul *et al.*, 2008; Metcalfe *et al.*, 2008; Moser *et al.*, 2014). In addition, studies exploring the effect of drought on root turnover have also found opposing results, ranging from no change to both negative and positive effects (Nobel, 1997; Leuschner *et al.*, 2001; Comas *et al.*, 2005; Meier & Leuschner, 2008b). These contrasting patterns may be explained by species variation in their drought response (Leuschner *et al.*, 2001) or the degree of water limitation (Zang *et al.*, 2014). Moreover, the distribution of water resources across soil depths could affect the extent to which fine root production is halted or relocated to deeper

soil layers (Peek *et al.*, 2006; Hodge, 2009). Soil water heterogeneity is more complicated to reproduce in greenhouse or outdoor experiments using pots, bins or rhizotron boxes. Yet, many drought studies on root growth dynamics have made use of these methods (Meier & Leuschner, 2008a; Zang *et al.*, 2014). There is a need for more field studies that can investigate the effect of seasonal water limitation on fine root production at the ecosystem-scale for longer periods of time.

In addition to root growth into areas of higher soil moisture, the lifespan of fine roots will influence soil resource acquisition. Fine root lifespan has been shown to be a plastic trait responding to changes in soil nutrient resources (Adams *et al.*, 2013). Yet, few studies have analyzed the effect of soil moisture levels on root longevity (Eissenstat *et al.*, 2013). Similar to fine root production, multiple variables such as the severity of whole-plant water stress and the degree of soil water heterogeneity may ultimately determine the effect of seasonal drought on root lifespan (McCormack & Guo, 2014). Hence, the number of field experiments testing the effect of decreasing water availability on root growth dynamics under different settings needs to increase so that we can get a more complete understanding of the factors influencing root responses to drought.

Lastly, drought-induced changes in root growth dynamics are likely to be influenced by forest species composition. Indeed, competition and identity of neighboring tree species have been shown to affect root carbon allocation (Gersani *et al.*, 2001; Semchenko *et al.*, 2007). In the context of competition for or sharing of soil water resources, various studies have found niche partitioning in rooting depth, root placement, root dynamics and water acquisition strategies in mixed plant species

stands (Buttner & Leuschner, 1994; Nobel, 1997; Fargione & Tilman, 2005; Kulmatiski & Beard, 2013). Under abiotic stress, facilitation is hypothesized to be more prevalent than competition (Callaway & Walker, 1997). This is an especially important hypothesis to test in Central European forests of *Fagus sylvatica* (European beech) and *Picea abies* (Norway spruce). These forests are heavily managed and drought-vulnerable monocultures of *P. abies* are abundant (Pretzsch *et al.*, 2014). Planting mixed forests may sustain the cultivation of *P. abies* trees (Río *et al.*, 2014). Nevertheless, few studies have tested this abiotic stress hypothesis in the context of belowground productivity. Moreover, species identity of neighboring trees is often ignored in explaining root responses to water limitation (Novoplansky & Goldberg, 2001). Since belowground competition is a ubiquitous feature of most ecosystems (Casper & Jackson, 1997), studying the effects of species interactions on root growth dynamics may enhance our understanding of forest carbon cycling in a changing climate.

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# CHAPTER 2: PHENOLIC ROOT EXUDATE AND TISSUE COMPOUNDS VARY WIDELY AMONG TEMPERATE FOREST TREE SPECIES AND HAVE CONTRASTING EFFECTS ON SOIL MICROBIAL RESPIRATION[1]

## *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.*,

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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 the 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 heat-treated montmorillonite clay granules (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 2.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. 2.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 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 ($\alpha$) 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 2.1). 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 2.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 2.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.

**Table 2.1** 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.

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. 2. S2). Within the flavonoid class, flavanols had higher abundance than any of the other sub-classes in both root exudate and tissue samples (Fig. 2.S3, 1.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. 2,S5).

**Table 2.2** 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 | $6.6 \pm 0.7^{b}$ | $1.3 \pm 0.1^{d}$ | $0.05 \pm 0.02^{d}$ | $2.1 \pm 0.5^{c}$ | $19.19 \pm 5.72^{b}$ |
| *Alnus rugosa* | Exudate | $28.9 \pm 1.6^{a}$ | $7.9 \pm 0.3^{a}$ | $0.85 \pm 0.01^{a}$ | $9.3 \pm 2.8^{b}$ | $4.06 \pm 1.24^{c}$ |
| *Fagus grandifolia* | Exudate | $2.9 \pm 0.4^{c}$ | $1.6 \pm 0.2^{d}$ | $0.17 \pm 0.05^{cd}$ | $0.8 \pm 0.2^{c}$ | $2.60 \pm 2.39^{c}$ |
| *Picea abies* | Exudate | $20.0 \pm 2.0^{a}$ | $3.2 \pm 0.3^{c}$ | $0.26 \pm 0.04^{bc}$ | $7.6 \pm 1.1^{b}$ | $23.54 \pm 5.15^{b}$ |
| *Pinus strobus* | Exudate | $26.8 \pm 1.5^{a}$ | $4.5 \pm 0.2^{b}$ | $0.34 \pm 0.2^{b}$ | $28.6 \pm 3.2^{a}$ | $116.06 \pm 15.65^{a}$ |
| *Quercus rubra* | Exudate | $4.12 \pm 0.5^{bc}$ | $1.3 \pm 0.2^{d}$ | $0.06 \pm 0.04^{d}$ | $0.2 \pm 0.0^{d}$ | $1.00 \pm 0.53^{c}$ |
| *Acer saccharum* | Tissue | $15.3 \pm 0.5^{b}$ | $4.3 \pm 0.3^{c}$ | $0.41 \pm 0.03^{d}$ | $1111 \pm 85^{b}$ | $1450 \pm 250^{b}$ |
| *Alnus rugosa* | Tissue | $18.4 \pm 1.7^{b}$ | $8.0 \pm 0.1^{a}$ | $0.85 \pm 0.01^{a}$ | $992 \pm 107^{bc}$ | $400 \pm 120^{c}$ |
| *Fagus grandifolia* | Tissue | $15.8 \pm 0.3^{b}$ | $4.3 \pm 0.2^{c}$ | $0.52 \pm 0.01^{c}$ | $1127 \pm 64^{b}$ | |
| *Picea abies* | Tissue | $16.5 \pm 0.3^{b}$ | $8.5 \pm 0.2^{a}$ | $0.82 \pm 0.01^{a}$ | $1127 \pm 105^{b}$ | $3010 \pm 300^{a}$ |
| *Pinus strobus* | Tissue | $22.8 \pm 0.7^{a}$ | $7.0 \pm 0.0^{b}$ | $0.64 \pm 0.01^{b}$ | $1956 \pm 59^{a}$ | |
| *Quercus rubra* | Tissue | $15.5 \pm 0.6^{b}$ | $4.7 \pm 0.2^{c}$ | $0.53 \pm 0.03^{c}$ | $685 \pm 68^{c}$ | $700 \pm 130^{bc}$ |

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.

*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. 2.1). 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. 2.1a). All species had their unique phenolic profiles in root tissue (Fig. 2.1b). 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. 2.S6).

Root exudate compound and class diversity were different among tree species ($p < 0.001$, Table 2.2). *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 ($p < 0.05$).

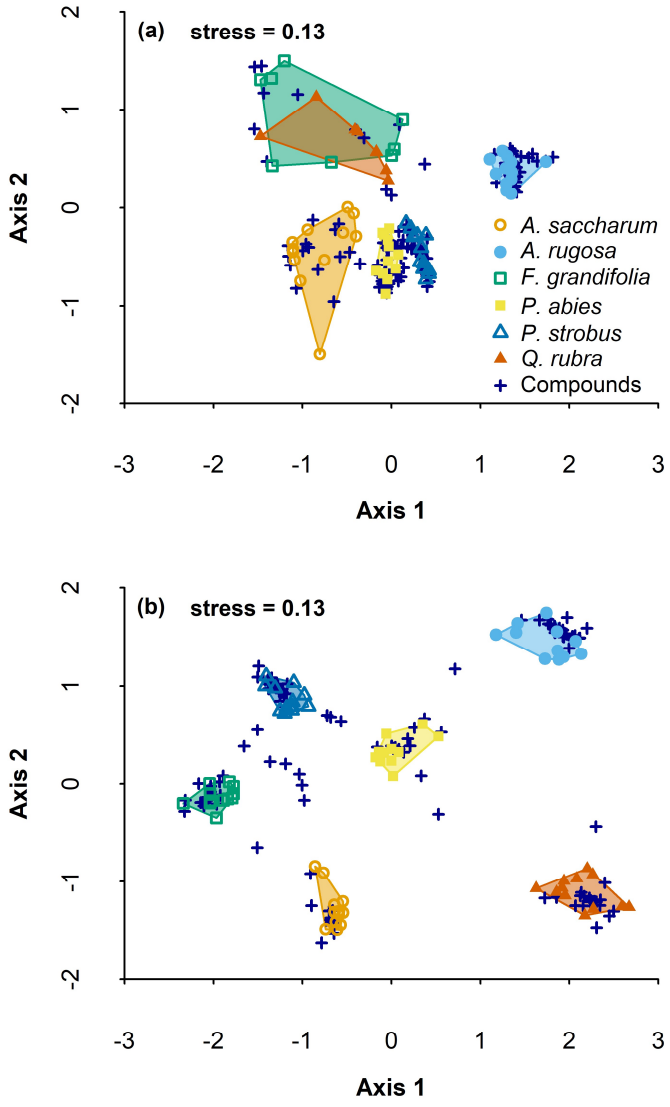


**Fig. 2.1** NMDS ordination of phenolic and diterpene composition of (a) root exudates and (b) fine root tissue for temperate forest tree species *A. saccharum* (orange), *A. rugosa* (light blue), *F. grandifolia* (green), *P. abies* (yellow), *P. strobus* (dark blue) and *Q. rubra* (dark red) (*N*=12 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.

The sum of relative root exudate abundances was also different among the tree species ($p < 0.001$, Table 2.2). *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 2.2) 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. 2.2) ranging from a 26% decrease to a 46% increase (Table 2.3). 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 2.3). 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. 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. 2.3). 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.

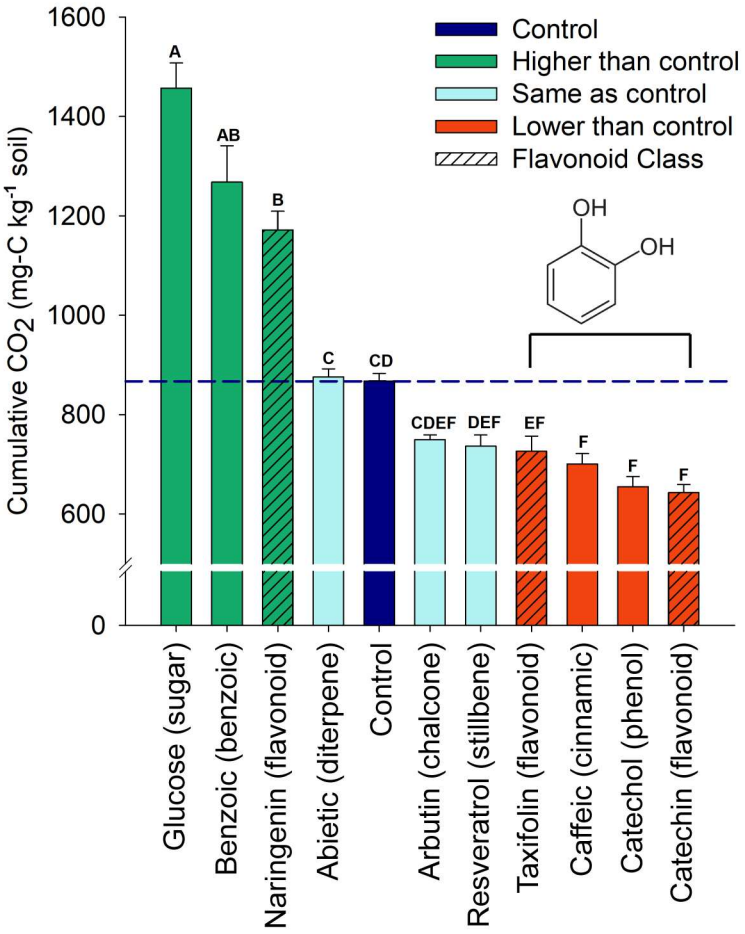


**Fig. 2.2** Cumulative respiration measured over a five-day period as influenced by phenolic and diterpene compounds belonging to different biosynthetic classes (means and standard errors, *N*=8). Biosynthetic classes are in parentheses. Different capital letters indicate significant differences ($p < 0.05$) 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).

**Table 2.3** 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.

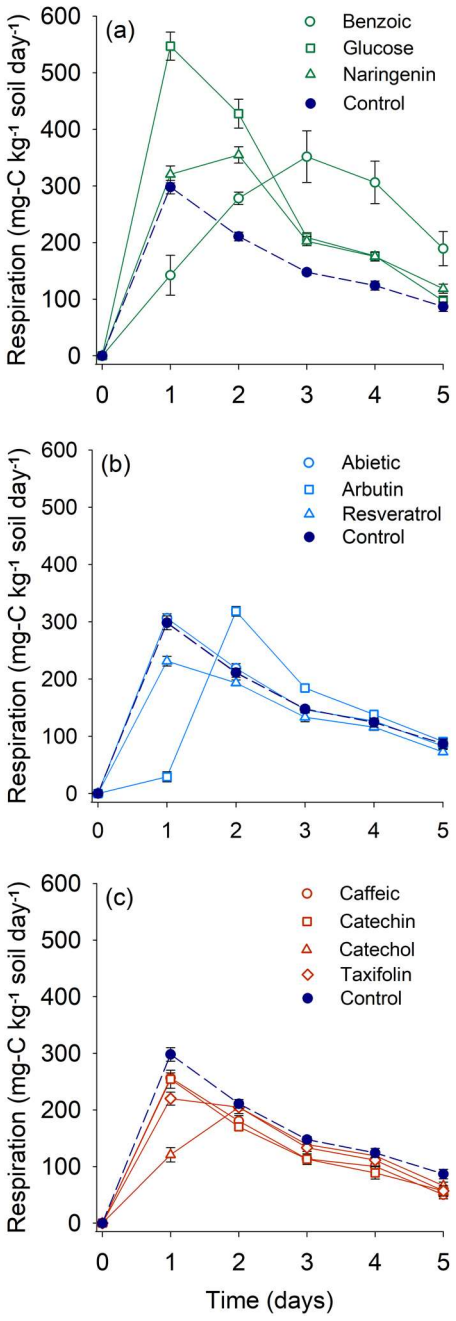


**Fig. 2.3** Daily respiration of soil-phenolic and -diterpene compound mixtures that (a) increased cumulative respiration relative to the control, (b) did not affect cumulative respiration relative to the control, and (c) decreased cumulative respiration relative to control (means and standard errors, *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. 2.2.

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. 2.3c).

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 to the control on the first day (Fig. 2.3a,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 community 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 research.

*Stimulatory phenolic effects on soil microbial respiration*

Two different C sources could have fueled the increase in cumulative respiration (Fig. 2.2): 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 2.3), 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. 2.3a) 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. 2.2), 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. 2.2). 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 2.1, Fig. 2.1) 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. 2.1) and that root compound diversity varies across tree species (Table 2.2). 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. 2.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 2.2). Moreover, flavanols dominated the chemical profiles of most of the studied tree species (Fig. 2.S3, 2.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. 2.2) 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 a 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 2.2). 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 an important factor to consider in rhizosphere dynamics. Yet, additional studies are essential to test root phenolic function in natural rhizospheres and at the forest ecosystem-scale.

*Phenolic release into soil*

The higher proportions of flavonoids, chalcones and diterpenes in root exudates in comparison to the root tissue (Fig. 2.S5) and overall higher tissue class diversity suggest that roots can partly control which chemicals and phenylpropanoid classes are exuded. Several forms of membrane transport have been proposed for flavonoids including active secretion (Buer *et al.*, 2007; Sugiyama *et al.*, 2007; Cesco *et al.*, 2010). Selective compound exudation hints that these chemicals perform a function for the tree such as chemo-attractants, toxins or inhibitors of particular microbial processes (Cesco *et al.*, 2012; Sugiyama & Yazaki, 2014; Venturi & Keel, 2016). Through actively changing the secretion of root exudates, plants may also influence microbial respiration. This raises the question to what extent individual plants can alter exudation as a function of their environment. While this has been shown in plant-symbiont interactions and during nutrient deficiency (van Scholl *et al.*, 2006; Badri *et al.*, 2009), future studies should examine how plants may alter secondary metabolite production in response to their soil environment to control the microbial metabolism of their rhizospheres.

The difference in phenolic composition between root exudate and fine root tissue samples (Fig. 2.S5) suggests that the compounds released during root exudation

and root turnover vary greatly. A recent meta-analysis by Huo *et al.* (2017) listed chemical variation as one of the reasons for why root exudates and plant litter may influence SOM decomposition differently. Besides differences in chemical composition, we expect that amount of phenolic compounds released during root exudation and turnover varies. However, quantifying root exudates is extremely challenging. Because we used a hydroponic setup, the low concentration of phenolics in the collection solution may have drawn a higher amount of root exudates from the root than under natural settings (Vranova *et al.*, 2013). This would be a more significant limitation for compounds that reach the rhizosphere through passive transport than for compounds transported by active secretion. Moreover, transferring the root systems from a solid medium to hydroponics may have caused disturbance and changed exudation rates. While we should be cautious about quantitative statements regarding root exudate concentrations, we can still conclude that root 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.

***Supporting Information***

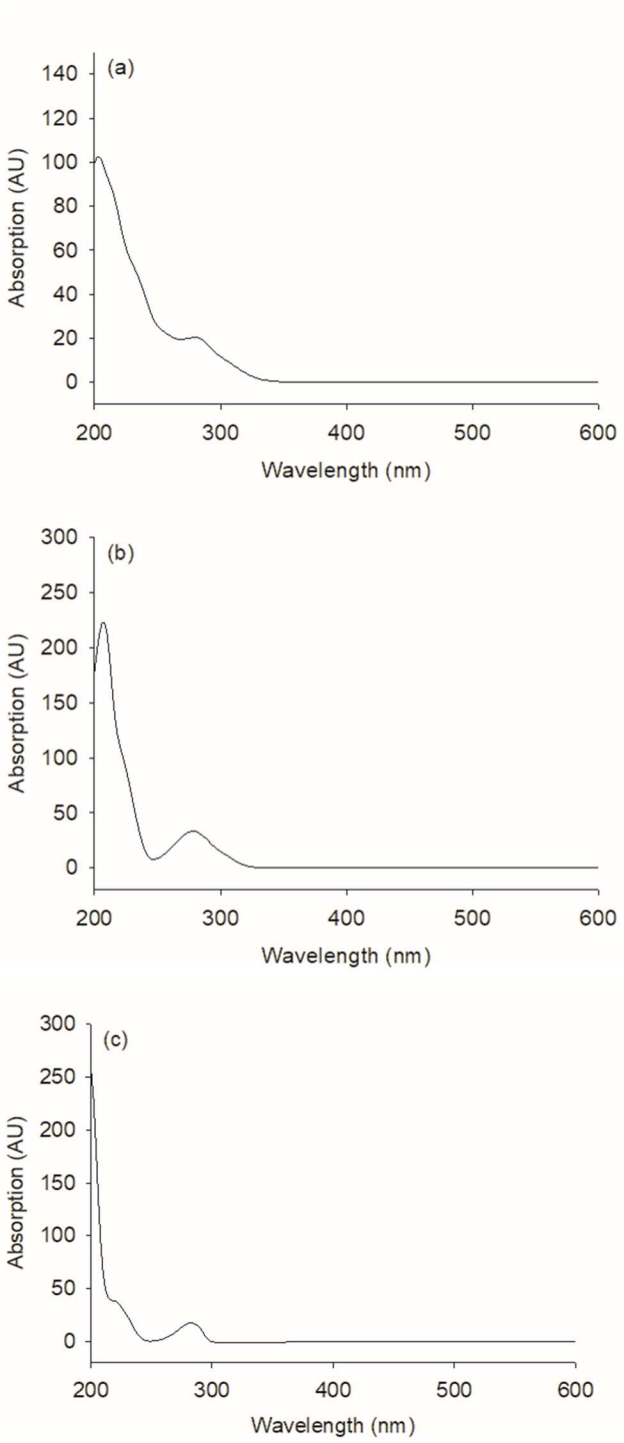


**Fig. 2.S1** UV-vis spectra of groups of unidentified compounds with similar absorption characteristics: (a) group A, (b) group B and (c) group C.

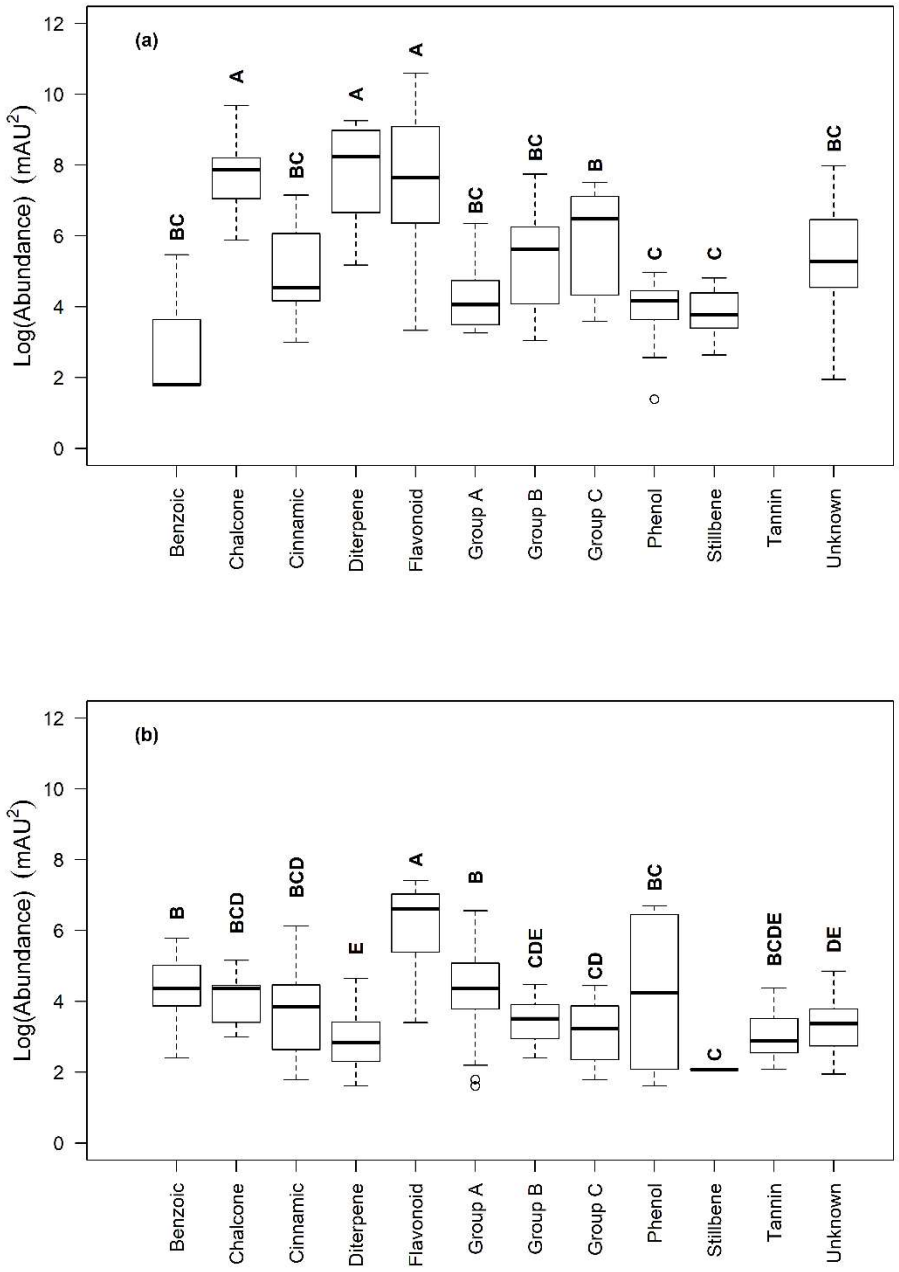


**Fig. 2.S2** Biosynthetic class relative abundance in (a) root exudates and (b) tissue extracts. Different letters indicate significant differences between classes as tested with Tukey HSD ($p < 0.05$). Median and inner quartiles (IQR) are represented by a box. Length of whiskers is determined by adding 1.5IQR to the maximum value of the third quartile and subtracting 1.5IQR from the minimum value of the second quartile. Values above or below this range are indicated as outliers with circles.

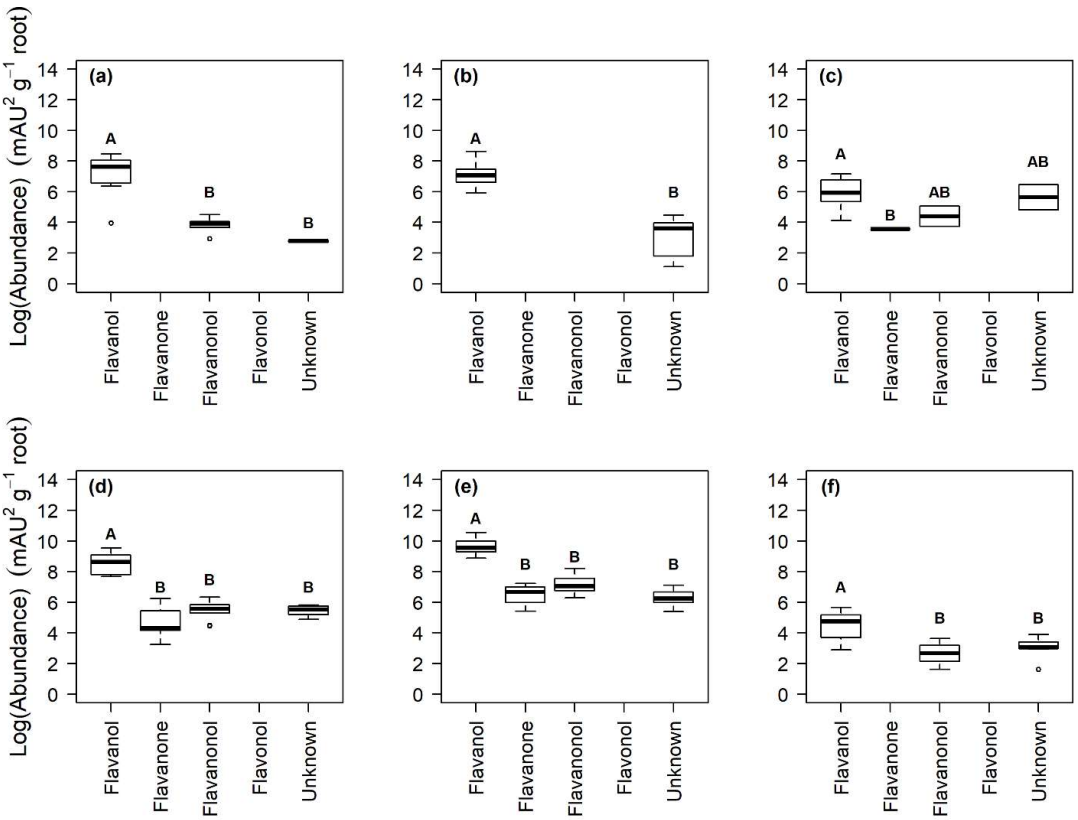


**Fig. 2. S3** Relative abundance of flavonoid sub-classes in root exudates by tree species: (a) *A. saccharum*, (b) *A. rugosa*, (c) *F. grandifolia*, (d) *P. abies*, (e) *P. strobus* and (f) *Q. rubra* (*N*=12 except for *F. grandifolia* and *Q. rubra* where N=8). Different letters indicate significant differences between sub-classes within species as tested with Tukey HSD ($p < 0.05$). Median and inner quartiles (IQR) are represented by a box. Length of whiskers is determined by adding 1.5IQR to the maximum value of the third quartile and subtracting 1.5IQR from the minimum value of the second quartile. Values above or below this range are indicated as outliers with circles.

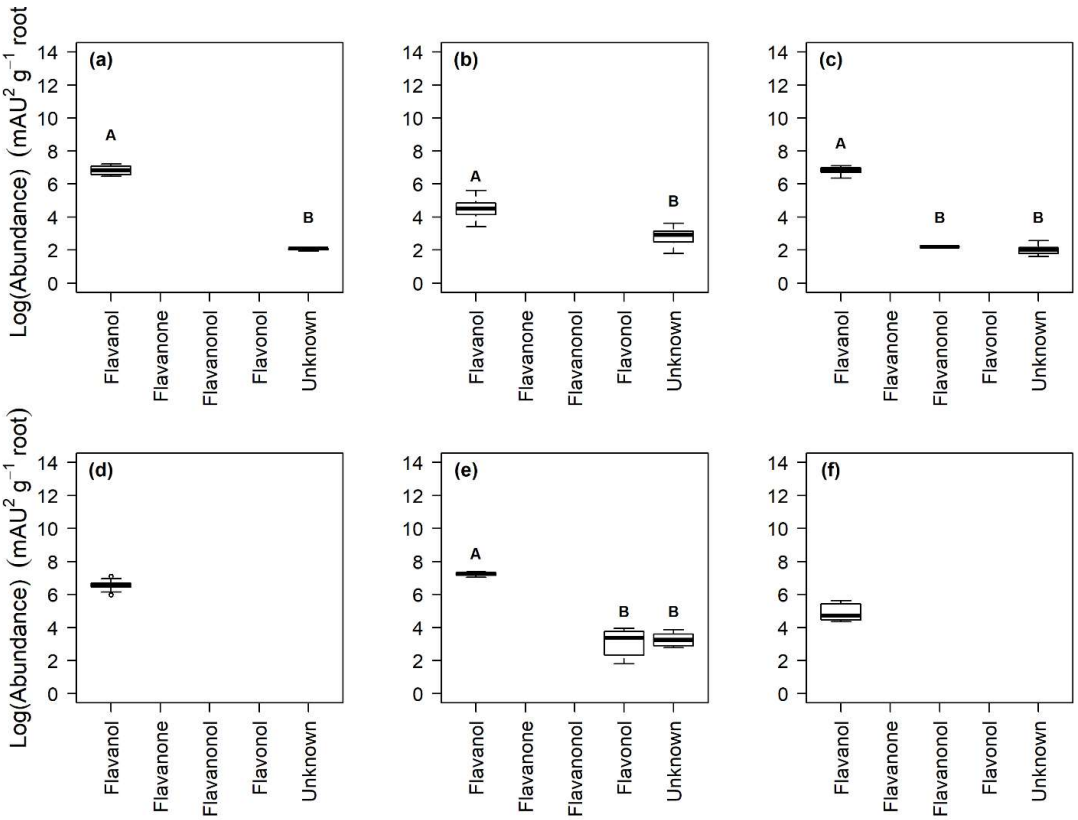


**Fig. 2.S4** Relative abundance of flavonoid sub-classes in root tissue by tree species: (a) *A. saccharum*, (b) *A. rugosa*, (c) *F. grandifolia*, (d) *P. abies*, (e) *P. strobus* and (f) *Q. rubra* (*N*=12 except for *F. grandifolia* and *Q. rubra* where *N*=8). Different letters indicate significant differences between sub-classes within one species as tested with Tukey HSD ($p < 0.05$). Median and inner quartiles (IQR) are represented by a box. Length of whiskers is determined by adding 1.5IQR to the maximum value of the third quartile and subtracting 1.5IQR from the minimum value of the second quartile. Values above or below this range are indicated as outliers with circles.

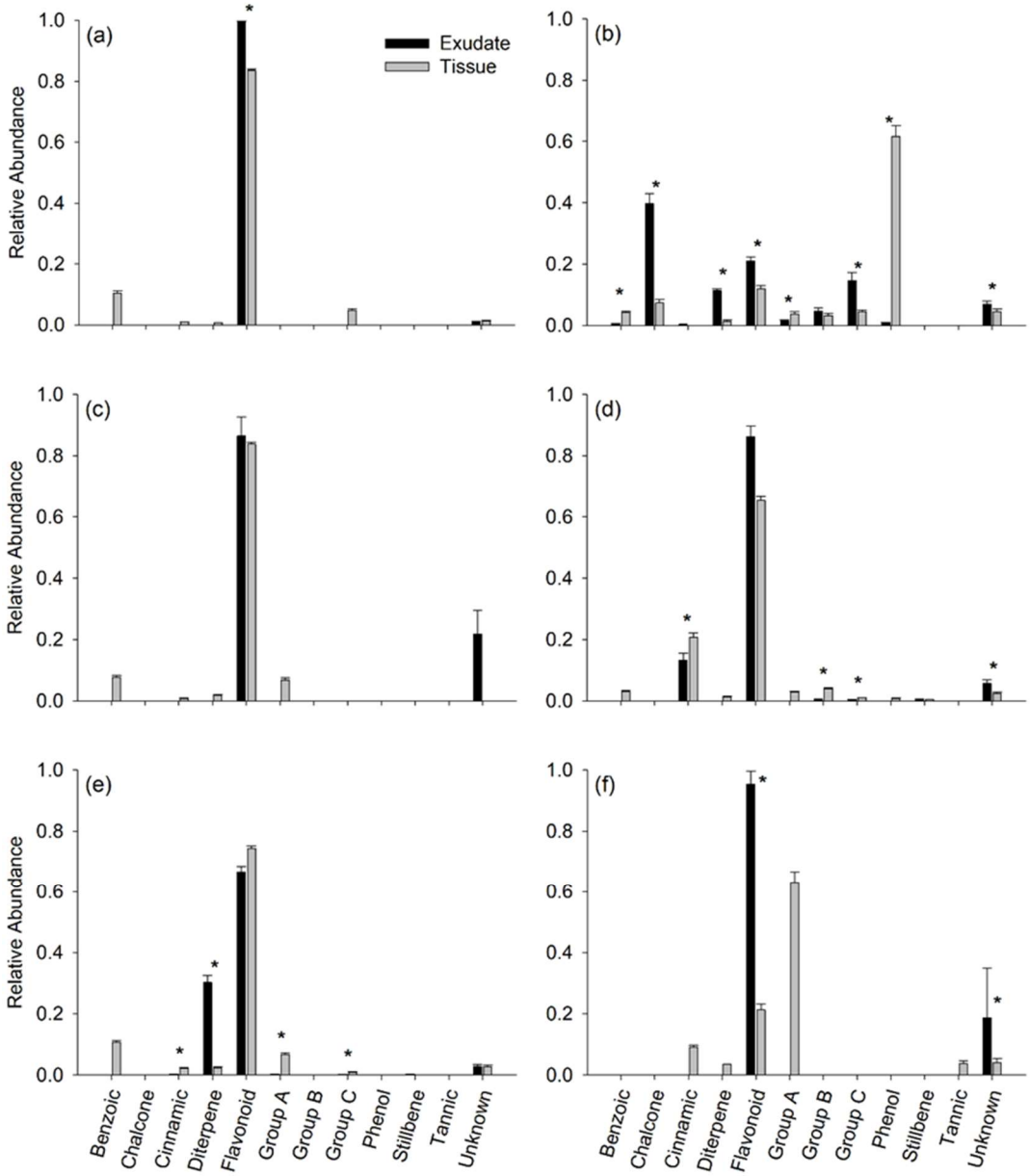


**Fig. 2.S5** Proportions of biosynthetic classes in the sum of relative abundances of root exudate and tissue samples by tree species: (a) *A. saccharum*, (b) *A. rugosa*, (c) *F. grandifolia*, (d) *P. abies*, (e) *P. strobus* and (f) *Q. rubra* ($N$=12 except for *F. grandifolia* and *Q. rubra* where $N$=8). A star indicates a significant difference between root exudate and tissue sample within one species as tested with Tukey HSD ($p < 0.05$). Means and standard errors are shown.

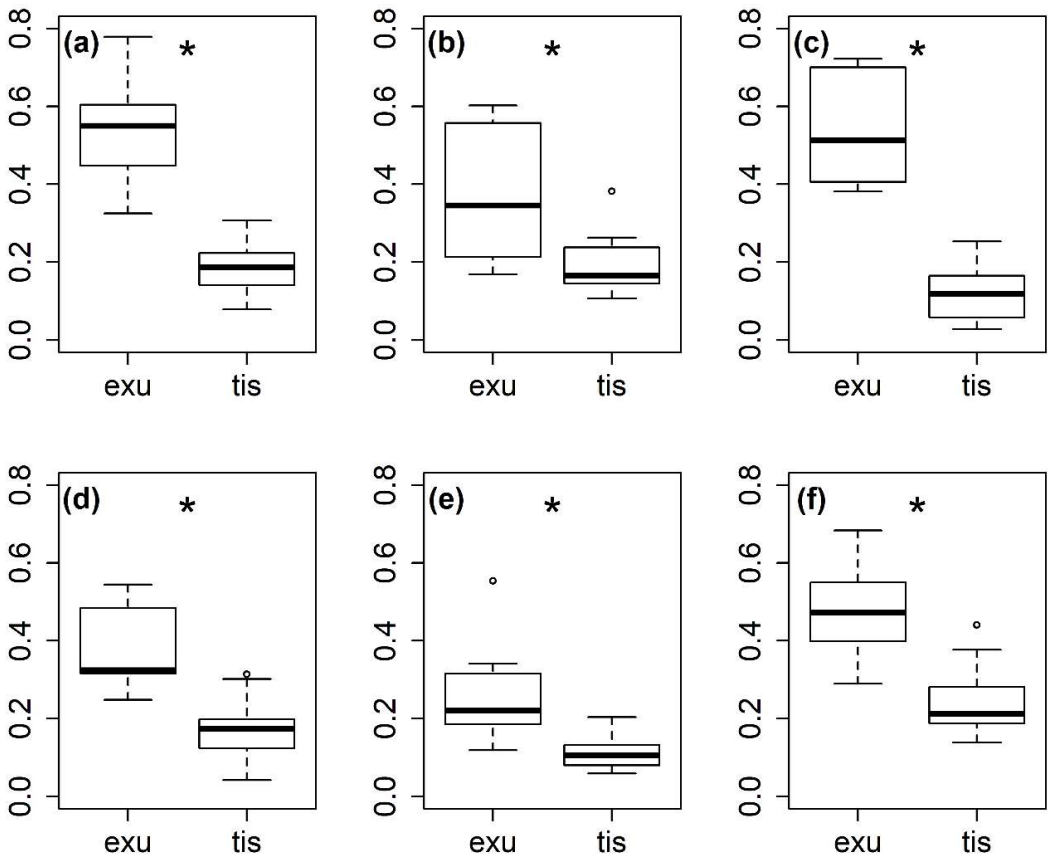


**Fig. 2.S6** Distance of species points in NMDS ordination plot to centroid of their respective clusters in the root exudate (exu) and tissue (tis) samples for the tree species (a) *A. saccharum*, (b) *A. rugosa*, (c) *F. grandifolia*, (d) *P. abies*, (e) *P. strobus* and (f) *Q. rubra* (*N*=12 except for *F. grandifolia* and *Q. rubra* where *N*=8). A star indicates a significant difference as tested with analysis of variance (ANOVA, $p < 0.05$). Median and inner quartiles (IQR) are represented by a box. Length of whiskers is determined by adding 1.5IQR to the maximum value of the third quartile and subtracting 1.5IQR from the minimum value of the second quartile. Values above or below this range are indicated as outliers with circles.

**Table 2.S1** Chemical standards used for compound identification and class assignment.

| Compound | Class | Sub-Class | Vendor (USA) | Catalog Number |
|---|---|---|---|---|
| Benzoic acid | Benzoic acid-derivatives | | Sigma Aldrich, St. Louis, MO | 242381-25G |
| Gallic Acid | Benzoic acid-derivatives | | Sigma Aldrich, St. Louis, MO | G7384-100G |
| Phthalic acid | Benzoic acid-derivatives | | Sigma Aldrich, St. Louis, MO | 402915-25G |
| Salicylic acid | Benzoic acid-derivatives | | Sigma Aldrich, St. Louis, MO | 247588-100G |
| Syringic acid | Benzoic acid-derivatives | | Sigma Aldrich, St. Louis, MO | S6881-5G |
| Arbutin | Chalcones | | Krackeler Scientific, Inc., Albany, NY | 66468-50MG |
| Phloridzin | Chalcones | | Krackeler Scientific, Inc., Albany, NY | 79589-50MG |
| Caffeic Acid | Cinnamic acid-derivatives | | Neta Scientific, Hainesport, NJ | CAYM-70602-10 |
| Chlorogenic acid | Cinnamic acid-derivatives | | Neta Scientific, Hainesport, NJ | CMX-21877-25G |
| Cinnamic acid | Cinnamic acid-derivatives | | Sigma Aldrich, St. Louis, MO | C80857-5G |
| *p*-Coumaric acid | Cinnamic acid-derivatives | | Sigma Aldrich, St. Louis, MO | C9008-10G |
| Ferulic acid | Cinnamic acid-derivatives | | Sigma Aldrich, St. Louis, MO | 128708-5G |
| Vanillic acid | Cinnamic acid-derivatives | | Sigma Aldrich, St. Louis, MO | 94770-10G |
| Umbelliferone | Coumarins | | Sigma Aldrich, St. Louis, MO | H24003 |
| Coumestrol | Coumestans | | Sigma Aldrich, St. Louis, MO | 27883 |
| Abietic acid | Diterpenes | | VWR International, Radnor, PA | 42582-MD |
| Pimaric acid | Diterpenes | | Santa Cruz Biotechnology, Inc., Dallas, TX | SC-296090 |
| Apigenin | Flavonoids | Flavone | Neta Scientific, Hainesport, NJ | SCBT-sc-3529 |
| (+)-Catechin hydrate | Flavonoids | Flavanol | Sigma Aldrich, St. Louis, MO | C1251-5G |
| (+)-Catechin | Flavonoids | Flavanol | Krackeler Scientific, Inc., Albany, NY | 43412-10MG |
| Chrysin | Flavonoids | Flavone | Neta Scientific, | CMX-21891 |

| | | | | |
|---|---|---|---|---|
| | | | Hainesport, NJ | |
| Daidzein | Flavonoids | Isoflavone | Sigma Aldrich, St. Louis, MO | D7802 |
| Daidzin | Flavonoids | Isoflavone | Sigma Aldrich, St. Louis, MO | 42926 |
| (-)-Epicatechin | Flavonoids | Flavanol | Krackeler Scientific, Inc., Albany, NY | 49045-U |
| Formomenetin | Flavonoids | Isoflavone | Sigma Aldrich, St. Louis, MO | 47752 |
| Genestein | Flavonoids | Isoflavone | Sigma Aldrich, St. Louis, MO | G6649 |
| Genestin | Flavonoids | Isoflavone | Sigma Aldrich, St. Louis, MO | 48756 |
| Naringenin | Flavonoids | Flavanone | Neta Scientific, Hainesport, NJ | CAYM-14173-10 |
| Quercetin | Flavonoids | Flavonol | Neta Scientific, Hainesport, NJ | CAYM-10005169-10 |
| Rutin | Flavonoids | Flavonol | VWR International, Radnor, PA | A13570 |
| Taxifolin | Flavonoids | Flavanonol | Neta Scientific, Hainesport, NJ | SCBT-sc-202828 |
| Benzoquinone | Simple phenols | | Krackeler Scientific, Inc., Albany, NY | B10358-5G |
| Catechol | Simple phenols | | Krackeler Scientific, Inc., Albany, NY | C9510-100G |
| Phenol | Simple phenols | | Krackeler Scientific, Inc., Albany, NY | P5566-25G |
| Resveratrol | Stillbenes | | Krackeler Scientific, Inc., Albany, NY | R5010-100MG |
| Tannic acid | Tannins | | Krackeler Scientific, Inc., Albany, NY | 403040-50G |

**Table 2.S2** The number of root exudate and tissue samples that showed an exact match with a phenolic reference during HPLC analysis. Samples are organized by tree species (*N*=12 except for *F. grandifolia* and *Q. rubra* where *N*=8).

| Species | Sample | Caffeic acid | Catechin | Gallic acid | Naringenin | Resveratrol | Syringic acid | Taxifolin |
|---|---|---|---|---|---|---|---|---|
| *Acer saccharum* | **Exudate** | | 4 | | | | | 4 |
| | **Tissue** | | 12 | | | | | |
| *Alnus rugosa* | **Exudate** | | 11 | | | | 1 | |
| | **Tissue** | | 11 | 9 | | | 9 | |
| *Fagus grandifolia* | **Exudate** | | 4 | | 1 | | | 2 |
| | **Tissue** | | | | | | | 1 |
| *Picae abies* | **Exudate** | | 12 | | | 5 | | 9 |
| | **Tissue** | | 11 | | | | | |
| *Pinus strobus* | **Exudate** | | 12 | | 11 | 3 | | 7 |
| | **Tissue** | 12 | | | | | | |
| *Quercus rubra* | **Exudate** | | 6 | | | | | 4 |
| | **Tissue** | | 12 | | | | | |

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# CHAPTER 3: DIFFERING FUNCTIONS OF ROOT PHENOLICS AND GLUCOSE HAVE INTERACTIVE EFFECTS ON SOIL ORGANIC MATTER DECOMPOSITION AND BACTERIAL COMMUNITY DYNAMICS IN A TEMPERATE FOREST SOIL[2]

## *Introduction*

Plant roots release a wide range of primary and secondary metabolites into soil (Shi *et al.*, 2012; Badri *et al.*, 2013; Haichar *et al.*, 2014), creating hotspots of biological activity (Kuzyakov & Blagodatskaya, 2015). The accelerated cycling of carbon (C) and nutrients in the rhizosphere may be responsible for up to one-third of the soil organic matter (SOM) mineralized in forest ecosystems (Finzi *et al.*, 2015), highlighting the importance of root-soil interactions to terrestrial biogeochemical cycles (Phillips *et al.*, 2011; Yin *et al.*, 2014; Meier *et al.*, 2017). While root exudates fuel the "priming" of SOM breakdown (Kuzyakov, 2002), they also facilitate belowground plant interactions with a diverse community of microorganisms (Philippot *et al.*, 2013). To date, few studies have simultaneously analyzed the role of root exudates as microbial substrate (Zhalnina *et al.*, 2018) and signaling compounds (van Dam & Bouwmeester, 2016). Hence, the multi-functionality of root exudates in the rhizosphere and their interactive effects on soil microbial composition and activity remain unclear. Considering the cocktail of diverse root compounds reaching and likely modifying the soil (Pinton *et al.*, 2009), it is crucial to enhance our

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understanding of the different mechanisms by which root exudates mediate soil biogeochemical processes (Hinsinger *et al.*, 2006).

One proposed mechanism that many root priming hypotheses have in common is that root exudates provide a labile source of C and energy to soil microbes increasing their activity and production of extracellular enzymes involved in the breakdown of SOM (Kuzyakov, 2010). Yet, the underlying biotic mechanisms of this increase in microbial activity remain disputed. Microbial growth (Blagodatskaya & Kuzyakov, 2008), triggering of microbial metabolism (Mason-Jones & Kuzyakov, 2017), and a shift in microbial community composition (Fontaine *et al.*, 2003) are all feasible explanations for a rise in soil microbial respiration and could even occur at the same time. This is where the difference in the chemical structure and function of root exudates may come into play. While soil microbes can efficiently incorporate labile compounds such as glucose into biomass (Strickland *et al.*, 2012; Bradford *et al.*, 2013), the uptake and metabolism of more complex substrates result in lower microbial growth because they require a larger number of enzymatic and metabolic pathways for degradation and assimilation (Manzoni *et al.*, 2012). In fact, some studies have shown that recalcitrant C sources like plant litter lead to greater priming effects than labile glucose compounds (Bingeman *et al.*, 1953; Wu *et al.*, 1993) possibly because extracellular enzymes produced for compound degradation are also involved in SOM breakdown. Moreover, recent studies identified chemical classes (Badri *et al.*, 2013) and functional groups (Zhalnina *et al.*, 2018) of root exudates as important drivers of rhizosphere community assembly. So far soil incubations testing and comparing the effect of different root exudates on soil microbial activity and C

cycling have mostly focused on primary metabolites like sugars and organic acids (Landi *et al.*, 2006; Renella *et al.*, 2007; Keiluweit *et al.*, 2015), disregarding the variety of secondary metabolites that may act through other mechanisms than just providing substrates for microbial respiration and growth.

Phenolic compounds in particular are an interesting set of secondary metabolites to rhizosphere processes. In addition to being a microbial food source, phenolic functions vary from signaling molecules (Hassan & Mathesius, 2012; Haichar *et al.*, 2014; Venturi & Keel, 2016) to inhibitors of enzyme activity (Salminen & Karonen, 2011) and toxins (Shaw *et al.*, 2006; Shaw & Hooker, 2008). In a previous study, we showed that the root phenolic profiles of two coniferous and four deciduous temperate forest tree species were highly species-specific (Zwetsloot *et al.*, 2018), providing evidence that phenolic exudates may drive differences in rhizosphere environments observed among plant species (Phillips & Fahey, 2006; Berg & Smalla, 2009; Ladygina & Hedlund, 2010; Shahzad *et al.*, 2015). In this same study, we incubated a forest soil amended with nine different phenolic compounds identified through root exudate analysis. Interestingly, we found contrasting phenolic effects on soil microbial respiration ranging from a 26% decrease to a 46% increase (Zwetsloot *et al.*, 2018). These results together with previous studies emphasize the importance of analyzing the mechanisms by which plant secondary metabolites influence soil microbes in order to obtain a more holistic view on rhizosphere biogeochemistry.

Studying root exudate-soil interactions is extremely challenging due to limited rhizosphere access and the diversity of chemicals simultaneously released into the soil (Shi *et al.*, 2013). Therefore, rhizosphere research requires a combined approach of

field, greenhouse and laboratory studies. On the one hand, field and greenhouse experiments can analyze rhizosphere processes under natural conditions (Haichar *et al.*, 2008; Shi *et al.*, 2012; Yin *et al.*, 2018). However, it is challenging to study the causal effects of root exudates on microbial-mediated rhizosphere processes under these circumstances because other root variables could be confounding the results and conclusions are mainly based on correlations. On the other hand, soil incubations mimicking a rhizosphere with the use of single exudate applications to a bulk soil are able to tackle questions about the direct role of root exudates in rhizosphere C and nutrient cycling (Kuzyakov *et al.*, 2007) but have the drawback of being more artificial. Such an experimental set-up also offers the unique opportunity to study the interactive effects of two root exudates with different functions on biogeochemical processes facilitated by soil microbes. Yet, laboratory studies have mainly focused on the priming effects of individually added labile compounds disregarding the extent by which secondary and primary metabolites may facilitate or inhibit each other's mode of action in the rhizosphere.

Few studies have explored this question (Hamer & Marschner, 2005) and we can only speculate about possible outcomes when a soil receives two root exudates with contrasting functions. Because phenolic compounds can decrease microbial activity (Zwetsloot *et al.* 2018), they could reduce the degradation and priming effects of labile compounds such as glucose. However, the potential role of phenolic compounds in rhizosphere community assembly (Badri *et al.* 2013) could also select for a larger abundance of aromatic compound (Chaudhry *et al.* 2005) and SOM degraders. In this case, the addition of glucose may increase their activity leading to a

greater priming effect. Considering the wide range of priming effects reported in the scientific literature (Huo *et al.*, 2017), it is crucial to further investigate the role of root exudate interactions in rhizosphere priming.

Therefore, the aim of this study was to analyze the mechanistic effect of soil amendments with glucose and phenolic compounds exuded by roots on microbial respiration of exudate- and soil-derived C by means of a 38-day soil incubation. We hypothesized that glucose functions as a food source quickly degraded by microbes, while phenolics are less easily broken down and have other mechanistic effects including signaling, toxicity, inhibition of enzyme activity, and mobilizing of soil resources. Therefore, our expectations were (1) if a phenolic compound increases soil respiration, it is more likely than glucose to induce priming of SOM decomposition, (2) microbial community composition is altered more by phenolics than by glucose, and (3) phenolics and glucose show interactive effects on soil microbial respiration and community composition.

## ***Materials and Methods***

### *Study design and experimental setup*

To evaluate how phenolics influence rhizosphere processes in the presence or absence of primary metabolites, we added a phenolic compound with or without glucose to a forest soil and measured their effect on soil $CO_2$ emissions, enzyme activity, microbial biomass and bacterial community composition (BCC) over a 38-day period. Adding root chemicals to soil to create a simplified or artificial rhizosphere is similar to the approach of other incubation studies analyzing the role of single root exudates on the

soil environment (Renella *et al.*, 2006; Keiluweit *et al.*, 2015). Because some of the phenolic compounds used in our study are not water-soluble, we applied the compounds in solid state to an air-dried soil at two different application rates.

The soil used in this study came from the Arnot Forest (Central NY, USA), is derived from bedrock and glacial till, and classified as acidic Dystrochrepts (Bohlen *et al.* 2004). After removing the organic horizon, we collected the soil from the first 0-15 cm mineral layers in an area of the forest dominated by *Acer saccharum* (sugar maple). Fine roots dominate this soil layer turning most of this soil into a large rhizosphere soil sample. Soils were air-dried and sieved (<2mm) removing rocks and roots as well as breaking up soil aggregates. Total soil organic C was 3.64 g $kg^{-1}$ and organic matter with loss on ignition was 8.74%. Total nitrogen and phosphorus were 0.29 g $kg^{-1}$ and 16.65 g $kg^{-1}$. Soil pH was 4.2 in water.

We used three different phenolic compounds for this study: benzoic acid, caffeic acid and catechin. These compounds were selected because our previous study showed they are exuded by tree roots and have contrasting effects on soil microbial respiration (Zwetsloot *et al.*, 2018). Phenolic effects were compared to an unamended control treatment. Half of the soil replicates including the untreated control also received a glucose application. Together this led to a full factorial design of eight treatments (4 x phenolic, 2 x glucose). In addition, we performed an experiment to test the extent by which application rate influences phenolic function in the rhizosphere. This experiment followed the same full factorial design as above, but the phenolic application rate was ten times lower while glucose was added at the same dose. The high dose was based on the catechin concentration of the fine root tissue (0.05 to 2.8

mg C $g^{-1}$) of the six tree species tested in our previous work (Zwetsloot *et al.* 2018). The lower rate was included because reported total carbon estimates of daily root exudation show approximately one to two magnitudes of variation (Brzostek *et al.*, 2013).

Soils received a one-time application of phenolic and/or glucose compounds both at a rate of 0.5 mg-C $g^{-1}$ soil, or 0.05 mg-C $g^{-1}$ soil for the low dose phenolic application. Glucose was only applied at the high dose. We used highly-labeled $^{13}C$-compounds (Sigma-Aldrich, St. Louis, MO, USA) to determine whether microbial respiration utilized C from native SOM or from the phenolic compound. In the soil treatments receiving a single phenolic or glucose amendment, the compound addition was labeled. For soils that received both a phenolic and glucose addition, only glucose was labeled. To create a label of 100 ‰ $\delta^{13}C$ with the highly-labeled $^{13}C$-compounds, we mixed the labeled chemicals with their natural abundance counterparts, for which benzoic acid and catechin were obtained from the same source and caffeic acid from Cayman Chemical (Ann Arbor, MI, USA). Because some of the phenolic compounds were insoluble, dilution of the label was achieved by combining the labeled and unlabeled compound with 20 g of air-dried soil from the Arnot forest, NY. This way, a sufficient volume was available to homogenize the labeled sample in a 60 ml qorpak jar (Qorpkac, Inc., Bridgeville, PA, USA). To achieve the desired compound:soil ratio for incubation, we added the labeled mixtures (above) to more air-dried soil in a new jar creating a large single sample for each of the soil treatments. After shaking thoroughly in 473 ml or 946 ml Mason jars (Newell Brands, Hoboken, NJ, USA), 15 g of each treatment mixture (7.5 mg substrate-C $jar^{-1}$, $N=5$) was weighed out into a 60

ml qorpak jar and placed in a 473 ml Mason jar with a lid and rubber septum.

At the start of the experiment, water was added to reach 60% water holding capacity (WHC) and the jars were put in a dark growth chamber at 20°C. Soil water content was checked regularly and maintained at 60% WHC through water additions. Soil respiration was monitored at intervals over the course of the experiment using potassium hydroxide (KOH) traps and headspace gas sampling. For the experiment with phenolic compounds added at a rate of 0.5 mg-C $g^{-1}$ soil, soils were destructively sampled on day 5, 15 and 38 for extracellular enzyme activity, microbial biomass and community composition analyses. For the lower phenolic application rates, soil samples were only taken at the end of the experiment on day 38. The soil samples were stored in a -20°C freezer until further analysis.

*Soil $CO_2$ measurements and stable-isotope analyses*

We made use of two different methods to measure soil microbial respiration, KOH traps and isotopic analysis. The KOH trap method is ideal for estimating the total amount of C respired over the course of the experiment due to its continuous presence in the jar, and interval-gas sampling is most effective for assessing hourly respiration rates and obtaining gas samples for isotopic analysis.

For the KOH method, a vial with 15 ml of 0.09 M KOH solution was placed in each replicate treatment jar. By means of an acid-base reaction, the KOH solution constantly absorbs respired $CO_2$ functioning as a trap (Zibilske et al. 1994). On days 1- 8, 11, 15, 21, 29 and 38, KOH traps were removed and assessed for total absorbed $CO_2$ by measuring the reduction in electrical conductivity (benchtop conductivity

meter Model 860032, Sper Scientific Direct, Scottsdale, AZ, USA). Clean vials with a new KOH solution were returned after each measurement to ensure maximum functionality of the trap. Jars injected with 2.5, 5, 10 or 20 mg-C of 100% $CO_2$ gas ($N$=2) functioned as calibration standards to calculate $CO_2$ concentration. In addition, we included a set of blank jars ($N$=5) with KOH traps to correct for atmospheric $CO_2$.

To measure short-term respiration rates and the isotopic signature of respired $CO_2$, we took three gas samples per replicate jar at a 2-hour time intervals on day 1, 2, 5, 15 and 38. During this time period, KOH traps were removed from the jars. Gas samples (15 mL) were transferred to septum-capped exetainer vials (SKU E2852, EA Consumables, Pennsauken, NJ, USA) that had been flushed with nitrogen gas and evacuated prior to use. Samples were submitted to Cornell University Stable Isotope Laboratory (COIL) for $CO_2$ concentration and C isotope ratios (GC Pal Autosampler, Agilent Technologies, Santa Clara, CA, USA) interfaced via a Gas Bench II (Thermo Scientific, Bremen, Germany) to a Thermo Scientific Delta V Advantage isotope ratio mass spectrometer. Using the Keeling plot approach, we calculated the respiration rate and $\delta^{13}C$ source (Pataki *et al.*, 2003). The proportion of respired C coming from the soil versus the labeled phenolic or glucose addition was determined by means of a mixing model (Dawson *et al.*, 2002). In the treatment where both a phenolic compound and glucose (labeled) were added, the mixing model calculated the respired C derived from the glucose versus the unlabeled phenolic compound plus SOM. To estimate the amount of soil-derived C in these treatments, we subtracted the amount of labeled phenolic C calculated as determined in the no-glucose treatment from the remaining C in the phenolic-glucose treatment.

To calculate the cumulative amount of exudate- and soil-derived C respired over the course of the experiment, we multiplied the exudate-C to soil-C ratio estimated by the stable isotope analysis at specific time points by the cumulative total respiration measured by the KOH traps over the time intervals where we did not take gas samples.

*Extracellular enzyme activity assay*

We measured the activity of five extracellular enzymes: β-1,4-glucosidase (BG), β-1,4-N-acetylglucosaminidase (NAG), acid phosphatases (PHOS), phenol oxidase (POX), and peroxidase (PER) (Saiya-Cork *et al.*, 2002) from the soil treatments sampled at day 5, 15 and 38. To analyze the activity of the hydrolytic enzymes, we used black 96-well plates and substrates with the fluorescing agent 4-methylumbelliferone. For the oxidative enzymes, we used clear 96-well plates and L-3, 4-dihydroxyphenylalanine as a substrate. Soil samples were thawed at 4°C overnight. After plate preparation, the BG, NAG and PHOS enzyme assays were incubated in the dark at 22 °C for 4 hours and the POX and PER assays for 24 hours. Hydrolytic enzyme activity was quantified using a fluorometer with 365 nm excitation and 450 nm emission filters and oxidative enzyme activity was estimated by measuring the absorbance of the samples at 450 nm with a microplate spectrophotometer (Synergy HT, BioTek Instruments, Winooski, VT, USA). Correcting for blanks and negative controls, we calculated extracellular enzyme activities in units of nmol $hr^{-1}$ $g^{-1}$ dry soil.

*Microbial DNA, microbial biomass and pH*

As a measure of microbial abundance on day 5, 15 and 38, we quantified soil DNA of samples using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen™, Thermo Fisher Scientific, Inc., Waltham, MA, USA ) (Sandaa *et al.*, 1998) after DNA from the soils was extracted (DNeasy PowerSoil Kit (QIAGEN, Germantown, MD, USA). DNA extracts were diluted 400 times in 1x TE buffer and pipetted into 96-well plates. Upon receiving a 200-fold dilution of PicoGreen, plates were incubated in the dark for 5 minutes. Fluorescence was measured with FilterMax F5 micro-plate reader (Molecular Devices, San Jose, CA, USA) with 485 nm excitation and 535 emission. 1x TE buffer blanks and standards were prepared and analyzed on the same plate.

At the end of the incubation (day 38), soil microbial C and nitrogen (N) were measured using the fumigation-extraction method (Brookes *et al.*, 1985; Vance *et al.*, 1987). Two samples of 5 g per replicate were weighed out into 50mL centrifuge tubes. The first sample immediately received 25 mL 0.05 M $K_2SO_4$, was shaken for 1 hour on an oscillating shaker, and filtered with a Whatman No. 1. The second sample was fumigated by pipetting 3 mL of chloroform onto two cotton balls in the headspace of the tube, and capping the tube (Allen & Schlesinger, 2004). After 24 hours, the cotton balls were removed and the centrifuge tubes were left open for 1 hour to let any of the remaining chloroform evaporate. The fumigated sample was extracted following the same steps as the non-fumigated sample. Samples were stored in at -20°C and analyzed for organic C and N with a Shimadzu TOC-L, TNM-L, ASI-L (Columbia, MD, USA).

Soil pH was measured in water at a ratio of 1:10 (w/w) with a benchtop pH

meter (Mettler Toledo Dl12 titrator with pH electrode (DG-111-SC), Columbus, OH, USA) at the end of the incubation.

*SSU rRNA Gene qPCR and Sequencing*

The V4 region of the 16S rRNA gene was amplified from soil DNA extracts on day 5, 15 and 38 by polymerase chain reaction (PCR) using dual-indexed barcoded 515f/806r primers as described by Kozich *et al*. (2013). Each 25 µL PCR reaction contained (i) 2 ng of DNA template; (ii) 12.5 µL of Q5 High Fidelity, Hot Start PCR Mastermix (New England Biolab, MA, USA); (iii) 1.25 µL of 10 µM primer; and (iv) a final concentration of 0.05x Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher Scientific, MA, USA). The following thermocycler conditions were used to amplify the 16S rRNA gene: initial denaturation at 95 °C for 2 minutes, then 30 cycles of 95 °C for 20 sec, 55 °C for 15 sec (annealing) and 72 °C for 10 sec (extension) with followed by a final extension 72 °C for 5 min. Amplicon libraries were produced in duplicate for every sample and the gene copy number was quantified during PCR using the CFX Connect Real-Time system (BioRad, CA, USA). Standard curves were prepared using the qPCR products from the bacteria *Burkholderia* sp. RP11 with a dilution series of Qubit-quantified DNA (ThermoFisher Scientific, MA, USA) and run in triplicate for every sample plate. There were no differences in PCR efficiency based on the slight differences in the GC content of 10 bp barcode sequence. When the $R^2$ of a standard curve was lower than 0.97, the plate was repeated. Copy numbers for each reaction were calculated with the relative fluorescent units (RFU) threshold set at 150 using Bio-Rad CFX manager and normalized to copies $g^{-1}$ dry weight of soil.

Duplicate PCR reactions were pooled, purified and normalized to a standard concentration using the SequalPrep normalization kit (Invitrogen, CA, USA).Samples were multiplexed and sequenced at the Biotechnology Resource Centre (Cornell University, Ithaca, NY, USA) on a single lane of Illumina MiSeq (2x250 paired-end) with an 8% addition of PhiX.

*Data analysis and statistics*

All statistical analyses were performed in R (R Core Team, 2017). We used the emmeans packages (Lenth, 2018) to interpret the interactions of linear regression models and to perform Tukey Honest Significance Difference (HSD) tests. For the management and analysis of the microbial sequencing data including Non-Metric Multi-Dimensional Scaling (NMDS), we used the phyloseq (McMurdie & Holmes, 2013) and vegan (Oksanen, 2017) packages. The Hmisc package (Harrel, 2018) helped us perform Spearman's correlations between respiration and other microbial parameters.

To analyze effects of phenolic and glucose amendments on soil respiration, enzyme activity, soil DNA, microbial C, microbial N and pH over time, we used linear regression models, analysis of variance (ANOVA) type-3 tables and Tukey HSD tests. Hourly respiration rates of total-C, exudate-C and soil-C as well as amount of soil DNA were log-transformed to satisfy the normality and equal variance of the residuals assumptions. Time (day 5, 15, 38) was handled as categorical variable. Besides comparing the fourteen treatments to each other, we also evaluated these phenolics effects within the groups where glucose was present or absent. We also individually

compared treatments to the control by means of Dunnett's multiple comparisons test.

Bacterial 16S rRNA gene amplicon sequence variant (ASV) libraries were filtered to remove contaminant present in no template controls (a 1.1% and 3.5% decrease in total sequences and number of ASVs, respectively) and low abundant and irregular ASVs, if they occurred in fewer than three samples or at a total relative abundance less than 0.01% of all samples. ASVs occurring in fewer than three samples at greater than 1% total relative abundance were retained (3 ASVs). Alpha-diversity estimates including richness and Shannon index (1948) were performed on rarified libraries ($N$=8,796 sequences per sample), while all other analyses were performed on counts normalized to total counts per thousand reads.

NMDS analysis using Weighted Unifrac distances (Lozupone & Knight, 2005) was used to estimate differences in BCC. In order to test the effect of phenolic and glucose addition on the shift in community composition relative to the control treatment, we calculated the mean Weighted Unifrac distance of each sample to the unamended soil replicates at its respective time point and performed a one-way ANOVA analysis on this distance across treatments. In addition, we correlated this measure of microbial community shift and the PicoGreen estimates of microbial abundance with respiration using Spearman's correlation tests. To evaluate the effect of phenolic compounds, glucose, time and their interactive effects on the relative abundance of phyla present in each sample, we performed a three-way ANOVA. Because we observed the greatest abundance changes in Proteobacteria, Acidobacteria and Firmicutes, we further explored treatment effects on relative abundance at genus level within these phyla.

## *Results*

### *Soil microbial respiration*

Phenolic applications led to contrasting trends in the respiration of exudate- and soil-derived C over time, while glucose amendments were quickly degraded, thereby increasing total respiration (Fig. 3.1). Peak respiration from soils with a high benzoic acid addition lagged one day behind all other phenolic treatments. Yet, despite this lag time, these soils had a significantly higher respiration rate by day 2 (Fig. 3.1a, $p < 0.05$). The glucose application increased total microbial respiration of all phenolic treatments. Except for the soil treatments with a high dose of caffeic acid and a low dose of catechin, the increase in total respiration caused by the glucose addition had leveled off by day 15.

Glucose was mostly respired on the first few days of the incubation with only benzoic acid lowering its degradation rate on day 1 (Fig. 3.1d, $p < 0.05$). Conversely, the degradation of phenolic C varied significantly among treatments over time (Fig. 3.1c, $p < 0.001$). Most phenolic C was respired from the benzoic acid treatments. By the fifth day, phenolic C was only detectable in the respiration samples from the treatments with high doses of benzoic acid or caffeic acid ($0.25 \pm 0.01$ and $0.14 \pm 0.07$ mg-C $hr^{-1}$ $kg^{-1}$ soil respectively, $p < 0.05$) at comparable amounts. Interestingly, we did not find evidence of catechin degradation at any time point ($p > 0.05$).

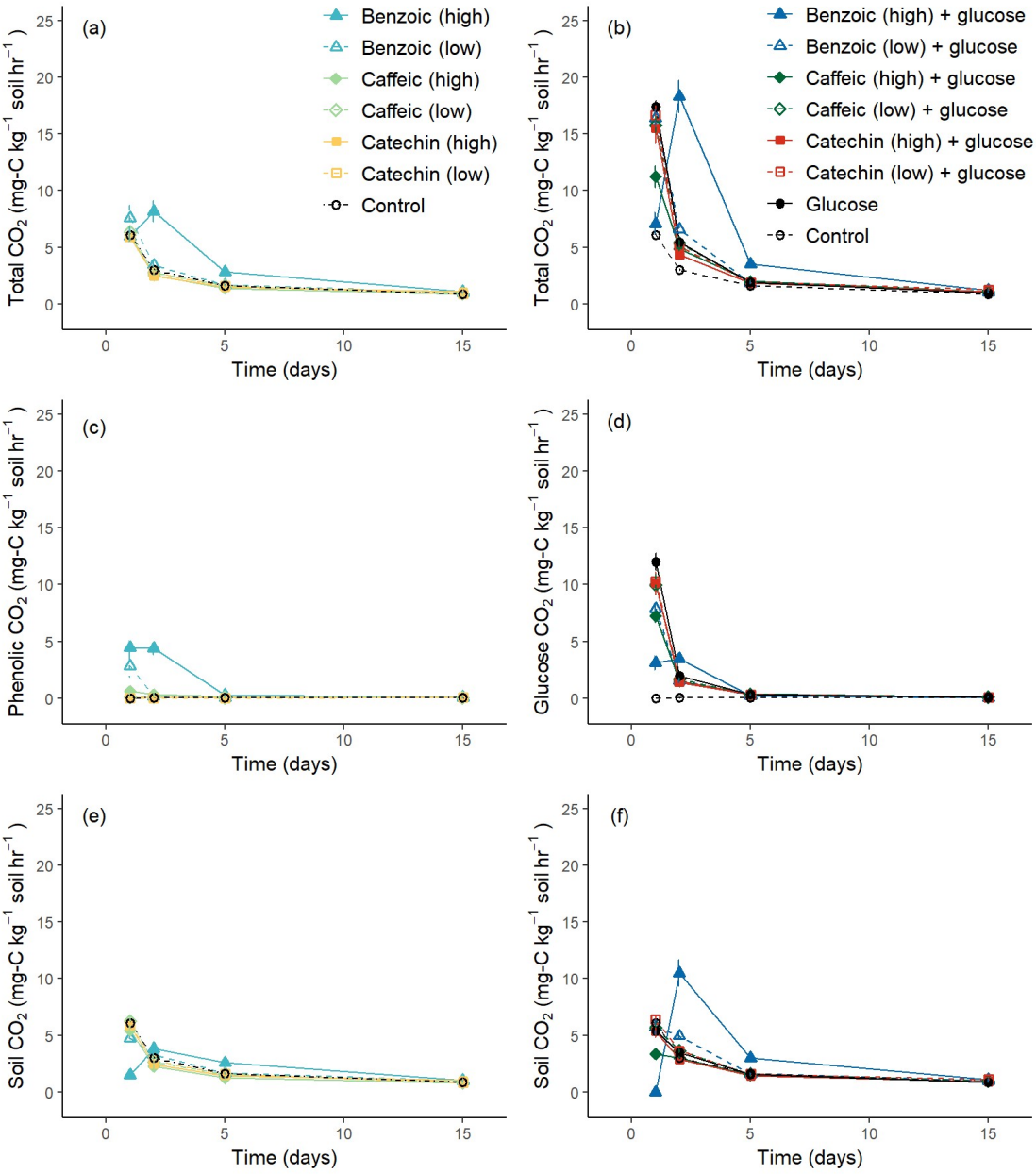


**Fig. 3.1.** Soil microbial respiration rates (mg-C $kg^{-1}$ soil $hr^{-1}$) shown as (a, b) total amount of carbon (C) respired, (c,d) amount of phenolic- or glucose-derived C respired, (e,f) amount of soil-derived C respired measured on day 1, 2, 5 and 15. Phenolic treatments without and with glucose are shown in the left and right panels respectively. Means and standard errors are shown ($N$=2-5). The compound treatments are indicated by different colors and symbols: control (black, open circle), glucose (black, closed circle), benzoic acid (light blue, triangle), benzoic acid with glucose (dark blue, triangle), caffeic acid (light green, diamond), caffeic acid with glucose (dark green, diamond), catechin (yellow, square), catechin with glucose (red, square). Phenolic application rates are 0.5 mg-C $g^{-1}$ soil (high, closed symbol with solid line) and 0.05 mg-C $g^{-1}$ soil (low, open symbol, dashed line).

The combined addition of benzoic acid and glucose primed the degradation of native SOM (Fig. 3.1f, $p < 0.05$). This trend was only detected for the low dose of benzoic acid on day 2, while the priming effect of the high dose persisted until day 5. Consistent with the trends in total respiration, the benzoic acid treatments reduced the respiration of soil-derived C on day 1 (Fig. 3.1.ef).

*Cumulative respiration and carbon partitioning*

After fifteen days, cumulative respiration was the greatest in soils amended with high doses of benzoic acid (Fig. 3.2, $p < 0.05$). Conversely, the high application of catechin suppressed net microbial activity, decreasing the cumulative $CO_2$ relative to the control and glucose treatments ($p < 0.05$). Low doses of phenolics did not elicit strong changes in microbial respiration.

The soils amended with a high dose of benzoic acid and glucose remained the most active throughout the 38-day incubation (Table 3.1, $p < 0.05$), while the total respiration from the other phenolic treatments without glucose did not significantly differ from controls. In terms of the cumulative respiration of soil-derived C, we found an interactive effect between glucose and both benzoic acid applications ($p < 0.001$). The joint addition of glucose and a high dose of benzoic acid led to the greatest mineralization of soil-derived C (40 % increase in soil-C respired), followed by the low benzoic acid treatment with glucose (22%). Even if we assumed all benzoic acid was consumed in the high application treatment with glucose, we would still observe an 18% priming effect. The remaining glucose treatments with and without phenolic amendments showed a 7-15% increase in SOM decomposition in comparison to the

control. Besides the high dose of benzoic acid (15%), phenolic treatments without glucose did not show a significant difference in cumulative soil-C respired.

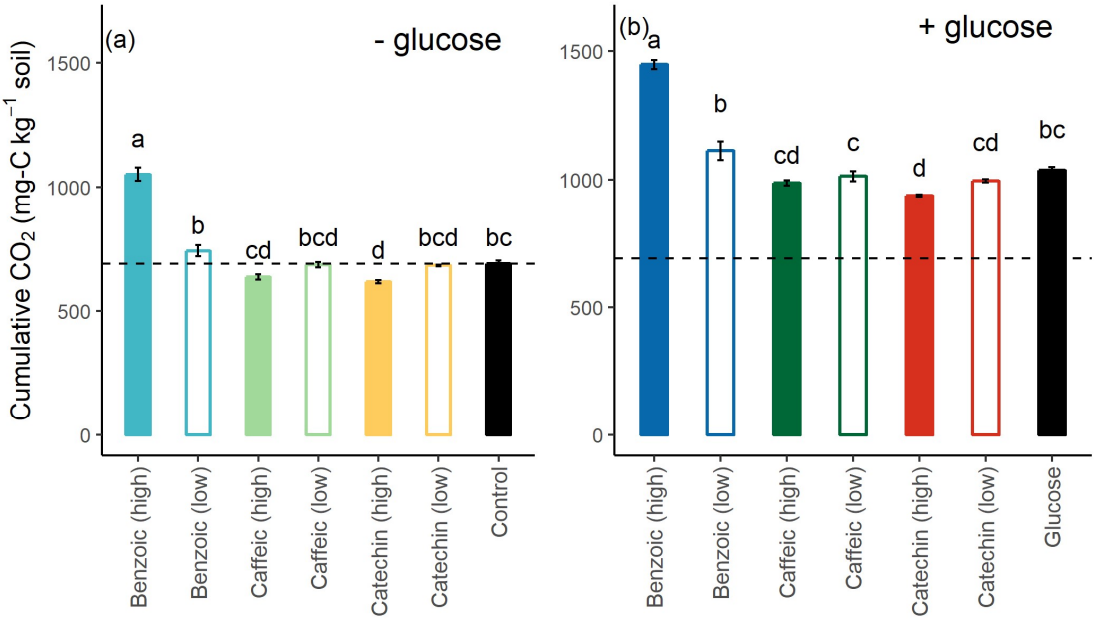


**Fig. 3.2** Cumulative respiration measured with potassium hydroxide (KOH) traps fifteen days after start of soil incubation with benzoic acid, caffeic acid and catechin at an application rate of 0.5 mg-C $g^{-1}$ soil (high) and 0.05 mg-C $g^{-1}$ soil (low). Results are grouped by (a) the absence or (b) presence of a 0.5 mg-C $g^{-1}$ soil joined glucose addition. Means and standard errors are shown (*N=5*). Contrasting letters indicate significant differences among treatments within their group ($p < 0.05$) as determined by the Tukey Honest Significant Difference test. Dashed line marks cumulative respiration from unamended control.

Phenolic amendments lowered the degradation of glucose ($p < 0.05$). Pure glucose amendments were respired at the highest rate, summing to 60% of the total C application. Amendments of glucose with high and low doses of caffeic acid ranked the second highest (52% and 51% of glucose, respectively). High and low doses of benzoic diminished the use of glucose to 40%. Catechin applications also decreased the amount of glucose respired but less so than the benzoic acid treatments, and likely for different reasons given the overall decline in respiration following catechin application. High doses of phenolic compounds were not as fully degraded as glucose

additions at the same concentration. The high dose of benzoic acid was respired the most (50%), followed by caffeic acid (18%) and lastly by catechin (9%). Low doses showed similar trends, but higher total amounts were respired (100%, 58% and 46%, respectively). While these differences were significant (Tukey HSD; $p < 0.05$), it is important to note that our calculations also indicated a small amount of $^{13}C$ respired from the control soils (38.2% of the low dose application).

**Table 3.1.** Estimation of the amount of $CO_2$ respired from phenolic or glucose compound addition (Compound-$CO_2$), soil organic matter (Soil-$CO_2$), and the total amount of C (Total-$CO_2$) respired over course of the 38-day incubation. Calculations used cumulative respiration measurements with KOH traps and soil-C to compound-C ratios from stable isotope analysis of gas samples. Mean and standard errors are shown (*N=5*). Contrasting letters indicate significant differences among treatments ($p < 0.05$) as determined with Tukey Honest Significant Difference test.

| **Root exudate treatments** | **Soil-$CO_2$ (mg-C kg$^{-1}$ soil)** | **Compound-$CO_2$* (mg-C kg$^{-1}$ soil)** | **Total-$CO_2$ (mg-C kg$^{-1}$ soil)** |
|---|---|---|---|
| Control | $1140 \pm 12^{e}$ | $19.1 \pm 0.2^{j}$ | $1159 \pm 13^{d}$ |
| Benzoic (high) | $1309 \pm 31^{c}$ | $252 \pm 7^{bc}$ | $1561 \pm 33^{bc}$ |
| Benzoic (low) | $1152 \pm 25^{de}$ | $50.7 \pm 0.8^{f}$ | $1203 \pm 25^{d}$ |
| Caffeic (high) | $1096 \pm 17^{e}$ | $87.8 \pm 1.3^{e}$ | $1184 \pm 18^{d}$ |
| Caffeic (low) | $1149 \pm \pm 14^{de}$ | $29.0 \pm 0.4^{h}$ | $1178 \pm 14^{d}$ |
| Catechin (high) | $1061 \pm 9^{e}$ | $43.4 \pm 0.5^{g}$ | $1105 \pm 9^{d}$ |
| Catechin (low) | $1132 \pm 3^{e}$ | $23.2 \pm 0.1^{i}$ | $1156 \pm 3^{d}$ |
| Glucose | $1243 \pm 8^{c}$ | $300 \pm 5^{a}$ | $1563 \pm 11^{bc}$ |
| Benzoic (high) + glucose | $1597 \pm 36^{a}$ | $192 \pm 3^{d}$ | $2041 \pm 37^{a}$ |
| Benzoic (low) + glucose | $1392 \pm 34^{b}$ | $209 \pm 7^{d}$ | $1652 \pm 41^{b}$ |
| Caffeic (high) + glucose | $1219 \pm 14^{c}$ | $259 \pm 3^{b}$ | $1565 \pm 15^{bc}$ |
| Caffeic (low) + glucose | $1280 \pm 17^{c}$ | $253 \pm 3^{b}$ | $1561 \pm 19^{bc}$ |
| Catechin (high) + glucose | $1191 \pm 16^{cd}$ | $241 \pm 3^{bc}$ | $1475 \pm 19^{c}$ |
| Catechin (low) + glucose | $1276 \pm 10^{c}$ | $235 \pm 5^{c}$ | $1534 \pm 11^{c}$ |

*Note that for the treatments with a phenolic and glucose application the amount of mg-C kg$^{-1}$ soil refers to the amount of glucose-derived C.

*Impact of Root Exudates on Microbial Biomass and Enzyme Activity*

At higher concentrations, caffeic acid and catechin reduced the amount of soil microbial DNA in the treatments with or without glucose (Fig. 3.3, $p < 0.05$). These patterns persisted from day 5 until the end of the incubation. The microbial abundance of soils amended with glucose and benzoic acid was not different from the control or the glucose-only treatment. Microbial abundance did not differ significantly between high and low doses on day 38.

The nitrogen content of microbial biomass and soil pH showed significant differences among treatments on day 38, while total microbial C did not show any differences (Table 3.2). Benzoic acid treatments without glucose had a lower amount of microbial N than all other treatments, though only significantly so in comparison to the glucose and high catechin amendment ($p < 0.05$). The addition of caffeic acid or catechin with glucose tended to increase microbial N content. Soil pH had shifted slightly among treatments by the end of the experiment (Table 3.2). The glucose treatments with a high dose of caffeic acid or catechin had a lower pH than the control soils as well as the soils amended with benzoic acid ($p < 0.05$).

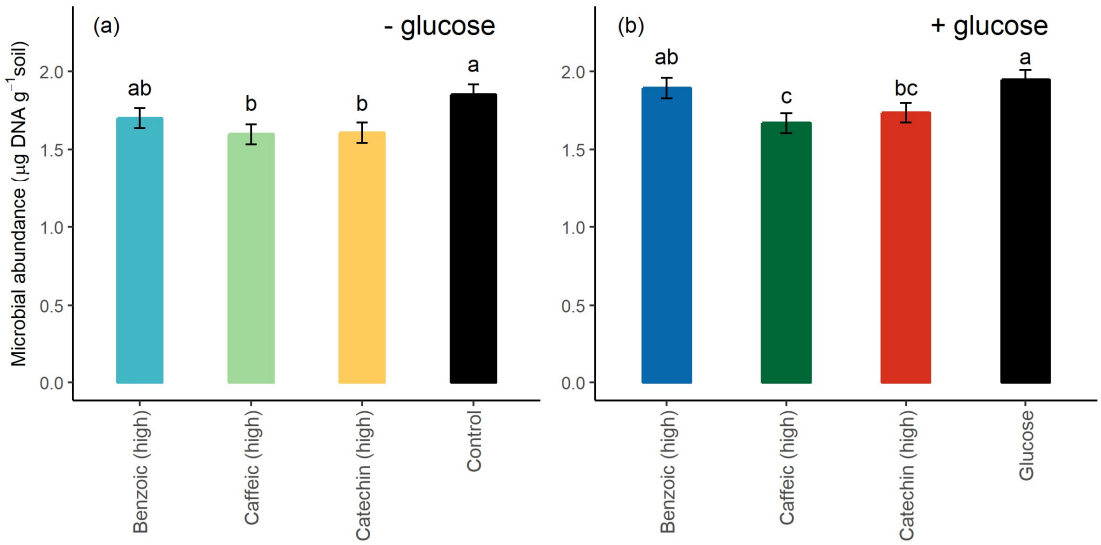


**Fig. 3.3** Microbial abundance measured at the fifteenth day of the soil incubation using Quant-iT™ PicoGreen™ dsDNA Assay Kit (Invitrogen™, Thermo Fisher Scientific, Inc., Waltham, MA, USA). Phenolic application of 0.5 mg-C $g^{-1}$ soil benzoic acid, caffeic acid, catechin or no phenolic addition are separated by (a) the absence or (b) presence of a glucose application at the same rate. Means and standard errors are shown (*N=5*). Contrasting letters indicate significant differences among treatments within their group ($p < 0.05$) as determined by the Tukey Honest Significant Difference test.

The increase in microbial respiration elicited by the phenolic and glucose additions was supported by observations of increased enzyme activity in amended soils (Fig. 3.4). The addition of glucose stimulated the activity of β-1,4-glucosidases (BG, $p = 0.03$), β-1,4-N-acetylglucosaminidases (NAG, $p = 0.04$) and acid phosphatases (PHOS, $p < 0.001$). High doses of phenolics significantly increased BG and NAG activity, but not PHOS. NAG activity was strongly stimulated by high doses of benzoic acid regardless of glucose additions relative to other treatments ($p < 0.05$). Catechin did not stimulate enzyme activity relative to glucose in any treatment. The heightened enzyme activity at day 15 in comparison to day 5 had subsided by the end of the incubation (Fig. 3.S1). Oxidative enzymes, phenol oxidase and peroxidase, were not affected in any treatment.

**Table 3.2.** Measurements of microbial carbon (C), microbial nitrogen (N), and soil pH in water at the end of the soil incubation (day 38) in the phenolic and glucose treatments. Means and standard errors are shown (*N=5* except for soil pH where *N*=4). Contrasting letters indicate significant differences among treatments ($p < 0.05$) determined by the Tukey Honest Significant Difference.

| **Root exudate treatments** | **Microbial C ($10^3$ mg-C $kg^{-1}$ soil)** | **Microbial N (mg-C $kg^{-1}$ soil)** | **$pH_{water}$** |
|---|---|---|---|
| Control | $1.42 \pm 0.06^{a}$ | $5.40 \pm 2.72^{ab}$ | $4.40 \pm 0.01^{ab}$ |
| Benzoic (high) | $1.55 \pm 0.15^{a}$ | $3.20 \pm 1.52^{b}$ | $4.44 \pm 0.07^{a}$ |
| Benzoic (low) | $1.77 \pm 0.18^{a}$ | $2.23 \pm 1.54^{b}$ | $4.42 \pm 0.09^{a}$ |
| Caffeic (high) | $1.81 \pm 0.26^{a}$ | $6.41 \pm 2.04^{ab}$ | $4.26 \pm 0.05^{bc}$ |
| Caffeic (low) | $1.64 \pm 0.19^{a}$ | $5.57 \pm 1.70^{ab}$ | $4.41 \pm 0.09^{ab}$ |
| Catechin (high) | $1.50 \pm 0.19^{a}$ | $4.00 \pm 1.20^{ab}$ | $4.33 \pm 0.05^{abc}$ |
| Catechin (low) | $1.75 \pm 0.27^{a}$ | $5.78 \pm 2.12^{ab}$ | $4.41 \pm 0.07^{ab}$ |
| Glucose | $1.68 \pm 0.30^{a}$ | $5.97 \pm 1.49^{ab}$ | $4.38 \pm 0.07^{ab}$ |
| Benzoic (high) + glucose | $1.59 \pm 0.16^{a}$ | $6.98 \pm 1.86^{ab}$ | $4.39 \pm 0.05^{ab}$ |
| Benzoic (low) + glucose | $1.55 \pm 0.20^{a}$ | $5.20 \pm 1.60^{ab}$ | $4.40 \pm 0.06^{ab}$ |
| Caffeic (high) + glucose | $1.67 \pm 0.26^{a}$ | $10.55 \pm 1.44^{ab}$ | $4.21 \pm 0.06^{c}$ |
| Caffeic (low) + glucose | $1.66 \pm 0.20^{a}$ | $4.67 \pm 2.08^{ab}$ | $4.40 \pm 0.01^{ab}$ |
| Catechin (high) + glucose | $1.64 \pm 0.14^{a}$ | $12.49 \pm 1.67^{a}$ | $4.20 \pm 0.06^{c}$ |
| Catechin (low) + glucose | $1.65 \pm 0.29^{a}$ | $7.35 \pm 2.04^{ab}$ | $4.38 \pm 0.04^{ab}$ |

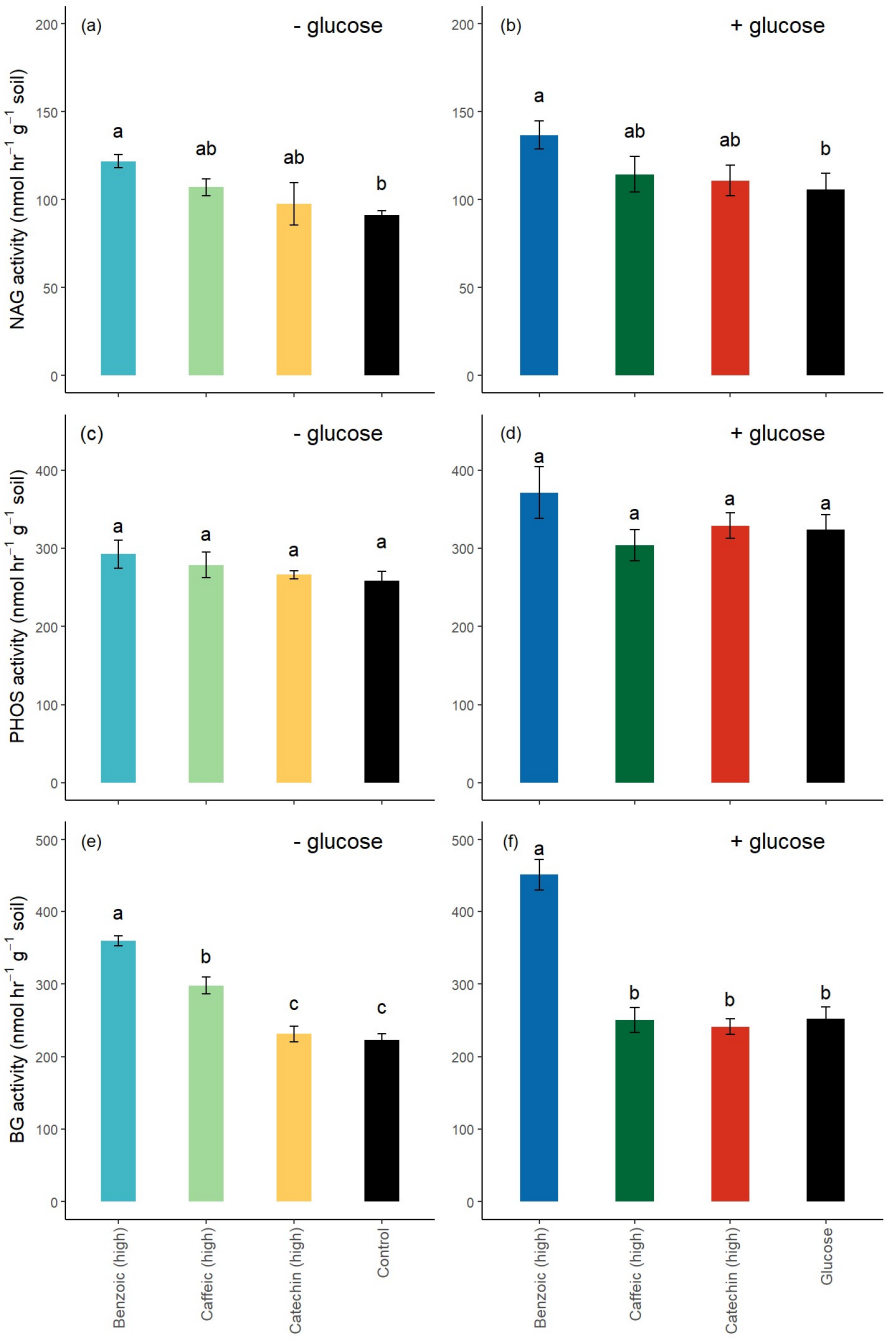


**Fig. 3.4** Potential enzyme activity of β-1,4-glucosidase (BG), β-1,4-N-acetylglucosaminidase (NAG) and acid phosphatases (PHOS) on day 15 of the soil incubation as influenced by the phenolics benzoic acid, caffeic acid and catechin applied at 0.5 mg-C g$^{-1}$ soil. Treatments are compared within groups of (a) the absence or (b) presence of a glucose applied at the same rate. Means and standard errors are shown (*N*=*5*). Contrasting letters indicate significant differences among treatments within their group ($p < 0.05$) determined by the Tukey Honest Significant Difference.

*Bacterial Community Composition*

Benzoic acid caused the most striking changes in bacterial community composition (BCC), consistent with measurements of respiration and enzyme activity. All substrate amendments influenced BCC in a time-dependent manner compared to unamended incubated soils (Fig. 3.5a). Incubation duration explained the greater differences in beta-diversity based on Weighted Unifrac dissimilarity (PERMANOVA; $R^2 = 0.34$, $p < 0.001$), followed by type of phenolic amendment ($R^2 = 0.18$, $p < 0.001$) and glucose amendment status ($R^2 = 0.02$, $p = 0.002$). Dose also had a significant effect on BCC based on data exclusively from day 38 (Fig. 3.S2, $R^2 = 0.06$, $p < 0.001$). We analyzed phenolic and glucose effects on BCC shifts from unamended control on day 5 because this was shortly after peak respiration and BCC differences were the greatest among treatments. Benzoic acid additions caused the greatest shift in BCC compared to unamended soil, followed by caffeic acid and lastly by catechin and glucose (Fig. 3.5b). Differences in BCC among treatments had already diminished by day 15. By the end of the incubation, we only found significant differences in BCC between benzoic acid dosed soils, catechin-only and control soils.

While bacterial richness was higher on day 15 and day 38 than on day 5 ($p < 0.05$), the glucose and phenolic additions did not influence richness ($p = 0.16$, $p = 0.23$, Table 3.S1). The root exudate treatments did significantly affect the Shannon index of the soil samples. On day 15, the control soils had a higher index than the soil replicates receiving a benzoic acid addition with or without glucose (Table 3.3, $p < 0.05$). As the incubation time increased, the Shannon index also became higher ($p < 0.05$).

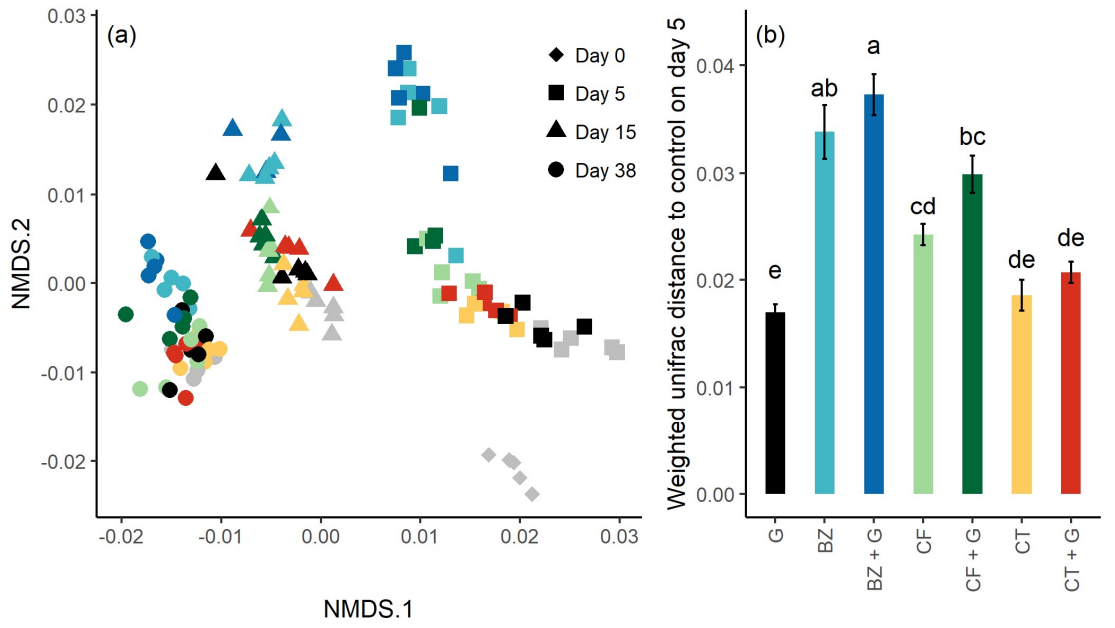


**Fig. 3.5** Phenolic- and glucose-driven shifts in bacterial community composition using Weighted Unifrac distances based on normalized count data analyzed and visualized with (a) non-metric multidimensional scaling (NMDS, stress = 0.08) and (b) one-way ANOVA of treatment effects on distance to control on day 5. The application treatments (*N*=5) are indicated by different colors: control (grey), glucose (G, black), benzoic acid (BZ, light blue), benzoic acid with glucose (BZ + G, dark blue), caffeic acid (CF, light green), caffeic acid with glucose (CF + G, dark green), catechin (CT, yellow), catechin with glucose (CT + G, red). Days are indicated by symbols: 0 (diamond), 5 (square), 15 (triangle) and 38 (circle). In panel b, means and standard errors are shown. Contrasting letters indicate significant differences among treatments ($p < 0.05$) as determined by the Tukey Honest Significant Difference test.

**Table 3.3.** Richness and Shannon diversity index calculated with filtered and rarified count data (permutations = 8,796) from treatment samples on day 15. Means and standard errors are shown (*N*=5). Contrasting letters indicate significant differences among treatments ($p < 0.05$) as determined by the Tukey Honest Significant Difference test.

| **Root exudate treatments** | **Richness** | **Diversity** |
|---|---|---|
| Control | $320 \pm 21^{a}$ | $4.99 \pm 0.05^{a}$ |
| Benzoic (high) | $334 \pm 24^{a}$ | $4.61 \pm 0.08^{bc}$ |
| Caffeic (high) | $319 \pm 58^{a}$ | $4.89 \pm 0.11^{ab}$ |
| Catechin (high) | $237 \pm 30^{a}$ | $4.74 \pm 0.10^{abc}$ |
| Glucose | $249 \pm 28^{a}$ | $4.70 \pm 0.07^{abc}$ |
| Benzoic (high) + glucose | $300 \pm 27^{a}$ | $4.43 \pm 0.10^{c}$ |
| Caffeic (high) + glucose | $332 \pm 33^{a}$ | $4.87 \pm 0.08^{ab}$ |
| Catechin (high) + glucose | $253 \pm 19^{a}$ | $4.73 \pm 0.05^{abc}$ |

Consistent with trends identified by PERMANOVA, BCC significantly changed over the duration of the incubation with broad changes occurring in all bacterial phyla except for the Actinobacteria (Fig. 3.6). Phenolic amendments had a greater effect than glucose on the relative abundances of phyla and there were no significant interactions between phenolic and glucose amendments. At the genus level, we explored the significant increases and decreases in relative abundance in comparison to the control for the phyla Acidobacteria, Firmicutes and Proteobacteria using Dunnett's multiple comparisons method (Fig. 3.7). The *Burkholderia and Parabulkholderia* genera from the Proteobacteria phylum showed the greatest increase in response to the addition of benzoic acid with glucose (207 ± 16 counts 1000 reads$^{-1}$), benzoic acid without glucose (187 ± 16 counts 1000 reads$^{-1}$), and caffeic acid with glucose (105 ± 16 counts 1000 reads$^{-1}$). The same treatments caused the largest decrease in relative abundance of *Tumebacillus* in the Firmicutes phylum: the benzoic acid application caused a decrease of 57 ± 14 counts 1000 reads$^{-1}$, the benzoic acid application with glucose a decrease of 63 ± 14 counts 1000 reads$^{-1}$, and caffeic acid with glucose a decrease of 58 ± 14 counts 1000 reads$^{-1}$. Interestingly, out of the 39 genera in the phyla Proteobacteria, Acidobacteria and Firmicutes that experienced a significant change in relative abundance, the glucose amendment without a phenolic addition showed only three significant changes in relative abundance relative to the control.

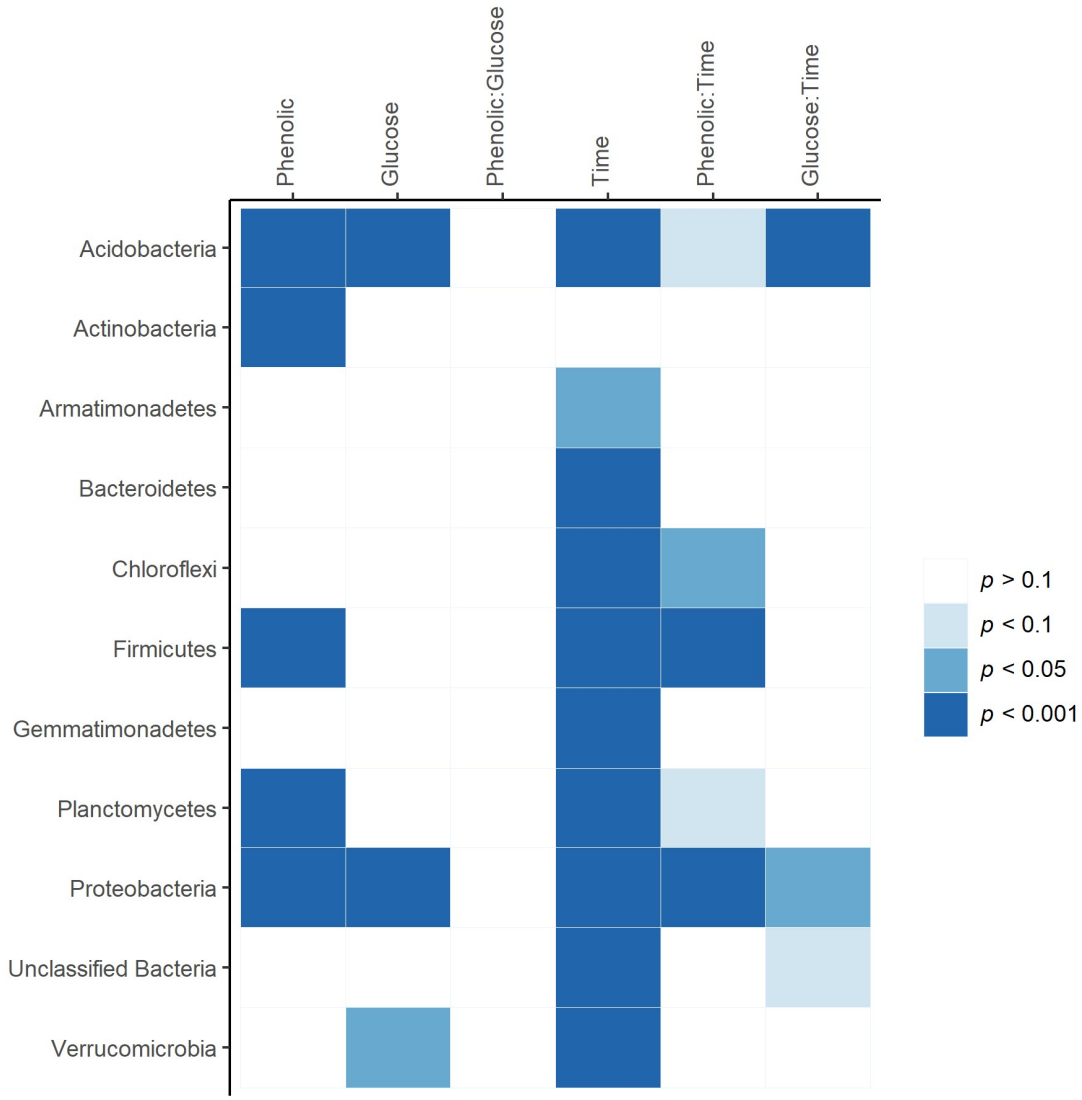


**Fig. 3.6** Analysis of variance (ANOVA) type-3 table showing the significance level (*p*-values) of the effects of phenolic compounds, glucose and incubation time on the relative abundance of each bacterial phyla.

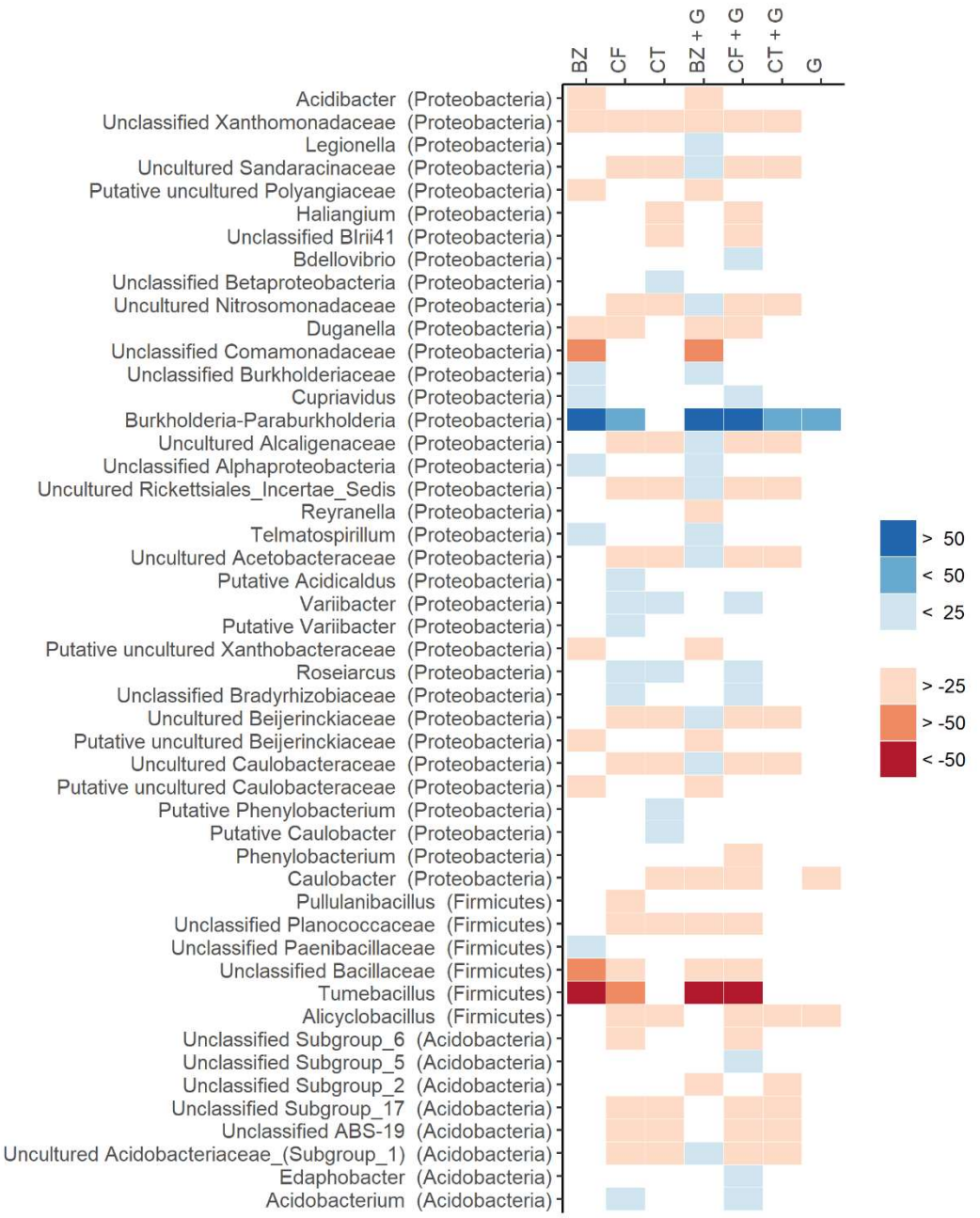


**Fig. 3.7** Heatmap of the genera in the Proteobacteria, Acidobacteria and Firmicutes phyla that showed a significant change in relative abundance (count 1000 reads$^{-1}$) in response to the phenolic and glucose additions averaged over the sampling days using Dunnett's multiple comparisons test with control as reference. Only significant changes are shown ($p < 0.05$). Direction of change is indicated by color: increase of 50 or more counts per thousand reads (dark blue), an increase of less than 50 (medium blue), an increase of less than 25 (light blue), no significant change (white), a decrease of less than 25 (light red), a decrease of less than 50 (medium red), a decrease of more than 50 (dark red) average counts 1000 reads$^{-1}$.

*Correlating respiration with microbial abundance and composition*

To determine whether exudate-induced changes in microbial respiration were linked to shifts in BCC (Weighted Unifrac distance to control) and changes in microbial abundance (µg DNA $g^{-1}$ soil),we used Spearman's non-parametric correlation test (Fig. 3.8). Weighted Unifrac distance representing shifts in BCC was correlated with changes in microbial respiration ($r_s = 0.53$, $p < 0.001$), while microbial abundance did not help explain the variation in respiration levels relative to the control ($p = 0.57$).

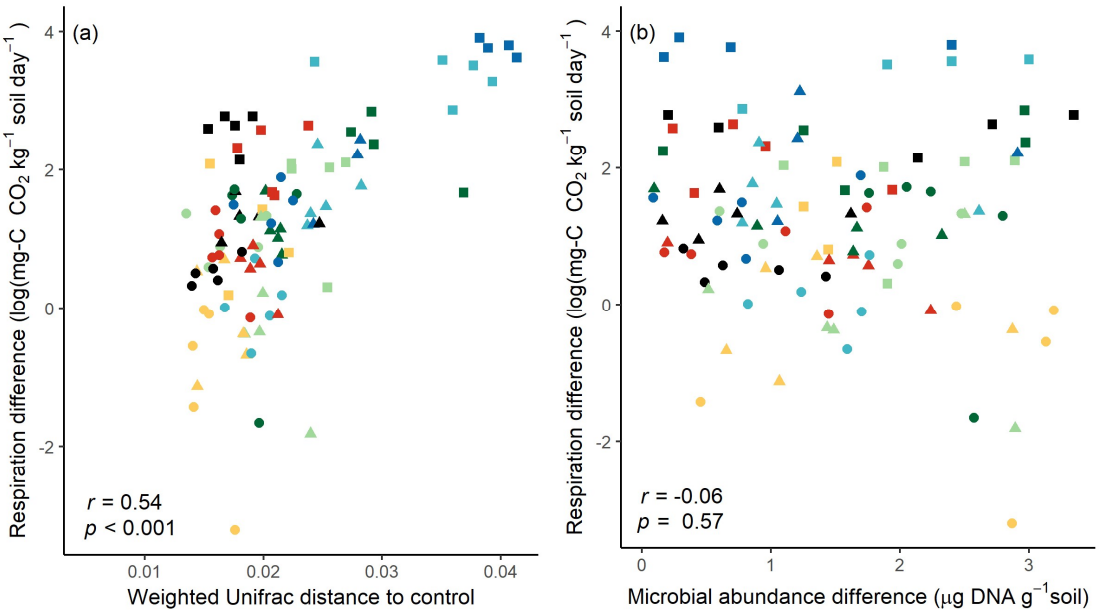


**Fig. 3.8** Relation between respiration difference between treatments and control and (a) Weighted Unifrac distance from control, and (b) difference in microbial abundance with control. Days are indicated by different symbols: day 5 (square), day 15 (triangle) and day 38 (circle). Colors indicate the different treatments: glucose (black), benzoic acid (light blue), benzoic acid with glucose (dark blue), caffeic acid (light green), caffeic acid with glucose (dark green), catechin (yellow), catechin with glucose (red).

## *Discussion*

### *Root phenolic function in soil*

As we hypothesized, root phenolic compounds degraded more slowly than glucose (Fig. 3.1) and we observed other potential phenolic functions besides microbial food source such as toxicity or signaling. Even by the end of the study, less than 20% of the C from the high application of caffeic acid and catechin had been respired (Table 3.1). We define toxicity as a reduction in growth upon compound addition and signaling as a root exudate effect on microbial community composition without immediate utilization of this compound as substrate (Venturi & Keel, 2016) and inhibitory effect on growth. The decrease in cumulative respiration (Fig. 3.2) and microbial abundance (Fig. 3.3) caused by caffeic acid and catechin provide evidence for toxicity mechanisms. Moreover, the lack of phenolic degradation makes it possible that signaling also played a role in changing the relative abundances of bacterial genera (Fig. 3.7) in these treatments. The delayed but efficient degradation of benzoic acid joined by an increase in respiration of soil-derived C (Fig. 3.1, Table 3.1) points at different mechanisms including the function of benzoic acid as food source and mobilizer of other labile SOM resources, which will be discussed in the next section. We did not find evidence of phenolics lowering soil extracellular enzyme activities or production (Fig. 3.4).

Previous studies have also found that phenolic compounds can reduce soil microbial abundance (Scalbert, 1991; Blum *et al.*, 1999; Inderjit *et al.*, 2009). Caffeic acid and catechin both contain a catechol functional group, which has been shown to be toxic to microorganisms (Capasso *et al.*, 1995; Boyd *et al.*, 1997). Inhibitory effects

of catechol-containing compounds on microbial growth and activity could include mechanisms of metal-chelation (Loper & Buyer, 1991), generation of phenoxyl radical and formation of reactive oxygen species (Schweigert *et al.*, 2001). However, toxicity depends on phenolic concentration (Siqueira *et al.*, 1991), which was also visible in this study. When only 10% of the initial compound addition was applied, caffeic acid and catechin did not reduce cumulative respiration in comparison to the control on day 15 (Fig. 3.2). Our application rates were similar to those used in other incubation studies investigating rhizosphere processes (Liu *et al.*, 2017). Hence, better estimates of both primary and secondary metabolites exuded by roots are necessary in order to determine the extent by which these toxicity mechanisms actually take place in the rhizosphere.

Most research on rhizosphere signaling has focused on a few plant symbiotic relationships with soil biota such as N-fixing rhizobial bacteria and arbuscular mycorrhizal fungi (Oldroyd, 2013). Yet, several scientific reviews discuss and hypothesize that other signaling mechanisms between plants and soil microorganisms exist (Bais *et al.*, 2006; Badri *et al.*, 2009). A recent study indeed found a new family of bacterial LuxR-proteins that bind and respond to plant chemicals (Subramoni & Venturi, 2009), but the range of root exudates that these receptors may interact with remains unclear. In particular, the slow and minimal degradation of catechin together with its effect on the relative abundance of a wide range of soil bacterial genera (Fig. 3.7) make it a potential candidate as a signaling molecule. However, catechin did demonstrate toxicity effects in the first fifteen days of the incubation, which makes it complicated to distinguish between these two different functions. Future studies

should further investigate the signaling role of catechin as well as the rhizosphere function of other plant secondary metabolites with low turnover rates.

*Potential priming mechanisms of benzoic acid*

The benzoic acid-induced increase in SOM decomposition (Fig. 3.1, Table 3.1) was associated with shifts in BCC (Fig. 3.5, 3.8). Conversely, benzoic acid did not induce microbial growth (Fig. 3.3) and microbial abundance was not correlated with respiration (Fig. 3.8). Acknowledging that these results are correlational, they support the hypothesis that phenolics influence soil C cycling through changing soil microbial community structures and not through increasing microbial abundance. Others have also proposed that root priming of SOM decomposition involves a shift in the microbial community towards SOM degraders in response to exudation (Fontaine *et al.*, 2003). In our experiment, benzoic acid dramatically increased the relative abundance of the *Paraburkholderia* and *Burkholderia* genera (Fig. 3.7), which lasted until day 38. Bacteria belonging to the *Burkholderia* genus have been detected in different soil types and in the rhizospheres of a variety plant species (Cello *et al.*, 1997; Viallard *et al.*, 1998; Richardson *et al.*, 2002; Woo *et al.*, 2014). Their genus-wide acid tolerance (Stopnisek *et al.*, 2014) might contribute to their competiveness in coniferous forest soils, where they are found in especially high relative abundance (Baldrian *et al.*, 2012). Besides their role in cellulose and lignin decomposition (McNamara & Leff, 2004; Štursová *et al.*, 2012; Woo *et al.*, 2014), *Burkholderia cepacia*, one of the species identified in this study, seems unaffected by the toxicity from phenolic compounds leaching from plant litter (McNamara & Leff, 2004). This

is an important trait to consider since the low respiration from the benzoic acid treatments on day 1 may indicate toxicity (Fig. 3.1). Moreover, *Burkholderia* spp. are efficient degraders of aromatic pollutants such as polychlorinated biphenyls and benzene (Leigh *et al.*, 2002; Chaudhry *et al.*, 2005; Ortega-González *et al.*, 2013), that have a benzene ring in common with benzoic acid. Hence, the ability of the *Burkholderia* and *Paraburkholderia* genera to degrade compounds with a benzene functional group, their tolerance of phenolic compounds and their involvement in organic matter breakdown probably explain how benzoic acid increased their abundance leading to accelerated rates in SOM decomposition.

The increase in potential BG enzyme activity in the benzoic acid treatments (Fig. 3.4) and the unaltered rates of phenol oxidase and peroxidase activity suggest that microbes were degrading labile C sources rather than recalcitrant organic matter. Glucosidase activity is associated with the breakdown of cellulose, chitin and storage carbohydrates in soils (Sinsabaugh *et al.*, 2002), which may have been the sources for the respiration of soil-derived C. The soil used in this study is highly aggregated with the largest abundance of macroaggregates (Yavitt *et al.* 2014). Our sieving step prior to the incubation could have broken down this large pool of macroaggregates making labile C easily accessible to microbes from the start of the experiment. Additionally, benzoic acid could have liberated soil mineral-protected C. Keiluweit *et al.* showed this alternate priming mechanisms for oxalic acid in a simulated rhizosphere (2015), but not for acetic acid and glucose. Future studies should investigate whether interactions between benzoic acid and mineral-organic matter associations contributed to the priming of SOM mineralization.

*Phenolic chemistry and substrate-preference as drivers of bacterial community composition*

As we expected, phenolic compounds caused a greater shift in BCC (Fig. 3.5) and affected the relative abundance of a larger number of bacterial phyla and genera than glucose (Fig. 3.7, 3.8). These effects were most pronounced for benzoic acid, followed by caffeic acid and lastly by catechin, which was consistent with their respective amounts of degradation (Table 3.2). These results suggest that shifts in BCC were primarily driven by phenolic chemical structure and microbial substrate-preference.

While glucose-induced respiration is used as a measure of soil microbial biomass because of its utilization by a broad range of microorganisms (Stenström *et al.*, 1998), microbial degradation of phenolic compounds requires more specialized enzymes and traits (Czekalowski & Skarzynski, 1948; Hinteregger *et al.*, 1992; Bending & Read, 1997). Presumably, benzoic acid led to a greater shift in BCC than glucose because the utilization of carbon and energy from this phenolic amendment were limited to the bacteria capable of benzoic acid breakdown. Recent evidence also demonstrated that the interactions between root exudate chemistry and substrate-preference influenced soil microbial community composition (Zhalnina *et al.*, 2018). In fact, this previous study specifically pointed out that rhizosphere microbes consumed aromatic compounds like shikimic, cinnamic and salicylic acid, which are all synthesized in phenylpropanoid pathway like the phenolic compounds tested in this incubation experiment. Another study also found that bacterial genes for the metabolism of aromatic compounds were upregulated in the rhizosphere (Matilla *et al.*, 2007).

In addition to substrate-preference, phenolic signaling and toxicity effects considered in the first section of this discussion may have supported the growth of specific bacterial species while limiting the abundance of others. Moreover, phenolics could have indirect effects on BCC through changing soil pH and resource availability. The soil treatments receiving a high dose of catechin and caffeic acid with glucose had lower pH than the control soils, while benzoic acid treatments without glucose showed an increasing yet non-significant trend in pH. The strong correlation between soil microbial community composition and pH (Högberg *et al.*, 2007; Lauber *et al.*, 2009) makes it probable that these differences in pH among treatments impacted soil BCC. Acknowledging that the greatest shifts in BCC were caused by phenolics that were also utilized as a food source, we also found support for the hypothesis that more subtle mechanisms contribute to rhizosphere BCC.

*Interactive effects of glucose and phenolic compounds on soil biogeochemistry*

Our results demonstrate interactions between glucose and phenolics influencing soil biogeochemistry, which is in line with our hypothesis and expectations. First of all, respiration of glucose-derived C was diminished when any level of phenolic exudates are added (Fig. 3.1, Table 3.1). Second, glucose amplified the benzoic acid-induced acceleration in SOM decomposition (Fig. 3.1, Table 3.1) if we assume benzoic acid degradation was the same in the benzoic-only and benzoic acid with glucose treatments. There is some evidence that glucose also augmented phenolic-driven increases in relative abundance of specific bacterial genera (Fig. 3.7), yet the addition of glucose did not significantly influence phenolic effects on BCC (Fig. 3.5b).

Different mechanisms could explain the decrease in the respiration of glucose in the presence of phenolic compound additions. In the case of the benzoic acid treatments, preferential substrate utilization (Kuzyakov, 2002) may have limited the amount of glucose consumption. This preferential substrate utilization hypothesis was initially proposed to explain a decrease in the rate of SOM decomposition upon substrate addition. However, microbial preference for benzoic acid could also explain a decrease in glucose degradation. Alternatively, microorganisms may not have had a preference, but rather the application of benzoic acid increased the availability of total carbon resources, diluting the $^{13}C$-signal from glucose detected in the respiration samples. For the caffeic acid and catechin treatments, their inhibitory effects on microbial abundance and activity over the first fifteen days of the experiment (Fig. 3.2, 3.3) could have limited the utilization of glucose for respiration. While we cannot detect significant cumulative toxicity effects on day 38, our calculations do suggest a lower degradation of glucose in these treatments (Table 3.1). Lastly, a higher incorporation of glucose-$^{13}C$ into microbial biomass would lead to a lower detection of glucose-derived $^{13}C$ in the respiration samples. Because we did not measure the carbon stable isotope ratios of the microbial biomass, and we did not find a significant increase in microbial-C on day 38 (Table 3.2), we have no evidence for this hypothesis so far.

Because this study found support for the hypothesis that glucose and phenolics have different functions in the rhizosphere, we will use this line of thought to explain the observed interactive effects between glucose and phenolics on SOM decomposition and BCC. The low dose of benzoic acid was fully consumed and

respired, while only 50% of the high benzoic acid dose was degraded. (Table 3.1). These results suggest that carbon resources were limited in the low benzoic acid treatment. If carbon and energy resources were limiting microbial activity, the primary function of glucose as energy and carbon source for microbes could have amplified the mechanistic effects of benzoic acid on SOM decomposition in the soil treatment receiving a low dose. In addition, benzoic acid could have increased the amount microbial substrates relative to the glucose-only treatment by functioning as a food source itself or liberating mineral-protected soil C. This same mechanism may explain the interactive effects of glucose and the high dose of benzoic acid, if benzoic acid could not be degraded quickly enough to provide microbial resources. While this explanation is speculative, so far few studies have explored interactive effects of root exudate on soil C cycling, emphasizing the need for future research on this topic.

It is important to note that our results on the interactive effects of glucose and benzoic acid on SOM decomposition include a level of uncertainty because our experimental design did not utilize stable isotope methods to distinguish between soil-derived and benzoic acid-derived C that was respired from the glucose-phenolic treatments. Instead, we subtracted the amount of phenolic C degraded in the individual treatments from the estimated respiration levels of the combined soil and phenolic C sources. This approach assumes that phenolic degradation was unaffected by the glucose application. Because the high dose of benzoic acid was only degraded for 50%, an increase in its consumption in the presence of glucose could decrease the stimulatory effect on SOM decomposition observed in this treatment. However, the glucose addition could not have increased the consumption of the low benzoic acid

dose because it was already fully degraded when added individually. If glucose did change the degradation of the low benzoic acid application, this effect could only have been inhibitory. An inhibitory effect on benzoic acid consumption would only lead to a greater rate of SOM decomposition. Therefore, for the low dose of benzoic acid, our conclusion that glucose and benzoic acid have an interactive and stimulatory effect on SOM decomposition still holds.

In line with the findings of Blum *et al.* (2000), we found little evidence of glucose influencing phenolic-driven shifts in soil BCC. This provides further evidence that phenolics and potentially other secondary metabolites play a dominant role in rhizosphere microbial community assembly. In a small number of cases, the joint addition of glucose and phenolic led to an increase in the relative abundance of Proteobacteria and Acidobacteria genera, which was not evident when added separately (Fig. 3.8). Interestingly, microbial N was substantially higher in the glucose treatment with a high dose of caffeic acid and catechin, and hence the microbial C:N ratio was lower. This could correlate with a shift towards a more bacterial-dominated community, which usually have a lower C:N ratios (Wallander *et al.*, 2003).

Overall, these findings show that the interactions between primary and secondary root metabolites can influence soil biogeochemical cycles through a variety of mechanisms. Considering that we only studied the interactive effects of two root exudates simultaneously, it is overwhelming to imagine the complex network of relations between root exudate chemistry, microbial community composition and rhizosphere C dynamics under more natural conditions. Yet, it will be crucial to verify and further investigate the processes observed in this study at the plant- and

ecosystem-scale in order to enhance our knowledge on root-soil interactions and their implications for the terrestrial C cycle.

***Supporting Information***

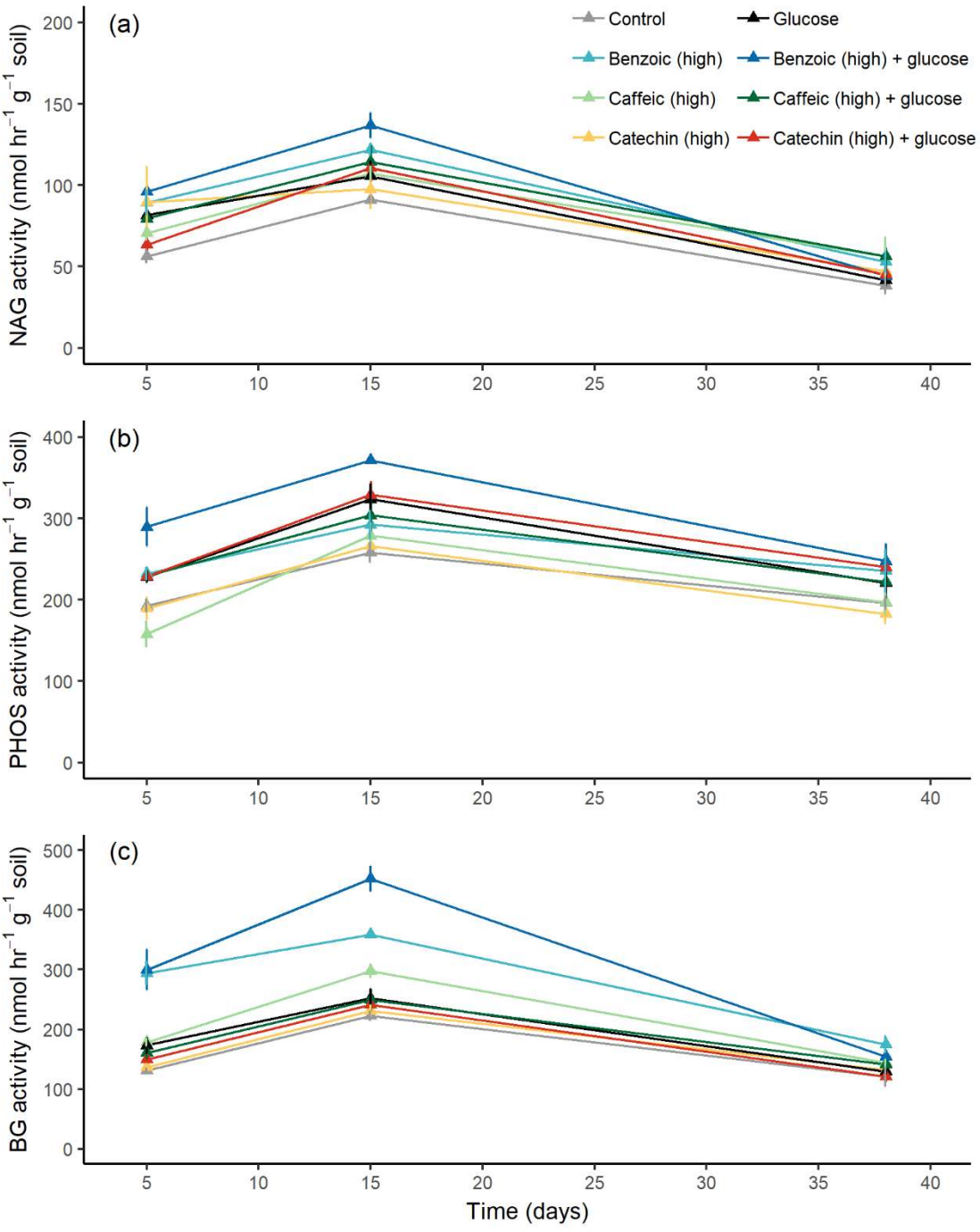


**Fig. 3.S1** Potential enzyme activity of (a) β-1,4-glucosidase (BG), (b) β-1,4-N-acetylglucosaminidase (NAG) and (c) acid phosphatases (PHOS) on day 5, 15 and 38 as influenced by the phenolics benzoic acid, caffeic acid and catechin applied at the high dose of 0.5 mg-C g$^{-1}$ soil. Means and standard errors are shown (*N*=*5*).

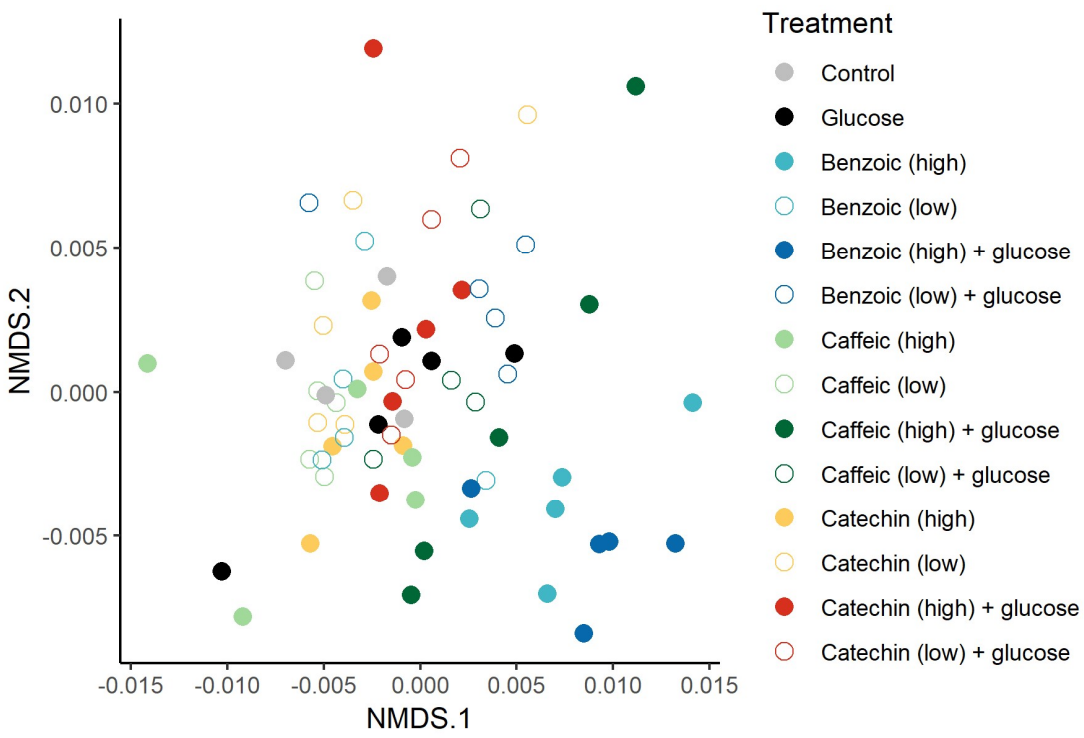


**Fig. 3.S2** Non-metric multidimensional scaling (NMDS) ordination of bacterial community composition on day 38 using Weighted Unifrac distances based on normalized count data (stress = 0.18). The application treatments (*N*=*5*) are indicated by different colors: control (grey), glucose (black), benzoic acid (light blue), benzoic acid with glucose (dark blue), caffeic acid (light green), caffeic acid with glucose (dark green), catechin (yellow), catechin with glucose (red). Two application rates are indicated for the phenolic compounds: 0.5 mg-C $g^{-1}$ soil (high, closed circle) and 0.05 mg-C $g^{-1}$ soil (low, open circle).

**Table 3.S1.** Richness and Shannon diversity index calculated with filtered and rarified count data (permutations = 8,796) from treatment samples on day 5 and 38. Means and standard errors are shown (*N*=5). Contrasting letters indicate significant differences among treatments ($p < 0.05$) as determined by the Tukey Honest Significant Difference test.

| **Root exudate treatments** | **Day** | **Richness** | **Diversity** |
|---|---|---|---|
| Control | 5 | 210 ± 37[a] | 4.08 ± 0.10[a] |
| Benzoic (high) | 5 | 234 ± 31[a] | 4.06 ± 0.07[a] |
| Caffeic (high) | 5 | 265 ± 38[a] | 4.16 ± 0.08[a] |
| Catechin (high) | 5 | 180 ± 27[a] | 4.10 ± 0.06[a] |
| Glucose | 5 | 212 ± 26[a] | 4.09 ± 0.08[a] |
| Benzoic (high) + glucose | 5 | 276 ± 59[a] | 4.00 ± 0.09[a] |
| Caffeic (high) + glucose | 5 | 206 ± 32[a] | 4.07 ± 0.07[a] |
| Catechin (high) + glucose | 5 | 215 ± 33[a] | 4.04 ± 0.04[a] |
| | | | |
| Control | 38 | 342 ± 36[a] | 5.12 ± 0.08[a] |
| Benzoic (high) | 38 | 329 ± 93[a] | 4.90 ± 0.11[a] |
| Caffeic (high) | 38 | 412 ± 93[a] | 5.19 ± 0.08[a] |
| Catechin (high) | 38 | 378 ± 38[a] | 5.15 ± 0.12[a] |
| Glucose | 38 | 374 ± 69[a] | 5.13 ± 0.13[a] |
| Benzoic (high) + glucose | 38 | 323 ± 29[a] | 4.82 ± 0.11[a] |
| Caffeic (high) + glucose | 38 | 275 ± 40[a] | 4.85 ± 0.11[a] |
| Catechin (high) + glucose | 38 | 273 ± 27[a] | 4.91 ± 0.10[a] |

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# CHAPTER 4: FINE ROOT RESPONSES TO FOUR YEARS OF REPEATED THROUGH-FALL EXCLUSION IN A MIXED-SPECIES FORESTS WITH EUROPEAN BEECH (*FAGUS SYLVATICA* [L.]) AND NORWAY SPRUCE (*PICEA ABIES* [L.] Karst)[3]

## *Introduction*

Fine root production and lifespan have important consequences for plant resource acquisition and biogeochemical cycles (Nadelhoffer & Raich, 1992; Eissenstat & Yanai, 1997; Bardgett *et al.*, 2014). Environmental changes in climate will influence plant resource availability and growth conditions in forest ecosystems around the world (Allen *et al.*, 2010; Lindner *et al.*, 2010; Williams *et al.*, 2013). Yet, the impact of environmental factors on root growth dynamics of different tree species remains largely unknown (McCormack & Guo, 2014). How fine roots respond to decreasing soil moisture is a particularly complicated question because reduced water availability affects both the local root environment as well as the aboveground assimilation of carbon resources needed for root production and maintenance. Furthermore, a depletion in water resources, may shift the nature and outcome of belowground species interactions (Callaway & Walker, 1997; Cavin *et al.*, 2013; Forrester, 2015). The degree of root plasticity by which roots can respond to the identity of different neighbors may be as important to plant performance and ecosystem productivity as their response to resource limitation (Callaway *et al.*, 2003; de Kroon, 2007; Tilman *et*

[3] A similar version will be submitted for publication under Zwetsloot MJ and Baurle TL

*al.*, 2012). Considering that the frequency, duration and severity of drought is predicted to increase even in areas where trees are typically not water-limited (Seneviratne *et al.*, 2012; Dai, 2013; Zeppel *et al.*, 2013), it is essential to study the role of root growth dynamics and belowground species interactions in plant adjustment to water limitation (Novoplansky & Goldberg, 2001; Anderegg, 2012; Brunner *et al.*, 2015). Moreover, fine roots and root-associated microorganisms are the primary source of soil organic matter (Rasse *et al.*, 2005; Clemmensen *et al.*, 2013) and previous research has emphasized the need for research on root-soil interactions to estimate forest ecosystem feedbacks to climate change (Norby & Jackson, 2000; Norby *et al.*, 2004; Warren *et al.*, 2015; Pausch & Kuzyakov, 2018). While the effect of higher atmospheric $CO_2$ on root-mediated soil biogeochemical processes in forests has received considerable attention (Pregitzer *et al.*, 1995; Heath *et al.*, 2005; Pritchard *et al.*, 2008; Phillips *et al.*, 2012), we know little about the patterns of drought-induced changes in root growth dynamics.

Root biomass generally shows a decrease in response to drought (Leuschner *et al.*, 2001, 2004; Moser *et al.*, 2014). Yet, in terms of fine root production, both increases (Leuschner *et al.*, 2001; Gaul *et al.*, 2008) and decreases (Mainiero & Kazda, 2006; Metcalfe *et al.*, 2008) have been reported under limiting soil water resources. These contrasting responses may be due to species physiological variation in their drought response (Leuschner *et al.*, 2001) and the severity of water limitation (Teskey & Hinckley, 1981; Zang *et al.*, 2014). Drought effects on root production could also vary by soil depth because water limitation is frequently higher in surface soil layers. Studies have indeed reported spatial shifts in root distribution during

surface soil drying with a relocation of root growth to deeper soil layers (Peek *et al.*, 2006; Hodge, 2009). In the case of seasonal or episodic water limitation, the ability of a tree to produce roots during wetter periods may be another important factor influencing plant performance during drought (Comas *et al.*, 2013). There is evidence of trees in tropical dry forests minimizing root production during the dry season while increasing root growth in the wet season (Rojas-Jimenez *et al.*, 2007). Yet, temperate forest tree species also show seasonal variation in root production (McCormack *et al.*, 2014). One study with different oak species suggested that low soil water potentials can limit fine root production when temperatures are higher than 2.5 °C, but not in winter when temperature limits growth (Reich, 1980). Yet, it is unclear to what extent temperate forest tree species can modify root production patterns to align with shifting spatio-temporal distribution of water resources, especially during repeated episodes of summer drought.

Resource acquisition is not only a function of root production but also depends on the lifespan of the roots present in a soil resource patch (Adams *et al.*, 2013). Fine root lifespan is a plastic trait influenced by belowground resource availability (Pregitzer *et al.*, 1993) and shows high between- and within-species variation ranging from days to weeks to years (Eissenstat & Yanai, 1997; McCormack *et al.*, 2012). In theory, root lifespan can be determined by root efficiency, the benefit of resource uptake relative to the production and maintenance costs of the root in carbon units. When analyzing this ratio over time, the point at which root efficiency is maximum marks the optimal point of root lifespan (Eissenstat *et al.*, 2000). The potential effects of water limitation on both the costs and benefits of fine roots make it difficult to

predict changes in root lifespan under drought. For example, the benefit of maintaining a root in a dry soil patch should be low. However, water limitation also influences the costs through changes in root morphology and maintenance respiration. Additionally, soil water heterogeneity and the extent by which drought limits whole-plant growth versus solely root growth complicate root lifespan predictions even further (McCormack & Guo, 2014). Even if we could calculate a theoretical optimum for root lifespan under drought conditions, this may not correspond with the degree of root plasticity a tree species possesses.

In practice, few studies have directly tested the effect of drought on root lifespan (Eissenstat *et al.*, 2013). Meier and Leuschner found that drought reduced the fine root lifespan of *Fagus sylvatica* (European beech) seedlings by 50% (2008a). Yet, these results may not translate to mature trees in forests where soil moisture is more heterogeneous and roots reach deeper into the soil. Bauerle *et al.* demonstrated that the root lifespan of grapevines was unaffected by drought due to hydraulic redistribution within the root system at night (2008). Furthermore, lifespan of fine roots produced at greater soil depth is often longer than of those in shallow soil layers (Coleman *et al.*, 2000; Baddeley & Watson, 2005; Peek *et al.*, 2006). If fine root growth shifts to deeper soil layers under drought, the final outcome may be an increase in lifespan of the whole root system.

Finally, root production and root lifespan also depend on available plant carbon resources (Eissenstat & Yanai, 1997), which is an especially important factor to consider during drought (Anderegg, 2012; Brunner *et al.*, 2015). While limitation in soil resources is expected to lead to increased carbon allocation to roots instead of

aboveground organs (Bloom *et al.*, 1985; McConnaughay & Coleman, 1999; Coomes & Grubb, 2000), this may not be the case during drought which could reduce carbon uptake through stomatal closure (McDowell & Sevanto, 2010). For example, a three-year-long field study with *F. sylvatica* showed that root biomass was reduced while leaf biomass stayed constant under water limitation (Meier & Leuschner, 2008b). Additionally, an experiment with seven-year-old *Picea abies* trees (Norway Spruce) found that severe drought decreased root nonstructural carbon reserves significantly (Hartmann *et al.*, 2013). If carbon resources for root production and maintenance are scarce, a new carbon allocation question arises. Is it worth investing into producing new roots or better to spend carbon resources on prolonging the lifespan of roots that are already built? Few studies have addressed this question so far.

At the ecosystem scale, fine roots may experience and respond to a depletion in water resources differently in mixed versus monotypic forest stands. Research performed in grassland ecosystems indeed showed that plant diversity increased resistance to negative drought effects on ecosystem productivity (Isbell *et al.*, 2015; Craven *et al.*, 2016). The abiotic stress hypothesis posed by Callaway and Walker (1997) states that facilitation is more prevalent under low resource availability while competition is more intense when resources are high. Few studies have tested this hypothesis in forest ecosystems but there is some evidence that diverse forest stands indeed show higher productivity relative to monotypic forest stands along decreasing water and nutrient gradients (Pretzsch *et al.*, 2010; Paquette & Messier, 2011; Prior & Bowman, 2014). However, most of these studies did not analyze belowground productivity.

Whether a positive species mixing effect on root growth dynamics and tree performance exists under drought is especially relevant to forests stands of *F. sylvatica* and *P. abies* found across Central Europe (Pretzsch *et al.*, 2013, 2014). *P. abies* is extensively grown in monoculture and expected to be highly vulnerable to drought. Mixed forest stands may allow for continued cultivation of *P. abies* by reducing drought impacts (Río *et al.*, 2014). Here, we present a four year-long throughfall-exclusion experiment in forest stands of *P. abies* and *F. sylvatica* under intraspecific and interspecific growing conditions. Our main research questions were: 1) to what extent drought influences fine root production and lifespan of *F. sylvatica* and *P. abies*, and 2) whether growing tree species in mixture buffers the seasonal drought impact on fine root production in comparison to the monospecific stands. In response to the first question, we hypothesized that tree carbon availability drives fine root production and lifespan patterns under drought. Secondly, we hypothesized that facilitation buffers drought impact on fine root growth dynamics under interspecific conditions. These hypotheses led us to predict that drought reduces fine root production but increase fine root lifespan to compensate for the low amount of new fine roots being built. Moreover, we expected that water limitation does not decrease fine root production in the mixed-species zone.

## ***Materials and methods***

### *Site characteristics and climatic conditions*

This research took place in the Kranzberg Forest (Pretzsch *et al.*, 2014), an experimental field site with *F. sylvatica* and *P. abies* trees, in southern Germany

(48°25'N, 11°39'E, 490 m a.s.l.) from March 2013 until December 2017. Average tree age determined in 2010 was 79±4 years for *F. sylvatica* and 59±2 years for *P. abies* (Pretzsch *et al.*, 2014). The forest consists of both monospecific *F. sylvatica* and *P. abies* zones as well as areas where both species are intermixed (Nickel *et al.*, 2018). The soil at the site is classified as luvisol which developed from loess over tertiary sediments, which extents until a hard clay-pan at 1m depth. Average soil texture from 10-80cm depth is 26.5% clay, 61.6 % silt and 11.9 % sand. With soil depth, clay and sand content increase while silt content decreases. From the surface to 56 cm soil depth, pH ranges from 4.10 to 4.32 in water, CEC from 142 to 53 μeq $g^{-1}$, soil density from 0.55 to 1.17 g $cm^{-3}$, and soil water holding capacity from 27.5 to 25.5 % (Göttlein *et al.*, 2012). From soil samples taken by our collaborators in 2014, we determined that soil total N and P nutrient levels vary significantly along a species gradient from monotypic *F. sylvatica* to monotypic *P. abies* with an interspecific zone in the middle. On average for the years 2014, 2015 and 2016, soil P levels were 1.50 g $kg^{-1}$ soil for the O horizon (0-5 cm) and 0.34 g $kg^{-1}$ for the A horizon (5-30 cm). Soil P content in the *F. sylvatica* zone was significantly lower than in the mix ($p < 0.05$). For total N, average soil content was 11.34 g $kg^{-1}$ and 3.94 g $kg^{-1}$ soil for the O and A horizon respectively. Total N content was the highest in *P. abies* zone ($p < 0.0001$), followed by the mix and the lowest in the *F. sylvatica* zone ($p < 0.1$). Soil ammonium and nitrate levels were higher in the *P. abies* zone than under *F. sylvatica* or the mixture ($p < 0.05$).

*Study design*

This study made use of ten clearly defined 100-200 $m^2$ research plots within the forest (Fig. 4.1). In 2010, these plots were trenched by digging down to 1m depth where a compacted clay-pan inhibits water transport from below. The trenches were lined with a low density poly-vinyl plastic and refilled with soil (Pretzsch *et al.*, 2016) preventing between-plot root interactions and lateral water transfer. The research plots were established in such a way that they contained three interaction zones: an intraspecific *F. sylvatica* zone, an intraspecific *P. abies* zone and an interspecific zone of both species in the central region of the plot. Each of the three zones contained a minimum of three trees.

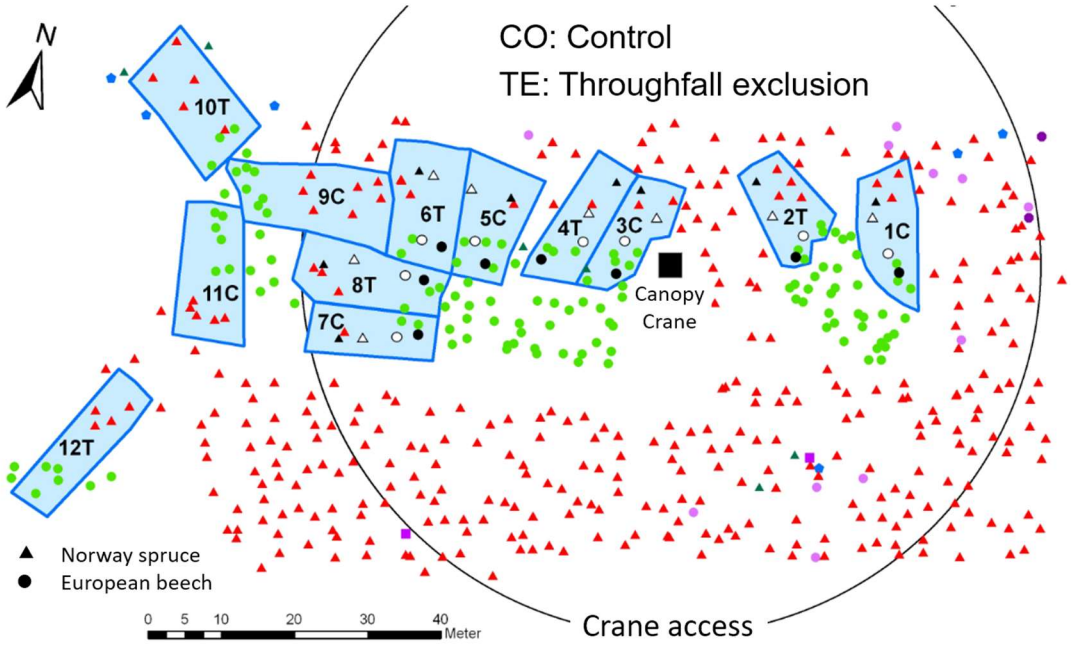


**Fig. 4.1** Map of Kranzberg Forest field site. Research plots 1-8 and 11-12 were used for this study. Throughfall-exclusion (T) and control (C) plots are indicated. *Fagus sylvatica* (green circle) and *Picea abies* (red triangle) are also marked.

In addition to the species interaction treatment, throughfall was excluded from half the research plots ($N$=5) from May 6 to December 9 in 2014, from March 10 to November 21 in 2015, from March 17 to November 3 in 2016 and from May 2 to November 7 in 2017. This was achieved through the installment of five retractable throughfall-exclusion roofs, which automatically closed during rain events. At all other times, roofs were open to minimize unwanted roof effects on plot conditions such as changes in temperature. Notably, soil moisture did not fully recharge in the shallow soil depths by the end of the roofless period (Fig. 4.2).

*Soil volumetric water content*

To monitor soil volumetric water content, time domain reflectometers (TDR, TDR-100, Campbell Scientific, North Logan, Utah) were installed at 0-7 cm, 10-30 cm and 30-50 cm soil depth in each of the three interaction zones of each plot. Soil volumetric water content was measured on a weekly basis.

*Root observation*

To monitor root growth dynamics, clear acrylic minirhizotron tubes (70 cm long, 6 cm outside diameter) were installed at an angle of $60^0$ from the horizontal, reaching a depth of 50 cm in 2010 (Paya, 2015). A total of six minirhizotron tubes were present in each plot: two in the monotypic *F. sylvatica* zone, two in the monotypic *P. abies* zone and two in the interspecific zone. Tubes were located at least one meter from the plot boundaries. In the mixed zone, tubes were positioned equidistantly from both species.

Root images were captured from March 29, 2013 until December 15, 2017. Using a specialized laparoscopic camera (BTC100X Camera, Bartz Technology, Carpinteria, California), 44 images were taken across the length of each minirhizotron tube every two weeks during the growing season from March until November. In winter (December-February), images were taken once a month. Image dimensions were 18 mm width and 15 mm height. All images were analyzed for fine root production, root mortality, root diameter and root order using WinRHIZO Tron MF (Regent Instruments Inc., Quebec, Canada). The mid-point between the appearance of a root and the previous image was identified as the birth date. Correspondingly, root death date was calculated as the mid-point between the death of a root (black, shriveled) and the preceding image session (Comas *et al.* 2005). We observed little new root growth from roots already marked as dead. Yet, in the case this did occur, this was corrected for. Roots that were present in one than more image were only counted once. Species were identified by visual inspection of root cortex coloration (*P. abies*: brown, *F. sylvatica*: reddish white), root tip branching patterns (*P. abies*: alternate branching, *F. sylvatica*: herringbone and often opposite branching), root tip morphology (*P. abies*: rounded tip and wider diameter, *F. sylvatica*: sharp tip and smaller diameter) (Paya, 2015).

*Data analysis and statistics*

All statistical analyses were performed in R (R Core Team, 2017).We used the lsmeans (Lenth, 2016), survival and coxme (Thernau & Grambsch) packages for Tukey Honest Significance Difference (HSD) tests and survival analyses. Plot was

included as random effect for the tests where there was significant between-plot variation. We determined p-values below 0.1 as significant but also indicated when p-values were lower than 0.05.

For each year and month, average soil moisture was determined by calculating the area under the soil water content curve over time in days for each replicate plot ($N$=5) at the depth levels of the TDR sensors. This number was then divided by the number of days from the first to last measurement of that year. To get a measure of the moisture levels that the observed roots were exposed to during their lifespan, we performed a similar calculation where we used the TDR sensor at the root's depth level and the birth and death date of roots as time points for the area under the curve approximation. This integral was then divided by the days that the root was alive.

Root tip production is expressed as number of roots $m^{-2}$ viewing window of the minirhizotron tube. For statistical analysis, we used the plot level as replicate ($N$=5) and averaged root tip production throughout the length of the window (50 cm) or by 10 cm depth increments over the two tubes in *the F. sylvatica*, *P. abies* and mix zone of each plot replicate. Because the random effect of plot was not significant, we used general linear models and Tukey HSD tests to analyze the effect of the drought, species interaction zone, soil depth and month of each year on root tip production. We log-transformed the number of root tips to satisfy the assumption of normality and equal variance of residuals. To calculate relative root production by depth level, we divided the number of root tips produced in each 10 cm soil layer by the total amount of root production for each tube. To evaluate the relationship between fine root production and soil moisture content, we calculated the Spearman's and Kendall's

rank correlation coefficients and associated p-values by year and month.

To analyze root survivorship of *F. sylvatica* and *P. abies* as influenced by drought, neighbor identity, root diameter, root depth and year, we used cox proportional hazard models (Cox, 1972). Roots that were still alive at the end of the study or disappeared before death (14.4 % of roots for *F. sylvatica* and 13.7% of roots for *P. abies*) were censored. In order to satisfy the cox proportional hazard assumptions, the fine roots in the model were stratified by 10cm soil depth layers, cohorts of roots produced in periods of open or closed rainfall exclusion roofs, and birth season of the root. The birth season cohorts were winter (December-February), spring (March-May), summer (June-August), and fall (September-November). Plot was included as random effect. We also used the Cox proportional hazard models to estimate the effect of the above-mentioned variables on root mortality. Root mortality and lifespan are negatively correlated: fine roots with a higher mortality risk have a shorter lifespan. Kaplan-Meier tests were used to estimate differences in root lifespan between the interspecific and intraspecific zone of the drought and control plots (Kaplan & Meier, 1958).

## *Results*

### *Soil moisture*

Overall, the throughfall-exclusion roofs reduced soil volumetric water content by 33% ($p < 0.001$, Fig. 4.2). This calculation includes soil moisture measurements from periods that the roofs were open. The reduction in soil moisture in the throughfall-exclusion treatment in comparison to the control was significant for each year of the experiment. Yet, soil water content also varied by year in both the control and throughfall-exclusion plots. Average soil moisture in 2015 and 2017 was lower than in 2014 ($p < 0.05$) while 2016 was not significantly different from any of the other years ($p > 0.05$).

Soil depth and species interaction zone also significantly influenced soil volumetric water content. The average soil moisture was different across the three depth levels with surface soil (0-7 cm) showing the lowest and the deepest soil layer (30-50 cm) the highest values under control and throughfall-exclusion treatment ($p < 0.001$, Fig. 4.2). In the control plots, soil moisture levels were the same in the intraspecific zone of *P. abies* and the interspecific zone throughout all years while the soil in the zone of *F. sylvatica* had higher soil moisture in 2014 and 2017 ($p < 0.05$, Fig. 4.3). In the throughfall-exclusion treatment, soil moisture in the interspecific zone was higher than in the *P. abies* zone in 2014 ($p = 0.01$), 2015 ($p < 0.001$) and 2016 ($p = 0.06$) but not in 2017 ($p = 0.99$). The *F. sylvatica* zone had higher soil water content than the *P. abies* zone except in 2017. Soil water content in the *F. sylvatica* zone and interspecific zone under throughfall-exclusion was similar for all years.

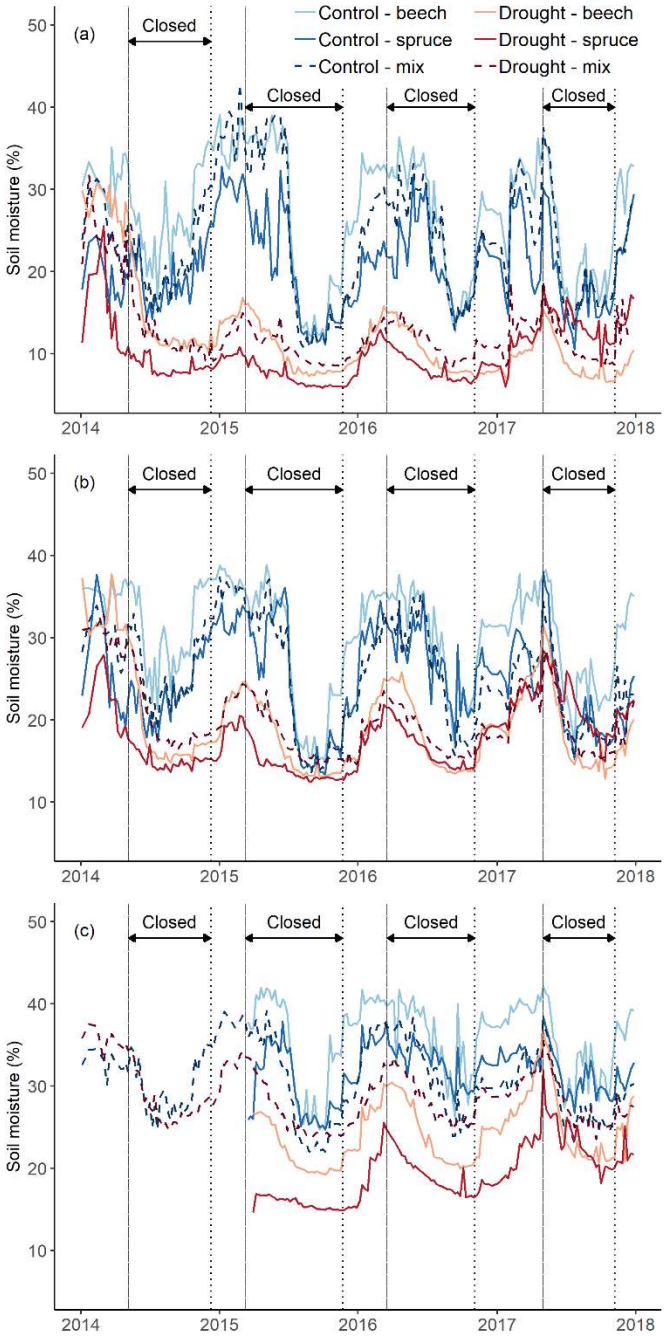


**Fig. 4.2** Mean volumetric soil water content over time at depth levels (a) 0-7 cm, (b) 10-30 cm, and (c) 30-50 cm under drought (red) or control (blue) conditions from 2014 until the end of 2017 (*N*=5). Soil moisture levels were measured under monotypic *Fagus sylvatica* (light blue/red), monotypic *Picea abies* (dark blue/red) and the mix of both species (dashed line). Time period of through-fall exclusion is indicated with vertical lines representing the dates on which roofs were closed (long dash) and re-opened (dotted) at the end of the growing season. Soil moisture measurements at 30-50 cm depth in the intraspecific zones started in 2015.

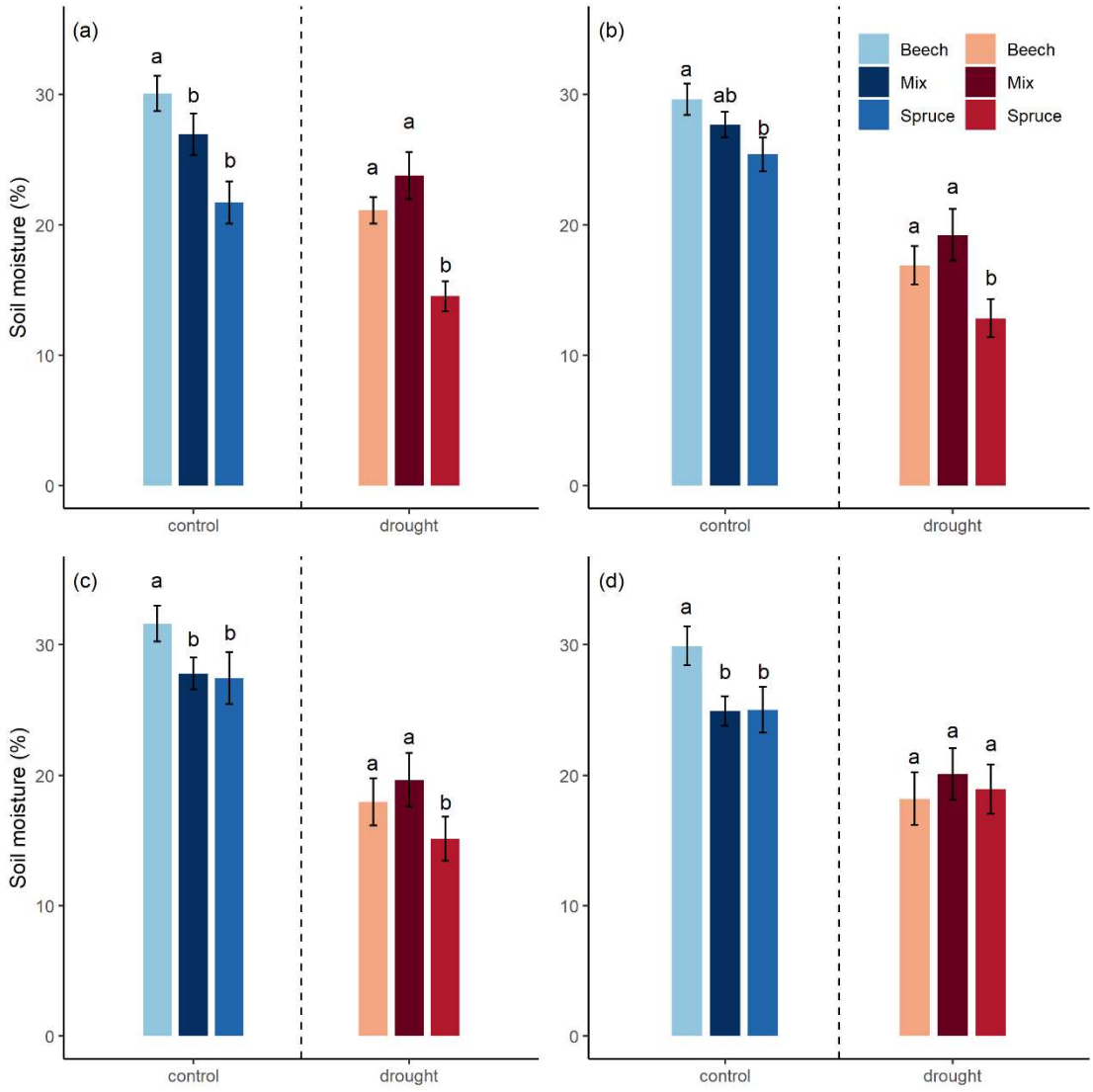


**Fig. 4.3** Average volumetric water content in monospecific *Fagus sylvatica* (Beech), monospecific *Picea abies* (Spruce) and interspecific zone in the control and drought treatment by the years (a) 2014, (b) 2015, (c) 2016, and (d) 2017. Mean and standard errors are shown ($N$=5). Contrasting letters indicate significant differences within control and drought treatment of that year as determined by Tukey's Honest Significant Difference (HSD) test ($p < 0.06$).

*Fine root production*

The four years of repeated throughfall-exclusion reduced root tip production of *P. abies* in the monospecific zone by 51% ($p < 0.05$). In the case of monospecific *F.*

*sylvatica*, root production was reduced by 48% ($p < 0.1$). Yet, throughfall-exclusion did not influence the number of root tips produced in the zones of the plots where both species were present ($p = 0.81$, Fig. 4.4).

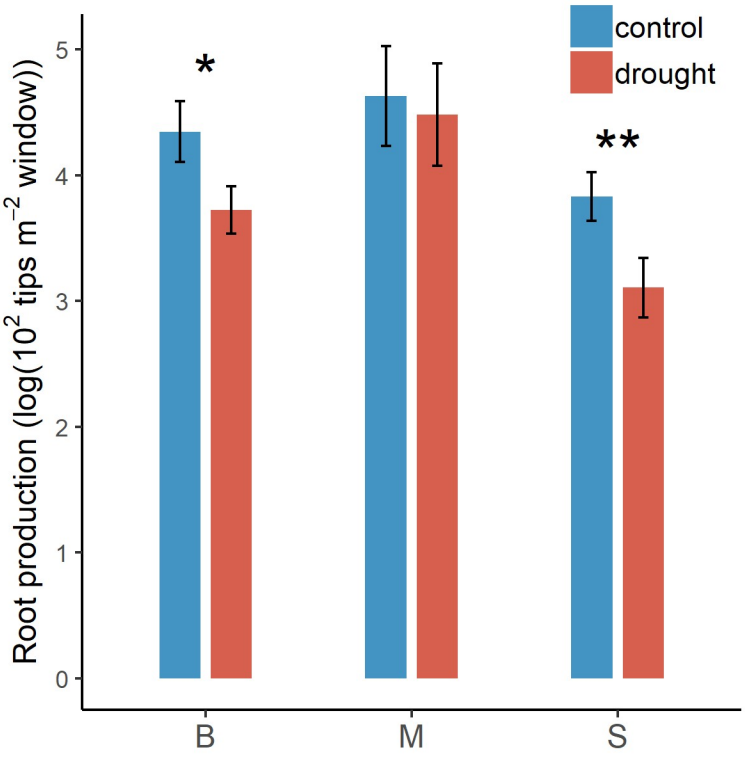


**Fig. 4.4** Log-transformed fine root production in the soils dominated by *Fagus sylvatica* (European beech, B), *Picea abies* (Norway spruce, S) and a mix of both species (M) under control (blue) and drought (red) conditions from March 2014 until December 2017. Mean and standard errors are shown ($N$=5). P-values below 0.1 (*) and 0.05 (**) are indicated as significant.

When analyzing the effect of drought on fine root production across soil depths in the interspecific and intraspecific zones, we found different patterns for *F. sylvatica* and *P. abies*. While throughfall-exclusion significantly reduced *P. abies* root production at the depths of 0-10 cm ($p < 0.1$) and 20-30 cm in the intraspecific zone ($p < 0.05$), there were no significant effects of throughfall-exclusion for *P. abies* in the mixture (Fig. 4.5a, 4.5c, 4.6a). Throughfall-exclusion did not have a contrasting effect

on *F. sylvatica* fine roots production in the interspecific or intraspecific zone (Fig. 4.6b). In the intraspecific zone, *F. sylvatica* produced fewer roots at 10-20 cm and 30-40 cm under throughfall-exclusion ($p < 0.05$, Fig. 4.5b). In the mixture, root production was only reduced at 10-20 cm ($p < 0.05$, Fig. 4.5d). While there was no significant difference in root tip production by depth in the intraspecific zones, soil depth had a significant effect on root production of both *F. sylvatica* and *P. abies* when grown intermixed ($p < 0.05$, Fig. 4.5). We also calculated the proportion of fine roots produced at each soil depth level. In terms of this relative distribution pattern of root production, the trend for *F. sylvatica* was the same as for the absolute numbers with a smaller proportion of roots present at 10-20 cm ($p < 0.001$) and at 30-40 cm depth ($p = 0.09$). For *P. abies*, throughfall-exclusion did not affect the relative distribution of root production by depth ($p = 0.29$).

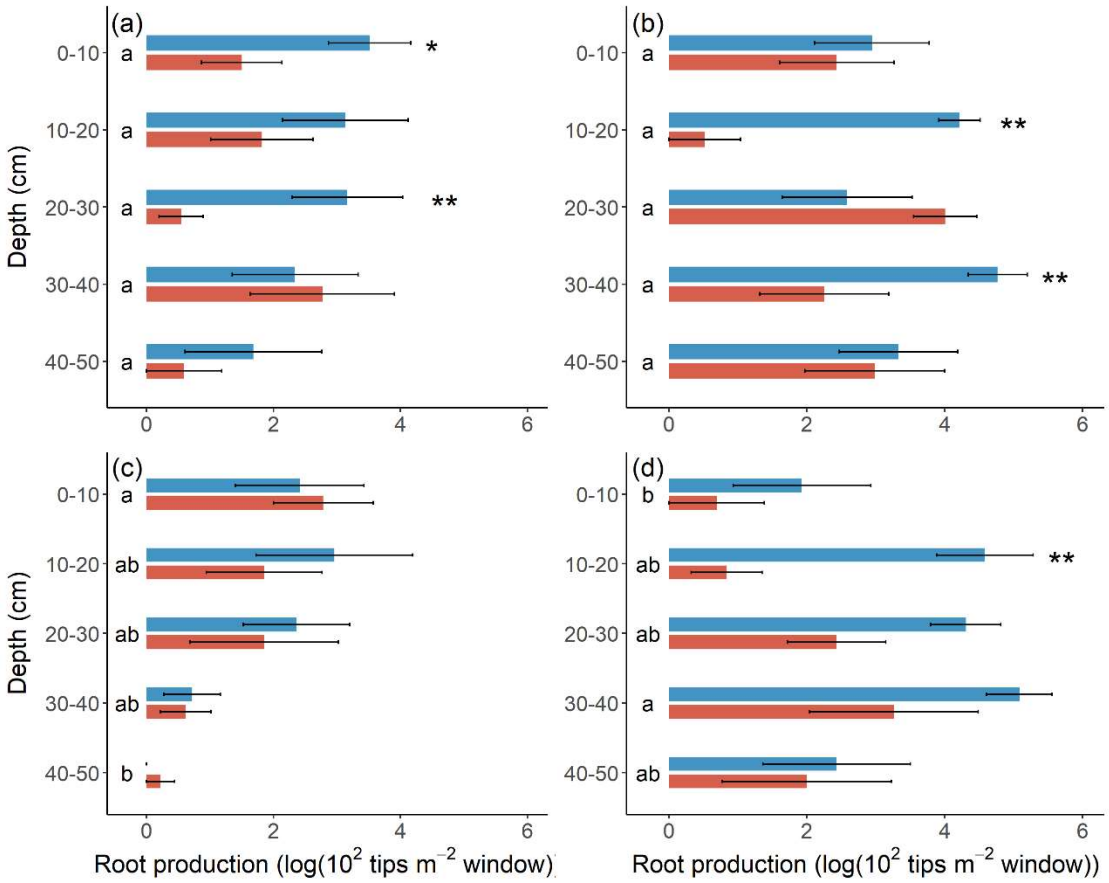


**Fig. 4.5** Log-transformed fine root production by 10 cm soil depth levels in the single-species and mixed-species zones of the forest under control (blue) and drought (red) conditions: (a) *Picea abies* in the intraspecific zone, (b) *Fagus sylvatica* in the intraspecific zone, (c) *Picea abies* in the interspecific zone, and (d) *Fagus sylvatica* in the interspecific zone. Mean and standard errors are shown ($N$=5). P-values below 0.1 (*) and 0.05 (**) are marked as significant. Contrasting letters indicate significant differences between depth levels as determined by Tukey's Honest Significant Difference (HSD) test ($p < 0.05$).

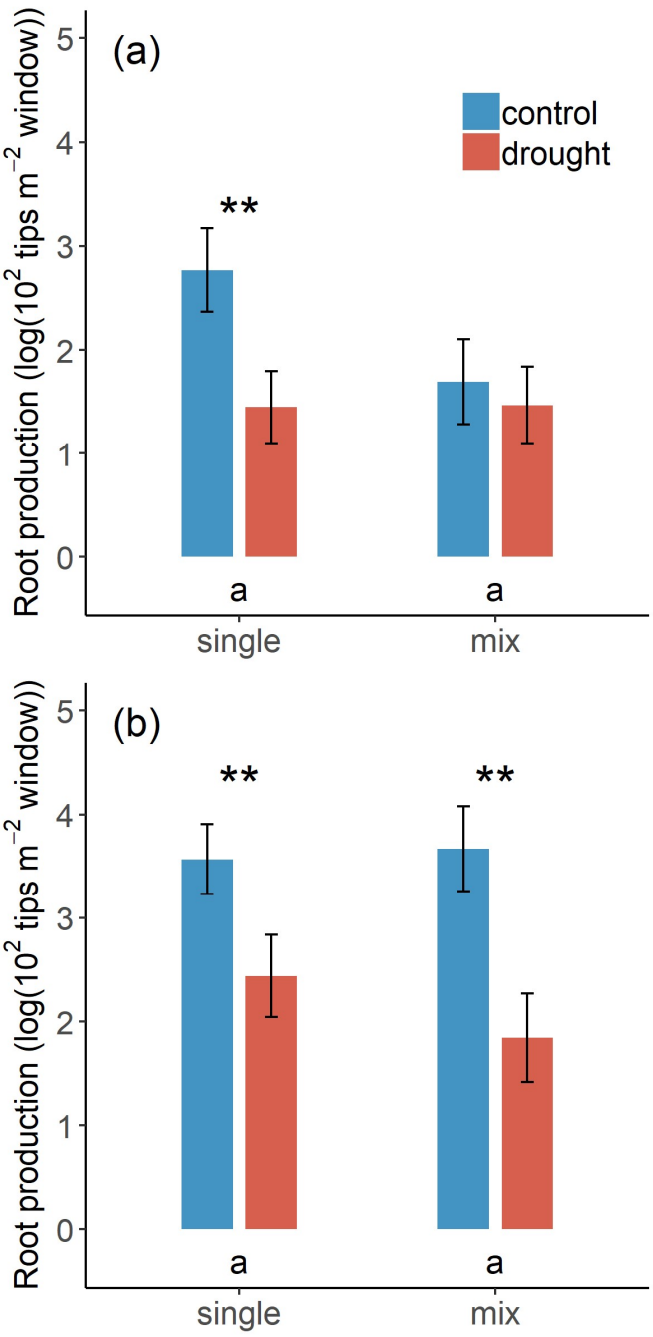


**Fig. 4.6** Log-transformed fine root production averaged over depth levels in the intraspecific (single) and interspecific (mix) zones for (a) *Picea abies* and (b) *Fagus sylvatica* under control (blue) and drought (red) conditions. Mean and standard errors are shown (*N*=5). P-values below 0.1 (*) and 0.05 (**) are indicated as significant.

The effect of throughfall-exclusion on fine root production varied by month and year (Fig. 4.7, 4.8). Every year, throughfall-exclusion reduced the production of *F.*

*sylvatica* fine roots in July regardless of the interaction zone ($p < 0.05$ or $p < 0.1$, Fig. 4.7). Depending on the year, we also observed lower *F. sylvatica* fine root production in May, June, August, September, October, November and December. Fine root production from January to April was largely unaffected by the experimental drought, which was also when the throughfall-exclusion roofs were open and some soil moisture recharge occurred (Fig. 4.2). The reduction in the monthly production of *P. abies* roots in the throughfall-exclusion plots was more pronounced in the intraspecific than in the interspecific zone (Fig. 4.8). Except for 2017, we found decreases in *P. abies* fine root production over the years from May to November and in February ($p < 0.05$ or $p < 0.1$).

Using Spearman's and Kendall's rank correlation tests, we found a positive relationship between the fine root production of *F. sylvatica* and soil volumetric water content (Table 4.1, $p < 0.001$). In the case of *P. abies*, this correlation was insignificant for yearly root production and average soil moisture and negative when we evaluated this relationship by month (Table 4.1).

**Table 4.1** Spearman's and Kendall's rank correlations of fine root production and average soil moisture content averaged by month and year for *Fagus sylvatica* and *Picea abies*. Fine root production was counted by depth levels of the soil moisture sensors (0-7 cm, 10-30 cm and 30-50 cm).

| Species | Time period | Spearman's ρ | Kendall's τ |
|---|---|---|---|
| *Fagus sylvatica* | Year | 0.47 ($p < 0.0001$) | 0.33 ($p < 0.0001$) |
| | Month | 0.13 ($p < 0.0001$) | 0.10 ($p < 0.0001$) |
| *Picea abies* | Year | -0.06 ($p = 0.383$) | 0.04 ($p = 0.391$) |
| | Month | -0.07 ($p = 0.003$) | 0.06 ($p = 0.003$) |

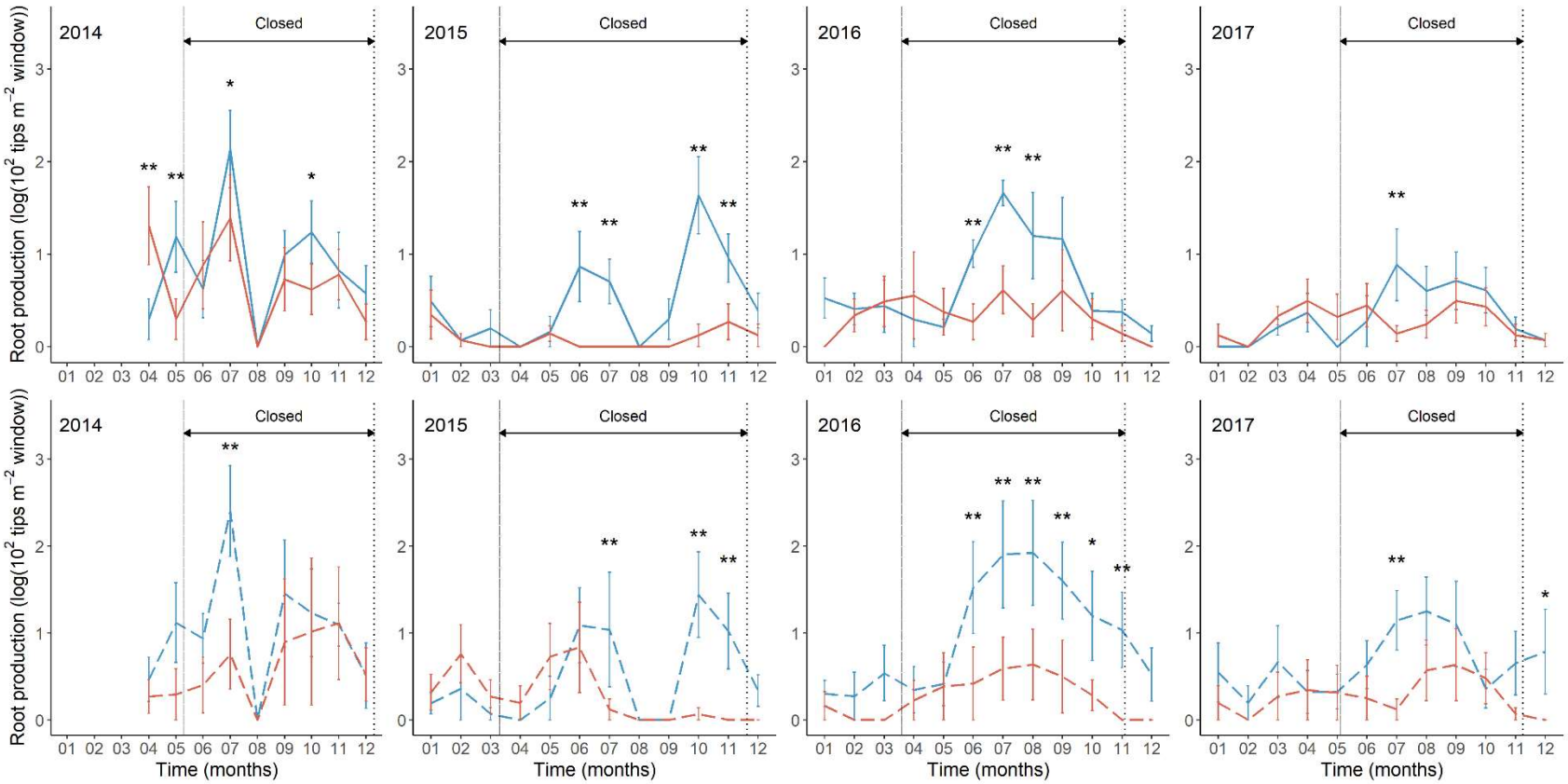


**Fig. 4.7** Log-transformed fine root production of *Fagus sylvatica* by month in the intraspecific (single, solid lines) and interspecific (mix, dashed lines) zones of the forest under control (blue) and drought (red) conditions over the years 2014, 2015, 2016 and 2017. Time period of through-fall exclusion is indicated with vertical lines representing the dates on which roofs were closed (long dash) and re-opened (dotted) at the end of the growing season. Mean and standard errors are shown (*N*=5). P-values below 0.1 (*) and 0.05 (**) are indicated as significant.

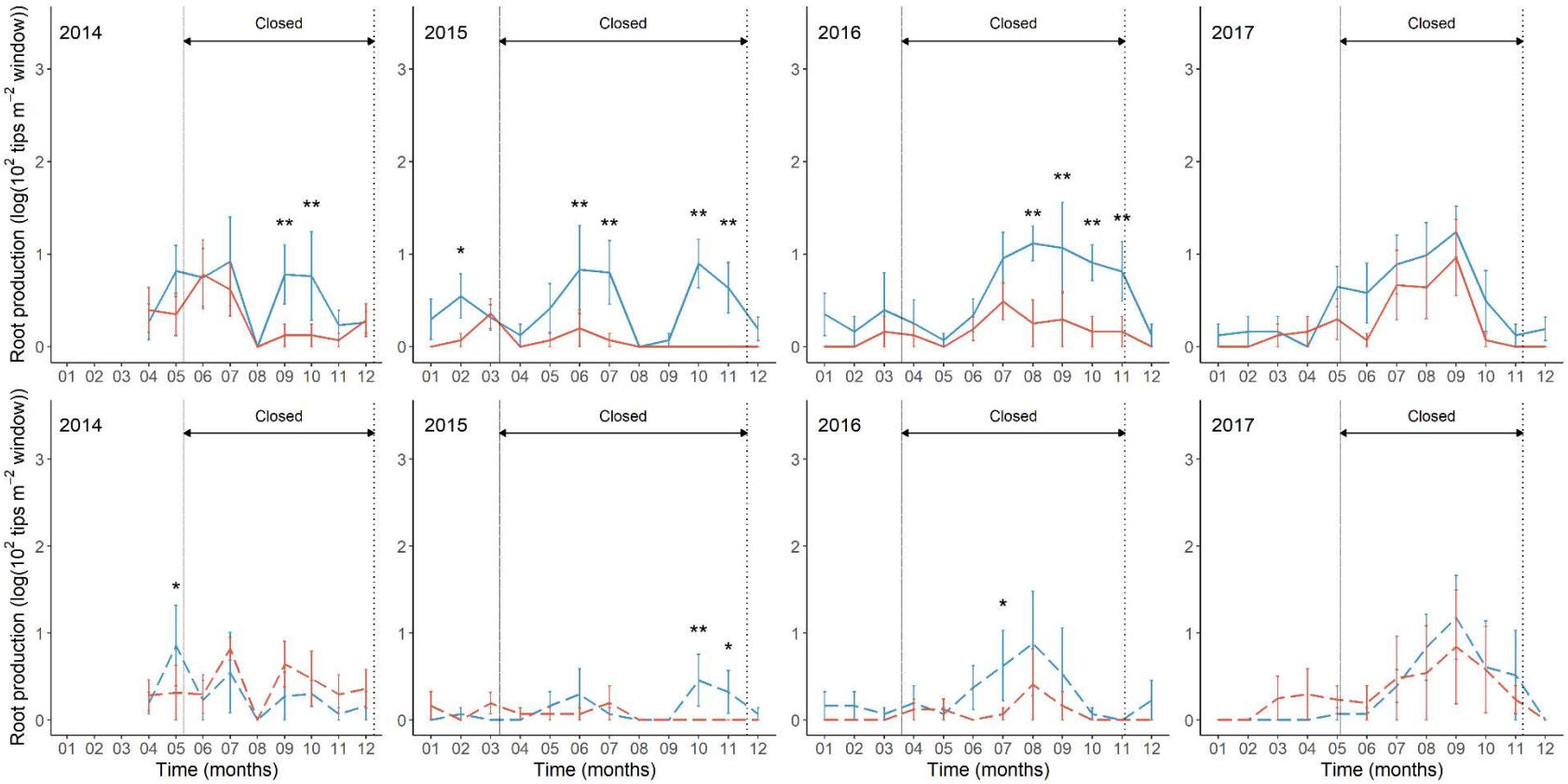


**Fig. 4.8** Log-transformed fine root production of *Picea abies* by month in the intraspecific (single, solid lines) and interspecific (mix, dashed lines) zones of the forest under control (blue) and drought (red) conditions over the years 2014, 2015, 2016 and 2017. Time period of through-fall exclusion is indicated with vertical lines representing the dates on which roofs were closed (long dash) and re-opened (dotted) at the end of the growing season. Mean and standard errors are shown (*N*=5). P-values below 0.1 (*) and 0.05 (**) are indicated as significant.

*Fine root lifespan and mortality*

According to the Cox proportional hazard (Table 4.2, 4.3) and Kaplan-Meier tests (Fig. 4.9), throughfall-exclusion increased the survivorship of *P. abies* and *F. sylvatica* first-order roots. A unit increase in the average soil water content (%) of the root environment increased the mortality risk of *F. sylvatica* fine roots by 2% (Table 4.2, $p = 0.038$), but did not have an effect on the root survivorship of *P. abies* (Table 4.2). In comparison to the intraspecific zone, the interspecific zone reduced the mortality risk of first-order *F. sylvatica* roots by 30% (Table 4.3). The Kaplan-Meier test also showed a significant difference between the median lifespan of *F. sylvatica* fine roots in the interspecific or intraspecific interaction zone ($p < 0.001$, Fig. 4.9). While the effect of species interaction on the survivorship of *P. abies* roots was not significant in the Cox proportional hazard model ($p = 0.98$), the median lifespan of *P. abies* first-order roots in the intraspecific zone was higher than in the interspecific zone according to the Kaplan-Meier test ($p < 0.001$, Fig. 4.9). A wider root diameter at birth increased median lifespan for both species ($p < 0.0001$), while root depth only had a significant effect on the survivability of *P. abies* roots (Table 4.2, 4.3). It is important to keep in mind that the model was stratified by 10 cm depth increments. Hence, the root depth variable was only tested within a 10 cm range.

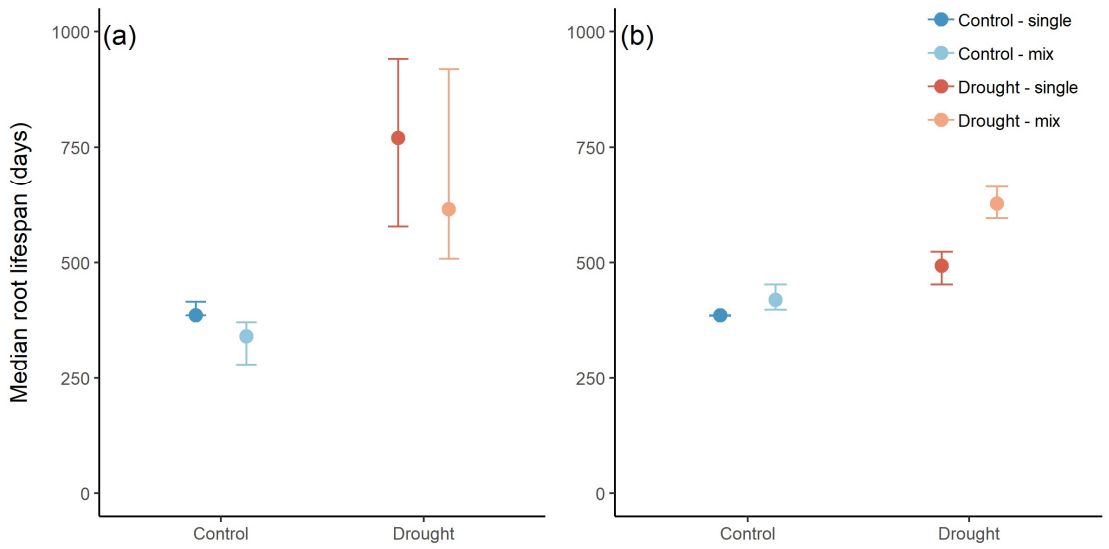


**Fig. 4.9** Median lifespan based on Kaplan-Meier estimates for (a) *Picea abies* and (b) *Fagus sylvatica* in the intraspecific (single) and interspecific (mix) zones of the forest under control and drought conditions. Bars show the 95% confidence intervals.

**Table 4.2** Cox-proportional hazard model output for the mortality risk of first-order fine roots of *Picea abies*. Coefficient estimates, standard error (SE), Wald Chi-Square statistic ($\chi^2$), hazard risk ratio and p-values are shown. For the categorical variables included in the model, their reference level (ref) is in parentheses. Significant p-values ($p < 0.05$) are in bold. A hazard ratio < 1 means a decrease in mortality risk, while a hazard ratio > 1 signifies an increase in mortality risk.

| Variable | Coefficient estimate | SE | $\chi^2$ | Hazard ratio | *P* |
|---|---|---|---|---|---|
| Treatment (ref = control) | | | | | |
| Drought | -1.379 | 0.387 | -3.56 | 0.25 | **< 0.0001** |
| Interaction (ref = single) | | | | | |
| Mix | -0.003 | 0.132 | -0.03 | 1.00 | 0.98 |
| Average daily water (% day$^{-1}$) | -0.001 | 0.02 | -0.11 | 1.00 | 0.91 |
| Diameter ($10^{-1}$ mm) | -0.237 | 0.048 | -4.93 | 0.79 | **< 0.0001** |
| Root depth (cm) | -0.127 | 0.021 | -5.85 | 0.88 | **< 0.0001** |
| Year (reference = 2014) | | | | | |
| 2015 | 0.109 | 0.189 | 0.58 | 1.12 | 0.56 |
| 2016 | 0.461 | 0.140 | 3.29 | 1.59 | **0.001** |
| 2017 | 0.785 | 0.290 | 2.71 | 2.19 | **0.0067** |
| Treatment:Year (ref = control 2014) | | | | | |
| Drought:2015 | 0.077 | 0.412 | 0.19 | 1.08 | 0.85 |
| Drought:2016 | -0.203 | 0.371 | -0.55 | 0.81 | 0.58 |
| Drought:2017 | 0.579 | 0.505 | 1.15 | 1.78 | 0.25 |

**Table 4.3** Cox-proportional hazard model output for the mortality risk of first-order fine roots of Fagus sylvatica. Coefficient estimates, standard error (SE), Wald Chi-Square statistic ($\chi^2$), hazard risk ratio and p-values are shown. For the categorical variables included in the model, their reference level (ref) is in parentheses. Significant p-values ($p < 0.05$) are in bold. A hazard ratio < 1 means a decrease in mortality risk, while a hazard ratio > 1 signifies an increase in mortality risk.

| Variable | Coefficient estimate | SE | $\chi^2$ | Hazard ratio | *P* |
|---|---|---|---|---|---|
| Treatment (ref = control) | | | | | |
| Drought | -0.845 | 0.235 | -3.60 | 0.43 | **< 0.0001** |
| Interaction (ref = single) | | | | | |
| Mix | -0.357 | 0.070 | -5.07 | 0.70 | **< 0.0001** |
| Average daily water (% $day^{-1}$) | 0.017 | 0.007 | 2.08 | 1.02 | **0.038** |
| Diameter ($10^{-1}$ mm) | -0.181 | 0.032 | -5.65 | 0.83 | **< 0.0001** |
| Root depth (cm) | -0.020 | 0.013 | -1.56 | 0.98 | 0.12 |
| Year (reference = 2014) | | | | | |
| 2015 | -0.184 | 0.079 | -2.33 | 0.83 | **0.02** |
| 2016 | -0.356 | 0.084 | -4.25 | 0.70 | **< 0.0001** |
| 2017 | 0.529 | 0.155 | 3.42 | 1.70 | **0.0006** |
| Treatment:Year (ref = control 2014) | | | | | |
| Drought:2015 | 0.345 | 0.230 | 1.50 | 0.23 | 0.13 |
| Drought:2016 | 1.177 | 0.186 | 6.34 | 0.19 | **< 0.0001** |
| Drought:2017 | 0.579 | 0.304 | 1.91 | 0.30 | **0.057** |

The survivorship of *F. sylvatica* and *P. abies* roots varied by year (Table 4.2, 4.3). Yet, these results should be interpreted with caution because significantly more roots were censored in 2016 and 2017 than 2014 ($p < 0.001$). Because survival analyses assume that censoring is independent of treatment, it is better to avoid between-year comparison. However, we can still compare the effect of throughfall-exclusion on the survival of fine roots within each year. Comparing throughfall-exclusion to control conditions over the years of the study indicates a positive effect of throughfall-exclusion on survivorship of *F. sylvatica* roots in 2014 and a negative effect on survivorship in 2016 (Fig. 4.10b). For *P. abies*, drought consistently

increased *P. abies* root survivorship in 2014, 2015 and 2016 (Fig. 4.10a).

The throughfall-exclusion caused higher root mortality in the control than the drought plots for *P. abies* ($p$ = 0.02). Yet, the number of possible deaths depends on the amount of root production, which was also higher in the control plots. When dividing the number of root deaths by the number of roots produced, there was no significant effect of throughfall-exclusion for *F. sylvatica* ($p$ = 0.90) or *P. abies* ($p$ = 0.50). Species interaction did not have a significant effect on root mortality or the mortality:production ratio of either species. Overall, *P. abies* and *F. sylvatica* did not show significant differences in monthly root mortality counts within in the control or throughfall-exclusion plots ($p$ = 0.39 and $p$ = 0.12), suggesting that root death events were rather constant throughout the year.

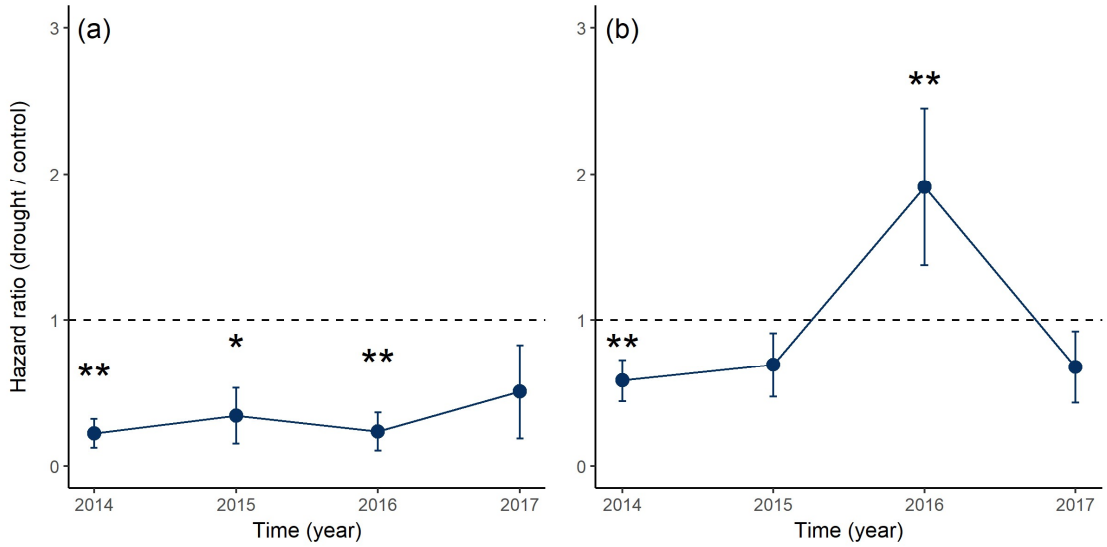


**Fig. 4.10** Drought to control hazard ratio by year calculated using a Cox proportional hazard model for the fine roots of (a) *Picea abies* and (b) *Fagus sylvatica*. Asterisks indicate a significant difference in mortality risk between the drought and control (** $p < 0.05$, * $p < 0.1$). A hazard ratio smaller than 1 (dashed horizontal reference line) means a lower mortality risk under drought than control conditions, while a hazard ratio greater than 1 means a higher mortality risk under drought.

***Discussion***

By means of a throughfall-exclusion experiment at the field scale, we explored the hypotheses 1) that tree carbon resource availability drives fine root production and lifespan patterns under drought, and 2) that facilitation buffers drought impacts on fine root growth dynamics under interspecific growing conditions. We found evidence supporting both predictions following from these hypotheses. First of all, throughfall-exclusion reduced fine root production in the monospecific areas of *F. sylvatica* and *P. abies* (Fig. 4.4, 4.5, 4.6), yet increased fine root lifespan of both species (Fig. 4.9). Second, fine root production was not decreased in the areas of the forest where both species were present (Fig. 4.4). In the first three sections of this discussion, we consider the results supporting our hypotheses and compare them to other studies. Moreover, we will use our findings to assess the degree of root plasticity of both species in response to water limitation and their neighbors. Lastly, we will discuss the implications of our results in the context of soil carbon cycling and tree cultivation in a changing climate.

*Explaining drought-induced reductions in fine root production*

Fine root production of *P. abies* and *F. sylvatica* decreased under throughfall-exclusion (Fig. 4.4, 4.5, 4.6), which is in line with our prediction. Other field studies also found a lower amount of root tips produced during drought (Mainiero & Kazda, 2006; Metcalfe *et al.*, 2008). A variety of complementary measurements in this experiment suggest that this decrease in fine root production can be explained by reduced plant carbon resources, supporting our hypothesis that plant carbon

availability drives shifts in fine root growth dynamics during drought. In 2014, 2015 and 2016, predawn leaf water potentials of *P. abies* and *F. sylvatica* were significantly lower in the throughfall-exclusion plots in comparison to the control, reaching down to values of -1.6 MPa in the summer of 2015 (Tomasella *et al.*, 2017). Leaf gas exchange was also reduced in both species; however, the reduction in saturated stomatal conductance at the leaf-level was greater for *P. abies* than for F. *sylvatica* (Goisser *et al*., unpublished). Moreover, a study conducted in 2015 and 2016 showed that throughfall-exclusion decreased phloem transport velocity and increased the mean residence time of photosynthates in the leaves of *F. sylvatica* (Hesse *et al.,* submitted), both of which indicate decreased C transport from leaves as has also been observed in other studies (Ruehr *et al.*, 2009).

These aboveground responses to drought are likely to affect the overall plant carbon budget, which could have limited the resources available for root production. Moreover, the fact that root production was not only reduced at the surface soil layers but also at 30-40 cm depth for *F. sylvatica* and 20-30 cm for *P. abies* (Fig. 4.5) where moisture levels were higher (Fig. 4.2) provides further evidence for our hypothesis that reduced carbon resources caused a decline in fine root production. As an alternative hypothesis, unfavorable soil moisture levels may have also contributed directly to reductions in fine root production. There would be little benefit in terms of resource uptake when growing a new root in a dry soil patch (Hodge, 2010). For *F. sylvatica*, there was indeed a positive correlation between root production and soil volumetric water content. However, in the case of *P. abies*, this correlation was insignificant or even slightly negative (Table 4.1). Future studies should focus on the

interplay of mechanisms leading to reduced fine root production under seasonal drought.

*Increased root lifespan as response to water limitation*

As we expected, median fine root lifespan of both *F. sylvatica* and *P. abies* increased significantly under throughfall-exclusion (Table 4.2, 4.3, Fig. 4.9). Although only a handful of studies have investigated the relationship between soil moisture deficit and root lifespan (Eissenstat *et al.*, 2013; McCormack & Guo, 2014), ours appears to be the first to report an increase in lifespan in response to seasonal drought. Some previous studies have found that drought increases root mortality (Mainiero & Kazda, 2006) and decreases root lifespan (Meier & Leuschner, 2008a), while others did not find an effect of water limitation on root growth dynamics (Bryla *et al.*, 1997; Joslin *et al.*, 2000; Bauerle *et al.*, 2008; Chen & Brassard, 2013).

A variety of factors influence fine root longevity. Ultimately, it is the balance between root carbon costs and the benefits of resource acquisition that determine the optimal root lifespan (Eissenstat & Yanai, 1997). If new root production costs are high, and root maintenance and uptake rates are low as, then root lifespan should be high (Eissenstat *et al.*, 2000). Under seasonal drought, such a scenario is possible. Several studies have reported lower root respiration rates and thus lower maintenance costs under water limitation (Bryla *et al.*, 1997; Burton *et al.*, 1998; Hasibeder *et al.*, 2015). Where water is limited, uptake of nutrients and water are reduced (Hu & Schmidhalter, 2005). This scenario suggests the optimal strategy for a tree may be to hold onto older roots until water and nutrient availability increase as opposed to

investing valuable carbon into new root production. While fine roots are vulnerable to injury under extreme soil moisture deficits (Mckay & White; Huang & Gao, 2000), this danger may be avoided by internal hydraulic distribution with water from areas with higher soil moisture (Bauerle *et al.*, 2008), relative increase of ectomycorrhizae with long rhizomorphs capable of long-distance water transport (Nickel *et al.*, 2018), and root structural changes to limit water loss (North & Nobel, 1992).

One of the greatest challenges of minirhizotron methods is the determination of root death solely based on visual indicators (Comas *et al.*, 2000). Hence, a logical question arising from our findings is whether we observed prolonged root lifespan or increased persistence of dead roots. Because of the positive correlation between mean annual rainfall and root decomposition rate (Silver & Miya, 2001), this is an important caveat to address. There are two pieces of evidence that suggest that the increased root lifespans in response to drought are real. First of all, minirhizotron methods generally underestimate root lifespan and overestimate root turnover (Tierney & Fahey, 2002; Strand *et al.*, 2008). Regardless of this bias, we detected a drought-induced increase in lifespan. Secondly, we only observed increased lifespans in the first few years of the study. We did not detect this increase in 2017 for *P. abies* and in 2015-2017 for *F. sylvatica* (Fig. 4.10), even though soil moisture was lower in 2017 than 2014 ($p < 0.05$). If we had been overestimating root lifespan due to drought-induced decrease in root turnover and root decomposition, longer root lifespans would be expected in 2017. Yet, we did not observe this.

The fact that the throughfall-exclusion did not lead to an increase in the fine root lifespan of *F. sylvatica* in the later years of the study (Fig. 4.10b) suggests that

prolonging root lifespan may not be a sustainable strategy under repeated episodes of drought. Presumably, carbon resources become more limited with the progression of drought and continuing trend of reduced gas exchange (Goisser *et al.* unpublished). Limited carbon availability may increase the relative costs of root maintenance making root shedding a more beneficial strategy under long-term or repetitive drought events.

*The buffering effect of species mixture on belowground productivity during drought*

In line with our hypothesis and prediction, the throughfall-exclusion treatment did not have a negative impact on fine root production in the zone of the forest where both species were present (Fig. 4.4). This is in contrast with the intraspecific zones of *F. sylvatica* and *P. abies* that did show a decrease in fine root production (Fig. 4.4). These results also provide evidence for Callaway and Walker's hypothesis (1997) stating that facilitation becomes more prominent under abiotic stress. Previous studies have shown that diversity increases aboveground productivity and buffers impacts of biotic stress (Morin *et al.*, 2011; Isbell *et al.*, 2015; Sun *et al.*, 2017; Oehri *et al.*, 2017). Yet, this is one of the first studies that shows that mixed-species compositions also dampen the negative effect on belowground productivity of forests.

Interestingly, *F. sylvatica* did show a reduction in fine root production in the interspecific zone of the throughfall-exclusion treatment, while *P. abies* did not (Fig. 4.5, 4.6). This result may be partly explained by the differences in soil water content between the interspecific and intraspecific zones of the forest. While soil moisture levels in the intraspecific zone of *F. sylvatica* were either the same or higher than in

the interspecific zone, the fine roots of *P. abies* experienced higher soil moisture in the mixed-species zone than in the single-species zone of the throughfall-exclusion plots (Fig. 4.3). This difference in soil water content between the interspecific and intraspecific areas of *P. abies* was not evident under control conditions.

One possible mechanism for similar belowground productivity under throughfall-exclusion in comparison to the control is more efficient sharing of water resources. In the control plots, we found a greater separation in rooting depth profiles between the two species. *P. abies* has a shallower root system and *F. sylvatica* more deep roots in the mixture (Fig. 4.5), which provides evidence for niche differentiation (Silvertown, 2004). However, only *P. abies* benefitted from the mixed-species composition under drought, suggesting that *F. sylvatica* may facilitate the fine root production of *P. abies* but not the other way around. Because the roots of *F. sylvatica* reach greater depth with higher moisture than the roots of *P. abies*, hydraulic redistribution (Dawson, 1993; Caldwell *et al.*, 1998) through roots of *F. sylvatica* may explain why *P. abies* is profiting from a higher soil water content in the mixed- vs. single-species zone. Alternatively, differences in tree species effects on soil biological, chemical and physical properties may lead to higher water availability in the interspecific zone than in the monospecific zone of *P. abies* (Augusto *et al.*, 2002; Pretzsch *et al.*, 2014). These mechanisms are speculative and warrant further research.

*Plastic root responses to water limitation and neighbors*

The reduced fine root production at 0-10 cm depth for *P. abies* and 10-20 cm depth for *F. sylvatica* (Fig. 4.5) provides evidence of root plasticity in both species in response

to drought. Decreasing root proliferation in the driest soil layers is a strategy of drought avoidance at the root level (Brunner *et al.*, 2015). Yet, because *F. sylvatica* also shows a reduction in fine root production at 30-40 cm where water availability is higher, it is difficult to know whether this is a result of limited carbon availability for root growth or selective root production to avoid drought and forage for water. While *F. sylvatica* did show a change in relative distribution of root production by depth in response to drought ($p = 0.03$), *P. abies* did not ($p = 0.29$). This suggests that *F. sylvatica* has a greater root system plasticity under drought than *P. abies*.

On a temporal scale, we found limited root production in the summer and fall months (Fig. 4.7, 4.8) when water limitation was the most severe (Fig. 4.2). This suggests that the timing of root production in both species can be selective by avoiding periods of severe water limitation, which has also been observed by studies in other ecosystems (Reich, 1980; Sayer & Haywood, 2006; Rojas-Jimenez *et al.*, 2007). However, there was no sign of increased root production in comparison to the control during the months where the throughfall-exclusion roofs were open or in early spring and summer. The lack of difference in root mortality over time and depth between the control and throughfall-exclusion treatments suggests root death was not used as drought avoidance strategy by *P. abies* or *F. sylvatica.*

The increase in root lifespan of both species in response to drought suggests that lifespan is a plastic trait under water limitation. However, for *F. sylvatica* this response appeared to be transient over the course of the four year experiment. The fine roots of *F. sylvatica* only showed an increase in lifespan under the throughfall-exclusion treatment in 2014 and this trend was even reversed in 2016 (Fig. 4.10b),

implying that this strategy cannot be maintained under repeated episodes of drought. *P. abies* maintained a longer root lifespan for the first three years, but not in 2017 (Fig. 4.10a). Yet, this may be due to the high amount of censoring of roots that were produced in 2017.

Root plasticity in the context of ectomycorrhizal colonization during drought also deserves attention. A previous study with *P. abies* and *F. sylvatica* highlighted the importance of considering mycorrhizal symbiosis as a plastic fine-root trait for resource uptake in environments with different resource availabilities (Weemstra *et al.*, 2017). At the Kranzberg experimental site, Nickel et al. found an increase in the relative abundance of ectomycorrhizae with long rhizomorphs for both species in the throughfall-exclusion treatment (2018). Hence, investing resources in mycorrhizae capable of water uptake and long-distance transport may be an important trait for tree species under drought stress.

Neighbor identity triggered a plastic root response in both species. *F. sylvatica* showed a shift in root production by depth (Fig. 4.5) as well as in lifespan (Table 4.3) under interspecific compared to intraspecific growing conditions. Neighbor identity did not change the root longevity of *P. abies* (Table 4.2), but we did observe a difference in spruce root production by depth in the single- and mixed-species zones of the forest (Fig. 4.4). A previous publication from this experiment discusses the mechanisms and implications of these belowground species interactions in more detail (Paya, 2015).

*Implications of seasonal drought for soil carbon storage and Norway spruce cultivation in mixed- and single-species forests*

Throughfall-exclusion reduced fine root production and increased lifespan of both *P. abies* and *F. sylvatica* (Fig. 4.5, 4.6, Table 4.2, 4.3). Acknowledging that we did not directly measure carbon fluxes from roots to soil, the decreased allocation of carbon resources belowground, together with a lower rate of root turnover, suggest that drought significantly reduces root carbon supply to soil. Drought-induced changes in this part of the carbon cycle – from plant root to soil - have received little attention and further experimentation is necessary in order to estimate drought effects on soil carbon storage in forests. In addition to the quantity of plant carbon received by soils under limited water conditions, the quality of plant organic matter also plays a role in soil carbon sequestration (De Deyn *et al.*, 2008; Castellano *et al.*, 2015). Together with the flux of carbon into soil, the $CO_2$ efflux from soil will determine soil carbon storage under future climate scenarios. So far both increases (Sowerby *et al.*, 2008; Cleveland *et al.*, 2010), decreases (Borken *et al.*, 2006; Sowerby *et al.*, 2008) and no changes (Borken *et al.*, 1999; Davidson *et al.*, 2004) in soil respiration have been found under decreasing water availability. Mean soil temperature, water content, amount of soil organic matter and biome of study site are all likely to play a role in explaining these contrasting results (Davidson *et al.*, 1998; Sowerby *et al.*, 2008; Liu *et al.*, 2016).

In contrast to the intraspecific zones of *F. sylvatica* and *P. abies*, interspecific areas did not show a reduction in fine root production under throughfall-exclusion (Fig. 4.4). This was mainly due to the unchanged production of fine roots by *P. abies* (Fig. 4.6), which experienced higher soil water contents in the interspecific than the

intraspecific zones during drought (Fig. 4.2). These results support the hypothesis that root carbon supply to soil will be higher in more diverse forests when precipitation is lower. Moreover, maintained fine root production of *P. abies* in more diverse forests during episodes of seasonal drought has important implications for its cultivation practices in Central European forests. Currently, drought-vulnerable *P. abies* trees are mainly grown in monoculture. If growing *P. abies* intermixed with *F. sylvatica* can increase soil water availability and sustain fine root production, this is likely to improve tree performance during drought. Limited evidence indeed shows that mixed-species compositions lower drought stress and increase tree growth relative to monocultures under drought (Pretzsch *et al.*, 2013). Yet, these trends have not yet been detected at the Kranzberg Forest field site and demand further experimentation.

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# CHAPTER 5: CONCLUSION

Root and rhizosphere interactions are an essential part of terrestrial biogeochemical cycles (Nadelhoffer & Raich, 1992; Bardgett *et al.*, 2014; Finzi *et al.*, 2015) and require in-depth study in order to improve global C models estimating forest ecosystem feedbacks to climate change (Drigo *et al.*, 2008; Warren *et al.*, 2015; Pausch & Kuzyakov, 2018). Because of the challenges associated with root and rhizosphere research (Brunner & Godbold, 2007; Hassan & Mathesius, 2012; Shi *et al.*, 2013), questions about the mechanisms by which root exudates influence soil carbon (C) cycling and root responses to changing environmental variables remain unknown. To reduce these knowledge gaps, the objectives of this dissertation were 1) to analyze the mechanistic effect of phenolic root exudate variation among tree species on rhizosphere dynamics, and 2) to study changes in fine root production and lifespan of temperate forest species in response to seasonal drought. In this conclusion chapter, I will summarize my research findings, discuss the implications of these results and talk about potential future studies that could follow from this dissertation research.

## ***Summary of results***

In the second and third chapter of this dissertation, I addressed the question to what extent species variation in phenolic root exudate profiles can influence rhizosphere C cycling and microbial community assembly. To start with the experiments from the second chapter, I modified a root exudate collection method for phenolic compounds and found that root phenolic profiles varied greatly among temperate forest tree

species (Fig. 2.1) by means of high-performance liquid chromatography (HPLC). As we hypothesized, root phenolic compounds had contrasting effects on soil microbial respiration ranging from a 26% decrease to a 46% increase when incubated with a forest soil for 5 days (Fig. 2.2). Interestingly, compounds belonging to the same biosynthetic class did not have similar effects on microbial activity, while compounds with the same catechol functional group consistently decreased microbial respiration.

For the third chapter, I designed a soil incubation with one-time artificial root exudate additions to further explore the mechanisms by which phenolics influence soil organic matter (SOM) decomposition and soil microbial community composition in the presence or absence of glucose, a proxy for primary root metabolites. Through using $^{13}C$-labeled root exudate compounds, I was able to distinguish between C respired from the exudate amendments and SOM sources. Measures of soil extracellular enzyme activity, microbial abundance, and bacterial community composition (BCC) allowed me to correlate changes in soil C cycling to microbial parameters. In line with our hypothesis, phenolics had other functions besides microbial food source and were less quickly degraded than glucose (Fig. 3.1). Moreover, benzoic acid and caffeic acid induced significantly greater shifts in BCC than glucose (Fig. 3.5). While caffeic acid and catechin additions led to a decrease in cumulative soil microbial respiration (Fig. 3.2) and lowered microbial abundance (Fig. 3.3) relative to the unamended control over the first 15 days of the incubation indicating toxicity, these inhibitory effects were not evident anymore on day 38. Contrary to my hypothesis, benzoic acid was almost as fully degraded as glucose by the end of the experiment (Table 3.1). Benzoic acid also had the greatest effect on

SOM decomposition and BCC, suggesting that its function as a food source may have contributed to these patterns. Changes in microbial respiration relative to the untreated control were correlated with shifts in BCC, but not with microbial abundance (Fig. 3.8). In particular, the relative abundance of *Burkholderia* and *Paraburkholderia* genera were increased in soils amended with phenolics and/or glucose, while the relative abundance of *Tumebacillus* genus was reduced by benzoic and caffeic acid additions. Remarkably, the greatest priming in SOM decomposition rate was observed when both benzoic acid and glucose were added at the same time (Fig. 3.1, Table 3.1). Yet, this result should be interpreted with caution because we estimated the amount of SOM breakdown in the treatments with $^{13}$C-labeled glucose and unlabeled phenolic amendment by subtraction. Because of the increased extracellular enzyme activity of β-1,4-glucosidase and the unaffected rate of phenol oxidase and peroxidase activity (Fig. 3.4), the soil-derived C respired most likely came from relatively labile SOM sources.

In the fourth chapter, the aim of the study presented was to investigate the effect of seasonal drought on fine root production and lifespan in a mixed-species forests of European beech (*Fagus sylvatica* [L.]) and Norway spruce (*Picea abies* [L.] Karst) by means of a four-year-long throughfall-exclusion experiment at the field scale. The throughfall-exclusion treatment reduced volumetric soil water content by 33% over the course of the experiment (Fig. 4.2, Fig. 4.3). Using minirhizotron methods, I observed that decreased water availability reduced fine root production in the monospecific stands of *F. sylvatica* and *P. abies*, but not in the interspecific stands where both species were present (Fig. 4.4, 4.5, 4.6). This is in line with Callaway and

Walker's abiotic stress hypothesis (1997), which states that facilitation among different plant species is more prominent when resources are limiting. The decrease in fine root production was primarily visible but not limited to the surface layers of the soil (Fig. 4. 5), where water was most limiting (Fig. 4.2). Potentially to counteract the effect of reduced fine root production on soil resource acquisition, the root lifespan of both species was increased in response to the throughfall-exclusion treatment (Fig. 4.9, Table 4.2, 4.3). While the increase in fine root lifespan persisted for *P. abies*, this trend was only significant in the first year of the throughfall-exclusion for *F. sylvatica* (Fig. 4.10), suggesting that this strategy may not be sustainable under repeated episodes of drought. Lastly, I did not find evidence for temporal shifts in fine root production towards periods of greater soil water availability when throughfall-exclusion roofs were open (Fig. 4.7, 4.8).

### *Implications and future research directions*

*The chemical composition of root exudates as driver of soil biogeochemistry*

Previous research has shown that the quantity of root exudates released into the soil influences SOM mineralization (Phillips *et al.*, 2011; Yin *et al.*, 2014). Now this dissertation adds another point to this statement; namely, that the quality of root exudates also affects soil microbes and the biogeochemical processes that they mediate. In the context of climate change, some efforts have been made to study how changing environmental factors influence soil C and nutrient cycling through altering root exudation patterns (Phillips *et al.*, 2006, 2009). However, so far these experiments have centered on the role of root exudate quantity and not quality. Because this

dissertation highlights the importance of root exudate chemical composition to soil biogeochemical cycles, future research should analyze to what extent root exudate composition and associated rhizosphere processes change in response to altered climate variables. Moreover, it is necessary to extent the phenolic root exudate analysis to including estimated total quantities of primary and secondary metabolites of mature trees through field experiments. This is an ambitious undertaking, which I did not manage to accomplish during my PhD. However, without this information, we cannot establish the relative importance of root exudate quantity versus quality as well as primary versus secondary metabolites in driving rhizosphere interactions.

In my dissertation research, I found that tree species show a wide variation in root phenolic profiles. Another study also concluded that there are species differences in root exudate chemistry (Gransee & Wittenmayer, 2000). While the amount of studies on species variation in root exudate composition is limited, many publications have postulated that root exudates are responsible for the observed differences in rhizosphere C cycling among plant species (Cheng *et al.*, 2003; Dijkstra & Cheng, 2007; Bengtson *et al.*, 2012; de Graaff *et al.*, 2014). This also makes one question to what extent root exudates can explain the biogeochemical variation observed in forests dominated by different species. So far these between- and within-forest differences in C and nutrient budgets have been ascribed to species variation in growth rates, soil resource acquisition strategies and plant-microbial interactions (reviewed in Phillips *et al.*, 2013), yet there is not enough information to test or include species differences in root exudate chemistry as a factor. Even though my findings support the hypothesis that species-specific root chemical profiles drive soil biogeochemical processes in

forests with different tree species, further research under more natural rhizosphere conditions and at the ecosystem-scale is necessary to test this statement. Considering that climate change may shift forest species composition through altering environmental conditions and resources (Iverson & Prasad, 2001), it will be even more crucial to study the root exudate mechanisms by which different tree species influence SOM mineralization in forests.

The theme of quality versus quantity was not only restricted to my root exudate work. Namely, I found that BCC (quality) was correlated with changes in microbial respiration, while microbial abundance (quantity) was not (Fig. 3.8). The relative increase in *Burkholderia* and *Paraburkholderia* genera was partly responsible for this trend (Fig. 3.7) and suggests that they played an active role in the priming of SOM decomposition (Fig. 3.1). Their tolerance towards phenolic compounds and low soil pH (Ortega-González *et al.*, 2013; Stopnisek *et al.*, 2014), their reputation as benzene and SOM degraders (McNamara & Leff, 2004; Ortega-González *et al.*, 2013) and their high abundance in forest soils (Baldrian *et al.*, 2012) fit in well with this hypothesis. To further test this, follow-up studies could use a nucleic acid stable isotope probing (SIP) approach (Chen & Murrell, 2010), which would allow for the identification of the microorganisms responsible for benzoic acid uptake.

Overall, the correlation between BCC and microbial respiration support the notion of including measures of microbial community composition and physiology into ecosystem models estimating terrestrial C cycling under different climate change scenarios, which has also been suggested by other studies (Six *et al.*, 2006; Allison *et al.*, 2010). While the soil-exudate incubation in this dissertation demonstrated that

microbial community composition matters to soil C cycling and identified two closely related bacterial genera that may be responsible for increased SOM decomposition upon the addition of phenolics and glucose, rhizosphere research is only at the beginning of linking microbial community composition to biological function.

Another important topic that did not get much attention in this dissertation is fungal abundance and community composition. The dominance of fungi in low pH and forest soils (Bååth & Anderson, 2003; Fierer *et al.*, 2005) provides a clear argument for including a fungal community composition analysis as part of the manuscript of chapter 3. This should give us a more complete picture of the microbial response to root exudates and their subsequent effects on SOM decomposition.

*Drought impact on root growth dynamics in monospecific and mixed-species forests*

The reduction in fine root production (Fig. 4.5, 4.6) and increase in root lifespan (Table 4.2, 4.3, Fig. 4.9) observed for *F. sylvatica* and *P. abies* under throughfall-exclusion suggest that root C inputs to soil will decrease in temperate forests subjected to repeated episodes of drought. Whether this will also influence the total amount of C stored in temperate forest soils depends on other factors that were not measured in this research. First of all, it should be established how changes in water limitation influence SOM decomposition since it is the input and output of C that determine the remaining soil C stock. To date, studies have observed both increases (Sowerby *et al.*, 2008; Cleveland *et al.*, 2010), decreases (Borken *et al.*, 2006; Sowerby *et al.*, 2008) and no changes (Davidson *et al.*, 2004; Borken *et al.*, 2006) in soil microbial respiration in response to water limitation. Considering that roots and associated

microorganisms are the primary soil C source in forests (Rasse *et al.*, 2005; Clemmensen *et al.*, 2013), future experiments should investigate the effect of drought on root C inputs, soil respiration and soil C storage simultaneously. Because my conclusions are entirely based on visual analyses, it will be important to include other methods allowing for quantitative estimates of drought-induced changes root production and turnover at the ecosystem scale.

Interspecific stands of *F. sylvatica* and *P. abies* buffered the negative drought impact on fine root production observed in the monospecific areas of the forest (Fig. 4.4). This was primarily due to the continued root growth of *P. abies* in the mixed-species composition (Fig. 4.6). Consistent with these results, I also found that soil volumetric water content was significantly higher in the interspecific area than in the monospecific *P. abies* area for all years except 2017 (Fig. 4.3). Conversely, I did not observe a significant difference in soil moisture between the interspecific and monospecific *F. sylvatica* zones (Fig. 4.3). These results suggest that *F. sylvatica* facilitated the production of *P. abies* root tips under decreasing water availability, which would be in line with Callaway and Walker's hypothesis (1997).

The implications of these findings are highly relevant to Central European forestry. *P. abies* is an important economic tree to this region, yet also extremely prone to drought stress (Pretzsch *et al.*, 2013, 2014). Currently, *P. abies* is mainly grown in monoculture. The question whether mixed-species arrangements with *F. sylvatica* and other tree species could allow for its continued cultivation under future climate scenarios by limiting drought impacts is an important item on the agenda of Central European foresters (Río *et al.*, 2014). This dissertation shows that mixed-

species composition at least buffer negative drought effects on belowground productivity. With this sustained pattern of root production and higher volumetric water content, it is likely that drought impacts on aboveground productivity and overall tree performance of *P. abies* are also reduced in mixed-species forest stands. In other forests sites, studies indeed found evidence for this hypothesis (Pretzsch *et al.*, 2013). However, at the Kranzberg Forest field site, a clear difference in tree growth of *P. abies* between the monospecific and interspecific stands under throughfall-exclusion has not been reported so far. Moreover, the aboveground and belowground responses to drought have not been clearly linked and tested at this site, which will be an important next step to undertake.

*Connecting root and rhizosphere processes in a changing climate*

The story about roots and the terrestrial C cycle presented in this dissertation may appear to have explored two opposing tales. On the one hand, roots can prime SOM decomposition rates (Kuzyakov, 2010) reducing soil C stocks. On the other hand, I have discussed studies demonstrating that roots are an important source of SOM (Rasse *et al.*, 2005; Clemmensen *et al.*, 2013) and used them as a justification to research root responses to drought. In this section, I will explain how these seemingly conflicting statements are not contradictory but subunits within a larger system that determines root effects on the terrestrial C cycle. In addition, I will suggest some next steps to study the connections between the root and rhizosphere processes addressed in this dissertation.

Estimating the ultimate effect of roots on soil C storage requires the

consideration of all root interactions combined and calculating the net outcome of these processes. Even though roots create hotspots in soils accelerating C and nutrient cycling (Kuzyakov & Blagodatskaya, 2015; Finzi *et al.*, 2015), their overall effect on soil C stocks could still be positive or neutral. While a proportion of labile root exudates are respired, the other part can efficiently be incorporated into microbial biomass (Manzoni *et al.*, 2012b) and eventually be stabilized by soil mineral interactions (Cotrufo *et al.*, 2013). Moreover, even if root exudates increase the rate of SOM mineralization, this may be compensated for by the turnover of fine roots and its associated micro-organisms that function also soil carbon inputs (Clemmensen *et al.*, 2013). Hence, it is the balance between all these root processes that determines the overall effect of roots on soil C cycling. While I studied some of these root processes individually, it will be crucial to connect them and estimate their overall role in root-facilitated C cycling in forests though experiments and modeling studies.

Two subjects for follow-up experiments joining the root processes that I studied in this dissertation are 1) the effect of drought on root priming, and 2) the relation between root lifespan and root exudation. Part of my dissertation work focused on developing and testing a method for root exudate collection and analysis. With this new tool, we can analyze how soil water availability or any other environmental factor influences root exudation patterns. Besides changes in root substrates, soil moisture will directly influence microbial dynamics too (Xiang *et al.*, 2008; Manzoni *et al.*, 2012a). Unraveling these interactions and their effects on soil C cycling will be challenging (Fuchslueger *et al.*, 2014), but an essential question to explore. The second research topic that could be based on this dissertation is to some

extent related to the first. In chapter 4 of this thesis, I conclude that root lifespan is a plastic trait responding to soil water availability. While there is some evidence that root age affects the physiochemical characteristics of the rhizosphere (Fischer *et al.*, 1989; Carminati, 2013), we know little about the qualitative changes in root exudation as a fine root gets older. Connecting these two root traits may help us better understand and predict how altered resource availability associated with global change influences root-mediated biogeochemical processes.

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