Journal of Plant Ecology Advance Access published December 7, 2014

Journal of
Plant Ecology

PAGES 1–11

doi:10.1093/jpe/rtu034

available online at
www.jpe.oxfordjournals.org

Nitrogen fertilization has minimal influence on rhizosphere effects of smooth crabgrass (*Digitaria ischaemum*) and bermudagrass (*Cynodon dactylon*)

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Abstract

Aims

Plants generally respond to nitrogen (N) fertilization with increased growth, but N addition can also suppress rhizosphere effects, which consequently alters soil processes. We quantified the influence of N addition on rhizosphere effects of two C_4 grasses: smooth crabgrass (*Digitaria ischaemum*) and bermudagrass (*Cynodon dactylon*).

Methods

Plants were grown in nutrient-poor soil for 80 days with either 20 or 120 μg NH_4NO_3-N g dry soil^{-1}. N mineralization rates, microbial biomass, extracellular enzyme activities and bacterial community structure were measured on both rhizosphere and bulk (unplanted) soils after plant harvest.

Important Findings

Fertilization showed nominal differences in net N mineralization, extracellular enzyme activity and microbial biomass between the rhizosphere and bulk soils, indicating minimal influence of N on rhizosphere effects. Instead, the presence of plant roots showed the strongest impact (up to 80%) on rates of net N mineralization and activities of three soil enzymes indicative of N release from organic matter. Principal component analysis of terminal restriction fragment length polymorphism (T-RFLP) also reflected these trends by highlighting the importance of plant roots in structuring the soil bacterial community, followed by plant species and N fertilization (to a minor extent). Overall, the results indicate minor contributions of short-term N fertilization to changes in the magnitude of rhizosphere effects for both grass species.

Keywords: rhizosphere effect, N fertilization, belowground carbon allocation, microbial biomass, extracellular enzyme, net N mineralization, microbial community composition

Received: 23 April 2014, Revised: 12 October 2014, Accepted: 27 October 2014

INTRODUCTION

Plants span a large area of soil that is impacted by the physical, chemical and biological properties of roots. This specific zone, termed the rhizosphere, is the narrow region of soil surrounding living roots. Ever since Lorenz Hiltner coined the term *rhizosphere* in 1904, numerous studies have reported higher microbial activity and different microbial community composition in the rhizosphere relative to the bulk soil (Cardon and Whitbeck 2007; Grayston *et al.* 1996; Lynch 1990). Such rhizosphere effects are mainly caused by root-derived substrate inputs (rhizodeposition), which may account for 17% of the carbon fixed by photosynthesis (Jones *et al.* 2004; Nguyen 2003). Moreover, root uptake of water and nutrients lead to changes in moisture dynamics and nutrient availability in the rhizosphere (Marschner *et al.* 1986).The rhizosphere thus functions as a hotspot of microbial activity and biogeochemical cycling in soils (Griffiths 1994; Pinton *et al.* 2007).

Greater attention has been given to belowground processes that impact ecosystem biogeochemistry; thus, 'rhizosphere effects' are being examined in detail. Plant rhizospheres generally have higher carbon availability (Cheng *et al.* 1996) and

higher biomass of soil microorganisms and fauna (Griffiths 1994) in comparison to bulk soils. The release of low molecular weight organic compounds such as organic acids from roots, and the uptake of anions or cations by roots, can change soil pH (Bardgett *et al.* 1999; Marschner *et al.* 1986) and microbial community composition (DeAngelis *et al.* 2009; Shi *et al.* 2011). The changes in microbial biomass and community composition can lead to further changes in microbial function in the rhizosphere (Schimel and Schaeffer 2012). Activities of extracellular enzymes for soil organic matter decomposition and nutrient cycling are often higher in the rhizosphere than in the bulk soil (Phillips and Fahey 2006; Priha *et al.* 1999), and microbial respiration and N mineralization tend to be higher in the rhizosphere (Kuzyakov 2002; Phillips and Fahey 2008; Zhu and Cheng 2011). These plant–microbe interactions in the rhizosphere have significant implications for soil carbon storage and nutrient availability to plants (Cheng *et al.* 2014; Grayston *et al.* 1996; Kuzyakov 2002; Sun *et al.* 2014).

Because rhizodeposition is the main driver of rhizosphere effects (Kuzyakov 2002; Paterson 2003), factors that regulate rhizosphere C fluxes may control the magnitude of rhizosphere effects (Jones *et al.* 2004). Higher soil fertility due to N fertilization may reduce relative belowground C allocation (Giardina *et al.* 2004; Phillips and Fahey 2007) and thus lead to lower rhizosphere effects (Ai *et al.* 2012; Blagodatskaya *et al.* 2014; Fontaine *et al.* 2011; Liljeroth *et al.* 1994; Phillips and Fahey 2008). However, neutral (Cheng *et al.* 2003) and even positive (Phillips and Fahey 2008) responses of rhizosphere effects to N fertilization have also been reported. These inconsistent results may be caused by differences in plant species and mycorrhizal association, soil type, the amount and chemical composition of fertilizers, and the timing and duration of fertilization in different studies. To better predict rhizosphere effects and to incorporate them into ecosystem process models (e.g. Perveen *et al.* 2014), we need more mechanistic studies of rhizosphere effects and their responses to environmental factors such as N addition.

In this study, we focus on grasses to better understand how grass rhizospheres impact soil processes under different N levels. Analysis of grass rhizospheres tend to be conducted in the field without a root exclusion treatment. In this study, we examined rhizosphere effects in a controlled growth chamber facility and assessed the role of N fertilization in modifying the responses. Nitrogen (N) addition can restrain a plant's ability to influence soil processes *via* its suppression on rhizosphere effects in soil. We hypothesized that N addition reduces the magnitude of rhizosphere effects including microbial biomass, microbial extracellular enzyme activity, N mineralization rates (N_{min}) and microbial community composition. To test this hypothesis, we grew two grass species in nutrient-poor forest soil receiving either low or high N addition from seed to flowering (~80 days) in a growth chamber. We measured plant biomass and a number of soil variables (pH, microbial biomass C and N, extracellular enzyme activity, N_{min} and bacterial community composition) in soils that were occupied by the plants (rhizosphere soil) or kept unplanted (bulk soil). Previous studies of rhizosphere effects focused on trees, crops and unmanaged natural grassland species, whereas these two grasses, annual smooth crabgrass *(Digitaria ischaemum* Schreb.) and bermudagrass *(Cynodon dactylon* L.), are important introduced species naturalized in urban and rural landscapes in North America. Here, we report the rhizosphere effects of grasses in comparison to results found in other plant species.

MATERIALS AND METHODS

Experimental design

The experiment was conducted in the Weil Hall controlled environment facility at Cornell University. We grew two grass species, annual smooth crabgrass *(D. ischaemum* Schreb.) and bermudagrass *(C. dactylon* L.). These species have been introduced to North America and are now naturalized in urban and rural landscapes. We collected surface (0–20 cm A-horizon, with little O-horizon) mineral soils (silt loam, Inceptisols) from a deciduous forest dominated by sugar maple (*Acer saccharum*) near Mount Pleasant farm of Cornell University, New York. Most urban grasslands are established on former forest and agricultural lands (Raciti *et al.* 2011). The soil has a pH of 4.61, 30.4 mg g^{-1} organic C and 2.3 mg g^{-1} total N. We sieved the soils through a 4-mm screen and filled 24 polyvinyl chloride pots (diameter 8 cm, height 32 cm) with 2146-g soil at a bulk density of 1.42 g cm^{-3}. Each pot was closed at the bottom with a rubber stopper and has an air inlet and an air outlet consisting of clear plastic tubing. We adjusted soil moisture content in each pot to 70% field capacity and pre-incubated the soils at room temperature (22°C) for 30 days. This pre-incubation is to minimize the potential impact of initial disturbance (e.g. sieving, packing, rewetting) on measured soil variables, as is commonly used in such experiments (Zhu and Cheng 2012).

All 24 pots were then moved to a growth chamber with 14-h light period (800 μmol m^{-2} s^{-1}, 25°C, 60% relative humidity, 400 p.p.m. CO_2) and 10-h dark period (20°C). Eight pots were planted with bermudagrass seeds (100 seeds per pot), eight pots were planted with crabgrass seeds (20 seeds per pot) and the other eight pots were kept unplanted. Both plants germinated within 2 weeks. Half of pots in each treatment (n = 4) received 20 (low N addition) and 120 (high N addition) μg N g $soil^{-1}$ as NH_4NO_3 solution during the first 2 weeks after germination. The low N addition was used to avoid severe nutrient deficiency of the plants growing in soils that were poor in mineral nutrients (<2 μg g^{-1} extractable N and P). The high N addition level reflected a typical fertilization concentration added to urban grasslands. The experiment is a complete factorial design with two main factors (planting and N addition) and four replicates. Soil moisture content was maintained at 70% field capacity (or 0.25 g water g^{-1} dry soil) throughout the experimental period by periodic weighing and watering with deionized water. Anaerobic conditions were avoided by circulating ambient air through each pot for

30 min twice a day using an aquarium pump. We randomly relocated the pots once a week to ensure similar growing conditions for the plants.

Measurements

All pots were destructively harvested after ~80 days of growth after germination. Both plants were at full flowering stage. Shoots were clipped at the soil surface for each planted pot, roots were carefully picked from each pot and then soils from both planted and unplanted pots were homogenized and subsampled for the following analyses.

Harvested plant shoots and roots were washed with deionized water and dried at 60°C to constant weight. Ground plant samples were processed for C (mg g^{-1}) and N (mg g^{-1}) on a Vario EL III elemental analyzer (GmbH, Hanau, Germany). Soil pH was measured using 10-g soil well mixed in 40-ml water.

Potential N_{min} were determined by extracting NH_4^+ and NO_3^- from soils before and after 10-day aerobic incubation at 22°C. Pre- and post-incubation soils (20 g) were extracted with 2 M KCl (50 ml), shaken for 1 h, filtered and frozen until analysis on a SEAL AQ2 analyzer (Seal Analytical Inc., Maquon, WI, USA). N_{min} was calculated as the change in extractable NH_4^+ and NO_3^- before and after the 10-day incubation (Phillips and Fahey 2006).

Microbial biomass C and N were measured by chloroform fumigation–extraction method (Wu *et al.* 1990). One subsample (20 g) was extracted with 50-ml 0.05 M K_2SO_4 solution, another subsample (20 g) was fumigated by ethanol–free chloroform in the dark for 48 h and then extracted with 50-ml 0.05 M K_2SO_4 solution. The concentration of total organic C and total N in each extract was analyzed using a Shimadzu TOC-V analyzer (Shimadzu Scientific Instruments, Columbia, MD, USA). Microbial biomass C and N were calculated as the difference between fumigated and unfumigated samples, adjusted by a proportionality coefficient (0.45) for both C and N (Jenkinson *et al.* 2004).

Potential activities of five hydrolytic enzymes (β-xylosidase, β-glucosidase, β-cellobiohydrolase, *N*-acetyl glucosaminidase [NAG] and leucine aminopeptidase [LAP]) were measured using 4-methylumbelliferone- (MUB) and 7-amino-4-methylcoumarin-(AMC) labeled substrates (200 μM), and potential activities of an oxidative enzyme (peroxidase) were assayed using L-3,4-dihydroxyphenylalanine (DOPA, 25 mM) substrate using protocols modified from previous studies (German *et al.* 2011; Saiya-Cork *et al.* 2002). Briefly, soil slurries were prepared by mixing 3 g of soil in 150-ml sodium acetate buffer (pH = 5.0, 50 mM) using a blender for 1 min. Hydrolytic enzyme assays were conducted in black 96-well microplates using a standard curve (soil slurry + MUB or AMC standard of 0, 2.5, 5, 10, 25, 50, 100 μ*M*) for each soil sample to minimize quenching effect. We pipetted 200 μl of soil slurry and 50 μl of MUB or AMC standards into wells of standard plate, and 200 μl of soil slurry and 50 μl of appropriate substrates into wells of substrate plate. We placed these plates in a dark incubator at 25°C for 3 h and measured fluorescence using a Synergy HT micromode microplate reader (BioTek Instruments, Winooski, VT, USA) with excitation wavelength at 365 nm and emission wavelength at 450 nm. Moreover, oxidative enzyme (peroxidase) assays were conducted in clear 96-well plates. In each plate, all columns (250-μl buffer, 200-μl buffer + 50-μl DOPA, 200-μl slurry + 50-μl buffer, 200-μl slurry + 50-μl DOPA) received 10-μl 0.3% H_2O_2 solution. We placed these plates in a dark incubator at 25°C for 24 h and measured absorbance at 460 nm using the same BioTek microplate reader. Phenol oxidase activity was undetectable in these soils. The activities were calculated based on equations shown in previous work (e.g. German *et al.* 2011; Saiya-Cork *et al.* 2002).

Bacterial community structure was characterized using terminal restriction fragment length polymorphism (T-RFLP). DNA was extracted from soil samples using the PowerSoil DNA isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA). For PCR, 50-μl reactions consisted of 10 μl of 5× GoTaq buffer (Promega Corporation, Middleton, WI, USA), 5 μl of 25-mM $MgCl_2$, 1.0 μl of 10-mM dNTP mix (Promega Corporation), 7.5 μl of 10-μM primer 8F, 2.5 μl of 10-μM primer 1492r, 0.6 μl of Go *Taq* enzyme (Promega Corporation), 1 μl of DNA template and water. The thermocycler settings were programmed as follows: 95°C for 3 min, 35 cycles of (95°C 30 s, 50°C 30 s and 72°C 45 s), final 72°C 12 min and cooled at 12°C. PCR products were cleaned using the QIAEX II gel extraction kit (Qiagen N.V., Netherlands) protocol for desalting and concentrating DNA solutions. The cleaned PCR products (400 ng) were digested in 50-μl reactions with 0.85 μl of HaeIII enzyme and 5 μl of 10× New England Biolabs buffer. The thermocycler program for restriction enzyme digestion was as follows: 6 h at 37°C, 20 min at 80°C, hold at 4°C. The digested samples were then added to 9.85 μl of Hi-Di formamide with 0.15 μl of Liz500 standard and analyzed by the Cornell Life Sciences Core Laboratory. The T-RFLP relative peak area profiles were analyzed using T-REX software (Culman *et al.* 2009).

Statistical analyses

Rhizosphere effect was calculated as the percent difference between rhizosphere soil (a homogenous sample from the planted pots after picking roots) and bulk soil (a homogenous sample from the unplanted pots) samples for each variable (Phillips and Fahey 2006; Zhu and Cheng 2011) under the same N treatment. A positive (or negative) rhizosphere effect means the variable is higher (or lower) in the rhizosphere than in the bulk soil. Independent samples *t*-test was used to compare each variable between rhizosphere soil and bulk soil to show the statistical significance of the calculated rhizosphere effect (Fig. 2). It was also used to compare each plant and soil variable between low and high N additions under each of the plant treatment (Fig. 1). For each plant species separately, we used two-way analysis of variance to assess the main effect of rhizosphere (planted vs. unplanted), the main effect of N addition (low N vs. high N) and their interaction on the soil variables (Table 2). For

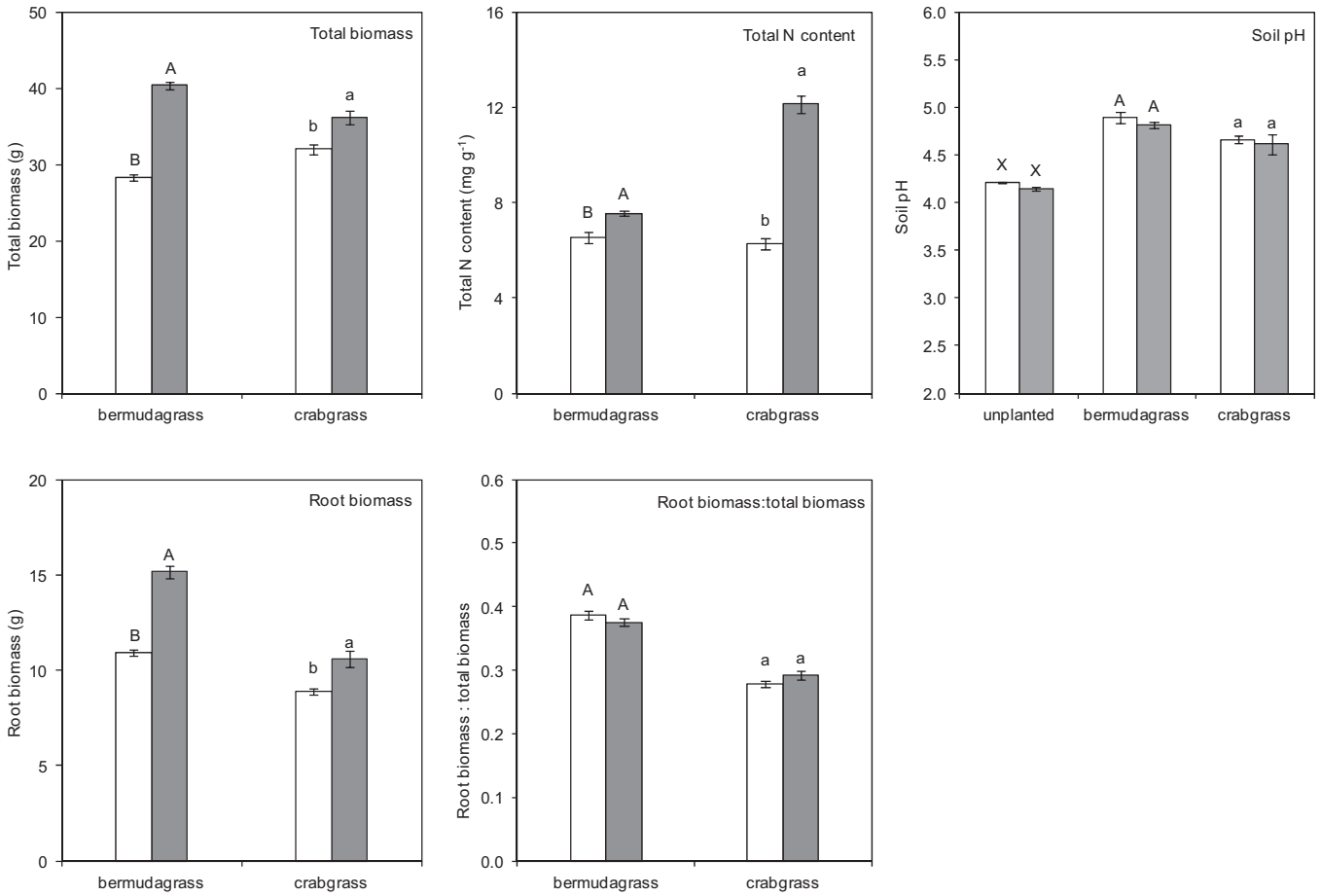

Figure 1: total and root biomass (g pot^{-1}), biomass-weighted total N content (mg g^{-1}), root biomass: total biomass ratio and soil pH (mean ± standard error, $n = 4$) measured after final harvesting. In each treatment (unplanted control, bermudagrass or crabgrass), bars with different letters are significantly different at $P < 0.05$ between low (white bars) and high N (gray bars) levels.

multivariate analysis of T-RFLP data, we performed principal component analysis on the standardized relative peak area of individual terminal restriction fragments (Fig. 3). All statistical analyses were done with PASW Statistics 18.

RESULTS

Total plant biomass ranged from 27.3 to 41.6 g pot^{-1} for bermudagrass and from 30.4 to 38.0 g pot^{-1} for crabgrass (Fig. 1), and root biomass was 10.5–16.0 and 8.6–11.8 g pot^{-1}, respectively. N addition significantly increased bermudagrass total biomass by 43% and crabgrass total biomass by 13% ($P < 0.05$, Fig. 1). Similar to total biomass, root biomass increased 39% in bermudagrass and 18% in crabgrass with N addition ($P < 0.05$, Fig. 1), whereas the root biomass: total biomass ratio was not significantly affected by N addition ($P > 0.05$, Fig. 1). Moreover, N addition strongly enhanced plant N content for both species ($P < 0.05$, Fig. 1). Compared to the low N treatment, biomass-weighted plant N content (mg g^{-1}) in the high N treatment increased 16% for bermudagrass and 93% for crabgrass ($P < 0.05$, Fig. 1).

Soil pH varied between 4.15 and 4.89 at the end of the experiment (Table 1). Compared to unplanted control soils, pH of the rhizosphere soils of bermudagrass and crabgrass were higher by 0.7 and 0.5 units, respectively ($P < 0.001$, Fig. 1). However, N addition did not affect pH of planted or unplanted soils ($P > 0.05$, Fig. 1).

Potential net N mineralization (N_{min}) was significantly higher in rhizosphere soils of both plants than in unplanted control soils ($P < 0.001$, Table 2). Rhizosphere effect on N_{min} was 42% (low N) and 39% (high N) for bermudagrass, and was 37% (low N) and 31% (high N) for crabgrass (Fig. 2). N addition decreased N_{min} to a similar extent between rhizosphere soil and bulk soil (Table 1). Therefore, N addition did not affect the rhizosphere effect of bermudagrass on N_{min} ($P > 0.10$, Fig. 2, Table 2), and only marginally inhibited the rhizosphere effect of crabgrass on N_{min} ($P = 0.08$, Fig. 2, Table 2).

Microbial biomass carbon (MBC) was significantly higher in rhizosphere soil than in bulk soil for both species ($P < 0.01$, Table 2) and was not affected by N addition ($P > 0.10$, Table 2). As a result, the positive rhizosphere effect on MBC was not affected by N addition ($P > 0.10$, Fig. 2, Table 2). In contrast, microbial biomass nitrogen (MBN) was significantly lower in rhizosphere soil than in bulk soil for both plants ($P < 0.01$, Table 2) and was not affected by N addition. The negative

Table 1: soil pH, N_{min} (μg N g soil^{-1} day^{-1}), MBC (μg C g soil^{-1}), MBN (μg N g soil^{-1}) and extracellular enzyme activities

			Microbial biomass		Extracellular enzyme activities					
Treatment	pH	N_{min}	MBC	MBN	BX	BG	CB	NAG	LAP	PER
Bermudagrass										
Low N	4.89 (0.06)	0.386 (0.005)	405.6 (3.2)	35.0 (0.4)	28.9 (1.2)	93.9 (4.6)	30.5 (1.4)	57.2 (2.4)	12.6 (0.3)	3.01 (0.10)
High N	4.81 (0.03)	0.348 (0.005)	417.8 (15.1)	36.5 (1.2)	27.3 (0.6)	95.6 (1.8)	28.2 (1.4)	52.5 (1.3)	10.9 (0.2)	2.46 (0.11)
Crabgrass										
Low N	4.66 (0.04)	0.372 (0.006)	408.9 (5.8)	36.0 (1.1)	30.3 (0.8)	105.3 (6.2)	33.4 (1.4)	57.9 (3.1)	11.8 (0.4)	2.67 (0.03)
High N	4.61 (0.10)	0.329 (0.005)	408.9 (4.6)	37.0 (1.3)	29.1 (1.6)	105.1 (4.2)	31.0 (1.5)	54.6 (1.2)	10.6 (0.2)	2.30 (0.07)
Unplanted										
Low N	4.21 (0.01)	0.272 (0.007)	373.0 (5.4)	42.0 (1.2)	27.2 (1.7)	83.5 (4.9)	26.4 (1.5)	35.1 (0.8)	9.8 (0.1)	2.17 (0.17)
High N	4.15 (0.02)	0.251 (0.005)	382.8 (5.6)	42.4 (0.7)	25.4 (1.8)	86.8 (3.7)	25.1 (2.3)	34.1 (2.4)	9.0 (0.2)	1.92 (0.12)

Abbreviations: BX = β-xylosidase; BG = β-glucosidase; CB = β-cellobiohydrolase; LAP, leucine aminopeptidase; NAG, β-1,4-*N*-acetyl-glucosaminidase; PER = peroxidase.
The five hydrolytic enzymes (nmol g soil^{-1} h^{-1}) include BX, BG, CB, NAG and LAP. The oxidative enzyme is PER (μmol g soil^{-1} h^{-1}). Values are the mean values of four replicates with standard errors in the brackets.

Table 2: two-way analysis of variance results of the main effects of rhizosphere (unplanted vs. planted), N (20 vs. 120 μg NH_4NO_3-N g soil^{-1}) and their interaction on each of the soil variables

	Bermudagrass			Crabgrass		
	Rhizosphere	N	Rhizosphere × N	Rhizosphere	N	Rhizosphere × N
pH	***	†	ns	***	ns	ns
N_{min}	***	***	ns	***	***	†
MBC	**	ns	ns	***	ns	ns
MBN	***	ns	ns	***	ns	ns
BX	ns	ns	ns	*	ns	ns
BG	*	ns	ns	**	ns	ns
CB	†	ns	ns	**	ns	ns
NAG	***	ns	ns	***	ns	ns
LAP	***	***	†	***	**	ns
PER	***	**	ns	**	*	ns

Abbreviations: BX = β-xylosidase; BG = β-glucosidase; CB = β-cellobiohydrolase; ns = not significant; LAP, leucine aminopeptidase; NAG, β-1,4-N-acetyl-glucosaminidase; PER = peroxidase.
The analyses were conducted for each species separately. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, †$P < 0.10$ and ns $P > 0.10$.

rhizosphere effect on MBN was not affected by N addition ($P > 0.10$, Fig. 2, Table 2).

The three extracellular enzymes involved in microbial degradation of soil organic carbon (i.e. β-xylosidase for hemicellulose degradation, β-glucosidase, and β-cellobiohydrolase for cellulose degradation) showed similar responses to rhizosphere effect and N addition. The presence of plants had minor effect on activities of these three enzymes compared to the unplanted control treatment, except that crabgrass showed positive rhizosphere effect on β-glucosidase (Fig. 2, Table 2). N addition did not significantly impact activities of these three enzymes (Table 2), and there was no interaction between rhizosphere and N on these enzymes (Fig. 2, Table 2).

The three extracellular enzymes involved in microbial degradation of soil organic N (i.e. NAG for chitin degradation, LAP for protein degradation and peroxidase for lignin degradation) were significantly higher in the rhizosphere soil of both plants than in the unplanted control soil ($P < 0.01$, Fig. 2, Table 2). The positive rhizosphere effect was stronger for NAG (54–65%) than for LAP (18–28%) and peroxidase (20–39%, Fig. 2). In addition, N addition significantly inhibited LAP and peroxidase activity ($P < 0.05$), but it had no effect on NAG activity ($P > 0.10$, Table 2). For all three enzymes and both plant species, there was no interaction between rhizosphere and N (Table 2), suggesting that the positive rhizosphere effects on these three enzyme activities were not responsive to N addition (Fig. 2).

Soil bacterial community composition was characterized by the T-RFLP approach (Fig. 3). Clearly, the presence of plants altered soil bacterial community composition, and this rhizosphere effect was stronger in bermudagrass than in crabgrass. However, N addition had minor effect on soil bacteria

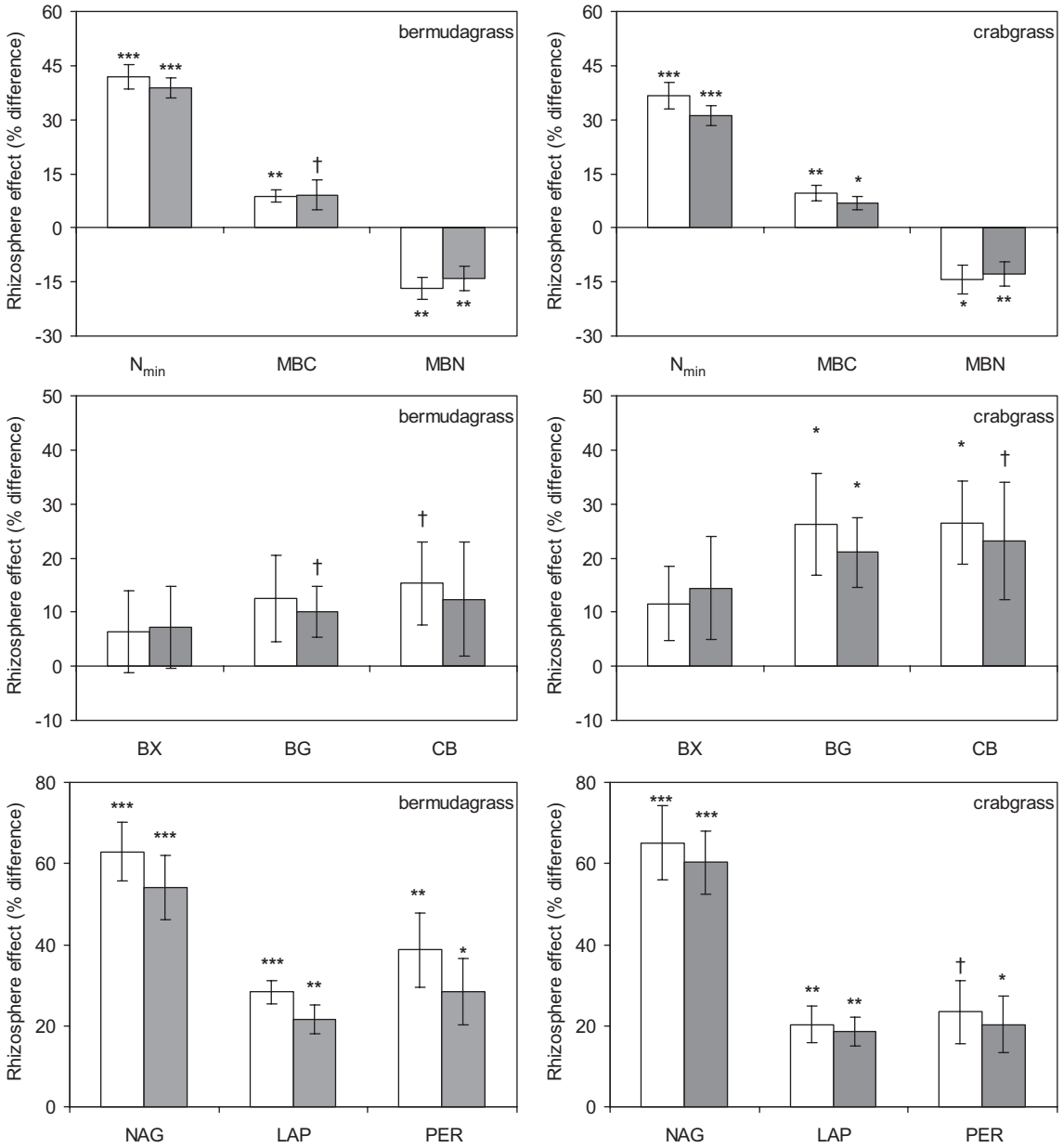

Figure 2: rhizosphere effects (mean ± standard error, $n = 4$) for N_{min}, MBC, MBN and activities of six extracellular enzymes (BX, BG, CB, NAG, LAP and PER). The symbols above each bar indicate significant differences in the variable between rhizosphere soil and bulk soil (i.e. the rhizosphere effect is significant): $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ and $^{\dagger}P < 0.10$. White and gray bars indicate low and high N treatments, respectively. BX, β-xylosidase; BG, β-glucosidase; CB, β-cellobiohydrolase; LAP, leucine aminopeptidase; NAG, β-1,4-*N*-acetyl-glucosaminidase; PER, peroxidase.

community composition, although the N effect appeared to be stronger in the rhizosphere of both plants than in the unplanted control soil (Fig. 3).

DISCUSSION

We had hypothesized that the presence of plants would enhance microbial biomass and enzyme activity and lead to faster soil C and N cycling in the rhizosphere, but the magnitude of rhizosphere effects would be reduced by N addition due to reduced belowground C supply. In fact, the results of this study showed that rhizosphere effects were mostly positive (except microbial biomass N) and significant (except C-degrading enzyme activities). N addition stimulated plant growth, suppressed net N mineralization and N-degrading enzyme activities, but had no significant impact on other soil variables. In contrast to our hypothesis, rhizosphere effects were not affected by the type, amount and duration of N addition (i.e. 100-μg NH_4NO_3-N g dry soil^{-1} for 80 days) in this study. We also detected shifts in soil bacterial community

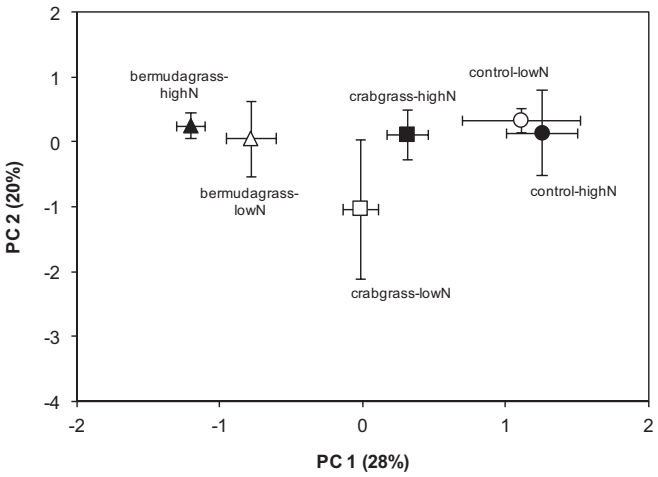

Figure 3: principle component analysis of bacterial T-RFLP profiles (means ± standard error, n = 3 or 4) of bermudagrass rhizosphere soils (triangles), crabgrass rhizosphere soils (squares) and unplanted bulk soils (circles). Empty and filled symbols indicate low and high N treatments, respectively. The first and second principal components account for 48% of the variability.

composition in the rhizosphere compared to bulk soil, but not with N addition. Taken together, these results suggested that rhizosphere effects of the two grasses on soil microbial biomass, activity and composition are minimally influenced by N addition.

Rhizosphere effects

In this study, we sampled rhizosphere soils in pots fully occupied by living roots of two grass species and bulk soils in unplanted control pots maintained at the same temperature and moisture levels. Many operationally defined methods have been used to sample a representative 'rhizosphere soil' versus a 'bulk soil' in order to study rhizosphere effect (Luster *et al.* 2009). For example, the commonly used methods include separating soil at difference distance from living roots (e.g. Cheng *et al.* 1996; Herman *et al.* 2006; Schenck zu Schweinsberg-Mickan *et al.* 2012), gentle shaking of freshly sampled soil attached (or not attached) to living roots (e.g. Phillips and Fahey 2006; Yin *et al.* 2012, 2014) and homogenizing soils from pots occupied by living roots or remained unplanted (Bardgett *et al.* 1999; Cheng *et al.* 2003; Zhu *et al.* 2014). We defined homogenized soils from the whole volume of planted pots after removing roots (but returning root-attached soils) as rhizosphere soils, which may dilute the intensity of rhizosphere effect as roots may not have fully occupied the soil volume. Therefore, the actual magnitude of rhizosphere effect in the "hotspot" may be stronger than reported here.

In general, the rhizosphere effects were significant and similar between the two grass species. Soil pH was 0.5–0.7 units higher in the rhizosphere compared to bulk soil, consistent with the results of some previous studies (e.g. Bardgett *et al.* 1999; Steer and Harris 2000). Root-induced changes in soil pH are affected by the relative uptake of anions (e.g. NO_3^-) versus cations (e.g. NH_4^+) by roots (Marschner *et al.* 1986). The lack of NO_3^- in the rhizosphere soil compared to bulk soil (0.6–0.8 versus 88.6 μg N g soil^{-1}) suggests that the release of OH^- or HCO_3^- during NO_3^- uptake and reduction may be one of the factors leading to the rise in rhizosphere pH (Steer and Harris 2000).

Microbial biomass C was 7–10% higher in the rhizosphere in this study. Generally, microorganisms are more C limited in the bulk soil compared to the rhizosphere (Cheng *et al.* 1996). Therefore, root-derived carbon input can increase microbial growth and biomass in the rhizosphere as shown in many studies (e.g. Griffiths *et al.* 1999; Phillips and Fahey 2006). Microbial biomass N, however, was 13–17% lower in the rhizosphere. This result is consistent with some previous studies (e.g. Zhu and Cheng 2012), but not with others (e.g. Herman *et al.* 2006; Phillips and Fahey 2008; Schenck zu Schweinsberg-Mickan *et al.* 2012). The lower microbial biomass N and lack of mineral N (ammonium and nitrate) in the rhizosphere at the end of this experiment suggest that the grasses may have outcompeted the microorganisms for the N mineralized from soil organic matter. Although microorganisms often outcompete plants for mineral N in the short term (hours and days, Jackson *et al.* 1989), plants can compete effectively for N with microorganisms in the long term (weeks and months) because of longer turnover time of roots (Frank and Groffman 2009; Kuzyakov and Xu 2013). Moreover, the higher microbial biomass C:N ratio in the rhizosphere may imply that the community was more dominated by microbial groups with high C:N ratios (e.g. fungi, Strickland and Rousk 2010). Although we did not quantify the relative proportion of fungi versus bacteria in this study, a few recent studies have provided evidence for higher relative abundance of fungi versus bacteria in soils receiving high-substrate loading rate (Griffiths *et al.* 1999) or in rhizosphere soils (Ai *et al.* 2012; Bardgett *et al.* 1999).

The activities of three hydrolytic enzymes degrading soil hemi-cellulose and cellulose showed minor responses to rhizosphere effect (6–27%, mostly not significant). In contrast, many studies have reported higher activities of these enzymes in the rhizosphere of trees and non-woody plants (Ai *et al.* 2012; Kaiser *et al.* 2010), and these higher enzyme activities may be linked to higher rates of microbial decomposition of soil organic matter in the rhizosphere (so-called 'rhizosphere priming effect', Cheng *et al.* 2014; Kuzyakov 2002; Sun *et al.* 2014). Further studies by labeling plants with enriched or depleted ^{13}C-CO_2 (e.g. Zhu and Cheng 2011) may help to understand whether the living roots of these two grasses enhanced microbial decomposition of soil organic matter.

In contrast to the three C-degrading enzymes, the three enzymes for N acquisition were significantly higher in the rhizosphere. The presence of roots of both plants elevated NAG activity by 54–65% and LAP and peroxidase activities by 18–39%, respectively. These results are consistent with the idea that root C inputs induce microorganisms to produce N-degrading extracellular enzymes to mine N from N-rich soil organic matter, particularly when soil mineral N

concentration is low (Fontaine *et al.* 2011; Kuzyakov 2002; Phillips and Fahey 2006; Zhu *et al.* 2014). We also found that potential N_{min} was 31–42% higher in the rhizosphere compared to the bulk soil, generally consistent with the higher activities of N-acquisition enzymes. The positive rhizosphere effects on microbial N-acquisition enzymes (chitin-, protein- and lignin-degrading enzymes) and gross or net N mineralization have also been observed in studies that sampled rhizosphere soils attached to living roots (e.g. Herman *et al.* 2006; Phillips and Fahey 2006; Yin *et al.* 2012), or compared planted versus unplanted soils (e.g. Cheng 2009; Dijkstra *et al.* 2009; Zhu *et al.* 2014).

We also detected shifts in soil bacterial community composition in the rhizosphere compared to the bulk soil based on T-RFLP analysis. Changes in bacterial community composition between rhizosphere and bulk soil have been shown using many microbial fingerprinting techniques (e.g. DeAngelis *et al.* 2009; Marschner *et al.* 2004; Peiffer *et al.* 2013). The shifts in bacterial community composition between rhizosphere and bulk soil may be associated with the changes in soil pH (and many other factors such as the quantity and quality of substrates, predation pressure), which has been identified as an important controlling factor of soil bacterial and fungal community composition (Lauber *et al.* 2009; Rousk *et al.* 2010). Although our T-RFLP results cannot give phylogenetic information of specific bacterial groups that changed in the rhizosphere, the shifts in bacterial (DeAngelis *et al.* 2009) and potentially fungal (Mouhamadou *et al.* 2013) community composition may contribute to the changes in enzyme activities and N_{min}. Further work should use more integrated metagenomic and functional analyses of soil microbial communities (e.g. Fierer *et al.* 2012; Zhou *et al.* 2012) to better link microbial community composition to function in the rhizosphere (Schimel and Schaeffer 2012).

N effects

N addition (100-μg NH_4NO_3-N g $soil^{-1}$) increased soil N availability, and not surprisingly, stimulated plant biomass (13–43%) and tissue N concentration (16–93%), which was expected in this nutrient-limited (<2 μg g^{-1} extractable N and P) soils. In contrast to the increased plant biomass, many soil variables were not responsive to or even suppressed by N addition. Soil pH, microbial biomass C and N, activities of three C-degrading enzymes and an N-degrading enzyme (NAG) were not significantly impacted by N addition, whereas rate of potential net N mineralization (N_{min}) and activities of two N-degrading enzymes (LAP and peroxidase) were inhibited by N addition. The lack of impact of N addition on soil pH, microbial biomass and C-degrading enzyme activities is unexpected given that N fertilization often reduces microbial biomass (Phillips and Fahey 2007; Treseder 2008) and increases microbial enzymes degrading hemi-cellulose and cellulose (Keeler *et al.* 2009; Saiya-Cork *et al.* 2002). The relatively short duration of the experiment (~80 days) and medium amount of N addition (100-μg NH_4NO_3-N g $soil^{-1}$) may prevent us from detecting statistically significant effects (Bardgett *et al.* 1999), and other nutrients (e.g. phosphorous) may be more limiting to these soil variables than N (Vitousek *et al.* 2010). Moreover, the suppression of N_{min} and microbial N-acquisition enzyme activities by N addition is consistent with the optimization theory that microorganisms reduce allocation to N-acquisition enzymes when there is freely available N in the soil (Allison and Vitousek 2005).

Although rhizosphere effects were mostly significant and positive for the soil variables measured in this study, they were not significantly affected by N addition. A few studies have reported inconsistent results on the influence of N fertilization on rhizosphere effects in forest, grassland and agricultural soils. Phillips and Fahey (2008) showed that N (as NO_3^-, and together with other micronutrients P, K, Ca and Mg) fertilization had positive, neutral or negative impact on rhizosphere effects, depending on the specific tree species and soil variables. Ai *et al.* (2012) reported that long-term addition of inorganic N fertilizers to a wheat–maize rotation field reduced rhizosphere effects on most extracellular enzyme activities. Perveen *et al.* (2014) showed that atmospheric N deposition suppressed rhizosphere priming effects using the SYMPHONY model. Liljeroth *et al.* (1994) found lower rhizosphere effects of wheat and maize on soil organic matter decomposition with N addition, whereas Cheng *et al.* (2003) did not find significant impact of N-P-K addition on rhizosphere effects of wheat and soybean. The impact of N fertilization on rhizosphere effects can be difficult to compare among different studies because many factors such as plant species, soil types, the amount and duration of N addition, and the chemical composition of fertilizers (e.g. N versus N-P-K, NH_4^+ versus NO_3^- versus urea) can all influence rhizosphere effects (Kuzyakov 2002). In this study, we added 100 μg of NH_4NO_3-N g $soil^{-1}$ over an 80-day experimental period. Root biomass: total biomass ratio was not significantly affected by N addition, whereas both root biomass and total biomass were increased by N addition to a similar extent for each species (39–43% for bermudagrass and 13–18% for crabgrass). Although we did not measure root-derived carbon inputs to soil (e.g. root exudates) in this study, belowground carbon allocation may not be significantly affected by the amount and duration of N addition in this study, which did not lead to a significant shift in rhizosphere effect (Cheng *et al.* 2003; Phillips and Fahey 2008).

In addition to microbial biomass, enzyme activities and N_{min}, we also measured soil bacterial community composition using T-RFLP technique. The presence of both grasses significantly shifted the bacterial community composition in the rhizosphere compared to unplanted bulk soil, whereas N fertilization had much lower impact on bacterial community composition. Although we could not quantify the rhizosphere effect for bacterial community composition as for other soil variables, it appears that N addition only had minor impact on soil bacterial community composition and its response to rhizosphere. Many studies have found shifts in soil bacterial and fungal community composition in response to N addition (e.g. Bardgett *et al.* 1999; Fierer *et al.* 2012; Frey *et al.* 2004; Marschner *et al.* 2004), but few studies (e.g. Mouhamadou *et al.* 2013; Zancarini *et al.* 2012)

have compared the N addition impact on soil microbial community composition between the bulk soil and the rhizosphere. Zancarini *et al.* (2012) found that N addition changed bacterial community composition in the rhizosphere of *Medicago truncatula* (but not in the bulk soil), whereas it had no significant impact on fungal community composition. Mouhamadou *et al.* (2013) detected significant differences in fungal community composition between rhizosphere and bulk soils within two grassland species (*Festuca paniculata* and *Dactylis glomerata*), but not between two N fertilization treatments. Although we did not measure soil fungal community composition in this study, the short-term N fertilization many not produce significant impact on fungal community composition (Mouhamadou *et al.* 2013; Zancarini *et al.* 2012).

CONCLUSIONS

In conclusion, our data suggest that rhizospheres of smooth crabgrass and bermudagrass had significant impacts on soil pH, microbial biomass, extracellular enzyme activities, N_{min} and bacterial community composition, whereas N fertilization suppressed N_{min} and two microbial N-acquisition enzyme activities and had no significant impact on other variables. Moreover, the direction and magnitude of rhizosphere effect was not significantly influenced by the type, amount and duration of N addition in this study. Taken together, these results suggest that rhizospheres of the two grasses exert a more important control of microbial community composition and function than soil fertility. If these results can be further generalized to more plant–soil combinations and to field conditions, they should improve our understanding of how plant–microbe interactions influence biogeochemical cycling.

FUNDING

United States Department of Agriculture National Institute of Food and Agriculture Hatch program (NYC-145403), the New York State Turfgrass Association and the US Department of Energy, Office of Science, Office of Biological and Environmental Research Terrestrial Ecosystem Science Program (DE-AC02-05CH11231).

ACKNOWLEDGEMENTS

We thank Sean Berthrong for help with T-RFLP methods and analysis, Guinevere Fredriksen for assistance with sample analysis and Sarah Carver, Stacey Lee, Lillian Aoki, Xiaoyun Gong, Michael Grome and Danielle Park for assistance with laboratory work. We also appreciate the insightful comments from two anonymous reviewers that improved this manuscript.

Conflict of interest statement. None declared.

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