Nitrogen fertilizer rate affects root exudation, the rhizosphere microbiome and nitrogen-use-efficiency of maize

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Abstract

The composition and function of microbial communities in the rhizosphere of crops has been linked to edaphic factors and root exudate composition. We examined the effect of N fertilizer (urea) rate on maize root exudation, the associated rhizosphere microbial community, and nitrogen-use-efficiency. Increasing N rate had a significant effect on root exudate quantity and composition. Specifically, the total abundance of sugars, sugar alcohols, and phenolics was positively correlated with N rate ($p < 0.005$). Similarly, the abundance of rhizosphere bacteria (16S rRNA copies g^{-1} soil FW) was enhanced with increasing N rate. Using PICRUST, we also explored the metagenomic contribution of bacterial OTUs to the abundance of N cycle-related genes in the maize rhizosphere. On a relative abundance basis, the nitrifying- (*pmoA-amoA*) and denitrifying-genes (*nirK* and *nosZ*) were significantly influenced by N rate ($p < 0.05$); whereas, the nitrogen fixing (*nifD* and *nifH*) and urease (*ureC*) genes were not influenced by N rate ($p > 0.05$). However, on a total abundance basis (gene copies g^{-1} soil FW) all N-cycle genes increased significantly with increasing N rate ($p < 0.05$). Percent N recovery from both soil and fertilizer sources showed a curvilinear response that was highest at intermediate N rates; whereas, fertilizer N lost from the system increased significantly at the two highest N rates ($p < 0.05$). In summary, our results show high N rates increase both root exudation and the abundance of soil bacteria, which may help explain the decline in fertilizer-use-efficiency and loss of N from the system at higher N rates.

Introduction

Maize (*Zea mays* L.) is one of the main crops widely used worldwide for food and bio-fuel. Increasing nitrogen (N) fertilizer applications has been a major management strategy to obtain high grain and biomass yields (Guo et al., 2010). However, N surplus combined with low N use efficiency have had negative consequences to the environment such as soil acidification, environmental pollution and decreased soil microbial activity (Chen et al., 2014; Guo et al., 2010; Ramirez et al., 2012). Thus, the reduction of N fertilizer inputs and improved N use efficiency are crucial for sustainable production of maize.

One alternative to reduce the application of N fertilizer could be the use of free-living N-fixing bacteria in maize agricultural systems (Cocking, 2003), which could improve crop production, reduce the overuse of synthetic fertilizers, and decrease greenhouse emissions (Kennedy et al. 2004). Benefits from N fixation have been demonstrated in sugarcane, rice, and graminaceous forage plants (App et al., 1984; Boddey and Victoria, 1986; Urquiaga et al., 1992). Many N-fixing bacteria have been reported in association with maize including *Azospirillum*, *Bradyrhizobium*, *Herbaspirillum*, *Ideonella*, *Klebsiella*, *Pantoea*, *Bacillus*, *Rhizobium etli*, *Burkholderia* and *Raoultella* (Chelius and Triplett, 2000; Palus et al, 1996; Di Cello et al., 1997, Perin et al, 2006; Dobbelaere et al., 2002; Roesch and Olivares, 2006; Roesch et al., 2008; Montanez and Sicardi, 2013). In addition, inoculation of maize with free living N-fixing bacteria has been shown to enhance crop yields through supplemental N input to the soil (Jacoud et al., 1998; Riggs et al, 2001; Sharma and Johri, 2003; de Garcia Salmone et al., 1996; Dobbelaere et al., 2002; Hungria et al., 2010).

The composition and function of microbial communities present in the rhizosphere can be affected by biotic and abiotic factors, including soil N content, soil type and host plant (Penanen et al., 1999). High levels of N-fertilization have been shown to negatively affect members of the diazotrophic community (Allison and Martiny, 2008, Roesch et al. 2008). For

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example, the activity of *Azospirillum lipoferum* and *Gluconacetobacter diazotrophicus* was inhibited by the supplementation of large quantities of N (Muthukumarasamy et al. 1999; Tsagou et al., 2003).

Other studies indicate that plants predominantly drive and shape the surrounding microbiome through the secretion of exudates that specifically stimulate or repress distinct microbial members of the soil (Haichar et al., 2008; Grayston *et al.*, 1998; Badri et al., 2013; Hartmann et al., 2009; Broeckling et al., 2008; Chaparro et al., 2012). Moreover, the secretion of root exudates is dependent on plant physiological status and nutrient availability. High nutrient availability favors the release of root exudates, whereas low nutrient availability restricts the allocation of plant resources towards root exudation (Neumann and Römheld, 2007). And, other studies have determined the effect of limited plant nutrition on root exudates and rhizosphere microbiology (Yang and Crowley, 2000; Liu et al., 2011). For example, maize secreted lower amounts of amino acids through roots under N deficiency, and these exudates suppressed numerous genes involved in protein synthesis in *Bacillus amyloliquefaciens* (Carvalhais et al., 2011, 2013). However, the effect of surplus N fertilization on root exudation and rhizosphere microbial communities in maize is not fully understood. Beginning to understand this complex process could reveal new methods for N application that would optimize the N balance through minimizing the losses of N and increasing microbe biological N fixation. In this study, we tested the effect of increasing levels of urea fertilization applied to maize on root exudation, the rhizosphere microbiome, and nitrogen-use-efficiency. We hypothesized that as N rates increase, root exudation will be modified along with (i) changes in microbial composition in the rhizosphere and a (ii) decrease in nutrient-use-efficiency.

Material and methods

Experimental design and growth conditions

Two separate experiments were designed to test the effect of increasing levels of urea fertilization applied to maize on root exudation and the rhizosphere microbiome as well as nitrogen-use-efficiency. For the rhizosphere microbiome and nitrogen-use-efficiency analysis, maize plants ($n = 15$) were grown at six different nitrogen (N) rates (0, 10, 30, 50, 80, and 160 mg N L⁻¹ soil). The N rates used in this study were determined based on a preliminary experiment that showed the biomass of corn was maximized between 50 and 80 mg N L⁻¹ soil (data not shown). The conversion of fertilizer rates between pot studies and field application rates is not a trivial matter; however, if we assume a plow layer of soil weighs 2000 metric tons per ha, then our highest N rate is approx. 320 kg ha⁻¹. Two independent trials were conducted for both the maize biomass and pyrosequencing analyses. Values for C and N analysis of shoot and soil in the present study were derived only from the second trial. For maize root exudate analysis, a separate trial was conducted with maize plants grown at four N rates (0, 30, 80, and 160 mg N L⁻¹). Each N rate was applied to 18 pots, the soil leachate from six replicate pots from each treatment were later pooled to generate three independent replicates per N rate. For all the above treatments, N was supplied as 90% urea (99%) and 10% urea-¹⁵N (99.7 atom %) to each pot (1 L soil pot⁻¹) as a single pre-planting application. The soil consisted of a mix of two parts sand (Play sand obtained from US Mix, Denver CO) and one part native soil collected from a Ponderosa pine forest near Young's Gulch, CO (pH 6.3; 1.4% organic matter; nutrient content: 2.6 mg/kg NH₄-N, 3.6 mg/kg NO₃-N, 3.0 mg/kg P, 136 mg/kg K, 1.1 mg/kg Zn, 16.7 mg/kg Fe, 2.5 mg/kg Mn, 1.3 mg/kg Cu). We selected a natural soil to rule out the potential interference of previous N fertilizer applications typical to agricultural soils. Two maize seeds [variety P1151HR (GMO) Pioneer] were sown in each pot. Once seedlings were established (~7 days) the plants were thinned to one plant per pot. All pots were placed into a rack in the greenhouse (12 h photoperiod with temperatures maintained below 35 °C) and rotated every week. Plants were irrigated once initially with 20 ml of

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full strength Hoagland's modified solution (Nitrogen-free medium) and then twice a week with water throughout the course of the experiment. When the maize plants reached the four leaf stage, they were sampled as described below.

Root exudates collection for GC-MS analysis

Root exudates were collected and prepared according to the method of Badri et al (2013) with a few modifications. Briefly, 200 ml of water was put into each pot and the flow-through was collected. A total of approx. 400 ml soil leachate was collected from each group (i.e., 6 pots) at the four leaf stage, similar to the biomass experiment above.

Root exudates were filtered by using 0.45 μm filter paper and 300 ml root exudates per replicate were freeze-dried into powder and weighed. The dried powder was dissolved in 5 ml of 80 % methanol and vortexed thoroughly to make sure that all powder dissolved completely. The solution was centrifuged at 8000 rpm for 15 minutes to pellet the undissolved particles; the clear liquid was transferred into new glass tubes and dried under nitrogen gas. Then, 1 ml of 80% methanol was added into the dried tube and vortexed thoroughly to dissolve all components and transferred into 1.5 ml Eppendorf tubes, which was centrifuged at 13,000 rpm for 15 minutes. Subsequently, the clear liquid was transferred into HPLC (brown) vials and dried under nitrogen gas. The samples were subjected to GC-MS analyses at the Genome Center Core Services, University of California Davis to identify the compounds present in the root exudates. Briefly, the root exudates were derivatized as described by Sana et al. (2010). A gas chromatograph (Agilent 6890, Santa Clara, CA) was used to run the samples controlled by Leco ChromaTOF software Version 2.32 (St. Joseph, MI). The resulting data files were exported to the data server with absolute spectra intensities and further processed by a filtering algorithm implemented in the metabolomics BinBase database (Skoger-son et al. 2011). Quantification was reported as peak height using the unique ion as default (Fiehn et al., 2008). Metabolites were unambiguously assigned by the BinBase identifier

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numbers using retention index and mass spectrum as the two most important identification criteria. Additional confidence criteria were used by giving mass spectral metadata using the combination of unique ions, apex ions, peak purity, and signal/noise ratios. All data entries in BinBase were matched against the Fiehn mass spectral library (<http://fiehnlab.ucdavis.edu/Metabolite-Library>). Data normalization was performed as described in Fiehn et al. (Fiehn et al., 2008) by using vector normalization.

For the GC-MS data of root exudates, we normalized the peak height of each compound with the sum of all peak heights for all identified metabolites for each sample. Relative peak heights were then used to calculate compound-specific concentrations ($\mu\text{g plant}^{-1}$) using the total exudate dry weights. Based on the chemical nature of the root exudates these were divided into seven categories (sugars, sugar alcohols, amines [amino acid, amino alcohols, etc.], carboxylic acids, polyols, phenolics, lipids [fatty acids, fatty alcohols, etc.], and others) with each value representing the sum of all compounds present in any given category. Significant differences in compound concentrations were analyzed using a one-way ANOVA using SigmaPlot 12.5 (Systat Inc.) and passed the default the default normality and homogeneity of variance (Wilks-Shapiro) tests performed by SigmaPlot.

Biomass, N analysis, and Microbial Community Assessment

Sampling

Biomass and soil microbial community assessment were done for both trials. For each trial, each plant ($n = 15$) was separated into above- (shoot) and below-ground (roots and rhizosphere soil) portions. Immediately after processing all samples were weighed to obtain fresh biomass. Samples were then placed into individual envelopes and dried at 60 °C for four days and reweighed to obtain dry biomass. In order to assess the microbial community, rhizosphere soils from each plant were collected by gently removing the plants from the pots and

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obtaining the soil attached to the roots. The rhizosphere soil from five plants were pooled into one sample and then stored at -20 °C. Each N rate treatment consisted of three samples. In order to obtain the necessary material for C and N analysis, five replicate shoot samples per treatment from the second trial were pooled and grounded to a fine powder. From each pot, a approx. 5 g bulk soil (0-10 cm depth) sample was also collected and pooled as described above.

C and N analysis

¹⁵N, C and N concentrations were determined for each pooled shoot ($n = 3$) and pooled bulk soil ($n = 3$) sample using a Costech ECS 4010 elemental analyzer (Costech Analytical Technologies, Valencia CA USA) coupled to a Delta V Advantage isotope ratio mass spectrometer (Thermo-Fisher Bremen, Germany).

qPCR and Pyrosequencing analysis

To characterize the soil microbial community affected by N rate, the rhizosphere soil sample (unground and stored at -20 °C) from both trials was analyzed separately. Total DNA was extracted from each rhizosphere sample ($n = 3$) by using a MoBio power soil DNA isolation kit (Mo Bio, Carlsbad, CA) according to the manufacturer's instructions. The DNA was quantified using a Nanodrop (Nanodrop Technologies, Wilmington, DE) spectrophotometer, and all DNA had an absorbance ratio (A_{260}/A_{280}) between 1.7 and 1.9. Quantitative PCR (qPCR) amplification of the bacterial 16S rRNA genes (V1-V3 hypervariable region) was performed with the 27F and 388R primers (Lane et al. 1985; Marchesi et al. 1998). Each reaction contained 2 μ l template DNA (diluted 1:20), 0.5 μ M of each primer, and 1X Maxima SYBRgreen master mix (cat # K0242, Thermo-Fisher Scientific); amplification was performed as follows: 1) 95 °C for 8.5 min, 2) 95 °C $\times$ 15 sec, 58 °C $\times$ 30 sec, 72 °C $\times$ 60 sec, repeated 35 times, 3) 72 °C $\times$ 5 min. Genomic DNA isolated from *Pseudomonas putida*

KT2440 was used as an external standard in order to calculate 16S rRNA copies per g soil FW extracted assuming a *P. putida* genome size of 3.174 fg and seven 16S rRNA copies per genome. qPCR efficiency was 93% and could detect as little as 100 *P. putida* genomes in a single PCR reaction. PCR amplicon products were purified using the Lonza FlashGel DNA Recovery System (Basel, Switzerland) and quantified using KAPA Biosystems qPCR kit following the manufacturer's instructions (Wilmington, Massachusetts). Amplified DNA samples were then pooled in equimolar ratios to create a single bacterial DNA library; the pooled library was re-quantified using the KAPA Biosystems qPCR kits and subjected to pyrosequencing at four copies per bead with the Roche GS Junior Sequencing System (Branford, Connecticut). All sequencing read editing and processing was performed with Mothur Ver. 1.32 (Schloss et al. 2009) using the default settings unless otherwise noted. Briefly, sequence reads were (i) trimmed (bdiff=0, pdiff=0, qaverage=25, minlength=100, maxambig=0, maxhomop=10); (ii) aligned to the bacterial-subset SILVA alignment available at the Mothur website (<http://www.mothur.org>); (iii) filtered to remove vertical gaps; (iv) screened for chimeras with UCHIME (Edgar et al. 2011); (v) classified using the RDP training set Vers. 9.75 and the naïve Bayesian classifier (Wang et al. 2007) embedded in Mothur, after which all sequences identified as chloroplast or mitochondria (<1% of the total sequences) were removed; (vi) sequences were screened (optimize=minlength-end, criteria=95) and filtered (vertical=T, trump=.) so that all sequences covered the same genetic space; and (vii) all sequences were pre-clustered (diff=2) to remove potential pyrosequencing noise and clustered (calc=onemap, coutends=F, method=average) into operational taxonomic units or OTUs (Huse et al. 2010). After processing, each 16S library rarefied to 1000 sequence reads in order to standardize sampling effort for each sample.

The abundance of nitrogen cycle genes, *nifD*, *nifH*, *ureC*, *nasA*, *nirA*, *pmoA-amoA*, *hao*, *nirK*, *norB*, *nosZ* (see Table 1 for more details), in each 16S library was predicted using PiC-

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RUST (Langille et al. 2013). Each edited and rarefied 16S library was (i) classified using the Green Genes database (Vers. 13_5_99) available from Mothur (classify.seqs, method=knn, numwants=1), (ii) normalized by copy number (PiCRUST: normalize_by_copy_number.py, and (iii) used to calculate metagenome contributions (PiCRUST: metagenome_contributions.py). Gene-specific relative abundances were calculated by dividing each gene count “CountContributedByOTU” by the total number of “normalized reads” for the sample of interest. Total gene-specific abundance (copies g⁻¹ soil FW) was calculated as the product of the relative abundance and the total 16S copies (copies g⁻¹ soil FW) determined from the initial sample qPCR.

Statistical analysis

Significant differences between different N rates on maize plant heights and biomass (root and shoot) and nitrogen content in shoots were analyzed using a one-way ANOVA using SigmaPlot (Systat Inc.). The normality and homogeneity of variances were tested prior to the one-way ANOVA. Nitrogen use efficiency (NUE) was calculated by the percentage recovery of fertilizer N with the equation from Varvel and Peterson (1990): $PFR = 100 \times [(NF) - (NC)] / R$, where PFR = percent fertilizer recovery, NF = total N uptake in corn from N fertilized pots, NC = total N uptake in corn from unfertilized pots, and R = rate of fertilizer N applied. The loss of fertilizer N was calculated by the equation: N lost = N applied – N uptake – N residual. The effect of N rate on bacterial community composition (16S rRNA clustered at 3% genetic distance) was visualized by partially constrained Principal Coordinates Analysis (capscale in R vegan package). Differences in nitrogen cycle related genes, *nifD*, *nifH*, *ureC*, *nasA*, *nirA*, *pmoA-amoA*, *hao*, *nirK*, *norB*, *nosZ*, in soil microbial communities was determined by the method of phylogenetic investigation of communities by reconstruction of unobserved states (PiCRUST) based on 16S input data (Langille et al., 2013). Two-way ANO-

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VA (SigmaPlot, Systat Inc.) was used to test the effects of trial and N rate on both relative and total N-cycle genes abundance.

Results

Effect of N rate on maize root exudates

The effect of the N application on the root exudates metabolome was analyzed by GC-MS. In total, we observed 124 putative compounds in the root exudates of all treatments. Among these, 75 compounds could be identified and were categorized based on their chemical nature into 14 sugars, 5 sugar alcohols, 7 amines, 21 carboxylic acids, 5 polyols, 5 phenolics, 15 lipids, and 3 others.

The total amounts of root exudates, obtained by soil leaching, increased with increasing N rates (Figure 1, Table 2). For the identifiable compounds, sugars increased approx. 11-fold (317 and 3459 $\mu\text{g plant}^{-1}$) and sugar alcohols increased approx. 30-fold (31 and 921 $\mu\text{g plant}^{-1}$) from the N0 to the N160 treatment. Phenolics increased approx. 7-fold (318 and 2327 $\mu\text{g plant}^{-1}$) from the N0 to the N160 treatment. No differences for the amines, carboxylic acids, polyols, or lipids were observed between the N0 and N160 rates. A total of 13 sugars, 4 sugar alcohols, 2 amines, 6 carboxylic acids, 3 polyols, 2 phenolics, and 5 lipids were significantly influenced by N rate (Table 2). In general, trends were consistent among all significantly changing compounds within a category. For example, of the 13 sugars and 4 sugar alcohols all significantly increased as N rates increased.

Effect of N rate on soil microbial composition

The influence of N application on maize rhizosphere soil microbial community was determined by 454 pyrosequencing analyses. Principal coordinates analysis (PCoA) indicated that the first two principal components accounted for 12.4 % of the total variation in the dataset (Figure 2). perMANOVA analysis showed that both trial ($p < 0.001$) and nitrogen fertilizer

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rate ($p = 0.006$) significantly altered soil microbial communities. Although both were significantly different, nearly twice as much of the variation in the microbial community was associated with trials (11.5%) as compared to N fertilizer rate (5.8%).

16S rRNA gene qPCR analysis demonstrated that total bacteria abundance significantly increased with N rate (from N50 to N160) compared with the control (Figure 3). Taxonomic analysis (order level) indicated that the relative abundance of Bacillales, Nitrosomonadales, and Rhodocyclales was significantly increased following the N rates (Table 3). In contrast, the relative abundance of Chloroflexales, TM7 (EW055), Gemmatimonadetes (N1423WL), and Phycisphaerae (WD2101) was significantly decreased with the increase of N rates. The relative abundance of Elusimicrobia (IIb) and Nitrospirales was significantly higher in the N80 treatment. The relative abundance of Elusimicrobiales and Cyanobacteria (MLE1-12) was significant higher in the N10 and N80 treatments, respectively.

The relationship between the relative or total abundances of N-cycle genes and N rate were predicted with the aid of PICRUSt. The relative abundance of *nirA*, *pmoA-amoA*, *nirK*, and *nosZ* significantly increased with N rate (Table 4); whereas, the total abundance of *ureC*, *nasA*, *nirA*, *pmoA-amoA*, *nirK*, *norB*, and *nosZ* increased with increasing N rates (Table 5). The total abundance of *nifD* and *nifH* was significantly increased at intermediate (N50 and N80) N rates.

Biomass

The height of maize was significantly augmented with the increase in N fertilization (from 10 to 80 mg/L) (Figure 4A, B). The fresh and dry biomass reached its maximum when maize was grown in pots supplied with N at a concentration of 80 mg/L, but decreased with N fertilization of 160 mg/L (Figure 4C, D). Significant differences in shoots and roots fresh and dry biomass were also found among the treatments (Figure 4C, D).

Effect of N rate on N-use efficiency

Nitrogen uptake by shoots significantly increased as N rate increase (Figure 5A). N uptake from the soil reached a maximum at the N50 rate; whereas, fertilizer-derived N increased with each successive N rate (Figure 5A). The total recovery of N by shoots from both soil- and fertilizer reached its highest level in the N50 treatment and decreased at higher N rates (Figure 5B). N recovery from soil was highest for the N50 treatment; whereas, N recovery from the fertilizer was highest in the N80 treatment (Figure 5B). There was not a significant build-up of the total C and N (data not show) in the soil with increasing N fertilization. We also calculated the amount of fertilizer N lost from the system ($N_{\text{applied}} - N_{\text{fert\_uptake}} - N_{\text{fert\_residual}}$). The amount of N lost increased significantly for the N80 and N160 treatments reaching nearly 30 mg pot⁻¹ or almost one-third of the amount of fertilizer applied (Figure 5C).

Discussion

Our main goal in this study was to explore the effect of N rate on maize root exudation and any associated changes in the microbial community; particularly those involved in the N cycle. We obtained root exudates using a soil leachate methodology from a soil with a biologically active microbial community. Although we use the term root exudates, we acknowledge that the soil leachate is ultimately derived from the combined activity of plant and microbial activity, which will consist of root exudates, microbial secretion, and other microbial activity (e.g., decomposition)..

It has been reported that the quantity and composition of root exudates is influenced by the nutritional status of a plant (Carvalhais et al., 2013; Baudoin et al., 2003; Haase et al., 2007). For example, under N-limited conditions a lower concentration of sugars was observed in bean plants (Haase et al., 2007), and lower concentrations of amino acids were secreted by roots of maize (Baudoin et al., 2003), pine (Bowen, 1969), potato (von Wirén et al.,

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2000) and bean (Haase et al., 2007). However, a systematic study comparing root exudation profiles for maize plants exposed to high N rates has not been reported. In this study, we found that the total amounts of maize root exudates increased with increasing N rates, especially the content of sugar, sugar alcohols, and phenolics.

Carbon-containing metabolites in plant root exudates have been shown to affect the rhizosphere microbiome (Berg and Smalla, 2009; Weller and Thomashow, 1994). In this study, increasing N fertilizer rates significantly increased the secretion of sugars, sugar alcohols, and phenolics by maize and resulted in a concomitant increase of bacteria in the rhizosphere.

Sugars and sugar alcohols serve as sources of energy for a broad range of microbes (Bera and Wagner, 1974) and also act as general chemotaxis substances (Badri et al., 2013; Baudoin et al., 2003; Eilers et al., 2010). In this study, we found that the relative abundance of Bacillales, Nitrosomonadales, and Rhodocyclales was correlated with higher N rates. Similarly, it has been reported that the abundance of Bacillales and Nitrosomonadales tend to increase when sugars are present (e.g., sugar-cane mills, Sharmin et al., 2013).

Phenolics are a dominant class of plant secondary metabolites (Hartley and Jones, 1997), which have been thought to serve as general antimicrobial compounds (de Weert, 2002; Rudrappa et al., 2008; Steinkellner et al., 2007; Zhang et al., 2009). In this study, the increase in phenolics correlated with a decreased abundance of Chloroflexales, EW055, Gemmatimonadetes, and Phycisphaerae. Overall, however, the effect of phenolics in this study appeared to have a negligible impact on total microbial abundance, which increased with N rate despite an increase in phenolic secretion. It is plausible that this is due to a rather limited phenolic composition in the root exudates (i.e., only five phenolic compounds were identified), of which, p-cresol was the most abundant. For example, p-cresol comprised approx. 88, 91, 93, and 94% of the phenolic content for the N0, N30, N80, and N160 treatments, respectively. The percent recovery of N in shoots was highest for the N50 treatment and decreased at high-

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er N rates. Previous studies have shown that nitrogen uptake efficiency depends on root biomass and function (Iversen et al., 2010; Pastor and Bridgham 1999). In this study, root biomass was significantly higher in the N80 treatment as compared to the N50 treatment, suggesting that the higher N recovery in the N50 treatment was not due to root biomass. Instead, we suggest that microbial immobilization of N, either by direct uptake of fertilizer N or through root exudate metabolism, have a significant impact on N uptake efficiency.

Most studies indicate that nitrate leaching from agricultural soils represents a substantial loss of fertilizer nitrogen (e.g., Lin et al., 2001). Nitrogen can readily leach out of the root zone in nitrate-N form, which can be moved downward freely with drainage (Lin et al., 2001). However, significant N leaching probably did not occur in the current pot study as watering was performed such that no drainage occurred from the pots. The denitrification of nitrate-N by microbes represents another potential N loss (Zumft, 1997) and subsequent decline in apparent plant N use efficiency. It has been well documented that N fertilizers promote ammonia oxidation and denitrification in soils (Mulvaney et al., 1997; Arcara et al., 1999). The community composition and specific N cycle gene changes observed in this study also suggest an increase in denitrification potential with increasing N rate. For example, we observed an increased abundance of Bacillales, and Rhodocyclales at high N rates; and some members of these orders have been reported to perform important steps in denitrification of soils (Hesselsoe et al., 2009; Segawa et al., 2014). Additionally, we observed an increase in the abundance of the Nitrosomonadales, which contains several genera (*Nitrosomonas*, *Nitrosospira*, *Nitrosovibrio*) capable of ammonia oxygenation. The mineralization of urea and subsequently nitrification should be mentioned, and discussed with the increases of *amoA* gene abundance. Consistent with the observed changes in taxonomy, the relative abundance of the nitrifying- (*pmoA-amoA*) and denitrifying-genes (*nirK* and *nosZ*), and the total abundance of the urea hydrolysis (*urea*), assimilatory nitrate reduction (*nasA* and *nirA*), nitrification

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(*pmoA-amoA*) and denitrification genes (*nirK*, *norB*, and *nosZ*) significantly increased with increasing N rate. The increase of these genes implies that the activity of ammonia-oxidizing and denitrification bacteria increased, which resulted in a greater loss of N fertilizer.

While it is clear from the above discussion that we observed significant changes in both root exudation and the abundance of several microbial taxa in response to N fertilizer rates our study design cannot separate the difference between direct and indirect effects of N fertilizer on microbial composition. For example, although our observations of specific root exudates and microbial taxa (indirect) are consistent with previous literature it is also possible that some of the changes in microbial taxa are directly associated with their ability to use urea as a C and/or N source. While the relative contribution of these two processes in determining microbial community structure is of interest and needs to be further explored using more controlled conditions (i.e., no plant controls, root exudate additions), the current design represents the effect of N rates under a more natural condition where both plant and microbe are allowed to interact freely.

Conclusion

The structure of rhizosphere microbial communities is the result of complex interactions between maize and N fertilization. When maize was supplied with increasing amounts of N, roots secreted more sugars, sugar alcohols, and phenolics, which subsequently altered the soil microbial community structure and abundance. In particular, high N rates appear to increase the activity of ammonia-oxidizing and denitrification bacteria leading to a decrease in nitrogen-use-efficiency. In summary, our results suggest that N has the ability to modify the composition and abundance of root exudates and to subsequently affect the rhizosphere microbial communities. As a result, excessive N fertilizer does not appear to benefit maize through the recruitment of beneficial microbes or the deterrence of undesirable microbes, but instead appears to lead to an overall increase in microbial abundance. Based on the current study, we

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suggest that it may be possible to identify maize cultivars that have reduced root exudation at high N rates resulting in increased NUE and crop yields by (i) reducing C and N losses through root exudation and (ii) minimizing N immobilization or N losses (e.g., denitrification) by soil microbes.

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Figure Captions

Figure 1. Root exudate compounds ($\mu\text{g plant}^{-1}$) of maize plants grown under different N rates (mg L^{-1}) and summed by chemical class.

Figure 2. Partially constrained Principal Coordinates Analysis depicting the effect of N rate on soil bacteria community composition. Analysis was conducted using Bray-Curtis distances from OTUs defined at a 3% genetic distance and subsampling to 1000 reads per sample. Symbols: Trial 1 (squares), Trial 2 (circles); N0 (black), N10 (red), N30 (green), N50 (blue), N80 (cyan), N160 (magenta). Ellipses show 95% confidence intervals of the standard deviation for each trial/N rate combination.

Figure 3. Effect of N rate on total bacteria abundance ($\times 10^8$ 16S rRNA copies g^{-1} soil FW) determined by 16S rRNA gene quantitative PCR. Bars with different letters are significantly different (Holm-Sidak post-hoc test, $p < 0.05$).

Figure 4. Biomass in maize (four-leaf stage) grown under increasing N fertilizer rates. **A)** Representative plants at the four leaf stage, **B)** Average heights (cm), **C)** Fresh biomass (g plant^{-1}) separated into shoot (gray) and root (white), **D)** Dry biomass (g plant^{-1}) separated into shoot (gray) and root (white). Bars are the LSmeans $\pm$ SE. Bars with different letters are significantly different (Holm-Sidak post-hoc test, $p < 0.05$).

Figure 5. Nitrogen uptake in maize (four-leaf stage) grown under increasing N fertilizer rates. **A)** Total nitrogen uptake (mg plant^{-1}) separated into fertilizer-derived (gray) and soil-derived (white) fractions, **B)** Total nitrogen recovery (%) separated into fertilizer-derived (gray) and soil-derived (white) fractions, **C)** Lost (unaccounted) fertilizer N (mg pot^{-1}). Lost fertilizer N = N applied – N uptake – N soil. Bars are the LSmeans $\pm$ SE. Bars with different letters are significantly different (Holm-Sidak post-hoc test, $p < 0.05$).

Table 1. KEGG orthologues selected for PICRUST analysis.

Process	Reaction	KEGG orthology		
		Entry	Gene	Definition
N-fixation	$\text{N}_2 \rightarrow \text{NH}_3$	K02586	<i>nifD</i>	nitrogenase molybdenum-iron protein α chain
		K02588	<i>nifH</i>	nitrogenase iron protein
Urea hydrolysis	$(\text{NH}_2)_2\text{CO} \rightarrow \text{NH}_3$	K01428	<i>ureC</i>	urease subunit alpha
ANR [†]	$\text{NO}_3^- \rightarrow \text{NO}_2^-$	K00372	<i>nasA</i>	Assimilatory nitrate reductase catalytic subunit
	$\text{NO}_2^- \rightarrow \text{NH}_3$	K00366	<i>nirA</i>	ferredoxin-nitrite reductase
Nitrification	$\text{NH}_3 \rightarrow \text{NH}_2\text{OH}$	K10944	<i>pmoA-amoA</i>	methane/ammonia monooxygenase subunit A
	$\text{NH}_2\text{OH} \rightarrow \text{NO}_2^-$	K10535	<i>hao</i>	hydroxylamine dehydrogenase
Denitrification	$\text{NO}_2^- \rightarrow \text{NO}^-$	K00368	<i>nirK</i>	nitrite reductase (NO-forming)
	$\text{NO}^- \rightarrow \text{N}_2\text{O}$	K04561	<i>norB</i>	nitric oxide reductase subunit B
	$\text{N}_2\text{O} \rightarrow \text{N}_2$	K00376	<i>nosZ</i>	nitrous-oxide reductase

[†]ANR – Assimilatory nitrate reduction

Table 2. Effect of N rate on root exudate compounds ($\mu\text{g plant}^{-1}$) obtained by soil leaching ($n = 3$). Only specific compounds with significant ANOVAs (Holm-Sidak post-hoc test, p -value < 0.05) are shown.

Category	Compound	N rate				<i>p</i> -value
		N0	N30	N80	N160	
Total dry weight	-	5566 b	4652 b	6433 ab	10799 a	0.010
Sugars	All sugars	317 b	167 b	344 b	3459 a	<0.001
	6-deoxyglucose	21 b	22 b	48 b	405 a	<0.001
	fructose	71 ab	8 b	17 b	862 a	0.005
	fucose	11 b	28 ab	56 a	53 a	0.009
	galactinol	2 b	5 b	12 ab	65 a	0.002
	galactose	108 b	13 c	24 c	1150 a	<0.001
	glucose	17 b	6 b	13 b	206 a	0.009
	glucose-1-phosphate	7 b	7 b	14 ab	36 a	0.005
	lactose	2 b	8 a	14 a	14 a	0.007
	levoglucosan	5 b	7 b	14 ab	34 a	0.007
	mannose	5 b	5 b	14 b	61 a	0.002
	sucrose	14 ab	6 b	21 ab	202 a	0.013
	threose	1 b	5 a	10 a	12 a	<0.001
	xylose	44 b	36 b	65 b	331 a	0.004
Sugar Alcohols	All sugar alcohols	31 b	31 b	64 b	921 a	<0.001
	erythritol	5 b	6 b	13 ab	35 a	0.002
	lyxitol	6 b	7 b	9 b	744 a	<0.001
	myo-inositol	5 b	5 ab	10 ab	34 a	0.039
	xylitol	12 b	6 b	21 ab	90 a	0.009
Amines (amino acids, amino alcohols, etc.)	All amines	125 a	63 a	92 a	161 a	0.333
	cyclohexamine	2 c	6 bc	15 b	70 a	<0.001
	glycine	2 b	7 ab	15 a	14 a	0.009
Carboxylic acids	All carboxylic acids	382 a	742 a	1116 a	799 a	0.202
	2-hydroxyvaleric acid	8 b	26 ab	44 a	61 a	0.007

	4-hydroxybutyric acid	3 b	6 ab	15 a	18 a	0.010
	azelaic acid	2 b	5 ab	12 a	12 a	0.010
	dihydroxymalonic acid NIST	2 b	5 ab	11 a	14 a	0.006
	phthalic acid	33 b	16 b	39 b	170 a	0.004
	quinic acid	3 b	7 ab	12 a	13 a	0.015
Polyols	All polyols	1945 a	458 b	928 ab	1595 a	0.006
	glycerol-3-galactoside	2 c	6 bc	8 b	43 a	<0.001
	phenylethanol NIST	4 b	11 ab	34 a	21 a	0.005
	stigmasterol	1 c	6 b	17 a	19 a	<0.001
Phenolics	All phenolics	348 b	1031 ab	2165 a	2327 a	0.003
	hydroquinone	3 b	17 ab	31 a	15 ab	0.014
	p-cresol	306 b	924 ab	2013 a	2199 a	0.002
Lipids (fatty acids, fatty alcohols, etc.)	All lipids	519 a	1115 a	1076 a	1312 a	0.319
	1-monopalmitin	13 b	25 ab	29 ab	35 a	0.021
	2-deoxyerythritol	8 b	10 b	17 ab	38 a	0.008
	2-monopalmitin	6 b	24 b	59 ab	73 a	0.016
	caprylic acid	6 b	15 ab	25 a	20 a	0.010
	palmitoleic acid	2 b	6 a	12 a	12 a	0.003

Table 3. Effect of N rates on Order relative abundance (count per 1000 reads) ($n = 3$). LSmeans with different letters are significantly different (Holm-Sidak post-hoc test, $p < 0.05$). For simplicity, only the LSmeans, p-values, and treatment groupings associated with N rate are shown.

Order	N rate						p-value
	N0	N10	N30	N50	N80	N160	
Bacillales	3.5 b	2.7 b	4.0 ab	3.5 a	2.0 a	5.5 a	0.043
Chloroflexales	1.3 a	0.5 b	0.3 b	0 b	1.0 a	0.3 b	0.037
TM7; EW055	4.0 a	0.7 b	0.8 b	0.8 b	0.5 b	0.5 b	<0.001
Elusimicrobiales	0 b	0.7 a	0.2 b	0 b	0.3 b	0 b	0.015
Elusimicrobia; IIb	0 b	0.2 b	0.2 b	0.5 b	1.0 a	0 b	0.011
Cyanobacteria; MLE1-12	0.8 b	0.8 b	0.8 b	2.5 a	1.3 b	0.2 b	0.045
Gemmatimonadetes; N1423WL	2.8 ab	4.0 a	1.5 bc	1.8 bc	2.0 bc	0.5 c	0.047
Nitrosomonadales	0.2 c	0.2 c	0.5 bc	1.0 bc	1.3 b	3.2 a	<0.001
Nitrospirales	1.8 b	0.3 b	2.7 ab	2.0 b	4.3 a	1.8 b	0.009
Rhodocyclales	0.7 b	0.5 b	0.3 b	0.5 b	0.8 b	3.5 a	<0.001
Phycisphaerae; WD2101	8.3 a	2.8 b	5.8 a	4.0 ab	5.5 ab	2.8 b	0.004

Table 4. Effect of N rate on relative abundance (%) of OTUs mapped to selected N cycle genes ($n = 3$). LSmeans with different letters are significantly different (Holm-Sidak post-hoc test, $p < 0.05$).

For simplicity, only the LSmeans and treatment groupings associated with N rate are shown.

N rate	<i>nifD</i>	<i>nifH</i>	<i>ureC</i>	<i>nasA</i>	<i>nirA</i>	<i>pmoA-amoA</i>	<i>hao</i>	<i>nirK</i>	<i>norB</i>	<i>nosZ</i>
0	17.8 a	17.7 a	35.8 a	44.5a	27.1 b	0.08 b	3.24 a	19.8 b	20.6 ab	9.49 b
10	16.6 a	16.4 a	36.3 a	42.6 a	29.9 ab	0.07 b	2.10 a	18.9 b	21.7 ab	9.18 b
30	16.1 a	16.2 a	35.6 a	39.9 a	32.0 a	0.23 b	3.27 a	17.7 b	18.1 b	7.96 b
50	19.3 a	19.2 a	36.2 a	46.1 a	26.8 ab	0.64 ab	3.30 a	20.7 ab	22.3 ab	10.6 ab
80	18.5 a	18.3 a	35.9 a	45.9 a	27.6 ab	0.81 ab	4.14 a	20.0 ab	22.7 ab	8.89 b
160	14.4 a	14.3 a	37.0 a	42.8 a	27.8 ab	1.44 a	3.93 a	24.7 a	25.3 a	12.6 a
Trial	<0.001	<0.001	<0.001	<0.001	<0.001	0.376	0.127	0.015	<0.001	0.196
N rate	0.096	0.103	0.977	0.271	0.022	<0.001	0.070	<0.001	0.008	0.001
Trial x N rate	0.337	0.419	0.494	0.208	0.157	0.168	0.209	0.198	0.069	0.017

Table 5. Effect of N rate on total abundance (gene copies g⁻¹ soil FW) of OTUs mapped to selected N cycle genes ($n = 3$). LSmeans with different letters are significantly different (Holm-Sidak post-hoc test, $p < 0.05$). For simplicity, only the LSmeans and treatment groupings associated with N rate are shown.

N rate	<i>nifD</i> (x10 ⁸)	<i>nifH</i> (x10 ⁸)	<i>ureC</i> (x10 ⁸)	<i>nasA</i> (x10 ⁸)	<i>nirA</i> (x10 ⁸)	<i>pmoA-amoA</i> (x10 ⁶)	<i>hao</i> (x10 ⁷)	<i>nirK</i> (x10 ⁸)	<i>norB</i> (x10 ⁸)	<i>nosZ</i> (x10 ⁷)
0	0.63 b	0.62 b	1.24 c	1.47 c	0.91 c	0.29 b	1.05 a	0.64 c	0.70 c	3.03 c
10	0.78 ab	0.77 ab	1.58 bc	1.84 c	1.28 bc	1.02 b	1.08 a	0.85 bc	0.98 bc	3.69 bc
30	0.80 ab	0.81 ab	1.70 bc	1.92 bc	1.60 ab	2.57 ab	1.90 a	0.85 bc	0.85 bc	3.73 bc
50	1.08 a	1.06 a	2.01 ab	2.57 ab	1.50 ab	4.37 ab	1.99 a	1.16 ab	1.25 ab	5.76 a
80	1.06 a	1.05 a	2.06 ab	2.72 a	1.56 ab	4.16 ab	2.54 a	1.14 ab	1.26 ab	5.44 ab
160	0.93 ab	0.92 ab	2.28 a	2.67 a	1.76 a	8.39 a	2.42 a	1.48 a	1.51 a	7.01 a
Trial	0.020	0.010	0.002	<0.001	<0.001	0.865	0.081	0.361	0.007	0.065
N rate	0.010	0.009	<0.001	<0.001	<0.001	0.006	0.008	<0.001	<0.001	<0.001
Trial x N rate	0.283	0.312	0.670	0.015	0.381	0.264	0.182	0.423	0.060	0.010

Figure 1. Root exudate compounds ($\mu\text{g plant}^{-1}$) of maize plants grown under different N rates (mg L^{-1}) and summed by chemical class.

Figure 2. Partially constrained Principal Coordinates Analysis depicting the effect of N rate on soil bacteria community composition. Analysis was conducted using Bray-Curtis distances from OTUs defined at a 3% genetic distance and subsampling to 1000 reads per sample. Symbols: Trial 1 (squares), Trial 2 (circles); N0 (black), N10 (red), N30 (green), N50 (blue), N80 (cyan), N160 (magenta). Ellipses show 95% confidence intervals of the standard deviation for each trial/N rate combination.

Figure 3. Effect of N rate on total bacteria abundance ($\times 10^8$ 16S rRNA copies g^{-1} soil FW) determined by 16S rRNA gene quantitative PCR. Bars with different letters are significantly different (Holm-Sidak post-hoc test, $p < 0.05$).

Figure 4. Biomass in maize (four-leaf stage) grown under increasing N fertilizer rates. **A)** Representative plants at the four leaf stage, **B)** Average heights (cm), **C)** Fresh biomass (g plant⁻¹) separated into shoot (gray) and root (white), **D)** Dry biomass (g plant⁻¹) separated into shoot (gray) and root (white). Bars are the LSmeans $\pm$ SE. Bars with different letters are significantly different (Holm-Sidak post-hoc test, $p < 0.05$).

Figure 5. Nitrogen uptake in maize (four-leaf stage) grown under increasing N fertilizer rates. **A)** Total nitrogen uptake (mg plant^{-1}) separated into fertilizer-derived (gray) and soil-derived (white) fractions, **B)** Total nitrogen recovery (%) separated into fertilizer-derived (gray) and soil-derived (white) fractions, **C)** Lost (unaccounted) fertilizer N (mg pot^{-1}). Lost fertilizer N = N applied – N uptake – N soil. Bars are the LSmeans $\pm$ SE. Bars with different letters are significantly different (Holm-Sidak post-hoc test, $p < 0.05$).