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Carbon availability mediates differences in rhizosphere denitrification potential between intercropped plants

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Denitrification is an important loss pathway for nitrogen (N) in agricultural systems; however, it is at times unpredictable. Some of the uncertainty surrounding denitrification rates is due to soil heterogeneity and spatial variability, especially with regard to carbon (C) availability, as it is the energy source fueling denitrification. Differences in C concentration and type are particularly large between the rhizosphere and bulk soil. The rhizosphere is a hotspot of microbial activity, where the chemical composition and quantity of C varies depending on both plant species and plant community diversity. However, we lack an understanding of how rhizosphere-driven differences in C impact denitrification in these different soil compartments. Here, we assessed denitrification and denitrifier gene abundance in bulk soils and rhizosphere soils of monoculture maize (*Zea mays* L.) and maize interseeded with annual ryegrass (*Lolium multiflorum* Lam.) and crimson clover (*Trifolium incarnatum* L.). In addition, we measured potential denitrification and gene abundance in rhizosphere soils of ryegrass and clover. We found that denitrification potential was enhanced in all rhizosphere soils compared to bulk soil and this effect varied depending on the species of plant, with significantly increased rates of denitrification in maize rhizosphere compared to clover rhizosphere. The availability of dissolved organic C, but not microbial biomass C, accounted for differences in denitrification rates among rhizosphere soils from different plants. Although not consistently enhanced in the rhizosphere, the reduction of N₂O in bulk soils was significantly lower when maize was intercropped with ryegrass compared to maize grown with clover or in monoculture, demonstrating the effects of increased plant diversity on denitrifiers. Of the denitrification genes assayed, the abundance of *nirS* was the most variable between soils, and gross N₂O production was positively correlated with *nirS* but not *nirK*. This suggests that *nirS* denitrifiers may be more responsive to differences in available C between soils compared to *nirK* denitrifiers. This study demonstrates how denitrification rates can differ significantly within the rhizospheres of different plants and highlights the importance of C availability as a driver of differences in denitrification rates in the rhizospheres of maize and cover crops.

KEYWORDS

denitrification, intercropping, N₂O reduction, niche differentiation, qPCR, root exudates

1 Introduction

Incomplete denitrification is responsible for much of the production of nitrous oxide (N_2O) from agriculture, but micro-scale variation and episodic fluxes make N_2O emissions difficult to predict (Groffman et al., 2009). Denitrification is an anaerobic respiratory process, wherein microbes from all three domains of life utilize various nitrogen-oxide species as terminal electron acceptors to generate N_2O (Shapleigh, 2013). Denitrification proceeds in a stepwise function and produces N_2O , which some denitrifiers (predominantly bacterial) will reduce further to N_2 (Butterbach-Bahl et al., 2013). The primary drivers of denitrification include C and nitrogen (N) availability as well as anaerobicity (Robertson and Groffman, 2015). These factors can vary greatly throughout the soil profile and over time, with for instance a greater portion of anaerobic regions following a large rainfall event or uneven distribution of C inputs from vegetation, including via root exudates. Understanding how these drivers of denitrification vary can improve predictions of N_2O emissions and facilitate the management of ecosystems to produce less N_2O .

The rhizosphere is the area of the soil that is in direct contact with plant roots and is a hotspot of microbial activity. This area of the soil receives large inputs of C from plants and is often anaerobic, due to high levels of respiration from roots and microbes utilizing root-derived C. It is therefore not surprising that past studies have demonstrated an increase in denitrification in the rhizosphere (Smith and Tiedje, 1979; Højberg et al., 1996; Philippot et al., 2006; Hamonts et al., 2013). Moreover, the rate of N_2O reduction has been shown to be driven by terminal electron-acceptor demand, where high availability of C and low oxygen availability can stimulate N_2O reduction to N_2 (Morley and Baggs, 2010; Gillam et al., 2011; Senbayram et al., 2012). These conditions of C availability and anaerobicity often prevail in the rhizosphere, providing an environment that should favor N_2O reduction. This rhizosphere effect is likely mediated by the provision of root exudates, which can vary in amount and composition between plants and even over the life cycle of a single plant (Badri and Vivanco, 2009; Jones et al., 2009). However, most studies have not assessed the rhizosphere effect on denitrification across multiple plant species in the same soil causing plant effects to be confounded with soil effects. Further, it is not clear which factors in particular drive an increase in denitrification within the rhizosphere. For instance, either changes in C and N availability, anaerobicity, or microbial community composition could all impact denitrification potential. The effect of these differences in the root zone of separate plants on denitrification requires further study in order to identify the mechanisms behind the rhizosphere effect on denitrification.

A recent meta-analysis found that the genetic potential for many N-cycling processes, including denitrification, is enhanced in the rhizosphere, and that this effect differs among plant types (Ling et al., 2022). This meta-analysis did not determine whether differences in genetic potential were related to increases in the functional potential of denitrifiers, and so how these community-level changes in the rhizosphere affect denitrification rates remains unknown. The genetic potential for denitrification is often assessed through two homologous genes for nitrite reduction, *nirK* and *nirS*, and by two clades of nitrous oxide reductase, *nosZ-I* and *nosZ-II* (Zumft, 1997; Jones et al., 2013). The two genes for nitrite reductase, copper-containing *nirK* and the heme-containing *nirS*, derive from distinct lineages (Jones et al., 2008), and it has long been speculated that the conserved differences between these two groups of denitrifiers is due to niche differentiation, where denitrifiers of one type are more suited to particular environments than the denitrifiers of the other type (Hallin et al., 2009; Jones and Hallin, 2010). In this

connection, studies have shown that *nirS* abundance is more strongly correlated with nitrate availability (Enwall et al., 2010; Xie et al., 2014; Assémien et al., 2019), while *nirK* is sometimes more responsive to concentrations of copper and/or organic C (Enwall et al., 2010; Ma et al., 2016). On the other hand, *nirS* denitrifiers have also been shown to respond directly to C additions (Langarica-Fuentes et al., 2018). Given the distinct environments between rhizosphere and bulk soil, we would expect to observe a difference in the abundance of specific denitrification genes between different soil compartments.

Intercropping is a practice where multiple plant species are grown in the same plot of land at the same time (Brooker et al., 2015). Interseeding cover crops into maize is emerging as a strategy for promoting cover crop establishment, particularly in regions where the window for seeding cover crops following maize harvest is short (Brooker et al., 2020; CTIC et al., 2020). By increasing plant diversity, interseeding can affect the functioning of the soil microbial community (Finney and Kaye, 2017; Curtright and Tiemann, 2021). Intercropping-mediated increases in microbial nutrient-cycling activity are not confined to the bulk soil and have been observed within the rhizosphere (Curtright and Tiemann, 2021). By altering the type and amount of C inputs to the soil, intercropping has the potential to impact denitrification rates in both rhizosphere and bulk soils (McGill et al., 2010; Leloup et al., 2018; Curtright et al., 2023). The intercropping effect has also been observed in the composition of rhizosphere microbial communities, with changes in the potential for various N cycling processes (Pivato et al., 2017); however, denitrification was not included among them. The effect of interseeding cover crops in maize on the functional and genetic potential for denitrification in bulk soil and the rhizosphere has not yet been described.

To assess how plant species identity and intercropping affect denitrifiers in the rhizosphere, we sampled bulk soil and rhizosphere soil from maize and two types of intercropped plants (ryegrass and clover). We performed denitrification potential assays and determined the abundance of key denitrification genes. We hypothesized that potential denitrification rates would be elevated in the rhizosphere and that this increase would vary between plant species as a result of differing root exudation patterns and N dynamics. Due to increased demand for N in the rhizosphere, we hypothesized that the potential for N_2O reduction would be greater, since denitrifiers would need to be more efficient in their use of N. Finally, we predicted that rhizosphere soils would enrich particular types of denitrifiers given the large differences in available C and N between the rhizosphere and bulk soil.

2 Methods

A two-year field experiment with cover crops interseeded into maize was initiated in June 2018 in East Lansing, Michigan, USA (42.7100° N, 84.4663° W). The soils at this location are an Aubbeenaubee-Capac sandy loam (fine-loamy, mixed, active, mesic Aeric Epiaqualf; fine-loamy, mixed, active, mesic Aquic Glossudalf), with a soil organic matter content of ~2.8% and pH between 5.8 and 7.6. Before maize planting in 2018, the field received conventional tillage, but the field was not tilled before maize planting in the second season (2019). No starter fertilizer was applied at planting in 2019, but urea was broadcast at 225 kg N ha⁻¹ at the V3 stage of corn growth. In both years, glyphosate was used to control weeds prior to planting and at the V3 stage of corn growth. The cover crop treatments included in the present study were annual ryegrass (*Lolium multiflorum* Lam.) and crimson clover (*Trifolium incarnatum* L.), which were

interseeded in June 2019 at the V3 stage of corn growth. A no-cover-crop control was established without cover crops. Replicate plots were established in four blocks. Further details of the intercropping experiment can be found in [Brooker et al. \(2020\)](#) and [Curtright et al. \(2023\)](#).

In August 2019, during the early reproductive phase of corn growth, bulk and rhizosphere soil samples were taken from all three interseeded cover-crop treatments including the no-cover-crop control. Rhizosphere soils from maize as well as the ryegrass and clover covers interseeded into maize were sampled. Rhizosphere soil samples were obtained by uprooting plants by digging approximately 10–20 cm from the base of the plant with a shovel, taking care to not sever below-ground roots. Plants were then vigorously shaken to remove loosely adhering soil, and the tightly adhering soil was sampled as “rhizosphere soil.” Bulk soil samples were also taken from all plots by sampling with a soil probe (1.9 cm diameter core, 10 cm depth) between maize rows and avoiding cover crop roots. Soils were particularly dry during sampling (~7% GWC). All soil samples were kept on ice until being returned to the lab for processing, where soils were sieved through a 4-mm mesh and large roots and plant material that did not pass through the sieve were removed.

Fresh soils were then stored at -80°C for later use in denitrification assays. Prior to assays, soils were thawed at 4°C over 3 days before weighing 5 g of soil into serum bottles, which were then covered and pre-incubated at room temperature for 4 days before the assay. Potential rates of denitrification and N_2O reduction were obtained by adding 15 mL of nitrate solution ($100\text{ mg N g}^{-1}\text{ soil}$) to serum bottles, following established protocols for determining denitrification enzyme activities ([Groffman et al., 1999](#)). Bottles were stoppered with rubber septa, evacuated, and flushed with N_2 . Either C_2H_2 (10% v/v final concentration) or N_2 was added to the headspace of jars to estimate denitrification and net N_2O production, respectively. It is noted that C_2H_2 can skew denitrification dynamics as it also inhibits nitrification and can potentially be used as a C source ([Malone et al., 1998](#); [Groffman et al., 2006](#)). However, given the non-limiting amount of nitrate provided and the relatively short timeframe of the assay, these were not considered to be significant limitations. Headspace samples were taken at 24, 26, and 28 h and analyzed on a GC equipped with TCD and ECD (TRACE 1310, Thermo Fisher Scientific, USA) to determine concentrations of CO_2 and N_2O , respectively.

Soil C and N measurements were made by performing soil extractions in $0.5\text{ M K}_2\text{SO}_4$ ($5\text{ mL g}^{-1}\text{ soil}$). Nitrate and ammonium were measured colorimetrically ([Doane and Horwath, 2003](#)). Dissolved organic C (DOC) and total dissolved N were determined on an elemental analyzer (vario TOC select, Elementar, Germany). Microbial biomass C (MBC) was determined by adding 1 mL of chloroform to 8 g of soil and incubating for 24 h. Soil extractions were then performed as described above, and the difference in DOC content between fumigated and non-fumigated samples was used to estimate microbial biomass by applying a correction factor of 0.45 to account for fumigation efficiency ([Jenkinson et al., 2004](#)).

Total N_2O production (“gross N_2O ”) was estimated from the amount of N_2O in jars that received C_2H_2 . The N_2O in jars that did not receive C_2H_2 was considered “net N_2O .” N_2 production was calculated by subtracting net N_2O production from gross N_2O production. The portion of total N_2O production that was not reduced to N_2 —referred to as dN_2O —was calculated by dividing net N_2O production by gross N_2O production. The rhizosphere effect, or the relative increase in denitrification activity compared to the surrounding bulk soil, was calculated from total N_2O and N_2 production. For estimating the rhizosphere effect, total N_2O and N_2 production in each rhizosphere sample was divided by total N_2O and N_2 production in the bulk soil sample taken from the same plot.

DNA for qPCR was collected from the same soil used for the denitrification potential assays. Only soil in the treatments without acetylene were used for DNA extraction. After the assay, jars were immediately frozen in liquid nitrogen and lyophilized. DNA was extracted from the lyophilized samples using the DNeasy PowerSoil Pro Kit (Qiagen, USA). Extracted DNA was quantified using the Quant-iT assay kit (Thermo Fisher Scientific). Denitrification genes were amplified with four sets of primers targeting *nirS*, *nirK*, *nosZ-I*, and *nosZ-II* ([Supplementary Table S1](#)). Standards for the qPCR assays were obtained by amplifying the respective gene product from a composite sample of the treatment soils and excising the bands with the expected length from an agarose gel using a GeneJET Gel Extraction Kit (Thermo Fisher Scientific). Initial amplifications were also used to determine optimal primer concentrations and melting points that minimized primer dimer formation. Following purification, the standard DNA templates were quantified using the Quant-iT assay kit and normalized to set concentrations. qPCR assays were performed under the following conditions: forward and reverse primers to concentrations specified in ([Supplementary Table S1](#)), 10 ng of template DNA, and 1x final concentration of PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) to a total reaction volume of 20 μL . PCR cycles included an initial melting stage at 95°C for 2 min followed by 35 cycles of the following steps: a melting stage at 95°C for 15 s, an annealing stage of 15 s at the primer-specific temperature specified in ([Supplementary Table S1](#)), and an elongation stage at 72°C for 1 min. All reactions were carried out in quadruplicate.

Statistical analyses were performed in R. Mixed models were constructed using the lmer function of the *lme4* package. Cover crop and soil compartment (i.e., bulk soil, maize rhizosphere, clover rhizosphere, ryegrass rhizosphere) were fixed effects, and field block and its interaction with fixed effects were coded as random effects in order to fully specify the structure of the mixed-effects model. Given the nature of the fixed effects, cover crop and soil compartment could not be fully crossed (i.e., while maize rhizosphere or bulk soil could be obtained from plots with or without cover crops, no treatments included ryegrass- and clover rhizosphere soil with other cover crops). Therefore, a two-step process was used for modelling soil compartment and cover crop effects. In the first step, we used a subset of the data that only included data from the maize rhizosphere and bulk soil (i.e., cover crop rhizosphere-soil data were excluded). We used these data to test for main and interactive effects of soil compartment (bulk versus maize rhizosphere) and cover crop (no cover, ryegrass, or clover) using a full-factorial two-way ANOVA. If no interaction was found between soil compartment and cover crop—meaning that the soil-compartment effect was not influenced by cover crop type—then cover crop was omitted from the model and only the effect of soil compartment was tested on the full dataset. If the interaction was significant, then all cover crop and soil-compartment treatment combinations were coded as a single factor for a one-way ANOVA. Significance of fixed effects were determined using the *lmerTest* package to perform F tests with denominator degrees of freedom estimated using the Kenward-Roger method. Treatment means were generated from the estimated marginal means derived from the linear models, and standard errors were calculated from the estimate of MSE. When fixed effects were significant at a Type I error rate of 0.05, means were separated using Fisher's LSD. A Tukey adjustment was used when comparing means without a significant fixed effect. This multiple comparisons approach was selected to afford greater statistical power when significant effects were present, while protecting against Type I errors when overall effects were not significant.

3 Results

3.1 Denitrification potential

Soil compartment (bulk versus rhizosphere of different plants) had a significant effect on potential gross N_2O production (Table 1). Interseeded cover crops did not have a significant interaction with the soil-compartment effect in maize rhizosphere and bulk soil; therefore, soil-compartment comparisons are presented as a single-factor analysis (where intercrops are combined for bulk soil and maize rhizosphere). The rate of potential gross N_2O production (Figure 1) was greatest in maize rhizosphere soil ($12.73 \pm 1.53 \text{ mg N}_2\text{O-N kg}^{-1} \text{ soil d}^{-1}$) which was nearly double the rate in bulk soil ($6.88 \pm 0.83 \text{ mg N}_2\text{O-N kg}^{-1} \text{ soil d}^{-1}$). Clover rhizosphere soil had a gross N_2O production rate of $9.13 \pm 1.22 \text{ mg}$

$\text{N}_2\text{O-N kg}^{-1} \text{ soil d}^{-1}$, which was significantly higher than bulk soil but significantly lower than maize rhizosphere soil. Gross production of N_2O from ryegrass rhizosphere soil was $12.06 \pm 1.61 \text{ mg N}_2\text{O-N kg}^{-1} \text{ soil d}^{-1}$, falling between the other rhizosphere soils.

We also investigated the proportion of N_2O that is produced and not ultimately reduced to N_2 (dN_2O). We found that dN_2O values were high in all soil samples, with the vast majority of N_2O production remaining unreduced (Figure 2). There was a significant interaction between soil compartment and intercrop (Table 1); therefore, means are presented for each combination of soil compartment and intercrop. Bulk soil with maize intercropped with ryegrass had the highest dN_2O value, $108 \pm 6\%$ (dN_2O values above 100% likely reflect variability and experimental error). This was significantly higher than bulk soil from the other intercropping treatments ($86 \pm 6\%$ and $87 \pm 6\%$ in non-intercropped and

TABLE 1 Type III ANOVA table of fixed effects.

Model	Gross N_2O		dN_2O		DOC		MBC	
	F	p	F	p	F	p	F	p
Model 1: subset								
Soil type	36.0	0.009	13.7	0.034	15.5	0.029	65.3	0.004
Cover crop	3.2	0.114	9.9	0.013	0.6	0.557	4.5	0.065
Soil type $\times$ cover crop	0.0	0.984	6.2	0.034	0.8	0.505	0.1	0.913
Model 2: all data								
Soil type	13.3	0.001	—	—	8.2	0.005	15.9	< 0.001
Soil type: cover crop	—	—	3.4	0.013	—	—	—	—

Model	NO_3		NH_4		Organic N	
	F	p	F	p	F	p
Model 1: subset						
Soil type	3.9	0.144	2.9	0.185	5.3	0.105
Cover crop	0.4	0.681	1.3	0.332	2.1	0.205
Soil type $\times$ cover crop	2.2	0.192	0.6	0.566	0.0	0.960
Model 2: all data						
Soil type	2.5	0.128	3.6	0.054	4.2	0.038
Soil type: cover crop	—	—	—	—	—	—

Model	<i>nirK</i>		<i>nirS</i>		<i>nosZ-I</i>		<i>nosZ-II</i>	
	F	p	F	p	F	p	F	p
Model 1: subset								
Soil type	0.1	0.827	15.2	0.002	2.3	0.139	1.1	0.339
Cover crop	0.9	0.441	5.6	0.016	4.1	0.038	1.3	0.362
Soil type $\times$ cover crop	1.0	0.408	3.0	0.080	0.7	0.498	1.0	0.442
Model 2: all data								
Soil type	0.1	0.947	—	—	0.9	0.464	2.1	0.117
Soil type: cover crop	—	—	5.8	0.001	—	—	—	—

Model 1 was performed on a subset of data that included only maize rhizosphere and bulk soil, allowing for the significance of the interaction between soil type and cover crop to be assessed in a two-way ANOVA. If the interaction was found to be significant, then a second model constructed from the full dataset incorporated each treatment combination of the two factors in a one-way ANOVA. If the interaction was not significant, then cover crop was omitted from the model and only the effect of soil type was tested.

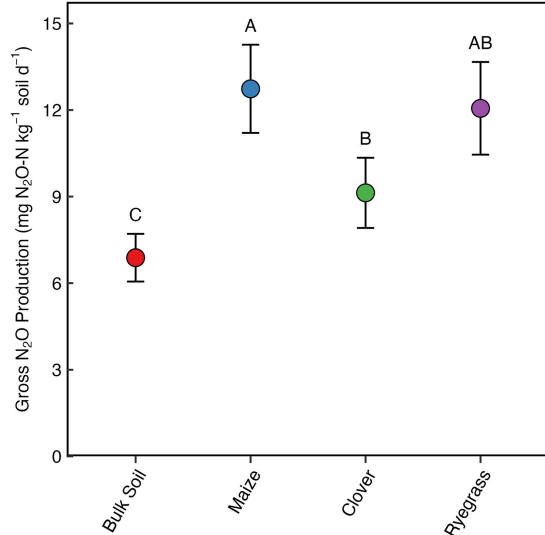


FIGURE 1
Differences in denitrification potential among soil compartments. Maize, clover, and ryegrass refer to rhizosphere soil from respective plant types. Error bars are standard error ($n = 4$). Letters indicate significant differences between means at $\alpha = 0.05$.

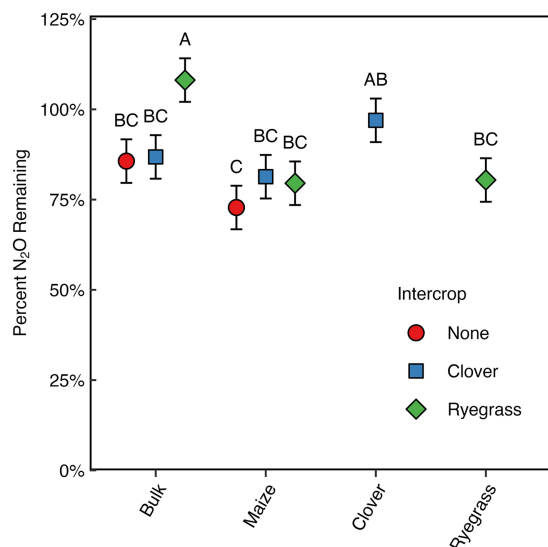


FIGURE 2
Differences in the percent N₂O remaining after N₂O reduction to N₂ occurs. Maize, clover, and ryegrass refer to rhizosphere soil from respective plant types. Letters indicate significant differences between means at $\alpha = 0.05$.

maize-clover intercropping, respectively). The dN₂O value was significantly lower in the maize and ryegrass rhizosphere soils, ranging between 73% and 80%. Apart from the maize-ryegrass intercropping treatment, apart from the maize-ryegrass intercropping treatment, only clover rhizosphere and maize rhizosphere without intercrop were significantly different from each other.

3.2 Soil dissolved organic C, inorganic N, and microbial biomass C

Soil compartment had a significant effect on DOC content, but there was no effect of intercrop type (Table 1). We found that

DOC was consistently higher in the rhizospheres of all plants (ryegrass, 105.1 ± 9.5 mg DOC-C kg⁻¹ soil; maize, 89.1 ± 6.4 mg DOC-C kg⁻¹ soil; clover, 82.4 ± 9.5 mg DOC-C kg⁻¹ soil) compared to bulk soil (61.8 ± 6.4 mg DOC-C kg⁻¹ soil). Microbial biomass C was also significantly affected by soil compartment (Table 1), but MBC was only significantly greater in the rhizosphere of maize compared to bulk soil (334 ± 16 versus 197 ± 16 mg MBC-C kg⁻¹ soil, Figure 3).

Inorganic N availability was relatively low, with no significant effect of soil compartment or intercrop (Table 1). Nitrate and NH₄⁺ tended to be highest in ryegrass rhizosphere (4.2 ± 1.5 and 2.7 ± 0.5 mg N kg⁻¹ soil, respectively) and lowest in maize rhizosphere (1.1 ± 1.2 and 0.9 ± 0.3 mg N kg⁻¹ soil, respectively). Concentrations of NO₃⁻ and NH₄⁺ in clover rhizosphere and bulk soil were between these two extremes. In contrast, soil compartment had a significant effect on organic N (Table 1). All rhizosphere soils (ryegrass, 8.9 ± 1.3 mg N kg⁻¹ soil; maize, 8.1 ± 1.0 mg N kg⁻¹ soil; clover, 7.0 ± 1.3 mg N kg⁻¹ soil) had significantly greater organic N concentrations than bulk soil (4.2 ± 1.0 mg N kg⁻¹ soil).

3.3 Abundance of denitrification genes

We found that only *nirS* gene abundance was significantly affected by soil compartment and intercrop type (Table 1). Maize rhizosphere soil with no intercrop had the highest abundance of *nirS*, with 773 ± 50 gene copies ng⁻¹ DNA (Figure 4). This was significantly higher than the maize rhizosphere soils with clover and ryegrass intercrop, as well as the bulk soil samples, which ranged between 287 and 425 gene copies ng⁻¹ DNA. The abundance of *nosZ-I* also varied between soil compartments, but not significantly. Within bulk soil and maize rhizosphere soil, *nosZ-I* tended to be less abundant in the intercropped treatments compared to the no-intercrop treatment. There was no effect of soil compartment or intercropping on the abundance of *nosZ-II* or *nirK*.

In comparing the homologous genes, *nirK* was more abundant than *nirS* (Figure 4). Gene counts of *nirK* varied between 735 and 1,006 gene copies ng⁻¹ DNA among all treatments, while the abundance of *nirS* varied between 287 and 773 gene copies ng⁻¹ DNA. For nitrous oxide reductase, *nosZ-II* (between 1785 and 3,204 gene copies ng⁻¹ DNA) was much more abundant than *nosZ-I* (between 98 and 161 gene copies ng⁻¹ DNA).

3.4 Correlations with denitrification rates

Denitrification potential was correlated with a number of edaphic and microbial factors. Both DOC and MBC were strongly and significantly correlated with denitrification ($r = 0.695$ and 0.656 , respectively; Table 2). However, the correlation of MBC with denitrification appeared to be driven by low biomass levels in bulk soil compared to rhizospheres, and it did not account well for differences between rhizosphere soils. When rhizosphere denitrification was expressed as a percentage of activity in the surrounding bulk soil (i.e., the rhizosphere effect), biomass itself was not a strong driver ($r = 0.073$) and only DOC explained a significant amount of variation ($r = 0.615$). Inorganic N did not explain a significant amount of variation in denitrification, but organic N content was significantly correlated with both gross N₂O production and the rhizosphere effect. Of the four denitrification genes assayed, only *nirS* abundance was significantly correlated with total N₂O production (Figure 5). No other denitrification gene was significantly correlated with total N₂O production.

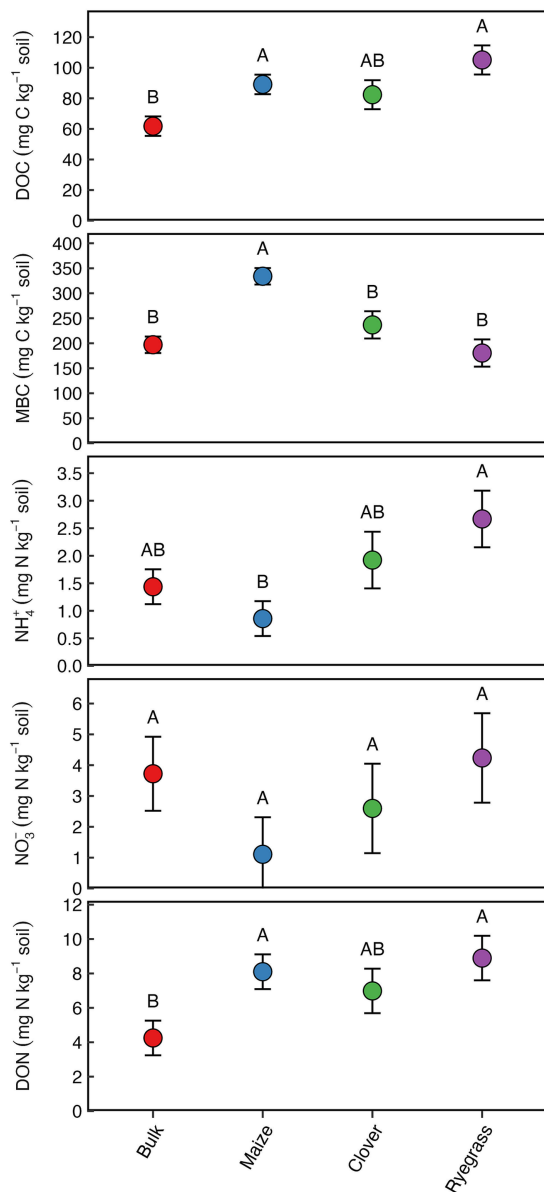


FIGURE 3
Differences in soil dissolved organic carbon (DOC), microbial biomass carbon (MBC), ammonium (NH₄⁺), nitrate (NO₃⁻), and dissolved organic nitrogen (DON) between soil compartments. Maize, clover, and ryegrass refer to rhizosphere soil from respective plant types. Error bars are standard error ($n = 4$). Letters indicate significant differences between means at $\alpha = 0.05$.

4 Discussion

4.1 Rhizosphere effect on denitrification

We hypothesized that denitrification would be enhanced in the rhizosphere due to root-derived C inputs and that this effect would vary between species. Indeed, rhizosphere soils had up to twice as much denitrification as bulk soil (Figure 1). This is in agreement with previous findings that have also found elevated rates of denitrification potential in plant rhizospheres (Smith and Tiedje, 1979; Højberg et al., 1996; Philippot et al., 2006; Hamonts et al., 2013), though this is not always the case (Graf et al., 2016;

Ai et al., 2017). Importantly, we demonstrate in this study that rates of denitrification differ among plant rhizospheres within the same soil: maize rhizosphere soil had significantly greater activity than clover (12.73 versus 9.13 mg N₂O-N kg⁻¹ soil d⁻¹). These plant-based differences could be due to differences in amounts of exudate C or unique root-exudation profiles between plants. The composition of carbohydrates, organic acids, amino acids, and secondary metabolites within root exudates often differs between, and even within, plant species (Badri and Vivanco, 2009; Jones et al., 2009). Previous work has shown that denitrifiers possess distinct rates of activity depending on the type of available C (Henry et al., 2008; Curtright and Tiemann, 2023). Accordingly, differences in denitrifier communities and *in situ* denitrification rates within rhizospheres have been correlated to root-exudate compositions (Wu et al., 2017; Maurer et al., 2021).

Differences in root-exudate composition can shape the structure of rhizosphere microbial communities (Zhalnina et al., 2018), and rates of denitrification could be determined in part by the unique composition of the denitrifier community within each rhizosphere. We found that the genetic potential for denitrification—specifically, *nirS* gene abundance—was greater in maize rhizospheres compared to bulk soil as well as between the rhizosphere of maize, clover, and ryegrass (Figure 4). However, we did not find any significant differences in the abundance of other denitrification genes between the rhizosphere and bulk soil. Other studies have shown differences in various denitrification genes in the rhizosphere compared to bulk soil, but the effects are inconsistent (Hamonts et al., 2013; Ai et al., 2020; Wang et al., 2021; Saghaei et al., 2022). In our study, *nirS* was the most dynamic denitrification gene of the four that we measured, varying the most between soil compartments. The cover crop treatment had a significant effect on *nirS* abundance in maize rhizosphere and bulk soil (Table 1). Others have also found that *nirS* is more responsive than its homolog *nirK* to changes in environmental conditions (Jones and Hallin, 2010; Hamonts et al., 2013; Ai et al., 2017; Langarica-Fuentes et al., 2018). This may be due to niche differentiation where denitrifiers carrying *nirS* are more responsive to the rhizosphere environment than other denitrifiers and better able to adjust to differing conditions between soil compartments.

Contrary to our hypothesis, we did not observe significantly lower dN₂O values in rhizosphere soils compared to bulk soils; dN₂O varied between 75 and 100% in both bulk soil and rhizosphere soil. These were relatively high dN₂O values, which are often observed under conditions with high O₂ or low pH (Cavigelli and Robertson, 2000; Senbayram et al., 2012; Qu et al., 2016). However, those conditions did not prevail in our study, which utilized denitrification potential assays. The response of N₂O reducers to rhizosphere conditions can be multifaceted. While studies have found that genes for N₂O reduction are often enriched in the rhizosphere (Ai et al., 2020; Saghaei et al., 2022), the potential for N₂O reduction is not always increased (Graf et al., 2016). In our study, we did not observe any differences in *nosZ-I* or *nosZ-II* between soils; however, *nosZ-II* was much more abundant than *nosZ-I* across all soils (Figure 4). N₂O-reducing organisms with *nosZ-II* more often lack the genes for generating N₂O (Graf et al., 2014; Hallin et al., 2018). A community composed primarily of *nosZ-II* organisms may therefore, under certain conditions, result in a decoupling of N₂O production and N₂O reduction that could result in a higher prevalence of incomplete denitrification;

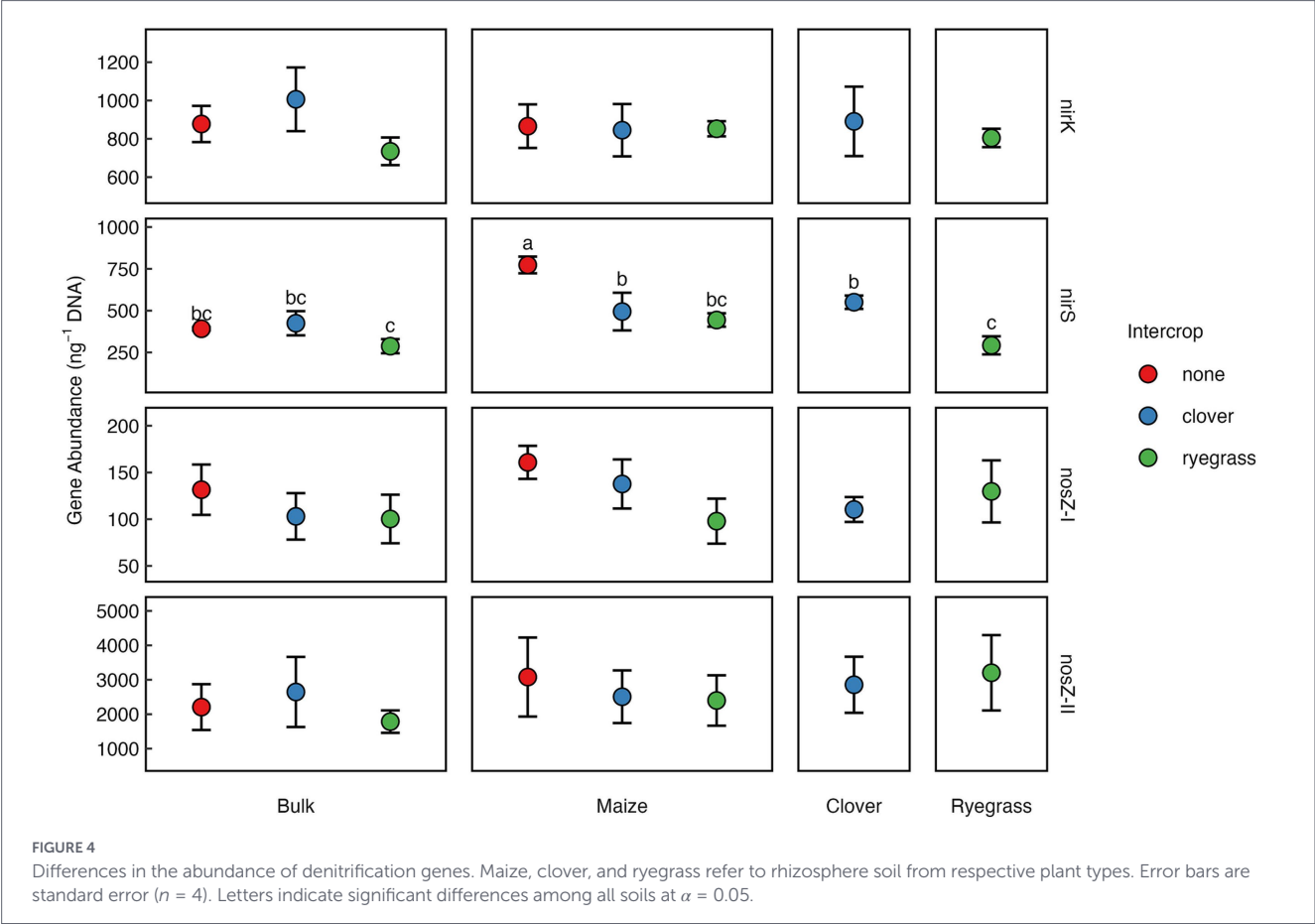


TABLE 2 Correlation coefficients between potential denitrification activities and soil chemical parameters.

Activity	DOC	MBC	NO ₃	NH ₄	DON
Total N ₂ O Production	0.695***	0.656***	−0.065	0.013	0.620***
dN ₂ O	−0.334	−0.402*	0.055	0.234	−0.231
Rhizosphere Effect	0.615**	0.073	0.252	0.211	0.526*

dN₂O is the proportion of N₂O remaining following N₂O reduction to N₂. The rhizosphere effect was the relative increase in total denitrification activity (N₂O production and N₂O reduction) in the rhizosphere compared to the surrounding bulk soil. Significance of the correlations is indicated by asterisks (*, p value < 0.05; **, p value < 0.01; ***, p value < 0.001). The rhizosphere effect was correlated against concentrations of C and N in the rhizosphere.

however, a correlation between *nosZ-II* organisms and a soil's N₂O sink potential has also been observed in other cases (Jones et al., 2014; Domeignoz-Horta et al., 2015).

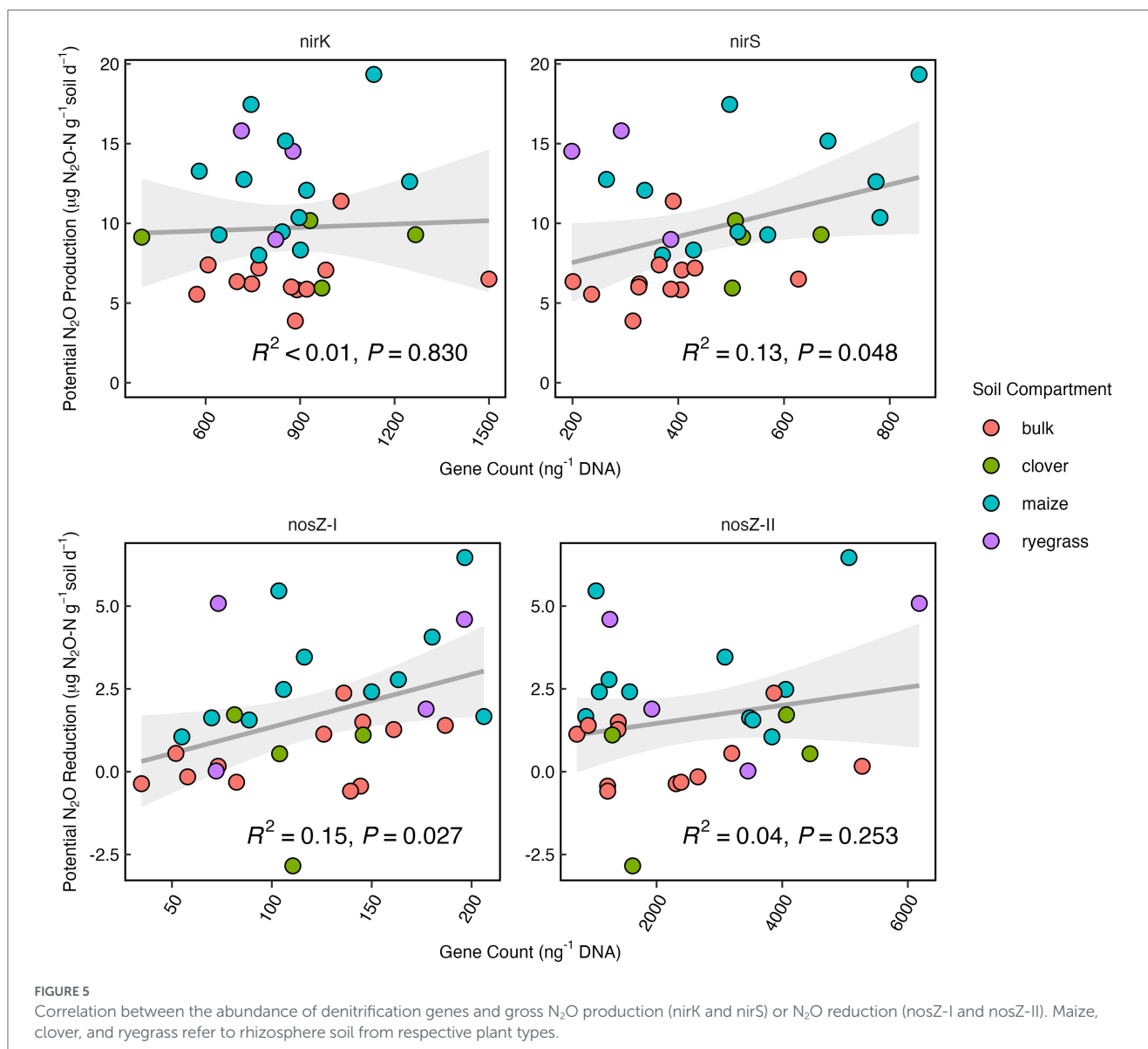
4.2 Factors affecting denitrification

We sought to determine the most important environmental factors that accounted for the increased denitrification rates in the rhizosphere. We found that, while MBC content explained much of the variation between bulk and rhizosphere soils, it was DOC that was able to account for differences between rhizosphere soils of different plants (Table 2). The increased content of MBC in the

rhizosphere is expected given how the rhizosphere is a hotspot that can stimulate microbial growth (Jones et al., 2009; Sasse et al., 2018). Further, the correlation between DOC and the rhizosphere effect between different rhizosphere soils supports the mechanism that DOC from root exudates have a large role in determining the activity of the rhizosphere community (Badri and Vivanco, 2009; Zhalnina et al., 2018).

Denitrification potential was positively correlated with the abundance of *nirS* (Figure 5). In previous studies, *nirS* tends to be more often correlated with denitrification rates than *nirK* (Enwall et al., 2010; Hamonts et al., 2013; Assémien et al., 2019). This could lend support to the idea that *nirS* denitrifiers are more active, even if they are lower in abundance than *nirK*-type denitrifiers (Hamonts et al., 2013; Ai et al., 2017). Correlations between denitrification gene abundance and process rates are only inconsistently observed (Rocca et al., 2015; Shan et al., 2021). In general, the link between gene abundance and process rates is indirect, especially in the soil microbial community (Prosser, 2015; Fierer, 2017). Complementary approaches, such as stable-isotope probing and transcriptomics, can provide more nuanced insight to the connection between genetic potential and denitrification activity (Hungate et al., 2015; Peng et al., 2024). Nevertheless, qPCR can still reveal important patterns in community assembly across varying conditions, especially in habitats that would promote rapid microbial growth, such as the rhizosphere (Fierer et al., 2021).

Competition for N is particularly strong in the rhizosphere (Kuznyakov and Xu, 2013), and we hypothesized that this would drive



an increase in N₂O reduction as a way of conserving scarce N. However, we found that inorganic N availability was not consistently lower in rhizosphere soil (Figure 3). In fact, concentrations of NH₄⁺ and NO₃⁻ were highest in ryegrass rhizospheres. Maize rhizospheres did tend to have the lowest concentrations of inorganic N, but this was not significantly different from bulk soil concentrations. This may have been due in part to dry soil conditions at the time of sampling, which could have reduced plant uptake of inorganic N (Comerford, 2005; Kuzyakov and Xu, 2013). While all soils were quite dry, the ryegrass rhizosphere soils were slightly drier than the others (4% vs. 7% GWC, data not shown). Ryegrass rhizosphere also tended to have the highest concentration of inorganic N and lowest abundance of *nirS* denitrifiers (Figures 3, 4), demonstrating a potential response to the somewhat drier conditions.

Across all soil compartments, inorganic N concentrations did not explain a significant amount of variation in dN₂O (Table 2). This is in contrast to previous studies that have shown that dN₂O is sensitive to soil NO₃⁻ concentrations (Firestone et al., 1979; Qin et al., 2017;

Senbayram et al., 2018). Although N availability may influence the potential for N₂O reduction, a strong concentration gradient did not exist between the different soils used in the study. Of course, it is also important to note that these assays were designed to measure denitrification potential, and non-limiting amounts of NO₃⁻ were added. Therefore, differences in dN₂O in this study would only reflect the potential for N₂O reduction; *in situ* efficiencies may be quite different under field concentrations of NO₃⁻.

Within maize rhizosphere and bulk soil, cover cropping had a significant effect on dN₂O, with bulk soil planted with ryegrass having the highest dN₂O values (Table 1; Figure 2). Perhaps relatedly, there was also a trend of ryegrass reducing the abundance of *nosZ-I* in maize rhizosphere and bulk soil, but these differences were not significant (Figure 4). Although we found that interseeded cover crops such as ryegrass tended to result in a lower proportion of N₂O being reduced, cover cropping reduced the overall denitrification potential, meaning that cover cropping will not likely increase the potential for net N₂O emissions. Intercropping

effects on denitrifiers can be mediated through changes to C and nutrient inputs (Finney et al., 2016) or by broader impacts on the microbial community, such as increased extracellular enzyme activity (Curtright and Tiemann, 2021). However, C and nutrient levels were not significantly affected by intercropping in this study (Table 2), making it difficult to identify the mechanisms behind the intercropping effect on dN_2O . Further work can explore these and additional possible drivers, such as C quality and chemical composition, micronutrient availability, anaerobicity, spatial heterogeneity, and microbial interactions.

5 Conclusion

Overall, we observed higher potential for N_2O production in the rhizosphere, with this effect differing among plant species. We also observed a greater abundance of *nirS* in maize rhizosphere compared to bulk soil. This provides further evidence for the importance of plant-derived compounds for influencing the rhizosphere denitrifier community. We also show that denitrification potential is significantly different among the rhizospheres of plant species intercropped together within the same plot. The availability of DOC was the strongest driver of this effect. dN_2O did not differ significantly between bulk and rhizosphere soils, but this value was affected by intercropping, where intercropping led to a less efficient denitrification pathway. In conclusion, this study demonstrates the importance of root-derived C inputs on increasing the potential for denitrification in a field system.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

AC: Writing – review & editing, Methodology, Writing – original draft, Visualization, Investigation, Conceptualization. LT: Supervision, Methodology, Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2026.1792407/full#supplementary-material>

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