

Short Communication

A distinctive root-inhabiting denitrifying community with high N₂O/(N₂O+N₂) product ratioChao Ai^a, Guoqing Liang^a, Xiubin Wang^a, Jingwen Sun^a, Ping He^{a,b}, Wei Zhou^{a,*}^a Ministry of Agriculture Key Laboratory of Plant Nutrition and Fertilizer, Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing, 100081, PR China^b International Plant Nutrition Institute China Program, Beijing, 100081, PR China

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ABSTRACT

Microbial denitrification in agriculture makes a considerable contribution to terrestrial nitrous oxide (N₂O) emissions, and the prevailing view is that this mainly occurs in soil. Here, we show the root N₂O emission capacity of wheat grown under three long-term (32-year) fertilization regimes, and compare root-, rhizosphere- and soil-inhabiting denitrifying microbial communities. The N₂O/(N₂O + N₂) product ratio of denitrification in the root was 0.5–9.2-fold higher than that in surrounding soil under fertilized conditions, especially manure application. Root N₂O/(N₂O + N₂) ratio was closely related to the proportion of two nitrite-reductase genes (*nirK/nirS*), with higher N₂O emission associated with increased *nirS* abundance. Rhodobacterales and Pseudomonadales dominated the root-associated *nirS* community. In contrast, soils showed a higher proportion of unclassified denitrifiers. Our results demonstrate the potential of wheat to emit N₂O from the roots that harbour low-complexity denitrifying communities distinct from those occurred in soils.

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Agricultural fields with high nitrogen (N) application rates are thought to be hot spots for N₂O emissions and contribute about 60% (5.3 Tg N year⁻¹) of the total anthropogenic emissions and 30% of the total terrestrial emissions (Syakila and Kroeze, 2011). The established wisdom is that N₂O is produced mainly by soil-based microorganisms such as nitrifying and denitrifying bacteria; the latter reduce soluble nitrate (NO₃⁻) or nitrite (NO₂⁻) to dinitrogen (N₂) through a series of gaseous intermediates, including nitric oxide (NO) and nitrous oxide (N₂O). Recent studies have shown that living plants are a potentially important source of N₂O emissions in agricultural systems (Yu and Chen, 2009). For example, a wheat field study indicated that N₂O emissions increased with planting density and plant development (Zou et al., 2005). These studies propose three potential mechanisms for the plant emissions. Firstly, plant roots impact N₂O production in the rhizosphere soil where root exudates stimulate or inhibit microbial populations and their activities (Bardon et al., 2014; Henry et al., 2008; Sun et al., 2016). Second, plants can act as a conduit for transport of N₂O from the soil (Bowatte et al., 2014). Finally, N₂O emissions are

directly detected in a particular plant organ or tissue. Recently, it was found that bacteria resident on plant leaves have the capacity to produce N₂O just as in soil (Bowatte et al., 2015). Similar to plant leaves, terrestrial plants harbour a root microbiome distinct from the complex microbial community present in surrounding soil (Bulgarelli et al., 2012; Lundberg et al., 2012). Surprisingly, a high number and diversity of denitrification genes have also been found inside plant roots by metagenomic analysis (Sessitsch et al., 2012). This might prompt us to ask whether the root-inhabiting denitrifiers have the capacity to contribute to terrestrial N₂O emissions as they do in soils.

Here we investigate the denitrification activity and N₂O emission potential of wheat root (*Triticum aestivum* L.), and compare root- and soil-inhabiting denitrifying microbial communities. We hypothesized that root had distinct denitrifying communities with different N₂O/(N₂O + N₂) product ratio. In doing so, wheat plants were grown in fertilized soils of contrasting geochemistry, designated control (infertile), NPK (inorganic nutrient-rich) or MNPK (organic matter-rich) soil (Table 1). The root and soil samples were collected 47 days after planting, when plants were in an active vegetative growth state. The bulk soil was sampled from the root-free compartments (Fig. S1). The rhizosphere soil was obtained from the middle compartment of the rhizobox. Root cleaning

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Table 1

Root and soil chemical characteristics. Data are means \pm SEM. Fertilization treatments: Control, non-fertilizer; NPK, inorganic N, P, and K fertilizer; MNPK, manure plus NPK fertilizer.

Treatments	Fractions	pH	Total C (g kg ⁻¹)	Total N (g kg ⁻¹)	C/N ratio	DOC (mg kg ⁻¹)	DON (mg kg ⁻¹)	NO ₃ ⁻ -N (mg kg ⁻¹)	NH ₄ ⁺ -N (mg kg ⁻¹)
Root	Control	–	418.58 \pm 0.88	8.21 \pm 0.11	50.96 \pm 0.61	–	–	495.51 \pm 55.05	363.33 \pm 40.43
	NPK	–	405.76 \pm 2.41	9.49 \pm 0.44	42.83 \pm 2.21	–	–	627.43 \pm 40.69	419.2 \pm 62.17
	MNPK	–	370.58 \pm 2.49	11.7 \pm 0.64	31.74 \pm 1.54	–	–	843.25 \pm 34.53	489.81 \pm 35.21
Rhizosphere	Control	8.12 \pm 0.02	7.39 \pm 0.14	0.84 \pm 0.03	8.8 \pm 0.17	49.89 \pm 1.58	14.81 \pm 0.79	2.97 \pm 0.18	3.51 \pm 0.12
	NPK	8.04 \pm 0.02	9.09 \pm 0.05	1.07 \pm 0.03	8.52 \pm 0.18	65.19 \pm 4.5	21.62 \pm 1.01	2.79 \pm 0.11	7.46 \pm 0.36
	MNPK	7.78 \pm 0.02	16.28 \pm 0.43	1.77 \pm 0.12	9.24 \pm 0.5	90.41 \pm 8.14	28.76 \pm 1.32	4.22 \pm 0.35	10.4 \pm 0.19
Bulk soil	Control	8.28 \pm 0.03	7.28 \pm 0.17	0.84 \pm 0.03	8.64 \pm 0.49	39.22 \pm 4.6	16.6 \pm 0.6	3.06 \pm 0.56	5.38 \pm 0.51
	NPK	8.05 \pm 0.02	8.18 \pm 0.08	0.93 \pm 0.03	8.81 \pm 0.17	34.73 \pm 1.84	16.47 \pm 0.59	4.51 \pm 0.22	5.82 \pm 0.24
	MNPK	7.82 \pm 0.03	16.92 \pm 0.15	1.71 \pm 0.04	9.91 \pm 0.27	56.28 \pm 3.56	28.52 \pm 0.21	12.07 \pm 1.06	8.67 \pm 0.5

procedure were performed in strict accordance with the method recommended by Lundberg et al. (2012). When tiny soil aggregates were disrupted and cleaned by vortexing and sonication, clean roots were either immediately (within 2 h) used to determine the total denitrification activity and potential N₂O emission, or frozen with liquid nitrogen, freeze-dried and then stored at -80 °C until molecular analyses. More details are presented in [Supplementary Information](#).

To determine whether root-inhabiting denitrifiers could produce N₂O, we calculated potential N₂O emission and total denitrification activity of sonication-cleaned root in anaerobic microcosms without and with acetylene, respectively (Fig. S2). The activity of denitrifying communities varied substantially across the different compartments, with values of 72.32–1662.83 and 11.76–75.99 ng N₂O-N g⁻¹ (root or soil) dry weight h⁻¹ for total denitrification activity and potential N₂O emissions, respectively. Organic treatment strongly enhanced the total denitrification activity by 9-fold in the bulk soil. The proportion of N₂O emitted by denitrification, calculated as the ratio of the rate of potential N₂O production and total denitrification activity (N₂O/(N₂O + N₂)) (Jones et al., 2014), was 1.2-fold higher in the root than in rhizosphere soil under no fertilization conditions (Fig. 1). The application of inorganic and organic manure led to a further 0.9–1.4-fold increase in root N₂O/(N₂O + N₂) compared with control, whereas decreasing trends were observed in rhizosphere and bulk soil when

organic manure was applied.

The abundance of organisms with potential to produce N₂O by denitrification were determined by quantitative real-time PCR of the *nirK* and *nirS* genes that encode two structurally different but functionally equivalent nitrite reductases (Wei et al., 2015). Across the different fertilization treatments, the abundances of *nirK* and *nirS* genes in the root ranged from 0.5 to 9.3 $\times 10^9$ copies g⁻¹ root dry weight, with *nirK* being 1.1–11.6 times more abundant than *nirS* (Fig. 2A and B). Fertilization practices, especially manure application, substantially increased root *nirS* gene abundance, resulting in distinctively different root *nirK/nirS* ratios of approximately 12.6, 6.8 and 2.1 in the control, NPK and MNPK treatments, respectively (Fig. 2C). In contrast, rhizosphere and bulk soils had lower ratios of *nirK* to *nirS* than the root in the control and NPK treatments, and no significant differences were observed among the different fertilization treatments. Microorganisms with the capacity to reduced N₂O were quantified by targeting the nitrous oxide reductase gene (*nosZ*), whose abundance was markedly increased in root by the application of inorganic and organic fertilizers (Fig. 2D). The ratio of total *nir* genes to *nosZ* in root ranged from 8.3 to 10.5 and was statistically significantly higher than that in rhizosphere and bulk soil ($P < 0.05$).

Multiple stepwise regression analysis indicated that high *nirS* abundance and low *nirK/nirS* ratio best explained the increase in root N₂O/(N₂O + N₂) ratio (Table 2), suggesting that the functional predominance of *nirS*-type denitrifiers in root might have substantial consequences for root N₂O production capacity. In contrast, the abundance of the *nosZ* gene in rhizosphere and bulk soil was the key and robust predictor that explained 79–82% of the variation in N₂O/(N₂O + N₂) ratio (Table 2; Fig. S3).

When finding root N₂O emission potential was strongly associated with the proportion of *nirK*- and *nirS*-type denitrifiers, we analysed the diversity of *nirK* and *nirS* genes by Illumina MiSeq sequencing. Compared with rhizosphere and bulk soils, the *nirS*-type communities in root showed significantly less diversity by both Shannon index and Invsimpson index (Fig. S4), but no significant differences in *nirK* diversity were obtained. We constructed two reference libraries of *nirK*- and *nirS*-type strain sequences retrieved from all draft and completed microbial genomes obtained from NCBI. These known sequences served as references within the phylogenetic pplacer program (Matsen et al., 2010) to classify approximately 41–50% of root *nirK* and *nirS* sequences to species level (i.e., terminal edges of the reference phylogeny; Figs. 3A and 4A). Order-level (Fig. 3B, C and D) and genus-level (Fig. S5) identification of these sequences revealed a specific composition of the *nirS*-type communities in the root. Pseudomonadales and Rhodobacterales were the dominant orders (from 19 to 76%) in the root, especially when organic manure was applied (Fig. 3B), whereas Rhizobiales (14–20%) predominated in rhizosphere and bulk soil

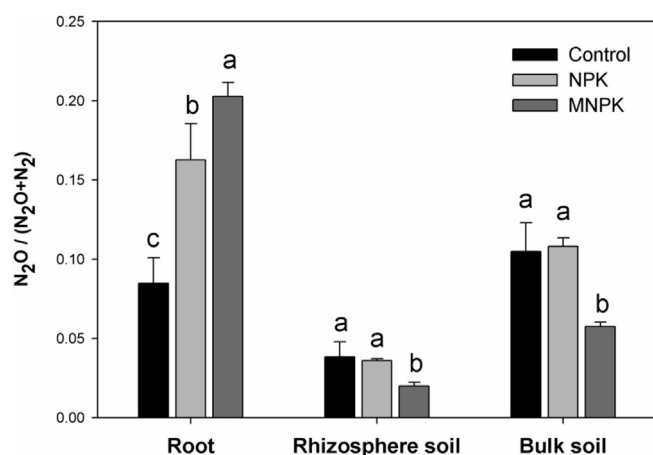


Fig. 1. High proportion of N₂O emitted by denitrification in the root. N₂O/(N₂O + N₂) is calculated as the ratio of the rate of potential N₂O emission and total denitrification activity (Fig. S2). The values given are the means \pm SEM of three separate treatments and different letters indicate significant differences among fertilizer treatments at $p < 0.05$, Fisher's LSD test. Fertilization treatments: control, no fertilizer; NPK, inorganic N, P, and K fertilizer; MNPK, manure plus NPK fertilizer.

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