Wheat leaves emit nitrous oxide during nitrate assimilation

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Nitrous oxide (N_2O) is a key atmospheric greenhouse gas that contributes to global climatic change through radiative warming and depletion of stratospheric ozone. In this report, N_2O flux was monitored simultaneously with photosynthetic CO_2 and O_2 exchanges from intact canopies of 12 wheat seedlings. The rates of N_2O-N emitted ranged from <2 pmol·m^{-2}·s^{-1} when NH_4^+ was the N source, to 25.6 ± 1.7 pmol·m^{-2}·s^{-1} (mean ± SE, n = 13) when the N source was shifted to NO_3^-. Such fluxes are among the smallest reported for any trace gas emitted by a higher plant. Leaf N_2O emissions were correlated with leaf nitrate assimilation activity, as measured by using the assimilation quotient, the ratio of CO_2 assimilated to O_2 evolved. ^{15}N isotopic signatures on N_2O emitted from leaves supported direct N_2O production by plant NO_3^- assimilation and not N_2O produced by microorganisms on root surfaces and emitted in the transpiration stream. *In vitro* production of N_2O by both intact chloroplasts and nitrite reductase, but not by nitrate reductase, indicated that N_2O produced by leaves occurred during photoassimilation of NO_2^- in the chloroplast. Given the large quantities of NO_3^- assimilated by plants in the terrestrial biosphere, these observations suggest that formation of N_2O during NO_2^- photoassimilation could be an important global biogenic N_2O source.

Plants play a critical role in regulating the chemical and physical state of the atmosphere through the exchange of biogenic greenhouse gases. Most notable are plant–atmosphere exchanges of CO_2, O_2, and H_2O, but leaves also emit a variety of carbon- and nitrogen-based trace gases involved in climate alteration processes (1). One such trace gas is nitrous oxide (N_2O). Plants—either aerenchymous (2) or nonaerenchymous (3, 4)—can serve as conduits for N_2O between the soil and atmosphere. They transpire significant quantities of N_2O when its concentration in the soil solution greatly exceeds the solution equilibrium concentration with ambient N_2O, currently at ≈315 nmol·mol^{-1} (5).

The global N_2O budget is beset by uncertainty, and sources of N_2O have historically fallen short of the primary sink, photolysis in the upper atmosphere (6–9). The primary biogenic N_2O sources are from soils (70%) and involve the microbial nitrogen transformations brought about by nitrification and denitrification (6). Although nitrification and denitrification are the major N_2O sources, several microbial organisms that do not nitrify or denitrify can also produce N_2O during NO_3^- assimilation (10, 11). These observations have led to the general hypothesis that any enzymatic nitrogen transformation through the +2 to +1 oxidation state may generate N_2O (12). One such transformation in higher plants is NO_2^- assimilation in chloroplasts. Nitrite assimilation in chloroplasts can generate intermediates capable of reacting to produce N_2O, including NO_2^- (as HNO_2) with hydroxylamine (13) or reaction of NO released during NO_2^- reduction (14, 15) with ascorbate (16). Nonetheless, early attempts to observe N_2O production by higher plant tissues were not successful (10) and were probably limited by lack of an analytical method capable of detecting plant N_2O emission at the exceptionally slow rates reported here. We developed an analytical approach by using cryogenic trapping (17) and gas chromatography coupled to high-precision isotope ratio mass spectrometry (18). This approach resolved leaf N_2O emissions at more than six orders of magnitude lower than photosynthetic gas exchanges of CO_2 and O_2 (Table 1), placing such fluxes among the smallest ever reported for any trace gas emitted by a higher plant (1).

Identifying and quantifying plant N_2O exchange is important. Atmospheric N_2O concentration is increasing at a rate of about 0.27% per year (19), and each mol of N_2O has ≈290 times the radiative forcing potential of CO_2 (20). Consequently, N_2O will account for as much as 7% of projected atmospheric warming (21). In addition to its greenhouse gas properties, photolytic reaction with excited oxygen [O(^{1}D)] in the upper atmosphere produces nitric oxide (NO), and NO, in turn, consumes stratospheric ozone (22). Biogenic and anthropogenic sources of N_2O are poorly constrained (23) and often do not account for the quantity of N_2O known to undergo photolysis in the upper atmosphere (6). Extreme heterogeneity of soil N_2O emissions largely contributes to such uncertainty, but unidentified hydrologic or biogenic sources may also play a role (7, 8). Kroeze *et al.* (24) have argued for closure of the global N_2O budget, but the theoretical uncertainty range for estimates of N_2O from agricultural soils is extreme [0.6–14.8 Tg N_2O-N y^{-1} (6)], and new evidence suggests that N_2O emissions from agricultural soils may be in the lower range of recent Intergovernmental Panel on Climate Change (IPCC) estimates (25). To moderate the atmospheric increases of N_2O and to better understand the role of the biosphere in its production, it is critical to identify all major N_2O sources and exchange pathways, including any potential contributions by plants.

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Methods

Wheat seeds (*Triticum aestivum* L. cv. Veery 10) were surface sterilized, germinated on rolled moist germination paper, and transferred to opaque hydroponics tanks containing dilute nutrient solutions (26). The hydroponics systems were kept in a controlled environment chamber (PGV36, Conviron, Winnipeg, MB, Canada) with a 25°C, 16-h, 600 μmol·m^{-2}·s^{-1} photosynthetic photon flux density day and a 20°C, 8-h night. After 10 days, when the plants had three true leaves, 12 individuals were transferred into a gas exchange chamber where their shoots produced a canopy with a leaf surface area of about 0.02 m^2. Roots were sealed into 12 individual gas-tight cuvettes that were connected to the lower surface of a platform over which the canopy chamber was sealed (27).

Net canopy CO_2 and O_2 exchanges were measured under steady-state conditions. A differential infrared gas analyzer (VIA-500R, Horiba, Sunnyvale, CA) monitored net CO_2 exchange. From a second parallel gas stream, a custom oxygen

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Abbreviation: IPCC, Intergovernmental Panel on Climate Change.

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Table 1. Shoot and rhizosphere gas exchange rates for 14-day-old wheat seedlings (*T. aestivum* L. cv. Veery 10)

N source	Photosynthesis			N_2O-N emissions	
	CO_2, $\mu mol \cdot m^{-2} \cdot s^{-1}$	O_2, $\mu mol \cdot m^{-2} \cdot s^{-1}$	AQ, CO_2/O_2	Shoot, $pmol \cdot m^{-2} \cdot s^{-1}$	Rhizosphere, $pmol \cdot g^{-1} \cdot s^{-1}$
50 μM $(^{15}NH_4)_2SO_4$	14.31 ± 1.32	12.06 ± 0.77	1.21 ± 0.06	ND	40.4 ± 2.1
100 μM $K^{15}NO_3$	15.32 ± 1.48	13.63 ± 0.84	1.13 ± 0.05	25.59 ± 1.68	304.1 ± 93.8

Shown for shoots are net photosynthetic CO_2 assimilation rates, net photosynthetic O_2 evolved, AQ (the ratio of CO_2 assimilated to O_2 evolved), and shoot N_2O emissions (mean ± SE, n = 11). Shown for the rhizosphere are N_2O production rates (n = 7).

analyzer that resolves a 2 $\mu mol \cdot mol^{-1}$ O_2 partial pressure difference on a background of 209,460 $\mu mol \cdot mol^{-1}$ monitored net O_2 exchange (28). Before passing through the O_2 electrodes, gases that can interfere with O_2 measurements, like water vapor and plant secondary carbon compounds, were cryogenically condensed to constant trace levels by using a liquid argon trap. The CO_2 concentration during the gas exchange measurements was sustained at 330 $\mu mol \cdot mol^{-1}$ and the N_2O concentration at 280 $nmol \cdot mol^{-1}$. A third parallel gas stream exiting the plant chamber flowed at 1.0–1.5 $dm^3\ s^{-1}$ through an ascarite filter to remove CO_2, a drierite filter to remove H_2O, and then a second cryogenic trap cooled with liquid argon to condense N_2O (17). This trap concentrated up to 10 $\mu mol \cdot mol^{-1}$ N_2O in 15 min when the N_2O concentration in the gas stream was at 280 $nmol \cdot mol^{-1}$. Gas trapped in the condenser was injected into a mass spectrometer tuned to determine the ratio of ion currents m/z at 45/44 and 46/44 (18). The rate of N_2O exchange by shoots ($mol \cdot m^{-2}\ s^{-1}$) was calculated as $F_{N_2O} = (J_l \cdot d_l \cdot C_l/A)$ (29). J_l is the flow rate of air through the plant chamber ($mol \cdot s^{-1}$). d_l is the mol fraction of N_2O proportional to canopy N_2O emission, calculated according to the isotopic enrichments in masses $^{45}N_2O$ and $^{46}N_2O$ (30). C_l is the concentration of N_2O in the gas stream exiting the chamber. A is leaf area (m^2). The theoretical detection limit for leaf N_2O emission by the mass spectrometer, on the basis of the observed variation in the mass ratios reported for a 1.5 $\mu mol \cdot mol^{-1}$ N_2O standard, was ≈2 $pmol \cdot m^{-2} \cdot s^{-1}$.

During the first 24 h of our experiments, roots received a nitrogen source containing 50 μM $(^{15}NH_4)_2SO_4$ (99.6 atom % ^{15}N), and shoot gas fluxes of CO_2, O_2 and N_2O were assessed during the final 6 h. This pretreatment allowed us to purge the xylem stream of NO_3^- and establish a baseline value for assimilation quotient (AQ, CO_2/O_2) when little NO_3^- was undergoing assimilation in leaves. The nitrogen source was then shifted to 100 μM $K^{15}NO_3$ (99.6 atom % ^{15}N) for 24 h, and shoot gas fluxes were again assessed during the final 6 h.

For rhizosphere N_2O production, the nutrient solution was delivered to roots by using a gas tight, continuous flow system (31). Fluxes of N_2O from the root cuvettes represent the production of N_2O by roots and any microbial organisms on the root surface capable of generating N_2O (32). Before passing through the root cuvettes, the solution concentrations of O_2 and N_2O were brought to their respective saturation concentrations. Dissolved oxygen concentration in the nutrient solution passing through the root cuvettes declined by only about 20% and the solutions were well stirred, so denitrification in the rhizosphere was minimized. During the experiments, two 5-ml samples of nutrient solution were collected into 15-ml septum bottles. One sample was collected before the nutrient solution entered the root cuvette and another after it exited the root cuvette. The head space gases from the septum bottles were injected into the mass spectrometer after equilibration at 22.5°C to determine the concentration of N_2O and the ratio of ion currents m/z at 45/44 and 46/44. The rate of N_2O production by the rhizosphere ($mol \cdot g^{-1} \cdot s^{-1}$) was calculated as $F_{N_2O} = (J_r \cdot d_r \cdot C_r/W)$. J_r is the flow rate of nutrient solution through the root cuvette ($liter \cdot s^{-1}$). d_r is the mol fraction of N_2O in the nutrient solution proportional to rhizosphere N_2O production, and calculated according to the isotopic enrichments in masses $^{45}N_2O$ and $^{46}N_2O$ (30). C_r is the total concentration of N_2O ($mol \cdot liter^{-1}$) in the nutrient solution (33). W is root dry mass.

For chloroplast assays, ≈40 g of fresh leaves from 2-week-old hydroponics-grown wheat plants was blended in a buffer solution containing 0.05 M K-Hepes (pH 7.3), 0.33 M Sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM Na_2EDTA, and 0.1% BSA. The extract was centrifuged at 3,000 × g for 5 min, resuspended in a 50/50 Percol gradient, and centrifuged at 7,000 × g for 10 min. Intact chloroplasts were then collected and washed with 0.05 M K-Tricine (pH 8.0) and 0.33 M Sorbitol. The washed chloroplasts were introduced into 5 ml of an incubation medium consisting of 0.3 mM $K^{15}NO_2$ (99.6 atom % ^{15}N) in 0.05 M K-Tricine (pH 8.0), 0.33 M sorbitol, and 0.3 mM $NaHCO_3$. The chloroplasts were incubated for 40 min in 15-ml septum bottles at 25°C and a light intensity of 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux density. At the end of the incubation period, the head-space gases were collected and injected into the mass spectrometer. The concentration of N_2O and ratio of ion currents m/z at 45/44 and 46/44 were used to calculate the quantity of N_2O produced, as previously described for the dynamic flow measurements.

For nitrate and nitrite reductase assays (34), 2 g of fresh leaves from 2-week-old hydroponics-grown wheat plants was ground in a mortar and pestle with cold-purified N-free sand in 8 ml of a chilled buffer solution. The buffer solution consisted of 0.05 M Tris (pH 8.5), 1 mM EDTA, 1 μM $NaMoO_4$, 10 μM FAD, 1 mM DTT, 10 μM leupeptin, and 1 $\mu g\ ml^{-1}$ pepstatin. The extract was centrifuged at 30,000 × g for 20 min. Then 0.05 ml of the supernatant was added to 0.95 ml of an assay solution containing 62.5 mM potassium phosphate buffer (pH 7.5), 0.7 mM $K^{15}NO_2$ (99.6 atom % ^{15}N), and 0.052 g ml^{-1} of methyl viologen in a 15-ml septum bottle. The reaction was initiated by injecting 0.2 ml of a solution containing 8.3 mg ml^{-1} $Na_2S_2O_4$, and incubated at 30°C for 15 min. For nitrate reductase, 0.05 ml of the supernatant was added to 0.95 ml of assay buffer containing 1.4 mM $K^{15}NO_3$ (99.6 atom % ^{15}N). The reaction was initiated by adding 0.2 ml of a solution containing 2 mg ml^{-1} NADH and incubated at 30°C for 15 min. The head-space gases from the nitrite and nitrate reductase assays were collected and analyzed on the mass spectrometer as previously described.

Results and Discussion

This is the first study, to our knowledge, to report quantitative leaf N_2O emissions under normal physiological conditions for an intact plant by using steady-state gas exchange methods. Earlier investigations have examined detached leaves or used static chamber methods, where CO_2, H_2O, and temperature change rapidly (3, 4, 10), and nitrogen source (NH_4^+ and NO_3^-) cannot be controlled. These preliminary studies were valuable in that they clearly demonstrated N_2O can move from soil to atmosphere via the plant transpiration stream, but they did not resolve the question of N_2O production by plant nitrogen metabolism.

In our investigation, leaves did not emit N_2O at a detectable rate while metabolizing $^{15}NH_4^+$. The AQ during $^{15}NH_4^+$ exposure averaged 1.21 ± 0.05 units (Table 1). When the N source was shifted to $^{15}NO_3^-$, canopy leaves emitted N_2O-N at an average rate of 25.6 ± 1.7 $pmol \cdot m^{-2} \cdot s^{-1}$, and the AQ declined to 1.13 ±

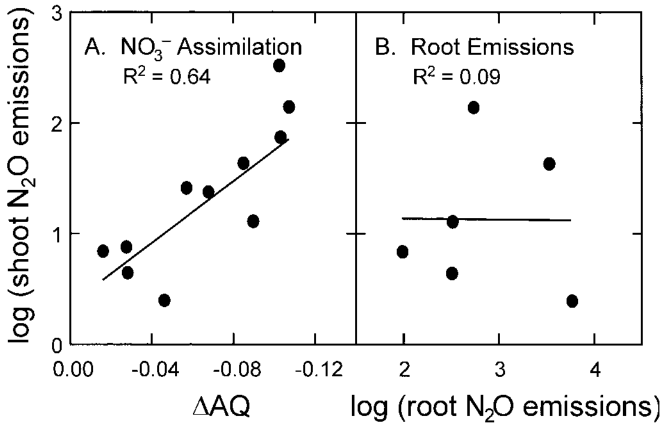

Fig. 1. The relationship between N_2O emission from wheat leaves (pmol N_2O-N $m^{-2}\cdot s^{-1}$) and (*A*) the change in assimilatory quotient (ΔAQ) when the nitrogen source was shifted from $^{15}NH_4^+$ to $^{15}NO_3^-$, or (*B*) N_2O production in the rhizosphere (pmol N_2O-N $g^{-1}\cdot s^{-1}$). Shown are the regression lines and the R^2 statistic. The rates of N_2O emission were normalized by using the $\log_{10}$ transformation.

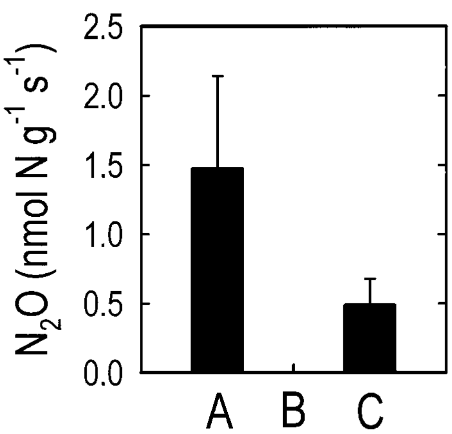

Fig. 2. N_2O production *in vitro* from (*A*) intact chloroplasts (nmol N_2O-N g^{-1} chlorophyll s^{-1}), (*B*) nitrate reductase (nmol N_2O-N g^{-1} protein s^{-1}), and (*C*) nitrite reductase (nmol N_2O-N g^{-1} protein s^{-1}). Intact chloroplasts, nitrate reductase, and nitrite reductase were extracted from fully expanded leaves of 2- to 3-week-old wheat plants (*T. aestivum* L. cv. Veery 10).

0.06 units (Table 1). Both observations are important. The change in AQ (ΔAQ) gives a nondestructive measure of NO_3^- assimilation under steady-state conditions. Net O_2 exchange provides a measure of photosynthetic electron transport, and either CO_2 or NO_3^- reduction can be coupled to such electron transfer (35). As NO_3^- photoassimilation increases, the AQ declines because reductant produced by photosynthetic electron transport increases to support NO_3^- and NO_2^- reduction in addition to CO_2 fixation (28, 36). Thus, not only did shoot N_2O emission become detectable when the N source was shifted to $^{15}NO_3^-$, but the strong correlation with ΔAQ (Fig. 1*A*) suggested that leaf N_2O flux was driven by leaf NO_3^- assimilation.

On the other hand, rhizosphere N_2O production also increased more than 7-fold (Table 1) with the change in N source from $^{15}NH_4^+$ to $^{15}NO_3^-$ (Table 1), raising N_2O concentration in the nutrient solution around roots to an average of 89.6 nmol·liter^{-1}. Such a concentration is an order of magnitude higher than the solution equilibrium concentration when N_2O in the shoot chamber is near ambient, so that movement of N_2O in the transpiration stream could have been significant. At least two experimental observations did not support this scenario. First, shoot N_2O emission during exposure to $^{15}NO_3^-$ was not correlated with root zone N_2O production (Fig. 1*B*). Second, leaf N_2O flux fell below detectable limits during exposure to $^{15}NH_4^+$ despite high rates of N_2O production by the rhizosphere (Table 1). The production of N_2O during $^{15}NH_4^+$ exposure comes from the activity of nitrifying bacteria on the root surface (37), an activity we were able to completely shut down by using the nitrification inhibitor nitrapyrin. Thus, the absence of a correlation between shoot N_2O emission and rhizosphere N_2O production provides further evidence that photoassimilation of NO_3^- was the major source of N_2O emitted from leaves in this investigation. For investigations where N_2O transpiration was responsible for leaf N_2O flux (6), the concentrations of N_2O in the soil solution were nearly four orders of magnitude higher, at ≈326 μmol·liter^{-1}, than N_2O concentrations observed in our nutrient solutions. This may help to explain why N_2O transpiration was not a factor in this investigation.

The isotopic composition of N_2O emitted from leaves provided further verification that shoot NO_3^- assimilation was largely responsible for the observed N_2O flux. In the course of the experiments, we used nitrogen sources in nutrient solutions highly enriched in ^{15}N (>99.6 atom % ^{15}N as NH_4^+ or NO_3^-). Thus, any N_2O produced by microbial metabolism in the rhizosphere, nitrification or denitrification, and subsequently transpired, would be predominantly composed of the $^{15}N^{15}N^{16}O$ or $^{46}N_2O$ isoform. We grew our wheat plants in a nutrient solution with 200 μM NH_4^+ plus 200 μM NO_3^- containing ^{15}N close to natural abundance levels (0.3674% $^{15}NH_4^+$ and 0.3665% $^{15}NO_3^-$, or > 99.6 atom % ^{14}N). These plants stored relatively large quantities of NO_3^- in their tissues (8.76 ± 2.23 mM, mean ± SE, n = 6), of which 2.25 ± 1.65 mM was withdrawn and assimilated during the first 24-h exposure to $^{15}NH_4^+$. N_2O emitted during the assimilation of this internal NO_3^- would not be detected during exposure of roots to $^{15}NH_4^+$, because the isotopic signature on such stored NO_3^- was very similar to that of N_2O in the background air. However, if stored NO_3^- were being mobilized and assimilated in leaves during the subsequent period of root exposure to $^{15}NO_3^-$, or chloroplast reactants were participating in N_2O production, then some of the N_2O emitted would contain the $^{45}N_2O$ isoforms of $^{15}N^{14}N^{16}O$ and $^{14}N^{15}N^{16}O$. Indeed, $^{45}N_2O$ was detected in the N_2O stream emitted from wheat leaves during exposure to $^{15}NO_3^-$. From the mass ratios of $^{44}N_2O$, $^{45}N_2O$, and $^{46}N_2O$ collected during such experiments, we estimated (30) that 5.19 ± 0.92 pmol·m$^{-2}\cdot$s^{-1} (mean ± SE, n = 13), or about 20%, of the N_2O-N emitted by leaves came from the ^{14}N stored in the plant. It is unlikely that bacterial mineralization of organic-N in the rhizosphere followed by nitrification and denitrification contributed to the $^{45}N_2O$ emitted. Roots treated with antibiotic cocktails (38) and nitrapyrin to eliminate rhizosphere microbial nitrogen transformations still emitted detectable quantities of $^{45}N_2O$. Nonetheless, the largest fraction of the observed leaf N_2O flux was clearly generated by plant NO_3^- assimilation and not from N_2O produced by microbial processes in the rhizosphere.

The two enzymes responsible for plant NO_3^- assimilation, NO_3^- reductase (NR) and NO_2^- reductase (NiR), are located in the cytoplasm and chloroplasts, respectively. To determine whether NO_3^- or NO_2^- reduction was responsible for the N_2O emitted, we extracted NR, NiR, and intact chloroplasts from wheat leaves and assayed for N_2O production *in vitro* as described. Whereas NR assays did not produce any detectable N_2O, NiR assays and intact chloroplasts did (Fig. 2). These experiments provide *in vitro* evidence that the N_2O emitted from wheat leaves was generated by NO_2^- reduction to NH_3 in the chloroplasts, where NO_2^- undergoes transformation through the +2 to +1 oxidation state, as predicted.

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The leaf N_2O emissions we measured were small (Table 1), and it is not currently known how important fluxes of this magnitude are in relation to the global N_2O budget. Some recent micrometeorological N_2O flux measurements over grass swards failed to detect a higher N_2O emission rate than that observed by using static chambers over soil alone (39). However, the error in such measurements was too large, with coefficients of variation for the chamber data ranging from 0.42 to 1.83 (40, 41), to resolve plant N_2O emissions of the size measured in this investigation. In support of a role for plant N_2O emission, Hutchinson and Mosier (42) found that aerodynamic flux measurements over an irrigated corn field were always higher than, although never exceeding twice the mean of, fluxes measured by using soil chambers. Some recent chamber studies found that ryegrass canopies were responsible for 21.1% of the total N_2O emissions (4), and that *Linum perenne* canopies contributed to as much as 50% of N_2O flux to the atmosphere (43). Our results indicated that 0.02–0.2% of the NO_3^--N assimilated by wheat was released as N_2O-N. Current estimates are that terrestrial land plants assimilate 1,200 Tg of N annually (44). Nearly half of this N is thought to be absorbed and assimilated as NO_3^- (45), of which 25–75% is assimilated in leaves (46). The NR and NiR enzymes involved are highly conserved among higher plants (47). Thus, if other terrestrial land plants behave as wheat does, we calculate that NO_3^- photoassimilation alone could produce from 0.03 to 0.9 Tg N_2O-N yr^{-1}. The uncertainty in the IPCC's estimates of global N_2O source emissions is substantial, with nearly two orders of magnitude in the range of estimated emission of N_2O from soils [0.6–14.8 Tg N y^{-1} (1)]. Robertson *et al.* (25) tracked N_2O emissions for 9 years from soils of six cropping systems, including a nitrogen-intensive corn rotation and four successional communities, and found emissions to be at the lower end of the IPCC's calculated emission factor, which is based on fertilizer application. Our estimates of plant N_2O emissions represent ≈5–6% of the total amount of N_2O-N thought to be emitted by agricultural plant–soil systems alone (1, 44). These approximations do not include the quantity of N_2O that might be conducted to the atmosphere via the plant transpiration stream. Thus, our results suggest that higher plants could play an intriguing role in N_2O exchange not previously considered in biosphere–atmosphere interactions.

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