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Evidence that Elevated CO₂ Levels Can Indirectly Increase Rhizosphere Denitrifier Activity†

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We examined the influence of elevated CO₂ concentration on denitrifier enzyme activity in wheat rhizoplanes by using controlled environments and solution culture techniques. Potential denitrification activity was from 3 to 24 times higher on roots that were grown under an elevated CO₂ concentration of 1,000 μmol of CO₂ mol⁻¹ than on roots grown under ambient levels of CO₂. Nitrogen loss, as determined by a nitrogen mass balance, increased with elevated CO₂ levels in the shoot environment and with a high NO₃⁻ concentration in the rooting zone. These results indicated that aerial CO₂ concentration can play a role in rhizosphere denitrifier activity.

The fate of nearly all nitrogen that cycles through terrestrial ecosystems (estimated at more than $1,200 \times 10^9$ kg annually; 23) depends on the activities of soil heterotrophic microorganisms (22). The metabolic activities of many heterotrophic microbes may be partially regulated by the flow of recently fixed photosynthate to the root zone (16). Photosynthate lost from roots to the rhizosphere, rhizodeposition, generally consists of high-energy-containing organic compounds that are easily sequestered and metabolized by microbial communities. Elevating atmospheric CO₂ concentration could increase rhizodeposition rates (13) and thus accelerate microbial nitrogen transformations that are dependent on carbon supply. Such nitrogen cycle processes might include mineralization (36), microbial mineral nitrogen assimilation, nitrogen fixation, and denitrification.

Denitrification depends on the availability of reduced organic compounds (4, 5, 8). As a consequence, denitrification activity is generally higher in the rhizosphere, where reduced organic carbon is readily available (10, 20, 28, 33). When resources other than carbon limit denitrification (e.g., NO₃⁻ or NO₂⁻ availability), rhizosphere carbon availability may have a smaller effect (11). Nonetheless, when conditions are favorable for denitrification, rhizodeposition should enhance it by supplying a source of reductant. Since elevated atmospheric CO₂ concentration appears to increase carbon rhizodeposition, elevated CO₂ could have a significant effect on denitrification activity. We examined this hypothesis by assessing potential denitrification enzyme activity on root surfaces and quantifying nitrogen loss from wheat canopies growing under ambient and elevated CO₂ concentrations.

We conducted our experiments in a 28-m³ controlled environment chamber with wheat canopies (*Triticum aestivum* cv. Veery 10) planted at 1,500 plants per m² (25). The chamber is used for fundamental research into bioregenerative life support systems (9). We examined two CO₂ concentrations and two root zone NO₃⁻ concentrations. The two CO₂ concentrations were 360 μmol of CO₂ per mol of air (360 μmol of CO₂ mol⁻¹), close to that in the current Earth atmosphere (6), and 1,000 μmol of CO₂ mol⁻¹, a concentration that saturates net

CO₂ assimilation rates for wheat canopies growing in such contained environments (14, 34). We used a flowing hydroponic culture for the root environments, with four 0.2-m² sub-canopies for each NO₃⁻ treatment (3). Each of the four sub-canopies was supplied from a 270-liter reservoir, with a constant circulation of 36 liters min⁻¹. The oxygen concentrations in the nutrient solutions were examined with a polarographic O₂ electrode placed directly into the solution at three depths. Oxygen concentrations averaged 76% of the saturation concentration at our elevation (220 μM) and did not differ significantly between the nutrient solutions used for the two aerial CO₂ treatments or between those used for the two NO₃⁻ treatments (21). The dissolved CO₂ concentration in the root zones was examined by headspace gas analyses. Briefly, 150 ml of nutrient solution was placed in a 250-ml Erlenmeyer flask that had been equilibrated with the CO₂ environment in the chamber, and the flask was covered with a latex stopper. The flask was placed on a shaker for 15 min to attain solution-gas phase equilibrium. Then two 3-ml gas samples were injected into an infrared gas analyzer calibrated against standards of 300, 560, 880, and 1,820 ppm of CO₂. The CO₂ concentration in the nutrient solution was calculated based on CO₂ solubility, temperature, and pressure (35). For elevated-CO₂ level tests, the solution CO₂ concentration was 36.8 ± 5.9 μM ($n = 4$; two from each NO₃⁻ treatment), less than 1.5 times higher than the expected concentration of approximately 29.7 μM at our elevation. For the ambient-CO₂ level treatment, it was 18.6 ± 7.3 μM ($n = 4$; two from each NO₃⁻ treatment), less than 2 times higher than the equilibrium value of about 10.7 μM. These values of O₂ and CO₂ concentrations indicated that, with the high velocity of nutrient solution flowing through the system (40 liters min⁻¹ m⁻² of canopy), and considering that root respiration consumes and produces large quantities of O₂ and CO₂, respectively, the nutrient solution was well aerated and mixed (27). So if anaerobic or microaerophilic sites that would favor denitrification were present, they probably existed mainly at the root surfaces, where oxygen consumption by roots and rhizoplane microorganisms can greatly reduce oxygen partial pressure (12).

The NO₃⁻ concentrations we used were 100 μM, a concentration slightly higher than the apparent K_m for net NO₃⁻ absorption by many cultivated temperate annuals (63.6 ± 19.5 μM [grand mean \pm standard error {SE}; $n = 8$]) (2, 24), and 1,000 μM, a NO₃⁻ concentration that saturates growth de-

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mands for our wheat canopies. Nitrate concentrations were held constant by two simultaneously operating control systems, in accordance with principles outlined by Bloom (1). The first system used the output signal from a NO_3^- -selective electrode to control metered additions (Razel Corp.; model A-99) of equal amounts of 1 to 2 M KNO_3 and 1 to 2 M $\text{Ca}(\text{NO}_3)_2$. The 1 M concentration was used during the first 10 days of the experiment when plant nitrogen demand was low. The higher concentration of 2 M was used during the final 13 days of the experiment, when plant nitrogen demand was high. The NO_3^- electrode was automatically calibrated every 24 h against standard solutions consisting of 100 or 1,000 μM NO_3^- by using an array of isolatching solenoid valves (General Valve; series 2 Iso-latch). The second system (Omega Engineering; model PHCN-36) used the output signal from a pH electrode suspended directly in the nutrient solution to control direct additions of a mixture of 50 mM HNO_3 plus 50 mM $\text{H}_2(\text{SO}_4)$. This system maintained H^+ concentration in the nutrient solution at $\text{pH } 5.8 \pm 0.1$. Having NO_3^- in the pH control system made the control response faster and more accurate than that used in earlier experiments that were conducted with only $\text{Ca}(\text{NO}_3)_2$ and KNO_3 as the NO_3^- sources. In those experiments, the nitrogen loss quantities we observed were not significantly different from those reported in Table 1 below. Using this dual-control array, we were able to sustain the low- NO_3^- treatment within about 20 μM of its set point concentration and the high- NO_3^- treatment within about 80 μM of its set point.

On the 15th day after germination, five intact plants were removed from each treatment. After shoot removal, N_2O evolution was measured in 250-ml Erlenmeyer flasks under anaerobic conditions, in the presence of C_2H_2 (20% of headspace gas) to block conversion of N_2O to N_2 , 1 g of chloramphenicol per liter to suppress microbial enzyme synthesis, 1.5 mM glucose, and 1.5 mM NO_3^- (31). Five 3-cm³ gas samples were withdrawn from the headspace at 10, 20, 40, 60, and 120 min. N_2O was detected in the samples with a Varian Inc. (model 3300) gas chromatograph equipped with a Poropak Q column maintained at 45°C and a ⁶³Ni detector maintained at 375°C. Total N_2O produced was determined by adding the quantity of N_2O in the headspace gas to that of N_2O dissolved in solution (31). We used the linear regression of total N_2O production over time to determine the rate of N_2O production. All of the regressions yielded significant correlation coefficients (r^2) of no less than 0.84. It has recently been found that chloramphenicol can mildly inhibit denitrification activity in denitrifier assays (19). Consequently, our assays may underestimate denitrification activity, but all of the assays were conducted under identical conditions with the same reagents so that comparisons among treatments were likely valid.

Nitrogen loss was estimated by using a nitrogen balance technique (18). The total amount of NO_3^- consumed by roots and root surface microbial communities was quantified by summing the NO_3^- -N added to the root zone by each control system and then adding that amount to the total amount of NO_3^- -N remaining in the nutrient solution at the end of each experiment (N consumed in Table 1). The biomass from each experiment was harvested, dried in a forced-air oven at 70°C, and weighed. The total amount of nitrogen assimilated in roots, shoots, and root surface microbial biomass plus the amount of NO_3^- stored in plants (N assimilated in Table 1) was determined by analyzing two finely ground root samples of 150 mg and two finely ground shoot samples of 150 mg from each block of each experiment ($n = 8$ for each treatment) in a total carbon, hydrogen, and nitrogen analyzer (LECO, Inc.; model CHN-1000). Nitrogen loss was estimated as the difference between N consumed and N assimilated.

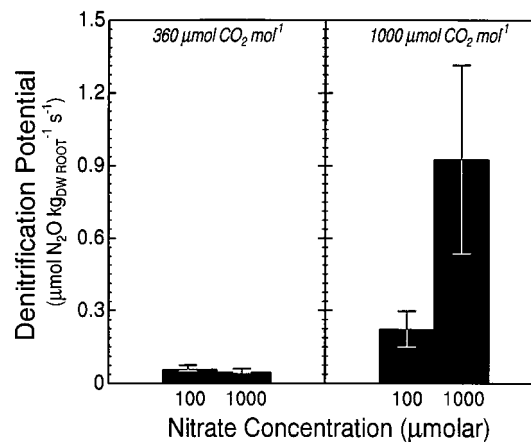


FIG. 1. Potential denitrifying enzyme activity (micromoles of N_2O per kilogram of root dry mass per second) on root surfaces of wheat plants grown under either 360 or 1,000 μmol of CO_2 mol^{-1} . We used two different NO_3^- concentrations under each CO_2 treatment as indicated. Each bar represents the mean \pm one SE for 12 to 16 observations taken from three experiments conducted under 360 μmol of CO_2 mol^{-1} and four experiments conducted under 1,000 μmol of CO_2 mol^{-1} .

We conducted three experiments under 360 μmol of CO_2 mol^{-1} and either 100 or 1,000 μM NO_3^- in the root zone and four experiments under 1,000 μmol of CO_2 mol^{-1} and either 100 or 1,000 μM NO_3^- . This allowed us to analyze data for potential denitrification activity and nitrogen loss according to a split-plot analysis of variance where CO_2 concentration represented the main-plot treatment and NO_3^- concentration represented the subplot treatment (GLM procedure; SAS Institute, Inc.).

Our hypothesis, i.e., that elevating atmospheric CO_2 would enhance rhizosphere denitrifier activity, was supported by a large increase in potential denitrification activity on root surfaces grown under elevated levels of CO_2 ($P = 0.044$; Figure 1). Under elevated levels of CO_2 , potential denitrification activity ranged from 3 times higher on roots from the 100 μM NO_3^- treatment to 24 times higher on roots from the 1,000 μM NO_3^- treatment, compared with the same NO_3^- treatments under ambient levels of CO_2 . We believe that the effect of CO_2 was indirect and was due to increased root nonstructural carbohydrate accumulation and consequent exudation. In our controlled environments, elevated levels of CO_2 increase shoot nonstructural carbohydrate content, and this translates into more than a 30% increase in total root nonstructural carbohydrate content (27). Under ambient levels of CO_2 , potential denitrification activities did not significantly differ between the two root zone NO_3^- treatments of 100 and 1,000 μM . Nonetheless, it appeared there may have been some enhancement of potential denitrification activity in high-concentration NO_3^- treatments for the elevated- CO_2 level condition (Fig. 1); however, the difference was only marginally significant ($P = 0.087$). That NO_3^- did not significantly influence denitrifier activity made sense in terms of the relative affinities that denitrifying microorganisms have for NO_3^- . The apparent K_m for denitrification is approximately 5 to 10 μM NO_3^- (32), 2 to more than 20 times lower than the range measured for plant roots (2, 24) and much lower than the concentrations we maintained in our hydroponics cultures. The above observations, i.e., that carbon rather than NO_3^- limited denitrification activity in these systems, was in general agreement with known resource limitations to denitrification activity in agricultural

TABLE 1. Nitrogen balance for wheat canopies grown in contained environments under two CO₂ concentrations and with two root zone NO₃⁻ concentrations^a

| Ambient CO ₂ concn (μmol of CO ₂ mol ⁻¹) | Nitrate concn (μM) | N consumed (g m ⁻²) ^b | N assimilated (g m ⁻²) ^b | N lost (g m ⁻²) ^c |
|--|--------------------|--|---|--|
| 360 | 100 | 39.12 ± 2.93 | 32.98 ± 1.78 | 6.13 ± 0.71 ^g |
| 360 | 1,000 | 39.56 ± 1.28 | 33.19 ± 1.66 | 6.96 ± 0.61 ^g |
| 1,000 | 100 | 45.69 ± 5.04 | 36.18 ± 3.25 | 9.51 ± 2.43 ^{gh} |
| 1,000 | 1,000 | 48.99 ± 4.60 | 38.21 ± 3.69 | 10.88 ± 1.19 ^h |

^a Shown are the means ± the SEs of the means for three 23-day experiments that were conducted under 360 μmol of CO₂ mol⁻¹ (*n* = 3) and four 23-day experiments conducted under 1,000 μmol of CO₂ mol⁻¹ (*n* = 4). The data were analyzed by analysis of variance by using a split-plot design where CO₂ concentrations represented the main-plot treatments and NO₃⁻ concentrations represented the subplot treatments.

^b The values in this column do not differ significantly (*P* ≥ 0.05).

^c Values in this column with the same superscript letter do not differ significantly (*P* ≥ 0.05).

ecosystems, where carbon rather than NO₃⁻ can be the primary limiting substrate (8).

The differences in denitrifier enzyme activity correlated well with the percentages of nitrogen that were lost from these systems (Table 1). Carbon dioxide fertilization increased biomass yields by 17.22% ± 2.93% (mean ± SE; *P* < 0.01), but NO₃⁻ concentration in the rooting zone did not influence yields. The increase in biomass under elevated levels of CO₂ resulted in an increase in the amount of nitrogen that was assimilated and the amount of nitrogen loss (Table 1). Nitrogen mass balances revealed that 15.67% ± 1.81% (mean ± SE) of the nitrogen consumed was lost from the plants treated with 100 μM NO₃⁻ when they were grown under the low-level CO₂ condition of 360 μmol of CO₂ mol⁻¹ and that 17.59% ± 1.54% of the nitrogen consumed was lost from the plants treated with 1,000 μM NO₃⁻ growing under low levels of CO₂ (Table 1). For the elevated-CO₂ level condition, 20.81% ± 5.32% and 22.21% ± 2.43% of the nitrogen consumed was lost from the plants treated with 100 μM NO₃⁻ and from the plants treated with 1,000 μM NO₃⁻, respectively (Table 1). These nitrogen deficits were most likely due to denitrification losses for several reasons. First, much smaller quantities of nitrogen are generally emitted through other canopy nitrogen loss pathways such as diffusive NH₃ exchange from wheat leaves (17). Our own preliminary measurements of canopy NH₃ emissions, obtained with an acid trap similar to that described by Farquhar et al. (7) and analyzed on a Lachat flow injection auto-analyzer (Lachat Chemicals, Mequon, Wis.), support this contention. These measurements have indicated that during the same 23-day growth period, only 1.2 or 1.4 mg of NH₃-N m⁻² h⁻¹ was volatilized from canopies grown in elevated- or ambient-CO₂ treatments, respectively (14a). These daily NH₃ loss rates agree with previous measurements for wheat canopies (17) and would account for no more than 5% of the total amount of N loss we measured, or on average less than 1% of the total N consumed. Second, bacterial population densities on wheat root surfaces (the rhizoplane) within these systems are high, (e.g., 10⁹ to 10¹² organisms per gram of dry root) (26)). Several taxa within the *Pseudomonas*, *Alcaligenes*, and *Flavobacterium* genera, which are capable of denitrification, are among many rhizoplane microbes consistently colonizing such wheat root surfaces (29). Many of these microbial taxa on wheat rhizoplanes in hydroponic culture are also common in soil ecosystems (8, 32). Third, the amounts of nitrogen lost from our canopies are similar to the amounts of ¹⁵NO₃⁻ di-

rectly denitrified from root zones in similar contained-environment wheat ecosystems (2a, 30). Finally, roots had high microbial population densities, and when we preincubated them for 3 h in a nutrient solution containing 20 mg of ampicillin plus 20 mg of trimethoprim per liter and then conducted the potential denitrification assays, we found that the rates of N₂O emission were reduced to levels that were not detectable under the experimental and analytical conditions used. Hence, it also appeared that practically all of the enzyme activity we measured was of microbial origin.

Among the many uncertainties concerning the influence of elevated atmospheric CO₂ concentration on biosphere processes, the influence of elevated levels of CO₂ on soil biota is probably least well understood (15). We have demonstrated under controlled laboratory conditions and by using hydroponic culture that aerial CO₂ availability can indirectly influence activities of root surface microbial communities responsible for denitrification, a key environmental nitrogen transformation that can be limited by organic carbon availability (8). Determining whether these results have important implications concerning nitrogen trace gas emissions and nitrogen loss rates from terrestrial ecosystems will require careful examination of the effect of elevated levels of CO₂ on soil microbial processes in agricultural ecosystems and natural ecosystems where denitrification plays an important role.

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