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Short Communication

Involvement of nitric oxide in the inhibition of nitrogenase activity by nitrate in *Lotus* root nodules

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ABSTRACT

Nitrogenase activity, as acetylene-reduction activity (ARA), in *Lotus* root nodules was clearly inhibited 27 h after the addition of nitrate. Nitric oxide (NO) production was detected at that time in nitrate-supplied root nodules using the NO-reactive fluorescent probe diaminofluorescein-2 diacetate. The involvement of NO production in the inhibition of nitrogenase activity by nitrate was investigated using the NO donor sodium nitroprusside (SNP) and the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO). SNP inhibited ARA at 1 mM, and c-PTIO suppressed the inhibition of ARA by nitrate. These results suggest that NO is involved in the inhibition of nitrogenase activity by nitrate in *Lotus* root nodules.

Introduction

Nitrate, the major form of inorganic nitrogen in the soil, is first reduced to nitrite by nitrate reductase (NR) and then assimilated into amino acids. Nitrogen from both symbiotic nitrogen fixation and the soil is necessary to raise the production of legume crops. However, root nodules cannot efficiently use nitrogen gas in the presence of significant levels of soil nitrate, since nitrate inhibits nodulation, nodule development, and nitrogenase activity (Streeter, 1988). Therefore, clarifying the mechanism involved in the inhibition of symbiotic nitrogen fixation by nitrate is important for agricultural production. In this study, we focused on the inhibition of nitrogenase activity by nitrate.

Nitrate metabolism in root nodules, rather than in roots, has been reported to be important in the nitrate-induced inhibition of nitrogenase activity (Takahashi et al., 1992). Although NR is present in the root nodules of both bacteroids and the cytosol (plant fraction), bacteroid NR is not involved in the inhibition of nitrogenase activity by nitrate (Streeter, 1985). In a NR-deficient pea mutant (Kaiser et al., 1997), the inhibition of nitrogenase activity by nitrate is partially suppressed. Thus, we focused on plant NR in the nodule cytosol (Kanayama et al., 1999; Kato et al., 2003).

Plant NR not only reduces nitrate to nitrite but also produces nitric oxide (NO) from nitrite using NADH (Yamasaki et al., 1999; Yamasaki and Sakihama, 2000). On the other hand, NO synthase-like activity, which synthesized NO from arginine, in roots and nodules of *Lupinus albus* were also reported (Cueto et al., 1996). Moreover, NO can also be produced by other enzymatic and nonenzymatic pathways (Bethke et al., 2004; Corpas et al., 2004; Neill et al., 2003). NO plays important roles as a signal molecule in plant growth, development, defense responses, programmed cell death, and stomatal closure (Lamattina et al., 2003; Neill et al., 2003). Therefore, NO produced from nitrate may be related to the inhibition of nitrogenase activity by nitrate in legume root nodules.

Leghemoglobin (Lb) plays an important role in providing oxygen to bacteroids, but Lb may have a higher affinity for NO than it does for oxygen (Maskall et al., 1977). Therefore, the formation of LbNO may inhibit the supply of oxygen to bacteroids (Arrese-Igor et al., 1998; Kanayama and Yamamoto, 1990; Vessey and Waterer, 1992). LbNO exists in intact root nodules of soybean plants grown in the absence of nitrate, as revealed by electron paramagnetic resonance (EPR) spectroscopy (Mathieu et al., 1998). NO production in functional root nodules in the absence of nitrogen supply during *Medicago truncatula–Sinorhizobium meliloti* symbiosis was detected using the NO-reactive fluorescent probe diaminofluorescein-2 diacetate (DAF-2DA) (Baudouin et al., 2006). Recently, the formation of LbNO was also reported in soybean root

Abbreviations: ARA, acetylene-reduction activity; c-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-2DA, diaminofluorescein-2 diacetate; Lb, leghemoglobin; NO, nitric oxide; NR, nitrate reductase; SNP, sodium nitroprusside

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nodules treated with nitrate (Meakin et al., 2007). However, information is lacking on NO production and its role in nitrate inhibition. Therefore, we here report the enhancement of NO production in *Lotus* root nodules by the addition of nitrate and the involvement of NO in the inhibition of nitrogenase activity by nitrate.

Materials and methods

Plant material

Japanese trefoil (*Lotus japonicus* (Regel) K. Larsen accession Gifu B-129) was sown on H_2O/agar medium and grown at 25 °C under an 18-h photoperiod. After 9 days, the plants were inoculated with *Mesorhizobium loti* strain New Zealand Palmerston 2235 and repotted in vermiculite. The plants were supplied with a nitrogen-free nutrient solution (Matsumoto et al., 1977). After 4 weeks, the plants were transferred onto filter paper and supplied with solutions containing various compounds. Nodulated root systems were supplied with 10 mM KNO_3 or 10 mM KCl (control).

Acetylene reduction assay

Nitrogenase activity (EC 1.19.6.1) was measured by acetylene reduction assays as described previously (Suganuma et al., 2003). The intact plants were placed in 15-mL vials in the presence of 10% acetylene and incubated at 25 °C. After 30 min, the amount of ethylene produced was determined by gas chromatography. Ethylene production is linearly related over 30 min assay period.

Visualization of NO production in root nodules

NO production was visualized using DAF-2DA (Daiichikagaku Chemical, Tokyo, Japan). Nodulated root systems were supplied with 10 mM KNO_3 or 10 mM KCl (control). After 22 h of incubation, they were transferred to loading buffer (20 mM HEPES–NaOH, pH 7.5) containing 12.5 μM DAF-2DA with 10 mM KNO_3 or 10 mM KCl (control). After 5 h, the excess DAF-2DA was removed by washing the nodulated root systems with loading buffer for 1 h. Images were taken using a confocal laser scanning microscope (FV1000; Olympus, Tokyo, Japan; excitation = 488 nm and emission = 510–550 nm). For figure preparation, photographs were processed using Adobe Photoshop 6.0 (Adobe Systems Inc., San Jose, CA, USA).

The influence of the NO donor or scavenger on nitrogenase activity in root nodules

Nodulated root systems were supplied with various concentrations of the NO donor sodium nitroprusside (SNP). After 27 h of incubation with SNP, acetylene-reduction activity (ARA) was assayed as described above. Nodulated root systems were also treated with the mixture of 1 mM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) (NO scavenger) and 1 mM SNP to clarify whether the influence of SNP on ARA was due to NO. The mixture was reacted under dark condition for 24 h in advance, and then added to nodulated root systems for 27 h.

The influence of the NO scavenger c-PTIO on the inhibition of ARA by nitrate was investigated. First nodulated root systems were supplied with 10 mM KNO_3 (nitrate) or 10 mM KCl (control) containing 1 mM c-PTIO for 27 h without pre-treatment, and the ARA was assayed. Next nodulated root systems were pretreated with 0.2 or 1 mM c-PTIO for 24 h and then supplied with 10 mM KNO_3 or 10 mM KCl (control) containing 0.2 or 1 mM c-PTIO for 27 h. The ARA was also assayed.

Results

Nitrate inhibition of nitrogenase activity in root nodules

ARA in nodules decreased by 34% after the addition of nitrate for 24 h and by 61% when incubated for 27 h (Fig. 1). Thus, a 27-h treatment with nitrate was used in all subsequent experiments.

Visualization of NO production in root nodules

The production of NO was clearly detected in root nodules supplied with nitrate using the NO-reactive fluorescent probe DAF-2DA (Fig. 2). NO-reactive fluorescence was visualized within the infected region of root nodules with nitrate, and it was also slightly detected in the infected region of control root nodules without nitrate. The NO-reactive fluorescence signals in roots did not differ between treatments with and without nitrate (data not shown). Fluorescence signals observed in the epidermal tissues of roots and root nodules were due to endogenous fluorescence (data not shown).

Influence of the NO donor and scavenger on nitrogenase activity in root nodules

The involvement of NO production in the inhibition by nitrate was investigated using the NO donor SNP. ARA decreased by 38% and 94% after the addition of 1 and 10 mM SNP, respectively (Fig. 3). On the other hand, ARA slightly increased following the addition of 0.1 mM SNP. The inhibition of ARA by 1 mM SNP was suppressed by 1 mM c-PTIO, a NO scavenger. This result indicates that the decrease in ARA caused by SNP was due to NO itself.

Influence of the NO scavenger on nitrogenase activity in root nodules supplied with nitrate

Because NO was produced following addition of nitrate, and a NO donor inhibited ARA, we assayed ARA in root nodules supplied with nitrate and the NO scavenger c-PTIO. ARA decreased by 61% after the addition of nitrate without c-PTIO, and by 49% with 1 mM c-PTIO (Fig. 4). The inhibition of ARA by nitrate was slightly sensitive to the addition of c-PTIO when root nodules were not pretreated with c-PTIO. Thus, root nodules were pretreated with c-PTIO as shown in

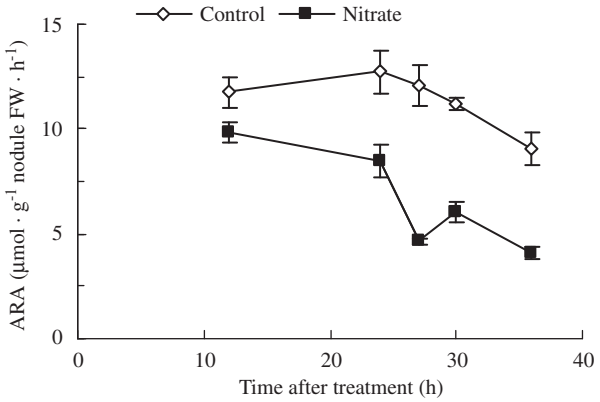

Fig. 1. Changes in acetylene reduction activity caused by addition of nitrate. Nodulated root systems were supplied with 10 mM KCl (control) or 10 mM KNO_3 (nitrate). Vertical bars indicate the standard error ($n = 11$).