Nitrous oxide (N\textsubscript{2}O) is a key atmospheric greenhouse gas that contributes to global warming and the destruction of stratospheric ozone (14, 46, 47). Agricultural land is a major source of N\textsubscript{2}O through the microbial transformation of nitrogen in the soil (13, 24, 58), and contributes significantly to the net increase in atmospheric N\textsubscript{2}O (46). Legume crops emit more N\textsubscript{2}O than non-legumes (10, 15, 32).

Yang and Cai (55) reported that the emission of N\textsubscript{2}O from a soybean field greatly increased in the late growth period. Soybean plants inoculated with nosZ-defective strains of *Bradyrhizobium japonicum* USDA110 (ΔnosZ, lacking N\textsubscript{2}O reductase) were grown in aseptic jars. After 30 days, shoot decapitation (D, to promote nodule degradation), soil addition (S, to supply soil microorganisms), or both (DS) were applied. N\textsubscript{2}O was emitted only with DS treatment. Thus, both soil microorganisms and nodule degradation are required for the emission of N\textsubscript{2}O from the soybean rhizosphere. The N\textsubscript{2}O flux peaked 15 days after DS treatment. Nitrate addition markedly enhanced N\textsubscript{2}O emission. A \textsuperscript{15}N tracer experiment indicated that N\textsubscript{2}O was derived from N fixed in the nodules. To evaluate the contribution of bradyrhizobia, N\textsubscript{2}O emission was compared between a nirK mutant (ΔnirKΔnosZ, lacking nitrite reductase) and ΔnosZ. The N\textsubscript{2}O flux from the ΔnirKΔnosZ rhizosphere was significantly lower than that from ΔnosZ, but was still 40% to 60% of that of ΔnosZ, suggesting that N\textsubscript{2}O emission is due to both *B. japonicum* and other soil microorganisms. Only nosZ-competent *B. japonicum* (nosZ\textsubscript{v} strain) could take up N\textsubscript{2}O. Therefore, during nodule degradation, both *B. japonicum* and other soil microorganisms release N\textsubscript{2}O from nodule via their denitrification processes (N\textsubscript{2}O source), whereas nosZ-competent *B. japonicum* exclusively takes up N\textsubscript{2}O (N\textsubscript{2}O sink). Net N\textsubscript{2}O flux from soybean rhizosphere is likely determined by the balance of N\textsubscript{2}O source and sink.

**Key words:** Nitrous oxide, *Bradyrhizobium japonicum*, Denitrification, nosZ gene, Soybean rhizosphere

A model system developed to produce N\textsubscript{2}O emissions from degrading soybean nodules in the laboratory to clarify the mechanism of N\textsubscript{2}O emission from soybean fields. Soybean plants inoculated with nosZ-defective strains of *Bradyrhizobium japonicum* USDA110 (ΔnosZ, lacking N\textsubscript{2}O reductase) were grown in aseptic jars. After 30 days, shoot decapitation (D, to promote nodule degradation), soil addition (S, to supply soil microorganisms), or both (DS) were applied. N\textsubscript{2}O was emitted only with DS treatment. Thus, both soil microorganisms and nodule degradation are required for the emission of N\textsubscript{2}O from the soybean rhizosphere. The N\textsubscript{2}O flux peaked 15 days after DS treatment. Nitrate addition markedly enhanced N\textsubscript{2}O emission. A \textsuperscript{15}N tracer experiment indicated that N\textsubscript{2}O was derived from N fixed in the nodules. To evaluate the contribution of bradyrhizobia, N\textsubscript{2}O emission was compared between a nirK mutant (ΔnirKΔnosZ, lacking nitrite reductase) and ΔnosZ. The N\textsubscript{2}O flux from the ΔnirKΔnosZ rhizosphere was significantly lower than that from ΔnosZ, but was still 40% to 60% of that of ΔnosZ, suggesting that N\textsubscript{2}O emission is due to both *B. japonicum* and other soil microorganisms. Only nosZ-competent *B. japonicum* (nosZ\textsubscript{v} strain) could take up N\textsubscript{2}O. Therefore, during nodule degradation, both *B. japonicum* and other soil microorganisms release N\textsubscript{2}O from nodule via their denitrification processes (N\textsubscript{2}O source), whereas nosZ-competent *B. japonicum* exclusively takes up N\textsubscript{2}O (N\textsubscript{2}O sink). Net N\textsubscript{2}O flux from soybean rhizosphere is likely determined by the balance of N\textsubscript{2}O source and sink.

Nitrous oxide (N\textsubscript{2}O) is a key atmospheric greenhouse gas that contributes to global warming and the destruction of stratospheric ozone (14, 46, 47). Agricultural land is a major source of N\textsubscript{2}O through the microbial transformation of nitrogen in the soil (13, 24, 58), and contributes significantly to the net increase in atmospheric N\textsubscript{2}O (46). Legume crops emit more N\textsubscript{2}O than non-legumes (10, 15, 32).

Yang and Cai (55) reported that the emission of N\textsubscript{2}O from a soybean field greatly increased in the late growth period, suggesting that senescence and the decomposition of roots and nodules contributed to emissions. Ciampitti et al. (7) also reported marked N\textsubscript{2}O emissions from a soybean field in the late growth period regardless of N fertilization. N\textsubscript{2}O emission from a field with nodulating soybeans was several times higher than that from a field with non-nodulating soybeans (27). N\textsubscript{2}O was emitted directly from degraded nodules of field-grown soybeans in the late growth period (20). Thus, soybean nodules emit N\textsubscript{2}O under field conditions, although the mechanism remains unresolved.

Microorganisms might be involved, as N\textsubscript{2}O can be generated by several microbial processes (4, 13). Using microbial community analysis, Inaba et al. (20) nominated potential N\textsubscript{2}O producers that increased in abundance in degraded nodules. Among them, *Bradyrhizobium japonicum* was one of the dominant microbes as endosymbionts of soybean nodules and rhizosphere soil bacteria (9, 29, 30, 33, 35, 39). It reduces nitrogen oxides during denitrification as

\[ \text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2, \]

where each step is catalyzed by specific reductases. These reductases are encoded, respectively, by *napA* (encoding periplasmic nitrate reductase), *nirK* (Cu-containing nitrite reductase), *norCB* (nitric oxide reductase), and *nosZ* (nitrous oxide reductase) (5). The aim of this study was to clarify the involvement of *B. japonicum* in the emission of N\textsubscript{2}O from the soybean rhizosphere. The N\textsubscript{2}O flux from denitrification mutants of *B. japonicum* was compared in the laboratory.

**Materials and Methods**

**Bacterial strains, plasmids, and media**

The bacterial strains and plasmids are listed in Table 1. *Bradyrhizobium japonicum* cells were grown at 30°C in HM salt medium (8) supplemented with 0.1% arabinose and 0.025% (w/v) yeast extract (Difco, Detroit, MI, USA). *Escherichia coli* cells used in transformation were grown at 37°C in Luria–Bertani medium (40). Antibiotics were added to the media at the following concentrations: for *B. japonicum*, 100 μg tetracycline (Tc) mL\textsuperscript{-1}, 100 μg spectinomycin (Sp) mL\textsuperscript{-1}, 100 μg streptomycin (Sm) mL\textsuperscript{-1}, 100 μg kanamycin (Km) mL\textsuperscript{-1}, and 100 μg polymyxin B (PolB) mL\textsuperscript{-1}, for *E. coli*, 50 μg Tc mL\textsuperscript{-1}, 50 μg Sp mL\textsuperscript{-1}, 50 μg Sm mL\textsuperscript{-1}, 50 μg Km mL\textsuperscript{-1}, and 50 μg ampicillin mL\textsuperscript{-1}.

**Construction of B. japonicum mutants**

Isolation of plasmids, DNA ligation, and transformation of *E. coli* were performed as described previously (40). DNA was prepared as described previously (43). A 5.6-kb BamHI/EcoRI fragment...
centrifuged at 5,555×g. The pellet was resuspended in 30 mL distilled water. The suspension was shaken for 10 min in centrifuge tubes and then washed twice with 30 mL distilled water to remove nitrate and nitrite. The washed material was then used for DNA extraction.

Preparation of soil suspension

Surface-sterilized soybean seeds (Glycine max cv. Enrei) were germinated in sterile vermiculite for 2 days at 25°C. The seedling was then transplanted into a Leonard jar pot (one plant per pot) (28, 53, 56), which contained sterile vermiculite and nitrogen-free nutrient solution (31, 34) (Fig. S1). The seedlings were then transplanted into a Leonard jar pot (one plant per pot) (28, 53, 56), which contained sterile vermiculite and nitrogen-free nutrient solution (31, 34) (Fig. S1). The seedlings were then transferred to the pot. The pots were left in a dark chamber for 15 days except for N2O determination for 15 days except otherwise indicated.

N2O determination

N2O flux was determined with a gas chromatograph (GC-14BpsE; Shimadzu, Kyoto, Japan) equipped with a 63Ni electron capture detector and a thermal conductivity detector. The N2O flux was determined by the analysis of N2O in air sampled from the rhizosphere of the plants.

Table 1. Bacterial strains and plasmids used in this study

| Strain or plasmid           | Relevant characteristicsa | Source or reference |
|-----------------------------|---------------------------|---------------------|
| 
| Strains                     |                           |                     |
| 
| Bradyrhizobium japonicum    |                           |                     |
| USDA110                     | Wild type, nosZ+          | 25                  |
| USDA110ΔnosZ                | USDA110 derivative, nosZ::del/ins Tc cassette; Te+ | 18                  |
| USDA110ΔnapAΔnosZ           | USDA110 derivative, napA::Ω cassette, nosZ::del/ins Tc cassette; Ssp’, Sm’, Te+ | 18                  |
| USDA110ΔnirK                | USDA110 derivative, nirK::Ω cassette; Ssp’, Sm’ | This study          |
| USDA110ΔnirKΔnosZ           | USDA110 derivative, nirK::Ω cassette, nosZ::del/ins Tc cassette; Ssp’, Sm’, Te+ | This study          |
| T9                          | Field isolate in Tokachi, Hokkaido, Japan, nosZ- | 42                  |
| 
| Escherichia coli            |                           |                     |
| DH5a                        | recA; cloning strain      | Toyobo              |
| 
| Plasmids                    |                           |                     |
| brp01958                    | pUC18 carrying nirK       | 25                  |
| pH43Ω                       | Plasmid carrying 2.1-kb Ω cassette; Ssp’, Sm’, Ap’ | 37                  |
| pK18mob                     | Cloning vector; pMB1ori Tc; Km’ | 44                  |
| pK18mob-nirK                | pK18mob carrying 5.6-kb nirK fragment; Km’ | This study          |
| pK18mob-nirK::Ω             | pK18mob carrying nirK::Ω cassette; Km’, Ssp’, Sm’ | This study          |
| pRK2013                     | ColE replicon carrying RK2 transfer genes; Km’ | 12                  |
| 
| a Ap’, ampicillin resistant; Te’, tetracycline resistant; Km’, kanamycin resistant; Ssp’, streptomycin resistant. |                     |                     |

Preparation of soil suspension

Soil was collected from an experimental field at Tohoku University (Kashimadai, Miyagi, Japan). This gray lowland soil had pH[H2O] 5.6, pH[KCl] 4.2, total C 1.37%, total N 0.132%, and C/N 7.6. Fresh soil (10 g) was extracted twice with 30 mL distilled water to remove nitrate and nitrite. The suspension was shaken for 10 min in centrifuge tubes and then centrifuged at 5,555×g for 15 min (Himac CR20E; Hitachi, Tokyo, Japan). The pelleted was resuspended in 30 mL distilled water.

Inoculation and plant cultivation

Surface-sterilized soybean seeds (Glycine max cv. Enrei) were inoculated with USDA110ΔnosZ. The seedling was then transplanted into a Leonard jar pot (one plant per pot) (28, 53, 56), which contained sterile vermiculite and nitrogen-free nutrient solution (31, 34) (Fig. S1). The seedlings were then transplanted into a Leonard jar pot (one plant per pot) (28, 53, 56), which contained sterile vermiculite and nitrogen-free nutrient solution (31, 34) (Fig. S1). The seedlings were then transplanted into a Leonard jar pot (one plant per pot) (28, 53, 56), which contained sterile vermiculite and nitrogen-free nutrient solution (31, 34) (Fig. S1). The seedlings were then transplanted into a Leonard jar pot (one plant per pot) (28, 53, 56), which contained sterile vermiculite and nitrogen-free nutrient solution (31, 34) (Fig. S1). The seedlings were then transplanted into a Leonard jar pot (one plant per pot) (28, 53, 56), which contained sterile vermiculite and nitrogen-free nutrient solution (31, 34) (Fig. S1). The seedlings were then transplanted into a Leonard jar pot (one plant per pot) (28, 53, 56), which contained sterile vermiculite and nitrogen-free nutrient solution (31, 34) (Fig. S1). The seedlings were then transplanted into a Leonard jar pot (one plant per pot) (28, 53, 56), which contained sterile vermiculite and nitrogen-free nutrient solution (31, 34) (Fig. S1). The seedlings were then transplanted into a Leonard jar pot (one plant per pot) (28, 53, 56), which contained sterile vermiculite and nitrogen-free nutrient solution (31, 34) (Fig. S1).

Fig. 1. Construction of a nirK insertion mutant of Bradyrhizobium japonicum USDA110. Cloned fragments in pK18mob derivatives are shown alongside the physical map of the nir gene cluster of Bradyrhizobium japonicum USDA110. See text for details.
detector and tandem columns packed with Porapak Q (80/100 mesh; 3.0 mm×1.0 m and 3.0 mm×2.0 m).

Model system for N$_2$O emission from degraded nodules

USDA110 (nosZ$^+$), USDA110$\Delta$nosZ (nosZ$^-$), and T9 (nosZ$^-$) were used as inoculants. Thirty days after inoculation, treatments were applied (Fig. 2). Ten days later, nodules were collected from soybean roots, washed with sterilized water, and weighed. The nodules were introduced into a 19-mL airtight vial. Gas in the vial was sampled 1, 2, and 3 h after the vials were sealed to determine N$_2$O concentration. This was the “excised nodule method” (Fig. 2).

Long-term N$_2$O monitoring

T9 was used as inoculum. Thirty days after inoculation, the D or DS treatment was applied. The N$_2$O flux from the pot was intermittently monitored during 2 months. On each measurement day, the pot was sealed with a lid with a gas sampling port (Fig. 2). After 5 h, the gas was sampled to determine N$_2$O concentration. After the gas sampling, the pot was returned to the phytotron. This was the “sealed jar method” (Fig. 2).

$^{15}$N$_2$ feeding and $^{15}$N determination

At 29 days after inoculation, a gas mixture (30% [v/v] $^{15}$N$_2$, 20% O$_2$, 50% Ar; SI Sciences, Tokyo, Japan) containing 32.2 atom% $^{15}$N$_2$ was supplied to the root zone of soybeans inoculated with USDA110$\Delta$nosZ in seven pots for 8 h (Fig. 2 and S1). The nodules from three plants were separately collected and dried at 80°C for 3 days. The $^{15}$N concentrations of the powdered nodules were determined by mass spectrometer (EA 1110 DeltaPlus Advantage ConFlo III; Thermo Fisher Scientific, Bremen, Germany). The other four pots received the DS treatment. Fifteen days later, the gas phase was sampled by the sealed jar method (Fig. 2). The $^{15}$N concentrations were determined by gas chromatography/mass spectrometry (GC/MS-QP2010 Plus; Shimadzu) (21, 22).

N$_2$O emission from degraded nodules with denitrification mutants

USDA110, USDA110$\Delta$nosZ, USDA110$\Delta$nirK$\Delta$nosZ, and USDA110$\Delta$nirK$\Delta$nosZ were used as inoculants. Thirty days after inoculation, D or DS treatment was applied (Fig. 2). Fifteen days later, the N$_2$O flux from the nodules was determined by the excised nodule method.

N$_2$O flux from soybean rhizosphere with denitrification mutants

USDA110 and its $\Delta$nosZ, $\Delta$nirK, and $\Delta$nirK$\Delta$nosZ mutants were used as inoculants to evaluate the effect of the $\Delta$nirK and nosZ genes on N$_2$O emission from the rhizosphere. The $\Delta$nirK mutation was selected as a nitrate-to-N$_2$O denitrification mutation, because the $\Delta$nirK mutant is not able to denitrify both nitrate and nitrite that exist in the rhizosphere (4). Thirty days after inoculation, DS treatment was applied. Fifteen days later, the N$_2$O flux from each pot was determined by the sealed jar method, 3 h after the pot was sealed. In addition, 50 mL of 5 mM KNO$_3$ solution was applied to each pot, the pots were immediately sealed, and the N$_2$O flux was determined as above.

Results

N$_2$O emission from degraded nodules

When B. japonicum USDA110 (nosZ$^+$) was used as the inoculum, N$_2$O was not emitted in any treatment (Fig. 3A). When USDA110$\Delta$nosZ or T9 (each nosZ$^-$) was used, the DS treatment induced marked N$_2$O emission, whereas the D and S treatments alone did not induce N$_2$O emission (Fig. 3B and C). Indeed, the nodules in the DS treatment were clearly degraded (Fig. S2), similar to those of field-grown soybean in the late growth period (20). On the other hand, the nodules in the S treatment stayed intact, and those in the D treatment looked slightly degraded (Fig. S2). These results indicate that both soil microbes and nodule degradation are required for N$_2$O emission. In addition, N$_2$O was emitted only from DS-treated nodules with nosZ$^-$ strains, suggesting that the B. japonicum nosZ gene is critical in the emission of N$_2$O from degraded nodules.

Long-term monitoring of N$_2$O flux from the soybean rhizosphere

Substantial N$_2$O was emitted from the rhizosphere of soybeans inoculated with T9 (nosZ$^-$) in DS treatment, but none was emitted in D treatment throughout the experimental period (5–63 days) (Fig. 4). This result is similar to the results in the excised nodule method (Fig. 3B and C). As the N$_2$O flux in the DS treatment peaked 15 days after the treatment was applied and then gradually decreased (Fig. 4), we measured N$_2$O flux at 15 days in later experiments.
Origin of N$_2$O-N

The profile of N$_2$O flux (Fig. 4) suggests that the source of N$_2$O was limited. Thus, we examined whether N$_2$O is derived from N fixed in the nodules by using $^{15}$N-labeled dinitrogen. The supply of $^{15}$N$_2$ to the root zone of USDA110ΔnosZ plants just before DS treatment produced $^{15}$N concentration in N$_2$O emitted 15 days later of 1.32±0.42 atom% excess (mean ± SD), similar to the concentration of nodule N (1.13±0.08 atom% excess). This result clearly indicates that the N$_2$O-N emitted from the soybean rhizosphere was derived from N fixed symbiotically in the nodules.

N$_2$O emission from degraded nodules with denitrification mutants

N$_2$O emissions from the nodules formed with USDA110 and its mutants were determined by the excised nodule method to reveal the involvement of bradyrhizobial denitrification (Fig. 5). Nodules inoculated with ΔnosZ, ΔnapAΔnosZ, and ΔnirKΔnosZ emitted marked amounts of N$_2$O in DS treatment. Nodules inoculated with USDA110 emitted negligible N$_2$O even in DS treatment (Fig. 5A).

Because the nosZ gene is responsible for the reduction of N$_2$O to N$_2$ (18, 43), and no N$_2$O was emitted from nosZ+ nodules (Figs. 3A and 5A), N$_2$O reductase encoded by nosZ is likely a sink for N$_2$O in the soybean rhizosphere. In the absence of nosZ, N$_2$O emission from nodules inoculated with double mutants (ΔnapAΔnosZ and ΔnirKΔnosZ) was lower than that from nodules with ΔnosZ, although there was no significant difference (Fig. 5B, C, and D, t-test [P<0.05]).

N$_2$O flux from the soybean rhizosphere with denitrification mutants

When soybean plants were inoculated with USDA110 and ΔnirK, a small quantity of N$_2$O was released (1.9–2.6 nmol h$^{-1}$ per pot; Fig. 6A). When plants were inoculated with ΔnosZ and ΔnirKΔnosZ, N$_2$O emission was significantly higher (16.7 and 9.9 nmol h$^{-1}$ per pot, respectively). These results strongly suggest that the nosZ gene of B. japonicum is involved in the uptake of N$_2$O that is released from degraded nodules. In Fig. 6A, the relative contribution of the
denitrifying pathway of difference is due to the loss of nitrite reductase in the $H_2$ two distinct sources; denitrification up to $N_2$ substrate for $N_2$. Therefore, the $N_2O$ flux from soybeans inoculated with USDA110 or its denitrification mutants ($\Delta nosZ$, $\Delta nirK$, $\Delta nirK\Delta nosZ$) in (A) the absence and (B) the presence of KNO$_3$. Bars indicate standard error with five biological replications. Differences in $N_2O$ flux are shown as follows: CZ1 and CZ2, contribution of $nosZ$ in $B. japonicum$; CK1 (41%) and CK2 (60%), relative contribution of $nirK$ under a $\Delta nosZ$ mutant background; CS1 (59%) and CS2 (40%), relative contribution of other soil organisms. Bars labeled with the same letter within a graph are not significantly different ($t$-test, $P<0.05$).

$nosZ$ gene to $N_2O$ flux is shown as “CZ1”. In the absence of $nosZ$, there was a significant difference in $N_2O$ flux between $\Delta nosZ$ and $\Delta nirK\Delta nosZ$ (CK1 in Fig. 6A). This difference is due to the loss of nitrite reductase in the denitrifying pathway of $B. japonicum$. Therefore, the $N_2O$ flux from soybeans inoculated with $\Delta nosZ$ could have had two distinct sources; denitrification up to $N_2O$ by $B. japonicum$ (CK1 [41%] in Fig. 6A), and other soil microbes (CS1 [59%] in Fig. 6A).

KNO$_3$ was added to the rhizosphere to clarify whether $N_2O$ is a precursor of N$_2$. When KNO$_3$ was supplied before $N_2O$ determination, the $N_2O$ flux from the pots with each inoculant was markedly enhanced, particularly from pots with $\Delta nosZ$ (78.1 nmol h$^{-1}$ pot$^{-1}$) and $\Delta nirK\Delta nosZ$ (31.3 nmol h$^{-1}$ per pot; Fig. 6B). This result confirms that $N_2O$ was produced from NO$_3$- through microbial denitrification. KNO$_3$ application also enhanced the contribution of $B. japonicum$ to $N_2O$ flux (60% [CK2, Fig. 6B] cf. 41% [CK1, Fig. 6A]). These results suggest that $B. japonicum$ prefers nitrate as a substrate for $N_2O$ production.

**Discussion**

The term “rhizosphere” was first coined in 1904 by Lorenz Hiltner in Germany, who had a special interest in complicated N transformations around leguminous nodules with higher N contents in fields (16). In a sense, the present study advances such historical work on leguminous rhizospheres.

The results show that $N_2O$ emission from degraded nodules in the soybean rhizosphere is due to $B. japonicum$ and other soil microbes. When plants were inoculated with $B. japonicum$ $nosZ$ strains and treated with shoot decapitation and soil addition (DS), $N_2O$ was markedly produced (Figs. 3, 4, 5, and 6). On the other hand, when plants were inoculated with a $nosZ$ strain, almost no $N_2O$ was emitted, even in DS treatment. These results suggest that $N_2O$ emission from degrading nodules formed with $nosZ$ strains was due to denitrification by both $B. japonicum$ ($nosZ$) and other soil microbes (Fig. 7). It is likely that $N_2O$ produced by soil microbes was offset by $nosZ$-competent $B. japonicum$ with its $N_2O$ reductase. In other words, both $B. japonicum$ and other soil microorganisms release $N_2O$ during nodule degradation ($N_2O$ source), and $nosZ$-competent $B. japonicum$ ($nosZ$ strains) takes up $N_2O$ ($N_2O$ sink) (Fig. 7).

What are these other soil microorganisms that emit $N_2O$ from degrading nodules? Prokaryotic denitrification, fungal denitrification, ammonium oxidation, and nitrate ammonification have been nominated as soil microbial sources of $N_2O$ (1, 14, 26, 38, 49, 50, 57). Community analysis specific to degrading nodules that emit $N_2O$ found many microorganisms that potentially produce $N_2O$ (20), including denitrifying bacteria such as *Acidovorax* (19) and *Enterobacter* (2); *Bradyrhizobium* (25), *Salmonella* (48), *Xanthomonas* (52), and *Pseudomonas* (36), which have functional genes and/or activities for denitrification; and *Fusarium*, a denitrifying fungus (43). Since *Fusarium* species generally lacks $N_2O$ reductase (51), it might be one of the key sources of $N_2O$ from degrading nodules.

The decline in $N_2O$ emission after the peak (Fig. 4) indicates that the source of N in the rhizosphere is limited. Indeed, the $^{15}N$ tracer experiment showed that nodule N is a major source of $N_2O$ emission from the soybean rhizosphere. Thus, complicated N transformation in the soybean rhizosphere would involve ammonification, nitrification, and denitrification.
KNO₂ addition enhanced N₂O emission (Fig. 6), supporting the idea that NO₂⁻ is a precursor of N₂O. When NH₄Cl was preliminarily added to the rhizosphere, the addition did not change N₂O emission (Inaba et al., unpublished data), suggesting that it is unlikely to be due to nitrification. KNO₃ addition also enhanced the contribution of _B. japonicum_ to N₂O emission in relation to the other soil microorganisms (Fig. 6). Nitrate might be more available to _B. japonicum_, whereas other microorganisms might prefer other substrates. In fact, nitrite is a better substrate for denitrifying fungi to produce N₂O (45). New approaches are needed to understand soil N₂O-producing microorganisms and N transformation from fixed nitrogen in the rhizosphere (4).

In soybean fields, it is likely that soybean roots are infected with multiple strains that differ in denitrifying activity. _nosZ_ strains of _B. japonicum_ that produce N₂O as the denitrification end product often dominate in agricultural fields (3, 6, 11, 41, 42, 54). Both N₂- and N₂O-producing strains occurred in paddy-upland rotation fields (3). Similarly, both _nosZ+_- and _nosZ–_ strains of _B. japonicum_ were isolated from soybean fields (41, 42). Thus, it is easily conceivable that both N₂- and N₂O-producing strains of _B. japonicum_ coexist in soybean fields. Consequently, the flux of N₂O from soybean fields during the late growth period may be partly determined by biotic factors, namely the balance between N₂O emission due to soil microorganisms and _B. japonicum (nosZ–)_ and N₂O uptake by _B. japonicum (nosZ+)_. (Fig. 7).

The use of _nosZ+_- strains of _B. japonicum_ as inoculants has been expected to reduce N₂O emissions from soybean fields (42, 43). Indeed, _nosZ+_- strains produced no N₂O and were able to take up N₂O from degraded nodules (Fig. 7). Recently, N₂O reduction by _nosZ_-carrying inoculants was shown in a soil-filled pot planted with soybeans (17). Thus, _B. japonicum_ mutants with increased N₂O activity (23) might be more effective to reduce net N₂O flux from soybean rhizosphere.

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