Nitrogen Cycling in Soybean Rhizosphere: Sources and Sinks of Nitrous Oxide (N₂O)

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Nitrous oxide (N₂O) is the third most important greenhouse gas after carbon dioxide and methane, and a prominent ozone-depleting substance. Agricultural soils are the primary anthropogenic source of N₂O because of the constant increase in the use of industrial nitrogen (N) fertilizers. The soybean crop is grown on 6% of the world’s arable land, and its production is expected to increase rapidly in the future. In this review, we summarize the current knowledge on N-cycle in the rhizosphere of soybean plants, particularly sources and sinks of N₂O. Soybean root nodules are the host of dinitrogen (N₂)-fixing bacteria from the genus *Bradyrhizobium*. Nodule decomposition is the main source of N₂O in soybean rhizosphere, where soil organisms mediate the nitrogen transformations that produce N₂O. This N₂O is either emitted into the atmosphere or further reduced to N₂ by the bradyrhizobial N₂O reductase (N₂OR), encoded by the nos gene cluster. The dominance of nos⁻ indigenous populations of soybean bradyrhizobia results in the emission of N₂O into the atmosphere. Hence, inoculation with nos⁺ or nos++ (mutants with enhanced N₂OR activity) bradyrhizobia has proved to be promising strategies to reduce N₂O emission in the field. We discussed these strategies, the molecular mechanisms underlying them, and the future perspectives to develop better options for global mitigation of N₂O emission from soils.

Keywords: *Bradyrhizobium*, soybean, rhizosphere, denitrification, N₂O reductase, nos regulation, greenhouse gas, mitigation strategies
supplies photosynthetically fixed carbon to the bacteria. Although rhizobia have been extensively studied as inhabitants of legume nodules, only few studies have focused on them as denitrifiers in the legume rhizosphere (Inaba et al., 2009, 2012; Shiina et al., 2014; Saeki et al., 2017).

Soybean [Glycine max (L.) Merr.] is grown on 6% of the world’s arable land. Its production increased from 17 to 230 million metric tons in the past 50 years, and is expected to increase rapidly in the future due to an increased demand for soybean meal and oil (Uchida and Akiyama, 2013). Soybean generally hosts rhizobia from the genus Bradyrhizobium (Argaw, 2014). In addition to fixing N₂, many soybean-associated Bradyrhizobium strains contain genes for some, or all of the four denitrification reductases. Denitrification is an alternate respiratory process in which the oxidized forms of N in the soil – nitrate (NO₃⁻) and nitrite (NO₂⁻) – are used as electron acceptors in oxygen limiting conditions. NO₂⁻ is reduced to nitric oxide (NO), nitrous oxide (N₂O), and N₂ gases, which are returned to the atmosphere (Figure 1). The complete denitrification pathway in soybean Bradyrhizobium requires four enzymes, periplasmic NO₂⁻ reductase (Nap), copper (Cu)-containing NO₂⁻ reductase (NirK), c-type NO reductase (cNor), and N₂O reductase (N₂OR) (Figure 1). Bradyrhizobial denitrification is functional under both free-living (for example, in the soybean rhizosphere) and symbiotic (inside the root nodules) conditions (Sameshima-Saito et al., 2006a; Sánchez et al., 2011; Inaba et al., 2012).

N₂O is the third most important greenhouse gas after carbon dioxide (CO₂) and methane (CH₄) and is currently the major ozone-depleting compound in the stratosphere (Hénault et al., 2012; Thomson et al., 2012). Terrestrial ecosystems are the main source of N₂O, primarily due to the use of industrial N fertilizers in agriculture (Hénault et al., 2012; Thomson et al., 2012; Intergovernmental Panel on Climate Change [IPCC], 2014). The soybean rhizosphere is a hotspot for N transformations including production and removal of N₂O. Nodule decomposition is a major source of N₂O, particularly in soybean ecosystems, compared to other possible sources including aboveground plant residues. Inaba et al. (2009) showed that N₂O is only emitted by decomposed nodules, but not by fresh nodules or roots. Studies showed that N₂O emission not only occurs from decomposed nodules after soybean harvesting, but also starts before the harvest till the late growth period (Yang and Cai, 2005; Inaba et al., 2012; reviewed by Uchida and Akiyama, 2013). Biological N₂ fixation is an important indirect source for N₂O during nodule decomposition. Indeed, a ¹⁵N tracer experiment revealed that the N₂O emitted from the soybean rhizosphere was almost entirely derived from N₂ fixed symbiotically in the nodules (Inaba et al., 2012; Figure 1). During nodule decomposition, rhizospheric microbes are essential for N₂O emission; organic N from the nodule is mineralized into ammonium (NH₄⁺, Figure 1); N₂O is then produced via nitrification and denitrification (Inaba et al., 2009, 2012; Figure 1). Although soybean Bradyrhizobium are important players in denitrification (responsible for ~41% of the total N₂O produced), but other denitrifying microorganisms are also important contributors (~59% of the total N₂O produced; Inaba et al., 2012). Populations of nematodes, protozoans, and fungi were markedly enhanced in the soybean rhizosphere of decomposing nodules, suggesting that these organisms contributed to the complex N transformation (Inaba et al., 2009). N₂O formed by denitrification is either emitted into the atmosphere or is further reduced to N₂ by N₂O reductase of soybean Bradyrhizobium (Sameshima-Saito et al., 2004, 2006b; Inaba et al., 2012; Figure 1). In soybean fields, both N₂- and N₂O-producing soybean Bradyrhizobium strains coexist; therefore soybean roots are infected with multiple Bradyrhizobial strains that differ in denitrifying activity (Sameshima-Saito et al., 2004, 2006b; Shiina et al., 2014). Thus, the flux of N₂O from soybean fields during nodule decomposition is partly determined by biotic factors like the balance between N₂O emission by soil microbes whose denitrification produce N₂O (including Bradyrhizobium) and N₂O uptake by soybean Bradyrhizobium that produce N₂O (Inaba et al., 2012; Figure 1).

**NITROUS OXIDE REDUCTASE: THE KEY ENZYME TO REDUCE N₂O EMISSION**

N₂OR is a Cu-containing enzyme that catalyzes the two-electron reduction of N₂O to N₂, which is the only known pathway for the removal of N₂O from ecosystems (Richardson et al., 2009). Therefore, the expression and activity of N₂OR is a natural target to mitigate N₂O emission from agricultural soils.

In *Bradyrhizobium diazoefficiens* (reclassified from *Bradyrhizobium japonicum* by Delamuta et al., 2013), the N₂OR (NosZ) and its accessory functions are encoded by nosRZDYFLX gene cluster (Velasco et al., 2004). The flavoproteins NosR and NosX form an electron transport pathway from the quinone pool to NosZ; NosR is also required for the transcription of nos genes (Velasco et al., 2004; Zumft and Krones, 2007). NosD, NosF, NosY, and NosL are involved in maturation of the Cu₂⁺ site of NosZ (Zumft and Krones, 2007). Although the reduction of N₂O to N₂ by N₂OR is integrated as the last step of the denitrification pathway, it can provide a benefit for N₂O respiration as a separate module. When N₂O is provided as the sole electron acceptor to *B. diazoefficiens*, anaerobic respiration and growth are sustained by reducing N₂O to N₂ (Zumft, 1997; Sánchez et al., 2013; Graf et al., 2014).

*Bradyrhizobium diazoefficiens* carries the nos gene cluster (nos+) and denitrifies NO₃⁻ to N₂, whereas other soybean Bradyrhizobium including *B. japonicum* lack the nos gene cluster (nos⁻) and cannot reduce N₂O to N₂ (Sameshima-Saito et al., 2004, 2006b; Inaba et al., 2012). Sameshima-Saito et al. (2006a) showed that soybean roots nodulated with *B. diazoefficiens* could scavenge very low concentrations of exogenous N₂O, equivalent to the natural concentration of N₂O in air (~0.34 ppm; Badr and Probert, 1992). Later, pot studies demonstrated that soybean roots inoculated with nos⁺ strains have the potential to reduce N₂O derived from decomposing nodules and other N sources from fertilizer and soil organic matter (Hénault and Revellin, 2011; Inaba et al., 2012; Uchida and Akiyama, 2013). Thus, *Bradyrhizobium nos⁺* strain inoculation is a promising strategy for mitigating N₂O emission at the field scale. This
Nitrogen cycling in soybean rhizosphere. Organic nitrogen in the nodule is mineralized into NH$_4^+$ that will be transformed into N$_2$O through nitrification and denitrification processes. Soybean bradyrhizobia (blue) and other microorganisms contribute to the denitrification process. The N$_2$O formed is either emitted into the atmosphere (red) or further reduced to N$_2$ exclusively by soybean bradyrhizobia that produce N$_2$O reductase (N$_2$OR; blue). Nase, nitrogenase; NR, dissimilatory nitrate reductase; Nap, periplasmic nitrate reductase; NirK, Cu-containing nitrite reductase; NOR, nitric oxide reductase; cNor, c-type nitric oxide reductase. See text for more details.

is likely effective in soybean soils that act as an N$_2$O source, a condition that potentially arises from several situations like (i) indigenous bradyrhizobia community being dominated by nos$^-$ species (Sameshima-Saito et al., 2004, 2006b; Shiina et al., 2014), (ii) anoxic conditions such as waterlogging that induce N$_2$O emissions from denitrification by *Bradyrhizobium* (Tortosa et al., 2016) and other microorganisms, and (iii) increased NO$_3^-$ supply as a consequence of heavy N fertilization leading to increased N$_2$O emission from intact soybean root systems via bradyrhizobial denitrification (Ciampitti et al., 2008; Hirayama et al., 2011; Inaba et al., 2012).

Among the N$_2$O-mitigation options for agricultural soils, the first biological method for the field scale was described by Itakura et al. (2013). Mutants of *B. diazoefficiens* USDA110 with a high nos expression and N$_2$O activity (nos$^{++}$ strains) were generated by a mutational strategy (Itakura et al., 2008). This strategy involved (1) introduction of a plasmid containing a mutated dnaQ gene (pKQ2) to enhance replication error on the *B. diazoefficiens* genome by disrupting the exonuclease proofreading activity of DNA polymerase, (2) enrichment culture under selection pressure favoring anaerobic N$_2$O respiration, and (3) elimination of the pKQ2 plasmid by nodulation. Thus, the resulting mutants were not genetically modified organisms (GMOs). The nos$^{++}$ mutants retained higher nos expression and N$_2$O activity in both free-living and symbiotic cells than the wild-type nos$^+$ strains (Itakura et al., 2013; Sánchez et al., 2014). Comparative analysis of the nos$^{++}$ mutant genomes revealed the mechanism underlying the nos$^{++}$ phenotype, a point mutation in nosS gene encoding the NO$_3^-$ sensor of the two-component NasST regulatory system (Sánchez et al., 2014, 2017), which will be discussed later.

The effectiveness of N$_2$O emission mitigation by the nos$^{++}$ mutant was first confirmed under simulated field conditions in a pot experiment with Andosol soil, which predominantly contains nos$^-$ bradyrhizobia (Itakura et al., 2013). N$_2$O emission from the Andosol soil inoculated with the nos$^{++}$ mutant strain was significantly reduced compared with that inoculated with wild-type nos$^+$ strain. Itakura et al. (2013) demonstrated that inoculation of nos$^{++}$ strains to growing soybean in Andosol soil reduced postharvest N$_2$O emission by 43% in the lysimeter study and by 54% in the farm-scale study. However, reduction in postharvest N$_2$O emission by inoculation with the nos$^{++}$ strains was not significant in a Gleysol soil, which predominantly contains nos$^+$ bradyrhizobia, although the nos$^{++}$ strains clearly showed higher N$_2$O-reducing potential than that of the nos$^+$ strains under laboratory conditions (Itakura et al., 2013; Shiina et al., 2014). Thus, some factors present in the soybean rhizosphere of Gleysol soil limited the potential N$_2$O mitigation ability of the nos$^{++}$ strains.

A recent report showed that inoculation of soil with a mixed and enriched culture of indigenous nos$^+$ strains of the *B. diazoefficiens* USDA110 group isolated from agricultural fields efficiently mitigated N$_2$O emission (Akiyama et al., 2016). As in the nos$^{++}$ approach above, inoculation with the mixed culture was successful in soils dominated by nos$^-$ bradyrhizobia. Additionally, this mixture is expected to be more competitive and
adaptable to changing environmental factors than a single strain (Akiyama et al., 2016). This method is an alternative to GMOs and overcomes the problem of strong opposition to them.

REGULATION OF N\textsubscript{2}O REDUCTASE GENES IN BRADYRHIZOBIA

Considering the importance of N\textsubscript{2}OR in N\textsubscript{2}O removal from ecosystems, significant progress has been made in understanding its genetic regulation in bacteria, especially in the denitrifying bacteria, Paracoccus denitrificans (reviewed by Gaimster et al., 2018), and B. diazoefficiens as a model for denitrification in legume-associated rhizobia. In the latter bacterium, the nos\textsubscript{R} gene is constitutively expressed at a low level from the promoter P\textsubscript{a} in aerobiosis, but is strongly induced from the promoter P\textsubscript{d} under denitrifying conditions (i.e., anoxia with NO\textsubscript{3}\textsuperscript{−} as electron acceptor), which is dependent on the oxygen-responsive regulatory cascade FixLJ–FixK\textsubscript{2} (Torres et al., 2016, 2017; Sánchez et al., 2017; Figure 2A). Decreasing oxygen level to 5% during a culture triggers ATP-dependent autophosphorylation of the heme-based sensor kinase, FixL to phosphorylate the response regulator FixJ, which activates FixK\textsubscript{2}, a transcriptional activator that directly interacts with the nos\textsubscript{R} promoter (Torres et al., 2016, 2017; Figure 2A). Although the FixLJ–FixK\textsubscript{2} cascade has been considered as the main regulator for nos genes in bradyrhizobia for a long time, the NasST two-component system has been revealed as an important regulator of nos transcription in response to NO\textsubscript{3}\textsuperscript{−} under both aerobic and anaerobic conditions (Sánchez et al., 2014, 2017; Figure 2A). The nasST operon encodes a NO\textsubscript{3}\textsuperscript{−} and NO\textsubscript{2}\textsuperscript{−} sensor/transcriptional antitermination regulatory system. This system was initially considered to be involved in the NO\textsubscript{3}\textsuperscript{−}/NO\textsubscript{2}\textsuperscript{−}-responsive regulation of the nos genes for the NO\textsubscript{3}\textsuperscript{−} assimilation pathway in bacteria, including B. diazoefficiens (Romeo et al., 2012; Wang et al., 2012; Luque-Almagro et al., 2013; Cabrera et al., 2016). A recent transcriptomic study using RNA-seq has shown that most of the genes whose expression changed in the B. diazoefficiens ΔnasT mutant are related to N metabolism, especially amino acid transport (Sánchez et al., 2019).

NasS contains a NO\textsubscript{3}\textsuperscript{−}/NO\textsubscript{2}\textsuperscript{−}-binding motif similar to that of NrtA, which is the periplasmic component of an ABC-type system for NO\textsubscript{3}\textsuperscript{−} and NO\textsubscript{2}\textsuperscript{−} uptake in cyanobacteria (Koropatkin et al., 2006). NasT is an ANTAR (AmiR and NasR transcription antitermination regulator)-family protein (Shu and Zhulin, 2002). NasS and NasT form a complex that dissociates when NasS senses NO\textsubscript{3}\textsuperscript{−} in micromolar concentrations (Luque-Almagro et al., 2013; Sánchez et al., 2014; Hidaka et al., 2016). When NO\textsubscript{3}\textsuperscript{−} is present, nos expression is markedly decreased (~70%) in the ΔnasT background. In absence of NO\textsubscript{3}\textsuperscript{−}, nos expression is induced in the ΔnasS background but such induction is abolished with the additional deletion of nasT. Thus, NO\textsubscript{3}\textsuperscript{−} counteracts the NasS-mediated inhibition of nos by allowing the dissociation of the antiterminator NasT from the NasS-NasT complex (Sánchez et al., 2014, 2017; Figure 2). Then, the application of nos\textsuperscript{++} mutants (carrying a mutation in nasS) may be more effective than that of wild type nos\textsuperscript{+}.

\begin{figure}[h]
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\caption{Regulation of N\textsubscript{2}O reductase genes in bradyrhizobia. (A) Environmental factors and regulatory proteins involved in the control of nos expression. (B) The mechanism for NasT-mediated transcriptional antitermination of the nos genes. In the absence of nitrate (NO\textsubscript{3}\textsuperscript{−}), NasT is sequestered by NasS; thus in the absence of NasT, the native conformation of the nos\textsubscript{R}-leader mRNA, which contains the H1 and H2 hairpins, is responsible for the termination of the nos transcription. When a certain level of NO\textsubscript{3}\textsuperscript{−} is sensed by NasS, NasT is dissociated from NasS–NasT complex; the binding of NasT to H1 (and likely to H3 region, in orange) results in a conformational change in the mRNA that allows the read-through transcription of nos genes.
} \label{fig:2}
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alone if NO$_3^-$ concentration in the rhizosphere is below the threshold for dissociation of the NasS-NasT complex. Although the concentration threshold in vitro is within the micromolar range (Hidaka et al., 2016), this concentration remains to be fixed under soil conditions.

When NasT is released from NasS, NasT interacts directly with a 5'-leader region of the nosR mRNA and interferes with the formation of a terminator structure, allowing a read-through of nos genes (Sánchez et al., 2017; Figure 2B). The transcription terminator located upstream of nosR comprises two RNA-hairpin structures (H1 and H2); the binding of NasT to H1 induces a conformational change in the terminator and facilitates read-through transcription to induce nos expression (Sánchez et al., 2017; Figure 2B). Deletion of either H1 or H2 increases nos expression in the absence or presence of NO$_3^-$ (Sánchez et al., 2014, 2017; Figure 2). Thus, theoretically, a B. diazoefficiens mutant defective in H1 (Figure 2B) would be an ideal nos$^{++}$ inoculant, because (i) it is expected to specifically induce nos genes, whereas nos$^{++}$ strains derived from nasS mutations affected other genes controlled by the NasST system, and (ii) nos induction is independent of soil NO$_3^-$ concentration (Itakura et al., 2013; Sánchez et al., 2017, 2019). Mutation of H1 may be applicable to other agriculturally important nos$^+$ bacteria such as Bradyrhizobium oligotrophicum S58, an endophyte of rice roots – where it potentially fixes N$_2$ (Ohta and Hattori, 1983; Okubo et al., 2013; Sánchez et al., 2017; Sánchez and Minamisawa, 2018).

Furthermore, studies on P. denitrificans have shown that reduction of N$_2$O to N$_2$ is dependent on Cu, a key cofactor of the Nos enzyme. Thus, bacterial cultures lacking Cu accumulate significant amounts of N$_2$O (Felgate et al., 2012). Cu deficiency results in a decreased expression of nosZ (Sullivan et al., 2013). Another key factor is the pH that significantly affects N$_2$O emission from microbes. The expression of functional N$_2$O-R is difficult at low pH (Bakken et al., 2012). Sinorhizobium meliloti, the alfalfa endosymbiont, is unable to express N$_2$O at pH 6 (Bueno et al., 2015). In P. denitrificans, pH has little effect on the transcription of the nosZ, but may have a direct posttranslational effect on the assembly and/or activity of the N$_2$O-OR holoenzyme (Bakken et al., 2012). The effect of Cu or pH on the reduction of N$_2$O to N$_2$ in B. diazoefficiens is currently unknown. Among the environmental factors that affect the bacterial N$_2$O-OR activity, very little is known about the effect of availability and redox state of carbon sources. In this context, the response regulator RegR of the RegSR two-component regulatory system appears to induce nos expression in B. diazoefficiens, most likely in response to redox state (Torres et al., 2016; Figure 2A).

FUTURE DIRECTIONS FOR PRODUCTION OF BRADYRHIZOBIAL INOCULANTS FOR N$_2$O MITIGATION

The understanding of N$_2$O production in the soybean rhizosphere has been significantly advanced. A variety of techniques, such as functional omics, $^{15}$N isotope analysis, and zymography, will facilitate a better understanding of the players and processes for N transformation in the soybean rhizosphere of degrading nodules. In addition, further studies on soil factors that control the amount and distribution of soybean bradyrhizobia in the rhizosphere are required because they are key determinants for the flux of N$_2$O during nodule decomposition (Inaba et al., 2012).

Shina et al. (2014) reported that the soil type determines the occurrence of B. diazoefficiens (nos$^+$) or B. japonicum (nos$^-$) in Japanese soybean fields; the nos$^+$ bradyrhizobia are predominant in Gleysol (wetland soils where water regime causes low-oxygen conditions), whereas the nos$^-$ bradyrhizobia are predominant in Andosols (volcanic soils containing porous sediments, resulting in more aerated conditions). Saeki et al. (2017) reported that the presence of nos in B. diazoefficiens confers a competitive advantage in flooded soils with low-oxygen conditions, similar to Gleysol soils. However, batch experiments suggested that B. japonicum may be less competitive compared to B. diazoefficiens due to energy depletion under anaerobic conditions, which is associated with a marked impairment of Nap activity in B. japonicum and not with the absence of nos (Siqueira et al., 2017). These findings emphasize the need for further research on how soil factors influence the relevance of the N$_2$O reduction step in bradyrhizobial competition.

Significant advances have led to the use of bradyrhizobial N$_2$OR as an N$_2$O sink in soybean ecosystems. Following the work done by Itakura et al. (2013) and Akiyama et al. (2016), promising strategies for production of rhizobial inoculants for N$_2$O mitigation would be the selection of superior native strains (in terms of adaptation to local environments and N$_2$-fixing symbiotic efficiency) and the optimization of N$_2$O reduction activity through appropriate genetic modification or management of soil chemical and physical properties. However, generating mutants requires more time, cost, and technical skill than isolating nos$^+$ strains from local soybean fields (Itakura et al., 2013; Akiyama et al., 2016). Moreover, inoculating a mixture of native strains provides more adaptability than a single strain (Akiyama et al., 2016). Thus, isolating nos$^+$ strains from local fields is more feasible for many soybean-producing countries and is potentially applicable to other ecosystems. Indeed, it has already been suggested the potential activity of Ensifer (formerly Sinorhizobium) meliloti, the alfalfa endosymbiont (Bueno et al., 2015).

AUTHOR CONTRIBUTIONS

Both authors have contributed equally to the discussion, writing, and approving the manuscript.

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