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Nitrogen Cycling in Soybean Rhizosphere: Sources and Sinks of Nitrous Oxide (N_2O)

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Nitrous oxide (N_2O) 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 N_2O 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 N_2O. Soybean root nodules are the host of dinitrogen (N_2)-fixing bacteria from the genus *Bradyrhizobium*. Nodule decomposition is the main source of N_2O in soybean rhizosphere, where soil organisms mediate the nitrogen transformations that produce N_2O. This N_2O is either emitted into the atmosphere or further reduced to N_2 by the bradyrhizobial N_2O reductase (N_2OR), encoded by the *nos* gene cluster. The dominance of *nos*$^-$ indigenous populations of soybean bradyrhizobia results in the emission of N_2O into the atmosphere. Hence, inoculation with *nos*$^+$ or *nos*$^{++}$ (mutants with enhanced N_2OR activity) bradyrhizobia has proved to be promising strategies to reduce N_2O 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 N_2O emission from soils.

Keywords: *Bradyrhizobium*, soybean, rhizosphere, denitrification, N_2O reductase, *nos* regulation, greenhouse gas, mitigation strategies

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NITROGEN TRANSFORMATIONS IN SOYBEAN RHIZOSPHERE: N_2O IS EMITTED DUE TO NODULE DECOMPOSITION

The term, "rhizosphere" was first defined by Lorenz Hiltner as the soil compartment influenced by plant roots. Since then, the rhizosphere has received widespread attention from scientists in different disciplines as a hotspot for intra-microbial and plant-microbe interactions (Hartmann et al., 2008; Bakker et al., 2013). Nitrogen (N), an essential component in living organisms, is the most common limiting nutrient for plant growth in agricultural soils (LeBauer and Treseder, 2008). Fixation of dinitrogen (N_2) into ammonia (NH_3) by legume-associated endosymbiotic bacteria, generally known as rhizobia, is a major source of N in soils, and is an agriculturally and ecologically crucial process to reduce plant dependence on industrial N fertilizers. Rhizobia are hosted within the nodules formed in the roots as the result of the symbiosis; inside the nodules, rhizobial cells encounter oxygen-limiting conditions that are required for the synthesis and activity of nitrogenase that converts N_2 into NH_3 (**Figure 1**). Thus, rhizobia provide N to the host plant, which in return,

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 N_2, 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 (NO_3^-) and nitrite (NO_2^-) – are used as electron acceptors in oxygen limiting conditions. NO_2^- is reduced to nitric oxide (NO), nitrous oxide (N_2O), and N_2 gases, which are returned to the atmosphere (**Figure 1**). The complete denitrification pathway in soybean bradyrhizobia requires four enzymes, periplasmic NO_3^- reductase (Nap), copper (Cu)-containing NO_2^- reductase (NirK), *c*-type NO reductase (cNor), and N_2O reductase (N_2OR) (**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).

N_2O is the third most important greenhouse gas after carbon dioxide (CO_2) and methane (CH_4) 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 N_2O, 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 N_2O. Nodule decomposition is a major source of N_2O, particularly in soybean ecosystems, compared to other possible sources including aboveground plant residues. Inaba et al. (2009) showed that N_2O is only emitted by decomposed nodules, but not by fresh nodules or roots. Studies showed that N_2O 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 N_2 fixation is an important indirect source for N_2O during nodule decomposition. Indeed, a ^{15}N tracer experiment revealed that the N_2O emitted from the soybean rhizosphere was almost entirely derived from N_2 fixed symbiotically in the nodules (Inaba et al., 2012; **Figure 1**). During nodule decomposition, rhizospheric microbes are essential for N_2O emission; organic N from the nodule is mineralized into ammonium (NH_4^+, **Figure 1**); N_2O is then produced via nitrification and denitrification (Inaba et al., 2009, 2012; **Figure 1**). Although soybean bradyrhizobia are important players in denitrification (responsible for ∼41% of the total N_2O produced), but other denitrifying microorganisms are also important contributors (∼59% of the total N_2O 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). N_2O formed by denitrification is either emitted into the atmosphere or is further reduced to N_2 by N_2O reductase of soybean bradyrhizobia (Sameshima-Saito et al., 2004, 2006b; Inaba et al., 2012; **Figure 1**). In soybean fields, both N_2- and N_2O-producing soybean bradyrhizobia 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 N_2O from soybean fields during nodule decomposition is partly determined by biotic factors like the balance between N_2O emission by soil microbes whose denitrification produce N_2O (including bradyrhizobia) and N_2O uptake by soybean bradyrhizobia that produce N_2OR (Inaba et al., 2012; **Figure 1**).

NITROUS OXIDE REDUCTASE: THE KEY ENZYME TO REDUCE N_2O EMISSION

N_2OR is a Cu-containing enzyme that catalyzes the two-electron reduction of N_2O to N_2, which is the only known pathway for the removal of N_2O from ecosystems (Richardson et al., 2009). Therefore, the expression and activity of N_2OR is a natural target to mitigate N_2O emission from agricultural soils.

In *Bradyrhizobium diazoefficiens* (reclassified from *Bradyrhizobium japonicum* by Delamuta et al., 2013), the N_2OR (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 Kroneck, 2007). NosD, NosF, NosY, and NosL are involved in maturation of the Cu_Z site of NosZ (Zumft and Kroneck, 2007). Although the reduction of N_2O to N_2 by N_2OR is integrated as the last step of the denitrification pathway, it can provide a benefit for N_2O respiration as a separate module. When N_2O is provided as the sole electron acceptor to *B. diazoefficiens*, anaerobic respiration and growth are sustained by reducing N_2O to N_2 (Zumft, 1997; Sánchez et al., 2013; Graf et al., 2014).

Bradyrhizobium diazoefficiens carries the *nos* gene cluster (*nos*$^+$) and denitrifies NO_3^- to N_2, whereas other soybean bradyrhizobia including *B. japonicum* lack the *nos* gene cluster (*nos*$^-$) and cannot reduce N_2O to N_2 (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 N_2O, equivalent to the natural concentration of N_2O 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 N_2O 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 N_2O emission at the field scale. This

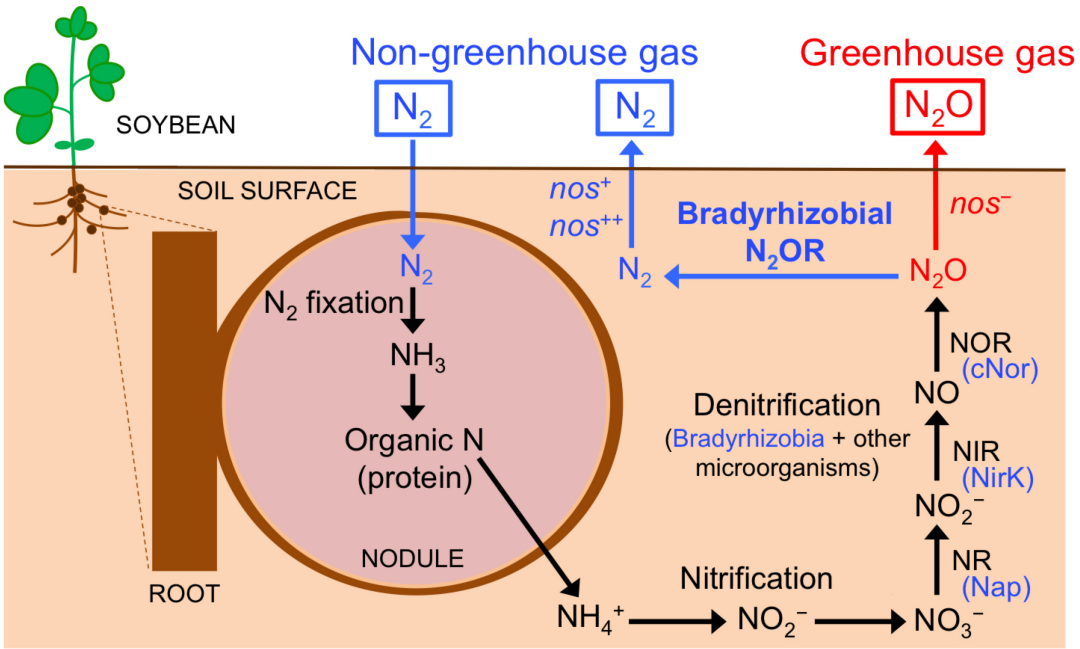

FIGURE 1 | Nitrogen transformations in soybean rhizosphere. Organic nitrogen in the nodule is mineralized into NH_4^+ that will be transformed into N_2O through nitrification and denitrification processes. Soybean bradyrhizobia (blue) and other microorganisms contribute to the denitrification process. The N_2O formed is either emitted into the atmosphere (red) or further reduced to N_2 exclusively by soybean bradyrhizobia that produce N_2O reductase (N_2OR; blue). Nase, nitrogenase; NR, dissimilatory nitrate reductase; Nap, periplasmic nitrate reductase; NIR, dissimilatory nitrite 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_2O 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_2O 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_2O emission from intact soybean root systems via bradyrhizobial denitrification (Ciampitti et al., 2008; Hirayama et al., 2011; Inaba et al., 2012).

Among the N_2O-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_2OR 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_2O 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_2OR 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 *nasS* 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_2O 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_2O 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_2O emission by 43% in the lysimeter study and by 54% in the farm-scale study. However, reduction in postharvest N_2O 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_2O-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_2O 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_2O 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_2O REDUCTASE GENES IN BRADYRHIZOBIA

Considering the importance of N_2OR in N_2O 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 *nosR* gene is constitutively expressed at a low level from the promoter P_a in aerobiosis, but is strongly induced from the promoter P_d under denitrifying conditions (i.e., anoxia with NO_3^- as electron acceptor), which is dependent on the oxygen-responsive regulatory cascade FixLJ–$FixK_2$ (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 $FixK_2$, a transcriptional activator that directly interacts with the *nosR* promoter (Torres et al., 2016, 2017; **Figure 2A**). Although the FixLJ–$FixK_2$ 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_3^- under both aerobic and anaerobic conditions (Sánchez et al., 2014, 2017; **Figure 2A**). The *nasST* operon encodes a NO_3^- and NO_2^- sensor/transcriptional antitermination regulatory system. This system was initially considered to be involved in the NO_3^-/NO_2^--responsive regulation of the *nas* genes for the NO_3^- 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* Δ*nasT* mutant are related to N metabolism, especially amino acid transport (Sánchez et al., 2019).

NasS contains a NO_3^-/NO_2^--binding motif similar to that of NrtA, which is the periplasmic component of an ABC-type system for NO_3^- and NO_2^- 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_3^- in micromolar concentrations (Luque-Almagro et al., 2013; Sánchez et al., 2014; Hidaka et al., 2016). When NO_3^- is present, *nos* expression is markedly decreased (~70%) in the Δ*nasT* background. In absence of NO_3^-, *nos* expression is induced in the Δ*nasS* background but such induction is abolished with the additional deletion of *nasT*. Thus, NO_3^- 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^{++} mutants (carrying a mutation in *nasS*) may be more effective than that of wild type nos^+

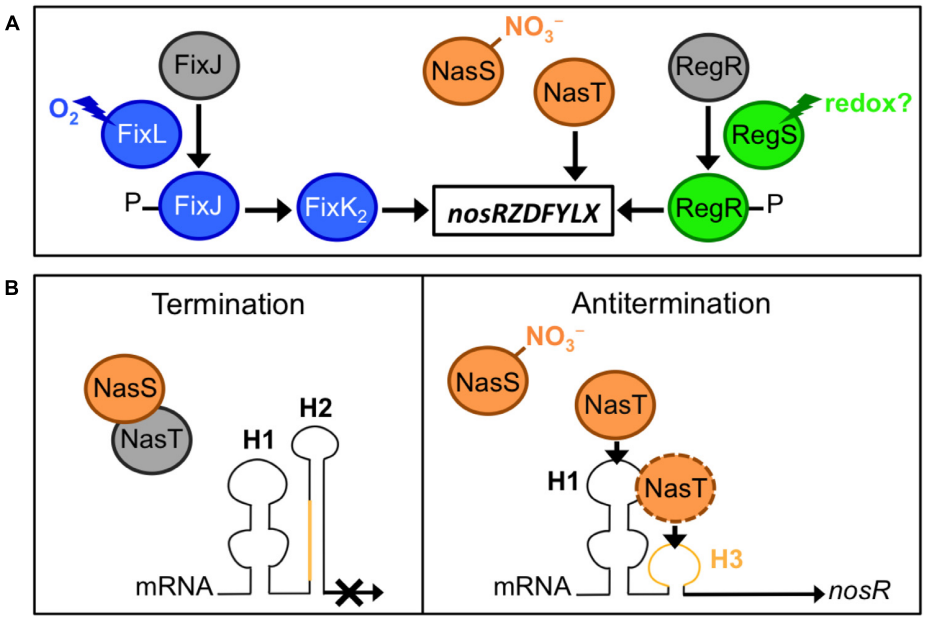

FIGURE 2 | Regulation of N_2O reductase genes in bradyrhizobia. **(A)** Environmental factors and regulatory proteins involved in the control of *nos* expression. **(B)** The mechanism for NasT-mediated transcriptional antitermination of the *nos* genes. In the absence of nitrate (NO_3^-), NasT is sequestered by NasS; thus in the absence of NasT, the native conformation of the *nosR*-leader mRNA, which contains the H1 and H2 hairpins, is responsible for the termination of the *nos* transcription. When a certain level of NO_3^- 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.

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 transcription 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_2O to N_2 is dependent on Cu, a key cofactor of the Nos enzyme. Thus, bacterial cultures lacking Cu accumulate significant amounts of N_2O (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_2O emission from microbes. The expression of functional N_2OR is difficult at low pH (Bakken et al., 2012). *Sinorhizobium meliloti*, the alfalfa endosymbiont, is unable to express N_2OR 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_2OR holoenzyme (Bakken et al., 2012). The effect of Cu or pH on the reduction of N_2O to N_2 in *B. diazoefficiens* is currently unknown. Among the environmental factors that affect the bacterial N_2OR 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_2O MITIGATION

The understanding of N_2O 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_2O during nodule decomposition (Inaba et al., 2012).

Shiina 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_2O reduction step in bradyrhizobial competition.

Significant advances have led to the use of bradyrhizobial N_2OR as an N_2O 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_2O 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_2O 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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