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SHORT COMMUNICATION

Role of Nitric Oxide of Bacterial Origin in the *Medicago truncatula–Sinorhizobium meliloti* Symbiosis

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Nitric oxide (NO) is a small ubiquitous gaseous molecule that has been found in many host-pathogen interactions. NO has been shown to be part of the defense arsenal of animal cells and more recently of plant cells. To fight this molecular weapon, pathogens have evolved responses consisting of adaptation to NO or degradation of this toxic molecule. More recently, it was shown that NO could also be produced by the pathogen and contributes likewise to the success of the host cell infection. NO is also present during symbiotic interactions. Despite growing knowledge about the role of NO during friendly interactions, data on the specificity of action of NO produced by each partner are scarce, partly due to the multiplicity of NO production systems. In the nitrogen-fixing symbiosis between the soil bacterium *Sinorhizobium meliloti* and the model legume *Medicago truncatula*, NO has been detected at all steps of the interaction, where it displays various roles. Both partners contribute to NO production inside the legume root nodules where nitrogen fixation occurs. The study focuses on the role of bacterial NO in this interaction. We used a genetic approach to identify bacterial NO sources in the symbiotic context and to test the phenotype in planta of bacterial mutants affected in NO production. Our results show that only denitrification is a source of bacterial NO in *Medicago* nodules, giving insight into the role of bacteria-derived NO at different steps of the symbiotic interaction.

Keywords: denitrification, legumes, nitric oxide, rhizobia, symbiosis

Nitric oxide (NO) is a signaling molecule found in almost all living organisms (Kolbert et al. 2019). Its small size and gaseous properties allow an easy and rapid diffusion of the molecule across cell membranes. When present in high concentration, NO is a toxic molecule and has long been described as a host defense in pathogenic interactions (Fang and Vázquez-Torres 2019). Mammalian cells can generate large quantities of NO in response to inflammatory stimuli and this molecule triggers many detrimental effects on bacterial metabolism, DNA replication and repair, as well as modification of regulatory proteins that coordinate bacterial virulence gene expression (Porrini et al. 2020). About 20 years ago, similar data were obtained on plants, where NO was proved to be a regulating agent during plant defense (Delledonne et al. 1998; Durner et al. 1998). Since then, the importance of NO in plant resistance has been well-documented. Pharmacological, biochemical, and genetic approaches have provided evidence that an early NO burst in plant cells after pathogen attack functions as a messenger in gene expression for developing a defense response and as a general key factor associated with basal resistance in various plant-pathogen systems (Bellin et al. 2013).

The main way to synthesize NO in mammalian cells is well-defined and consists in a NO synthase (NOS), generating NO and citrulline from arginine, whereas the situation is more complex in higher plants, in which NOS genes have not been found (Jeandroz et al. 2016). Instead, NO production is dependent upon several enzymatic pathways, such as nitrate reductase, mitochondrial electron transport chain–dependent nitrite reductase activity, and polyamine oxidases (Astier et al. 2018; Jeandroz et al. 2016; Kolbert et al. 2019). This diversity of NO sources in plants increases the difficulty for researchers to impair NO production with the goal to study the impact of NO on plant-microbe interactions. In bacteria, NO is produced by at least two different ways, depending upon the considered species. NO is an intermediate of the denitrification pathway, which involves the dissimilatory reduction of nitrate (NO_3^-) to dinitrogen through a series of intermediates that include nitrite (NO_2^-), NO, and nitrous oxide (N_2O) (Ruiz et al. 2021; Shapleigh 2006). Also, several bacterial species, primarily gram-positive, harbor a gene encoding a simple form of NO synthase (bNOS) (Adak et al. 2002; Choi et al. 1997; Crane et al. 2010; Hutfless et al. 2018; Santolini 2019). While eukaryotic NOS possess an oxygenase and a reductase domain within the same polypeptide, the bacterial NOS are truncated and contain only an oxygenase domain. Despite this difference, they function as mammalian NOS and can catalyze the conversion of arginine into citrulline and NO.

In the combat between pathogens and host, the speed of response of the foes is a major component. Microbes infecting other organisms have selected NO in highly effective defensive and offensive strategies. These mechanisms can involve enzymes responsible for resistance to nitrosative stress by means of the expression of detoxifying systems, such as NO reductases or

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flavohemoglobins involved in NO degradation (Fang and Vázquez-Torres 2019). More recently, NO production by pathogens was also described as an emerging strategy for successful infection (Campos et al. 2019; Chung et al. 2013; Kinkel et al. 2016; Mogen et al. 2017). Emerging functions of NO synthesized by bacteria include its ability to respond as a cellular antioxidant, to modulate bacterial respiration once inside the host, and to post-transcriptionally regulate the function of several bacterial or host proteins. NO production has also been demonstrated in many diverse plant pathogens, including bacteria, fungi, and oomycetes, and there is increasing evidence that the pathogen-derived NO is an important regulatory molecule involved in pathogen virulence and its survival in the host (Arasimowicz-Jelonek and Floryszak-Wieczorek 2014; Jedelská et al. 2021; Martínez-Medina et al. 2019; Samalova et al. 2013). This is an interesting challenge to understand, as the same molecule could favor both the invader and the host.

For about 15 years, the presence of NO has also been shown in mutualistic plant-microbe interactions, such as the legume-rhizobium symbioses or mycorrhizal symbioses (Baudouin et al. 2006; Berger et al. 2019, 2020a; Calcagno et al. 2012; del Giudice et al. 2011; Hichri et al. 2016; Martínez-Medina et al. 2019; Nagata et al. 2008; Sánchez et al. 2010; Signorelli et al. 2020). The establishment of the legume-rhizobium symbiosis requires recognition between the rhizobia and the legume and the formation of nodules, new plant organs hosting the rhizobia, where the nitrogen fixation takes place thanks to a bacterial nitrogenase (Lindström and Mousavi 2019; Oldroyd et al. 2011; Schwember et al. 2019). Following the recognition step, rhizobia induce a curl on the plant root hair and the bacteria enter the root and will be delivered in the cortical cells of the developing nodule, thanks to a tubular structure called an infection thread. Once inside the plant cells, the bacteria will differentiate into bacteroids, which will fix nitrogen to the benefit of the plant. Reactive oxygen species, such as hydrogen peroxide, and reactive nitrogen species, particularly NO, are known to play an important role in the establishment and functioning of the rhizobium-legume symbiosis (Pauly et al. 2006; Puppo et al. 2013). NO is present at every step of the interaction, plant-rhizobium recognition, root hair curling, along the infection thread, in the developing as well as the mature nodules (Berger et al. 2021). During symbiosis, either successively or simultaneously, NO has been shown to regulate gene expression (Berger et al. 2020b; Boscari et al. 2013; Sánchez et al. 2010) and to act as a metabolic intermediate in energy regeneration processes via phytoglobin-NO respiration (Berger et al. 2020a and b, 2021; Horchani et al. 2011). It has also been shown that NO can modulate enzyme activities by mean of post-translational modifications (Kato et al. 2010; Melo et al. 2011; Sainz et al. 2015) and that *Sinorhizobium meliloti* could control these modifications by modulating NO levels inside nodules (Blanquet et al. 2015). In addition, proteins from *S. meliloti* have also been found to be possible targets of NO-mediated post-translational modifications (Cazalé et al. 2020).

It has been demonstrated that NO is necessary for the establishment of the symbiosis (Berger et al. 2020b; del Giudice et al. 2011; Leach et al. 2010) and for the nitrogen fixation (Berger et al. 2020a and b, 2021), and it was also observed to locally induce nodule senescence in *Medicago truncatula* or *Lotus japonicus* (Bruand and Meilhoc 2019; Cam et al. 2012; Fukudome et al. 2019; Sun 2018).

Interestingly NO is also produced by rhizobia during their interaction with legumes. Indeed, inside *Medicago* nodules, *S. meliloti* contributes to about 30% of the NO production, while it can reach up to 90% in other symbiotic interactions, such as *Bradyrhizobium* and the soybean when grown in the presence of nitrate (Horchani et al. 2011; Salas et al. 2019; Sánchez et al. 2010). These data raised the burning question of what could be the role played by the NO originated from the bacteria in a friendly interaction?

The strategy to answer this question was to generate bacterial mutants that do not produce NO and observe the phenotype in planta of these mutants to determine whether they have a role at one or both the early and late steps of the symbiotic interaction. We have shown that *S. meliloti* does not possess a bNOS gene or NOS activity (Ruiz et al. 2021) and that the main way to produce NO in this bacterium is via the denitrification pathway (Ruiz et al. 2019). Nitrate is reduced into nitrite and into NO thanks to the successive actions of a nitrate reductase encoded by *nap* genes (*napEFDABC* operon) and a nitrite reductase encoded by *nir* genes (*nirKV* operon). The nitrate assimilation pathway composed of nitrate reductase NarB and nitrite reductase NirBD contributes to NO production in free-living *S. meliloti* but only in conditions in which denitrification is active (i.e., oxygen limitation and presence of nitrate).

Previous studies have shown that *narB* and *nirBD* genes were expressed in the nitrogen fixation zone of *M. truncatula* nodules (Roux et al. 2014; Ruiz et al. 2019). To determine whether the nitrate assimilation pathway is involved in NO production inside nodules, we made use of a *nirBDnarB S. meliloti* mutant strain and we constructed multiple mutants in which the *napEFDABC* operon (referred to as *nap* mutant), *nirKV* operon (referred as *nir* mutant), and one or both *nap* and *nir* and *nirBDnarB* operons have been deleted. We inoculated *M. truncatula* plantlets with these mutants or the wild-type (WT) strain and quantified NO inside the nodules 3 weeks postinoculation. As controls, we also constructed and tested mutants in which only *nap* or *nir* operons were deleted. The NO content was compared with that obtained with a WT strain and the results are shown in Figure 1.

A 22 to 36% decrease in NO production was measured in nodules occupied by *nap*, *nir*, or *nap nir* mutants relative to nodules occupied by the WT strain. This level is in the same order of magnitude as that measured previously in nodules induced by *napA* or *nirK* transposon insertion mutant (30%) (Horchani et al. 2011), thus confirming that *S. meliloti* contributes to NO production through denitrification in nodules. Interestingly, when the *nirBDnarB* operon involved in the nitrate assimilation pathway was deleted, there was no significant difference in the NO level measured in the nodules, as compared with a WT strain. Also, deletion of *nirBDnarB* in the *nap* or *nir* mutant strains did not further reduce the NO content of nodules induced by these strains. Altogether, these results indicate that the nitrate assimilation pathway encoded by *nirBDnarB* does not play any detectable role in NO production in nodules, unlike what was described in free-living cells (Ruiz et al. 2019). Therefore, denitrification remains the main way to produce NO in mature nodules.

NO is required for an optimal establishment of the interaction between rhizobia and legumes. The treatment of soybean roots inoculated with *Bradyrhizobium japonicum* with a NOS inhibitor resulted in a 70% reduction of the nodule number, suggesting the contribution of a plant NOS-like enzyme (Leach et al. 2010). A second study performed on *M. truncatula* showed that two nitrate reductases, MtNR1 and MtNR2, might have a specific role as NO sources during symbiosis establishment (Berger et al. 2020a). Interestingly NO is present in the root hair curl of *M. truncatula* in which a *S. meliloti* microcolony is entrapped as well as along the infection thread (del Giudice et al. 2011). We made the hypothesis that if *S. meliloti* cells were responsible for NO production at these steps, a mutant strain that does not produce NO would be less competitive than the WT strain to infect plant cells and occupy a nodule. To test this hypothesis, we made use of the mutant deleted for both *nap* and *nir* operons. *M. truncatula* plants were inoculated with a mixture (1:1) of the WT and mutant strains. Two to three weeks postinoculation,

nodules occupied with either the WT or the mutant strain were counted. To differentiate nodules occupied by each strain, one strain displayed a constitutive and strong green fluorescence from a green fluorescent protein, while the second strain displayed a red fluorescence from a red fluorescent protein, both plasmid-encoded. If both strains are similarly competitive, half of the nodules are expected to be occupied by each strain. To avoid a bias due to the expression of different fluorescent proteins, the experiments were also performed after swapping the fluorescent proteins produced by the strains. The results are presented in Figure 2.

When the competition experiment was performed with two WT strains, each carrying a different fluorescent protein, half of the nodules counted were occupied by each strain, confirming the validity of the experiment (competitiveness index [CI] = 1). Interestingly, when the competition assay was made with a WT strain and a *nap nir* mutant, no significant difference in the strain competitiveness was observed, suggesting that a strain that does not produce NO is as competitive as a WT strain for nodule occupancy. At this stage, we can conclude that, at early steps of the interaction, either NO is produced by the plant or NO is produced by the bacteria but is not important for bacterial infection. In a previous work, inoculation of *M. truncatula* plantlets with a *S. meliloti* strain overexpressing *hmp* (involved in NO degradation) resulted in delayed nodulation and reduced competitiveness, indicating that NO was important at the first steps of the interaction (del Giudice et al. 2011). The apparent discrepancy between both sets of results can be explained by a drastic effect of *hmp* overexpression on the global level of NO, including NO from plant origin. Interestingly, a recent study (Achouak et al. 2019) found that denitrification and *nirK* expression on the root system of *M. truncatula* were both very low, which could indirectly support the idea that NO is not produced by rhizobia on *M. truncatula* roots.

In addition, we performed a new set of experiments in which nitrate, the substrate of denitrification pathway, at concentrations not inhibiting nodulation (0.5, 1, and 1.5 mM) was added on the roots. The results obtained were comparable to those obtained in the absence of nitrate (data not shown), indicating that the presence of low nitrate concentrations does not modify the competitiveness of the *nap nir* mutant as compared with the WT strain.

We also tested the competitiveness for nodule occupancy of a *nirBDnarB* mutant strain. Surprisingly the WT strain was outcompeted by the mutant strain, as it occupied only about 30% of the nodules (CI = 2.79) (Fig. 2). The nitrate assimilation pathway does not contribute to NO production in nodules, which

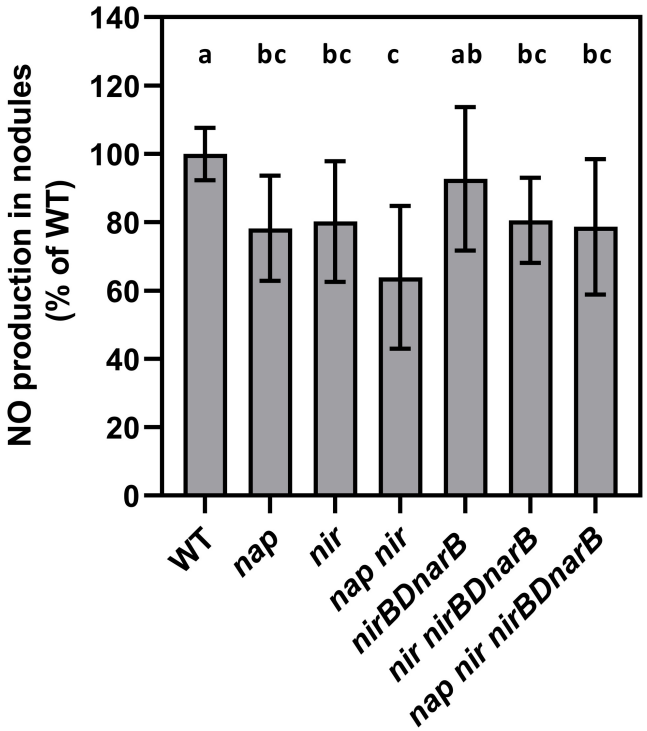


Fig. 1. Nitric oxide (NO) production in *Medicago truncatula* nodules occupied by a *Sinorhizobium meliloti* wild-type (WT) or different mutant strains. The fluorescence intensity of the NO production by nodules was measured 3 weeks postinoculation, using the 4,5-diaminofluorescein probe (Sigma-Aldrich). Four independent series of plants were analyzed. The mean of values obtained for the WT strain was calculated for each series. In a same series, each value obtained for a WT or a mutant is normalized with this WT mean value. Data are means ± standard deviation ($n = 4$). Letters above bars indicate statistically significant differences according to one-way analysis of variance and Tuckey post-hoc test ($n = 4$; $P < 0.05$). *nap* = *napEFDABC* deletion; *nir* = *nirKV* deletion; *nap nir* = *napEFDABC* and *nirKV* deletions; *nir nirBDnarB* = *nirKV* and *nirBDnarB* deletions; and *nap nir nirBDnarB* = *nap EFDABC*; *nirKV* and *nirBDnarB* deletions.

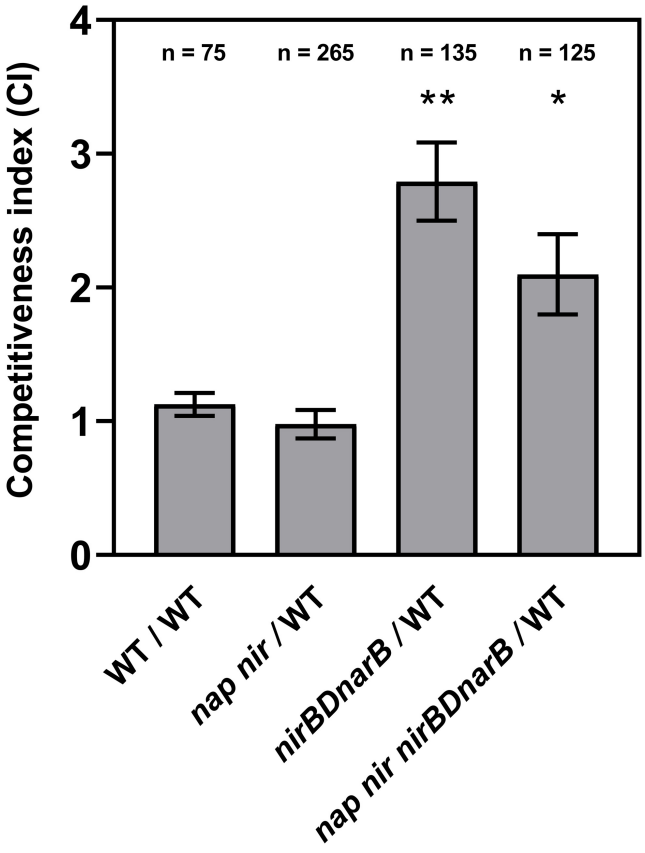


Fig. 2. Nodulation competitiveness of *Sinorhizobium meliloti* wild-type (WT) and different mutant strains. Each plant was inoculated with a mixture of two strains, i.e., a WT and a mutant strain (50 µl, 1:1 strain mixture, optical density at 600 nm = 0.01). Each strain contains a plasmid carrying either a green or a red fluorescent protein–encoding gene. Nodules stained in red and green were counted 2 to 3 weeks postinoculation. For each series of plants, a competitiveness index (CI) was calculated. CI is defined as the ratio of nodule number occupied by a mutant to the nodule number occupied by a WT strain, divided by the ratio of mutant colony forming units (CFU) to WT CFU in the inoculum. Data are means ± standard error of the mean from three to nine independent series. CI = 1 indicates that the competitiveness of both strains is identical. Statistical analysis was performed by a one-sample *t* test. Asterisks (* and **) indicate CIs that are significantly different from 1 ($P < 0.05$ and 0.01, respectively). n = total number of plants tested; *nap nir* = *napEFDABC* and *nirKV* deletions; *nap nir nirBDnarB* = *nap EFDABC*, *nirKV*, and *nirBDnarB* deletions.

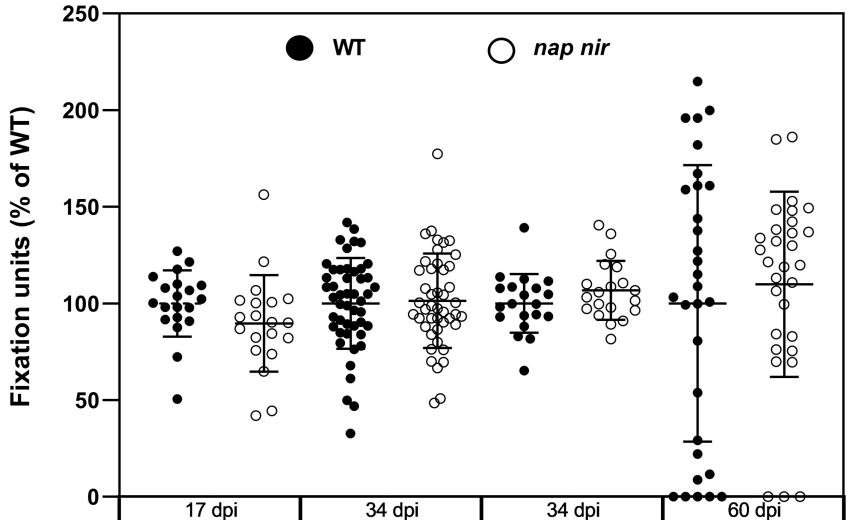
makes unlikely that a decrease of NO would be responsible for this increased competitiveness. But the absence of assimilation likely makes more nitrate available as substrate for denitrification, which may contribute to more NO synthesis and could make the strain more competitive. However, a similar competitiveness was measured with a strain in which both pathways are inactivated (Fig. 2), showing that bacterial NO synthesis is not involved in the observed phenotype. Although this finding is interesting, it remains puzzling. As the main reaction product of the nitrate assimilation pathway is ammonium (NH_4^+), it is tempting to speculate that NH_4^+ could have a negative effect on the first steps of the symbiotic interaction, as has been suggested before (Dusha 2002; Patriarca et al. 2002).

Finally, to test whether bacterial NO has a role in late steps of the interaction, we inoculated *M. truncatula* plantlets either with the WT or with the mutant strain deleted for all the genes encoding the nitrate (*napEFDABC*) and nitrite reductases (*nirKV*). We tested the nitrogen fixation efficiency by means of the acetylene reduction assay and we assessed plant fitness by measuring the dry weight of the plant shoots at 17, 34, and 60 days postinoculation (dpi). The results shown in Figure 3 indicate that neither the plant fitness nor the nitrogen fixation efficiency is affected by inactivation of the bacterial NO sources. Addition of nitrate in the plant culture medium at a concentration not affecting nodulation (0.5 mM) did not change the observation. Nodule senescence was also assessed by monitoring a color shift from pink to green at the bases of the nodules over a period of 60 dpi (Blanquet et al. 2015). The senescence kinetics of both WT and mutant strains were comparable, with all nodules being senescent at 60 dpi (Supplementary Fig. S1). Altogether, these results indicate that there is no major or specific biological function affected by bacterial NO that could affect the late steps of symbiosis.

In summary, we have shown that the denitrification pathway is the main source of NO synthesis by *S. meliloti* during its symbiotic life but that NO of bacterial origin does not have a specific or major role, either at early stages of the plant microbe-interaction or at later stages when nitrogen fixation occurs. Recent research has suggested that beneficial and pathogenic associations share common molecular mechanisms underlying the way the microbes function with their hosts. In this line, NO is a key signal in the establishment and fine-tuning of both mutualistic and pathogenic interactions. NO was described as being a weapon for the host, but there is also growing evidence that NO produced by pathogenic bacteria and fungi is essential for a successful infection of plants. Our work suggests that microbial NO production might not have such a role in beneficial interactions. NO is also a component in the establishment of plant fungal mutualistic interactions, such as the arbuscular mycorrhizal symbiosis (Martínez-Medina et al. 2019) or in another type of symbiosis (i.e., *Vibrio fischerii* and the little Squid *Euprymna scolopes* [Davidson et al. 2004; Mandel and Dunn 2016]). However, data concerning the production of NO by these mutualistic microbes and its role in these interactions are still too scarce and further work will be necessary to develop accurate models.

Another conclusion from our work is that the denitrification pathway does not have a major role during the symbiosis between *S. meliloti* and *M. truncatula*. As the genes encoding the different enzymes in this pathway have been shown to be expressed in the nodules and the enzymes were shown to be functional, it was suspected that denitrification might have a role in one or both respiration and energy production in the oxygen-limiting conditions prevailing inside the nodules. Our results lead to the conclusion that denitrification is not determinant in the symbiotic lifestyle of *S. meliloti*. These results are also supported by a recent work whose aim was to determine the minimal gene set from *S. meliloti* required for efficient symbiosis with *Medicago* spp. In that study, only 58 genes located on the megaplamid pSymA were found to be essential, excluding genes involved in denitrification (Geddes et al. 2021).

One of the denitrification enzymes, i.e., the NO reductase, involved in the reduction of NO into N_2O displays a moderate role in symbiosis. Indeed, a mutant deleted for the *nor* operon and a mutant deleted for the *nap nir* and *nor* operons displayed a small decrease in nitrogen fixation efficiency (Supplementary Fig. S2) and an increase in the number of nodules on the plant roots (significant in the *nor* mutant). This is in accordance with



Number of plants	20	20	48	45	20	20	30	30
KNO_3	-	-	-	-	+	+	-	-
Nodules per plant	7.6 (± 3.0)	8.2 (± 3.5)	8.3 (± 2.8)	9.0 (± 3.4)	7.1 (± 3.9)	6.7 (± 2.3)	13.5 (± 3.8)	12.9 (± 3.4)
Dry weight (mg)	ND	ND	16.4 (± 3.2)	16.9 (± 3.0)	22.4 (± 3.6)	23.0 (± 3.0)	28.3 (± 4.1)	29.0 (± 2.9)

Fig. 3. Nitrogenase activity and shoot dry weight of *Medicago truncatula* plants inoculated with a wild-type (WT) or a mutant *Sinorhizobium meliloti* strain. Plants were tested at the indicated timepoints (in days postinoculation [dpi]) for nitrogen fixation efficiency, using an acetylene reduction assay (ARA) and dry weight of the aerial part of the plants. The mean of ARA values obtained from the WT strain was calculated for each series. In a same series, each value obtained for a WT or a mutant is normalized with this WT mean value. Each value obtained is represented on the graph. Two to four independent series were performed. Statistical analysis was performed by an unpaired *t* test followed by Welch's correction, if necessary. The mean (± standard deviation) for the dry weight of the shoots and the number of nodules per plant is shown in the table under the graph. When indicated, plants were grown in the presence of KNO_3 (0.5 mM). *nap nir* = *napEFDABC* and *nirKV* deletions.

a role of Nor in limiting the total amount of NO found in the nodule. However, one or both a decrease in N_2O and a decrease in energy production linked to this reaction could also explain the observed phenotype.

Denitrification is also involved in NO production in free-living *S. meliloti* bacteria (Ruiz et al. 2019). Our findings do not exclude the idea that denitrification and bacterial NO might have a role in plant-microbe communications underground. The rhizosphere is not only a source of nutrients and support for the plants, it is an ecosystem with diverse groups of microorganisms that are useful or harmful for the plants. Plant-microbe interactions occur via intricate communication networks comprising complex mechanisms of recognition of friends and foes. In this context, NO might play a role in mediating the communication between plants and microbes in the rhizosphere (Pande et al. 2021). Pedospheric NO production mostly originates from denitrification (Hu et al. 2021). Interestingly high denitrification rates were reported in the legume rhizosphere (Achouak et al. 2019; Kilian and Werner 1996). Mechanisms governing plant-microbe interactions in the rhizosphere have gained interest in the last decade and the exact role of NO will have to be looked at deeper in the future.

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