

# Both Plant and Bacterial Nitrate Reductases Contribute to Nitric Oxide Production in *Medicago truncatula* Nitrogen-Fixing Nodules<sup>1[W][OA]</sup>

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Nitric oxide (NO) is a signaling and defense molecule of major importance in living organisms. In the model legume *Medicago truncatula*, NO production has been detected in the nitrogen fixation zone of the nodule, but the systems responsible for its synthesis are yet unknown and its role in symbiosis is far from being elucidated. In this work, using pharmacological and genetic approaches, we explored the enzymatic source of NO production in *M. truncatula*-*Sinorhizobium meliloti* nodules under normoxic and hypoxic conditions. When transferred from normoxia to hypoxia, nodule NO production was rapidly increased, indicating that NO production capacity is present in functioning nodules and may be promptly up-regulated in response to decreased oxygen availability. Contrary to roots and leaves, nodule NO production was stimulated by nitrate and nitrite and inhibited by tungstate, a nitrate reductase inhibitor. Nodules obtained with either plant nitrate reductase RNA interference double knockdown (*MtNIR1/2*) or bacterial nitrate reductase-deficient (*napA*) and nitrite reductase-deficient (*nirK*) mutants, or both, exhibited reduced nitrate or nitrite reductase activities and NO production levels. Moreover, NO production in nodules was found to be inhibited by electron transfer chain inhibitors, and nodule energy state (ATP-ADP ratio) was significantly reduced when nodules were incubated in the presence of tungstate. Our data indicate that both plant and bacterial nitrate reductase and electron transfer chains are involved in NO synthesis. We propose the existence of a nitrate-NO respiration process in nodules that could play a role in the maintenance of the energy status required for nitrogen fixation under oxygen-limiting conditions.

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Nitric oxide (NO) is a gaseous intracellular and intercellular signaling molecule in mammals with a broad spectrum of regulatory functions in various physiological processes (Ignarro, 1999). There is increasing evidence of a role for this molecule in plant growth and development (del Río et al., 2004; Delledonne, 2005). NO appears to have signaling functions in the induction of cell death, defense genes, and interaction with reactive oxygen species during plant defense against pathogen attack (Wendehenne et al., 2001; Delledonne, 2005). Similarly, there is evidence of a role for NO in plants exposed to abiotic stress such as osmotic stress, salinity, or high temperature (Gould et al., 2003). However, NO synthesis in plants is still a matter of debate (Besson-Bard et al., 2008; Corpas et al., 2009; Moreau et al., 2010). Possible generating systems have been proposed, such as NO synthase-like proteins (Corpas et al., 2009), nitrate reductase (NR; Dean and Harper, 1988; Rockel et al., 2002), polyamine oxidase (Yamasaki and Cohen, 2006), and nitrite (NO<sub>2</sub><sup>-</sup>)-NO reductase (Stöhr and Stremmler, 2006), but convincing evidence of

their involvement in the purposeful generation of NO in vivo is still lacking (Zemojtel et al., 2006; Moreau et al., 2010).

Contrary to that in pathogenic situations, the interaction between legumes and soil bacteria of the Rhizobiaceae family leads, after extensive recognition of both partners, to the establishment of a symbiotic relationship characterized by the formation of new differentiated organs called nodules, which provide a niche for bacterial nitrogen fixation. Functional nodules result from the combination of developmental and infectious processes: bacteria released in plant cells differentiate into bacteroids with the unique ability to fix atmospheric nitrogen via nitrogenase activity (Oldroyd and Downie, 2008). As nitrogenase is strongly inhibited by oxygen, nitrogen fixation is made possible by the microaerophilic conditions prevailing in the nodule (Millar et al., 1995).

Several lines of evidence have demonstrated the occurrence of NO production during the legume-rhizobium symbiosis. NO was transiently observed in *Lotus japonicus* and *Medicago sativa* roots within a few hours after infection with *Mesorhizobium loti* and *Sinorhizobium meliloti* strains, respectively (Shimoda et al., 2005; Nagata et al., 2008). NO has also been involved in the auxin-controlled formation of *M. sativa* and *Medicago truncatula* nodules (Pii et al., 2007). NO formation has also been detected in functional *M. truncatula*-*S. meliloti* nodules: NO detection was restricted to the fixing zone of the nodule (Baudouin et al., 2006). Finally, NO has been shown to be produced by mature *Glycine max*-*Bradyrhizobium japonicum* nodules in response to flooding conditions (Meakin et al., 2007; Sánchez et al., 2010). A wide modulation of NO-responsive genes has also been detected during the establishment of a functioning nodule, pointing to a possible contribution of NO in nodule metabolism (Ferrari et al., 2008). Moreover, it has been shown that leghemoglobin (Lb), the hemoprotein ensuring an oxygen flux to the bacteroids in the microaerophilic conditions of the nodule, has the capacity to scavenge NO (Herold and Puppo, 2005), which suggests that it participates in the protection of bacteroids against the inhibition of nitrogenase by NO (Trinchant and Rigaud, 1982; Shimoda et al., 2009; Kato et al., 2010). In the same way, NO has been shown to induce the expression of nonsymbiotic hemoglobin genes in *L. japonicus* (Shimoda et al., 2005), and overexpression of a class 1 plant hemoglobin gene appeared to enhance symbiotic nitrogen fixation activity between *L. japonicus* and *M. loti* (Shimoda et al., 2009). Similarly, *M. truncatula* or *M. sativa* nodules induced by a *S. meliloti* mutant strain deficient in the flavohemoglobin Hmp, a well-known NO-degrading enzyme, showed a decreased nitrogen fixation efficiency (Meilhoc et al., 2010). The sources of NO in symbiotic nodules are still unclear. In bacteria, the denitrification pathway is the main route for NO production identified to date (Zumft, 1997), and it was assumed that it could be a source of NO in

symbiotic nodules. This was recently demonstrated by Delgado's team, which found that *B. japonicum* periplasmic nitrate ( $\text{NO}_3^-$ ) and  $\text{NO}_2^-$  reductase are the main source of NO production in soybean (*Glycine max*) nodules in response to flooding (Sánchez et al., 2010). On the plant partner side, a NO synthase-like activity has been measured in lupine (*Lupinus albus*) nodules (Cueto et al., 1996), and it was suggested that such an enzyme could be responsible for NO production in nodule infected cells (Baudouin et al., 2006). On the other hand, it has been known for a long time that nodules, grown aseptically in the absence of a source of combined nitrogen, exhibit high NR activity (Cheniae and Evans, 1960), and it was asked whether nodule NR activity could be involved in functioning nodules (Kato et al., 2010). However, no evidence for the contribution of either a nitric oxide synthase (NOS)-like enzyme or the NR to NO production in the plant partner was provided to date.

As underground organs, nodules may be subjected to flooding or drought episodes. Hypoxia is a major determinant in the adverse effects of waterlogging on plants (Mommer et al., 2004). Hypoxic stress has pronounced effects on mitochondrial function, both from the perspective of oxygen limitation and from increased production of compounds that compete at the oxygen-binding site. Among these compounds, NO has been demonstrated to be produced in hypoxic roots through a mechanism called "nitrate-NO respiration," which involves the nonsymbiotic hemoglobin, the NR, and the electron transfer chain (ETC; Gupta et al., 2005; Igamberdiev and Hill, 2009). Under these conditions,  $\text{NO}_2^-$  acts as a final electron acceptor instead of oxygen. In the ETC,  $\text{NO}_2^-$  is reduced to NO, which diffuses to the cytosol, where it is oxidized by the nonsymbiotic hemoglobin to  $\text{NO}_3^-$ .  $\text{NO}_3^-$  is then reduced to  $\text{NO}_2^-$  by cytosolic NR.

In this work, using pharmacological and genetic approaches, we explored the enzymatic source of NO production in *M. truncatula*-*S. meliloti* nodules under normoxic and hypoxic conditions. We report that both plant and bacterial NR and ETC are involved in NO synthesis, and we propose the existence of a  $\text{NO}_3^-$ -NO respiration process in nodules.

## RESULTS

### Hypoxia Triggers Overproduction of NO by Nodules

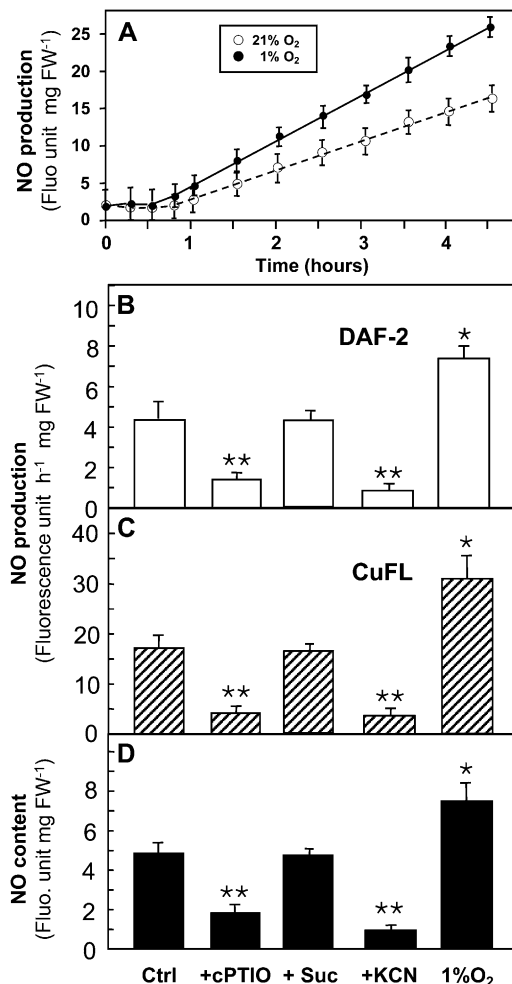
To investigate the production of NO by symbiotic nodules, *M. truncatula* plants were inoculated with *S. meliloti* and grown in normoxic conditions for 4 to 5 weeks. NO production by *M. truncatula*-*S. meliloti* nodules was analyzed using the 4,5-diaminofluorescein (DAF-2) probe. In the presence of oxygen, NO autooxidizes to nitrous anhydride, which reacts with DAF-2 to form a highly fluorescent triazolofluorescein (DAF-2T; Kojima et al., 1998). In previous reports, the production of NO in root nodules was investigated using sliced nodules (Baudouin et al., 2006; Shimoda

et al., 2009; Kato et al., 2010). However, as NO was shown to be produced in response to excision and mechanical stress (Arasimowicz and Floryszak-Wieczorek, 2007), we decided to measure the NO produced and released from entire nodules with a less invasive method to keep the nodule structures intact and maintain the microaerophilic conditions inside the nodule. Entire nodules were taken from the roots and incubated in a detection medium containing the DAF probe. In these conditions, after a 45- to 60-min transient equilibration period, the production of NO, when measured under either 21% or 1% oxygen, was found to be linear for at least 4 h (Fig. 1A). Consequently, NO

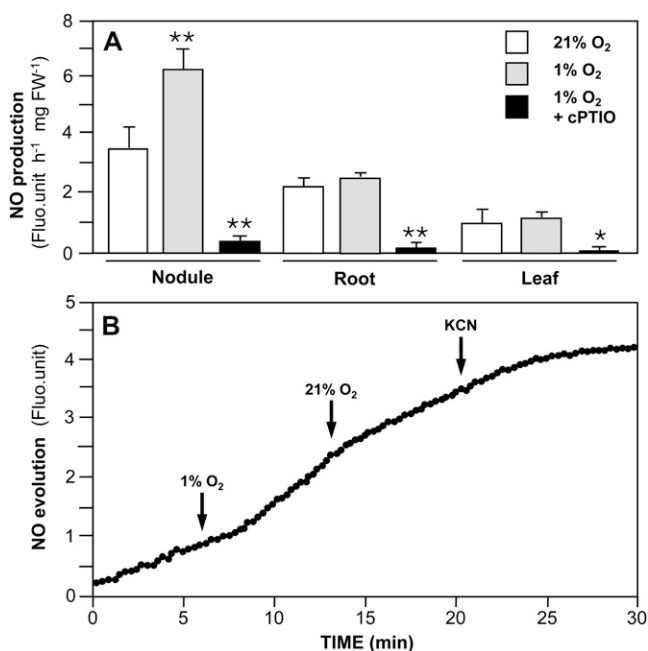
production was routinely measured between 2 and 4 h of incubation.

To test the experimental protocol of NO measurement and the specificity of DAF for NO, nodule NO production was analyzed in various conditions (Fig. 1B). When measured in the presence of 2-[4-carboxyphenyl]-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO; a NO scavenger), DAF fluorescence was 73% inhibited compared with the control, showing that the major part of the DAF fluorescence was due to the production of NO itself and not to products interacting with DAF-2 (Planchet and Kaiser, 2006). The presence of 50 mM Suc in the incubation medium did not modify NO production, indicating that nodules were not sugar starved during the experiments. In the presence of KCN, a lethal and potent inhibitor of many heme-containing enzymes, NO production was 83% inhibited (Fig. 1B). The remaining NO production measured with KCN treatment (about 1 fluorescence unit  $\text{h}^{-1} \text{mg}^{-1}$  fresh weight) was considered as the background in our experimental system and was subtracted in the following experiments. Under 1% oxygen (12  $\mu\text{M}$  oxygen), NO production was 1.7-fold increased compared with the control (Fig. 1, A and B), indicating that DAF fluorescence may be used for NO measurement in hypoxic conditions. Moreover, measurement of NO production by samples containing 5 to 120 mg of fresh nodules showed that neither oxygen nor DAF-2 was limiting in our experimental conditions (data not shown). In addition, to further test our experimental system, NO production was also analyzed with the Cu(II) fluorescein (CuFL) fluorescent probe, which is known to react rapidly and specifically with NO itself (Lim et al., 2006). The absence of CuFL toxicity for nodules was first verified (see "Materials and Methods"), and NO production was then measured in the conditions tested above. The results were similar to those obtained with the DAF probe (Fig. 1C). Finally, NO production was compared with the amount of NO measured in the extracts of nodules crushed immediately after a 4-h incubation period with the above-tested effectors. NO measured in tissue extracts (Fig. 1D) was found to correlate NO production (Fig. 1, B and C), which confirmed whole organ assays. Taken together, these data show that DAF fluorescence may be considered as a good indicator of NO produced and released from nodules and that the protocol used in this study is efficient to assess NO production by symbiotic nodules. A similar protocol was recently used to estimate nodular NO accumulation in soybean nodules (Sánchez et al., 2010). All the experiments of NO production reported in this work were first carried out with DAF-2 and then repeated with CuFL with similar results. For simplicity, only the data obtained with DAF-2 are presented below.

The production of NO in normoxic and hypoxic conditions was tested also in root segments (without nodule) and leaf discs. In normoxia, NO production of roots and leaves was 35% and 70% lower, respectively, than that found with nodules, and this production was



**Figure 1.** NO production and content in *M. truncatula* nodules. A, Time course of NO production measured using DAF-2 under either 21% or 1% oxygen. B and C, Nodule NO production measured using either DAF-2 (B) or CuFL (C) probes. D, Nodule NO content. NO production and content are expressed as relative fluorescence units. Nodules were incubated under 21% oxygen (control [Ctrl]), in the presence of either 100  $\mu\text{M}$  cPTIO, 50 mM Suc (Suc), or 300  $\mu\text{M}$  KCN, or under 1% oxygen. FW, Fresh weight. Data are means  $\pm$  SD of five (A and B), four (C), and three (D) independent experiments assayed in duplicate. Asterisks indicate significant difference (\*  $P = 0.05$ , \*\*  $P = 0.01$ ) when compared with the control according to Student's *t* test.

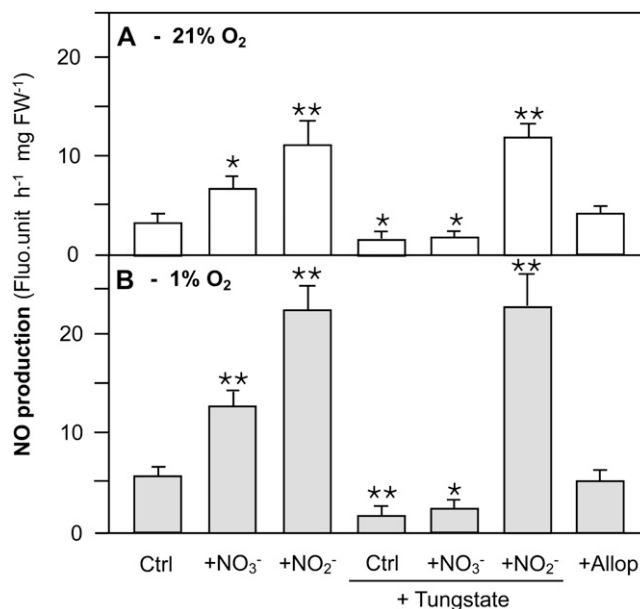


**Figure 2.** NO production by *M. truncatula* nodule, root, and leaf. A, Nodules, root segments, or leaf discs were incubated under 21% oxygen, 1% oxygen, or 1% oxygen plus 100  $\mu\text{M}$  cPTIO. NO production was expressed as relative fluorescence units. FW, Fresh weight. Data are means  $\pm$  SD of six (nodule) or three (root and leaf) independent experiments assayed in duplicate. Asterisks indicate significant difference (\*  $P = 0.05$ , \*\*  $P = 0.01$ ) when compared with the control according to Student's  $t$  test. B, Six nodules were fixed in a fluorescence cuvette, and the time course of NO evolution was recorded using DAF-2 under different oxygen conditions. Starting  $p\text{O}_2$  was 21% oxygen; KCN was 300  $\mu\text{M}$ . This experiment was reproduced four times with similar results.

not increased in hypoxic conditions (Fig. 2A). It may be noted that, whatever the organ, fluorescence was 90% to 95% reduced when measured at 1% oxygen in the presence of 100  $\mu\text{M}$  cPTIO, which indicates that most of the fluorescence was related to NO production. These data show that, contrary to roots and leaves, nodules are able to overproduce NO within hours following the transition from normoxia to hypoxia. To assess the sensitivity of the nodules to changing partial oxygen pressure ( $p\text{O}_2$ ) conditions, nodule NO production was measured during rapid normoxic (21% oxygen) to hypoxic (1% oxygen) transition, and vice versa. As reported in Figure 2B, NO production rate exhibited a 2-fold increase within 2 to 4 min after 21% to 1% oxygen transition and decreased within 5 min upon return to 21% oxygen conditions. As observed previously (Fig. 1), the addition of 300  $\mu\text{M}$  KCN to the incubation medium almost totally abolished the production of NO (Fig. 2B). These results underline the flexibility and the reversibility of nodule NO production regarding the oxygen environment and indicate that nodules are able to quickly respond to changes in  $p\text{O}_2$ .

## NR Activity Is Involved in Nodule NO Production

DAF fluorescence was analyzed with nodules incubated in the presence of NR effectors under either 21% or 1% oxygen. As shown in Figure 3, in the presence of 10 mM  $\text{NO}_3^-$ , the substrate of NR, NO production was 2.2-fold increased both in normoxia and hypoxia, suggesting that NR is possibly involved in NO production. To further test this hypothesis, NR activity was inhibited with the use of tungstate (Tg), an inhibitor of NR (Harper and Nicholas, 1978). In these conditions, Tg significantly reduced NO production both in the control and in the presence of  $\text{NO}_3^-$  (Fig. 3). This means that NR is involved in the production of NO either directly or indirectly via the production of  $\text{NO}_2^-$ , the product of NR. Moreover, on the basis of Tg inhibition results, it may be concluded that the increase in NO production observed under hypoxia was due to NR activity, since NO production was inhibited to similar values (1.5 fluorescence units  $\text{h}^{-1} \text{mg}^{-1}$  fresh weight) both in normoxia and hypoxia (Fig. 3). This indicates that NR activity contributes more importantly to NO production under hypoxia than under normoxia. In the presence of 1 mM  $\text{NO}_2^-$ , nodule NO production increased 3.6- and 4.0-fold under normoxic and hypoxic conditions, respectively (Fig. 3). However, it was not inhibited by Tg in the presence of  $\text{NO}_2^-$ , which indicates that NR is involved in NO production through the reduction of  $\text{NO}_3^-$  in  $\text{NO}_2^-$ .



**Figure 3.** Effects of NR effectors on nodule NO production. NO production, expressed as relative fluorescence units, was measured under either 21% (A) or 1% (B) oxygen. Effector concentrations were 10 mM  $\text{NaNO}_3$  ( $\text{NO}_3^-$ ), 1 mM  $\text{NaNO}_2$  ( $\text{NO}_2^-$ ), and 1 mM NaTg. FW, Fresh weight. Data are means  $\pm$  SD of five independent experiments assayed in duplicate. Asterisks indicate significant difference (\*  $P = 0.05$ , \*\*  $P = 0.01$ ) when compared with the control (Ctrl) according to Student's  $t$  test.

but does not produce NO directly. In addition to NR, xanthine oxidase (a MoCo enzyme like NR) has been reported to reduce  $\text{NO}_2^-$  into NO (Millar et al., 1998; Li et al., 2001). As xanthine oxidase is also inhibited by Tg, the question arose whether the production of NO, and its inhibition in the presence of Tg, could be due to xanthine oxidase activity rather than that of NR. To answer this question, we analyzed the effect of allopurinol, an inhibitor of xanthine oxidase (Atkins et al., 1988), on NO production. As reported in Figure 3, allopurinol did not modify NO production under either 21% or 1% oxygen, which excludes the contribution of xanthine oxidase in the synthesis of NO.

The involvement of NR activity in the generation of NO has already been investigated in various plant organs and tissues, and it was concluded that it contributes, directly or indirectly, to NO production in roots and leaves (Dean and Harper, 1988; Rockel et al., 2002; Gupta et al., 2005; Planchet et al., 2005). Thus, to assess the possible contribution of NR in NO production in other *M. truncatula* organs than nodules, root segments and leaf discs were also analyzed for NO production in the presence or absence of NR effectors. It resulted that, under either 21% or 1% oxygen, the production of NO was not affected by the addition of  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ , or Tg in the incubation medium (Supplemental Fig. S1). This means that, contrary to what happens in nodules, NR is not involved in the production of NO in the roots and leaves of *M. truncatula* plants.

To further investigate the differences between nodules, roots, and leaves with regard to NR-dependent NO production, we analyzed NR and nitrite reductase (NiR) activities in these organs. When expressed as a function of fresh weight, NR activity was found to be 3- and 6-fold higher in nodules than in roots and leaves, respectively (Table I). These activities are of the same order of magnitude than those measured in the nodules of other legumes such as yellow lupine (Polcyn and Lucinski, 2001), faba bean (*Vicia faba*) and pea (*Pisum sativum*; Chalifour and Nelson, 1987), or soybean (Arrese-Igor et al., 1998). In the three organs, NiR activity was higher than that of NR (Table I). It has long been known that  $\text{NO}_2^-$  is cytotoxic for plants, although the molecular mechanism is still obscure, and a higher NiR versus NR activity, which avoids  $\text{NO}_2^-$  accumulation in the tissues, was classically observed in plants (Luciński et al., 2002). Interestingly, the NiR-to-NR activity ratio was found to be about 2 in nodules and 8 in roots and leaves (Table I). This indicates that the  $\text{NO}_2^-$  production versus  $\text{NO}_2^-$

utilization capacity is significantly higher in nodules than in roots and leaves and underlines a possible specific function of NR in the nodules.

### Both Plant and Bacteroid NRs Contribute to NO Production in Nodules

In symbiotic nodules, NR activity has generally been found, with some exceptions, in both the plant and the bacteroid partners (Luciński et al., 2002). In this work, to determine if the NR-dependent production of NO observed in the nodules was due to either one or both of the partners, we used a mutant approach.

Two NR genes have been identified in *M. truncatula*, NR1 (TC137636; Mtr.10604.1.S1\_at) and NR2 (TC130773; Mtr.42446.1.S1\_at), which are both expressed at detectable levels in  $\text{N}_2$ -fixing nodules (data not shown). To date, the main function of NR identified in plants is its key role in the  $\text{NO}_3^-$ -to- $\text{NH}_4^+$  reduction pathway, which controls nitrogen metabolism (Campbell, 1999). Thus, to assess the involvement of NR in the production of NO by the nodules, without affecting the nitrogen metabolism in the whole plant, we used a nodule-targeted RNA interference (RNAi) strategy. An RNAi *M. truncatula* *MtNR1/2* double knockdown was constructed under the control of the zone III-specific promoter MtNCR001 (Mergaert et al., 2003; Supplemental Fig. S2A). In this way, NR expression level was only affected in the  $\text{N}_2$ -fixing zone (zone III) of the nodule, avoiding any other effect that could affect plant and nodule growth at early stages of development. Four weeks after inoculation, *MtNR1/2* RNAi transgenic roots did not show significantly modified phenotypes compared with the GUS RNAi control for plant growth and nodulation events (data not shown), but nodule size was 30% to 40% reduced in the *MtNR1/2* RNAi roots (Supplemental Fig. S2, B and C). Measurement of the NR activity in this knockdown mutant line showed a 40% decrease compared with the GUS RNAi control nodules (Table II). In the *MtNR1/2* RNAi nodules, the production of NO was found to be 46% decreased compared with that of control nodules when measured under either 1% oxygen (Fig. 4; Table II) or 21% oxygen (Supplemental Fig. S3). In addition, for both *MtNR1/2* RNAi and GUS RNAi control nodules, the production of NO was found to be increased by  $\text{NO}_2^-$  and inhibited by Tg under 1% oxygen (Fig. 4) or 21% oxygen (Supplemental Fig. S3). These results clearly indicate that the decrease in NO production in knockdown nodules was related to the decrease in the plant NR activity and

**Table I.**  $\text{NO}_3^-$  and  $\text{NO}_2^-$  reductase activities in nodule, root, and leaf of *M. truncatula* plants

Data are means  $\pm$  SD of six (nodule) or three (root and leaf) independent experiments.

NR or NiR	Nodule	Root	Leaf
NR (nmol min <sup>-1</sup> g <sup>-1</sup> fresh weight)	9.7 $\pm$ 1.2	3.3 $\pm$ 1.3	1.7 $\pm$ 0.6
NiR (nmol min <sup>-1</sup> g <sup>-1</sup> fresh weight)	18.5 $\pm$ 2.3	26.1 $\pm$ 8.3	13.2 $\pm$ 5.0
NiR-NR	1.9	7.9	7.8

**Table II.** NR activity and NO production in *M. truncatula*-*S. meliloti* control and mutant nodules

*M. truncatula* control (GUS) and *MtNR1/2* RNAi (*MtNR1/2*) plants were inoculated with *S. meliloti* 2011 or *napA* strains. NO production, expressed as relative fluorescence units, was measured under 1% oxygen. Data are means  $\pm$  SD of four independent measurements. Asterisks indicate significant difference (\**P* = 0.05, \*\**P* = 0.01) when compared with the control (GUS/2011) according to Student's *t* test.

Variable	GUS/2011	MtNR1/2/2011	GUS/ <i>napA</i>	MtNR1/2 <i>napA</i>
NR activity (nmol NO <sub>2</sub> <sup>-</sup> min <sup>-1</sup> g <sup>-1</sup> fresh weight)	6.56 $\pm$ 0.87	3.94 $\pm$ 0.87*	4.69 $\pm$ 0.76*	3.08 $\pm$ 0.57**
Percent of control	100	60	71	47
NO production (fluorescence units h <sup>-1</sup> mg <sup>-1</sup> fresh weight)	4.65 $\pm$ 0.84	2.54 $\pm$ 0.71*	2.81 $\pm$ 0.39*	1.33 $\pm$ 0.37**
Percent of control	100	54	60	29

that the remaining NO production was dependent on bacteroid and/or residual plant NR activities.

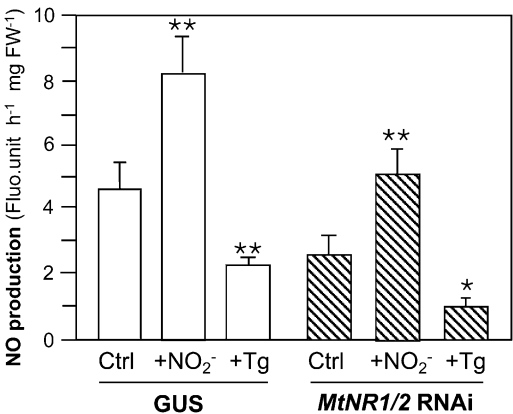
In bacteria such as *S. meliloti*, the denitrification process is known to generate NO as an intermediate of NO<sub>3</sub><sup>-</sup> reduction to N<sub>2</sub>. NO<sub>3</sub><sup>-</sup> is first reduced to NO<sub>2</sub><sup>-</sup> by NR, and NO<sub>2</sub><sup>-</sup> is then reduced to NO by NiR. To investigate the involvement of the bacteroid denitrification pathway in the generation of NO, we analyzed NO production in nodules formed upon root infection with *S. meliloti napA* and *nirK* mutant strains, which are impaired in NR and NiR activity, respectively. As reported in Table III, NR and NiR activities were found to be 37% and 38% reduced, respectively, in *napA* and *nirK* nodules compared with wild-type ones. As a control, no NR or NiR activity was found in the bacteroid fractions extracted from *napA* and *nirK* mutant nodules, respectively (Table III), which confirms the absence of NR or NiR activity in the mutant strains. In both *napA* and *nirK* mutant nodules, the production of NO was decreased by about 35% compared with that of the wild-type control when measured under either 1% oxygen (Fig. 5) or 21% oxygen (Supplemental Fig. S3). Moreover, as observed in wild-type nodules, NO production was stimulated by NO<sub>2</sub><sup>-</sup> and inhibited by Tg when measured under either 1% oxygen (Fig. 5) or 21% oxygen (Supplemental Fig. S3). These results indicate that the decrease in NO production in *napA* and *nirK* mutant nodules was related to the absence of bacteroid NR and NiR activities, respectively, and that the remaining NO production was dependent on the plant partner NR and other potential plant or bacteroid NO-producing activities.

*MtNR1/2* and GUS RNAi transgenic roots were inoculated with *S. meliloti* wild type and *napA* mutant strains to test for a possible additive effect of plant and bacterial NR mutations on NO production. In agreement with the data presented above (Figs. 4 and 5), NR activity and NO production were decreased in both *MtNR1/2* RNAi and *napA* mutant nodules (Table II). The effects of the plant NR-silencing and bacteroid NR mutations were found to be partially additive in the *MtNR1/2* RNAi/*napA* nodules, where NR activity and NO production were decreased to 47% and 29% of their respective controls (Table II). Despite the absence of fully additive effects at NR activity and NO production levels, which probably can be explained by the up-regulation of complementary systems, these data confirm that NO production in nodules is essentially related to the activity of NR.

Taken together, our data showed that, in *M. truncatula*-*S. meliloti* nodules, (1) both the plant and the bacteroid partners produce NO through NR-dependent processes, (2) NO is mainly produced by the plant partner, and (3) around one-third of the NO generated by the nodule is produced by the bacteroid denitrification pathway.

**Mitochondrial and Bacteroid ETCs Are Involved in Nodule NO Production**

The maintenance of NO production, in the presence of both NO<sub>2</sub><sup>-</sup> and Tg (Fig. 3), indicated that NR does not produce NO directly but more likely produces NO<sub>2</sub><sup>-</sup>, which in turn is reduced to NO. Beside NR, root mitochondria have been reported to be able to reduce NO<sub>2</sub><sup>-</sup> to NO at the expense of NADH under anoxic conditions but not in air (Gupta et al., 2005; Planchet et al., 2005; Gupta and Kaiser, 2010). Here, we investigated the involvement of the mitochondria in NO production through the use of various mitochondrial and denitrification pathway inhibitors. As reported in Figure 6A, under either 21% or 1% oxygen, NO production was 40% inhibited by rotenone, an inhibitor of



**Figure 4.** NO production by *M. truncatula* GUS and *MtNR1/2* RNAi nodules. *M. truncatula* control (GUS) and *MtNR1/2* RNAi plants were inoculated with *S. meliloti* 2011 strain. NO production, expressed as relative fluorescence units, was measured under 1% oxygen. Effector concentrations were 1 mM NaNO<sub>2</sub> (NO<sub>2</sub><sup>-</sup>) and 1 mM NaTg. FW, Fresh weight. Data are means  $\pm$  SD of three independent experiments assayed in duplicate. Asterisks indicate significant difference (\**P* = 0.05, \*\**P* = 0.01) when compared with the control (Ctrl) according to Student's *t* test.

**Table III.**  $\text{NO}_3^-$  and  $\text{NO}_2^-$  reductase activities in nodules and bacteroids of *M. truncatula* plants

*M. truncatula* wild-type plants were inoculated with *S. meliloti* 2011, *napA*, or *nirK* strains. The inhibition of NR activity by Tg in nodule and bacteroid preparations was checked after a 4-h incubation period in the presence of the inhibitor. nd, Not detected. Asterisks indicate significant difference (\* $P = 0.05$ ) when compared with the control (2011) according to Student's *t* test.

NR or NiR	Nodule			Bacteroid		
	2011	<i>napA</i>	<i>nirK</i>	2011	<i>napA</i>	<i>nirK</i>
	<i>nmol NO<sub>2</sub><sup>-</sup> min<sup>-1</sup> g<sup>-1</sup> fresh wt</i>			<i>nmol NO<sub>2</sub><sup>-</sup> min<sup>-1</sup> mg<sup>-1</sup> protein</i>		
NR						
Control	9.7 ± 1.2	6.1 ± 1.2*	8.5 ± 0.8	0.13 ± 0.02	nd	0.11 ± 0.03
+1 mM NaTg	1.4 ± 0.3	–	–	0.01 ± 0.01	–	–
NiR	18.5 ± 2.3	16.0 ± 4.3	11.5 ± 2.7*	1.12 ± 0.07	1.25 ± 0.08	nd

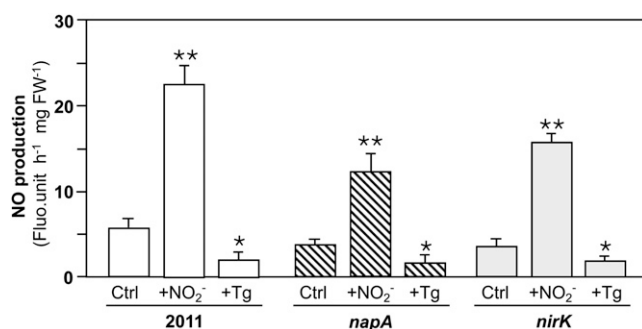
the mitochondrial complex I and of the bacteroid NADH-quinol oxidoreductase. In the presence of antimycin A and myxothiazol, two inhibitors of the complex III, NO production was 50% to 55% and 80% inhibited in normoxic and hypoxic conditions, respectively (Fig. 6A). The NO production insensitive to the inhibitors (approximately 2 fluorescence units  $\text{h}^{-1} \text{mg}^{-1}$  fresh weight in both conditions) accounted for the residual part of NO, the production of which does not depend on ETC functioning. This means that in normoxia, the production of NO largely depends on mitochondrial and bacteroid ETC functioning and that the increase in NO production observed in hypoxic versus normoxic conditions was essentially contributed by mitochondrial and bacteroid ETCs. Furthermore, NO production was found to be insensitive to the uncoupler carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP; Fig. 6A), indicating that it does not depend on the presence of the transmembrane electrochemical proton gradient. Similarly, NO production was found to be insensitive to propylgalate, an inhibitor of the mitochondrial alternative oxidase (AOX), which indicates that AOX probably does not contribute to NO production (data not shown). When nodules were incubated in the presence of  $\text{NO}_2^-$  in the incubation medium, NO production was increased in the control condition, as already shown in Figure 3, and was inhibited by the above-tested inhibitors to the same extent as in the absence of  $\text{NO}_2^-$  (Supplemental Fig. S4). Moreover, as these inhibitors are not specific for either mitochondria or bacteroid ETC, the production of NO was also analyzed in nodules issued from *M. truncatula* inoculated with *S. meliloti nirK* mutants, where bacteroid ETC presumably does not produce NO. As shown in Figure 6B, the effects of all the inhibitors tested on NO production were similar to that observed with wild-type nodules under 21% as well as 1% oxygen.

Taken together, these data show that nodule NO production is (1) strongly (80%) or partially (60%) inhibited by ETC inhibitors in hypoxia and normoxia, respectively, (2) independent of the transmembrane electrochemical proton gradient, (3) stimulated by  $\text{NO}_2^-$  supply, and (4) similarly inhibited by ETC inhibitors both in wild-type and *nirK* mutant nodules. This means, first, that mitochondrial and bacteroid

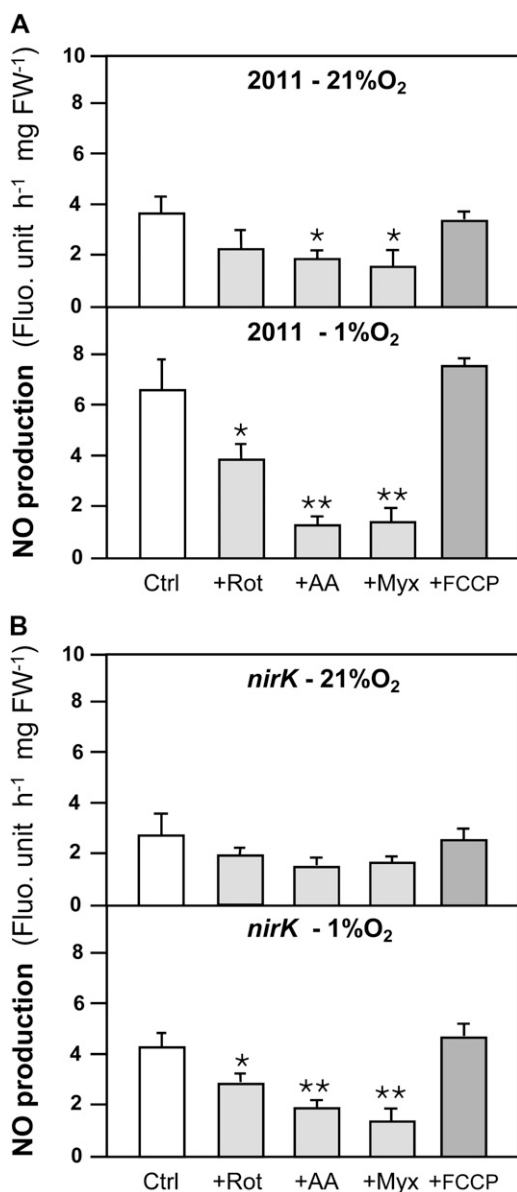
ETC are directly involved in the production of NO in functioning nodules, and second, that NO is essentially produced through both mitochondrial and bacteroid ETC in hypoxic conditions.

#### NR Activity Is Necessary to Maintain Nodule Energy Status

In the roots of plants subjected to hypoxia, a  $\text{NO}_3^-$ -NO respiration, involving the sequential reduction of  $\text{NO}_3^-$  into  $\text{NO}_2^-$  and then NO via NR and mitochondrial ETC, was proposed to contribute to energy supply under microaerobic conditions (Igamberdiev and Hill, 2009). The above data demonstrating the involvement of NR in the production of NO raised the hypothesis of a role of NR in the energy functioning of symbiotic nodules. To test this hypothesis, we analyzed the energy state (i.e. the ATP-ADP ratio) of nodules incubated in the presence of NR effectors (Fig. 7). Under 21% oxygen, ATP-ADP ratio was high (close to 6–7) in the control nodules, indicating that ATP-regenerating processes were not limited. In the pres-



**Figure 5.** NO production by *S. meliloti* 2011, *napA*, and *nirK* nodules. *M. truncatula* wild-type plants were inoculated with *S. meliloti* 2011, *napA*, or *nirK* strains. NO production, expressed as relative fluorescence units, was measured under 1% oxygen. Effector concentrations were 1 mM  $\text{NaNO}_2$  ( $\text{NO}_2^-$ ) and 1 mM NaTg. FW, Fresh weight. Data are means ± SD of four independent experiments assayed in duplicate. Asterisks indicate significant difference (\* $P = 0.05$ , \*\* $P = 0.01$ ) when compared with the control (Ctrl) according to Student's *t* test.



**Figure 6.** Effects of ETC effectors on NO production of *M. truncatula*-*S. meliloti* nodules. *M. truncatula* wild-type plants were inoculated with *S. meliloti* 2011 (A) or *nirK* (B) strains. NO production, expressed as relative fluorescence units, was measured under either 21% or 1% oxygen. Effector concentrations were 10  $\mu$ M rotenone (Rot), 25  $\mu$ M antimycin A (AA), 25  $\mu$ M myxothiazol (Myx), and 10  $\mu$ M FCCP. FW, Fresh weight. Data are means  $\pm$  SD of four (2011) and two (*nirK*) independent experiments assayed in duplicate. Asterisks indicate significant difference (\*  $P = 0.05$ , \*\*  $P = 0.01$ ) when compared with the control (Ctrl) according to Student's *t* test.

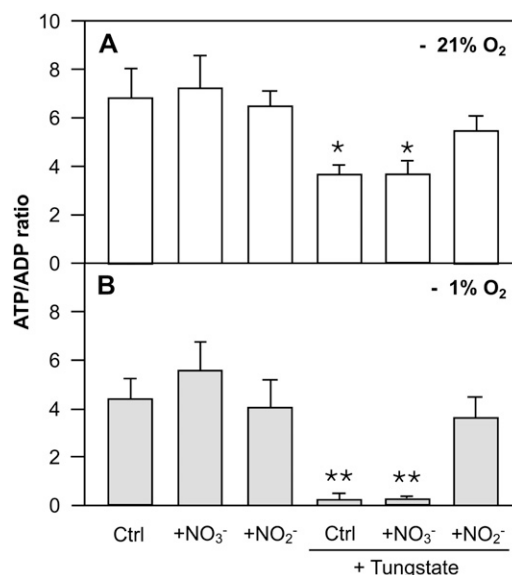
ence of  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ , or Tg plus  $\text{NO}_2^-$ , the ATP-ADP ratio was not significantly modified, but it was 50% decreased when nodules were incubated with Tg only or Tg plus  $\text{NO}_3^-$  (Fig. 7A). This means that the inhibition of NR partially affects the energy state of the nodule even in normoxic conditions. Under 1% oxygen, the ATP-ADP ratio of control nodules was

close to 4 (Fig. 7A), which indicates that the decrease in  $p\text{O}_2$  from 21% to 1% significantly affects the energy status of the nodules but maintains it compatible with nodule functioning. In these conditions, the presence of  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ , or Tg plus  $\text{NO}_2^-$  did not significantly modify the energy status of the nodules, but Tg only or Tg plus  $\text{NO}_3^-$  triggered a dramatic fall (95%) of the ATP-ADP ratio. These data clearly mean that, under 1% oxygen, ATP-regenerating processes almost entirely depend on the functioning of NR activity.

## DISCUSSION

### Plant and Bacteroid NR and ETC Contribute to NO Production

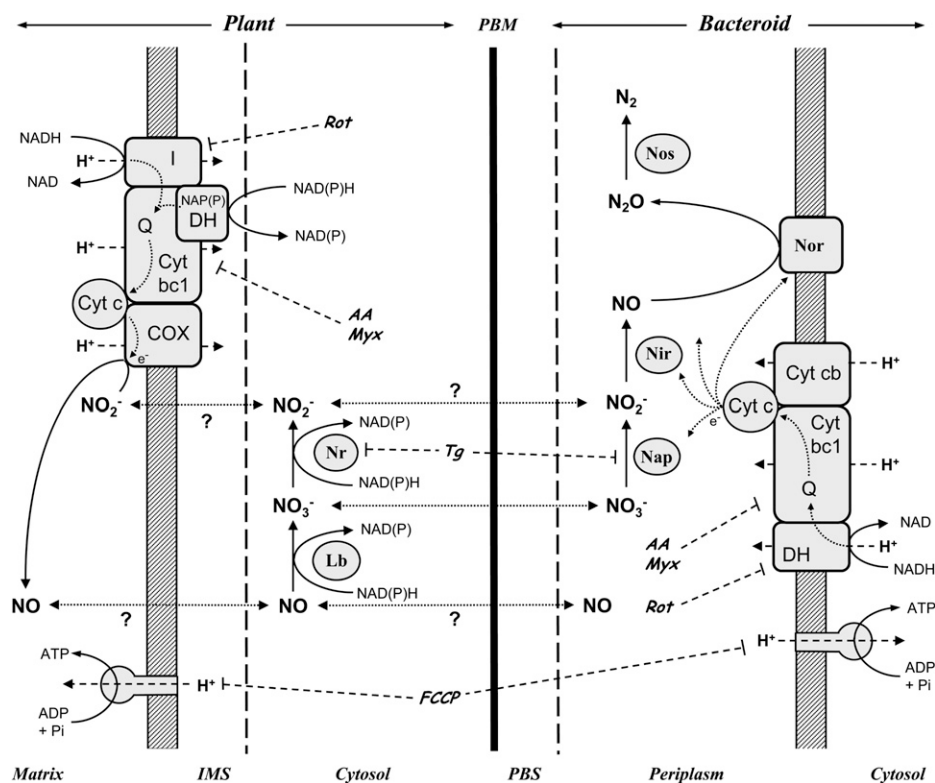
NO synthesis in plants has been reported to occur via different routes such as NR,  $\text{NO}_2^-$ -NO reductase, mitochondrial ETC, NOS-like, nonenzymatic reduction, and potentially an as yet unidentified polyamine oxidation pathway (Besson-Bard et al., 2008; Moreau et al., 2010). In bacteria, the main route for NO production identified to date is the denitrification pathway, which supplies energy to the cell under hypoxic conditions (Zumft, 1997), although NOS enzymes (Sudhamsu and Crane, 2009) or NOS-like activities (Pii et al., 2007) are also present. The specificity of the nodule is to gather plant and bacteroid partners into the same organ (Fig. 8), which complicates the analysis of the NO source. In this work, both pharmacological and genetic approaches were used to analyze the



**Figure 7.** Effects of NR effectors on nodule ATP-ADP ratio. Adenine nucleotides were measured under either 21% (A) or 1% (B) oxygen. Effector concentrations were 10 mM  $\text{NaNO}_3$  ( $\text{NO}_3^-$ ), 1 mM  $\text{NaNO}_2$  ( $\text{NO}_2^-$ ), and 1 mM NaTg. Data are means  $\pm$  SD of two (21% oxygen) or three (1% oxygen) independent experiments assayed in duplicate. Asterisks indicate significant difference (\*  $P = 0.05$ , \*\*  $P = 0.01$ ) when compared with the control (Ctrl) according to Student's *t* test.

potential role of NR in the production of NO. First, the increase in NO production upon nodule feeding with either  $\text{NO}_3^-$  or  $\text{NO}_2^-$ , and its inhibition by Tg (Fig. 3), and second, the lower level of NO production in *MtNR1/2* RNAi (Fig. 4) and *napA* and *nirK* (Fig. 5) mutant nodules, provide strong evidence for a  $\text{NO}_2^-$ -dependent NO synthesis via the activity of NR. However, the relief of the Tg-related inhibition of NO production by  $\text{NO}_2^-$  (Fig. 3) clearly indicates that NR does not produce NO by itself but  $\text{NO}_2^-$ , which is then reduced into NO. The inhibition of NO production by ETC inhibitors such as rotenone, antimycin A, and myxothiazol (Fig. 6), but not by FCCP or propylgalate, indicates that mitochondrial and bacteroid ETCs are directly involved in the reduction of  $\text{NO}_2^-$  into NO, probably at the cytochrome oxidase site. Thus, in *M. truncatula* nodules,  $\text{NO}_3^-$  may be reduced into NO in a two-step mechanism involving successively NR and ETC activities (Fig. 8).

The use of either plant *MtNR1/2* RNAi or bacteria *napA* and *nirK* mutants showed that both the plant and the bacteroid partners are involved in the production of NO in the nodule. Indeed, both plant and bacterial mutants exhibited decreased NO production (Figs. 4 and 5), and these effects were found to be additive in the *MtNR1/2* RNAi/*napA* nodules (Table II). The production of NO by the bacteroid partner was expected. Indeed, denitrification activity has been shown to occur in *S. meliloti* bacteroids (O'Hara et al., 1983), and NO is a well-known intermediate product of the denitrification pathway (Zumft, 1997). Moreover, it was recently described that bacteroid NR and NiR, products of the *nap* and *nir* genes, contribute to the major part of the NO formed in soybean nodules, particularly under hypoxic conditions (Meakin et al., 2007; Sánchez et al., 2010). However, evidence for the involvement of the plant partner in NO production by nodules was still lacking. The sensitivity of *nirK* mu-



**Figure 8.** Representation of putative mitochondrial  $\text{NO}_3^-$ -NO respiration and bacteroid denitrification pathway operation in hypoxic nodules. On the plant side, mitochondrial internal dehydrogenase (complex I [I]) and external dehydrogenases (NAD[P] DH) oxidize matricial and cytosolic NADH and NADPH, respectively. For simplicity, NADH and NADPH dehydrogenases are represented as only one complex. Electrons are successively transferred to ubiquinone (Q), cytochrome  $bc_1$  (Cyt  $bc_1$ ), cytochrome c (Cyt c), and COX.  $\text{NO}_2^-$  is reduced into NO at the COX site. NO diffuses into cytosol, where it is oxidized into  $\text{NO}_3^-$  by Lb. NR (Nr) reduces  $\text{NO}_3^-$  into  $\text{NO}_2^-$ , which is translocated into mitochondria, reducing power, issued from NADH oxidation by NADH-quinol oxidoreductase (DH), is supplied to each denitrification step via cytochrome c.  $\text{NO}_3^-$  is successively reduced into  $\text{NO}_2^-$ , NO,  $\text{N}_2\text{O}$ , and  $\text{N}_2$  by NR (Nap), NiR (Nir), NO reductase (Nor), and nitrous oxide reductase (Nos). NO and  $\text{NO}_2^-$  exchange mechanisms between matrix, cytosol, and periplasm are still unidentified. In both plant and bacteroid partners, ATP is synthesized due to a transmembrane electrochemical gradient generated by proton ( $\text{H}^+$ ) pumping at the different sites of the ETCs. AA, Antimycin A; IMS, mitochondrial intermembrane space; Myx, myxothiazol; PBM, peribacteroid membrane; PBS, peribacteroid space; Rot, rotenone.

tant nodules to ETC inhibitors (Fig. 6) indicates that the mitochondrial ETC is significantly involved in NO production. This observation is consistent with the fact that root mitochondria of several species have been shown to be able to reduce  $\text{NO}_2^-$  into NO under anoxic or strongly hypoxic conditions (Gupta et al., 2005; Stoimenova et al., 2007; Gupta and Kaiser, 2010).

Taken together, our data show that in *M. truncatula* nodules,  $\text{NO}_3^-$  reduction into  $\text{NO}_2^-$ , and  $\text{NO}_2^-$  reduction into NO, via the mitochondrial and bacteroid NR and ETC pathways (Fig. 8), constitute the main route for NO synthesis under hypoxic conditions and contribute to this synthesis in normoxic ones (Figs. 3 and 6). They also point to the possible involvement of other systems in NO generation. On the plant side, a NOS-like activity (Cueto et al., 1996; Baudouin et al., 2006), a polyamine oxidase (Yamasaki and Cohen, 2006), or a plasma membrane-bound  $\text{NO}_2^-$ -NO reductase (Stöhr and Stremlau, 2006), which was recently hypothesized to be involved in physiological processes including anoxia and mycorrhizal symbiosis (Moche et al., 2010), could be good candidates. Other bacteroid systems, such as NOS (Pii et al., 2007; Sudhamsu and Crane, 2009), also cannot be excluded.

#### Is NO Production Part of an Alternative Respiratory Pathway?

NO production has been shown to be induced in the roots of plants subjected to hypoxia (Dordas et al., 2003, 2004), and this production was supposed to be linked with improved capacity of the plants to cope with hypoxic stress and to maintain cell energy status (Igamberdiev and Hill, 2009). Functional nodules of *L. japonicus* (Shimoda et al., 2009), soybean (Meakin et al., 2007), and *M. truncatula* (Baudouin et al., 2006) have been shown to produce NO, and this production was increased in the soybean nodules when the roots were subjected to a 1-week hypoxia treatment in the presence of  $\text{NO}_3^-$  (Meakin et al., 2007; Sánchez et al., 2010). Because of nitrogenase sensitivity to oxygen, legume nodules are naturally hypoxic organs, with  $\text{pO}_2$  in the range of nanomolar concentrations in the infected region (Layzell and Hunt, 1990; Sung et al., 1991). Thus, the question was raised whether nodule NO production is related to hypoxic conditions prevailing in nodules. In this work, using two different NO probes (DAF and CuFL), we showed that *M. truncatula* nodules produced NO at a higher level than leaves or roots and that this production may be stimulated upon the transition from normoxic to hypoxic conditions, contrary to what was observed in leaves and roots (Fig. 2A; Supplemental Fig. S1). Considering the rapidity of the nodule response to hypoxia (within a few minutes; Fig. 2B), such an increase can hardly be explained by an up-regulation of gene expression but clearly indicates that NO production capacity is already present in functioning nodules and may be promptly up-regulated to face a sudden decrease in oxygen availability.

The data presented in this study raise the question of the role of such a NO production process in microoxic symbiotic nodules. The presence of a gaseous diffusion barrier in the inner cortex of the nodule and the respiration of bacteroids maintain naturally a low oxygen pressure (5–60 nM oxygen) within the infected cells of the nodules (Layzell and Hunt, 1990; Millar et al., 1995), and the  $\text{pO}_2$  value can even fall to the nanomolar level in the infected zone of nodules when the plants experience hypoxic environmental conditions. Under conditions that limit oxygen availability, oxygen-dependent respiration of root mitochondria declines below the oxygen level required to saturate AOX and cytochrome *c* oxidase (COX). The AOX  $K_m$  value for oxygen is in the micromolar range (Millar et al., 1994; Affourtit et al., 2001), precluding AOX function under low oxygen pressure, whereas that of COX is in the range of 80 to 160 nM (Hoshi et al., 1993; Millar et al., 1994), which makes respiration possible under moderate hypoxic conditions. In symbiotic nodules, Lb provides oxygen to bacteroids and host cell mitochondria, which contain a specific COX pathway with high apparent affinity for oxygen ( $K_m$  of 50 nM; Millar et al., 1995). However, considering the oxygen dissociation constant value of hemoglobin (2 nM; Duff et al., 1997) and the very low oxygen concentration (nanomolar range) prevailing in infected cells, the question arises whether oxygenic respiration can still fulfill the ATP requirements for metabolic and biosynthetic purposes in nodules subjected to hypoxia. It may be suggested that, under the microaerobic conditions prevailing in nodules, the  $\text{NO}_3^-$ -NO respiratory pathway (Igamberdiev and Hill, 2009; Igamberdiev et al., 2010, and refs. therein) may contribute to energy supply in symbiotic  $\text{N}_2$ -fixing nodules. Several lines of evidence argue in favor of this hypothesis. In this work, we show that NRs and ETCs contribute to NO production, via  $\text{NO}_3^-$  and  $\text{NO}_2^-$  reduction, particularly under hypoxic conditions. Similarly, in soybean nodules, bacteroidal NR and NiR have been involved in NO production in response to flooding conditions (Sánchez et al., 2010). Moreover, it has been shown that oxyLb, like plant and animal class 1 hemoglobin, has the capacity to efficiently react with NO to produce  $\text{NO}_3^-$  with an elevated rate constant (Herold and Puppo, 2005). The NO generated at either mitochondrial or bacteroidal ETC level, therefore, may be oxidized by Lb into  $\text{NO}_3^-$ . It should be mentioned that, considering the complexity of NO chemistry (Stamler et al., 1992), the chemical forms and the mechanisms of NO diffusion or transport between the different compartments (matrix, cytosol, periplasm, etc.) are still unknown. In the plant partner particularly, the nature and the importance of the NO flux between its production (COX in the mitochondria) and oxidation (Lb in the cytosol) sites remained to be formally established and estimated. However, different experiments carried out with yeast, mammal, or plant mitochondria (Castello et al., 2006; Stoimenova et al., 2007; Gupta and Kaiser, 2010) showed that the NO produced by COX, in

hypoxia or anoxia, may be detected by conventional methods and partly quantified. The exchange of NO between mitochondrial matrix and cytosol, or between the plant and bacteroid partners, may be reasonably hypothesized. Thus, as summarized in Figure 8, in parallel to the bacteroidal denitrification process, a plant  $\text{NO}_3^-$ -NO respiration could be of importance in the microoxic nodules, particularly under hypoxic conditions such as flooding, to maintain cell energy status and  $\text{N}_2$ -fixing metabolism when oxygen supply becomes limiting. The occurrence of such a mechanism is strongly supported by the data on ATP and ADP measurements (Fig. 7), which show that the energy status of the nodules depends either significantly, or almost entirely, on NR functioning under normoxic or hypoxic conditions, respectively.

The possible occurrence of the  $\text{NO}_3^-$ -NO respiration highlights potential new functions for Lb and NR in  $\text{N}_2$ -fixing nodules. Thus, in addition to its role in nitrogenase protection against inhibition by NO (Herold and Puppo, 2005; Shimoda et al., 2009; Sánchez et al., 2010), Lb could not only scavenge NO but oxidize it into  $\text{NO}_3^-$  to feed cytosolic NR and the denitrification pathway with  $\text{NO}_3^-$ . Similarly, it is well established that many symbiotic associations between legumes and rhizobia are characterized by high NR activity (Cheniae and Evans, 1960; Luciński et al., 2002), and it was asked whether and how nodule NR activity could be involved in functioning nodules (Luciński et al., 2002; Kato et al., 2010). Considering that the main route for nitrogen reduction in nodules is the bacteroid nitrogenase and not the NR-NiR pathway (Vance, 1990), an important function of the plant NR in the nodule could be the reduction of  $\text{NO}_3^-$  into  $\text{NO}_2^-$  in the cytosol to supply mitochondria and COX with  $\text{NO}_2^-$ . The aim of future projects will be to demonstrate the functioning of  $\text{NO}_3^-$ -NO respiration in  $\text{N}_2$ -fixing nodules and the role of Lb and NR in this process as well as to consider the interplay between oxygen-dependent and  $\text{NO}_3^-$ -NO respirations for energy regeneration processes in symbiotic nodules subjected to varying  $\text{pO}_2$  conditions.

## MATERIALS AND METHODS

### Biological Material and Growth Conditions

*Medicago truncatula* 'Jemalong' seeds were scarified in 1 M  $\text{H}_2\text{SO}_4$  (6 min), rinsed several times, and imbibed in sterile distilled water for 3 h. Germination was carried out for 3 d on 0.4% agar plates in the dark at 16°C. Seedlings were transplanted in planters containing a mixture of vermiculite and perlite (2:1, v/v) and watered for the first time with 500 mL of nutritive solution (Frendo et al., 1999) containing 4.4 mM  $\text{NO}_3^-$  (as a nitrogen starter to initiate plant growth). Plants were then watered every 3 to 4 d, two times with water and one time with nitrogen-free nutritive solution. Plantlets were grown in a climatic chamber as described (Frendo et al., 1999) and inoculated 1 week after transplanting with either wild-type *Sinorhizobium meliloti* 2011 or different *S. meliloti* 2011 derivatives: 2011 Tn5-STM-1.13.B08 (*nirK::mTn5*) and 2011 Tn5-STM-3.02.F08 (*napA::mTn5*; Pobigaylo et al., 2006). Locations of mTn5 insertions were verified by PCR.

Nodule, root, and leaf samples were collected 4 to 5 weeks after inoculation and either immediately processed for NO quantification or frozen into liquid

nitrogen and stored at  $-80^\circ\text{C}$  for further analysis. Bacteroids were prepared as described previously (Trinchant et al., 2004).

### Construction of a Binary Vector for Hairly Root Transformation

For the RNAi construct, the cauliflower mosaic virus 35S promoter (P35S) in pK7GWIWG2D(II),0 vector was replaced by the MtNCR001 promoter (Mergaert et al., 2003). Following the nomenclature described for these binary vectors (<http://www.psb.ugent.be/gateway/index.php>), we named our construction pK7GWIWG5D(II), where 5 is assigned for the promoter PMtNCR001. *SacI* and *SpeI* restriction sites were added to PMtNCR001 by a PCR amplification with PMtNCR001*SacI*-F and PMtNCR001*SpeI*-R primers, using as a template pENTL4L1-PMtNCR001. The resulting 2,634-bp PCR product was subcloned in pGEM-T vector (Promega). The insertion of this promoter was done in three sequential subcloning steps. First, a 2,472-bp *SacI*-P35S::ccdB: intron-*MluI* from pK7GWIWG2D(II),0 vector was subcloned in a modified  $\Delta\text{EagI}$  pGEM-T vector without the *SpeI* site. Second, P35S was replaced by PMtNCR001 into *SacI*-*SpeI* sites. Finally, pK7GWIWG5D(II) was obtained by insertion of the *SacI*-PMtNCR001::ccdB:intron-*MluI* cassette back into the original pK7GWIWG2D(II),0 vector. The primers used were PMtNCR001*SacI*-F (5'-GAGAGCTCGTTGTCCTTATTAGAGCGCCTA-3') and PMtNCR001*SpeI*-R (5'-GACTAGTCTTAGACCTTTGAACGTACTAAA-GAGATT-3').

Using *M. truncatula* cDNA as template, 432- and 441-bp fragments of *MtNR1* (TC137636; Mtr.10604.1.S1\_at) and *MtNR2* (TC130773; Mtr.42446.1.S1\_at) genes, respectively, were obtained via PCR with specific primers: NR1F (5'-CGGGATCCCCACTGGCAGTTACTCCTCAC-3'), NR1R (5'-GGGGTACCTTGAGCCAATAGGCATTGAA-3'), NR2F (5'-CGGAATTCTCTCCGATTGTCATTACCC-3'), and NR2R (5'-GGGGTACCTCCGGTTGCATAAACACA-3').

PCR products were independently ligated into pGEM-T Easy vector (Promega) and subsequently subcloned into pENTR4 vectors in *Bam*HI-*Kpn*I restriction sites for *MtNR1* and in *Eco*RI and *Kpn*I restriction sites for *MtNR2*. The pENTR4 vector carrying the *MtNR1* or the *MtNR2* fragment was recombined with pK7GWIWG5D(II) vector using the LR clonase enzyme mix (catalog no. 11791-019; Invitrogen) to create the RNAi expression vectors. Constructs were checked by sequencing.

### *Agrobacterium rhizogenes* Root Transformation and Inoculation

The constructs pK7GWIWG5D-MtNR1/2 (RNAi::MtNR1/2) were introduced into *A. rhizogenes* strain Arqua1 (Quandt et al., 1993). *M. truncatula* plants were transformed with *A. rhizogenes* according to Vieweg et al. (2004). Control plants were transformed with *A. rhizogenes* containing the pK7GWIWG5D empty vector. Selection of hairy roots based on the fluorescent marker took place 21 d after transformation. The roots were rapidly examined with a fluorescence stereomicroscope (Leica MZFL III), and the composite plants harboring transgenic roots were used for the inoculation with the appropriate rhizobial strain.

### Measurement of NO Production

Ten to 20 mg of detached nodules (about 15–30 nodules), 100 to 200 mg of root segments (1 cm long), or four to five leaf discs (5 mm diameter) were incubated in the dark at 23°C in Eppendorf tubes containing 500  $\mu\text{L}$  of detection medium (10 mM Tris-HCl, pH 7.5, 10 mM KCl) in the presence of 10  $\mu\text{M}$  DAF-2 (Coger) fluorescent probe. When using nodules obtained with RNAi::MtNR1/2 plants, nodules issued from at least two transgenic roots were pooled and used in each assay. For NO measurement under hypoxic conditions, the detection buffer was first equilibrated, and mutant bacteria were then maintained throughout the experiments at 1% oxygen with a 1%:99% (v/v) oxygen: $\text{N}_2$  gas mixture. The mean value of 1% oxygen (1 kPa) for hypoxia treatment was chosen on the basis that  $\text{pO}_2$  in most waterlogged soils ranged from 5 to 0 kPa (Gibbs and Greenway, 2003). The NO produced by the tissues and released into the detection medium was measured using the fluorescence of the DAF probe. At various times, aliquots of the incubation medium were sampled, and the fluorescence of DAF-2T, the reaction product formed from DAF-2 and NO, was measured using a microplate reader spectrofluorimeter (Cary Eclipse; Varian), with excitation at 495 nm and emission at 515 nm. In

these conditions, NO production and release were found to be linear between 1 h and at least 4 h of incubation. Assay blanks contained detection buffer and DAF, without nodules. Alternately, NO production was measured in the same experimental system through the use of the CuFL fluorescent probe (Strem Chemicals) instead of DAF-2 in the detection buffer. As CuFL is known to be a cell-permeant probe (Lim et al., 2006), its capacity to penetrate into nodule cells and its cytotoxicity were analyzed. After a 2 h-incubation period of entire nodules in the presence of 5  $\mu\text{M}$  CuFL, nodules were excised into 100- $\mu\text{m}$ -thick slices with a Vibratom 1000 Plus (Labonord) and analyzed with a LSM 500 confocal laser microscope (Carl Zeiss) as described (Baudouin et al., 2006). No fluorescence could be detected in nodule cells (Supplemental Fig. S6A), indicating that CuFL probe, or its *N*-nitrosamine FL-NO derivative, did not penetrate into the nodule and could be used to measure the NO in the incubation medium. To test CuFL toxicity, the effects of increasing concentrations (0, 2, 5, 10, and 20  $\mu\text{M}$ ) of CuFL were analyzed after 2 h of incubation on the nodule energy state (ATP-ADP ratio being used as a marker of cell viability). Adenine nucleotides were extracted and analyzed as described below. No effect was observed on ATP-ADP ratio (Supplemental Fig. S6B), which means that, in these conditions, CuFL was not toxic to nodule cells. Thus, when assayed with CuFL, NO production was routinely measured for 2 h with a probe concentration of 5  $\mu\text{M}$ .

For rapid  $\text{pO}_2$  transition (between 21% and 1% oxygen) experiments, four to six nodules were set in a fluorescence cuvette containing 1 mL of detection medium, and NO production was continuously measured on a kinetic mode using a Xenius spectrofluorimeter (SAFAS).  $\text{pO}_2$  in the incubation medium was imposed by a permanent bubbling of either ambient air or a 1%:99% oxygen: $\text{N}_2$  (v/v) gas stream. Incubation medium was continuously homogenized using noninvasive stirring equipment during the assay.

## Measurement of NO Content

Ten to 20 mg of nodules, either freshly detached or incubated for 4 h in the presence or absence of effectors, was crushed with mortar and pestle in 200 to 300  $\mu\text{L}$  of detection medium in the presence of 10  $\mu\text{M}$  DAF probe. The extract was centrifuged at 4°C for 10 min, and the fluorescence of the supernatant was immediately measured as described above.

## Effects of Effectors on NO Production

The effectors tested on NO production were routinely used at the following concentrations: 10 mM  $\text{NaNO}_3$ , 1 mM  $\text{NaNO}_2$ , 1 mM NaTg, 1 mM allopurinol, 50 mM Suc, 300  $\mu\text{M}$  KCN, 100  $\mu\text{M}$  cPTIO, 10  $\mu\text{M}$  rotenone, 25  $\mu\text{M}$  antimycin A, 25  $\mu\text{M}$  myxothiazol, 1 mM propylgallate, and 10  $\mu\text{M}$  FCCP. The effectors were added to the detection buffer at the same time as nodules, and their effects on NO production were measured after 2 to 4 h of incubation as described above.

## Enzymatic Activity Measurements

Tissue samples were crushed at 4°C using an extraction buffer containing 25 mM Tris-HCl, pH 8.5, 1 mM EDTA, 20  $\mu\text{M}$  FAD, 1 mM dithiothreitol, 20  $\mu\text{M}$  L-transepoxysuccinyl-leucylamide-[4-guanidino]butane, and 2 mM phenyl-methylsulfonyl fluoride. The extracts were centrifuged at 15,000g for 15 min and used for  $\text{NO}_3^-$  and  $\text{NO}_2^-$  reductase activities.

NR activity was assayed at 28°C by measuring  $\text{NO}_2^-$  production. The reaction medium (1 mL) contained enzymatic extract, 0.2 M HEPES, pH 7.0, 15 mM  $\text{KNO}_3$ , and 250  $\mu\text{M}$  NADH. Reaction was stopped after 30 min by boiling the sample at 100°C for 3 min. The  $\text{NO}_2^-$  produced was measured with the addition of the  $\text{NO}_2^-$  reagent (Miranda et al., 2001): 250  $\mu\text{L}$  of 1% (w/v) sulfanilamide in 1 N HCl plus 250  $\mu\text{L}$  of 0.01% (w/v) *N*-1-naphthylethylenediamine dihydrochloride in water. After incubation for 30 min at ambient temperature, samples were centrifuged for 10 min at 13,000g, and the absorbance of the supernatant was read at 540 nm. Assay blanks contained enzymatic extracts boiled at 100°C for 3 min before the addition of  $\text{KNO}_3$  and NADH. To measure the inhibition of NR activity by Tg, enzymatic extracts were first preincubated with NaTg for 15 min at ambient temperature before activity measurement. On the basis of inhibition experiment data (Supplemental Fig. S5), a concentration of 1 mM NaTg was routinely used for in vivo and in vitro experiments. To assess the effectiveness of Tg in vivo, nodules or bacteroids were incubated for 4 h in the presence of 1 mM NaTg, proteins were extracted, and NR activity was measured as described above.

NiR activity was assayed at 28°C by following  $\text{NO}_2^-$  consumption from the assay mixture using the dithionite-methyl viologen method. The reaction

medium (1 mL) consisted of 20 mM potassium phosphate (pH 7.3), 1 mM  $\text{NaNO}_2$ , 40  $\mu\text{M}$  methyl viologen, and the sample to be assayed. The reaction was started by the addition of 10  $\mu\text{L}$  of 100 mM sodium dithionite in 200 mM sodium bicarbonate. Samples were maintained under anaerobiosis. After 30 min of incubation, 20- $\mu\text{L}$  aliquot fractions were sampled, diluted in 480  $\mu\text{L}$  of water, and shaken energetically for 30 s. A 500- $\mu\text{L}$  aliquot of the  $\text{NO}_2^-$  reagent (Miranda et al., 2001) was then added, and the  $A_{540}$  was measured after 30 min. Assay blanks contained enzymatic extracts plus reagents except dithionite.

## Extraction and Measurement of Adenine Nucleotides

All extraction steps were carried out at 4°C. Frozen nodules (10–30 mg) were crushed in liquid nitrogen with 300  $\mu\text{L}$  of perchloric acid solution, containing 7% (v/v)  $\text{HClO}_4$  and 25 mM  $\text{Na}_2\text{EDTA}$ , with a mortar and pestle. After thawing, the extract was taken and the mortar was rinsed with 200  $\mu\text{L}$  of perchloric acid solution, which was then pooled with the extract. The sample was centrifuged for 5 min at 13,000g. The supernatant was quickly and carefully neutralized at pH 5.6 to 6.0 using a 2 M KOH-0.3 M MOPS solution.  $\text{KClO}_4$  precipitate was discarded by centrifugation (5 min, 13,000g). Adenine nucleotides of the supernatant were measured in a luminometer (Bio-Orbit) using the ATPlite one-step assay system (ATPLT1STP-0509; Perkin-Elmer) according to the manufacturer's instructions.

## Protein Measurements

Soluble proteins were quantified on clarified extracts using  $\gamma$ -globulin as a standard (Bradford, 1976).

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Effects of NR effectors on *M. truncatula* leaf and root NO production.

**Supplemental Figure S2.** Histochemical analysis of *MtNCR001* expression in nodules, and phenotype of *MtNR1/2* RNAi nodules.

**Supplemental Figure S3.** NO production by *M. truncatula* GUS and *MtNR1/2* RNAi and by *S. meliloti* 2011, *napA*, and *nirK* nodules under 21% oxygen.

**Supplemental Figure S4.** Effects of ETC effectors on NO production of *M. truncatula*-*S. meliloti* nodules in the presence of  $\text{NO}_2^-$ .

**Supplemental Figure S5.** Effects of NaTg on nodule NR activity.

**Supplemental Figure S6.** Histochemical analysis and toxicity test of CuFL-treated nodules.

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