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 (MtNR1/2) 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.
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 (Pi 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 (Ferrarini 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, 2008). 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, NO acts as a final electron acceptor instead of oxygen. In the ETC, NO is reduced to NO, which diffuses to the cytosol, where it is oxidized by the nonsymbiotic hemoglobin to NO. NO is then reduced to NO 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 NO synthase (NOS)-like enzyme or the NR to NO production in the plant partner was provided to date.

**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 autoxidizes 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 h$^{-1}$ mg$^{-1}$ fresh weight) was considered as the background in our experimental system and was subtracted in the following experiments. Under 1% oxygen (12 μM 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
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 μM 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 (pO2) 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 μM 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 pO2.

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 NO3−, 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 NO3− (Fig. 3). This means that NR is involved in the production of NO either directly or indirectly via the production of NO2−, 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 h⁻¹ 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 NO2−, 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 NO2−, which indicates that NR is involved in NO production through the reduction of NO3− in NO2−.
but does not produce NO directly. In addition to NR, xanthine oxidase (a MoCo enzyme like NR) has been reported to reduce 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 NO$_2^-$, NO$_3^-$, 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 NO$_2^-$ is cytotoxic for plants, although the molecular mechanism is still obscure, and a higher NiR versus NR activity, which avoids NO$_2^-$ accumulation in the tissues, was classically observed in plants (Lucinski 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 NO$_2^-$ production versus 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 (Lucinski 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 N$_2$-fixing nodules (data not shown). To date, the main function of NR identified in plants is its key role in the NO$_3^-$-to-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 MiNR1/2 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 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, MiNR1/2 RNAi transgenic roots did not show significantly modified phenotypes compared with the GUS RNAi control for plant growth and nodule formation events (data not shown), but nodule size was 30% to 40% reduced in the MiNR1/2 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 MiNR1/2 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 MiNR1/2 RNAi and GUS RNAi control nodules, the production of NO was found to be increased by NO$_3^-$ 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>
<thead>
<tr>
<th>Table I. NO$_3^-$ and NO$_2^-$ reductase activities in nodule, root, and leaf of M. truncatula plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Data are means ± SD of six (nodule) or three (root and leaf) independent experiments.</td>
</tr>
<tr>
<td>NR or NiR</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>NR (nmol min$^{-1}$ g$^{-1}$ fresh weight)</td>
</tr>
<tr>
<td>NiR (nmol min$^{-1}$ g$^{-1}$ fresh weight)</td>
</tr>
<tr>
<td>NiR-NR</td>
</tr>
</tbody>
</table>
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 NO3− reduction to N2. NO3− is first reduced to NO2− by NR, and NO2− 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 NO2− 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.

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

<table>
<thead>
<tr>
<th>Variable</th>
<th>GUS/2011</th>
<th>MtNR1/2/2011</th>
<th>GUS/napA</th>
<th>MtNR1/2 napA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR activity (nmol NO2−·min−1·g−1 fresh weight)</td>
<td>6.56 ± 0.87</td>
<td>3.94 ± 0.87*</td>
<td>4.69 ± 0.76*</td>
<td>3.08 ± 0.57**</td>
</tr>
<tr>
<td>Percent of control</td>
<td>100</td>
<td>60</td>
<td>71</td>
<td>47</td>
</tr>
<tr>
<td>NO production (fluorescence units h−1·mg−1 fresh weight)</td>
<td>4.65 ± 0.84</td>
<td>2.54 ± 0.71*</td>
<td>2.81 ± 0.39*</td>
<td>1.33 ± 0.37**</td>
</tr>
<tr>
<td>Percent of control</td>
<td>100</td>
<td>54</td>
<td>60</td>
<td>29</td>
</tr>
</tbody>
</table>

*Significant difference (*P = 0.05, **P = 0.01) compared with the control (GUS/2011) according to Student’s t test.

Mitochondrial and Bacteroid ETCs Are Involved in Nodule NO Production

The maintenance of NO production, in the presence of both NO3− and Tg (Fig. 3), indicated that NR does not produce NO directly but more likely produces NO2−, which in turn is reduced to NO. Beside NR, root mitochondria have been reported to be able to reduce NO3− 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...
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 NO$^-$-NO respiration, involving the sequential reduction of NO$^-_3$ into 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-

Table III. NO$^-_3$ and 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.

<table>
<thead>
<tr>
<th>NR or NiR</th>
<th>Nodule 2011</th>
<th>Bacteroid 2011</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>napA</td>
<td>nirK</td>
</tr>
<tr>
<td>NR Control</td>
<td>9.7 ± 1.2</td>
<td>6.1 ± 1.2*</td>
</tr>
<tr>
<td>+1 mM NaTg</td>
<td>1.4 ± 0.3</td>
<td>nd</td>
</tr>
<tr>
<td>NiR</td>
<td>18.5 ± 2.3</td>
<td>16.0 ± 4.3</td>
</tr>
</tbody>
</table>

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 h$^{-1}$ 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 NO$^-_3$ 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 NO$^-_3$ (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 NO$^-_3$ supply, and (4) similarly inhibited by ETC inhibitors both in wild-type and nirK mutant nodules. This means, first, that mitochondrial and bacteroid

The 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 NaNO$^-_2$ (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 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 NaNO$^-_2$ (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.](image-url)
ence of NO$_3^-$, NO$_2^-$, or Tg plus 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 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 pO$_2$ 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 NO$_3^-$, NO$_2^-$, or Tg plus NO$_2^-$ did not significantly modify the energy status of the nodules, but Tg only or Tg plus 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, NO$_3^-$-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...
potential role of NR in the production of NO. First, the increase in NO production upon nodule feeding with either NO$_3^-$ or 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 NO$_2^-$-dependent NO synthesis via the activity of NR. However, the relief of the Tg-related inhibition of NO production by NO$_2^-$ (Fig. 3) clearly indicates that NR does not produce NO by itself but 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 NO$_2^-$ into NO, probably at the cytochrome oxidase site. Thus, in M. truncatula nodules, 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-
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 NO$_3^-$ 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, NO$_3^-$ reduction into NO$_2^-$, and 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 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 NO$_3^-$ (Meakin et al., 2007; Sánchez et al., 2010). Because of nitrogenase sensitivity to oxygen, legume nodules are naturally hypoxic organs, with 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 respiratory 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 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 NO$_3^-$-NO respiratory pathway (Igamberdiev and Hill, 2009; Igamberdiev et al., 2010, and refs. therein) may contribute to energy supply in symbiotic 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 NO$_3^-$ and 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 NO$_2^-$ 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 NO$_2^-$, and NO$_2^-$ must be reduced to NO before it can be utilized by worm, 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 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 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 NO$_3^-$-NO respiration highlights potential new functions for Lb and NR in N$_2$-fixing nodules. Thus, in addition to its role in nitrogen protection against inhibition by NO (Herold and Pupo, 2005; Shimoda et al., 2009; Sánchez et al., 2010), Lb could not only scavenge NO but oxidize it into NO$_2^-$ to feed cytosolic NR and the denitrification pathway with NO$_3^-$. Similarly, it is well established that many symbiotic associations between legumes and rhizobia are characterized by high NR activity (Cheniæ and Evans, 1960; Lucirski et al., 2002), and it was asked whether and how nodule NR activity could be involved in functioning nodules (Lucirski 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 NO$_3^-$ into NO$_2^-$ in the cytosol to supply mitochondria and COX with NO$_2^-$. The aim of future projects will be to demonstrate the functioning of NO$_3^-$-NO respiration in 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 NO$_3^-$.NO respiration during energy regeneration processes in symbiotic nodules subjected to varying pO$_2$ conditions.
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, the presence of 5 μM CuFL, nodules were excited into 100-μm-thick slices with a Vibratome 1000 Plus (Labinord) and analyzed with a LSM 510 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-nitroamine 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 μM) 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 for nodule cells. Thus, when assayed with CuFL, NO production was routinely measured for 2 h with a probe concentration of 5 μM.

For rapid pO2 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). pO2 in the incubation medium was imposed by a permanent bubbling of either ambient air or a 1%-99% oxygen:N2 (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 μL of detection medium in the presence of 10 μM 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 NaNO3, 1 mM NaNO2, 1 mM NaTg, 1 mM allopurinol, 50 mM Suc, 300 mM KCN, 100 mM cPTIO, 10 μM rotenone, 25 mM antimycin A, 25 mM myxothiazol, 1 mM propylgalate, and 10 μM 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 mM FAD, 1 mM dithiothreitol, 20 μM L-transpeptidasecyl-cysteinyl-lycuxamido-[4-guanidino]butane, and 2 mM phenylmethylsulfonyl fluoride. The extracts were centrifuged at 15,000g for 15 min and used for NOX1 and NOX2 reductase activities.

NR activity was assayed at 28°C by measuring NO2- production. The reaction medium (1 mL) contained enzymatic extract, 0.2 mM HEPES, pH 7.0, 15 mM KCNO, and 250 mM NADH. Reaction was stopped after 30 min by boiling the sample at 100°C for 3 min. The NO2- produced was measured with the addition of the NO2- reagent (Miranda et al., 2001): 250 μL of 1% (w/v) sulfanilamide in 1 n HCl plus 250 μL of 0.01% (w/v) N-1-naphthylenediimin dihydrochloride in water. After incubation for 30 min at ambient temperature, samples were centrifuged for 10 min at 13,000 g, 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 KNO3 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 test 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.

NR activity was assayed by 28°C by following NO2- consumption from the assay mixture using the diithionite-methyl violoen method. The reaction medium (1 mL) consisted of 20 mM potassium phosphate (pH 7.3), 1 mM NaNO3, 40 μM methyl viologen, and the sample to be assayed. The reaction was started by the addition of 10 μL of 100 mM sodium dithionite in 200 mM sodium bicarbonate. Samples were maintained under anaerobiosis. After 30 min of incubation, 20-μL aliquot samples were fractioned, diluted in 480 μL of water, and shaken energetically for 30 s. A 300-μL aliquot of the NO2- reagent (Miranda et al., 2001) was then added, and the A540 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 μL of perchloric acid solution, containing 7% (v/v) HClO4 and 25 mM Na2EDTA, with a mortar and pestle. After thawing, the extract was taken and the mortar was rinsed with 200 μL 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% (w/v) KOH-0.3 M MOPS solution. KClO4 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 (ATPLITISTP-0509; Perkin-Elmer) according to the manufacturer’s instructions.

### Protein Measurements

Soluble proteins were quantified on clarified extracts using γ-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 MmNR001 expression in nodules, and phenotype of MNRI/2 RNAi nodules.

Supplemental Figure S3. NO production by M. truncatula GUS and MNRI/2 RNAi and by S. meliloti 1101, 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 NO3-.

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

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

### ACKNOWLEDGMENTS

We thank Sarra Mselhi (University of Tunis) for her dedicated technical assistance in the construction of binary vectors. We are grateful to Julie Hopkins (Institut Agribiotech, Sophia Antipolis) for critical reading of the manuscript and to Anke Becker (University of Freiburg) for providing S. meliloti mutant strains.

Received September 16, 2010; accepted November 29, 2010; published December 7, 2010.

### LITERATURE CITED


Atkins CA, Sanford PJ, Storer PJ, Pate JS (1988) Inhibition of nodule
functioning in cowpea by a xanthine oxidoreductase inhibitor, allopurinol. Plant Physiol 88: 1229–1234
Delledonne M (2005) NO news is good news for plants. Curr Opin Plant Biol 8: 390–396
Delledonne M (2005) NO news is good news for plants. Curr Opin Plant Biol 8: 390–396
Pobigaylo N, Wetter D, Szymczak S, Schiller U, Kurtz S, Meyer F,


