***Rhizobium*-Initiated Rice Growth Inhibition Caused by Nitric Oxide Accumulation**

Francine M. Perrine-Walker,^{1,2} Elena Gartner,^{1,2} Charles H. Hocart,^{1,2} Anke Becker,³ and Barry G. Rolfe^{1,2}

¹ARC Centre of Excellence for Integrative Legume Research, ²Genomics Interaction Group, Research School of Biological Sciences, Australian National University, GPO Box 475, Canberra, ACT 2601, Australia; ³Fakultät für Biologie, Universität Bielefeld, 33594 Bielefeld, Germany

Submitted 10 October 2005. Accepted 11 October 2006.

Isolates of *Rhizobium leguminosarum* bv. *trifolii* (the clover root-nodule endosymbiont) from the Nile River delta have been found to infect rice roots and colonize the intercellular spaces of the rice roots. Some of these isolates inhibit rice seedling growth but one in particular, R4, has been found in rice roots which develop and grow normally. We present evidence that the induced growth inhibition is due to a toxic accumulation of nitric oxide (NO), from the reduction of nitrate, and suggest that the reason that R4 does not inhibit rice root growth is because it is capable of completing the reduction of NO through to nitrogen gas. Thus, strain R4 is a candidate for engineering into a future biological nitrogen fixation system within these roots.

Additional keywords: denitrification, nitrate reduction, nitrite.

Nonlegumes such as, wheat, rice, maize, oat, sorghum and sugarcane form the major crops of the planet. Various soil bacteria have been examined for their potential as reliable inoculant strains for nonlegume crop plants (Gutiérrez-Zamora and Martínez-Romero 2001; Ladha et al. 1997; Prayitno et al. 1999; Rolfe et al. 1997; Yanni et al. 1997, 2001). A recent study using a variety of green fluorescent protein (*gfp*)-labeled *Rhizobium* strains has also demonstrated the utility of some rhizobial strains as biofertilizers (Chi et al. 2005). Plant growth-promoting rhizobacteria were examined and tried as inocula to enhance crop growth (Kloepper et al. 1992). Yanni and associates (1997) identified *Rhizobium leguminosarum* bv. *trifolii* strains, which could nodulate berseem clover (*Trifolium alexandrinum* L.) and colonize the roots of rice grown in the Nile delta region of Egypt. In Egypt, rice and berseem clover have been grown in rotation annually for more than 700 years; therefore, the finding of a population of rhizobia in the soils used for rice growth was not unusual. Agriculturists in Egypt have tested growing rice, which was inoculated with rhizobia, and various levels of nitrogen fertilizer in an attempt to enhance the rice growth with reduced levels of added fertilizer (Yanni et al. 2001). A variety of strains that could associate closely with both rice and clover were isolated from rice plants. We have tested some of these rhizobia along with other *Rhizobium* strains and have classified them into three general groups: strains that inhibited rice seedling growth, those that promoted growth, and strains that had no influence in their association with rice seedlings (Perrine et al. 2001, 2005; Prayitno et al. 1999). To study this interaction further, we

examined growth responses of rice seedlings (*Oryza sativa* L. cv. Pelde) to specific *Rhizobium* strains and their mutants.

The inoculation experiments showed that there are genes on certain plasmids of *R. leguminosarum* bv. *trifolii* that affect the growth and development of rice root morphology (Perrine et al. 2001). Subsequent studies found that bacteria can associate intimately with rice seedling roots by an alternative mechanism to that encoded by the symbiotic (pSym) plasmid, and some of the bacterial strains examined behaved as endophytes, entering and growing in the intercellular regions of rice root tissue (Perrine et al. 2001, 2005; Prayitno et al. 1999). Under the growth conditions used, this entry occurred most frequently with *R. leguminosarum* bv. *trifolii* R4, which multiplied and migrated inside the rice root tissues to form long lines of individual bacterial cells along the inside of the growing lateral roots (Perrine et al. 2001, 2005; Prayitno et al. 1999). Strain R4 did not inhibit rice seedling growth and development but did enhance growth with different cultivars of rice (Perrine et al. 2001; Prayitno et al. 1999).

The *Rhizobium*-rice interaction can be influenced by different media and the interaction of ions and particular rhizobia strains (Prayitno et al. 1999). The inhibition of both lateral root growth and general root development could be induced by the addition of exogenous auxin, or by supplementing liquid nitrogen-free modified Fahraeus medium (NFM) with a high concentration of nitrate (Perrine et al. 2001). These observations suggested that the phytohormone auxin and nitrate could influence this *Rhizobium*-rice interaction. Both nitrate and auxin have been shown to affect lateral root development in legumes and nonlegumes and nodulation in legumes (Perrine et al. 2001).

Nitrogen (N) is the major limiting nutrient for most plant species and nitrate is the major N source available in aerobic soils (Luciński et al. 2002). N is also one limiting factor in the generation of high yields from modern N-fertilizer-responsive rice cultivars (Stoltzfus et al. 1997), making it the single most important commercial input into rice production (Dawe 2000). However, successful nodulation in legumes by *Rhizobium* spp. only occurs under nitrogen-limiting conditions (Stougaard 2000). Nitrate retards the development of nodules on legumes nodulated with *Rhizobium* strains (Gibson and Pagan 1977) and nitrate has been shown to inhibit nitrogenase activity in legume plant nodules due to accumulation of nitrite (Luciński et al. 2002). Furthermore, Trinchant and Rigaud (1980) demonstrated that nitrite, the reduced product of nitrate, inhibited bacteroid nitrogenase activity. The enzyme nitrate reductase (NR) catalyses the reduction of nitrate to nitrite. Nitrate is believed to inhibit nodulation by autoregulation (Caba et al. 2000; Caetano-Anolles and Gresshoff 1991) by increasing the auxin sensitivity of root cortical cells and thus, in the presence of nitrate, cortical cells are

strongly prevented from sensing the Nod-factor-related auxin decrease (Caba et al. 2000). Nitrate metabolism involves many genes such as nitrate reductases, dissimilatory nitrate reductases, periplasmic nitrate reductases, σ^{54} factor required for nitrogen assimilation, and a global nitrogen regulatory (*ntr*) system (Merrick and Edwards 1995) in the bacterium and the plant and can influence final root organogenesis. The work by Zhang and colleagues (Zhang and Forde 2000; Zhang and Hasenstein 1999; Zhang et al. 1999) demonstrated that nitrate is a key component in the signal transduction pathway regulating lateral root initiation, proliferation, and elongation, because there is an overlap between the nitrate and auxin response pathways in the control of lateral root elongation (Zhang and Forde 2000). Nitrate reduction has three different purposes: the utilization of nitrate as a nitrogen source for growth (nitrate assimilation), the generation of metabolic energy by using nitrate as a terminal electron acceptor (nitrate respiration), and the dissipation of excess reducing power for redox balancing (nitrate dissimilation) (Moreno-Vivian et al. 1999).

Plants, fungi, and algae reduce nitrate to nitrite with NR and the nitrite then is converted to NH_4^+ via nitrite reductase (NiR) (Barlaan et al. 1998; Campbell 2001; Galván and Fernández 2001) (Fig 1). NR in plants is regulated by nitrate, light, growth hormones, reduced metabolites, and phosphorylation (Crawford 1995; Mohr et al. 1992). Rice, grown under flooded and anaerobic conditions, assimilates nitrate as a source of N (Barlaan et al. 1998). Nitrate is usually reduced in the aerial parts of most plants (Barlaan et al. 1998) within the plastids (Mohr et al. 1992).

Nitrate at high concentrations (10 to 50 mM) inhibits lateral root development in *Arabidopsis* (Zhang et al. 1999). *Arabidopsis* seedlings treated with 50 mM potassium nitrate developed short lateral roots (Zhang et al. 1999) and also were sensitive to the inhibitory effect of a high concentration of nitrate, but only during a specific stage of lateral root development; just after emergence and before maturation (Zhang et al. 1999).

Nitrite is generally regarded as being toxic to plant growth and metabolism (Klepper 1974). Lee (1979) showed that the sensitivity of plants to nitrite increased at low oxygen concentrations (Lee 1979).

In this article, we investigated whether genes related to nitrate metabolism found on the pSymA megaplasmid of the *Sinorhizobium meliloti* 1021 are involved in the inhibition of rice seedlings. We had shown that strain 1021 inhibited rice growth but that 1021 cured of its pSymA plasmid did not inhibit rice seedling growth (Perrine et al. 2005). This article will also present a hypothetical model for the rice growth inhibition by rhizobia and the reason why strain R4 is potentially an ideal candidate for an inoculant strain of rice and perhaps other nonlegume crop plants.

RESULTS

Nitrate reduction under conditions ranging from microaerophilic to anaerobic.

To investigate the ability of strains *S. meliloti* and its pSymA and pSymB mutants and *Rhizobium* R4 (Table 1) to reduce nitrate to nitrite under conditions ranging from microaerophilic

Fig. 1. Proposed model for the observed inhibition of rice growth initiated by inoculation with *Sinorhizobium meliloti* strains. **A**, Nitrate metabolism in rice seedling and *S. meliloti* Sm1021 in Magenta jars containing liquid nitrogen-free modified Fahraeus medium supplemented with 10 mM KNO_3 ; nitrate reductase (NR); nitrite reductase (NiR); nitrate reduction (1); nitrate assimilation (2); unidentified nitrite reductase (nir); nitric oxide reductase (nor); nitrous oxide reductase (nos); and NAD(P)H nitrite reductase (nirB). **B**, Reduction of nitrate to nitrite and subsequent accumulation of nitric oxide (NO) inhibits plant growth when inoculated with Sm1021. Lack of inhibitory plasmid-associated genes in pSymA-cured strain Sm818 means that little or no NO is accumulated and rice growth is normal. The presence of the inhibitory associated genes in pSymB-deleted derivatives (transporters and cell replication) results in some NO being formed and a consequent partial inhibition of plant growth. Strain R4 is capable of reducing nitrate to N_2 gas with no accumulation of NO, so that rice growth is normal.

to anaerobic, bacterial strains were grown in tryptone yeast (TY) liquid medium in the presence and absence of 10 mM KNO₃. *Pseudomonas aeruginosa* PAO5 (Table 1) was used as a positive control, because *Pseudomonas* spp. are known to be able to reduce nitrate to nitrite (Bedzyk et al. 1999). At 7 days, all test tubes were turbid except for the uninoculated control test. In test tubes containing only TY liquid medium, no nitrite was detected. The results from TY supplemented with 10 mM KNO₃ at 7 days are tabulated in Table 2. At 7 days, strain Sm2011 reduced nitrate whereas the closely related strain Sm1021 did not, suggesting that one of the differences between Sm2011 and Sm1021 is in nitrate metabolism (Table 2). As all the pSymA and pSymB deleted and cured derivatives were derived from Sm2011, they were compared with the wild-type strain Sm2011. All pSymA mutants of Sm2011 reduced nitrate except for SmA144. None of the pSymB-deleted derivatives could reduce nitrate (Table 2). *Rhizobium* sp. strain R4 was able to reduce nitrate completely to N₂ (gas) and no nitrite or nitrate was detected after the addition of zinc. *P. aeruginosa* PAO5 completely reduced the supplied nitrate to nitrogen gas.

Chromosomally located gene, *RpoN*, in *S. meliloti* Sm2011 is not involved in rice growth inhibition.

The role of the sigma factor, σ^{54} , encoded by the *rpoN* (*ntrA*, *glnF*) gene in rice growth inhibition, was investigated by inoculating rice seedlings of cv. Pelde with strain Sm5422 (the RpoN Tn5 mutant of Sm2011). This strain was tested because

the inhibition of rice growth by strain Sm1021 and Sm2011 was influenced by the form and concentration of nitrogen. Furthermore, RpoN (σ^{54}) is a sigma factor required for the transcriptional activation of the nitrogen regulation (NTR) system, the latter being involved in sensing the availability of nitrogen (Merrick and Edwards 1995; Ronson et al. 1987). At 21 days, all rice plants inoculated with strain Sm5422 and the wild-type strain Sm1021 were inhibited in growth.

NapA, NapF, and NirV periplasmic nitrate reductase located on pSymA are not involved in rice growth inhibition.

Periplasmic nitrate reductase mutants were used to determine the inhibitory role of nitrate in the interaction of rice seedlings with wild-type strain Sm1021 (Sm2011). Mutants in the genes *NapA*, needed for in vivo activity; *NapF*, involved in the assembly of the iron-sulfur center of *NapA*; and *NirV*, the periplasmic nitrate reductase, were chosen so as to disrupt the reduction of nitrate. All of these genes are located in the 400-kb region of deleted DNA of the noninhibitory pSymA-deleted derivative, strain SmA146. Strains M1, M2, M3, M4, M17, and M18 (Table 1) were used as inoculants, as well as the wild-type strain Sm1021 and Sm2011, and were tested individually on rice seedlings. At 21 days, wild-type strains Sm1021 and Sm2011 inhibited rice growth; similarly, the strains M1 and M2 (NapA mutants) inhibited the growth of cv. Pelde rice seedlings. Strains M3 and M4 (NapF mutants) inhibited rice seedling growth while strains M17 and M18 (NirV

Table 1. Bacterial strains

Strain	Characteristics ^a	Reference, source ^b
<i>Rhizobium leguminosarum</i>		
bv. <i>trifolii</i> strains		
TA1	Wild type, Nod ⁻ on cv. Woogenellup, Nod ⁺ Fix ⁺ on other subterranean clover cultivars	Gibson 1968
ANU794	Sm ^r derivative of <i>R. leguminosarum</i> bv. <i>trifolii</i> strain TA1; Nod ⁺ Fix ⁺ on white and subterranean clovers	J. Vincent
R4	<i>R. leguminosarum</i> bv. <i>trifolii</i> from rice root	Yanni et al. 1997
<i>Sinorhizobium meliloti</i>		
Sm2011	Spontaneous streptomycin-resistant derivative of <i>S. meliloti</i> SU47, wild type, Nod ⁺ , Fix ⁺ on alfalfa	J. Dénarié
Sm1021	Spontaneous streptomycin-resistant derivative of <i>S. meliloti</i> SU47, wild type, Nod ⁺ , Fix ⁺ on alfalfa	Meade et al. 1982
SmA818	2011 pRm2011a cured, Nod ⁻ , Fix ⁻	Oresnik et al. 2000
SmA146	2011 carrying a 750-kb deletion in pRm2011a, Nod ⁻ , Fix ⁻	Oresnik et al. 2000
SmA144	2011 carrying a 650-kb deletion in pRm2011a, Nod ⁻ , Fix ⁻	Perrine et al. 2005
SmA225	2011 carrying a 500-kb deletion in pRm2011a, Nod ⁻ , Fix ⁻	Perrine et al. 2005
SmA838	2011 carrying a 850-kb deletion in pRm2011a, Nod ⁻ , Fix ⁻	Perrine et al. 2005
SmA845	2011 carrying a 850-kb deletion in pRm2011a, Nod ⁻ , Fix ⁻	Perrine et al. 2005
G270	pRmeSU47b deletion derivative, $\Delta\Omega 5033-5007::Tn5-233 \Delta\Omega 5085-5047::Tn5$ -Tp, Gm ^r -Sp ^r Tp ^r	Charles and Finan 1991
G271	pRmeSU47b deletion derivative, $\Delta\Omega 5033-5007::Tn5 \Delta\Omega 5149-5079::Tn5-233 \Delta\Omega 5085-5047::Tn5$ -Tp, Nm ^r Gm ^r -Sp ^r Tp ^r	Charles and Finan 1991
G277	pRmeSU47b deletion derivative, $\Delta\Omega 5020-5011::Tn5-oriT \Delta\Omega 5149-5079::Tn5-233 \Delta\Omega 5085-5047::Tn5$ -Tp, Nm ^r Gm ^r -Sp ^r Tp ^r	Charles and Finan 1991
RmF909	$\Delta\Omega 5085-5047::Tn5$ V, Nm ^r Gm ^r -Sp ^r	Charles and Finan 1991
Sm5422	Rm1021, <i>ntrA75::Tn5</i>	Finan et al. 1988
M1	SMa1236G1_32D09_01.01, NapA periplasmic nitrate reductase	Becker
M2	SMa1236G1_32D09_01.02, NapA periplasmic nitrate reductase	Becker
M3	SMa1240G1_32D10_01.01, NapF Ferredoxin component of periplasmic nitrate reductase	Becker
M4	SMa1240G1_32D10_01.02, NapF Ferredoxin component of periplasmic nitrate reductase	Becker
M7	SMb20436G1_37F12_01.01, nitrate transporter protein	Becker
M8	SMb20436G1_37F12_01.02, nitrate transporter protein	Becker
M11	SMa0583G1_31D01_01.01, NrtB, nitrate transport permease protein	Becker
M12	SMa0583G1_31D01_01.02, NrtB, nitrate transport permease protein	Becker
M17	SMa1247G1_32D12_01.01, NirV periplasmic nitrate reductase	Becker
M18	SMa1247G1_32D12_01.02, NirV periplasmic nitrate reductase	Becker
M15	SMb20078G1_35H07_01.01, nitrate/nitrite response regulator protein	Becker
M16	SMb20078G1_35H07_01.01, nitrate/nitrite response regulator protein	Becker
M19	2011mTn5STM.1.13.B08 nirK, nitrite reductase	Becker
M20	GIPELR32E1 nirK, nitrite reductase	Becker
<i>Pseudomonas aeruginosa</i>		
PAO5	Rif ^r Tp ^r , opportunistic human pathogen	M. Nayudu

^a Nod⁺ = ability to nodulate, Nod⁻ = inability to nodulate, Fix⁺ = ability to fix nitrogen, Fix⁻ = inability to fix nitrogen, Δ = defined deletions, and Ω = transposon insertions.

^b Becker = Becker at Bielefeld University.

mutants) also stunted the growth of rice seedlings at 21 days. Uninoculated rice seedlings had green leaves and showed a normal pattern of root growth and development at 21 days (Fig. 2A).

Nitrate transport permease located on pSymA is not involved in the inhibition of rice growth.

The nitrate transport permease (NtrB), which is located outside the deleted region of the pSymA-deleted derivative SmA146, was used to investigate the role of nitrate in the inhibition of rice seedling growth. The aim was to disrupt the transport of nitrate inside *S. meliloti* Sm2011 and, thus, make nitrate unavailable for nitrate reduction. Strains M11 and M12 (ntrB mutants) were tested on rice seedlings of cv. Pelde. At 21 days, rice seedlings inoculated with strain M11 and M12 were stunted in growth.

Copper-containing nitrite reductase located on pSymA is not involved in the inhibition of rice growth.

Strains M19 and M20, copper-containing nitrite reductase (nirK) mutants, were tested on rice seedlings of cv. Pelde (Fig. 2A). It was thought that *nirK* may be responsible for the production of NO from the reduction of nitrite and that, by knocking out this gene, inoculated plants would be able to grow normally. However, at 21 days, rice seedlings inoculated with these strains were stunted in growth and, indeed, chlorosis was noted in the shoots at 7 days.

Nitrate/nitrite response regulator protein and the nitrate transporter protein located on pSymB are not involved in rice growth inhibition of Sm1021.

Perrine and associates (2005) have shown that pSymB-deleted derivatives of Sm2011 inhibited rice growth. It was

Table 2. Characterization of bacterial strains for their ability to reduce nitrate in test tubes after 7 days in liquid tryptone yeast (TY) medium

Bacterial strain	At 7 days in TY liquid medium + 10 mM KNO ₃		
	Bacterial growth (turbidity) ^a	Presence of nitrite ^b	Presence of nitrate ^b
Control (uninoculated)	–	–	+++
<i>Sinorhizobium meliloti</i>			
Sm1021	+++	–	+++
Sm2011	+++	+++	ND
SmA818	+++	+	ND
SmA146	+++	++	ND
SmA144	+++	–	+++
SmA225	+++	++	ND
SmA845	+++	+	ND
SmA838	+++	+	ND
G270	+++	–	+++
G271	+++	–	+++
G277	+++	–	+++
SmF909	+++	–	+++
Sm5422	+++	–	+++
<i>Rhizobium leguminosarum</i> bv. <i>trifolii</i>			
R4	+++ (mucoid)	–	–
<i>Pseudomonas aeruginosa</i>			
PAO5 (positive control)	+++	–	–

^a Bacterial growth was monitored by the turbidity of the medium; – = no growth and +++ = very turbid.

^b Amount of nitrite production was monitored by the amount of red pigment produced compared with the standard curve: – = no red pigment, nitrite absent; + = 0.005 mM nitrite; ++ = 0.01 mM nitrite; +++ = greater or equal to 0.1 mM nitrite; ND = not determined. Results were an average from three test tubes.

Fig 2. Growth of Pelde rice seedlings in liquid nitrogen-free modified Fahraeus medium supplemented with 10 mM KNO₃ 21 days after inoculation (six seedlings per pot, five replicates). **A**, Only the uninoculated control and seedlings inoculated with either strain 818 or R4 grew normally. **B**, After 7 days, the normally growing plants were able to tolerate the accumulation of nitrite in the medium.

also shown that the removal of key regions of pSymB in some pSymB deletion mutants (for example, strains G270 and G271) affected the level of inhibition. Strains M7, M8, M15, and M16 (Table 1) were then used to inoculate rice seedlings as described by Perrine and associates (2005). At 21 days, all rice plants were inhibited in growth and development similarly to Sm1021- and Sm2011-treated rice seedlings.

Strain Sm1021 with nitrite as the only nitrogen source inhibited the growth of rice seedlings.

To confirm that nitrate is not the sole cause of rice growth inhibition, nitrite, at concentrations of 0.1, 1, 2, 5, and 10 mM NaNO₂, was used as a source of nitrogen for the growth of rice seedlings. Also, the inhibitory strain Sm1021 was used to inoculate rice seedlings to investigate the effect of nitrite on the interaction of *S. meliloti* with rice. Rice seedlings were inoculated with Sm1021 and then grown as described by Perrine and associates (2001). In NFM supplemented with 10 mM KNO₃ (F10) liquid medium at 21 days, Sm1021-inoculated plants were inhibited in growth and lateral root development, whereas uninoculated rice seedlings had green shoots and showed a normal pattern of root development (Table 3; Fig 2A). Magenta jars containing liquid NFM supplemented with either 2 or 5 mM NaNO₂ appeared to contain the optimal amount of nitrite to support the growth of uninoculated rice seedlings and the rice seedlings had a normal pattern of growth compared with uninoculated rice seedlings grown in F10 at 21 days (Table 3). In the case of rice plants grown in NFM supplemented with 0.1 mM NaNO₂, they were slightly inhibited in growth; however, this may be attributed to an inadequate nitrogen supply and growth could not be supported by the medium for the full 21 days. Sm1021-treated rice seedlings grown in the NFM supplemented with 2 or 5 mM NaNO₂ were stunted in growth (Table 3). In NFM medium supplemented with 10 mM NaNO₂, the growth of uninoculated and Sm1021-treated rice seedlings were inhibited by day 21 (Table 3).

Nitrate reduction occurs in absence of rhizobia and nitrite concentration is higher in Magenta jars containing rice plants unaffected in growth by rhizobia.

To investigate whether nitrate reduction did occur in the Magenta jars in the presence of rice seedlings and rhizobia, the media was assayed for nitrite. At 21 days, Magenta jars containing uninoculated rice seedlings were found to contain nitrite (Table 4). Much less nitrite was found in Magenta jars containing inhibitory strains Sm1021, TA1, and ANU794 (Table 4). Previous work by Perrine and associates (2001) demonstrated that the use of tetracycline affected the interaction of rhizobia with rice by inhibiting the growth of inhibitory strains TA1 and ANU794. Therefore, another experiment was set up using TA1, ANU794, and R4 as a positive control to investigate the production of nitrite in Magenta jars in the presence of rice seedlings and tetracycline (2 mg ml⁻¹). At 21 days, all rice seedlings inoculated with inhibitory strains ANU794 and TA1 were stunted in their growth (Table 4). In contrast, rice seedlings inoculated with strain R4 had green shoots and root

growth appeared normal, similar to uninoculated rice seedlings (Table 4). In the presence of tetracycline, rice seedlings inoculated with inhibitory strains ANU794 or TA1 or the noninhibitory strain R4 had a pattern of growth similar to that of uninoculated rice seedlings at 21 days (Table 4). The level of nitrite detected in the Magenta jars containing ANU794- and TA1-inoculated rice seedlings treated with tetracycline was higher compared with those not treated with antibiotic (Table 4). There was no difference in the nitrite level found in Magenta jars containing rice seedlings treated with strain R4 with or without tetracycline, and the plants appeared normal (Table 4).

A quantitative time course study of the nitrite and nitrate concentrations in the F10 medium (Fig. 2B) showed that, for the normally growing plants (inoculated with either R4 or 818 or uninoculated), medium nitrite concentrations increased rapidly after 7 days to 5.3 μM (R4 and 818) and 1.2 μM (uninoculated). The nitrite levels for all the growth-inhibited plants ranged from 0.53 to 0.72 μM at 7 days and did not change significantly thereafter. The medium containing the normally growing plants (uninoculated, or inoculated with 818 or R4) experienced a net decrease in nitrate concentrations, as a consequence of growth, of 540, 744, and 672 μM, respectively.

Light intensity, however, affected the inhibitory phenomenon of strain Sm1021 on rice seedlings grown in F medium supplemented with 10 mM KNO₃. When the level of the photon flux density was reduced from 600 to 300 μmol m⁻² s⁻¹, the inhibition of strain Sm1021 on rice seedlings at 21 days was abolished (data not shown).

DISCUSSION

We previously have established that the growth of rice seedlings inoculated with most *Rhizobium* strains is inhibited when nitrate is added to the medium (Perrine et al. 2001; Prayitno et al. 1999). The inhibiting strains caused a marked reduction in root and lateral root growth and, as a result, the extent of entry and multiplication of the rhizobia inside the rice roots was greatly reduced. However, one strain in particular, strain R4, did not cause root inhibition and actually stimulated the growth of rice seedlings and formed long lines of bacterial cells within the growing lateral roots (Perrine et al. 2001; Prayitno et al. 1999). We were able to show that inoculation of rice seedlings with less than 10 bacterial cells of an inhibiting strain was sufficient to cause inhibition and also induce inhibition in mixed-cell inocula containing both inhibitory and non-inhibitory strains (Perrine et al. 2005). Furthermore, we showed that the *Rhizobium*-rice interaction could be influenced by different media, ion concentrations, and the intensity of the light conditions used to grow the rice plants. Additional experiments suggested that the phytohormone auxin and nitrate could have an effect on lateral root development (Perrine et al. 2001). The problem, then, was to separate out the funda-

Table 3. Effect of nitrite on the growth of rice seedlings (cv. Pelde) at 21 days postinoculation in liquid nitrogen-free modified Fahraeus medium^a

	KNO ₃		NaNO ₂ (mM)			
	10 mM	1	2	5	10	
Control	+++	++	++	++	—	
Sm1021	—	—	—	—	—	

^a Control = uninoculated. Normal growth = +++ and complete inhibition of growth = —; 0.1 mM NaNO₂ was inadequate to support growth for the full 21 days.

Table 4. Nitrate reduction in medium

Strains	Plant growth	Nitrite concentration ^a
Control (uninoculated)	+++	+++
M12	—	+++
Sm1021	—	+
TA1	—	+
ANU794	—	+
R4	+++	+++
TA1 + tetracycline	+++	+++
ANU794 + tetracycline	+++	+++
R4 + tetracycline	+++	+++

^a Optical density: water = 0.000, + = 0.000 to 0.18, ++ = 0.18 to 0.36, and +++ = 0.36 to 0.54.

mental influence and determine whether auxin or nitrate was the major cause of defective root growth. The model micro-symbiotic strain *S. meliloti* 1021 and its closely related strain Rm2011 also were shown to inhibit rice seedling growth (Perrine et al. 2005). The advantage of using the strain 1021 was the availability of a complete genome sequence (Barnett et al. 2001) and the large number of available mutant strains.

Experiments using a series of plasmid-cured and plasmid-deleted derivatives of *S. meliloti* Rm2011 showed that the pSymA plasmid encoded the genes associated with the growth inhibition of rice seedlings (Perrine et al. 2005). Some deletions in the pSymB plasmid also could partially relieve inhibition of rice growth. Additional studies showed that pSymA encoded genes required for indole-3-acetic acid (IAA) synthesis and nitrate metabolism and that the pSymA was associated with rice growth inhibition. This inhibited growth phenotype was induced by environmental nitrate. Perrine and associates (2005) concluded, "The rice growth inhibition phenomena could represent the outcome of a complex interaction between nitrate, nitrite, and perhaps IAA."

Nitrate is not the cause of rice growth inhibition in rice seedlings.

Our current studies suggest that the individual genes involved in nitrate reduction via the periplasmic nitrate reductase and nitrate transport via the *nttB* gene, both located on the Sm1021-pSymA megaplasmid, did not play any role in the Sm1021-induced inhibition of rice seedling growth. Similar results were observed with the nitrate/nitrite response regulator protein and the nitrate transporter protein located on the Sm1021 (Rm2011) pSymB megaplasmid, suggesting that they also were not involved in the inhibition of rice growth (Table 5). Furthermore, it is important to note that rice plants also contain nitrate reductase activity which can reduced nitrate to nitrite (Barlaan et al. 1998). This was evident when nitrite was found in the Magenta jars containing uninoculated rice seedlings (Table 4). The use of nitrite instead of nitrate as sole source of N in rice plant medium did not abolish the inhibitory effect of Sm1021 on cv. Pelde rice seedlings (Table 4). Therefore, if the *nap* genes were not functional in the *S. meliloti* mutants, the rice plants would still have been able to reduce the nitrate to nitrite, making it available for use by the mutants to cause rice growth inhibition. Thus, nitrite and not nitrate is the cause of rice growth inhibition.

In the presence of 10 mM NaNO₂ and the absence of Sm1021, rice seedling growth was inhibited (Table 3). For nitrite to be the cause of this inhibition, nitrite production by Sm1021 and Rm2011 would need to reach a concentration of at least 10 mM nitrite (Table 3; nitrite inhibits at 10 mM but not at 5 mM). Nitrite at high concentration is toxic to the growth of bacteria and bacteroids (Casella et al. 1988). Exposure to more than 1.0 mM nitrite for 24 h greatly inhibited nitrogenase activity (Casella et al. 1988). Micromolar nitrite concentrations also reduced cell yields and inhibited oxygen respiration in free-living bacteria (Casella et al. 1988). Accumulation of nitrite in plant tissues is also toxic due to the production of nitric oxide (NO), either nonenzymatically through

light-mediated conversion by carotenoids or enzymatically through NADPH nitrate reductase (Durner and Klessig 1999). NO has been shown to be involved in root organogenesis (Pagnussat et al. 2002) but also functions as a signal in plant defense (Durner and Klessig 1999; Klessig et al. 2000). Rhizobia and other denitrifiers have the ability to produce NO due to respiratory nitrite reductase, *nirK* (Moreno-Vivian et al. 1999). This type of respiratory process permits cell growth of *Bradyrhizobium japonicum* and *R. sphaeroides* under microaerophilic conditions (Jain and Shapleigh 2001; Velasco et al. 2001).

Growth studies showed that the bacterial strains Sm1021 and its pSymA-cured derivative SmA818 grew in F10 liquid containing rice seedlings (data not shown). Through the generation of NO or the generation of NH₄⁺, SmA818 and Sm1021 could maintain their growth in the Magenta jars. Nitrite can be converted to ammonium or to NO by different types of nitrite reductases. In *Escherichia coli*, the NADH-dependent *nirB* gene catalyses the reduction of nitrite to ammonium, thus detoxifying nitrite accumulated in anaerobically grown cells (Moreno-Vivian et al. 1999). In denitrifying bacteria, two different types of respiratory enzymes reduce nitrite to nitric oxide. They are *nirS*, found in *Pseudomonas* spp. and most denitrifiers, and the *nirK* gene encoded copper nitrite reductase, found in other bacteria (Jain and Shapleigh 2001; Toffanin et al. 1996; Velasco et al. 2001; Zumft 1997).

Little is known as to the possible function of the pSymA-located *nap* genes in *S. meliloti* 1021. In the genomic sequence of *S. meliloti* 1021, the *napCBADFE* operon is located on the pSymA megaplasmid near the *nos* and *nor* operons involved in nitric oxide and nitrous oxide metabolism, respectively. This suggests that the *nap* genes in *S. meliloti* may have a similar function to those in the *Pseudomonas* sp. strain G179. The *nirB* gene is located on the pSymB megaplasmid, whereas the *nirK* copper nitrite reductase was found on the pSymA megaplasmid near the *nap*, *nos*, and *nor* operons, suggesting that Sm1021 can reduce nitrite to ammonium or NO.

In growth studies with strains in which *nirK* was disabled, plant growth was inhibited; however, this loss of function may be compensated for by one or more as yet functionally unidentified genes. This is consistent with the comment by Jain and Shapleigh (2001) that multiple forms of all the reductases are known except for NO (Zumft 1997). It was also noted recently by Baudouin and associates (2006) that *S. meliloti* mutants of *nirK* and *norD* were still capable of producing NO, and that denitrification was not a major source of NO in nodules. In addition, Bethke and associates (2006) presented evidence for nonenzymic synthesis of NO from apoplastic nitrite.

Is the inhibition of rice growth by Sm1021 or Rm2011 due to accumulation of NO in plant tissues or in the medium?

When nitrate or nitrite is the sole N source for rice seedlings and Sm1021, the rice seedling and Sm1021 assimilated nitrate for their growth, thus increasing nitrite concentration within the plant tissue, medium, and bacteria. The accumulation of nitrite may lead to NO formation in the rice plant tissues or cause Sm1021 to reduce nitrite to NO so as to facilitate survival during temporary hypoxia or anoxia. Based on the fol-

Table 5. Nitrate related genes on pSymB megaplasmid of *Sinorhizobium meliloti* 1021^a

Name	Accession number	Protein description
Y20078	SMb20078 AA	Nitrate/nitrite response regulator protein
Y20436	SMb20436 AA	Nitrate transporter protein
NtrA	SMb21114 AA	Putative nitrate transport protein
NarB	SMb20986 AA	Putative nitrate reductase, large subunit
Y20997	SMb20997 AA	Putative membrane protein, similar to periplasmic nitrate reductase NnuR

^a DNA searches were done using the sequence of *S. meliloti* 1021.

lowing, we propose that the formation of NO is the cause of rice growth inhibition by inhibitory strains of rhizobia (Fig. 1).

First, the form of nitrate in the plant medium (Perrine et al. 2005) did affect the interaction of Sm1021 with cv. Pelde rice seedlings by affecting the plant, the bacteria, or both. The use of NH_4NO_3 and $(\text{NH}_4)_2\text{SO}_4$ as a nitrogen source in the plant medium prevented Sm1021 from killing the treated rice seedlings compared with when KNO_3 and $\text{Ca}(\text{NO}_3)_2$ were used as an N source (Perrine et al. 2005). This is evidence for NH_4^+ preventing the nitrate- or nitrite-dependent rice growth inhibition. In fungi, NH_4^+ represses nitrate and nitrite transport and reduction (Galván and Fernández 2001). Furthermore, the inhibitory effects caused by the NH_4^+ ion upon nitrate uptake by roots of higher plants, such as barley and rice, have been studied (Colmer and Bloom 1998; Fried et al. 1965; Kronzucker et al. 1999). This makes the rice plants less susceptible to inhibition via nitrite in the presence of NH_4^+ . Also, NH_4^+ can affect the growth of Sm1021 by repressing the conversion of nitrate to ammonium, and switch to the use of the Ntr system to sustain its growth.

Second, the level of nitrite was lower in jars containing rice seedlings inoculated with viable inhibitory rhizobia compared with those treated with the bacteriostatic antibiotic, tetracycline (Table 4). This could be due to the reduction of nitrite to NO by viable inhibitory rhizobia.

Third, in plants, nitrate and nitrite reductases and nitrate transport are coordinated with respect to light (Galván and Fernández 2001). Work by Sawhney and Naik (1972) demonstrated that light was necessary for the synthesis of nitrate reductase and nitrite reductase in the shoot and excised embryos of rice seedlings. Also, by the use of colored cellophane papers filters to achieve different wavelengths of light, the synthesis of both rice reductases was affected (Sawhney and Naik 1972). When light intensity was less than optimal, the growth of Sm1021-treated rice seedlings was not inhibited, which suggests that light may have affected how the rice plant responded to the presence of nitrate or nitrite in its cells.

Fourth, strain R4 was shown to be capable of reducing nitrate to N_2 (gas) under the range of microaerophilic to anaerobic conditions present, as could the *Pseudomonas* PAO5 (Table 1). Nevertheless, nitrite was detected in Magenta jars containing R4-inoculated rice seedlings at 21 days and the rice seedlings were not inhibited in growth (Table 4). Other strains of rhizobia could reduce nitrate or were deficient in nitrate reduction under such conditions and all inhibited rice growth (Table 4). Therefore, the ability and efficiency of reductase enzymes to metabolize nitrate to N_2 (gas) in the *Rhizobium*-rice interaction influenced growth or inhibition of rice roots.

Fifth, when nitrite in the NFM medium was the only source of nitrogen in the presence of Sm1021, rice seedlings were still inhibited in growth (Table 3). This suggests that nitrate itself is not the cause of rice growth inhibition in the presence of rhizobia. It is possible that the metabolism of nitrite under such conditions by rhizobia could be producing an inhibitory compound that would affect plant growth and development. The fact that untreated rice seedlings could grow in F medium supplemented with 1, 2, and 5 mM NaNO_2 suggested that the nitrite probably was being reduced to NH_4^+ by nitrate reductase in rice plants and this, in turn, was being used for the making of amino acids essential for sustaining growth. It was not until the nitrite concentration was too high and toxic (10 mM NaNO_2) that the untreated rice plants were inhibited in growth and development. This phenomenon also could be mimicked by the rice seedlings grown in F medium supplemented with 1, 2, and 5 mM NaNO_2 in the presence of Sm1021. Recent literature suggests the possible role of NO in plant signaling and in plant defense responses (Beligni and Lamattina 2001; Crawford

and Guo 2005; Wendehenne et al. 2004; Zeidler et al. 2004; Zeier et al. 2004). NO has been shown to play a role in root organogenesis (Pagnussat et al. 2002) and in determining lateral root development in tomato (Correa-Aragunde et al. 2004). Interestingly, work by Pagnussat and colleagues (2003, 2004) has demonstrated that NO is a messenger and mediates the indole-acetic-induced adventitious rooting process in cucumber.

Two independent studies investigating the formation of NO in plants may help our understanding of how rhizobia could be affecting plant growth and development. First, Modolo and associates (2005) demonstrated that NO synthesis was triggered when *Arabidopsis thaliana* leaves were inoculated with an avirulent strain of *P. syringae* pv. *maculicola*. Their data suggested that nitrite was the major source for NO production during this plant-microbe interaction (Modolo et al. 2005). However, the article failed to explore the possibility of NO synthesis by that strain and its role in NO-induced responses in its tested plant host. Second, Creus and colleagues (2005) showed that NO was involved in the *Azospirillum brasilense* Sp245-induced lateral root formation. They also found that aerobically grown *A. brasilense* produced nitric oxide in pure culture. Both studies suggest the possibility that NO produced by plant-colonizing bacteria may directly or indirectly affect the growth of their host.

We propose that *Rhizobium* spp. may have similar a effect on the growth of rice plants. The infecting rhizobia produce NO and, as NO increases in concentration, it could act as a signal and thus regulate the indole acetic pathway in the rice plant or act as a toxic compound on the rice (Crawford and Guo 2005). Perrine and associates (2001) demonstrated that exogenous auxins could inhibit rice growth and development. The pattern of rice growth inhibition by auxin-treated rice plants was similar to that of rice plants inoculated with inhibitory rhizobia strains (Perrine et al. 2001, 2004, 2005).

We have shown conclusively that certain strains of rhizobia have the ability to infect rice roots and colonize the intercellular spaces of the rice roots without inhibiting their growth and the development. Thus, the presence of noninhibitory rhizobia in rice roots provides an opportunity to build a biological nitrogen fixation system within these roots. *R. leguminosarium* bv. *trifolii* R4 is an ideal candidate as a future rice inoculant strain because it contains these characteristics plus the ability to completely reduce nitrate to N_2 (gas) without accumulating toxic concentrations of NO. Further development will be necessary before achieving a long-term goal of some form of biological nitrogen fixation in rice. Moreover, strain R4 could be a suitable candidate for the inoculation of maize plants, like some of the *R. etli* bv. *phaseoli* isolates obtained from maize roots which did provide plant growth promotion effects (Gutiérrez-Zamora and Martínez-Romero 2001).

MATERIALS AND METHODS

Plant growth media.

Rice plants (*Oryza sativa* L. cv. Pelde) were grown in NFM (Rolfe et al. 1980; Rolfe and McIver 1996; Vincent 1970) and F10 (Perrine et al. 2005) media. Magenta jars containing the liquid medium were wrapped in a black plastic jacket to keep the roots in darkness. This system was devised to mimic the conditions in which rice is grown in paddy fields. In some experiments, these jars were supplemented with various concentrations of sodium nitrite.

Bacterial strains and plant inoculation.

All bacterial strains used in this study are listed in Table 1. Routine subculturing of rhizobia was done on Bergensen's modified medium (BMM) or TY plates at 28°C and *E. coli* on

Luria-Bertani medium at 37°C (Table 1). Antibiotics were incorporated into plates whenever rhizobia and *P. aeruginosa* strains carried a marker. For rice plant assays, strains were grown on BMM or TY for 3 days and *E. coli* strains overnight on Luria-Bertani medium with appropriate antibiotic markers. Colonies were resuspended in sterile water to an optical density at 600 nm of 0.1 to inoculate rice plants, as described by Perrine and associates (2004). For nodulation assays, colonies were grown overnight to an optical density at 600 nm of 0.2 to 0.3 in BMM liquid medium. Plasmid integration mutants were constructed basically as described by Luo and associates (2005) and Pobigaylo and associates (2006). Internal fragments of the target genes were amplified by PCR using primers flanked by *Hind*III or *Bsr*GI restriction sites (5' extensions of the specific primer sequences: GGTTCACGTAAGC TTCC, GCGATTACCCTGTACACC, restriction sites are underlined). *Hind*III- and *Bsr*GI-restricted fragments were inserted into suicide plasmid pK19mob2ΩHMB (Pobigaylo et al. 2006; Luo et al. 2005). The resulting plasmids were transferred to wild-type *S. meliloti* Rm1021 by *E. coli* S17-1-mediated conjugation (Simon et al. 1983). Transconjugants carrying the plasmid integrated into the genome were selected for neomycin resistance and were confirmed by PCR. Sequences of PCR fragments used for mutagenesis are available on the Universität Bielefeld's public *Sinorhizobium meliloti* 1021 genome browser.

The strains were used to check the effects of the assimilatory nitrate reduction, where nitrate is reduced to ammonia (NH₄⁺) via nitrite and the assimilatory nitrate reductase which operates under both aerobic and anaerobic conditions (Konings and Boonstra 1977). NH₄⁺ is the preferred source of nitrogen for growth; therefore, where nitrate is the only N source, the rhizobia uses nitrate to produce NH₄⁺ by first reducing nitrate to nitrite (Patriarca et al. 2002). We tested the two types of dissimilatory nitrate reductases; the Nar system is associated with denitrification and anaerobic nitrate respiration and the Nap system is widespread among gram-negative bacteria and normally is unaffected by ammonium and oxygen (Moreno-Vivian et al. 1999; Payne 1981).

Bacterial nitrate and nitrite assay.

A loopful of bacteria was added to 5 ml of TY or TY supplemented with 10 mM KNO₃ in 13-ml test tubes. The inoculated test tubes were incubated at 29 or 37°C for 7 days. The test tubes were not shaken and they were sealed using Nescofilm so as to create conditions ranging from microaerophilic to anaerobic. Seven days after inoculation, 0.5 ml each of solution A (1%, wt/vol, sulphanilamide dissolved in 3 M HCl) and solution B (0.02%, wt/vol, *N*-naphthyl-ethylenediamine-hydrochloride dissolved in water) were added to the test tubes (Skotnicki and Rolfe 1980). An immediate red color indicated the presence of nitrite. No color meant that nitrite was absent. To confirm that a negative reaction was due to nitrate being completely reduced to nitrogen gas, 2 mg of zinc dust was added to the test tubes. Zinc reduced the nitrate in the solution to nitrite and N₂ gas, and formed a red color. If no color appeared within 10 min of zinc addition, it indicated that nitrate was absent and that it had been completely reduced to N₂ gas. The amount of nitrite produced was quantified using TY supplemented with sodium nitrite at the following concentrations: 10, 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, and 0 mM NaNO₂.

Plant nitrate and nitrite assay on F10 in Magenta jars.

A qualitative nitrate or nitrite assay was carried out in Magenta jars containing rice seedlings of cv. Pelde grown in F10 media, which had been inoculated with various *Rhizobium*

strains at 21 days. The assay was as described above, except that 1 ml each of solution A and solution B were added to each Magenta jar and results recorded after 10 min. These assays were done in duplicate.

Nitrate and nitrite concentrations were quantified by flow injection analysis on a Lachat Instruments QuikChem 8500 analyzer. Nitrite was estimated by sulphanilamide colorimetry and total NO_x concentrations after cadmium reduction. Nitrate concentrations then were calculated from the total NO_x by subtraction of the nitrite concentration. Samples were frozen and kept at -20°C until analyzed.

Application of nitrite as an alternative nitrogen source.

To investigate the possible role of nitrite on the interaction of rice plants with rhizobia, liquid NFM was supplemented with 0.1, 1, 2, 5, and 10 mM sodium nitrite. Rice seed were germinated, inoculated with *S. meliloti* Sm1021, and then transferred to Magenta jars containing the liquid NFM supplemented with various concentrations of sodium nitrite. Experiments were performed in duplicate, with each treatment consisting of six rice seedlings per Magenta jar. The plants were harvested after 21 days and the dry weights or shoot and root characteristics recorded.

Screening of *S. meliloti* 1021 transconjugants derived from plasmid integration mutagenesis on rice growth.

S. meliloti 1021 transconjugants (Table 2) were used as inoculants on rice seedlings in F10 medium for 21 days as described by Perrine and associates (2005) to determine the specific nitrate-related genes involved in the rice-*Rhizobium* sp. interaction. Experiments were performed in duplicate, with each treatment consisting of six rice seedlings per Magenta jar.

Statistical analysis.

Experiments were arranged as randomized block designs. Data were subjected to analysis of variance with treatment means compared at the 95% probability level ($P < 0.05$). Significant results of treatment means from analysis of variance tests were analyzed further with the least significant difference at the 95% level. All statistical calculations were done using Genstat (sixth edition, version 6.1; VSN International Ltd, Hemel Hempstead, U.K.).

ACKNOWLEDGMENTS

We thank R. Philips, CSIRO Analytical Services, for the nitrate and nitrite analyses and J. Weinman for compilation of Figure 2. The project was supported by an Australian National University (ANU) grant and F. Perrine-Walker was supported by an ANU Ph.D. scholarship.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Sinorhizobium meliloti strain 1021 Genome Project: bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/public
 Universität Bielefeld's public *Sinorhizobium meliloti* 1021 genome browser:
www.cebitec.uni-bielefeld.de/groups/nwt/sinogate