Effects of Different Sources of Nitrogen on Endophytic Colonization of Rice Plants by *Azospirillum* sp. B510

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*Azospirillum* sp. B510, a free-living nitrogen-fixing bacterium isolated from the stems of rice (*Oryza sativa* cv. Nipponbare), was investigated to establish effective conditions for the colonization of rice plants. We analyzed the effects of the nitrogen sources KNO₃, NH₄Cl, urea (CO[NH₂]₂), and NH₄NO₃ at different concentrations (0.01–10 mM) on this colonization. Nitrogen promoted plant growth in a concentration-dependent manner, with minor differences being observed among the different nitrogen sources. Bacterial colonization was markedly suppressed on media containing NH₄⁺ concentrations higher than 1 mM. Since concentrations of up to and including 10 mM NH₄⁺ did not exhibit any antibacterial activity, we analyzed several factors affecting the NH₄⁺-dependent inhibition of endophytic colonization, including the accumulation of the reactive oxygen species H₂O₂ and the secretion of the chemotactic substrate malic acid. The accumulation of H₂O₂ was increased in rice roots grown on 1 mM NH₄Cl. The amounts of malic acid secreted from NH₄⁺-grown rice plants were lower than those secreted from plants grown without nitrogen or with KNO₃. Although the bacterium exhibited chemotactic activity, moving towards root exudates from plants grown without nitrogen and KNO₃-grown plants, this activity was not observed with root exudates from NH₄⁺-grown plants. NH₄⁺, but not NO₃⁻, caused the acidification of growth media, which inhibited plant bacterial colonization. These NH₄⁺-dependent phenomena were markedly suppressed by the stabilization of medium pH using a buffer. These results demonstrate that the type and concentration of nitrogen fertilizer affects the colonization of rice plants by *Azospirillum* sp. B510.

Key words: *Azospirillum* sp. B510, rice, endophytic colonization, nitrogen, acidification

Biofertilizers have been widely used in many countries as an alternative to chemical fertilizers in order to increase soil fertility and crop production for sustainable farming. The application of beneficial microbes can enhance plant growth and resistance to adverse environmental stresses, such as water and nutrient deficiencies and heavy metal contamination. One group of these microbes is referred to as plant growth-promoting rhizobacteria (PGPR), and some are commercially used as biofertilizers (6, 37).

*Azospirillum* sp., a well-studied member of PGPR, has been found in association with some of the world’s most staple food crops, including rice, maize, sorghum, wheat, and millet (14, 19, 28, 34). Members of the genus *Azospirillum* are widespread in soil and their inoculation on cereals and forage crops results in yield increases in many field experiments, not only due to nitrogen fixation, but also through the production of plant growth-promoting substances, such as the phytohormones indole-3 acetic acid (IAA) and gibberellic acid (2, 22). The inoculation of *A. brasilense* into *Zea mays* and *Sorghum bicolor* has been reported to enhance the uptake of mineral ions (NO₃⁻, K⁺, and H₂PO₄⁻) (28). The uptake of NH₄⁺ and PO₄⁻ was also enhanced in rice plants after an inoculation with *A. lipoferum* under hydroponic conditions (31).

*Azospirillum* sp. B510 (B510) is a diazotrophic endophyte that has been isolated from the stems of a rice plant (*Oryza sativa* cv. Nipponbare) (13). Increased seed production by B510-colonized rice plants was demonstrated under greenhouse, paddy field, and laboratory conditions (7, 21, 37). Moreover, rice plants inoculated with this bacterium had induced resistance against rice blast disease and rice blight disease (42). Kaneko et al. (23) determined the complete genome sequence of B510; it has a single chromosome and six plasmids encoding 3,416 putative proteins, including putative genes encoding enzymes related to IAA biosynthesis and the reduction of host ethylene levels. A comparative metabolomic analysis revealed that rice plants inoculated with B510 induced a modified metabolic response in shoots and roots, suggesting that this bacterium triggers a systemic response against pathogens (7). Consequently, the beneficial effects of B510 colonization in rice make it a useful biofertilizer. However, these beneficial effects were found to vary in field experiments, depending on both the rice genotype and nitrogen level (37). Moreover, neither the mechanism by which this bacterium colonizes host plants nor the relationship between colonization and environmental conditions around the roots has been elucidated.

Nitrogen fertilizers, particularly ammonium, the form of nitrogen favored by rice, are widely used in rice cultivation; however, the influence of nitrogen fertilizers on the establishment of endophytic colonization by bacteria, such as B510, has not yet been clarified. Thus, to understand the relationship between nitrogen nutrition and endophytic colonization by B510 in rice plants, we performed physiological, histochemical, and microscopic analyses on rice-*Azospirillum* interactions. We found that high concentrations of NH₄⁺ exert indirect suppressive effects on the colonization of rice plants by B510. We also propose a model to explain the mechanisms responsible for the effects of high NH₄⁺ concentrations on the plant redox state, rhizosphere acidification, bacterial chemotaxis, and host colonization by B510.

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Materials and Methods

Bacterial strains and growth conditions

The bacterial strains used in the present study are listed in Table S1. Azospirillum sp. B510 was grown at 28°C in nutrient broth (NB) medium (Eiken Chemical, Tokyo, Japan) with appropriate antibiotics (50 μg mL⁻¹ polymyxin and 50 μg mL⁻¹ streptomycin). Escherichia coli strain S17-1 was grown at 37°C in Luria-Bertani (LB) medium supplemented with 50 μg mL⁻¹ ampicillin. DsRed-labeled Azospirillum sp. B510 was constructed using the plasmid pBjGroEL4::DsRed2, as described by Hayashi et al. (18).

Plant growth conditions and endophytic inoculation

Seed coats were removed from rice seeds (O. sativa cv. Nipponbare), which were then surface-sterilized with 70% ethanol for 30 s and shaken in 5% (w/v) sodium hypochlorite (Wako Pure Chemical Industries, Osaka, Japan) for 10 min. Seeds were then washed with sterilized distilled water three times for 10 min each time.

Regarding seed inoculation, B510 was grown on NB medium including 50 mg L⁻¹ polymyxin B (Wako Pure Chemical Industries) at 28°C, harvested at 30 h, and washed twice with sterilized distilled water. Bacterial cells were resuspended in sterilized distilled water to a final density of 10⁸ colony-forming units (CFU) mL⁻¹. Appropriate dilutions. After an incubation at 28°C for 3 d, the number of colony-forming units (CFU) mL⁻¹ was counted. The seedlings were then quickly washed five times with sterilized distilled water and placed into 30 mL Milli-Q water in a 50-mL Falcon™ tube for 2 d in the growth chamber. After the removal of seedlings, the solution was filtered with a 0.22-μm membrane filter (Millex-GP; Merck Millipore), lyophilized, and stored at –80°C. The lyophilized sample was dissolved in 1 mL Milli-Q water before being analyzed.

Measurement of malic acid in root exudates

Rice plants were grown in RM medium inoculated with or without B510 for 10 d. Each seedling was washed gently with sterilized distilled water and placed into 30 mL Milli-Q water in a 50-mL Falcon™ tube for 2 d in the growth chamber. After the removal of seedlings, the solution was filtered with a 0.22-μm membrane filter (Millex-GP; Merck Millipore), lyophilized, and stored at –80°C. The lyophilized sample was dissolved in 1 mL Milli-Q water before being analyzed.

A malic acid analysis was performed using the series LC-20AC HPLC system (Shimadzu, Kyoto, Japan) with a Shodex RSPAK KC-811 (300-8 mm) analytical column and KC-811 pre-column (Showa Denko K.K., Tokyo, Japan) run with 0.1% phosphate buffer at a flow rate of 0.25 mL min⁻¹. The column temperature was set to 40°C. Malic acid was detected and quantified using a UV detector set at 210 nm. The peaks obtained were compared with an array of standard malic acid peaks run under the same conditions. The major peak was identified by comparing the retention time with that of the matching standard. Standard malic acid (Sigma-Aldrich) and root exudates (25 μL of each) were sequentially injected into the chromatographic system and run under the same conditions with four replicates per sample. Malic acid in the root exudate was identified by comparisons with the retention times of standard samples.

Chemotaxis assay

The drop assay was performed as described in de Weert et al. (10) with slight modifications. B510 was grown in NB medium at 28°C and 160 rpm until the logarithmic phase (OD₆₀₀ of 0.8) was obtained. Bacterial cells were washed and resuspended in sterilized distilled water to an OD₆₀₀ of 2.0 and 1% hydroxypropylmethylcellulose solution (Sigma-Aldrich) was then added (final concentration, 0.25%). The cell suspension was transferred to a 90-mm Petri dish, on which it formed a 3-mm-thick layer. Concentrated (50-fold) root exudates or individual 100 mM organic acid components were added to the center of the dish as a 10-μL drop. After an incubation at room temperature for 15 min, the plates were inspected for the appearance of a clear zone surrounding the drop.

Biofilm assay

The production of biofilms by Azospirillum sp. B510 was measured in vitro using a PVC microtiter plate assay (40). Five μL of B510 cultures adjusted to OD₆₀₀=0.01 were inoculated into 95 μL of NB medium a 96-well microtiter plate (TPP Techno Plastic Products AG, Trasadingen, Switzerland), which was then incubated without shaking at 28°C for 48 h. In the quantification of biofilm development, 25 μL of 1.0% crystal violet solution (Wako Pure Chemical Industries) was added to the wells. After an 30-min incubation, unbound crystal violet in each well was gently removed with a pipette and the wells were washed with distilled water, followed by 70% ethanol, and then distilled water. Crystal violet in each well was solubilized by adding 100 μL of 100% ethanol and quantified by absorbance at 550 nm.
pH measurement

The pH of the cultivation medium was adjusted to 5.5 before cultivation. After cultivation for 7 d, RG medium was removed from the plants and mixed thoroughly. The pH of the medium was measured using a pH meter (Horiba, Kyoto, Japan). RG medium containing 0.05% bromophenol blue (BPB, Wako Pure Chemical Industries) was used to assess pH. To maintain pH in RG medium at 5.5, 100 mM of 2-morpholinoethanesulfonic acid monohydrate (MES) (Wako Pure Chemical Industries) was added to RG medium containing NH₄Cl.

Statistical analysis

Statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls (SNK) test. Different lower-case letters represent significantly different values (P<0.05).

Results

Effects of various nitrogen sources on endophytic colonization of rice by Azospirillum sp. B510

Seedlings of O. sativa cv. Nipponbare were inoculated with the DsRed-labeled B510 strain to establish optimum conditions for endophytic colonization. Plants were grown in RG medium containing different concentrations (0.01–10 mM) of each nitrogen source (KNO₃, urea, NH₄Cl, or NH₄NO₃). In most cases, rice roots inoculated with DsRed-labeled B510 showed strong red fluorescence under the fluorescence microscope (Fig. 1). Marked differences were not observed between 0.01 and 0.1 mM of each nitrogen source. Rice plants grown in more than 1 mM KNO₃ or urea exhibited strong fluorescence, indicating well-established bacterial colonization on root surfaces (epiphytic colonization). In contrast, red fluorescence was not detected in plants grown at NH₄Cl and NH₄NO₃ concentrations of 1 mM or higher, suggesting that NH₄⁺ suppressed bacterial colonization (Fig. 1). To further confirm these results, we counted the number of B510 cells on root surfaces. The number of B510 cells on root surfaces was 100-fold lower in 1 mM NH₄Cl-treated plants and 10,000-fold lower in 10 mM NH₄Cl-treated plants than in control plants without NH₄Cl (Fig. S1).

We then examined the endophytic colonization of rice by B510 by counting the number of colonies within surface-sterilized whole plants 10 d post-inoculation (Fig. 2). In rice plants grown on KNO₃, the number of B510 cells became higher as the KNO₃ concentration increased (Fig. 2A). Although the root length was very short in RG medium containing 10 mM urea, endophytic colonization was clearly detected (Fig. 2B and S2). B510 colonized the inside of host roots with 10⁴–10⁶ CFU g⁻¹ fresh weight at low concentrations (0.01–0.1 mM) of NH₄Cl. However, endophytic colonization by B510 was markedly suppressed at high concentrations (higher than 1 mM) of NH₄Cl with no detection (Fig. 2C). B510 also colonized host roots with 10³–10⁶ CFU g⁻¹ fresh weight at a low NH₄NO₃ concentration (0.01–0.1 mM), and only 10² CFU g⁻¹ fresh weight or no detection at 1 or 10 mM NH₄NO₃, respectively. These results indicate that high concentrations of both forms of ammonium (NH₄Cl and NH₄NO₃) have a negative effect on endophytic colonization by B510.
Since a high concentration of NH$_4^+$ is known to have a toxic effect on bacteria (3, 29), we investigated whether NH$_4^+$ inhibits the growth of B510 at the concentrations used in the colonization experiments. B510 was incubated in RG medium adjusted to pH 5.5 containing each concentration (0.01, 0.1, 1, and 10 mM) of NH$_4$Cl. No significant differences were observed in bacterial growth among these concentrations of NH$_4$Cl, demonstrating that NH$_4^+$ did not exhibit any antimicrobial activity against B510, which is in contrast to findings obtained using other bacteria (Table S3). These results indicate that endophytic colonization by B510 was inhibited by high concentrations of NH$_4^+$, but also that high concentrations of NH$_4^+$ did not inhibit B510 growth in vitro. Bacterial exopolysaccharides (EPS) are important for the attachment of the endophyte to the root surface (30). The establishment of a biofilm structure involves bacterial cells and EPS, which produce an optimal biosphere for the conversation of genetic material between cells. Thus, biofilms are of great importance in the plant-microbe interaction. The quantity of EPS in biofilms may comprise approximately 50–90% of organic compounds (11). However, B510 produced EPS or biofilms at similar levels regardless of the nitrogen source (NH$_4^+$ or NO$_3^-$; Fig. S4A and B). This result suggests that EPS or biofilm-mediated attachment is not a key step in the NH$_4^+$-dependent suppression of colonization.

NH$_4$Cl induces H$_2$O$_2$ accumulation in rice roots

Plant-associated bacteria need to cope with host defense responses during infection. Plant defense responses to pathogen infection involve the production of reactive oxygen species (ROS), including H$_2$O$_2$. H$_2$O$_2$ directly inhibits the growth of bacterial and fungal pathogens (41). Since infection by B510 was suppressed at 1 mM NH$_4^+$, we measured the accumulation of H$_2$O$_2$ in the roots of infected rice plants grown without nitrogen (control) and with 1 mM KNO$_3$ and 1 mM NH$_4$Cl using the DAB staining method (Fig. 3). The brown color that formed with DAB, which correlates with H$_2$O$_2$ accumulation, was more intense in roots that were grown in NH$_4$Cl than in KNO$_3$ and the control (Fig. 3B). Quantitative measurements of H$_2$O$_2$ revealed that NH$_4$Cl induced significantly greater H$_2$O$_2$ accumulation than KNO$_3$ (Fig. 3A). These results suggest that H$_2$O$_2$ accumulated with the NH$_4$Cl treatment, but that its production was slightly reduced by the inoculation with B510.

Chemotactic responses to root exudates from plants grown with KNO$_3$ or NH$_4$Cl

Azospirillum strains display chemotactic responses to specific substrates, such as root exudates, including organic acids (33). To analyze chemotactic responses to root exudates secreted from rice roots grown in RG media without nitrogen (control) and with KNO$_3$ and NH$_4$Cl, we performed a taxis assay (Fig. 4). Root exudates extracted from control and KNO$_3$-grown roots resulted in the formation of a bacterial ring in the plate assay, revealing a positive chemotactic reaction. However, the bacterial ring was not observed when root exudates from NH$_4$Cl-grown roots were used (Fig. 4). The bacterial rings formed by the root exudates of B510-inoculated plants were slightly larger than those of the non-inoculated control, suggesting that the B510 inoculation stimulated chemotactarctant production by roots (Fig. 4, lower panels).

Fig. 3. Qualitative and quantitative assessments of H$_2$O$_2$ in B510-inoculated rice grown on RG medium.
DAB staining was performed 10 d after the inoculation of rice seeds with B510. (A) The accumulation of H$_2$O$_2$ was quantified in the roots using standard H$_2$O$_2$ concentrations to calibrate data during optical density (OD) measurements. Plants were grown in RG medium including 1 mM of KNO$_3$, 1 mM NH$_4$Cl, 1 mM NH$_4$Cl+100 mM MES, or without nitrogen (control), inoculated with B510 (+) or not inoculated (–). Error bars indicate standard deviations (n=3). (B) H$_2$O$_2$ levels correlate with color intensity (brown). The arrow indicates root curling. Scale bar=0.5 cm.

Fig. 4. Chemotactic response of B510 towards rice root exudates.
The chemotactic response of B510 was analyzed using a negative control (water), positive control (100 mM Malic acid), and root exudates from rice roots. Rice seedlings were grown in RG media including control, 1 mM KNO$_3$, 1 mM NH$_4$Cl, or 1 mM NH$_4$Cl+100 mM MES for 10 d without an inoculation or an inoculation with B510. Before transplantation, seedlings were washed twice in sterilized distilled water and transplanted to 30 mL sterilized distilled water for 2 d. A 50-fold concentrated root exudate (10 μL) was added to the center of each Petri dish (90 mm). The bacterial chemotactic response was triggered after an incubation at room temperature for 10 min. A response correlated with the appearance of a ring of turbidity near the center of each Petri dish. Scale bars=1 cm. The experiment was repeated three times with similar results.
Nitrogen and Azospirillum Colonization

It may be taken up by plants as a cation (NH$_4^+$) or anion (NO$_3^-$). Previous studies demonstrated that plants supplied with NO$_3^-$ counterbalance the corresponding excess of negative charges by releasing equivalent amounts of OH$^-$ or HCO$_3^-$ into the rhizosphere, thereby increasing rhizosphere pH (9, 27). Plants receiving NH$_4^+$ counterbalance the corresponding excess of positive charges by releasing equivalent amounts of H$^+$ into the rhizosphere, thereby decreasing rhizosphere pH (20). Thus, we examined whether a pH change in the rhizosphere using different nitrogen sources affects the B510-endophytic colonization of rice plants. Before planting the seeds, the pH of RG medium was adjusted to 5.5. The acidification of RG medium after plant growth was clearly visualized using BPB, which changes from purple-blue to colorless when pH is between 3.0–4.6 (Fig. 6). The pH of control medium (without nitrogen) decreased slightly to pH 5.2 7 d after planting (Fig. 6). In the cases of KNO$_3$ and urea, pH increased to 6.2 and 8.4, respectively. However, in the cases of NH$_4$NO$_3$ and NH$_4$Cl (in which H$^+$ is released), pH markedly decreased to pH 3 and RG medium de-colorized (Fig. 6). The inoculation with B510 did not significantly affect pH (Table S2). These results indicate that NH$_4^+$-induced acidification around the rice rhizosphere. Therefore, the low pH caused by NH$_4^+$ may inhibit endophytic colonization by B510.

To analyze the effects of NH$_4^+$-induced acidification on endophytic colonization by B510, we tested the addition of MES buffer to RG medium, which was expected to prevent a decrease in pH. Without MES buffer, the pH of RG medium containing NH$_4$Cl decreased to 3.4 (Fig. 6). In RG medium containing NH$_4$Cl and MES, pH was maintained at 5.5 (Fig. 6) and endophytic and root surface colonization by B510 was not inhibited under higher concentrations of NH$_4^+$ tested (Fig. 1 and 2E). Furthermore, the accumulation of H$_2$O$_2$ decreased in rice roots grown with NH$_4$Cl and MES (Fig. 3). A chemotactic response by B510 was observed with exudates extracted from roots grown with NH$_4$Cl and MES (Fig. 4). In addition, malic acid secretion was increased by the B510 inoculation in exudates from roots grown with NH$_4$Cl and MES (Fig. 5). These results indicate that the pH decrease induced by NH$_4^+$ inhibited endophytic colonization by B510. We then performed a bacterial growth test using NB media adjusted to different pH levels (pH 3–8). The results obtained indicated that acidic media with pH<4 suppressed the growth of B510 (Table S4). These results imply that acidification directly affects the bacterial growth of as well as endophytic colonization by B510.

**Discussion**

Endophytic bacteria invade internal plant tissues through sites of injury in the epidermis, root tips, and root cracks formed at the sites of lateral roots, and some endophytic bacteria spread to distant plant organs (5, 36). *Azospirillum* sp. B510 was isolated from the stems of rice (13). A previous study demonstrated that B510 colonized the interior of rice roots using orthogonal optical sections (7). In the present study, B510 endophytic colonization of rice plants was negatively affected by a pH decrease induced by NH$_4^+$, as we demonstrated in our acidification test using NG medium and bacterial growth test using NB media. These results suggest that NH$_4^+$-induced acidification around the rice rhizosphere directly affects the bacterial growth and endophytic colonization by B510.
concentrations of NH$_4^+$ contribute to the inhibition of bacterial colonization. Plants maintain a neutral intracellular pH (pH 6–7) even when the rhizosphere is acidic (25, 32). However, in the present study, endophytic colonization by B510 was not observed when the rhizosphere was acidic. This result suggests that B510 resides in the intercellular spaces of host roots rather than intracellularly and, thus, bacterial infection is affected by extracellular pH changes. The optimal pH for the growth of B510 is pH 5–7, as is that of other Azospirillum sp. strains (Table S4; [12]).

Host-derived ROS are key factors controlling the bacterial infection of host plants. Endophytic bacteria possess ROS-scavenging enzymes to allow successful infection. Genome data shows that B510 has two superoxide dismutases (sod1: AZL_024870, sod2: AZL_014560) and a glutathione reductase (NADPH: AZL_04110). These two enzymes are essential for endophytic colonization by Gluconacetobacter diazotrophicus PAL5 (1). In the present study, rice plants constitutively produced the ROS, H$_2$O$_2$, at higher levels under high NH$_4^+$ concentrations (Fig. 3). This result implies that B510 overcomes transient and low-level ROS production by the host plant using these types of ROS-scavenging enzymes, but also that it cannot overcome constitutive and high-level ROS production by the host under high NH$_4^+$ concentrations. However, the accumulation of H$_2$O$_2$ was significantly decreased in rice roots grown with NH$_4$Cl and MES (Fig. 3), and endophytic colonization by B510 was not suppressed in these rice roots. These results suggest that high NH$_4^+$ concentrations inhibit colonization by B510 not only through acidification, but also with the accumulation of high levels of ROS production.

![Diagram](image.png)

**Fig. 7.** Proposed model for the suppression of bacterial colonization of rice roots by acidification with NH$_4^+$. Following the application of NH$_4^+$, rice plants induce a H$^+$ efflux to maintain the pH inside their cells. The consequent acidification of the rhizosphere supresses the secretion of chemotactic substrates, including malic acid, by the roots. As a result, the growth of bacteria, such as B510, is markedly suppressed. Moreover, acidification induces the production of ROS, including H$_2$O$_2$, by the plant roots. This suppresses colonization by endophytic bacteria, such as B510.
In the present study, we demonstrated that high concentrations of NH$_4^+$ inhibited the endophytic colonization of rice by B510. We propose a model to explain the mechanisms by which this phenomenon occurs (Fig. 7). In this model, root-mediated pH changes, caused by the application of NH$_4^+$, result in acidification around the rice rhizosphere. Low pH (<5.0) decreases root growth, increases H$_2$O$_2$ accumulation (45), and inhibits the secretion of chemotactic organic acids in root exudates, thereby suppressing the growth of and colonization by this bacterium. H$_2$O$_2$ production is reduced in plants grown in the presence of NH$_4^+$ and inoculated with B510 (Fig. 3). The biosynthesis of malic acid was increased in plants grown in the presence of NO$_3^-$ and inoculated with B510 (Fig. 5), and their root exudates exhibited enhanced chemotactic activity (Fig. 4). This result indicates additional benefits to those identified in previous studies, in which B510 enhanced plant growth and plant disease resistance (21, 42). These beneficial traits make B510 a superb source of biofertilizer.

Recent studies reported that rice plants fertilized with nitrogen contribute to environmental pollution (such as eutrophication) by emitting ammonia in Japanese rice paddies, which is caused by NH$_4^+$ (16, 17). In the present study, endophytic B510 cells were maintained at high numbers by controlling the pH of the rice rhizosphere using a buffer, and the roots also secreted more chemotactic compounds. Future studies will be directed towards the identification of the optimal combination of nitrogen fertilizers and B510 inoculation to promote the sustainable production of crops such as rice.

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References

1. Alqueres, S., C. Meneses, L. Rouws, M. Rothballer, I. Baldani, M. Schmid, and A. Hartmann. 2013. The Bacterial superoxide dismutase and glutathione reductase are crucial for endophytic colonization of rice roots by Gluconacetobacter diazotrophicus PAL5. Mol. Plant Microbe Interact. 26:937–945.
2. Bashan, Y., and L.E. de-Bashan. 2010. How the plant growth-promoting bacterium Azospirillum promotes plant growth—a critical assessment. Adv. Agron. 108:77–136.
3. Bédard, C., and R. Knowles. 1989. Physiology, biochemistry, and specific inhibitors of CH$_4$, NH$_4^+$, and CO oxidation by methanotrophs and nitrifiers. Microbiol. Rev. 53:68–84.
4. Böhm, M., T. Hurek, and B. Reinhold-Hurek. 2007. Twitching motility is essential for endophytic rice colonization by the N$_2$-fixing endophyte Azorarcus sp. strain BH12. Mol. Plant Microbe Interact. 20:526–533.
5. Carvalho, T.L.G., E. Balsemão-Pires, R.M. Saraiva, P.C.G. Ferreira, and A.S. Hemerly. 2014. Nitrogen signalling in plant interactions with associative and endophytic diazotrophic bacteria. J. Exp. Bot. 65:5631–5642.
6. Cassán, F., and M. Díaz-Zorita. 2016. Azospirillum sp in current agriculture: From the laboratory to the field. Soil Biol. Biochem. 102:117–130.
7. Chamam, A., H. Sanguin, F. Bellvert, G. Meirifen, G. Comte, F. Wisnieciski-Dyé, C. Bertrand, and C. Prigent-Combaret. 2013. Plant secondary metabolite profiling evidences strain-dependent effect in the Azospirillum-Oryza sativa association. Phytochemistry 87:65–77.
8. Comant, S., C. Clément, and A. Sessitsch. 2010. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biol. Biochem. 42:669–678.
9. Crawford, N.M., and B.G. Forde. 2002. Molecular and developmental biology of inorganic nitrogen nutrition. Arabidopsis Book. 1:e0011.
10. de Weert, S., H. Vermeiren, I.H. Mulders, I. Kuiper, N. Hendrickx, G.V. Bloemberg, J. Vanderleyden, R. De Mot, and B.J. Lugtenberg. 2002. Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by Pseudomonas fluorescens. Mol. Plant Microbe Interact. 15:1173–1180.
11. Donlan, R.M. 2002. Biofilms: Microbial Life on Surfaces. Emerg. Infect. Dis. 8:881–890.
12. Eckert, B., O.B. Weber, G. Kirchhof, A. Halbritter, M. Stoffels, and A. Hartmann. 2001. Azospirillum doebereinerae sp nov., a nitrofen-fixing bacterium associated with the C4-grass Miscantus. Int. J. Syst. Evol. Microbiol. 51:17–26.
13. Elbeltaygy, A., K. Nishioka, T. Sato, H. Suzuki, B. Ye, T. Hamada, T. Isawa, H. Mitsui, and K. Minamisawa. 2001. Endophytic colonization and in planta nitrogen fixation by a Herbaspirillum sp isolated from wild rice species. Appl. Environ. Microbiol. 67:5285–5293.
14. Fallik, E., and Y. Okon. 1996. Inoculants of Azospirillum brasilense: Biomass production, survival and growth promotion of Setaria italica and Zea mays. Soil Biol. Biochem. 28:123–126.
15. Fester, T., and G. Hause. 2005. Accumulation of reactive oxygen species in arbuscular mycorrhizal roots. Mycorrhiza 15:373–379.
16. Hayashi, K., S. Nishimura, and K. Yagi. 2006. Ammonia volatilization from the surface of a Japanese paddy field during rice cultivation. Soil Sci. Plant Nutr. 52:545–555.
17. Hayashi, K., S. Nishimura, and K. Yagi. 2008. Ammonia volatilization from a paddy field following applications of urea: Rice plants are both an absorber and an emitter for atmospheric ammonia. Sci. Total Environ. 390:485–494.
18. Hayashi, M., S. Shiro, H. Kanamori, et al. 2014. A thaumatin-like protein, Rj4, controls nodule symbiotic specificity in soybean. Plant Cell Physiol. 55:1679–1689.
19. Heulin, T., A. Guckert, and J. Balandreau. 1987. Stimulation of root exudation of rice seedlings by Azospirillum strains: carbon budget under gnotobiotic conditions. Biol. Fertil. Soils 4:9–14.
20. Hinsinger, P., C. Plassard, C. Tang, and B. Jaillard. 2003. Origins of root-mediated pH changes in the rhizosphere and their responses to environmental constraints: A review. Plant Soil 248:43–59.
21. Isawa, T., M. Yasuda, H. Awaizaki, K. Minamisawa, S. Shinozaki, and H. Nakashita. 2010. Azospirillum sp strain B510 enhances rice growth and yields. Microbes Environ. 25:58–61.
22. Janzen, R.A., S.B. Rood, J.F. Dormaar, and W.B. Megill. 1992. Azospirillum brasilense produces gibberellic in pure culture on chemically-defined medium and in co-culture on straw. Soil Biol. Biochem. 24:1061–1064.
23. Kaneko, T., K. Minamisawa, T. Isawa, et al. 2010. Complete genomic structure of the cultivated rice endophyte Azospirillum sp B510. DNA Res. 17:37–50.
24. Kirk, G.J.D. 2001. Plant-mediated processes to acquire nutrients: carbon uptake by rice plants. Plant Soil 232:117–134.
25. Kosegarten, H., F. Grolik, J. Wienceke, G. Wilson, and B. Hoffmann. 1997. Differential ammonia-elicted changes of cytosolic pH in root hair cells of rice and maize as monitored by 2,7’-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-fluorescence ratio. Plant Physiol. 113:451–461.
26. Kronzucker, H., M.Y. Siddiqi, A.D.M. Glass, and G.J.D. Kirk. 1999. Nitrate-ammonium synergism in rice. A subcellular flux analysis. Plant Physiol. 119:1041–1045.
Le Bot, J., D.J. Pilbeam, and E.A. Kirkby. 1994. Plant mineral nutrition in crop production, p. 33–72. In A.S. Basra (ed.), Mechanisms of Plant Growth and Improved Productivity, Marcel Dekker Inc., New York.

Lin, W., Y. Okon, and R.W.F. Hardy. 1983. Enhanced mineral uptake by zea-mays and sorghum-bicolor roots inoculated with *Azospirillum brasilense*. Appl. Environ. Microbiol. 45:1775–1779.

Liu, P.V., and H.C. Hsieh. 1969. Inhibition of protease production of various bacteria by ammonium salts: its effect on toxin production and virulence. J. Bacteriol. 99:406–413.

Meneses, C.H.S.G., L.F.M. Rouws, J.L. Simões-Araújo, M.S. Vidal, and J.I. Baldani. 2011. Exopolysaccharide production is required for biofilm formation and plant colonization by the nitrogen-fixing endophyte *Gluconacetobacter diazotrophicus*. Mol. Plant Microbe Interact. 24:1448–1458.

Murty, M.G., and J.K. Ladha. 1988. Influence of *Azospirillum* inoculation on the mineral uptake and growth of rice under hydroponic conditions. Plant Soil 108:281–285.

Pittman, J.K. 2012. Multiple transport pathways for mediating intracellular pH homeostasis: the contribution of H+/ion exchangers. Front. Plant Sci. 3:11.

Reinhold, B., T. Hurek, and I. Fendrik. 1985. Strain-specific chemotaxis of *Azospirillum* spp. J. Bacteriol. 162:190–195.

Reynders, L., and K. Vlassak. 1982. Use of *Azospirillum brasilense* as biofertilizer in intensive wheat cropping. Plant Soil 66:217–223.

Rudrappa, T., K.J. Czymmek, P.W. Parè, and H.P. Bais. 2008. Root-secreted malic acid recruits beneficial soil bacteria. Plant Physiol. 148:1547–1556.

Santoyo, G., G. Moreno-Hagelsieb, M.C. Orozco-Mosqueda, and B.R. Glick. 2016. Plant growth-promoting bacterial endophytes. Microbiol. Res. 183:92–99.

Sasaki, K., S. Ikeda, S. Eda, *et al.* 2010. Impact of plant genotype and nitrogen level on rice growth response to inoculation with *Azospirillum* sp. strain B510 under paddy field conditions. Soil Sci. Plant Nutr. 56:636–644.

Vessey, J. 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255:571–586.

Vrzheshch, P.V., N.A. Akovbian, S.D. Varfolomeyev, and V.V. Verkhusha. 2000. Denaturation and partial renaturation of a tightly tetramerized DsRed protein under mildly acidic conditions. FEBS Lett. 487:203–208.

Wilson, C., R. Lukowicz, S. Merchant, *et al.* 2017. Quantitative and qualitative assessment methods for biofilm growth: A mini-review. Res. Rev.: J. Eng. Technol. 6:1–25.

Wu, G., B.J. Shortt, E.B. Lawrence, E.B. Levine, K.C. Fitzsimmons, and D.M. Shah. 1995. Disease resistance conferred by expression of a gene encoding H$_2$O$_2$-generating glucose oxidase in transgenic potato plants. Plant Cell 7:1357–1368.

Yasuda, M., T. Isa, W. Shinozaki, K. Minamisawa, and H. Nakashita. 2009. Effects of colonization of a bacterial endophyte, *Azospirillum* sp B510, on disease resistance in rice. BioSci. Biotechnol. Biochem. 73:2595–2599.

Yasuda, M., H. Miwa, S. Masuda, Y. Takebayashi, H. Sakakibara, and S. Okazaki. 2016. Effector-triggered immunity determines host genotype-specific incompatibility in Legume-Rhizobium symbiosis. Plant Cell Physiol. 57:1791–1800.

Zachow, C., G. Jahanshah, I. de Bruin, *et al.* 2015. The novel lipopeptide poaeamide of the endophyte *Pseudomonas poae* RE star 1-1-14 is involved in pathogen suppression and root colonization. Mol. Plant Microbe Interact. 28:800–810.

Zhang, Y.K., D.F. Zhu, Y.P. Zhang, H.Z. Chen, J. Xiang, and X.Q. Lin. 2015. Low pH-induced changes of antioxidant enzyme and ATPase activities in the roots of rice (*Oryza sativa* L.) seedlings. PLoS One 10:e0116971.