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Root Colonization, Systemic Spreading and Contribution of *Herbaspirillum seropedicae* to Growth of Rice Seedling

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Abstract

To study the benefit and contribution of bacterial nitrogen fixation to grass plants growth we used a Nif$^+$ (LR15) and a Nif$^-$ (IM40) mutant strain of the diazotrophic bacterium *Herbaspirillum seropedicae* wild type SmR1. These two strains were inoculated in rice seedlings grown in an axenic system and the effects were compared to uninoculated plants, without or with addition of fixed nitrogen. The Nif$^+$ (*nifH::gusA*) mutant strain was also used to follow the expression of the *nifH* gene during the association of the diazotroph with rice. Our data show that *Herbaspirillum seropedicae* has the potential for biological nitrogen fixation on and inside gramineous plant tissues, although no difference in colonization and plant growth promotion was observed upon inoculation with the Nif$^+$ or Nif$^-$ strains.

Keywords: Biological nitrogen fixation (BNF), diazotrophs, *nifH*, GUS, rice, *Herbaspirillum seropedicae*

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1. Introduction

Nitrogen is a crucial element required for plant development. Shortage of nitrogen is often the main factor limiting plant growth in soils. Several gramineous plants have been described to be associated with nitrogen-fixing bacteria some of which grow endophytically inside roots and stems (James et al., 1994; Hurek et al., 1994). Therefore biological nitrogen fixation may contribute substantially to plant nutrition in gramineous systems.

The ability of diazotrophic bacteria to colonize the rhizosphere and to enter host grasses was described by Schloter and Hartmann (1998), Olivares et al. (1997), and Reinhold-Hurek and Hurek (1998) for the diazotrophic endophytes *Azospirillum brasilense* Sp245, *Herbaspirillum rubrisubalbicans* M4 and *Azoarcus* sp. BH72, respectively. However, the knowledge and understanding of plant-microbe interactions are limited, mainly due to difficulties in studying bacterial populations at root surfaces or enclosed in plant tissues. Since gramineous plants do not form nitrogen-fixing structures such as root nodules, it is crucial for the understanding of the mechanism of interactions to know the sites of bacterial colonization and expression of bacterial genes, especially structural genes of nitrogenase.

Herbaspirillum seropedicae is an endophytic nitrogen-fixing bacterium, microaerophilic and gram-negative, which can be found in tissues of several plants, including Poaceae, like rice, sorghum and sugarcane (Baldani et al., 1986; Olivares et al., 1996; Cruz et al., 2001). *H. seropedicae* does not survive well in soil and colonizes roots, stems and leaves of various gramineous plants without producing disease symptoms in the associated plants (Olivares et al., 1996).

H. seropedicae has been proposed to benefit plant growth (Boddey et al., 1995; Baldani et al., 2001). Research has focused on the question of whether species of *Herbaspirillum* really fix nitrogen within plants. There has been few indirect evidence of nitrogen fixation of *Herbaspirillum* in grass plants, such as acetylene reduction activity in inoculated sugarcane (James et al., 1994), reaction with nitrogenase antibody in sorghum (James et al., 1997) and rice (James et al., 2002; Gyaneshwar et al., 2002), and incorporation of $^{15}N_2$ into *Oryza officinalis* inoculated with *Herbaspirillum* sp. strain B501 (Elbeltagy et al., 2001).

In order to determine the relative contribution of bacterial nitrogen fixation for plant growth, we used *H. seropedicae* as a model endophytic organism. In this work, the expression of bacterial *nif* genes inside rice plants was studied using a transcriptional *nifH::gusA* fusion in *H. seropedicae* strain SmR1. We also evaluated the role of *H. seropedicae* in rice growth by correlating the bacteria inoculation with root dry mass, in the presence and absence of nitrogen fertilization.

2. Materials and Methods

Herbaspirillum seropedicae strains and construction of nifH::gusA fusion

The bacterial strains used in this study are listed in Table 1. NFb medium was used for growing *H. seropedicae* (30°C) (Döbereiner et al., 1995). The *nifH::gusA* mutant was obtained by insertion of a *gusA*-kanamycin cassette into the *nifH* gene of *H. seropedicae* strain SmR1, which is a spontaneous streptomycin-resistant mutant (Souza et al., 2000) derivative of the wild type strain Z78 (Baldani et al., 1986). The construction *nifH::gusA* was integrated into the chromosome of *H. seropedicae* strain SmR1 by homologous recombination after electroporation (Klassen et al., 1999), resulting in the mutant *H. seropedicae* strain LR15. The *gusA* insertion into the chromosome was confirmed by DNA hybridization analysis, as the product of a single crossover event. Thus, the LR15 strain showed a Nif^+ phenotype.

Germination, inoculation and growth of seedlings

Surface sterilization of *Oryza sativa* seeds was carried out as described previously (Döbereiner et al., 1995). The sterile seedlings were transferred to glass tubes containing 4 g of sterilized vermiculite and 3 ml of plant medium (pH 6.8 with 0.2 mM or 4 mM of NH_4NO_3, without an added carbon source) (Egener et al., 1999). The tubes were inoculated with 10^7 bacterial cells per g of vermiculite. Plants were grown in a greenhouse with 16 hours of light per day, at 28°C. Plants were harvested at 5, 10 and 30 days post inoculation (dpi) for scanning electron microscopy (SEM), histochemical observations and plant growth measurements, respectively.

Plant parameter measurements, experimental design and statistical analysis

The dry mass of rice roots was quantified 30 dpi in randomized plant growth experiments conducted with five replicates. Each replicate consisted of three plants. The dry weights of rice roots are given as the average of total weight per replicate. Significance is given by $P<0.01$. The most probable number (MPN) was used to count bacterial cells (Döbereiner et al., 1995).

Preparation of plants for histochemical analysis

For histochemical detection of GUS activity, the roots and leaves were incubated for 2 h in 50 mM sodium cacodylate buffer (pH 7.5) with 0.5 mg/ml 5bromo-4chloro-3indolyl β-D-glucuronide (X-gluc) at 45°C (Hurek et al., 1994).

Table 1. Characteristics of *Herbaspirillum seropedicae* strains used in this study.

Characteristic	SmR1 wild type	LR15 mutant	IM40 mutant
Acetylene reduction in semi-solid culture (nmol C_2H_4/min/mg protein)	3.34 ± 1.31	5.26 ± 1.34	nd
Antibiotic resistance*	Sm^R	Sm^R; Km^R	Sm^R; Km^R
Nitrogenase structural genes	*nifHDK* present	*nifHDK* present	*nifH* disrupted
Reporter fusion	–	*nifH::gusA*	*nifH::lacZ*
Reference	Souza et al. (2000)	This study	Machado et al. (1999)

*(Sm^R) streptomycin-resistant; (Km^R) kanamycin-resistant; (nd) not detected.

SEM (scanning electron microscopy)

For scanning electron microscopy, samples of inoculated plant roots were fixed with 0.25% (v/v) glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 30 min. The fixed material was dehydrated in a graded ethanol series, dried in a critical point dryer (CPD010, Balzers Union, FL, USA) in a CO_2 atmosphere, affixed to aluminum stubs with silver paste, and finally coated with ionized gold film (SCD030 Balzers Union, FL, USA) (Nowell and Parules, 1980). The samples were examined in a Phillips SEM 505.

3. Results and Discussion

Rice root surface, endophytic colonization and expression of nifH gene of H. seropedicae

The root surface colonization by *H. seropedicae* was examined by SEM. Results indicated that bacteria were attached and distributed uniformly or in cellular clumps at the surface of epidermal cells of rice roots (Figs. 1A and 1B). The identity of the diazotroph bacterial cells was confirmed by immunostaining using rabbit raised polyclonal antibodies against whole cells of *H. seropedicae* (data not shown).

The interaction between gramineous plants and the *H. seropedicae* mutant LR15 was studied with whole plant samples at different time intervals after inoculation and by comparison with uninoculated plants. Rice roots were intensely colonized, as shown by Toluidine blue and immunostaining. The systemic occupation was observed in the roots and stems cortical layers at intercellular spaces and in the vascular tissues (Figs. 1C and 1D).

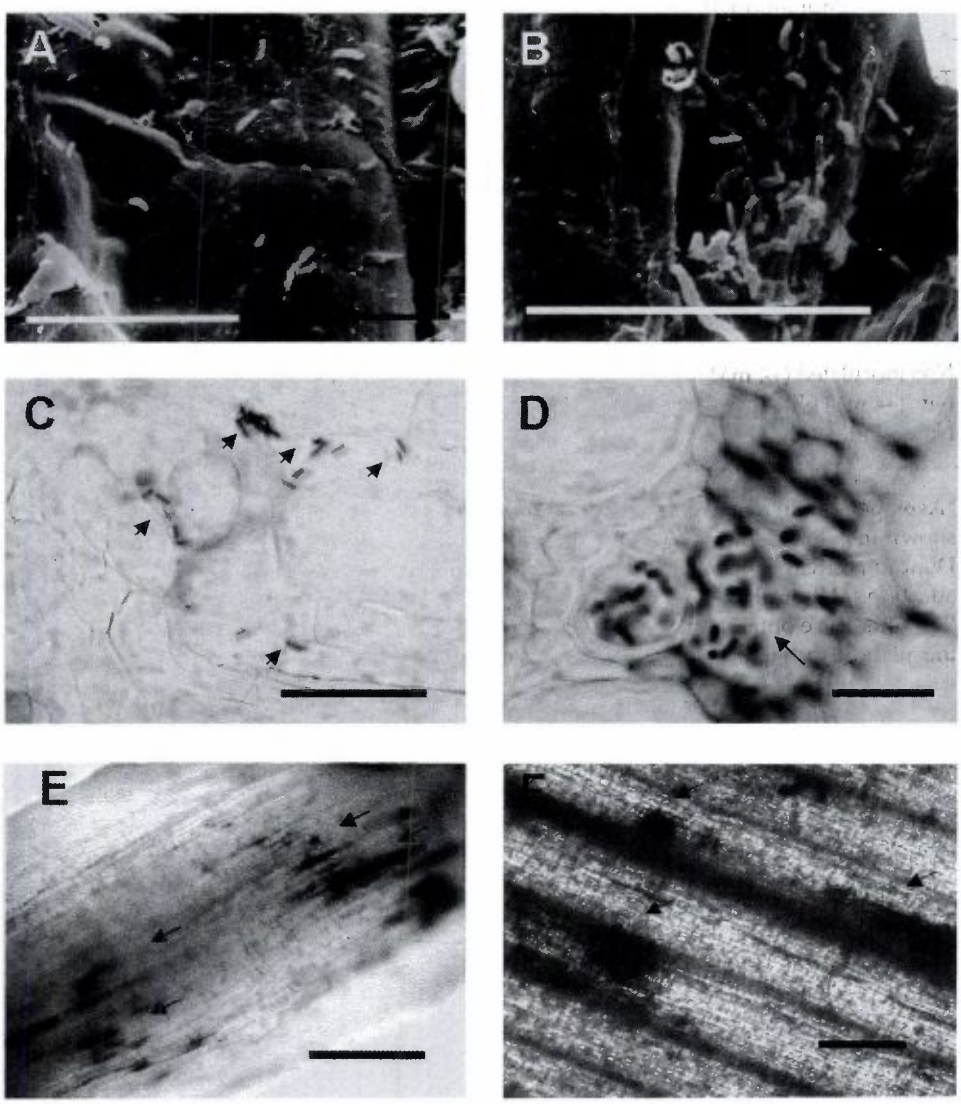

Figure 1. Localization of *H. seropedicae* strain LR15 in rice roots. (A) Scanning electron micrograph of surface of rice root, 5 days after inoculation. (B) Bacterial clumps at rice root epidermis, 10 days after inoculation. (C) Bacterial cells localized at intercellular spaces at secondary root emergence point (arrows) of rice. (D) Bacterial occupation of stem xylem vessel (arrow). (E and F) Expression *nifH::gusA* by *H. seropedicae* on rice roots and leaves, respectively, 10 days after inoculation. C and D, Toluidine blue staining. E and F, β-glucuronidase activity. Bars represent 10 μm (A–D) and 50 μm (E and F).

Table 2. Root dry biomass of rice inoculated with *Herbaspirillum seropedicae* Nif$^+$ mutant (LR15) and Nif$^-$ mutant (IM40) strains.

Treatment	Dry weight of roots (mg)*	MPN**
Non inoculated (–N)	55.0 ± 7.4 bc	nd
Nif$^+$ (–N)	72.5 ± 0.6 a	1.8×10^5
Nif$^-$ (–N)	68.8 ± 8.8 a	5.0×10^5
Non inoculated (+0.2 mM N)	43.7 ± 9.4 c	nd
Nif$^+$(+0.2 mM N)	67.7 ± 9.4 a	3.0×10^4
Nif$^-$ (+0.2 mM N)	66.8 ± 12.6 ab	4.0×10^4
Non inoculated (+4 mM N)	51.0 ± 8.1 c	nd
Nif$^+$ (+4 mM N)	55.0 ± 7.6 bc	1.9×10^4
Nif$^-$ (+4 mM N)	45.3 ± 13.7 c	0.8×10^4

*Average of total dry weight of rice roots per replicate (three plants/vase) is given. Values shown in column followed by the same letter are not significantly different. Analyzed by Duncan method, $P<0.01$. Measurements were 30 days after inoculation, without nitrogen addition (–N), in the presence of 0.2 mM (+0.2 mM N) or 4 mM (+4 mM N) of NH_4NO_3. **Counts were obtained using MPN estimation in semi-solid NFb medium, 20 days after inoculation. (nd) not detected by reporter enzyme activity assay.

Similar to *Azoarcus* sp. BH72 (Egener et al., 1999), *H. seropedicae* attached to rice root expressed *nifH*::*gusA*, often in the emerging zones of secondary roots (Fig. 1E). The blue color of GUS staining was also detected in the leaves of rice (Fig. 1F). Expression of nitrogenase by *H. seropedicae* was also shown in leaves and xylem of sorghum (James et al., 1997). Internally, the expression of *nifH*::*gusA* was observed mainly in the vascular system of stems and leaves (not shown).

Effect of H. seropedicae inoculation on rice

The present study evaluated the contribution of N_2-fixation to the growth of rice seedlings. We measured the root and shoot mass of greenhouse-grown plants 30 dpi, and compared this parameter with plants inoculated with the Nif$^-$ *H. seropedicae* strain IM40 (Machado et al., 1999). The Nif$^-$ strain has a Km::*lacZ* cassette insertion in *nifH* gene and is unable to fix N_2 in culture (Machado et al., 1999) (Table 1).

Under N-limiting conditions, root dry weights were significantly greater in rice plants inoculated with *H. seropedicae* than uninoculated plants (Table 2).

An equivalent increase was observed when plants were inoculated with either the Nif$^+$ (LR15) or the Nif$^-$ strain (IM40), indicating similarities in plant response to both strains. These results suggest that the biological nitrogen fixation is not the only factor involved in the contribution to growth of rice plants by *H. seropedicae*.

Similar results have also been observed with other bacteria associated with grasses, such as *Azospirillum brasilense* and *Azoarcus* sp., in which Nif$^-$ mutant strain had wild type ability to enhance plant growth (Bashan and Levanony, 1989; Hurek et al., 1994). In contrast, inoculation of Nif$^-$ mutants of *Gluconacetobacter diazotrophicus* did not improve growth over that of uninoculated plants, under N-limiting conditions (Sevilla et al., 2001).

When no N was added, plants inoculated with *H. seropedicae* showed an increase in root dry mass up to 30% compared to uninoculated plants. This increase was up to 50% if low N (0.2 mM) was added. However, in the presence of excess N, plants inoculated with Nif$^+$ or Nif$^-$ strains or uninoculated plants had similar root dry masses (Table 2). These plants were also shorter than plants that received low N addition (data not shown). Presumably, *H. seropedicae* improved the plant ability to utilize the available resources.

There were no differences in the ability of the *H. seropedicae* Nif$^+$ and Nif$^-$ to colonize rice plants. Bacterial cell counting by MPN of Nif$^+$ and for the Nif$^-$ mutant strain showed the same size of population in inoculated rice plants (Table 2). The similarity of population size suggests that nitrogen fixation is not essential for endophytic colonization of *H. seropedicae*. This observation agreed with the results of Sevilla et al. (2001) where no difference in colonization of sugarcane was found between the *G. diazotrophicus* wild type and a Nif$^-$ mutant strain.

Muthukumarasamy et al. (1999) and Reis Junior et al. (2000) showed that the population of *Herbaspirillum* spp. in sugarcane did not decrease due to N-fertilization. These reports agreed with our results, indicating that N-fertilization does not affect the bacterial population size. However, Muthukumarasamy et al. (1999) suggest that the physiological state of the plant is altered by nitrogen, which subsequently affects the benefits of the associated diazotrophic microorganisms. Vose et al. (1981) demonstrated that high levels of mineral N caused a significant decrease in the acetylene reduction activity (ARA). This effect is probably due to the inhibition of nitrogenase synthesis. Qualitative changes in the associations may apparently reduce the benefit of the endophyte to the plant. Our results show that addition of high concentration of N abolished β-glucuronidase activity in roots of rice inoculated with LR15, and that inoculation of plants grown under N excess did not improve growth. Since the Nif$^-$ strain had a positive effect on plant growth under N-limiting conditions, additional factors, such as phytohormones, produced by *H. seropedicae* are also important contributors to

the improvement of plant growth. These additional factors either are produced by the bacteria or have positive effect only under N limitation.

Shoot mass and seedling height were also measured but consistent significant differences between inoculated and uninoculated plants were not observed 30 dpi (data not shown). *H. seropedicae* was reisolated from roots, stems and leaves of inoculated plants, and it was not isolated from uninoculated control plants.

In summary, the results of this study show that *H. seropedicae* LR15 was able to colonize the root surfaces and inner tissues of rice plants. The inoculation of *H. seropedicae* enhanced root growth of the rice in greenhouse conditions. Although the mechanism of stimulation of rice growth by *H. seropedicae* has not been identified, the results presented here suggest that the contribution of *H. seropedicae* to growth of grass seedling is not due only to the N_2-fixation process, although the bacteria express *nifH* gene in association with rice, suggesting that rice tissues are suitable for active expression of *H. seropedicae* *nif* genes. Additionally, only a fraction of the colonizing bacteria within the same region showed production of the GUS stain. Since an axenic system was used and the effect of inoculation was evaluated in not fully developed plants, future studies are necessary to establish the conditions under which *nif* genes are expressed *in planta* and to identify additional factors promoting efficient vegetal growth.

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REFERENCES

Baldani, J.I., Baldani, V.L.D., Seldin, L., and Döbereiner, J. 1986. Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a root-associated nitrogen fixing bacterium. *International Journal of Systematic Bacteriology* 36: 86–93.

Baldani, V.L.D., Baldani, J.I., and Döbereiner, J. 2001. Inoculation of rice plants with the endophytic diazotrophs *Herbaspirillum seropedicae* and *Burkholderia* spp. *Biology and Fertility of Soils* 30: 485–491.

Bashan, S.M. and Levanony, H. 1989. Contribution of *Azospirillum brasilense* Cd to growth of tomato seedlings is not through nitrogen fixation. *Canadian Journal of Botany* **62**: 2429–2434.

Boddey, R.M., Oliveira, O.C., Urquiaga, S., Reis, V.M., Olivares, F.L., Baldani, V.L.D., and Döbereiner, J. 1995. Biological nitrogen fixation associated with sugar cane and rice: contributions and prospects for improvement. *Plant and Soil* **174**: 195–209.

Cruz, L.M., Souza, E.M., Weber, O.B., Baldani, J.I., Döbereiner, J., and Pedrosa, F.O. 2001. 16S Ribosomal DNA characterization of nitrogen-fixing bacteria isolated from banana (*Musa* spp.) and pineapple (*Ananas comosus* (L.) Merril). *Applied and Environmental Microbiology* **67**: 2375–2379.

Döbereiner, J., Baldani, V.L.D., and Baldani, J.I. 1995. Como isolar e identificar bactérias diazotróficas de plantas não-leguminosas. EMBRAPA, Brasilia. 60 p.

Egener, T., Hurek, T., and Reinhold-Hurek, B. 1999. Endophytic expression of *nif* genes of *Azoarcus* sp. strain BH72 in rice roots. *Molecular Plant-Microbe Interactions* **12**: 813–819.

Elbeltagy, A., Nishioki, K., Sato, T., Suzuki, H., Ye, B., Hamada, T., Isawa, T., Mitsui, H., and Minamisawa, K. 2001. Endophytic colonization and *in planta* nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. *Applied and Environmental Microbiology* **67**: 5285–5293.

Gyaneshwar, P., James, E.K., Reddy, P.M., Reinhold-Hurek, B., and Ladha, J.K. 2002. *Herbaspirillum* colonization increases growth and nitrogen accumulation in aluminum tolerant rice varieties. *New Phytologist* **154**: 131–146.

Hurel, T., Reinhold-Hurek, B., Van Montagu, M., and Kellenberg, E. 1994. Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. *Journal of Bacteriology* **176**: 1913–1923.

James, E.K., Gyaneswar, P., Mathan, N., Barraquio, W.L., Reddy, P.M., Iannetta, P.P.M., Olivares, F.L., and Ladha, J.K. 2002. Infection and colonization of rice seedlings by the plant growth-promoting bacterium *Herbaspirillum seropedicae* Z67. *Molecular Plant-Microbe Interactions* **15**: 894–906.

James, E.K., Reis, V.M., Olivares, F.L., Baldani, J.I., and Döbereiner, J. 1994. Infection and colonization of sugarcane by nitrogen-fixing bacterium *Acetobacter diazotrophicus*. *Journal of Experimental Botany* **45**: 757–766.

James, E.K., Olivares, F.L., Baldani, J.I., and Döbereiner, J. 1997. *Herbaspirillum*, an endophytic diazotroph colonizing vascular tissue in leaves of *Sorghum bicolor* L. Moench. *Journal of Experimental Botany* **48**: 785–797.

Klassen, G., Pedrosa, F.O., Souza, E.M., Yates, M.G., and Rigo, L.U. 1999. Sequence and functional analysis of *nifENXorf1orf2* operon from *Herbaspirillum seropedicae*. *FEMS Microbiology Letters* **181**: 165–170.

Machado, I.M., Yates, M.G., Machado, H.G., Souza, E.M., and Pedrosa, F.O. 1999. Cloning and sequencing of nitrogenase structural genes *nifHDK* of *Herbaspirillum seropedicae*. *Brazilian Journal of Medical and Biological Research* **29**: 1599–1602.

Muthukumarasamy, R., Revathi, G., and Lakshminarasimhan, C. 1999. Influence of N fertilization on the isolation of *Acetobacter diazotrophicus* and *Herbaspirillum* spp. from indian sugarcane varieties. *Biology and Fertility of Soils* **26**: 157–164.

Nowell, J.A. and Parules, J.B., eds. 1980. *Preparation of Experimental Tissue for Scanning Electron Microscopy*. v. 2. AMSO-HARE, Chicago, IL, USA.

Olivares, F.L., James, E.K., Baldani, J.I., and Döbereiner, J. 1997. Infection of mottled stripe disease-susceptible and resistant sugar cane varieties by the endophytic diazotroph *Herbaspirillum*. *New Phytologist* **135**: 723–737.

Olivares, F.L., Baldani, V.L.D., Reis, V.M., Baldani, J.I., and Döbereiner, J. 1996. Occurrence of endophytic diazotrophs *Herbaspirillum* spp. in roots, stems and leaves predominantly of Gramineae. *Biology and Fertility of Soils* **21**: 197–200.

Reinhold-Hurek, B. and Hurek, T. 1998. Life in grasses: diazotrophic endophytes. *Trends in Microbiology* **6**: 139–144.

Reis Junior, F.B., Reis, V.M., Urquiaga, S., and Döbereiner, J. 2000. Influence of nitrogen fertilization on the population of diazotrophic bacteria *Herbaspirillum* spp. and *Acetobacter diazotrophicus* in sugarcane (*Saccharum* spp.). *Plant and Soil* **219**: 153–159.

Schloter, M. and Hartmann, A. 1998. Endophytic and surface colonization of wheat roots (*Triticum aestivum*) by different *Azospirillum brasilense* strains studied with strain-specific monoclonal antibodies. *Symbiosis* **25**: 159–180.

Sevilla, M., Gunapala, N., Burris, R.H., and Kennedy, C. 2001. Enhancement of growth and N content in sugarcane plants inoculated with *Acetobacter diazotrophicus*. *Molecular Plant-Microbe Interactions* **14**: 358–366.

Souza, E.M., Pedrosa, F.O., Rigo, L.U., Machado, H.B., and Yates, M.G. 2000. Expression of the *nifA* gene of *Herbaspirillum seropedicae*: role of the NtrC and NifA binding sites and of the -24/-12 promoter element. *Microbiology* **146**: 1407–1418.

Vose, P.B., Ruschel, A.P., Victoria, R.L., and Matsui, E. 1981. Potential N_2-fixation by sugar cane, *Saccharum* sp. in solution culture. I. Effect of NH_4^+vs. NO_3^-, variety and nitrogen level. In: *Associative N_2-Fixation*. vol II, P.B. Vose and A. P. Ruschel, eds. CRC Press, Boca Raton, pp. 119–123.