BRAZILIAN JOURNAL
OF MEDICAL AND BIOLOGICAL RESEARCH
www.bjournal.com.br

ISSN 0100-879X
Volume 43 (3) 182-267 March 2011

BIOMEDICAL SCIENCES
AND
CLINICAL INVESTIGATION

Braz J Med Biol Res, March 2011, Volume 44(3) 182-185

doi: 10.1590/S0100-879X2011007500004

Evidence for the endophytic colonization of *Phaseolus vulgaris* (common bean) roots by the diazotroph Herbaspirillum seropedicae

M.A. Schmidt, E.M. Souza, V. Baura, R. Wassem, M.G. Yates, F.O. Pedrosa and R.A. Monteiro

Brazilian Journal of Medical and Biological Research (2011) 44: 182-185
ISSN 0100-879X

Evidence for the endophytic colonization of *Phaseolus vulgaris* (common bean) roots by the diazotroph *Herbaspirillum seropedicae*

M.A. Schmidt[1], E.M. Souza[1], V. Baura[1], R. Wassem[2], M.G. Yates[1], F.O. Pedrosa[1] and R.A. Monteiro[1]

[1]Departamento de Bioquímica e Biologia Molecular, [2]Departamento de Genética, Universidade Federal do Paraná, Curitiba, PR, Brasil

Abstract

Herbaspirillum seropedicae is an endophytic diazotrophic bacterium, which associates with important agricultural plants. In the present study, we have investigated the attachment to and internal colonization of *Phaseolus vulgaris* roots by the *H. seropedicae* wild-type strain SMR1 and by a strain of *H. seropedicae* expressing a red fluorescent protein (DsRed) to track the bacterium in the plant tissues. Two-day-old *P. vulga*ris roots were incubated at 30°C for 15 min with 6 x 10^8 CFU/mL *H. seropedicae* SMR1 or RAM4. Three days after inoculation, 4 x 10^4 cells of endophytic *H. seropedicae* SMR1 were recovered per gram of fresh root, and 9 days after inoculation the number of endophytes increased to 4 x 10^6 CFU/g. The identity of the recovered bacteria was confirmed by amplification and sequencing of the 16SrRNA gene. Furthermore, confocal microscopy of *P. vulgaris* roots inoculated with *H. seropedicae* RAM4 showed that the bacterial cells were attached to the root surface 15 min after inoculation; fluorescent bacteria were visible in the internal tissues after 24 h and were found in the central cylinder after 72 h, showing that *H. seropedicae* RAM4 is capable of colonizing the roots of the dicotyledon *P. vulgaris.* Determination of dry weight of common bean inoculated with *H. seropedicae* SMR1 suggested that this bacterium has a negative effect on the growth of *P. vulgaris.*

Key words: *Herbaspirillum seropedicae*; *Phaseolus vulgaris*; Confocal microscopy

Introduction

Herbaspirillum seropedicae is a diazotrophic endophyte of the β-proteobacteria (1), which colonizes internal tissues of maize, rice, wheat, sorghum, and sugar cane (2,3). This organism has also been isolated from plants other than Poaceae such as banana and pineapple (4,5). *H. seropedicae* may stimulate plant growth by supplying fixed nitrogen to the plant, producing and secreting phytohormones or protecting the host against pathogenic microorganisms (6,7). The bacterial association with poaceous crops apparently initiates with attachment to root surfaces followed by proliferation at the emergence points of secondary roots and penetration through discontinuities in the epidermis. Rapid occupation of root intercellular spaces then occurs, and the bacteria spread to aerenchyma, xylem vessels and aerial portions (8-10).

Olivares et al. (11) described the isolation of *H. seropedicae* from gramineae and also from roots of a legume species (*Cajanus cajan*). However, the authors suspected that fragments of maize roots might have contaminated the legume sample. Later, Valverde et al. (12) isolated a new species of this genus from the nodules of *Phaseolus vulgaris*. Based on genotypic and phenotypic characterization, the new isolates were classified as a novel species for which the authors proposed the name *H. lusitanum* sp nov.

The aim of the present study was to determine if *H. seropedicae* is able to establish an endophytic relationship with *P. vulgaris.*

Material and Methods

H. seropedicae SMR1 is a spontaneous streptomycin-resistant derivative of the wild-type strain Z78 (13). RAM4 is a strain tagged with the *dsred* gene, which expresses the red fluorescent protein, to allow the monitoring of single bacterial cells in *P. vulgaris* roots (10). Seeds of *P. vulgaris* (cv. Uirapuru) were surface-sterilized with 70% ethanol (J.T. Baker, Mexico) for 5 min and shaken in a 2% sodium hypochlorite (Bond

Correspondence: R.A. Monteiro, Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, 81531-990 Curitiba, PR, Brasil. E-mail: roseadele@ufpr.br

Received June 16, 2010. Accepted December 15, 2010. Available online January 14, 2011. Published March 7, 2011.

Carneiro, Brazil) containing 0.02% Tween-20 (United States Biochemical, USA) solution for 20 min at 30°C. Seeds were then washed five times with sterile distilled water by shaking for 15 min each time and germinated at 25°C in the dark for 48 h. *P. vulgaris* roots were then incubated at 30°C for 15 min with 6 x 10^8 colony forming units (CFU)/mL SMR1 (wild-type) or RAM4 (*dsred*) grown in NFbHPN medium (14). After incubation, the plantlets were placed in wells of a 96-well block containing filter paper soaked with plant medium (15) and incubated for a 14-h light period at 25°C.

The roots of 3-day-old plantlets of *P. vulgaris* were analyzed at 0, 3, 7, and 9 days after inoculation with 6 x 10^8 CFU/mL *H. seropedicae* strain SMR1. After these intervals, approximately 0.05 g fresh roots was surface-sterilized by sequentially washing with 70% ethanol (1 min), 1% sodium hypochlorite containing 0.01% Tween-20 (1 min) and three times with sterile water (5 min). The roots were homogenized using a sterile pestle and mortar, and the extracts diluted in 1 mL sterile saline (0.9% NaCl). The diluted extracts were plated onto solid NFbHPN medium in the absence or presence of 80 µg/mL streptomycin (Sigma, USA). The number of CFU was determined after 24-48 h of incubation at 30°C. The identity of the recovered bacteria was determined by amplification and sequencing of the 16SrRNA gene (16).

The colonization pattern of *P. vulgaris* roots by *H. seropedicae* was observed by inoculating *P. vulgaris* roots with the RAM4 strain followed by confocal microscopy. Root samples were collected 1, 2 or 3 days after inoculation, washed with water, hand cut, mounted on a microscope slide and immediately examined under a BioRad Confocal Radiance 2001-Eclipse E800 Nikon Microscope (USA) equipped with an HeNe laser (DsRed: excitation, 543 nm; emission filter LP, 560 nm).

We measured the dry weight of inoculated plants grown in medium containing 0, 0.2, and 4 mM ammonium nitrate (Merck, Germany) and compared it to that of non-inoculated plants grown in the same medium. The bean seeds were sterilized, germinated and inoculated as described above. Ten days after inoculation the roots were dried and the weight was determined.

Results

The results indicated that *H. seropedicae* colonized *P. vulgaris* roots progressively from the first to the 9th day after inoculation. The number of bacteria recovered from surface sterilized roots increased from 4 x 10^4 per gram fresh root 3 days after inoculation to 4 x 10^6 bacteria 9 days after inoculation (Figure 1). To confirm that the recovered bacteria were indeed *H. seropedicae*, the 16SrRNA gene of randomly selected colonies was amplified and sequenced. All sequences were 100% identical to those of *H. seropedicae* SMR1.

Under the confocal microscope *H. seropedicae* expressing the DsRed protein showed bright red fluorescence, easily distinguishable from the diffuse fluorescence background of the contour of the plant cells. After 15 min of incubation with the RAM4 strain, cell clusters were found on the root hairs (Figure 2). These are probable entry sites used by the bacteria, possibly due to a higher concentration of carbon sources at these points (17).

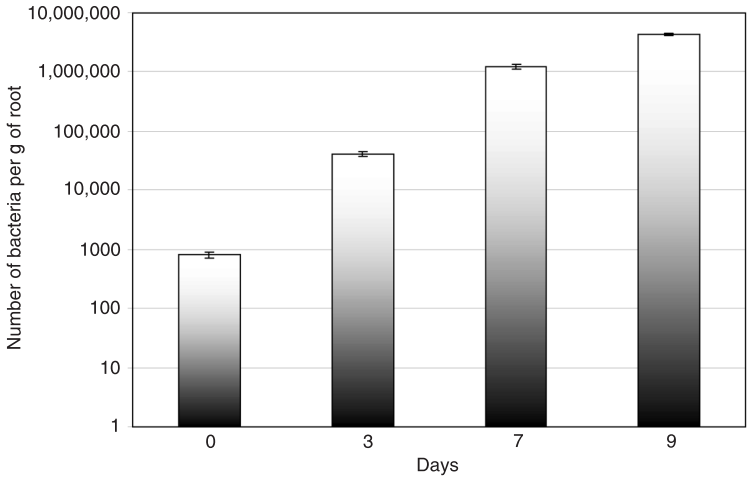

Figure 1. Internal colonization of *Phaseolus vulgaris* roots by *Herbaspirillum seropedicae*. The extent of internal colonization was determined 15 min after inoculation (day 0) and on days 3, 7, and 9. Data are reported as CFU/g (mean ± SD) fresh roots from 3 plants per treatment.

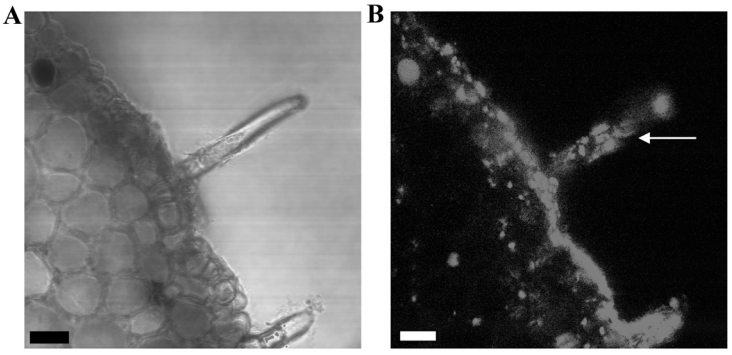

Figure 2. Photomicrographs of a cross-section of *Phaseolus vulgaris* root 15 min (A, B) after inoculation with *Herbaspirillum seropedicae* strain RAM4. *Left*, Transmitted light microscopy. *Right*, Confocal fluorescence microscopy. Arrow shows bacteria accumulating in the lateral root. Magnification bars: 15 µm.

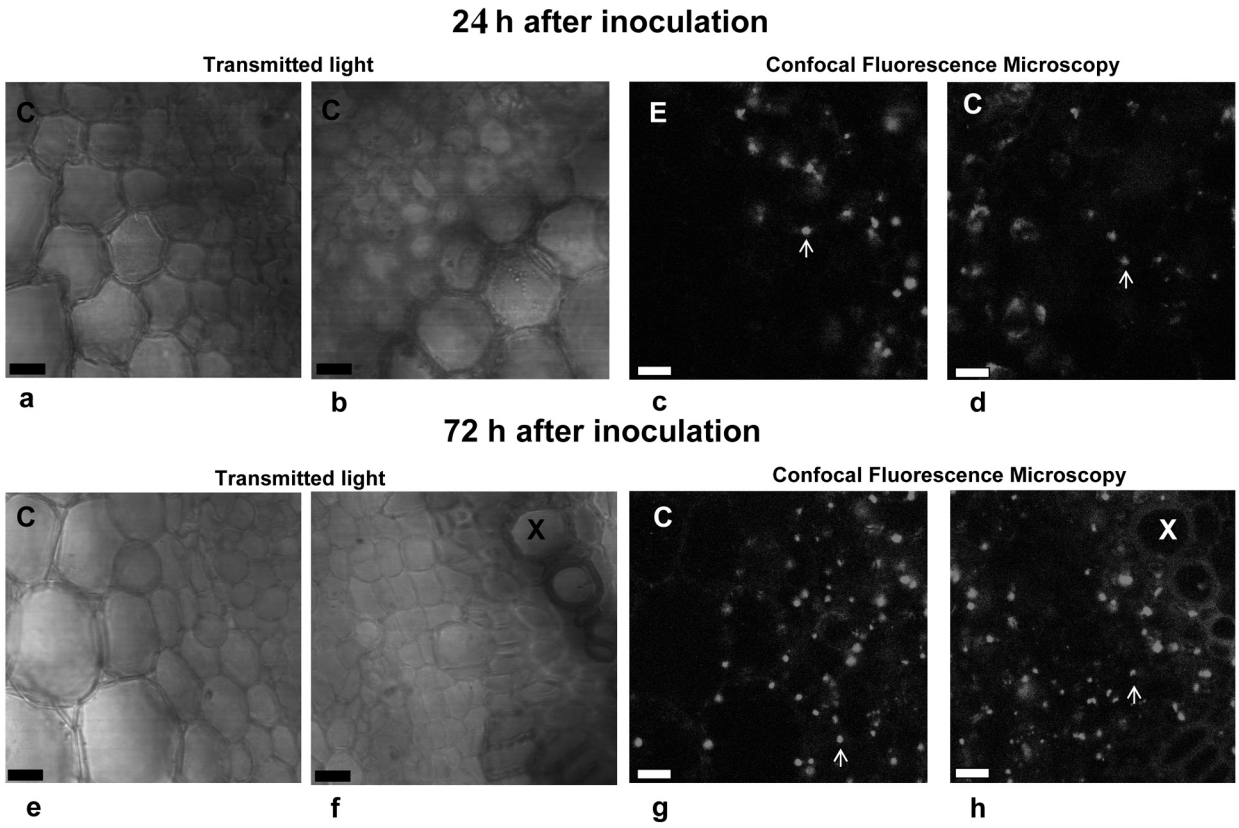

Figure 3. Transmitted light and confocal fluorescence microscopy of a cross-section of *Phaseolus vulgaris* root 24 (a-d) or 72 h (e-h) after inoculation with *Herbaspirillum seropedicae* strain RAM4. The images were recorded in two different regions of the cross-section, i.e., the epidermis (a, c, e, and g) and the central cylinder (b, d, f, and h). Transmitted light microscopy (a, b, e, and f); confocal fluorescence microscopy (c, d, g, and h). C = cortex; E = epidermis; X = xylem vessels. Arrows indicate the fluorescent bacteria. Magnification bars = 50 µm.

Confocal microscopy images of different regions of a cross-section of *P. vulgaris* roots inoculated with RAM4 showed that the bacteria had started to invade the root internal tissues 24 h after inoculation. After 72 h the number of bacterial cells increased progressively from the epidermis to the central cylinder (Figure 3).

To investigate the effect of *H. seropedicae* on *P. vulgaris* growth, plantlets were incubated for 15 min with 1 mL of a fresh culture containing 10^8 CFU, and transplanted to vermiculite in the presence of different NH_4NO_3 concentrations. After 10 days, the dry weight of the inoculated plants was significantly lower than that of the non-inoculated plants under all conditions (Table 1), suggesting that *H. seropedicae* has a negative effect on the growth of *P. vulgaris* (cv. Uirapuru).

Table 1. Dry weight of *Phaseolus vulgaris* roots inoculated or not with *Herbaspirillum seropedicae* SMR1.

NH_4NO_3 (mM)	Dry weight (g)	
	Inoculated	Uninoculated
0.0	11.8 ± 2.5	28.2 ± 7.7*
0.2	31.8 ± 1.2	41.6 ± 4.7*
4.0	35.6 ± 9.5	58.2 ± 2.6*

Data are reported as means ± SD for 3 plants per treatment. Plants were grown without nitrogen or in the presence of 0.2 or 4 mM NH_4NO_3 and analyzed 10 days after inoculation. The total dry weight of bean roots inoculated or not with *H. seropedicae* SMR1 is shown. *P < 0.05 compared to inoculated (Student *t*-test).

Discussion

The results showed that *H. seropedicae* is able to adhere to and colonize *P. vulgaris* roots internally. *H. lusitanum* was also recovered from bean roots 2 weeks after inoculation (12).

The *H. seropedicae* colonization process on and in *P. vulgaris* roots appears to occur in a pattern similar to that

of Poaceae (9,10): the bacteria invade the intercellular spaces and disperse in the cortex, eventually reaching the xylem vessels. A notable difference was a lower number of bacterial cells visualized at the time tested, when compared with that of maize (10).

We also evaluated the effect of *H. seropedicae* inoculation on the growth of common bean seedlings (Table 1). The result showed that *H. seropedicae* has a negative effect on *Phaseolus* growth, since the root dry weights of plants inoculated with *H. seropedicae* were lower than those of uninoculated plants. This is in contrast to the growth-promoting effect of *H. seropedicae* on rice plants (9), indicating that *H. seropedicae* has different effects on different plants. A strong interaction between the plant genotype and the rhizobacteria inoculated has been documented, with effects ranging from variable growth promotion (18,19) to a slight reduction in yield (20,21). The negative effect of *H. seropedicae* on the growth of *Phaseolus* may be due to interaction between the plant and bacterial molecular factors such as those secreted by the type three secretion system (T3SS) of *H. seropedicae*. In *Rhizobium* NGR234, the inactivation of T3SS leads to an increase in the number of root nodules in *P. vulgaris* cv. BAT93, suggesting that the secretion of certain effector proteins may negatively affect the interaction with *Rhizobium* NGR234 (Lariguet P, unpublished results).

The present results show that *H. seropedicae* is not an exclusive endophyte of gramineous plants, but is capable of colonizing other plant types such as the common bean, indicating that *H. seropedicae* is a broad host-range endophyte. Whether this association benefits the development of common beans under specific conditions is yet to be established.

References

1. Baldani JI, Baldani VLD, Seldin L, Döbereiner J. Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a new root-associated nitrogen-fixing bacterium. *Int J Syst Bacteriol* 1986; 36: 86-93.
2. Baldani JI, Baldani VLD, Sampaio MJAM, Döbereiner J. A fourth *Azospirillum* species from cereal roots. *An Acad Bras Cienc* 1984; 56: 365.
3. Boddey RM, Oliveira OC, Urquiaga S, Reis VM, Olivares FL, Baldani VLD, et al. Biological nitrogen fixation associated with sugar cane and rice: contributions and prospects for improvement. *Plant Soil* 1995; 174: 195-209.
4. Cruz LM, Souza EM, Weber OB, Baldani JI, Döbereiner J, Pedrosa FO. 16S ribosomal DNA characterization of nitrogen-fixing bacteria isolated from banana (*Musa* spp) and pineapple (*Ananas comosus* (L.) Merril). *Appl Environ Microb* 2001; 67: 2375-2379.
5. Weber OB, Baldani VLD, Teixeira KRS, Kirchhof G, Baldani JI, Döbereiner J. Isolation and characterization of diazotrophic bacteria in banana and pineapple plants. *Plant Soil* 1999; 210: 103-113.
6. Bashan Y, Holguin G, de-Bashan LE. *Azospirillum*-plant relationships: physiological, molecular, agricultural, and environmental advances (1997-2003). *Can J Microbiol* 2004; 50: 521-577.
7. Baldani JI, Pot B, Kirchhof G, Falsen E, Baldani VL, Olivares FL, et al. Emended description of *Herbaspirillum*; inclusion of [Pseudomonas] *rubrisubalbicans*, a milk plant pathogen, as *Herbaspirillum rubrisubalbicans* comb. nov.; and classification of a group of clinical isolates (EF group 1) as *Herbaspirillum* species 3. *Int J Syst Bacteriol* 1996; 46: 802-810.
8. James EK, Gyaneshwar P, Mathan N, Barraquio WL, Reddy PM, Iannetta PP, et al. Infection and colonization of rice seedlings by the plant growth-promoting bacterium *Herbaspirillum seropedicae* Z67. *Mol Plant Microbe Interact* 2002; 15: 894-906.
9. Roncato-Maccari LD, Ramos HJ, Pedrosa FO, Alquini Y, Chubatsu LS, Yates MG, et al. Endophytic *Herbaspirillum seropedicae* expresses nif genes in gramineous plants. *FEMS Microbiol Ecol* 2003; 45: 39-47.
10. Monteiro RA, Schmidt MA, Baura VA, Balsanelli E, Wassem R, Yates MG, et al. Early colonization pattern of maize (*Zea mays* L. Poales, Poaceae) roots by *Herbaspirillum seropedicae* (Burkholderiales, Oxalobacteraceae). *Genet Mol Biol* 2008; 31: 932-937.
11. Olivares FL, Baldani VLD, Reis VM, Baldani JI, Döbereiner J. Occurrence of endophytic diazotrophs *Herbaspirillum* spp. in roots, stems and leaves predominantly of Gramineae. *Biol Fert Soils* 1996; 21: 197-200.
12. Valverde A, Velazquez E, Gutierrez C, Cervantes E, Ventosa A, Igual JM. *Herbaspirillum lusitanum* sp. nov., a novel nitrogen-fixing bacterium associated with root nodules of *Phaseolus vulgaris*. *Int J Syst Evol Microbiol* 2003; 53: 1979-1983.
13. Pedrosa FO, Teixeira KRS, Machado IMP, Steffens MBR, Klassen G, Benelli EM, et al. Structural organization and regulation of the *nif* genes of *Herbaspirillum seropedicae*. *Soil Biol Biochem* 1997; 9: 843-846.
14. Klassen G, Pedrosa FO, Souza EM, Funayama S, Rigo LU. Effect of nitrogen compounds on nitrogenase activity in *Herbaspirillum seropedicae* SMR1. *Can J Microbiol* 1997; 43: 887-891.
15. Broughton WJ, Dilworth MJ. Control of leghaemoglobin synthesis in snake beans. *Biochem J* 1971; 125: 1075-1080.
16. Magnani GS, Didonet CM, Cruz LM, Picheth CS, Pedrosa FO, Souza EM. Diversity of endophytic bacteria in Brazilian sugarcane. *Genet Mol Res* 2010; 9: 250-258.
17. Bennett RA, Lynch JM. Bacterial growth and development in the rhizosphere of gnotobiotic cereal plants. *J Gen Microbiol* 1981; 125: 95-102.
18. Roesch LFW, Camargo FAO, Selbach PA, Sá ELS, Passaglia LMP. Identificação de cultivares de milho eficientes na absorção de nitrogênio e na associação com bactérias diazotróficas. *Cienc Rural* 2005; 35: 924-927.
19. Baldani VLD, Baldani JI, Döbereiner J. Inoculation of rice plants with endophytic diazotrophs *Herbaspirillum seropedicae* and *Burkholderia* spp. *Biol Fertil Soil* 2000; 30: 485-491.
20. Didonet AD, Lima OS, Candaten AA, Rodrigues O. Realocação de nitrogênio e de biomassa para os grãos, em trigo submetido a inoculação de *Azospirillum*. *Pesq Agropec Bras* 2000; 35: 401-411.
21. Garcia de Salomone I, Döbereiner J. Maize genotype effects on the response to *Azospirillum* inoculation. *Biol Fertil Soil* 1996; 21: 193-196.