Endophytic *Herbaspirillum seropedicae* expresses *nif* genes in gramineous plants

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Received 17 December 2002; received in revised form 14 March 2003; accepted 8 April 2003

First published online 8 May 2003

Abstract

The interactions between maize, sorghum, wheat and rice plants and *Herbaspirillum seropedicae* were examined microscopically following inoculation with the *H. seropedicae* LR15 strain, a Nif<sup>+</sup> (*nifH::gusA*) mutant obtained by the insertion of a *gusA*-kanamycin cassette into the *nifH* gene of the *H. seropedicae* wild-type strain. The expression of the *nifH::gusA* fusion was followed during the association of the diazotroph with the gramineous species. Histochemical analysis of seedlings of maize, sorghum, wheat and rice grown in vermiculite showed that strain LR15 colonized root surfaces and inner tissues. In early steps of the endophytic association, *Herbaspirillum* colonized root exudation sites, such as axils of secondary roots and intercellular spaces of the root cortex; it then occupied the vascular tissue and there expressed *nif* genes. The expression of *nif* genes occurred in roots, stems and leaves as detected by the GUS reporter system. The expression of *nif* genes was also observed in bacterial colonies located in the external mucilaginous root material, 8 days after inoculation. Moreover, the colonization of plant tissue by *H. seropedicae* did not depend on the nitrogen-fixing ability, since similar numbers of cells were isolated from roots or shoots of the plants inoculated with Nif<sup>+</sup> or Nif<sup>−</sup> strains.

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Keywords: *Herbaspirillum seropedicae*; Nitrogen-fixing bacterium; Plant colonization; Endophyte; *nif* gene; GUS

1. Introduction

Endophytes are microorganisms that spend most of their life cycle inside plants [1]. Various endophytic nitrogen-fixing bacteria, named endophytic diazotrophs, have been identified associated with cereals and grasses. These endophytes do not cause damage to the host organism; on the contrary, they promote plant growth by one or more of three factors: the production and secretion of plant growth regulators, antagonistic activity against phytopathogens and the supply of biologically fixed nitrogen [2–4]. Effective stimulation of plant growth, fixation of atmospheric nitrogen and its supply to the host depend on an efficient colonization [5–7]. However, the nature of such a contribution by the endophyte to the associated plant is not clear, due mainly to difficulties in assessing nitrogen fixation in colonized plants and dispersion of the bacteria throughout the plant body as opposed to a localized colonzation as in legume–rhizobium nodules.

*Herbaspirillum* spp. is an endophytic diazotroph found in roots, stems and leaves of several plants, including economically important gramineous species, such as rice and sugarcane, which can contain high numbers of the bacterium. These organisms are also found in forage grasses and tropical species, such as pineapple and banana [8–10]. *Herbaspirillum seropedicae* was the first species reported [8]; it does not survive well in soil [9] and usually does not produce disease symptoms in the associated plants [9,11–14]. Subsequently, *Pseudomonas rubrisubalbicans* was reclassified as *Herbaspirillum rubrisubalbicans*.
A new species, *Herbaspirillum frisingense*, isolated from C₄ fiber plants, was proposed recently [15]. A fourth species, termed *Herbaspirillum ‘species 3’*, has been reported mainly isolated from clinical human material, although a few strains were also isolated from sugarcane, maize and sorghum [13].

*H. rubrisubalbicans* has been reported to cause mottled stripe disease in sugarcane and red stripe disease in sorghum. However, inoculation of sugarcane and sorghum with *H. seropedicae* produced either mild or no symptoms of these diseases. Olivares et al. [16] and James et al. [17] showed that *H. rubrisubalbicans* colonized intercellular spaces and xylem vessels of sugarcane and sorghum leaves, respectively. *H. seropedicae* also colonized the xylem vessels of sorghum leaves [17] and sugarcane roots [18].

A considerable amount of work has been published on the interaction between *Herbaspirillum* spp. and rice [19–21]. Elbeltagy et al. [19] isolated *Herbaspirillum* sp. from wild rice *Oryza officinalis*. Gyaneshwar et al. [20] demonstrated that *H. seropedicae* Z67 colonization increased growth and nitrogen accumulation in aluminum-tolerant rice varieties. In wetland rice, cvs. IR72 and IR42, James et al. [21] described the ‘crack entry’ process of infection by *H. seropedicae* Z67, and the rapid colonization of the intercellular spaces, aerenchyma, and xylem of the roots and aerial parts.

Olivares et al. [16], James et al. [17] and James and Olivares [18] evaluated nitrogenase expression of endophytic *Herbaspirillum* spp. by immunodetection. Dinitrogen reductase was localized in cells of *H. rubrisubalbicans* colonizing sugarcane and sorghum leaves. *H. seropedicae* cells colonizing the roots and aerial parts of rice also contained intracellular inclusions reactive to an antibody against component II of the iron protein, although most bacteria were not labeled [21]. These studies suggested that nitrogenase was expressed by the endophytes. A problem with the immunolabeling technique is that the expression of nitrogenase proteins by bacteria in planta appears to be low compared to free-living bacteria [22] and that the immunogold signal is not always distinguishable from background labeling [23]. Moreover, a Nif⁻ *H. rubrisubalbicans* mutant was not used to assess cross-reactivity of the antibodies.

Although the interaction between *H. seropedicae* and rice, sugarcane or sorghum plants has been well studied, very little is known on the association of *H. seropedicae* with other grass species, such as maize and wheat.

In this work a Nif⁺ (Pnif::gusA) mutant LR15 was obtained by insertion of a gusA-kanamycin cassette into the nifH gene of the *H. seropedicae* wild-type strain SmR1. The expression of the Pnif::gusA fusion was followed during association of the diazotroph with gramineous species, enabling the simultaneous identification of sites of colonization and nif operon expression by *H. seropedicae*. Plant–bacterial interactions were followed by both optical and scanning electron microscopy (SEM), and histochemical detection of GUSA activity. The results showed heavy colonization of *H. seropedicae* and expression of the Pnif on root surfaces and within root and aerial tissues of maize and wheat in addition to rice and sorghum, expanding the range of hosts for *H. seropedicae*.

2. Materials and methods

2.1. Bacterial strains and media

*Escherichia coli* strains were routinely grown at 37°C in LB medium and *H. seropedicae* at 30°C in Nfb medium [8,24]. Indicator plates for bacterial strains carrying gusA fusions contained 50 μg ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-d-glucuronide (X-gluc) [25]. *H. seropedicae* SMR1 [26] is a spontaneously streptomycin-resistant derivative of the wild-type strain Z78 [8] and was used to construct the mutant Pnif::gusA LR15. *H. seropedicae* IM40 is a Nif⁻ mutant which carries a chromosomal nifH::lacZ fusion [27].

2.2. Construction of the Pnif::gusA fusion *H. seropedicae* strain

The nif genes of *H. seropedicae* are found in two separate regions. Region I contains the contiguous nifA and nifB genes [28]. Region II contains the largest nif cluster including the nifHDKENX operon [28,29]. Transcriptional analysis revealed that the whole nifHDKENX operon is transcribed from a single NifA-dependent, −24/−12 promoter located upstream of the nifH gene [28,29]. To obtain the transcriptional fusion Pnif::gusA a 1.75-kb EcoRI fragment of plasmid pGK3 [30], containing part of the nifH gene and the nifD gene, was deleted by digestion with NruI and SmaI and ligated to produce plasmid pLAU1, which contains the promoter region and a 5’ portion of the *H. seropedicae* nifH gene. A 4-kb BamHI fragment of plasmid pWM6 [31], carrying the promoterless *E. coli* gusA gene, was cloned into the BamHI site of pLAU1, to create a transcriptional Pnif::gusA fusion. A colony with the construction in the desired orientation (pLAU25) was selected by restriction analysis. To confirm that transcription of the gusA gene in this construction was under control of the nif promoter, plasmid pH11 (Klebsiella pneumoniae nifA gene) [32] was co-introduced in *E. coli* and the transformants plated on LA medium with X-gluc [25]. *K. pneumoniae* NifA activated the Pnif::gusA promoter fusion of *H. seropedicae* (not shown).

The Pnif::gusA fusion was integrated into the chromosome of *H. seropedicae* strain SmR1 by homologous recombination after electroporation of pLAU25, resulting in the mutant *H. seropedicae* strain LR15. The insertion by single cross-over into the chromosome was confirmed by DNA hybridization analyses using plasmid pLAU25 labeled with [³²P] as DNA probe [33]. The *H. seropedicae*
LR15 strain is Nif⁺. When *H. seropedicae* LR15 was grown in semi-solid medium in the presence of 20 mM NH₄Cl no β-glucuronidase activity was detected. In the absence of fixed nitrogen, the β-glucuronidase-specific activity [34] observed for the mutant was 830 nmol p-nitrophenol phosphate produced min⁻¹ mg protein⁻¹ but was still undetectable for the wild-type strain.

2.3. Germination, inoculation and growth of seedlings

Seeds of *Oryza sativa* (upland cv. IAPAR-64), *Triticum aestivum* (cv. BRS-49), *Zea mays* (cv. BR-3133) and *Sorghum bicolor* (commercial forage cultivar, Agroceres) were surface-sterilized with 70% ethanol for 1 min and shaken in 1% sodium hypochlorite and 0.01% Tween 20 solution for 30 min at 30°C. Seeds were then washed three times with sterilized distilled water by shaking (15 min each). The seedlings produced were transferred to glass tubes containing 4 g of sterilized vermiculite and 3 ml of plant medium (pH 6.8 with 0.2 mM NH₄NO₃) [35], without an added carbon source. The tubes were inoculated with 10⁷ bacterial cells g⁻¹ of vermiculite. Plants were grown in a greenhouse with 12 h of light per day.

2.4. Bacterial counting

Aerial parts (stems and leaves) and roots were sampled 10 days after inoculation. All plant parts, stems, leaves and roots were surface-sterilized with 70% ethanol for 5 min, followed by 1% sodium hypochlorite and 0.01% Tween 20 solution for 1 min and washed three times in distilled water, before maceration in saline (0.9% NaCl). Homogenates were serially diluted and plated on NFb malate medium [24] plus 0.5 mM sodium glutamate, and either X-gluc (30 μg ml⁻¹) or X-gal (5-bromo-4-chloro-3-indolyl β-d-galactopyranoside) (30 μg ml⁻¹). Kanamycin (100 μg ml⁻¹) was also added to the plates. Bacterial colonies were counted after 2–3 days incubation at 30°C.

2.5. Histochemical analysis

To verify internal colonization, plants were resin-embedded into Leica Historesin (Leica Microsystems), sectioned to 4–7 μm by microtome CUT 4055 Olympus (Olympus Americana) and toluidine blue-stained, which stains *H. seropedicae* cells a purple color [16].

For histochemical detection of GUS activity, the roots were incubated for 2 h in 50 mM sodium cacodylate buffer (pH 7.5) with 0.5 mg ml⁻¹ X-gluc at 45°C [36].

2.6. SEM

The roots inoculated with *H. seropedicae* were fixed with 0.25% (v/v) glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 1 h. The fixed material was dehydrated in a graded ethanol series. The samples were dried in a critical point drier (CPD010, Balzers Union, FL, USA) in a CO₂ atmosphere. The dried samples were affixed to aluminum stubs with silver paste, and finally coated with ionized gold film (SCD030, Balzers Union, FL, USA) [37]. The samples were examined by a Philips SEM 505. As a control we examined the cellular morphology of *H. seropedicae* in liquid culture.

3. Results

3.1. Root surface distribution and colonization by *H. seropedicae*

Maize, sorghum and wheat roots inoculated with *H. seropedicae* LR15 were examined by SEM. The results indicated that *H. seropedicae* attached and colonized maize root surface progressively from 3 to 15 days after inoculation (Fig. 1A–D). A similar pattern of colonization was also observed with sorghum (not shown). 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).

Three days after inoculation, the population of *H. seropedicae* was composed mainly of single cells or small colonies embedded in a thick mucilaginous film (Fig. 1A,B). Small colonies were visible 5–8 days after inoculation on all plant species (Fig. 1C). Cells were connected to a sheath by fibrils on maize and sorghum roots (Fig. 1B,C, arrows). After 12 days, an increased number of *H. seropedicae* cells were detected on inoculated maize and sorghum plants (Fig. 1D–F). A halo of mucilage surrounding the bacteria was detected in many areas on maize and sorghum roots (Fig. 1A) but no halo was observed on rice or wheat. The nature and origin of these halos and fibrils present on inoculated maize and sorghum roots are yet unknown. The possibility of an artifact caused by SEM sample preparation of maize or sorghum roots was discarded because identical SEM sample preparation of either non-inoculated roots or inoculated roots of rice and wheat yielded no halos.

3.2. Localization of endophytic *H. seropedicae* in tissues of gramineous plants

The interaction between gramineous plants and the *H. seropedicae* mutant LR15 was studied at different times after inoculation with whole plant samples and by comparison with uninoculated plants. No symptom of disease was noticed in any plant during the experimental period. To determine whether nitrogen fixation influenced colonization the strains of *H. seropedicae* Nif⁺ (LR15, Pnif⁺::gusA) or Nif⁻ (IM40, nifH::lacZ) were used to inoculate maize, sorghum or wheat and the bacteria were re-isolated from surface-sterilized tissues. Both strains were recovered in high numbers 10 days after inoculation from roots,

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stems and leaves of the species studied (Table 1), indicating internal colonization. Sorghum was more heavily colonized than maize or wheat. Moreover, similar numbers of *Herbaspirillum* were isolated from roots or from shoots (stems plus leaves) of plants inoculated with Nif$^+$ or Nif$^-$ strains (Table 1). In all three gramineous species, the bacterial population in the shoots was lower than that in the roots. No *H. seropedicae* could be isolated from the uninoculated control plants.

Sections of colonized rice roots showed a large number of bacterial cells visible on the axes of the root surface surrounded by a mucilaginous material (Fig. 2A). Surface colonization was followed by cortical occupancy, mainly in the intercellular spaces of the inner cortex (Fig. 2B).

### Table 1

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Strain</th>
<th>CFU per g of dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Root</td>
</tr>
<tr>
<td>Wheat</td>
<td>LR15</td>
<td>$3.9 \times 10^6$</td>
</tr>
<tr>
<td></td>
<td>IM40</td>
<td>$5.7 \times 10^6$</td>
</tr>
<tr>
<td>Sorghum</td>
<td>LR15</td>
<td>$3.3 \times 10^6$</td>
</tr>
<tr>
<td></td>
<td>IM40</td>
<td>$1.3 \times 10^6$</td>
</tr>
<tr>
<td>Maize</td>
<td>LR15</td>
<td>$3.6 \times 10^6$</td>
</tr>
<tr>
<td></td>
<td>IM40</td>
<td>$3.3 \times 10^6$</td>
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</tbody>
</table>

The results are from a representative experiment with three replicates (CV = 11%). Two independent experiments produced similar results.

*Stems plus leaves.

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Fig. 1. Adhesion and colonization of the epidermis of gramineous roots by *H. seropedicae* LR15. A–D: Time course of colonization of maize inoculated with H. *seropedicae* ($10^7$ cells g$^{-1}$ vermiculite) and analyzed by SEM. A: Bacterial cells surrounded by a halo (arrow) adhered to maize roots, 5 days after inoculation. B,C: Bacterial colonies attached to the maize root surface by fibrils (arrows), 8 days after inoculation. D: Root epidermal cells of maize showing heavy colonization 12 days after inoculation. E,F: *H. seropedicae* colonization of sorghum and wheat roots, respectively, 12 days after inoculation. Bars represent 10 μm (A–E) and 100 μm (F).
Sections of colonized rice root showed that bacteria were located adjacent to the aerenchyma spaces and in the intercellular spaces next to the central cylinder (Fig. 2C).

Deep colonization of maize and wheat aerial tissues by *H. seropedicae* was confirmed by stele and xylem occupation (Fig. 2D–H). Transverse sections of wheat and maize stem segments revealed that bacteria were also located adjacent to the aerenchyma (Fig. 2D), and had occupied the intercellular spaces of the vascular parenchyma (Fig. 2E) located near the xylem, which was heavily colonized by *H. seropedicae* (Fig. 2F). Longitudinal sections of wheat leaves showed that bacteria were also dispersed in the intercellular spaces of the leaf parenchyma (Fig. 2G), and also in the xylem (Fig. 2H).

### 3.3. Expression of *Pnif::gusA* by *H. seropedicae LR15 on the root surfaces of gramineous plants

Bacteria expressing *Pnif::gusA* were attached to maize, sorghum, wheat and rice roots, often in the emerging zones of secondary roots (Fig. 3A, B). Usually, *Pnif* expression occurred in clusters of cells localized particularly in the grooves located between epidermal cells on the root surface. Expression of *Pnif::gusA* on root surfaces was variable. This was expected since only bacterial cells in the center of colonies or at regions of low pO2 and limiting NH₄⁺ concentration can express the *nif* promoter. Interestingly, expression of the *nif* promoter was also observed in bacterial colonies located in the external...
mucilaginous root material of rice, 8 days after inoculation (Fig. 3C). Presumably the mucilaginous material protected the cells against O2 repression of nif expression. GUSA activity was not detected on or in any of the uninoculated control plants.

3.4. Expression of the Pnif::gusA by H. seropedicae LR15 within rice, wheat and maize tissues

To verify internal nif promoter expression, stained rice, wheat and maize plants were resin-embedded in historesin and sectioned. GUS activity was detected in roots, stems and leaves. Bacterial cells expressing the nif operon were visible in the intercellular spaces of root cortex (Fig. 3D,E), but no GUS activity was detected in the aerenchyma spaces, probably due to high O2 repressing nif gene expression.

Bacteria expressing the nif gene were seen within the vascular tissue in sorghum (not shown), maize and rice stems (Fig. 3F,G), a site distant from the root site of inoculation. H. seropedicae LR15 expressing the fusion Pnif::gusA was also easily detected within the wheat leaf tissue, in the intercellular spaces of mesophyll cells and vessels, 10 days after inoculation (Fig. 3H). The root cortical layers and vascular tissues of the gramineous plants were intensely colonized, as shown by toluidine blue staining, although only a fraction of the colonizing bacteria within the same region produced β-glucuronidase (compare Figs. 2 and 3).

4. Discussion

4.1. Infection and colonization

Internal colonization by Herbaspirillum was demonstrated in sugarcane and sorghum leaves [16,17] and roots and shoots of rice [19–21]. Here we show that H. seropedicae also colonizes the root surface and the interior tissues of wheat and maize as well as rice and sorghum.
Our results distinguish consecutive stages of the interaction between H. seropedicae and gramineous roots. The initial step consists of the attachment of the bacteria to the root surface. SEM revealed the presence of filaments with attached bacteria adhering to the rhizoplane (Fig. 1), resembling cellulose fibrils developed after the attachment of the phytopathogen Agrobacterium tumefaciens [38,39] or fibrillar material anchoring Azospirillum sp. to roots [38,39,40]. At this stage, the predominant localization of colonizing cells at the intercellular junctions of epidermis roots is consistent with the suggestion that micro-colonies develop where bacteria find an increased concentration of carbon sources, thus explaining the preference of bacteria for this region of the root tissue [41,42].

Root surface colonization was followed by a second stage, characterized by cortical infection, and a third stage, which consisted of stele and xylem invasion. The Herbaspirillum invasion seems to occur primarily through lateral root emergence (Fig. 2). Previously, Gyaneshwar et al. [20] and James et al. [21] documented Herbaspirillum invasion of rice at lateral root cracks. A similar pattern of invasion has also been described for sugarcane infection by Gluconacetobacter diazotrophicus [43] and also for non-diazotrophic endophytic bacterial infections, such as Pseudomonas solanacearum of dicotyledonous plants [44–46].

The intercellular infection of the cortex suggests apoplastic dispersion of the bacteria. However, to reach the vascular cylinder, the bacteria have to pass through the endodermis which with suberized walls and storage of phenolic compounds may constitute a barrier for bacterial dispersion [44]. In our observations, the endodermis of wheat, sorghum and maize was never disrupted in plants colonized by H. seropedicae, except on root sites where this physical barrier is not yet fully differentiated, or reoriented at lateral root growth. We have observed H. seropedicae often colonizes sites of secondary root, suggesting that these sites offer an opportunity for H. seropedicae to occupy the vascular cylinder after cortical invasion. The lateral root colonization by H. seropedicae of rice plants has been comprehensively illustrated by James and co-workers [21]. Our results suggest that this process is also used by H. seropedicae to colonize other grasses. There are similarities in the pathways, and perhaps also in the mechanisms of infection between phytopathogenic and endophytic bacteria. Entry of Azospirillum spp. and P. solanacearum near lateral roots has also been observed [38,44], suggesting that taxonomically unrelated bacteria might share similar mechanisms of entry into both monocotyledonous and dicotyledonous plants [22].

4.2. Does the colonization of plant tissue by H. seropedicae depend on the nitrogen-fixing ability?

H. seropedicae Nif⁺ and Nif⁻ colonized maize, sorghum and wheat plants equally, as evidenced by the number of bacteria recovered from inoculated plants 10 days after inoculation. Bacterial cell counting of Nif⁺ (LR15) and Nif⁻ (IM40) strains revealed the same size of population in inoculated plants (Table 1), suggesting that nitrogen fixation is not required for endophytic colonization. This observation agrees with the results of Sevilla et al. [47] where no difference in colonization of sugarcane was found between the G. diazotrophicus wild-type and a Nif⁻ mutant strain. In contrast a Nif⁻ mutant of Azooarcus spp. did not persist in the rhizosphere and could not colonize Kallar grass as well as the wild-type [48].

4.3. Expression of nif gene and nitrogen fixation

The present study shows that the nifHDKENX operon is expressed on and inside the tissues of the four gramineous species. Direct evidence of nif expression by Herbaspirillum during association with host plants is scarce and consists of the reaction with polyclonal antibody against the iron protein of Rhodospirillum rubrum nitrogenase inside leaves of sugarcane [16], sorghum [17] and rice [21]. In this study we showed the expression of the H. seropedicae nif promoter on and inside roots, leaves and stems of rice, maize, wheat, and sorghum plants using a Pnif::gusA fusion inserted into the chromosome of H. seropedicae. Expression of the nif gene in the colonized plants suggests strongly that the infected tissues provide an environment suitable for nitrogen fixation. Consistent with this result, incorporation of 15N has been used to show substantial nitrogen fixation of H. seropedicae colonizing rice [19–21]. Furthermore, expression of K. pneumoniae NiFH in maize roots but not in stem sections was demonstrated using antibody to purified NiFH [49]. Our evidence of Pnif::gusA in roots and stems of maize inoculated with H. seropedicae reinforces the suggestion that maize–bacteria associations and in planta nif expression may occur naturally.

We observed that H. seropedicae nif promoter expression occurred only in a limited number of bacterial cells within the roots and aerial tissues of maize, wheat and sorghum. A similar result was found for the H. seropedicae NiFH protein in the roots of rice plants [20,21]. Several studies indicated that N₂ fixation in plants is carbon-limited. In addition, gramineous species inoculated with Herbaspirillum and others diazotrophic bacteria (Azospirillum, Klebsiella, Serratia) had acetylene reduction activity only in the presence of added carbon source [20,36,50,51]. Such carbon limitation would restrict O₂ uptake, allowing free O₂ to inhibit nif expression and nitrogenase activity. These results suggest that the relatively low expression of Pnif::gusA fusion of H. seropedicae LR15 may be due to carbon limitation under our experimental conditions, and possibly under field conditions. From this conclusion it follows that growth benefits occurring as a result of inoculation with diazotrophs can be partially independent of nitrogen fixation, e.g., through phytohormone-dependent growth stimulation, since H. seropedicae has been shown to produce indole acetic acid and gibberellins [52].
In summary, the results of this study show that *H. seropedicae* LR15 is capable of colonizing the root surfaces and inner tissues of whole plants, and to express *nif* genes in roots and aerial parts of maize, sorghum, wheat and rice, suggesting that grass tissues contain suitable environments to allow bacterial proliferation and the expression of *H. seropedicae* *nif* genes. Whether this expression leads to sufficient nitrogen fixation to enhance maize, sorghum and wheat growth under field conditions remains to be established.

Acknowledgements

We are grateful to EMBRAPA and IAPAR for the plant seeds and to Centro de Microscopia Eletrônica-UFPF (Curitiba, PR, Brazil) for the excellent help with the scanning electron microscopy. The authors thank Dr. Iara Machado for the Nif \(^{\text{a}}\) strain IM40. We also thank Roseli Prado, Valter A. Baura, Julieta Pie, Cândido J.T. Pereira and Nilson Belem Filho for technical assistance. This work was supported by the Brazilian Research Council (CNPq), PRONEX (FINEP/MCT/CNPq) and Fundação Araucária/Paraná Tecnologia.

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