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Short Communication

Early colonization pattern of maize (*Zea mays* L. Poales, Poaceae) roots by *Herbaspirillum seropedicae* (Burkholderiales, Oxalobacteraceae)

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Abstract

The bacterium *Herbaspirillum seropedicae* is an endophytic diazotroph found in several plants, including economically important poaceous species. However, the mechanisms involved in the interaction between *H. seropedicae* and these plants are not completely characterized. We investigated the attachment of *Herbaspirillum* to maize roots and the invasion of the roots by this bacterium using *H. seropedicae* strain SMR1 transformed with the suicide plasmid pUTKandsRed, which carries a mini-Tn5 transposon containing the gene for the *Discosoma* red fluorescent protein (Dsred) constitutively expressed together with the kanamycin resistance gene. Integration of the mini-Tn5 into the bacterial chromosome yielded the mutant *H. seropedicae* strain RAM4 which was capable of expressing Dsred and could be observed on and inside fresh maize root samples. Confocal microscopy of maize roots inoculated with *H. seropedicae* three days after germination showed that *H. seropedicae* cell were attached to the root surface 30 min after inoculation, were visible in the internal tissues after twenty-four hours and in the endodermis, the central cylinder and xylem after three days.

Key words: confocal microscopy, diazotroph, endophytic, *Herbaspirillum seropedicae*.

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Bacterial colonization of plant internal tissue has been described in many plant species. Although many plant-colonizing bacteria are phytopathogenic, the majority of such interactions are either neutral or beneficial to the host plant. The first studies on the interactions of diazotrophic bacteria with poaceous plants involved *Azotobacter paspali* isolated from the soil or from plant rhizospheres (Döbereiner, 1966) and later it was shown that nitrogen-fixing *Azospirillum brasilense* colonized both the rhizosphere and the root interior of several forage grasses and cereals (Baldani *et al.*, 1986a) of the family Poaceae. In the last decade, new nitrogen-fixing genera have been identified that colonize internal plant tissues, including members of the genera *Acetobacter*, *Burkholderia*, *Herbaspirillum* and *Pseudomonas* (Lodewyckx *et al.*, 2002*).* These bacteria are capable of occupying the intercellular spaces and the vascular tissue of host plants and are referred to as endophytic bacteria (James, 2000). It has been shown that the endophytic interaction can enhance plant growth, increase plant resistance to pathogens and specific xenobiotics (Reiter *et al.*, 2002; Siciliano *et al.*, 2001) and possibly transfer fixed nitrogen to the host (James and Olivares, 1998).

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The endophytic nitrogen-fixing bacterium *Herbaspirillum seropedicae* (Burkholderiales, Oxalobacteraceae) associates not only with economically important poaceous plants such as maize, sugar cane and sorghum (Baldani *et al.*, 1986b) but also agriculturally important tropical plants such as banana and pineapple (Cruz *et al.*, 2001). This bacterium is known to colonize maize, rice, sorghum, sugar cane and wheat, first invading the intercellular spaces of the roots and then occupying the xylem vessels (James and Olivares, 1998; Roncato-Maccari *et al.*, 2003) and the aerial parts of the plant involved (James *et al.*, 2002; Roncato-Maccari *et al.*, 2003). Although great numbers of *H. seropedicae* cells can be found in internal plant tissues, the mechanisms of infection and colonization are not completely understood, and a better understanding of these processes is needed to ensure that *H. seropedicae* and other endophytes can be the efficiently used as biofertilizers.

Identifying and tracking endophytic bacteria within the host plant is a necessary step in understanding colonization mechanisms. Microscopy and tagging bacteria with reporter genes such as *gusA* can be used to investigate colonization but these techniques can produce artifacts during sample preparation. However, such problems can be avoided by marking endophytic bacteria with genes that allow the expression of fluorescent proteins which provide a unique visual marker in fresh plant tissue samples and the application of confocal microscopy, a technique which allows visualization of bacteria in thick samples and is tolerant to the background autofluorescence which often occurs with plant tissue. Assmus *et al.* (1995) reported using a similar strategy involving fluorescently-labeled rRNA probes to detect *A. brasilense* in the rhizosphere of wheat.

During the study described in this paper, we used confocal microscopy of transverse sections of fresh maize (*Zea mays* L. Poales, Poaceae) roots to examine the progression of the early events of maize colonization by *H. seropedicae* strains expressing the coral-derived *Discosoma* red (DsRed) fluorescent protein (Matz *et al.*, 1999), and to characterize the distribution and organizational patterns of the bacterial cells within the plant tissues.

We electro-transformed the spontaneous streptomycin-resistant (SmR) *H. seropedicae* strain SmR1 (Pedrosa *et al.*, 1997) with the pUTKandsRed plasmid (Tolker-Nielsen *et al.*, 2000) and obtained 45 kanamycin-resistant recombinants, which expressed DsRed and were able to grow on nitrate and had nitrogenase activity (12 nmol ethylene/min.mg protein) and growth rates (growth rate coefficient $\mu = 0.28\ h^{-1}$) comparable to the wild-type strain. For the study described in this paper, we used a DsRed tagged recombinant colony, colony number four, which was assigned the code RAM4, and which produced a reddish color on NFbHPN medium agar plates (Klassen *et al.*, 1997) after 2-3 days incubation at 30 °C.

Bacterial attachment studies were carried out using *H. seropedicae* strains SmR1 and RAM4, each of which were grown in shake-flask cultures in 125 mL flasks containing 25 mL of NFbHPN medium (Klassen *et al.*, 1997) (pH 6.5) at 30 °C for 16 h and an agitation rate of 120 revs min^{-1}. The control for artifactual entry of bacteria during root manipulation was *Escherichia coli* strain CC118 (Tolker-Nielsen *et al.*, 2000) containing plasmid pUTKandsRed (*E. coli* CC118-DsRed), which had been grown in shake-flask cultures in 125 mL flasks containing 25 mL of LB media (pH 6.8) at 30 °C for 16 h and an agitation rate of 120 revs min^{-1}. For use in the experiments, the number of colony forming units (CFU) was determined using serial dilution and plate counts, and standardized using calibrated optical density curves (data not shown).Seeds of the maize cultivar BR-3133 (bought from Santa Helena Sementes Ltda, Brazil) were sanitized by washing in 70% ethanol for 1 min, followed by shaking for 20 min at 30 °C in sodium hypochlorite (2% w/w available chlorine) solution supplemented with 0.02% (v/v) Tween 20 (USB, USA) and then washing for 30 min by gently shaking in sterile distilled water, the washing being repeated five times (modified from Roncato-Maccari *et al.*, 2003). The sanitized seeds were transferred to 96-well deep-well blocks (Greiner Bio-One, Austria), each well being 9 mm x 9 mm x 35 mm, and containing 3M filter paper plus 2 mL of plant medium (Egener *et al.*, 1999) and grown for three days at 25 °C using a 12 h light period and 1250 lux illumination provided by white-type fluorescent tubes (Aqua Glo, Japan). After three days of growth, seedlings were incubated with 1 mL of cell suspension containing 6×10^9 CFU mL^{-1} of *H. seropedicae* strain SmR1 or RAM4 or the *E. coli* control. After 15 min of incubation at 30 °C, roots were washed twice in sterile NaCl 0.9%, cut, weighed and vortexed for 20 s in 1 mL of NaCl 0.9%. The supernatant was used to determine the number of bacteria attached by plate counting. The diluted extracts were plated on solid NFbHPN medium (Klassen *et al.*, 1997). After 24-48 h incubation at 30 °C, the number of colony forming units (CFU) was determined, further samples being taken every 15 min for one hour. Three replicated experiments were performed, with the roots from three plants being analyzed in each experiment.

For internal colonization studies, three-day old plantlets were inoculated with 6×10^9 CFU mL^{-1} for 30 min and were then transferred to sterile filter paper moistened with sterile plant medium (Egener *et al.*, 1999) where they remained until 96 h after inoculation, after which the roots were surface sterilized as described by Roncato-Maccari *et al.* (2003), triturated in saline and the endophytic bacterial population determined on NFbHPN medium (Klassen *et al.*, 1997). Confocal microscopy using a BioRad Confocal Radiance 2100-Eclipse E800 Nikon microscope (BioRad, USA) was used to examine maize roots, which were uninoculated or which had had been exposed to single-strain cultures of *H. seropedicae* strain RAM4 or the *E. coli* CC118-DsRed control. The bacteria were detected by exciting the DsRed protein with a helium-neon laser (BioRad, USA) with an excitation wavelength of $\lambda = 543$ nm in conjunction with an emission LP 560 filter (Nikon, Japan). For these experiments, maize roots were exposed to bacteria as described above, and then washed three times in water and transverse cross sections, approximately 25 µm thick, were hand cut about 3 cm from the root tip, mounted on a microscope slide, and examined immediately. Three independent experiments were performed, and roots from three plants were analyzed for each data.

In the bacterial attachment studies, after 15 min incubation we recovered approximately 7×10^5 CFU g^{-1} of root tissue, on a fresh weight basis (fwb), for both strain SmR1 and RAM4. The number of CFU attached reaching a maximum of 1.5×10^7 g^{-1} of root tissue (fwb) after one hour of incubation, indicating that the capacity of strain RAM4 to attach to maize roots was similar to that of the SmR1 parent

strain. For the internal colonization studies, on the fourth day after inoculation of the plantlets, we recovered approximately 6 x 10^6 CFU g^{-1} of root tissue (fwb) for both strain SmR1 and RAM4, indicating that strain RAM4 was not impaired in its capacity to colonize maize.

Using confocal microscope, *H. seropedicae* strain RAM4 showed bright red fluorescence and were visually distinct from the diffuse fluorescent background of the contour of the plant cells. After thirty minutes, the root surface showed dense agglomerates of strain RAM4, with the highest density of bacteria occurring at the intercellular spaces and, especially, at the emergence of root hairs, these points being probable routes used by the bacteria to invade the plant tissue. The early accumulation of bacteria at these points suggests that strain RAM4 was rapidly attracted, and apparently firmly attached, to discontinuities in the epidermis (Figure 1). Although a large number of bacteria were found attached to the surface after thirty minutes, there was no evidence of internal colonization. Incubation of the roots with strain RAM4 for 1 h produced essentially the same results, with an apparently higher number of cells attached. The preference of *H. seropedicae* strain RAM4 for the intercellular regions and lateral root emerging zones is probably related to a higher concentration of carbon sources at these points (Bennett and Lynch, 1981). Our finding agrees with studies that indicate that the lateral root emergence points are highly susceptible to disruption (Agarwhal and Sende, 1987). No agglomerates of *E. coli* CC118-dsRed was observed attached to the root surface (Figure 1) and no internal colonization was found, strongly indicating that the presence of fluorescent *H. seropedicae* in the maize root tissues represents true internal colonization.

Twenty-four hours after inoculation, the bacteria started to invade the internal tissues of the roots. Confocal microscopy of different regions of cross-sections of maize roots inoculated with strain RAM4 showed colonization of the apoplast near the root surface (Figure 2). The bacterial population decreased progressively from the epidermis to the central cylinder, but fluorescent bacteria were clearly observed in the cortex, endodermis and xylem regions. This pattern of colonization suggests that *H. seropedicae* can rapidly penetrate the plant tissues and infect the xylem vessels.

The pattern of maize root colonization three days after inoculation showed a clear increase in internal colonization (Figure 2), with a higher number of strain RAM4 in the endodermis and the central cylinder. The bacteria were also dispersed in the intercellular spaces, mostly as isolated cells (Figure 2, arrow). This colonization pattern was observed in three independent inoculation experiments and was also observed in separate experiments involving *H. seropedicae* strains ZM152 and ZA95 (data not shown).

Our results suggest that *H. seropedicae* penetrates to the interior of maize roots by entering via cracks at the lateral root junctions. After entering, the bacteria rapidly colonize intercellular junctions and are distributed to the cortex, endoderm cells, and xylem vessels of maize root tissues.

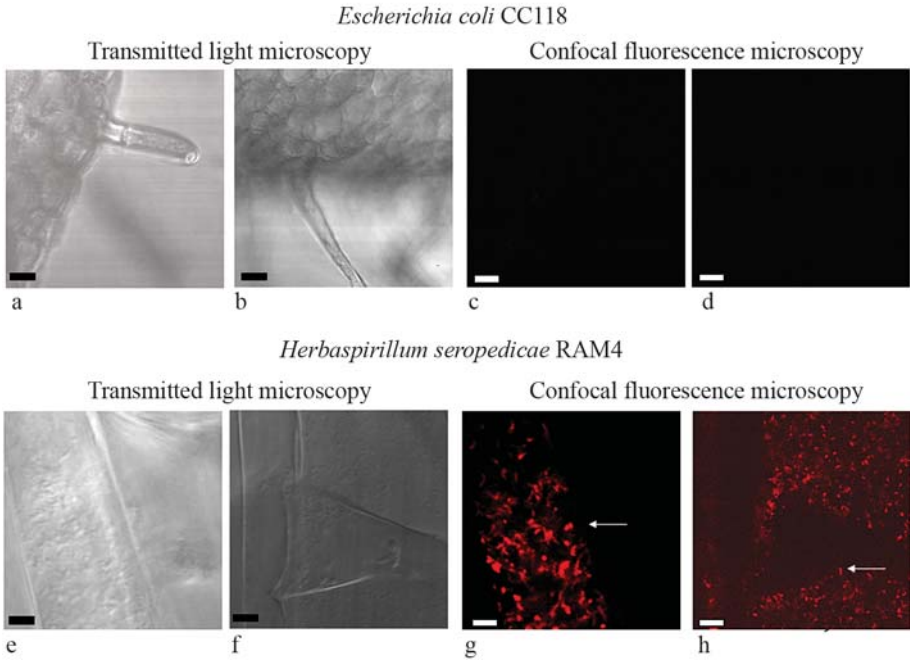

Figure 1 - Maize root tissue thirty minutes and one hour after inoculation with *Herbaspirillum seropedicae* strain RAM4 or *E. coli* CC118 (control), both expressing red fluorescent protein. Photomicrographs of a cross-section of maize root thirty minutes (a, c, e and g) and one hour (b, d, f and h) after inoculation with *E. coli* CC118 (upper panel) or *H. seropedicae* strain RAM4 (bottom panel). Left – transmitted light microscopy, righ – confocal fluorescence microscopy. Arrows show bacteria accumulating in the lateral root. After one hour clumps of bacteria can be seen at the lateral root junctions and spreading into the epidermis. Bars: a = 15 μm; b = 50 μm.

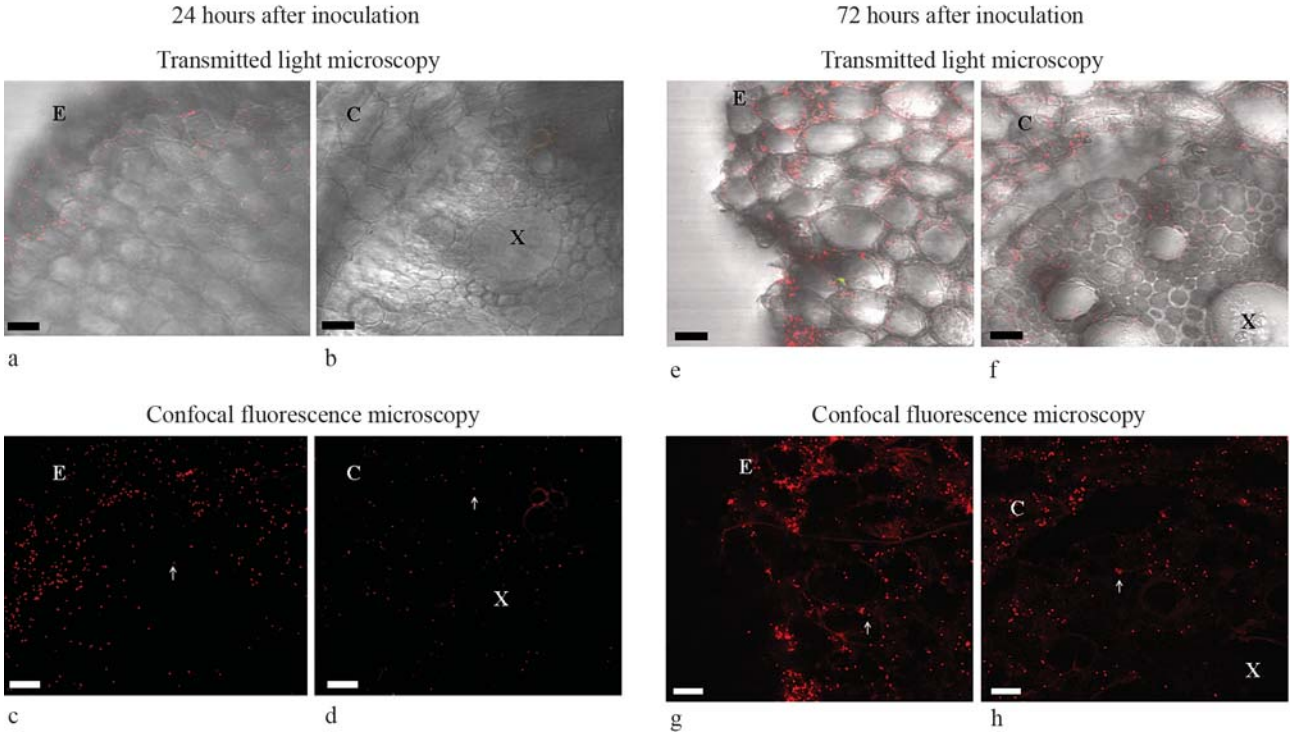

Figure 2 - Transmitted light and confocal fluorescence microscopy of a cross-section of maize root 24 (a to d) and 72 h (e to h) after inoculation with *Herbaspirillum seropedicae* strain RAM4. The images were recorded in two different regions of the cross-section: the epidermis (a, c, e and g) and the central cylinder (b, d, f and h). Key: E = epidermis; X = xylem vessels; C = cortex. Arrows indicate the fluorescent bacteria. Bars = 50 µm.

Accumulation of *Herbaspirillum* spp cells has previously been shown to occur at the lateral root emerging points two days after inoculation for rice (James *et al.*, 2002), four days after inoculation for sugar cane (James and Olivares, 1998; Olivares and James, 2000) and five days after inoculation for rice, sorghum, maize and wheat (Roncato-Maccari *et al.*, 2003).

The data from our study indicates that the *H. seropedicae* strain RAM4 colonization and invasion process is very rapid. Thirty minutes after maize root inoculation, we observed a high density of strain RAM4 at the lateral root junctions, which increased substantially after one hour. This finding agrees with studies that indicate that these parts of the roots are highly susceptible to disruption (Agarwhal and Sende, 1987). A similar pattern of root colonization by other diazotrophs has been reported for *Azhorhizobium caulinodans*, which also colonized lateral root cracks of *Arabidopsis thaliana* and *Oryza sativa* (Gough *et al.*, 1997; Gopalaswamy *et al.*, 2000), and *Azoarcus* sp. strain BH72 labeled with green fluorescent protein (GFP), which was found in high number in rice lateral roots (Egener *et al.*, 1998). In addition, beta-glucuronidase staining (GUS) of rice roots has shown lateral root junction colonization by *H. seropedicae* strain Z67 (James *et al.*, 2002). Recently, Liu *et al.*, (2006) also reported accumulation of GFP-labeled *Bacillus megaterium* at the lateral roots cracks of maize and rice. It is possible that the bacteria secrete cell wall-degrading enzymes, such as cellulases and pectinases, at these susceptible root regions to facilitate entry.

As mentioned above, 24 h after the start of the experiment we found *H. seropedicae* strain RAM4 in all parts of the root tissues, with this internal distribution increasing further by the 72 h after inoculation. Roncato-Maccari *et al.*, (2003), using normal microscopy of root sections, also found *Herbaspirillum* distributed throughout the roots of maize five days after inoculation. Our results show that by using bacteria labeled with red fluorescent protein it is possible to verify that only 24 h after inoculation the root, including the xylem, is completely colonized by the bacteria. Colonization of the vascular system of the plant allows the bacteria to distribute throughout the plant, indicating that invasion is a very rapid process, and occurs in the absence any visible defense response by the plant.

In our experiments, the presence of a large number of bacteria in the intercellular spaces was probably due to the fact that there is more space and a higher concentration of carbon source in the apoplast (Bennett and Lynch, 1981). The presence of the bacteria in the cortex, endoderm cells and xylem vessels indicate that these bacteria probably utilize plant nutrients and, in turn, can secrete metabolites that can be used by the plant (Grayston *et al.*, 1997).

It is known that *H. seropedicae* has a low survival rate in sterile soils, suggesting that this organism is highly specialized in colonizing plants and supporting the efficient colonization pattern observed by us. However, it is impor-

tant to note that our study was carried out under axenic culture in the absence of competing bacteria. The introduction of competition may substantially change the pattern observed, since fewer attachment points may be available, competing bacteria may secrete anti-bacterial substances and the plant may elaborate a defense against the mixed bacterial population. Other authors have reported infection and colonization of plant tissues by *H. seropedicae* using light and electron microscopy and the beta-glucuronidase *gusA* reporter gene (James and Olivares, 1998; James *et al.*, 2002; Roncato-Maccari *et al.*, 2003).

Our study indicates that the use of fluorescent marked proteins and confocal microscopy of fresh plant tissue has several advantages as an investigative tool in that it reduces procedural artifacts, allows very rapid (within minutes) detection and quantification of the bacteria and facilitates the design of competition experiments involving one or more competing pathogenic or non-pathogenic microorganisms. This combination of techniques is an important tool for furthering understanding of plant-microbe interactions.

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