

STRUCTURAL INTERACTION BETWEEN GFP-LABELED DIAZOTROPHIC ENDOPHYTIC BACTERIUM *Herbaspirillum seropedicae* RAM10 AND PINEAPPLE PLANTLETS 'VITÓRIA'

Lílian Estrela Borges Baldotto^{1*}; Fábio Lopes Olivares²; Ricardo Bressan-Smith³

¹ Universidade Federal de Viçosa, *Campus* de Florestal, Florestal, MG, Brasil; ² Laboratório de Biologia Celular e Tecidual, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, RJ, Brasil; ³ Laboratório de Melhoramento Genético Vegetal, Centro de Ciências e Tecnologias Agropecuárias, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, RJ, Brasil.

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ABSTRACT

The events involved in the structural interaction between the diazotrophic endophytic bacterium *Herbaspirillum seropedicae*, strain RAM10, labeled with green fluorescent protein, and pineapple plantlets 'Vitória' were evaluated by means of bright-field and fluorescence microscopy, combined with scanning electron microscopy for 28 days after inoculation. After 6 hours of inoculation, *H. seropedicae* was already adhered to the roots, colonizing mainly root hair surface and bases, followed by epidermal cell wall junctions. Bacteria adherence in the initial periods occurred mainly in the form of solitary cells and small aggregates with pleomorphic cells. Bacteria infection of root tissue occurred through the cavities caused by the disruption of epidermal cells during the emergence of lateral roots and the endophytic establishment by the colonization of intercellular spaces of the cortical parenchyma. Moreover, within 1 day after inoculation the bacteria were colonizing the shoots. In this region, the preferred sites of epiphytic colonization were epidermal cell wall junctions, peltate scutiform trichomes and non-glandular trichomes. Subsequently, the bacteria occupied the outer periclinal walls of epidermal cells and stomata. The penetration into the shoot occurred passively through stoma aperture followed by the endophytic establishment on the substomatal chambers and spread to the intercellular spaces of spongy chlorenchyma. After 21 days of inoculation, bacterial biofilm were seen at the root hair base and on epidermal cell wall surface of root and leaf, also confirming the epiphytic nature of *H. seropedicae*.

Key words: *Ananas comosus*, plant-growth promoting bacteria, microscopy.

INTRODUCTION

Diazotrophic bacteria have been isolated from various plant species and contribute particularly to promote the growth of the host plant (1). The first diazotrophic bacteria with endophytic characteristics isolated were initially described as

Azospirillum seropedicae (3). The bacteria were isolated from roots of sorghum, maize and rice, and later re-classified based on studies of DNA homology into a new genus, *Herbaspirillum*, and renamed *Herbaspirillum seropedicae* (2). This gram-negative bacterium is rod-shaped, has polar flagella and low survival in soil (2, 23). Bacteria of this genus are

*Corresponding Author. Mailing address: Universidade Federal de Viçosa, *Campus* de Florestal (UFV), Rodovia LMG, 818, km 6 – 35690-000, Florestal, Minas Gerais, Brazil.; Email: liestrelaborges@gmail.com

found on roots, stems and leaves of various grasses (2, 11, 23) and also on tropical fruits such as banana and pineapple (9, 36).

The potential to promote plant growth of *H. seropedicae* has been evaluated mainly in species of the Poaceae family, with initially unsatisfactory results (27), and later, by the selection of strains from *in vitro* plants (4, 26), positive results were obtained for rice (4), maize (29), and sugarcane (26). The mechanisms responsible for plant growth promotion by *Herbaspirillum* are not yet fully elucidated and include not only biological nitrogen fixation (34), but also the biosynthesis of plant hormones (28) and influence the activity of ACC deaminase (31).

The steps of the structural interaction between *H. seropedicae* and the host plant have been evaluated by artificial inoculation and subsequent microscopic analysis in rice plants (11, 14, 30), sorghum (16), maize (20) and sugarcane (15, 22, 24, 25, 32). In sugarcane, Olivares (24) showed elegantly, by means of conventional techniques of light and electron microscopy combined with immunolabelling, that the penetration of *H. seropedicae* through the cavity formed by the rupture of epidermal cells by the emergence of lateral roots is passive, and that the endophytic establishment occurs through the colonization of intercellular spaces of cortical parenchyma and the xylem lumen. Currently, with the advent of recombinant DNA technology, mutant strains of *H. seropedicae* are obtained with insertion of genes that express fluorescent proteins, e.g., the green fluorescent protein (GFP), enabling studies of the bacteria-plant interaction in real-time (11, 22).

For being stable and fluorescence-emitting when directly excited by UV light, GFP can be considered a tool for easy detection by fluorescence and confocal microscopy and, unlike the conventional techniques of microscopy and immunolabelling, requires no chemical reagents, which minimizes the effects of artifacts and allows *in situ* space-time studies of the plant-microorganism interactions (11, 22).

The intensification of the use of plant growth-promoting bacteria, such as *H. seropedicae*, in agricultural systems, depends on knowledge about the structural and physiological

mechanisms of interaction. In pineapple, for example, different strains of diazotrophic endophytic bacteria have been isolated and identified (9, 36) with plant growth-promoting potential (6, 35), but there are no data on the structural events of the interaction.

Therefore, the objective of this study was to investigate the events of the structural interaction between the GFP-labeled bacteria *H. seropedicae* RAM10 and pineapple plantlets 'Vitória' propagated *in vitro* over time.

MATERIALS AND METHODS

Plant Material

Pineapple plantlets (*Ananas comosus* L. Merrill) 'Vitória' (13) propagated by *in vitro* culture in baby-food glass pots was provided by the Laboratory of Biotechnology Biomudas and maintained in MS medium (21) without addition of growth regulators and vitamins. The *in vitro* plantlets were maintained in a growth chamber with photosynthetic photon flux of 25 $\mu\text{mol m}^{-2} \text{s}^{-1}$, at 25 ± 2 °C and 16 h photoperiod. Every three months, the plantlets were transferred to a new MS medium. For the subsequent experimental stages, plantlets with about 1.5 g fresh weight, number of leaves about 10, size about 8 cm long, were selected and transferred separately to test tubes containing 20 mL 1/10 solution of MS medium (21) without addition of growth regulators, vitamins or agar and pH adjusted to 5.8.

Bacterial growth and inoculation

The bacteria *Herbaspirillum seropedicae* strain RAM10, with GFP gene insertion by transposon Tn5, was used. This construction was kindly provided by Dr. Rose Adele Monteiro (Department of Biochemistry and Molecular Biology, Federal University of Paraná, Brazil), and had been originally derived from the strain *H. seropedicae* ZA95 isolated from rice (2). The inoculum was prepared by growing the bacteria in liquid medium DYGS (10) for 24 h, 30 °C, 120 rpm. The inoculation was performed by transferring the selected plantlets to the test tubes (containing 20 mL 1/10 solution of MS medium as

described above) and applying 30 μL of bacterial solution ($\text{DO}_{440} = 1.0$) in the liquid medium near the roots with a automatic pipette. As control, 30 μL of the autoclaved medium DYGS was inoculated.

Fluorescence microscopy

The microscopic observations began 6 h after inoculation and were continued on the 1st, 2nd, 3rd, 7th, 14th, 21st and 28th day, using three different plants on each date. Entire leaves and roots, as well as transverse and longitudinal hand sections of leaves and roots, were placed on glass slides with distilled water, covered with coverslips and observed under a fluorescence microscope Axioplan (Zeiss) with BP (band-pass) filters with an excitation wavelength between 460 and 490nm and LP (long-pass) emission wavelength between 510 and 550. The photographs were taken by a digital camera Canon Power Shot A640 coupled to the microscope and analyzed using software Zoom Browser EX.

Bright field microscopy

Fragments of leaf blades (0.5 – 1.0 cm^2) and roots (1.0 cm long) were fixed in a solution containing 2.5% glutaraldehyde, 4.0% formaldehyde and 0.05 mol L^{-1} phosphate buffer at pH 7.0, for 2 h. Subsequently, the samples were washed 3 times in the same buffer and post-fixed in 1% osmium tetroxide solution in water, at room temperature, for 2 h. The material was washed 3 times with the same buffer and dehydrated in a graded acetone series (30, 50, 70, 90, 3 x 100% at 1 h each). After dehydration the samples were gradually embedded in Epon resin. The individual samples were transferred to microtubes containing the resin, and subsequently polymerized at 60 ° C for 48 h. Semi-thin sections (0.8 - 1.0 μm) were cut with a glass knife on a Reichert Ultracuts Ultramicrotome. The semi-thin cuts were placed on glass slides with a drop of water, fixed on a heated metal plate and stained with 0.1 % toluidine blue in an aqueous solution of 1 % sodium tetraborate. The slides were examined and images captured using the above-cited microscope.

Scanning electron microscopy

Leaf blade and root samples were fixed, post-fixed and dehydrated as described above for light microscopy. Then the samples were dried with CO_2 using a Critical Point Dryer apparatus BAL-TEC CPD 030, mounted on aluminum stubs and gold-sputtered with a Sputter Coater apparatus BAL-TEC SCD 050, as proposed by Baldotto and Olivares (5). Thereafter, the samples were observed at 15 and 25 kV under a scanning electron microscope ZEISS DSEM 962. For each time under investigation, 3 samples from leaf blades and roots were fully scanned by SEM.

Bacterial Counts

The number of bacteria present on the pineapple plantlets was performed by the technique of the Most Probable Number (10). Plantlet samples of 1 g were macerated in 9 mL saline solution (NaCl , 8.5 g L^{-1}) and from this dilution (10^{-1}) serial dilutions were made, taking 1 mL of the original dilution in 9 mL of saline solution until 10^{-10} . Aliquots of 100 μL of the dilutions were transferred to glass vials containing 5 mL of semi-solid JNFb medium. The vials were incubated at 30 ° C for 7 days. After this period, bacterial growth was evaluated based on the presence of a white film on the medium surface. The number of bacteria was obtained by consulting the Table of McCrady for 3 replicates per dilution. The identity of the re-isolates was confirmed by observations of the cell shape and fluorescence emission using a fluorescence microscope Axioplan (Zeiss).

RESULTS

Root colonization

The green fluorescence from the GFP-labeled bacterium *H. seropedicae* strain RAM10 could be easily distinguished from the yellow autofluorescence emitted by pineapple roots tissue by fluorescence microscopy (Figure 1). This difference in color facilitated observations in the early stages of the interaction between the bacterium and the host plant.

After 6 h of inoculation, the bacteria were observed along the entire root length, particularly in the piliferous zone, on the cell wall surface of root hairs, with apolar adhesion, arranged individually or in small aggregates (Figure 1A). The movement of the bacteria was intense and the shape were filamentous, characteristic of the culture in stationary phase grown in complex DYGS medium (Figure 1A).

After 1 day of inoculation a population increase was recorded and *H. seropedicae* was present on the root hair basis (Figure 1B) and on epidermal cell wall junctions. Already in

this period, bacteria in the curved rod shape typical of *Herbaspirillum* were also observed, indicating the proliferation of bacteria in the new growth condition - diluted MS medium associated to exudates of the pineapple plant. From the 2nd day of bacterium-plant interaction, it was possible to observe increased bacteria distribution in the form of aggregates of different sizes, ranging from 20 to 100 μm in length (Figure 1C, 1D) on the root hairs. Bacteria in rod-shape predominated (Figure 1D), with a cell length of approximately 2 μm and slow movement.

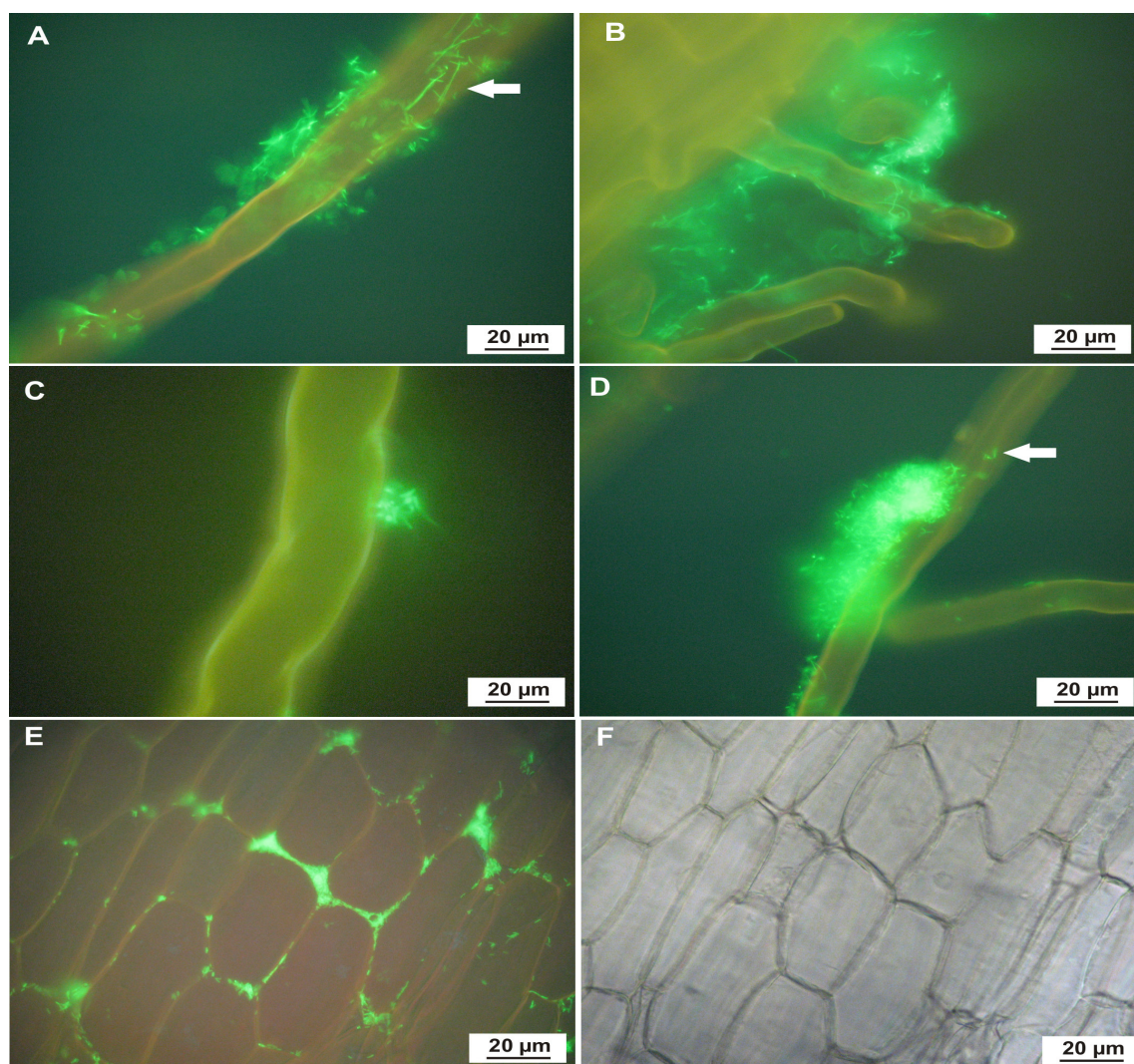


Figure 1. Fluorescence microscopy (A, B, C, D, E) and bright-field microscopy (F) of the initial stages of interaction between *H. seropedicae* RAM10 and pineapple plantlet roots. (A) 6 h after inoculation, predominance of bacteria in filamentous shape (arrow) colonizing preferentially the root hair surface, solitary or in small aggregates. (B) 1 day after inoculation, the bacteria also colonized the root hair base. (C, D) 2 days after inoculation, the bacteria were arranged on the root hair surface in the form of aggregates of different sizes and rod-shaped bacteria were predominant (arrow). (E, F) 7 days after inoculation, bacteria colonized epidermal cell walls junctions.

Between 3 and 7 days after inoculation colonization on the root hairs decreased and bacteria predominated at the root hair basis and on the epidermal cell walls junctions (Figure 1E, 1F). This shift of the predominant colonization site was maintained 14 days after inoculation, where the bacteria showed dominant epiphytic colonization of the entire outer periclinal wall of epidermal cells (Figure 2A) specifically present in the regions close to the emergence of lateral roots (Figure 2B), while

colonization all along the length of the root axis was no longer observed. These observations suggest that the cavities (Figure 2C) formed by the disruption of epidermal cells during the emergence of lateral roots represent a natural opening through which the passive penetration and endophytic colonization of *H. seropedicae* RAM10 occurs in roots of pineapple plantlets. After infection, the endophytic bacteria was established in the intercellular spaces of cortical parenchyma (Figure 2D).

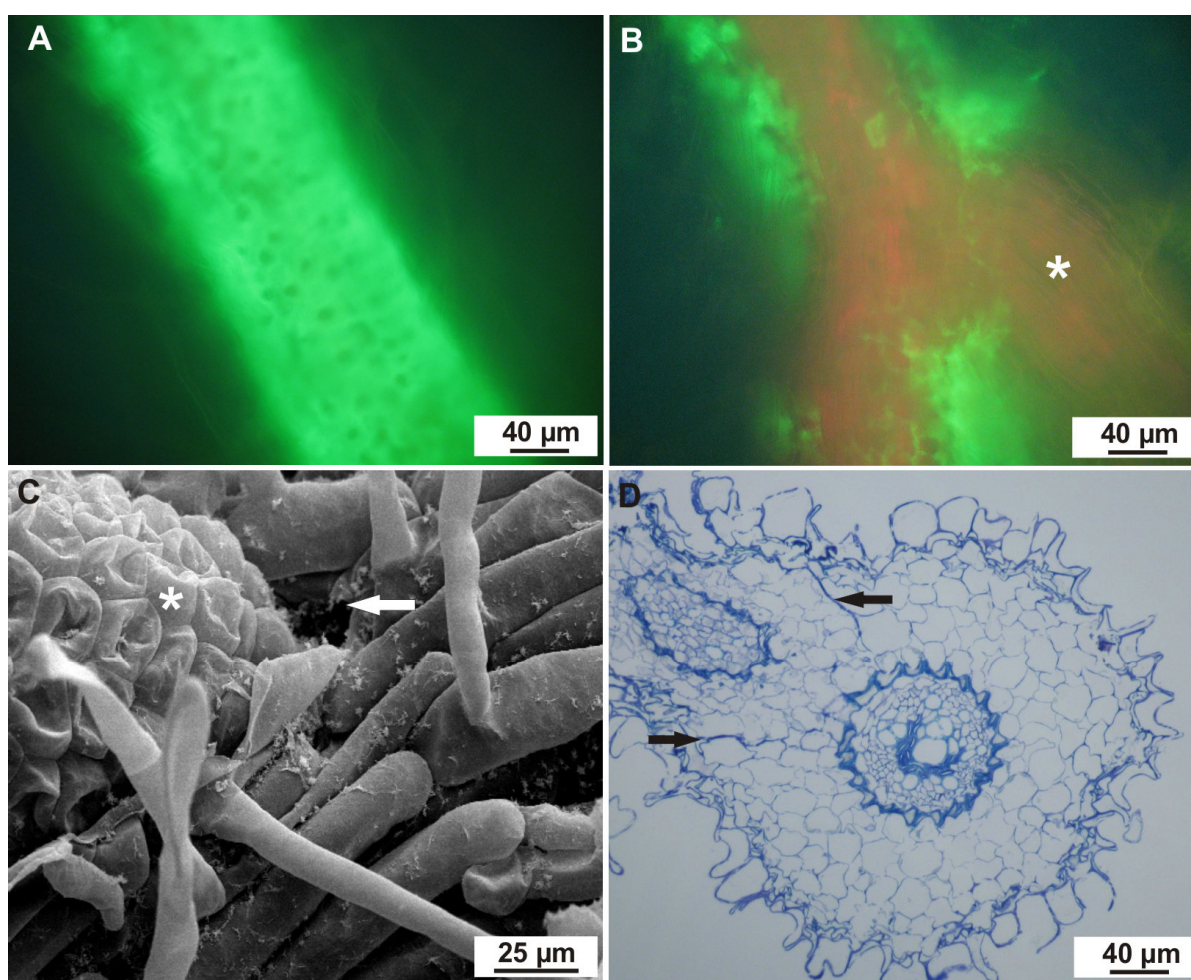


Figure 2. Fluorescence microscopy (A, B), scanning electron microscopy (C) and bright-field microscopy (D) showing the epiphytic colonization, infection and endophytic colonization of *H. seropedicae* RAM10 on pineapple plantlets roots 14 days after inoculation. (A) Intense epiphytic bacterial colonization on the periclinal wall of epidermal cells. (B) Colonization mainly in the regions of emergence of lateral roots (asterisk). (C) Bacterial infection through the cavity (arrow) resulting from the rupture of epidermal cells during the emergence of lateral roots (asterisk). (D) Endophytic colonization (arrow) in the intercellular spaces of cortical parenchyma.

In the more advanced periods of interaction (between 21 and 28 after inoculation), *H. seropedicae* RAM 10 was structured predominantly like biofilm, i.e., large populations with bacteria adhered to one another and to the plant surface by an extracellular matrix, mainly at the root hair base (Figure 3A) and on the outer periclinal wall of epidermal cells (Figure 3B). Endophytic colonization was restricted to the apoplastic compartment with bacteria present in the intercellular spaces of cortical parenchyma, where no bacteria were seen in the vascular cylinder. It is emphasized that from the 21 day after inoculation onwards, no fluorescence from the bacteria was detected and the observations were based on scanning electron

microscopy and bright-field microscopy. Although the bacteria did not emit fluorescence *in situ*, they reassumed fluorescence emission when re-isolated in semi-solid JNFb medium.

During the 28 days of the experiment no changes in pigmentation, morphology and matter gain was detected in the pineapple plantlets. No structural change was also detected in the pineapple plantlets inoculated with *H. seropedicae* RAM 10, in comparison with non-inoculated plantlets. Regarding the means of cultivation, no change in color and turbidity was identified with the naked eye, although there was a decrease in pH to values between 2.8 to 3.5.

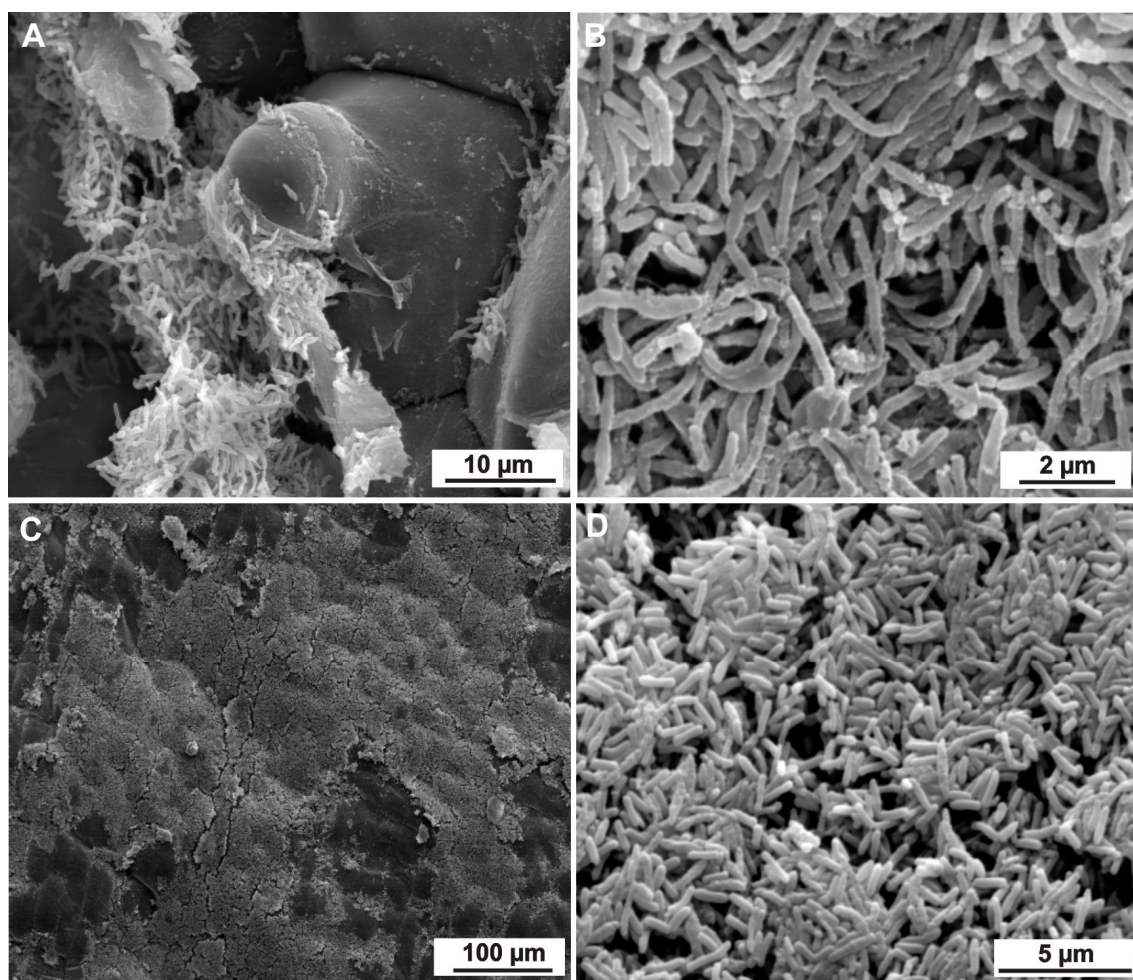


Figure 3. Scanning electron microscopy of biofilms of *H. seropedicae* RAM10 located epiphytically on the root and shoot surface of pineapple plantlets 21 days after inoculation. (A) Biofilms on the root hairs and (B) on the periclinal wall of root epidermal cells. (C) Biofilms on the outer periclinal wall of epidermal leaf cells and (D) detail of the bacterial cells forming the biofilm.

Leaf Colonization

The green fluorescence from the GFP-labeled bacterium *H. seropedicae* RAM10 was also easily distinguished from the red autofluorescence from chloroplasts in the pineapple shoots and leaf blade by fluorescence microscopy (Figure 4).

After 6 h of inoculation, few bacteria were seen in filamentous shape, adhered apolarly to the outer periclinal wall

of epidermal cells. In periods of greater interaction (1 and 3 days after inoculation), the bacteria inhabited preferentially non-glandular trichomes (Figure 4A, 4B), peltate scutiform trichomes (Figure 4C, 4D, 4E, 4F) and the epidermal cell wall junctions (Figure 5A, 5B, 5C), arranged individually or in small aggregates.

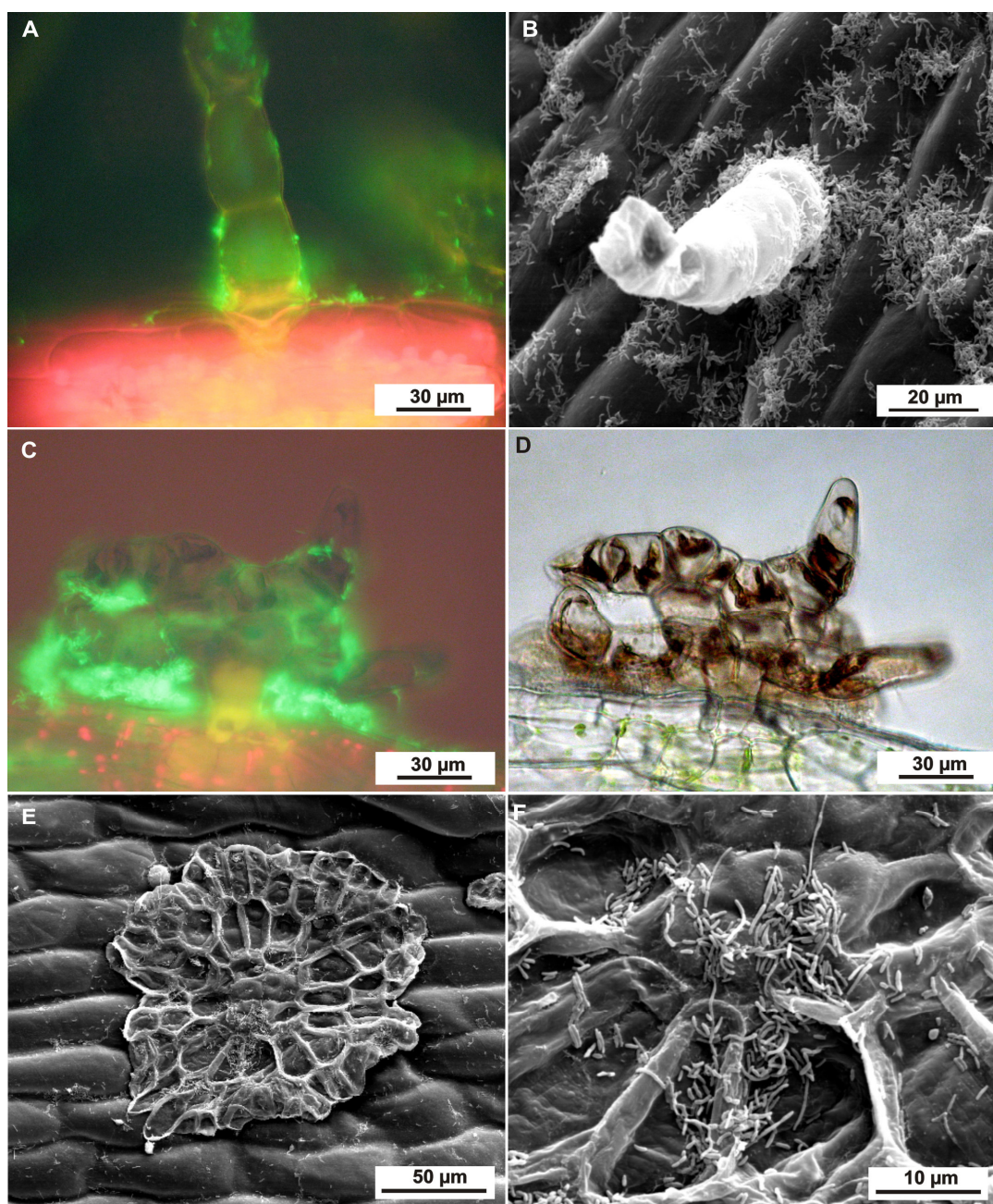


Figure 4. Fluorescence microscopy (A, C), scanning electron microscopy (B, E, F) and bright-field microscopy (D) of leaf epiphytic colonization of *H. seropedicae* RAM 10 preferably on the trichomes of pineapple. (A, B) Bacterial colonization on and at the base of non-glandular trichomes. (C, D, E, F) Bacterial colonization on and at the basis of peltate scutiform trichomes.

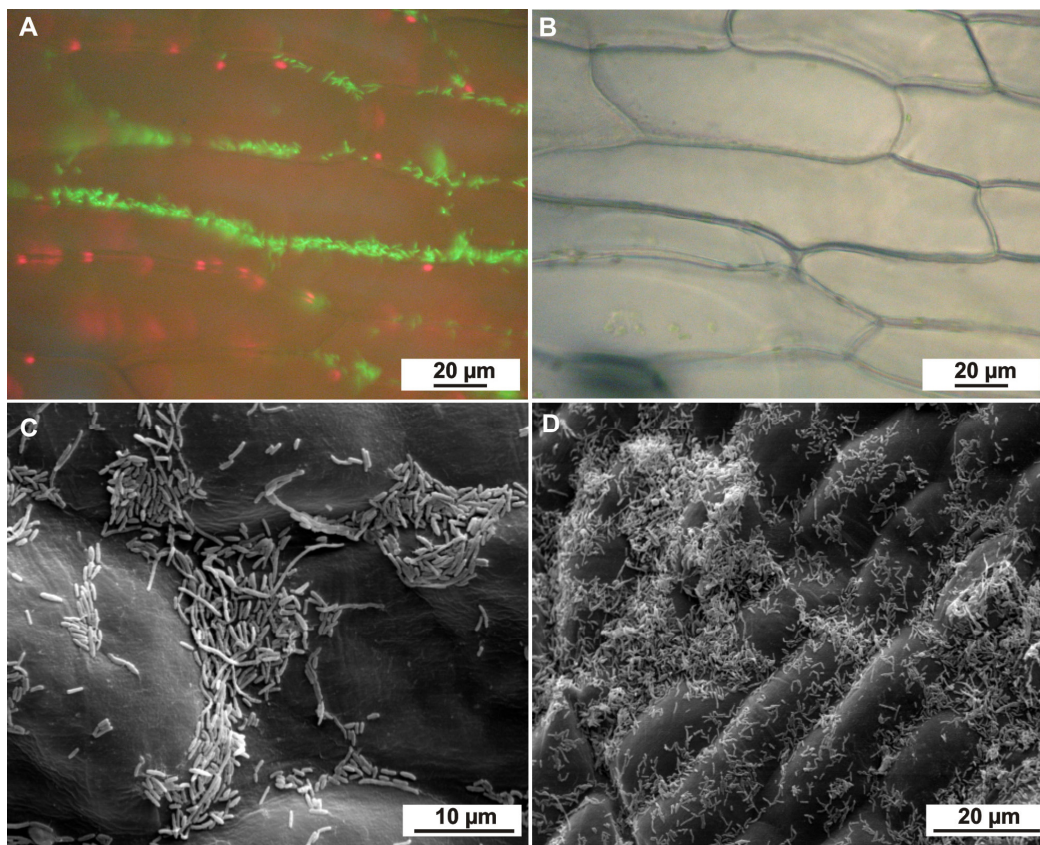


Figure 5. Fluorescence microscopy (A), bright-field microscopy (B) and scanning electron microscopy (C, D) of leaf epiphytic colonization of *H. seropedicae* RAM 10. (A, B, C) Bacterial colonization in epidermal cell walls junctions and (D) on the outer periclinal wall of epidermal cells.

Seven and 14 days after inoculation, larger epiphytically bacterial aggregates were observed not only on the trichomes and epidermal cell wall junctions, but also on the outer periclinal wall of the epidermal cells (Figure 5D) and in the vicinity of the stomatal complexes (Figures 6A, 6B). It was found that the penetration of *H. seropedicae* RAM10 into shoots of pineapple plantlets occurs passively via stoma (Figure 6B, 6C). The endophytic colonization however begins in the substomatal chamber (Figure 6C) and spread through the intercellular spaces of spongy chlorenchyma of the leaf mesophyll (Figure 6D). There was no bacterial colonization on the vascular bundles of the leaf.

On the 21 and 28th day after inoculation, scanning electron microscopy showed bacterial biofilm on the outer periclinal wall of epidermal cells (Figure 3C, 3D). All images of the first 3 days after inoculation were taken at the basal region of the

leaf blade, thereafter (7 to 28 days after inoculation), bacterial colonization was also observed in the median region of the leaf blade, indicating a base-to-apex direction of epiphytic colonization along the longitudinal leaf axis.

Population dynamics

The *H. seropedicae* RAM10 population increased in the first seven days after inoculation, reaching a maximum of approximately 10^{10} cells per gram fresh weight of pineapple plantlet on the 3rd day after inoculation (Figure 7). Subsequently, the population declined until the 20th day after inoculation and the bacteria were established on the host plantlet with a population of approximately 10^6 cells per gram fresh weight of pineapple 'Vitória'. No bacterial growth was detected on the control plantlets.

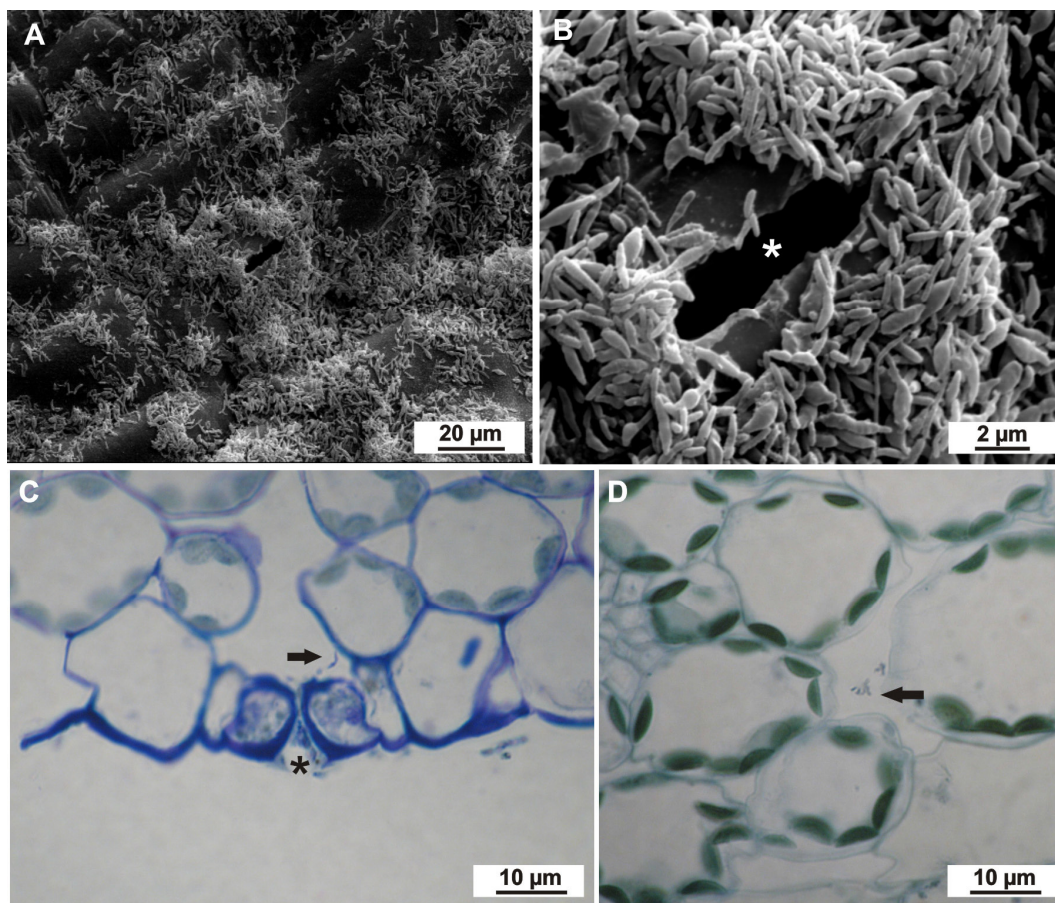


Figure 6. Scanning electron microscopy (A, B) and bright-field microscopy (C, D) of epiphytic colonization, infection and endophytic colonization of *H. seropedicae* RAM 10 in shoots of pineapple plantlets. (A) Epiphytic colonization of bacteria on the surface of the outer periclinal walls of epidermal cells. (B) Bacterial colonization on the stomatal complex and infection via stoma (asterisk). (C) Bacterial infection through stoma (asterisk) and endophytic colonization in the substomatal chamber (arrow). (D) Endophytic bacterial colonization in the intercellular spaces of the chlorophyll parenchyma (arrow).

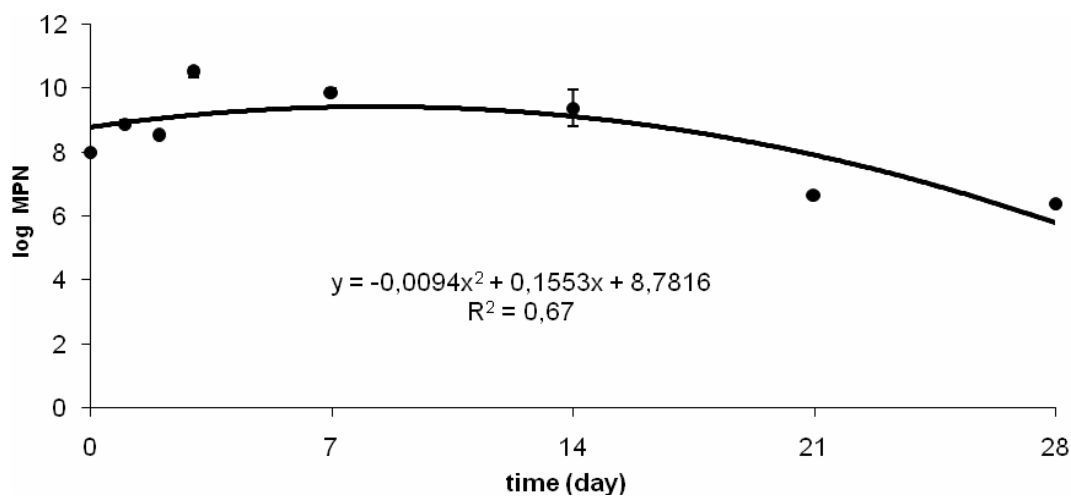


Figure 7. Log of the most probable number (MPN) of *H. seropedicae* RAM10 on pineapple plantlets 'Vitória' in response to time.

DISCUSSION

Through different microscopic techniques this study identified the stages of the structural interaction between *H. seropedicae* RAM10 and *in vitro* pineapple plantlets. The initial colonization (6 h after inoculation) of *H. seropedicae* on the roots of pineapple plantlets occurs preferentially in the piliferous zone with predominantly apolar bacterial adherence to root hairs and subsequent formation of small aggregates. This step was transient, because after the 3rd day of inoculation the bacteria were no longer easily detected on the root hair surface, but rather at their basis and mainly on the epidermal cell walls junctions. The cell walls junctions (middle lamella) that are pectin and calcium-rich sites favor bacterial adherence, since calcium is the mediator between the negatively charged bacteria and plant surfaces, an adhesion mechanism already described for nitrogen-fixing bacterium *Rhizobium leguminosarum* (33).

Later (7 to 14 days after inoculation), *H. seropedicae* epiphytically colonized predominantly the outer periclinal wall of epidermal cells in regions near the emergence sites of lateral roots. These regions are infection sites widely reported in the literature for *H. seropedicae* in association with plants of the family Poaceae, as reported in maize after 30 minutes of inoculation (20), in rice after 2 days of inoculation (14), in sugarcane after 4 days of inoculation (15), and in rice, sorghum, maize and wheat after 5 days of inoculation (30). However, root colonization by *H. seropedicae* does not occur in all host plants. Ebeltagy *et al.* (11), for example, observed that in rice *H. seropedicae*, strain B501, colonizes only seeds of *Oriza sativa* cv. Sasanishiki and preferably the shoot and not the roots of *Oriza officinalis*, indicating that the structural interaction depends on the bacterial strain as well as on the genotype of the host plant.

After penetration of *H. seropedicae* into roots of pineapple plantlets the endophytic establishment occurs only in the intercellular spaces of cortical parenchyma. No bacterial colonization was observed in the stele, indicating that in this case the endodermis was an effective barrier to the radial

bacteria spreading. James *et al.* (14) also reported that the endophytic colonization of *H. seropedicae* in rice roots occurs preferentially in the intercellular spaces of the cortical parenchyma and aerenchyma, and rarely in the stele. In maize, Monteiro *et al.* (20) also reported that *H. seropedicae* colonizes the apoplastic of the cortical parenchyma, but unlike other studies, the authors found that 3 days after inoculation the bacteria had colonized the endodermis and the xylem vessels.

On the pineapple shoots, *H. seropedicae* initially colonizes the epidermal cell walls junction, peltate scutiform trichomes and non-glandular trichomes. At these sites the bacteria is better protected against the hostile conditions of the leaf surface and the nutrient availability is greater. In fact, Baldotto and Olivares (5) investigated microbial colonization on the phylloplane of 47 plant species in tropical environment and found that the presence of trichomes is the most important anatomical feature that favor epiphytic bacterial establishment. This colonization pattern is not exclusive to *H. seropedicae*. Biosensors that detect sucrose and fructose show that the epidermal cell walls junctions, trichomes, veins and stomata are the preferred sites for the carbon metabolism of *Erwinia herbicola* on the phylloplane of *Phaseolus vulgaris* (18).

Thereafter, *Herbaspirillum seropedicae* colonized the outer periclinal walls of epidermal cells, the surroundings of the stomatal complexes and penetrated passively into the leaves via stoma aperture. The endophytic colonization began in the substomatal chamber and spread to the intercellular regions of spongy parenchyma of the pineapple leaves. In rice, Ebeltagy *et al.* (11) observed that the penetration of *Herbaspirillum* sp. inoculated artificially on seeds occurs in young, not yet fully expanded leaves and that endophytic colonization occurs via apoplast. Olivares *et al.* (24), however, reported a hypersensitivity response to the *H. seropedicae* inoculate in sugarcane shoots.

After 21 and 28 days of inoculation bacterial biofilm were observed on root and leaf surface of pineapple plantlets also showing the (*rhizo* and *phyllo*) epiphytic nature of *H. seropedicae*. The formation of biofilm contributed to the bacterial persistence on the plant surface (7) since the bacteria

take advantage from the processes of cooperation through the *quorum sensing* system (37, 8). According to Monier and Lindow (19), the survival of *Pseudomonas syringae* on the phylloplane of *Phaseolus vulgaris* under different moisture conditions was higher for bacteria arranged in aggregates than of solitary cells.

In terms of population dynamics of *H. seropedicae* RAM10 on pineapple plantlets an initial population increase and subsequent decline were observed, and a stabilization 22 days after inoculation, at values of approximately 10^6 cells per gram fresh weight. This evidence is based on a study of James *et al.* (14), in which the colonization of *H. seropedicae* Z67 in rice plantlets had the same behavior, 5 to 7 days after inoculation increments of 10^6 and then decreased to values between 10^3 and 10^4 log CFU per gram fresh weight. It is possible that this population dynamics reflects the non-pathogenic nature of *H. seropedicae* and the capacity of each plant genotype to host them.

In this study it was observed that 21 days after inoculation the bacteria no longer emitted fluorescence *in situ*, but reassumed fluorescence emission when re-isolated in semi-solid JNFb medium. Along with the loss of fluorescence, there was a decrease in pH of the culture medium, reaching values below 3.5. The acidification of the culture medium is probably due to exudation of organic acids by pineapple roots (17) and also by the metabolism of the bacteria growing in the medium (28). With the pH decrease the GFP chromophore is protonated, and remains in the non-fluorescent form (12). However, when the bacterium was re-isolated in the semi-solid JNFb medium, where the initial pH of 5.8 gradually increased with bacterial growth, the chromophore was deprotonated and fluorescence consequently detected.

This study describes over the course of time of the interaction between *H. seropedicae* RAM 10 and pineapple plantlets the structural events: adherence, epiphytic colonization, infection and endophytic colonization in shoot and roots. Knowledge of the colonization strategy of *H. seropedicae* on pineapple plantlets is essential for studies that aim to intensify the real use of plant growth-promoting bacteria

in agricultural systems.

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