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Endophytic bacteria in rice seeds inhibit early colonization of roots by *Azospirillum brasilense*

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Abstract

From the rhizoplane of *Oryza sativa,* vars. Morelos A-88 and Apatzingan, rice plantlets, we isolated two bacterial strains: *Corynebacterium flavescens* and *Bacillus pumilus.* By scanning electron microscopy, endophytic bacteria were frequently identified at the base of secondary roots, between the epidermis and the mucilaginous layer. Endophytes were also identified in the intercellular spaces when the mucilaginous layer was disrupted. These endophytic bacteria were not pathogenic when assayed on tobacco leaves. Plantlets from the rice varieties cultured gnotobiotically under hydroponic conditions were inoculated with *Azospirillum brasilense,* 6-81 or UAP-154 strains. Control experiments were performed using non-inoculated plantlets or plantlets previously treated with nalidixic acid. Comparison of the length of inoculated or nalidixic acid-treated plantlets, with non-inoculated plantlets revealed a significant ($p < 0.05$) promotion of the growth of the shoots at 15 days of culture in plantlets colonized exclusively by endophytes. *A. brasilense* seems to be excluded from the rhizoplane by the endophytic bacteria, suggesting that endophytes compete with *Azospirillum,* and also that *A. brasilense* inhibits growth of rice. Our results indicate that endophytic bacteria could participate in the growth and development of rice plants.

Keywords: Endophytic bacteria; *Corynebacterium flavescens; Bacillus pumilus; Oryza sativa* seeds; *Azospirillum brasilense*

1. Introduction

Lower and higher plants have endophytes that can be found intracellularly, such as the endosymbiotic rhizobia, either as obligate or in facultative association, as for example, arbuscular micorricic fungi (Harley, 1983; Hinton and Bacon, 1985). In many cases, plant-endophytic bacteria do not damage the host organism (Khush and Bennett 1992; Misaghi and Donndelinger, 1990; Quispel, 1992); sometimes, endophytic bacteria are even potential sources of resistance against pathogenic agents, such as fungi (Benhamou et al., 1996).

To understand the biology of plants and their microbial ecology, many studies performed with endophytes have focused on evaluating the colonization pattern of vegetative tissues, as well as the effects of endophytes on plant growth (Patriquin and Dobereiner, 1978). These studies have been performed by inoculating the host plants with endophytic bacteria, and comparing the inhibition of disease symptoms (Poon et al., 1977). Therefore, endophytic bacteria have been considered as potential biocontrol agents (Chen et al., 1995). The exact mechanism by which bacteria induce protection in the host plants remains unclear, although production of siderophores, metabolites with anti-fungal activity, or competition for nutrients and exclusion from the ecological niche of colonizing microorganisms have been suggested as possible mechanisms (Chen et al., 1995).

Inoculating seeds or plants with microorganisms has been successfully used for the control of pathogens. Effective fixing of atmospheric nitrogen to the plant and promotion of plant growth inducers depend on an efficient colonization of the rhizosphere (Bull et al., 1991; Van Peer and Schippers, 1989; Weller, 1988). The use of plant growth promoting rhizobacteria (PGPR), such as *Azospirillum, Pseudomonas,* and bacilli, as bio-fertilizers. has been

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inconsistent (Boddey and Dobereiner, 1982; Elmerich, 1984). These inconsistencies could result from the competition between the inoculated colonizing bacteria and the pathogenic or saprophytic organisms present in the soil environment (Basham and Holguin, 1997; Benhamou et al., 1996; Hurek et al., 1994; Mundt and Hinkle, 1996; Weller, 1988). In the present work we identified endophytic bacteria in rice seeds and evaluated their effects on early colonization of *Azospirillum.*

2. Material and methods

2.1. Seeds and bacteria

The Departamento de Microbiologia, Universidad Autonoma de Puebla, donated *Azospirillum brasilense,* 6-81 strain and wild strain UAP-154. The rice, *Oryza sativa,* seeds, vars. Morelos A-88 and Apatzingan were from INIFAP-Zacatepec, Morelos. Potted plants of *Nicotiana tabacum* petit Havana SRI were obtained at the Escuela Nacional de Ciencias Biologicas, IPN, Mexico.

2.2. Cultivation of plants

Seeds of *O. sativa,* var. Morelos and Apatzingan, were superficially sterilized by vigorous shaking at 25°C for 7 min in a solution containing 30 ml of commercial sodium hypoclorite, 1 g of Na_2CO_3, 30 g of NaCl, and 1.5 g NaOH per liter of distilled water (Hurek et al., 1994). The seeds were then washed several times with sterile water, and treated with 0.5% $HgCl_2$ in water for 7 min at 25°C under vigorous shaking (Sriskandarajah et al., 1993), washed with distilled water and then incubated with 1% chloramine in water during 25 min; afterwards, they were washed with sterile water and imbedded in sterile water during 24 h at 25°C. To allow germination and to detect the presence of microorganisms, seeds were aseptically transferred to agar plates containing a medium with the following composition (per liter): 250 g potato (boiled and filtrated); 10 g D-glucose; 10 g peptone and 15 g agar. For seedling germination the agar plates were cultured at 28°C for three days in the dark, and the plantlets were aseptically transferred to a hydroponic system of culture with Hoagland's (Hoagland, 1975) nutrient solution diluted 1:2 and containing (per liter): 0.12 g $NH_4H_2PO_4$; 1.4 g $Ca(NO_3)_2 \cdot 4H_2O$; 0.2 g $CaCl_2 \cdot H_2O$; 0.5 g $MgSO_4 \cdot 2H_2O$; 0.6 g KNO_3; 3 mg H_3BO_3; 4 mg $CuCl_2 \cdot 2H_2O$; 0.6 mg $ZnCl_2$; 0.003 mg $NaMoO_4 \cdot 2H_2O$; 6.7 mg EDTA; 5 mg $FeSO_2 \cdot 7H_2O$. All solutions prepared for hydroponic cultures were sterilized to keep the plants under gnotobiotic conditions.

2.3. Bacterial identification

Plantlets from *O. sativa,* vars. Apatzingan and Morelos, in axenic conditions were cultured for 15 days. Roots were then washed under aseptic conditions with sterile water three times, weighed and then transferred individually to thread cap tubes containing 16 ml sterile water, the roots were shaken at 100 rpm, 1 h at 25°C, in an orbital shaker. Samples of 50 µ1 of the supernatant of this root-water suspension were cultured in plates containing Py culture medium (Sigma Fine Chem., St. Louis, MO, USA) at 28°C. To quantify and characterize the bacteria, the plates were read at 24 and 48 h, a third reading was performed two weeks after culturing the bacteria. Identification of the isolated strains of *Bacillus* and *Corynebacterium* from roots, was confirmed by different parameters: *Bacillus* was identified by the Graber's criterion and confirmed with API 50 CBH (Graber, 1970). *Corynebacterium* was identified by catalase and oxidase activity, and oxidation-fermentation according to previously described procedures (Barrow and Feltham, 1993; Jones and Collins, 1986; Misaghi and Donndelinger, 1990).

2.4. Participation of endophytes in radical colonization

The plantlets from each rice variety were inoculated with *A. brasilense* 6-81 or *A. brasilense* UAP-154 grown obtained at exponential growth phase in Py culture medium at 108 CFU/ml of Hoagland's medium. Inoculated plants and controls (without *A. brasilense*) were maintained for 15 days under a 12 h dark/light cycle at 26-30°C. The length of the shoots was measured and then, the roots were rinsed in sterile water and transferred individually to thread cap tubes containing 16 ml sterile water. For identification of bacterial strains present in the rhizoplane, the roots were shaken at 100 rpm 1 h at 25°C in an orbital shaker, and 50 µl of the supernatant of this root-water suspension was cultured in plates containing Py culture medium at 28°C, the plates were read at 48 h, and the bacteria were identified as described previously. Participation of endophytes in each experiment was determined by identifying and counting the microorganisms present in the rhizoplane, results are reported in the number of CFU/g of wet root. Control experiments were performed with non-inoculated groups of plantlets, and plants cultured in Hoagland's nutrient solution supplemented with 35 ppm of nalidixic acid to identify plant growth in the absence of endophytic bacteria. Each experiment was performed in triplicate. We assessed rhizospheric competence, as an indicator of radical colonization, between the inoculated and the endophytic bacteria (Hozore and Alexander, 1991). For this purpose we evaluated characteristics and number of bacteria present in the rhizoplane of the rice plantlets inoculated or not with *Azospirillum* 15 days after culture.

2.5. Scanning electron microscopy *(SEM)*

The roots from rice at 15 days of culture were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 2 h at 4°C, and postfixed in 1 % (w/v) osmium tetroxide in the same buffer for 2 h at 4°C. The fixed roots were dehydrated in a graded ethanol series. Then the samples

Table 1
Presence of bacteria on the rhizoplane and comparison of the growth of the plantlet shoots from *O. sativa* var. Apatzingan (* *statistical significance ($p < 0.05$) when compared with the average length of non-inoculated plants. Inoculations were performed with 10^8 CFU/ml of *A. brasilense* 6-81 or UAP-154 in Hoagland's medium)

	Plants inoculated								
	A. brasilense 6-81			*A. brasilense* UAP-154			Non-inoculated		
Shoot length (cm)[a]	19.5**	20.7	21.2	16.3**	16.6**	19.6**	25.2	25.8	27.5
Presence of bacteria[b]									
C. flavescens	10^4	10^6	ND	ND	10^7	10^4	10^7	10^8	10^8
B. pumilus	10^6	ND	10^7	10^4	ND	10^7	10^9	10^6	10^7
A. brasilense	10^7	10^6	10^5	10^8	10^7	10^3	ND	ND	ND

[a] Results are the average of the length of three plantlets.
[b] To quantify and characterize the bacteria, the roots were weighed and transferred to tubes containing sterilized water, and shaken at 100 rpm 1 h at 25°C. 50 µl of this root-water suspension were cultured in plates containing Py culture medium at 28°C for 48 h. Identification and counting of bacterial species were performed as indicated in Section 2. Results are reported as CFU bacteria/g fresh root. ND, non-detected bacteria after 48 h of culture in PY culture medium. Control experiments with plantlets cultured in the presence of nalidixic acid showed average lengths of 13.2 ± 0.6 cm, and no bacteria were detected.

were treated with CO_2 and mounted on an aluminum cylinder with silver paste, and finally covered with a steam of carbon and ionized gold (Nowell and Parules, 1980). The samples were examined under a SEM (Zeiss DSM-950 operated at 25 kV at an 8-10 mm distance). Cellular morphology of endophytes and of inoculated *Azospirillum* was determined by SEM in previously purified groups of bacteria.

2.6. Pathogenicity test

The pathogenic activity of isolated *C. flavescens* and *B. pumilus* was evaluated according to Klement (1963). To induce infection, we infiltrated 10^7 CFU of each bacterium in the intercellular spaces of an old leaf from *Nicotiana tabacum*, and evaluated the presence of necrotic zones after two weeks. Control tests of pathogenicity were performed using *Pseudomonas syringae*, pv. syringae at the same cell density. Statistics were calculated using one-way ANOVA.

3. Results

3.1. Isolation and characterization of endophytic bacteria

Axenic plantlets were obtained from *O. sativa*, vars. Morelos A-88 and Apatzingan, seeds that had been disinfected superficially and cultured gnotobiotically for several time intervals up to 30 days. Two endophytic bacteria were isolated from the Py nutrient culture medium used. These bacteria were isolated from the rhizoplane and identified by biochemical tests, as *Corynebacterium flavescens* and *Bacillus pumilus*. *Bacillus* was identified by the Graber's criterion and confirmed with API 50 CBH, indicating that bacteria were positive for milk peptonization, utilization of citrate, and for reduction of arabinose, glucose, and mannitol. Starch hydrolysis and nitrate reduction were negative for the identified group of Bacillus. *Corynebacterium* was glucose positive; however, arabinose, xylose, rhamnose, lactose, maltose, sucrose, trehalose, raffinose, salicin, and starch were negative. The methyl red test was positive, and esculin and urea hydrolysis were negative. Culture of plantlets was repeated under the same conditions using several

Table 2
Presence of bacteria on the rhizoplane and comparison of the growth of the plantlets shoots from *O. Sativa*, var. Morelos A-88 (**statistical significance ($p < 0.05$) when compared with the non-inoculated plants. Inoculations were performed with 10^8 CFU/ml of *A. brasilense)*

	Plants inoculated								
	A. brasilense 6-81			*A. brasilense* UAP-154			Non-inoculated		
Shoot length (cm)[a]	14.0**	16.2**	17.2**	17.5**	19.6	21.8	24.3	24.6	25.0
Presence of bacteria [b]									
C. flavescens	ND	10^4	ND	ND	ND	10^5	10^7	10^8	10^8
B pumilus	ND	10^6	10^7	ND	10^9	10^8	ND	10^6	10^7
A. brasilense	10^8	10^4	10^5	10^8	10^5	10^4	ND	ND	ND

[a]Results are the average of the length of three plantlets.
[b] Identification and counting of bacterial species were performed as indicated in Section 2. For details see Table 1. ND, non-detected bacteria after 48 h of culture in PY culture medium. Control experiments performed with plantlets cultured in the presence of nalidixic acid showed average shoot lengths of 12.8 ± 0.2 cm, and no bacteria were detected.

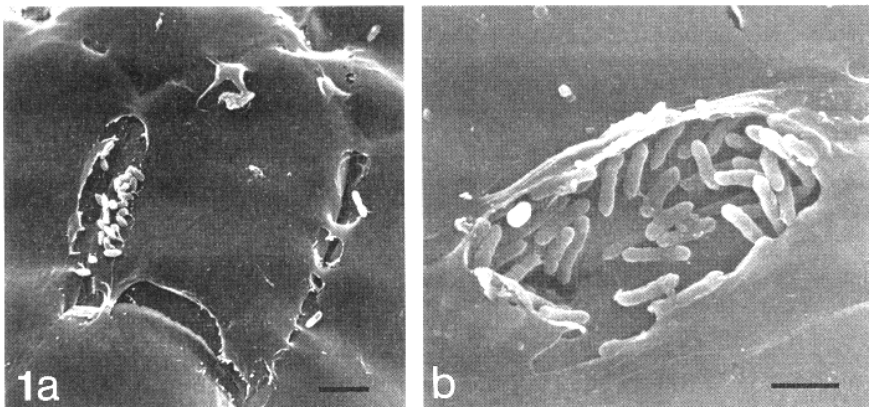

Fig. l . Location of endophytic bacteria on the rice of non-inoculated plants. (a) Bacteria were observed in disrupted zones of the mucigel covering the grooves formed by the junctions among the epidermal cells. Bar = 5 μm. (b) High magnification of endophytic bacteria localized between the mucigel coat and the epidermis of secondary roots. Bar = 2 μm. SEM.

sources of the Morelos and Apatzingan varieties of rice: results were consistent, yielding *C. flavesceus* and *B. pumilus* in all assays.

3.2. Participation of endophytes in radical colonization

To evaluate the role of endophytic bacteria in early events of colonization by *A. brasilense* and their participation in the growth of the rice plant, we measured the length of the plant shoots after 15 days, and compared this parameter with the presence of *Azospirillum* and endophytes in the rhizoplane of the plants. As shown in Tables 1 and 2, presence of *A. brasilense* induced a significant inhibition ($p < 0.05$) in rice plantlets growth. There were also differences in the inhibitory effect for *A. brasilense* in the rice varieties: the Morelos variety is more sensitive to the inhibitory effects of *A. brasilense* A 6-81 (Table 1), whereas *A. brasilense* UAP-154 is a stronger inhibitor for the Apatzingan variety plantlets (Table 2). Control experiments performed with axenic plants cultured with Hoagland's solution supplemented with nalidixic acid revealed no bacteria in the rhizosphere, but these plantlets showed a lower growth rate ($p < 0.05$) than plants containing two endophytic groups of bacteria or non-inoculated groups of plants (Tables 1 and 2).

3.3. Localization and distribution of endophytic bacteria on roots

The rice roots from 3 to 15 days old plantlets were examined by SEM. Results indicate that in non-inoculated plants, endophytic bacteria were localized on the grooves formed by the junctions among the epidermal cells, beneath the mucigel coat that frequently showed disrupted zones (Fig. la); the rhizoplane of secondary roots is covered by endophytes masked by mucigel coat (Fig. 1 b). Three days later, the plants inoculated with *A. brasilense* showed clusters of these bacteria in the emerging zones of the secondary roots, which induced disruption of cortical and epidermal tissues (Fig. 2a). At day 15 after inoculation, the effect of

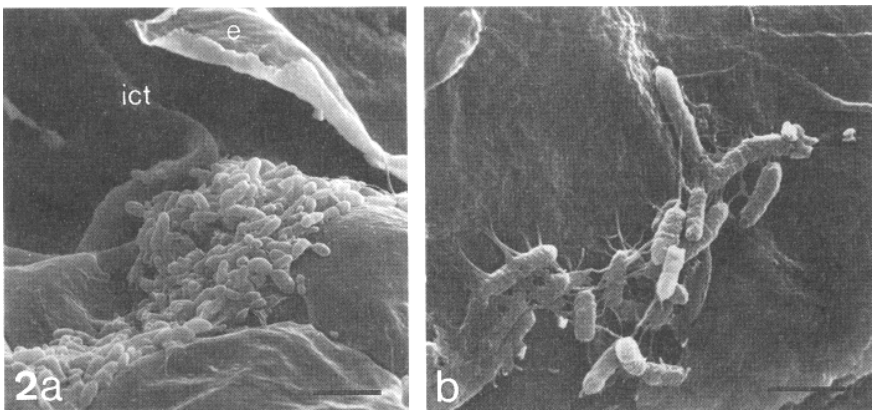

Fig. 2. Location of endophytic bacteria and *Azospirillum brasilense* on the rice roots of inoculated plants. (a) At three days old inoculated rice plants, clusters of *A. brasilense* were localized on the base of emerged secondary roots. Epidermis of primary root (e) and cortical tissues (ict) were also observed. Bar = 5 μm. (b) Fifteen days after culture, the roots were colonized preferentially by endophytic bacteria, which showed adhesion filaments among them, and with the rhizoplane. Bar= 2 μm. SEM.

endophytes on plant growth was inhibited by competence with *A. brasilense.* Moreover, endophytic bacteria adhered among them and attached to the rhizoplane through slender, probably, mucilaginous filaments (Fig. 2b).

3.4. Pathogenicity test

Results indicate the absence of hypersensitivity in the tobacco plant to the infection by endophytic bacteria isolated from rice seeds. No visible necrotic zones were identified in leaves two weeks after inoculation; however, *P. syringae* pv syringae, used as control, induced necrotic zones on the leaves two days after infection with this bacterium.

4. Discussion

In this study we confirmed the presence of *B. pumilus* and *C. flavescens* as endophytic bacteria in rice seeds. Bacteria emerge to the rhizosphere even during early germination of the seeds. These endophytes were not pathogenic for tobacco leaves and played a relevant role in rice root colonization. Indeed, the endophytic bacterium showed potential capacity to promote growth of the rice plantlets. The presence of these endophytes seems to exert in rice, and very probably in plants in general, a regulatory function on the interaction of the plant with other components of the rhizosphere (Hallmann et al., 1997); furthermore, our results strongly suggest that *A. brasilense* inhibits growth of rice.

According to our results, endophytic bacteria seem to be uniformly distributed on the rhizoplane of the root; although, we identified the greatest density of bacteria at the intercellular junction. This finding is probably due to the fact that intercellular regions represent more space and opportunity for the movement of endophytes; besides, very probably the mucilaginous layer, which covers the epidermis of the root, has a lower tension in these regions (Bowen, 1979). Previous reports indicate that, at the intercellular regions, there is an important increase in the concentration of carbon as a source of energy, thus explaining the preference of bacteria for this part of the root (Bennett and Lynch, 1981; Bowen, 1979). It has been suggested that microcolonies could develop on the surface of the epidermal cells and on the cellular junctions (Bowen, 1979).

Results indicate the presence of many filaments cross-linking the endophytic bacteria and with the rhizoplane, suggesting a structural compatibility between endophytes and the vegetal cell wall. Evidence of a specific interaction of cyanobacteria with plant roots has been found with Nostoc 259B. This bacterium specifically interacts with wheat roots through a sequence of three neutral sugars and glucuronic acid; this interaction allows for an efficient colonization and exclusion of other colonizing cyanobacteria (Gantar et al., 1995). We identified high densities of endophytic bacteria in emerging zones from the lateral roots and, particularly, in the basal parts. This finding agrees with other studies indicating that these parts of the roots are highly susceptible to disruption, causing the release of endophytes (Agarwhal and Shende, 1987; Jacobs et al., 1985).

Up to date, much research has been focused on the effects induced by plant growth promoting rhizobacteria (Anderson, 1983; Kloepper and Beauchamp, 1992); information concerning the role of endophytic bacteria in plant growth indicates that their beneficial effects are due to the antagonic activity exerted against bacterial pathogens (Basham and Holguin, 1997; Benhamou et al., 1996; Van Buren et al., 1993), and by stimulating vegetal growth (Hallmann et al., 1997; Hurek et al., 1994). In this study, we evaluated the role of endophytes in rice growth and the effect of *A. brasilense* by correlating the bacteria identified in the rhizoplane with the length of the plantlet shoot of non-inoculated or inoculated with *A. brasilense* plantlets. Control experiments were performed with plantlets cultured in the presence of nalidixic acid in order to eliminate the effect of endophytic bacteria. As the results indicate, *A. brasilense* seems to inhibit plant growth, since addition of this bacterium induced a significantly lower growth than in non-inoculated plants. Plantlets treated with nalidixic acid showed the lowest growth rate, strongly suggesting that plant growth is positively stimulated by the presence of endophytes (Kloepper and Beauchamp, 1992).

It is important to note that the presence of two bacterial strains was optimal for plant growth, in hydroponic conditions. This effect could be related to the cellular density; at high densities the beneficial effects of bacteria cannot be developed. The presence of colonizing bacteria seems to represent a competition for the microhabitat as well as for nutrients with endophytic bacteria; hence a combination of colonizing and endophytic bacteria induces a reduction in plant growth. Besides, the possibility exists that endophytic bacteria could be preferentially attracted by root exudates produced by the rice plants, resulting in specific interactions between rice and endophytic bacteria (Bacilio, 1997). The ability of the exogenous bacterium, *Azospirillum*, to influence plant growth seems to depend on the amount of rhizospheric populations present in the rhizoplane, although we emphasize the fact that this colonizing bacteria seems to inhibit rice growth (Bacilio, 1997; Fallik et al., 1988; Patriquin et al., 1983). The exact mechanism used by the endophytic bacteria to penetrate and colonize the endorhizosphere of the rice seeds remains unclear, and more studies are needed to understand better the mechanism of penetration as well as the exact role of endophytic bacteria. In plants, such as *Pisum sativum*, *B. pumilus* has been demonstrated to be an endophyte of the roots, acting as a defensive barrier at the cell wall level, and inducing production of an antifungal environment (Benhamou et al., 1996). SEM confirmed the presence of endophytic bacteria, able to colonize in great extent the root surface. The present results clearly indicate the need to identify the presence and participation of endophytic bacteria in seeds

used for extensive culture before adding colonizing bacteria to induce plant growth in hydroponic conditions (Bruijn et al., 1995).

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