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Ecology of a plant growth-promoting strain of *Pseudomonas fluorescens* colonizing the maize endorhizosphere in tropical soil

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***Pseudomonas fluorescens* strain BR-5 stimulated the growth of maize in a natural soil and inhibited fungal root pathogens *in vitro*. Strain BR-5 was detected inside plant cells, indicating that it is able to colonize the endorhizosphere. No significant effect was detected on soil or ectorhizosphere microbial population after inoculation of strain BR-5 onto seeds.**

Key words: Maize growth, *Pseudomonas fluorescens*, rhizosphere.

Fluorescent *Pseudomonas* spp. are prevalent in the rhizosphere of plants (Schroth & Hancock 1982; Lambert *et al*. 1987; Lemanceau *et al*. 1995). Certain bacteria of this group are called plant growth promoting rhizobacteria (PGPR) (Schroth & Hancock 1982) because they are able to promote plant growth. These bacteria can colonize different parts of roots, including internal tissues such as the cortex (Darbyshire & Greaves 1971). Araújo *et al*. (1994, 1995) reported that fluorescent *Pseudomonas* are prevalent in the rhizosphere of maize grown in a tropical soil. They isolated several strains of *P. fluorescens* and *P. putida* and tested them for colonization and survival in the rhizosphere. A rifampicin-resistant mutant derivative of *P. fluorescens* designated BR-5, chosen as a model for risk assessment in tropical soils, survived and colonized bulk soil and maize rhizosphere (Araújo *et al*. 1994, 1995). Tropical soils are typically acidic and have a high concentration of Al^{+3} and low concentrations of Ca^{+2} and Mg^{+2}.

Fluorescent *Pseudomonas* spp. are important for biological control. Certain strains can suppress diseases caused by phythopathogenic fungi (Weller & Cook 1983; Thomashow & Weller 1988) and are candidates as hosts for the delivery of genes, such as biocontrol toxins, to the plant rhizosphere (Obukowicz *et al*. 1986; van Elsas *et al*. 1991; Araújo *et al*. 1995). In temperate soils *P. fluorescens* controlled phytopathogenic root diseases (Thomashow & Weller 1988; Pierson & Weller 1994). Nevertheless, little is known about the behaviour of these bacteria in tropical soils. Some isolates of *P. fluorescens* from the northeastern region of Brazil suppressed damping-off caused by *Rhizoctonia solani* of cowpea (*Vigna unguiculata*). The CR-20 isolate was the most efficient against the disease even in field conditions (Barbosa *et al*. 1995). Since the incidence of root diseases is greater in tropical soils than in temperate soils, the utilization of PGPR could have applications in agriculture. However, it is important to understand the effect of these bacteria as inoculants and to know whether they cause changes in the microbial community structure in the rhizosphere and the soil.

In this study we analysed a *P. fluorescens* strain BR-5 for its effect on maize growth, its localization in root tissues, the effect of inoculation on soil microbial populations and the capacity of inhibiting phytopathogenic fungi *in vitro*.

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Materials and Methods

The effect of P. fluorescens *BR-5 on plant growth and soil microbial communities*

The soil experiment was performed in 12 plastic pots each containing 5 kg of yellow red Latosol from Rio de Janeiro in a

randomized experimental design over a period of 90 days and exposed to weather changes. Soil analysis were performed to determine fertilizers and lime necessities. There were two systems: maize seeds (Brazilian cultivar BR-106) inoculated with strain BR-5 and uninoculated. For each system, six replicates were used. Seeds were surface-sterilized by immersion in 95% ethanol (1 min), $HgCl_2$ 1:500 (3 min) and 10 washes with sterile distilled H_2O. Seeds were coated with an inoculum peat that was prepared as follows: *P. fluorescens* BR-5 was grown in 50 ml of Luria–Bertani medium for 48 h at 28 °C and 20 ml was transferred to 50 g of sterile peat. It was incubated at 28 °C for 48 h. Seeds were coated with the inoculum containing 1×10^8 c.f.u. of BR-5/g of peat and dried for 2 h. An uninoculated system was used as the control. The seeds were coated with sterile peat. Each pot received 10 maize seeds and after 7 days only six healthy plants/vessel were maintained.

The effect of introduction of BR-5 on rhizosphere microbial populations was determined by sampling 10 g of rhizosphere soil (root and root-adhered soil) in 90 ml of a solution containing 0.1% sodium pyrophosphate and 0.1% Tween 80 (Wollum 1982) by stirring. Heterotrophic bacteria and *Pseudomonas* spp. were enumerated on plate colony counts of trypticase soy agar and S_1 medium (Gould *et al*. 1985). Nitrifying and cellulolytic populations were enumerated by MPN counts (Alexander 1982; Wollum 1982). Dehydrogenase enzyme activity, an indirect measure of microbial activity, was determined as described by Tabatabai (1982).

Plant growth was evaluated as foliar dry weight after drying for 48 h at 60 °C. Growth measurements and microbiological studies were conducted at the 0, 30, 60 and 90 days after planting and data were analysed by Statistix 4.0 software.

Association of P. fluorescens *BR-5 with plant root tissue*

For optical microscopy, disinfected maize seeds inoculated with 1×10^8 c.f.u./g of *P. fluorescens* BR-5 in peat, or not inoculated, were planted in sterilized pots containing vermiculite and incubated in a controlled growth chamber at 25 ± 1 °C with a 16:8 h photoperiod (light/dark). After 21 days, 2-cm long root segments near the seeds were washed with sterile distilled H_2O and incubated in 0.15% tetrazolium solution (Patriquin & Döbereiner 1978) for 24 h, thin sections were cut and stained with Astra blue to observe red-marked bacteria inside plant tissues.

For transmission electron microscopy studies, 2-cm long root segments near the seeds were washed with sterile distilled H_2O, fixed with glutaraldehyde, post-fixed with osmium tetroxide and dehydrated with acetone. The specimens were embedded in Spurr resin, ultrathin sections made and stained by uranyl acetate solution and lead citrate and observed with a Philips 301 electron microscope.

Liquid scintillation analysis was used to detect strain BR-5 inside the root tissues. Two systems were used in sterilized pots containing vermiculite: disinfected seeds inoculated with strain BR-5 labelled by growing with [^{35}S] methionine and an another system with unlabelled strain BR-5.

Bacteria for inoculation were grown in 20 ml of minimal medium. The bacterial cells were labelled with [^{35}S] methionine at a final concentration of 50 μCi. Bacterial cultures were grown for 48 h at 28 °C and then centrifuged three times at 5000 rev/min in sterile 0.85% NaCl. The pellets were analysed in a liquid scintillator (Beckman LS 6000 SC). 1 ml of each pellet was added to 9 ml of 1% carboximethylcellulose. Labelled bacteria were inoculated on seeds and the pots were incubated in a controlled growth chamber at 25 °C ± 1 °C with a 16:8 h photoperiod (light/dark). After 35 days, the presence of bacteria was detected in 2 cm root segments by radiation analysis in a liquid scintillator (Beckman LS 6000 SC) with Aquasolve scintillation solution (PPO, 5.0 g; POPOP, 0.1 g; Triton X, 100, 333 ml; toluene, 667 ml).

In vitro *antagonistic activity of* P. fluorescens *BR-5 against phytopathogenic fungi*

In vitro antagonistic activity of *P. fluorescens* BR-5 was tested by methods described by Carruthers *et al*. (1994) and Panthier *et al*. (1979). The methodology of Panthier *et al*. (1979) was modified by changing the top actinomycetes medium to PDA potato dextrose agar) medium and using the following phytopathogenic fungi: *Fusarium oxysporum*, *Fusarium graminearum*, *Aspergillus parasiticus* and *Chaetomium globosus*.

Results and Discussion

Inoculation with *P. fluorescens* BR-5 had a significant effect on maize growth (Table 1). Foliar dry weight is a simple way of evaluating plant development according to their capacity to accumulate biomass because more developed plants have a higher photosynthetic rate.

Inoculation with *P. fluorescens* BR-5 had no significant effect on soil microbial communities or on soil dehydrogenase enzyme activity (Figure 1). No deleterious effect of *P. fluorescens* BR-5 as a seed inoculant was detected, suggesting that the equilibrium in the rhizosphere habitat was not affected by the presence of BR-5, even at a high population.

The increase observed in the analysed heterotrophic, *Pseudomonas* spp., nitrifying and cellulolytic communities and dehydrogenase activity could be caused by BR-5 stimulation of plant growth (Figure 1). More developed plants increase root exudate and consequently stimulate communities which are in the rhizosphere.

The recovered population of *P. fluorescens* BR-5 was less than 1×10^4 c.f.u./g dry soil in soil experiments. A low recovered population was expected because it is known that introduced microorganisms do not survive very long in soil (Gaskins *et al*. 1985; van Elsas 1992). High temperature and low rainfall during the experiment (summer 1994/1995) could have stressed strain BR-5 and caused the low recovery. Hartel *et al*. (1994) observed that a *P. putida* strain and its genetically modified derivative populations decreased when they were submitted to high temperature and desiccation. At 35 °C,

Table 1. Plant growth promotion by inoculation with *Pseudomonas fluorescens* strain BR-5 (g dried foliar weight).

Days After planting	Uninoculated seed	Inoculated seed
30	0.92	0.93
60	7.10	10.97*
90	0.16	4.34*

* Tukey test, difference at the 5% level.

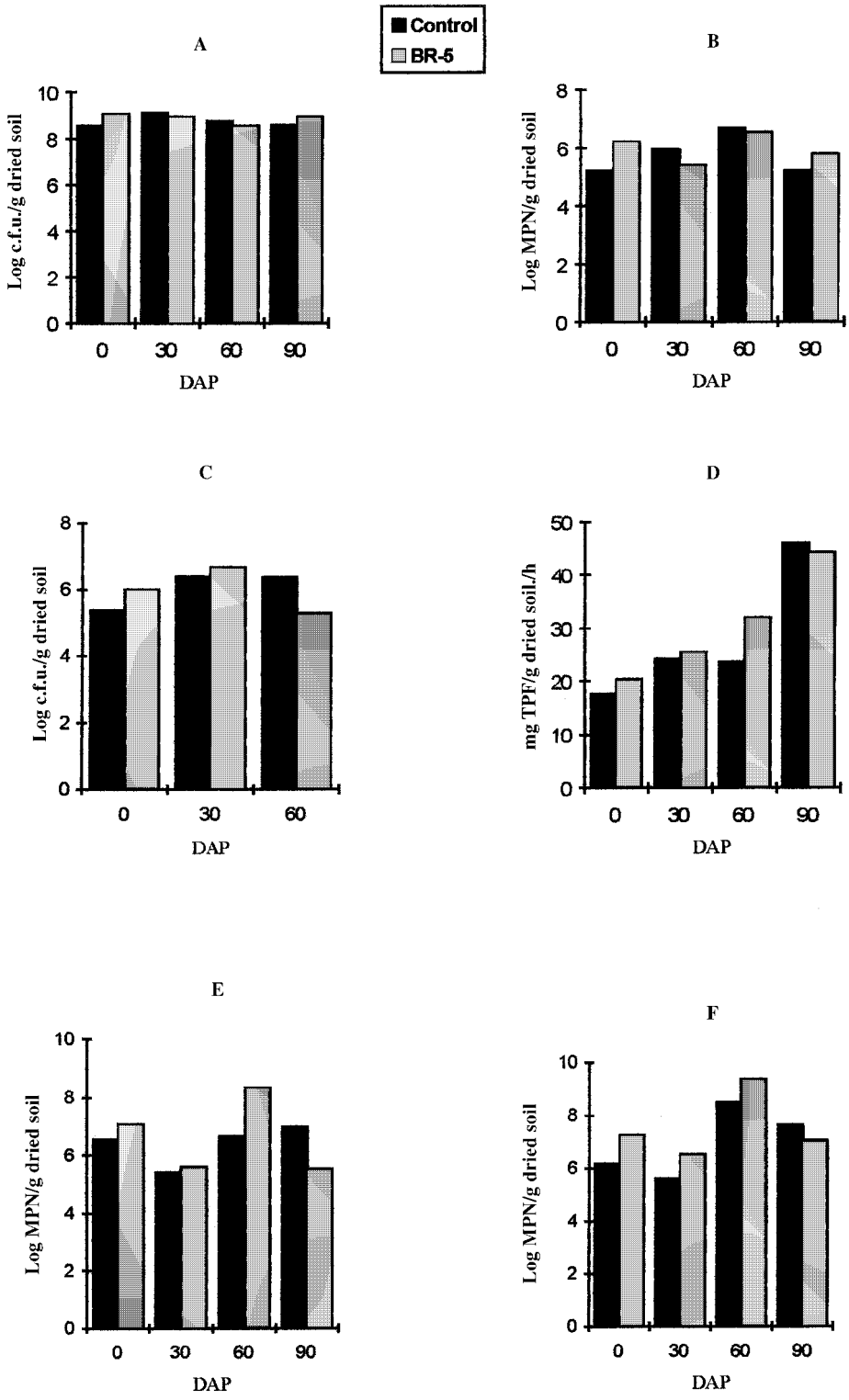

Figure 1. The effect of *P. fluorescens* BR-5 inoculation on microbial communities. (A) Heterotrophic bacteria; (B) cellulolytic bacteria; (C) *Pseudomonas* spp.; (D) soil dehydrogenase enzyme activity; (E) ammonium-oxidizing bacteria; (F) nitrite-oxidizing bacteria. c.f.u, Colony forming units; TPF, triphenylforman; MPN, most probable number; DAP, days after planting.

the original strain density decreased from 1×10^8 to 1×10^3 c.f.u./g dry soil and its derivative population decreased from 1×10^8 to less than 10 c.f.u./g dry soil.

Araújo *et al*. (1994, 1995) have observed that *P. fluorescens* BR-5 and its genetically modified derivative, BR-12 which received Tn*5*:TOX-cryIVB (van Elsas *et al*. 1991), had good survival and colonized maize roots in soil microcosms. This agreed with our results which proposed to investigate whether a root colonizer could also be a plant growth stimulator. The inoculation effect of the BR-5 population on plant growth was observed beginning with the early growth. However, it became statistically significant only at 60 and 90 days after plating (DAP) (Table 1), especially at 60 DAP when plants had reached maximum development.

The low BR-5 population in the rhizosphere and its plant growth stimulation suggested that it could colonize the inner tissues of the plant. Optical and transmission electron microscopy of internal plant anatomy showed gaps in the cortex of inoculated plants (Figure 2). The gaps could be explained by the increase of plant respiratory rate of inoculated roots. The gaps could be used to store air to supply the respiratory demand of roots or they could be formed by the release of substances similar to plant hormones or their forerunners by inoculated bacteria, as known for some *Pseudomonas* species (Ankenbauer & Cox 1988).

Transmission electron microscopy indicated the presence of BR-5 in the root tissues (Figure 3) and the level of radioactivity along the roots from seeds inoculated with

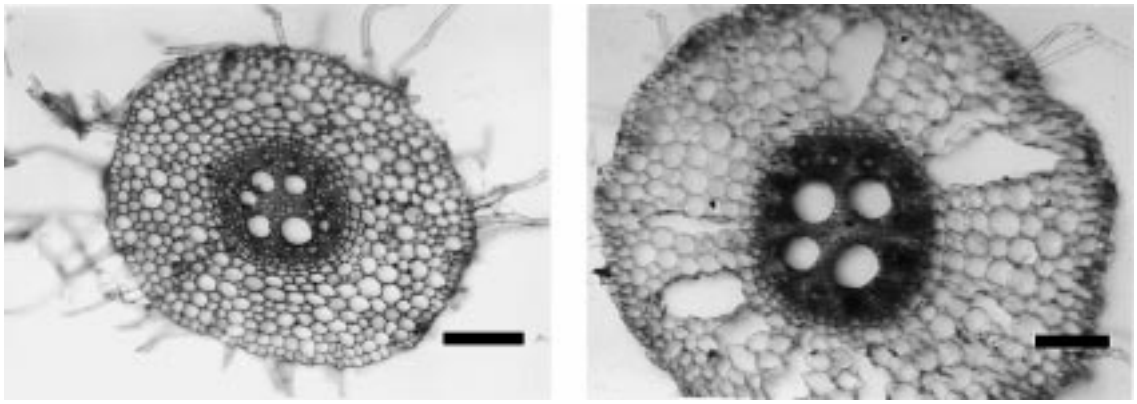

Figure 2. Anatomic differences between inoculated and uninoculated systems (left, uninoculated system; bar = 68.3 μm; right, inoculated system; bar = 68.2 μm).

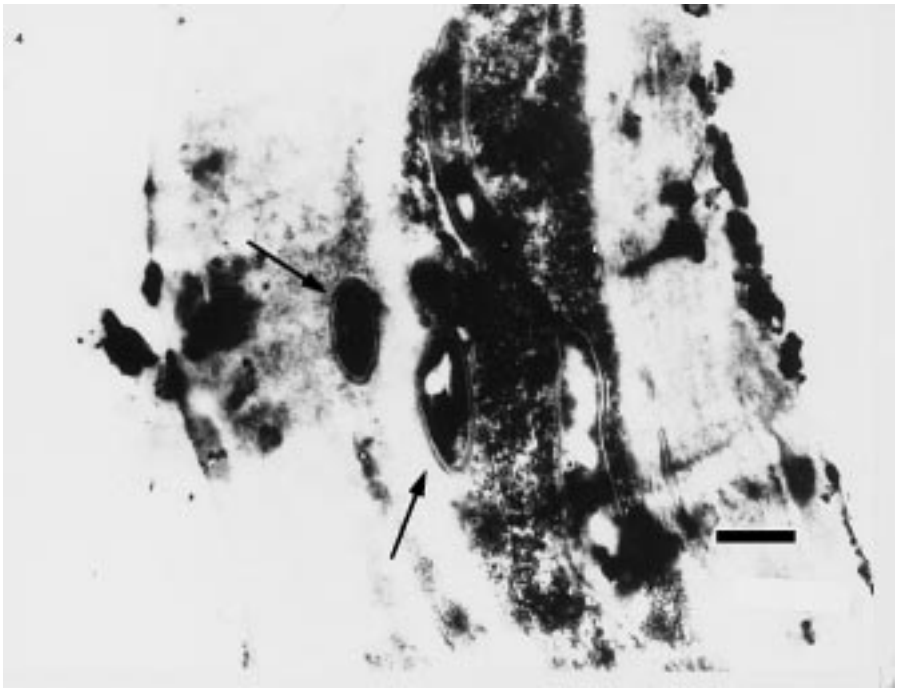

Figure 3. The presence of bacteria on root tissues of inoculated systems (transmission electron microscopy) (bar = 0.4 μm).

labelled BR-5 confirmed its presence (Table 2). This confirmed that *P. fluorescens* BR-5 was present inside root tissues in sterilized systems. Since the strain was capable of entering root tissues, metabolic exchange between the plant and the bacteria could explain the growth-promoting effect in inoculated plants.

The initial analysis of the *P. fluorescens* BR-5 biocontrol capacity was performed *in vitro*. Bacterial effect on development of the tested fungi was detected. It was observed that strain BR-5 was able to inhibit *F. oxysporum*, *F. graminearum*, *A. parasiticus* and *C. globosus* development *in vitro* (Table 3). This suggested that strain Br-5 can produce fungistatic or fungicidal substances *in vitro* which inhibited these fungi. The mechanism could be siderophore production (Kloepper *et al*. 1980) because of the observed heavy production of characteristic pigment on S_1 medium (Gould *et al*. 1985) plates.

The ability to inhibit phytopathogenic fungi that was noted for *P. fluorescens* strain BR-5 *in vitro* may be another mechanism stimulating maize growth. This strain could decrease the density of phytopathogens and nutrient competitors, supporting better development of the plants (Weller & Cook 1983; Thomashow & Weller 1988; Carruthers *et al*. 1994; Pierson & Weller 1994).

Table 2. Distribution of [^{35}S] methionine-labelled *Pseudomonas fluorescens* strain BR-5 in maize root segments after 35 days growth.

Root sections (cm)	Radioactivity (%) *
0–2	0
2–4	ND
4–6	ND
6–8	ND
8–10	11
10–12	3
12–14	ND
14–16	2
16–18	19
18–20	9
20–22	56

* Radioactivity = 8×10^3 d.p.m. inoculated seed. ND Not detected.

Table 3. *In vitro* inhibition of phytopathogenic fungi by *P. fluorescens* strain BR-5.

Fungi	BR-5 Innoculation density (c.f.u./ml)		
	0	10^4	10^7
A. parasiticus	3*	0	1
F. oxysporium	3	0	1
F. graminearum	3	0	2
C.globosus	3	1	3

*Inhibition degree: 0, total inhibition; 1, > 50% inhibition; 2, < 50% of inhibition; 3, no inhibition.

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