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Colonization of pepper roots by a plant growth promoting *Pseudomonas fluorescens* strain

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Abstract The effect of a *Pseudomonas fluorescens* strain (Aur 6) isolated from *Lupinus hispanicus* on pepper seedlings (*Capsicum annum* cv. Roxy) was studied. This strain had already shown its capacity as a plant growth promoting bacteria (PGPB) on seedlings of *Lupinus albus*, *Pinus pinea* and *Quercus ilex*. Strain Aur 6 significantly enhanced all biometric parameters measured: fresh weight, height, neck root diameter and slender index (height/neck root diameter). The PGPB effects could be related to auxin and siderophore production, as strain Aur 6 produced substances of both classes in pure culture. For a further monitoring of Aur 6 in the rhizosphere, strain-specific monoclonal antibodies were developed. Using the strain-specific it could be proofed that Aur 6 is an effective and persistent colonizer of pepper roots, but it is not able to behave as an endophyte.

Keywords Plant growth promotion · Pepper · *Pseudomonas* · Monoclonal antibodies · Biofertiliser

Introduction

Since 1978, when Kloepper and Schroth (1978) reported that fluorescent pseudomonads promoted early growth of radish and potatoes by up to 500%, numerous examples of plant growth stimulation have been reported (Brown et al. 1964; Merriman et al. 1975; Kloepper et al. 1980; Chanway et al. 1991; Probanza et al. 1996; Cattelan et al. 1999). Current environmental criteria designed to improve crop and forest productivity, together with the

development of a sustainable agriculture and silviculture (Swaminathan 1991; Van Elsas et al. 1998), and the intended reduction of the use of fertilizers and pesticides have produced an ongoing increase in scientific interest and practical use of plant growth promoting bacteria (PGPB) as inocula.

These effects are due mostly to a non-infective interaction of root-associated bacteria with plants, which has some symbiotic features. The interaction between *Azospirillum* and some plants is one of the most important ones (Bashan and Holguin 1997). The specificity of this non-infective symbiosis is not absolute, and therefore, a certain strain can behave as a PGPB in different plants such as pine (Probanza et al. 2001, 2002), or pepper (Bashan 1998; Govedarica et al. 1997), which is certainly an advantage in the successful use of PGPBs as inocula.

Saprophytic *Pseudomonas* are common root-colonizing bacteria that can improve plant growth or health. Efficient exploitation of these bacteria in agriculture requires knowledge of traits that enhance ecological performance in the rhizosphere (Rainey 1999).

However, in some cases, the use of bacteria in agriculture has not had the success expected, since some bacteria may find the environment different and hostile. Therefore, before the deliberate use of a PGPB-bacterium as an inoculum or biocontrol agent, it is necessary to know some key parameters such as root colonization capacity, location of infection and degree of persistence of the inoculum (Wiehe and Höflich 1995). These parameters must be studied under the most realistic conditions possible.

The aim of this work was to study the colonization capacity, persistence and colonization pattern (rhizosphere and endorhizosphere) of a PGPB *Pseudomonas fluorescens* strain isolated from roots of *Lupinus albus* on pepper (*Capsicum annum* cv. Roxy), using strain-specific monoclonal antibodies and to correlate colonization behaviour of this strain and enhanced plant growth. The strain used in this work, Aur 6, already showed its capacity to promote the growth of *L. albus* seedlings in gnotobiotic conditions (Gutierrez Mañero et al. 2003).

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Nevertheless, these conditions were removed from real conditions where the inoculum strain has to compete with a wide variety of soil microorganisms. Therefore, experiments under real conditions are necessary to clarify if the strain is able to promote the growth of plants under real soil conditions and can be applied as an inoculum.

Materials and methods

Bacterial strain

The bacterium used in this study is deposited in the Colección Española de Cultivos Tipo (CECT 5,398). Strain Aur 6 was isolated from the rhizosphere of *L. albus* (Gutiérrez Mañero et al. 2003); it was selected among 576 strains sampled from the rhizosphere of *L. albus*, *Lupinus luteus*, *Lupinus angustifolius* and *Lupinus hispanicus* (Lucas García et al. 2001) for the strong growth stimulation exerted by the bacterium growth culture media free of bacterium on *L. albus* var. Multolupa seedlings (Gutiérrez Mañero et al. 2003). Aur 6 was identified as a strain of *P. fluorescens* biotype C by fatty acid methyl ester patterns (Sherlock Microbial Identification System, Microbial ID, Newark, N.J.).

For the determination of the specificity of the raised monoclonal antibodies, reference strains were obtained from the Colección Española de Cultivos Tipo CECT and *Pseudomonas* sp. isolates from the culture collection of the University of San Pablo CEU. All strains were grown on nutrient broth medium (Difco).

Plant growth promoting capacity

Aur 6 was tested for auxin production [indole acetic acid (IAA)-like substances], using the method of Benizri et al. (1998). Siderophore production was measured using the chrome azurol S approach (Alexander and Zuberer 1991). Phosphate solubilization capacity (de Freitas et al. 1997) and hydrolysis of 1-aminocyclopropane-1-carboxylate (ACC) (Glick et al. 1995) were tested accordingly.

Production of strain-specific monoclonal antibodies against *P. fluorescens* Aur 6

The production of strain-specific monoclonal antibodies was performed according to Schlöter and Hartmann (1996). Female BALB/c mice, 6–8 weeks old, were immunized by intraperitoneal injection of 10^8 bacteria, which had previously been inactivated by UV treatment. Three similar injections were made every 10 days. X63Ag8 cells were fused with spleen lymphocytes from one of the immunized mice. Culture supernatants were screened for the content of antibodies against Aur 6 by enzyme-linked immunosorbent assay (ELISA) 14 days after the fusion. Cells from positive wells were recloned and the culture supernatants were tested again. Hybridomas showing consistent production of specifically reacting antibodies were subcloned.

Specificity of the monoclonal antibodies

Cross reactivity tests were performed with hybridoma cell lines showing promising antibody production, according to Schlöter and Hartmann (1996) and Schlöter et al. (1997), using the ELISA procedure with a peroxidase-coupled secondary antibody and 2, 2'-azino-di[3-ethylbenzthiazoline sulphonic acid] (Boehringer Mannheim) as substrate. An ELISA plate photometer (OPSYS MR Dynex Technologies, USA) at a wavelength of 405 nm was used.

Plant cultivation and inoculation

Two hundred pepper (*Capsicum annum* cv. Roxy, provided by Almeriplant and manufactured by Syngenta) seedlings (one seedling per pot), which showed fully developed cotyledons with no true leaves and all of the same height, were inoculated with 1 ml of Aur 6 suspension at 10^8 colony-forming units (cfu). This suspension was prepared from a stationary phase bacterial culture of Aur 6 growing in nutritive broth (Difco), in which the bacterium concentration reached 10^{10} cfu. The enumeration of the inoculum and calculations were carried out following the drop method (Hoben and Somasegran 1982). The culture was centrifuged (350 g for 10 min), washed with sterile distilled water, and pellets were resuspended in sterile distilled water to achieve 10^8 cfu ml⁻¹. A further 100 seedlings were not inoculated and were used as a control. Plants were grown in pots (45 cm³) containing blond peat, the same peat used by companies that produce plants for fruit production. Plants were maintained under controlled conditions (25/20°C, 14/10 h light/dark, achieving 350 $\mu\text{E m}^{-2} \text{s}^{-1}$) in a culture chamber (Sanyo MLR-350H).

Harvest and preparation of rhizosphere and endorhizosphere

Fifteen, 30 and 45 days after inoculation, plants were harvested. At each sampling time ten plants among those inoculated were harvested at random. Five were used to quantify the inoculated bacterium in the rhizosphere soil and the other five to quantify the inoculated bacterium in the endorhizosphere. Each plant was considered a replicate.

Roots were shaken in PBS for 10 min to separate the soil adhering to them (rhizosphere soil). Afterwards, rhizosphere soil was sonicated in an ultrasonicator Selecta at 19 kHz for 7 min. To determine the number of bacteria in the root tissue (endorhizosphere), the root was incubated for 5 min in 1% chloramin T solution and washed overnight in PBS at 4°C (Baldani et al. 1986; Schlöter and Hartman 1998). Afterwards, roots were homogenized in distilled water.

At the end of the experiment (45 days after inoculation) all plants were harvested and the following data were collected: fresh weight, height, neck root diameter and slender index (height/neck root diameter).

This experimental design was repeated twice, and the data shown are the means of the two experiments.

Quantification of cfu in the rhizosphere and endorhizosphere

cfu were determined on nutrient agar (Difco) plates after an appropriate dilution in sterile-distilled water and counted after 24 h incubation at 28°C. Three plates per dilution were prepared and the dilution with 20–100 colonies per plate was used.

Determination of *P. fluorescens* Aur 6

Colonies of the plates indicated above were individually sown on plates with nutrient agar medium (Difco). To reduce the number of colonies to be tested in ELISA, strains were classified by Gram-stain into Gram-positive and Gram-negative prior to the ELISA procedure. Bacteria which showed a Gram-negative phenotype were further investigated by ELISA as described above.

Statistical analysis

Unidirectional ANOVA were made on each biometrical parameter, and when differences were significant, the least significant difference test was performed (Sokal and Rohlf 1979).

Results and discussion

Metabolic capacity of *Pseudomonas fluorescens* (Aur 6)

Auxin production of strain Aur 6 after growth in NB medium was estimated to $1.48 \mu\text{g ml}^{-1}$ IAA-like. This strain was able to mobilize iron from chelating agents added to solidified culture media. However, Aur 6 was not able to solubilise calcium phosphate or to use ACC on the only N source. Although the mechanisms by which PGPRs promote plant growth are not yet fully understood, it is clear that auxin and siderophore production are two different mechanisms of plant growth stimulation (Klopper et al. 1986; Patten and Glick 1996; Gutierrez Mañero et al. 1996; Cattelan et al. 1999).

Validation and cross reactivity of the monoclonal antibodies

Overall more than 50 antibody producing cell lines could be established. All obtained monoclonal antibodies (mAbs) were tested against a variety of *Pseudomonas* sp. isolates. These isolates were selected from 576 bacteria that were grouped into clusters with at least 90% similarity indexes, using the molecular markers PCR-RAPDs (Lucas García et al. 2001) and *Pseudomonas* sp. strains provided by the Colección Española de Cultivos Tipo CECT. Two hybridoma cell lines produced strain-specific antibodies against Aur 6 (Au.mAK3.2 and Au.mAK4.15). These antibodies gave a clear signal in ELISA only with the immunogen *P. fluorescens* Aur 6. All other obtained mAbs showed different degrees cross-reactivities with non-target microbes. It seems that Au.mAK3.2 and Au.mAK4.15 bind to strain-specific epitopes of *P. fluorescens* Aur 6. The overall signal in ELISA with the immunogen for Au.mAK3.2 was higher compared to Au.mAK4.15. If this enhanced signal strength of Au.mAK3.2 is due to a higher affinity of the mAb to the antigen or due to different binding epitopes of Au.mAK3.2 and Au.mAK4.15 this has to be investigated in further experiments. For the investigations described in this paper, Au.mAK3.2 was chosen.

Colonization capacity

Figure 1 shows the log of total cfu in the rhizosphere and endorhizosphere and the log of cfu of the inoculated bacterium. There were no differences in the total number

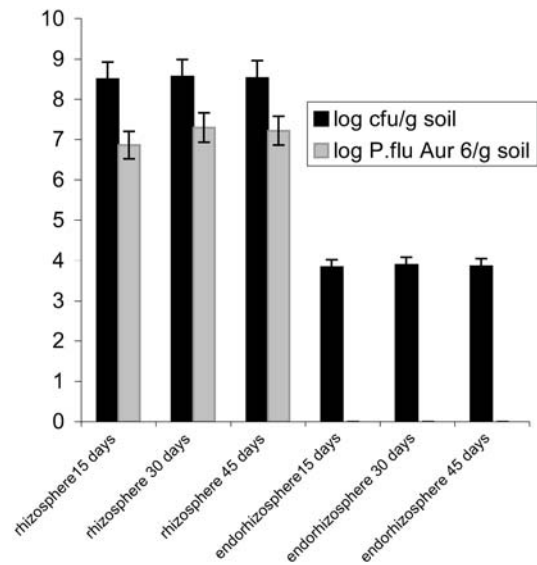


Fig. 1 Log of total colony-forming units (cfu)/g soil and log cfu of inoculated plant growth promoting bacteria [*Pseudomonas fluorescens* (*P.flu*) Aur 6] per gram of soil in the rhizosphere and endorhizosphere of pepper grown on peat

of cfu among different sampling times. However, over time there was an increase in the number of inoculated bacterium. Root colonization capacity and persistence of the inoculum are very important parameters in determining the capacity of a bacterium to be used as a PGPB (Wiehe and Höflich 1995; Chiarini et al. 1998). Our results show that Aur 6 is able to colonize the rhizosphere of pepper and is even able to develop in this environment because we found an increase in the number of cfu from the first sampling time (15 days after inoculation) until the last sampling time (6 weeks after inoculation). Schloter and Hartman (1998), studying the colonization of wheat roots by different strains of *Azospirillum brasilensis* with strain-specific monoclonal antibodies, found that the highest numbers of bacteria were obtained 4 weeks after inoculation, decreasing afterwards. The same results were found by Remus et al. (2000), working with *Pantoea agglomerans* and *Klebsiella pneumoniae* in wheat root, using polyclonal antibodies.

Effects of inoculated bacterium on growth of pepper

Aur 6 increased all biometrical parameters measured. Table 1 shows the results obtained on the growth of pepper inoculated with Aur 6. Inoculated plants showed a

Table 1 Biometrical data on effects of inoculation with plant growth promoting bacteria *Pseudomonas fluorescens* Aur 6 on growth parameters of pepper seedlings. Within a column data followed by different letters are significantly different at $P < 0.05$

	Fresh weight (g)	Height (cm)	Neck root diameter (mm)	Slender index (height/neck root diameter)
Control	4.24±0.25 a	22.26±0.69 a	3.75±0.10 a	6.00±0.19 a
Pepper	5.66±0.20 b	28.30±0.49 b	4.30±0.10 b	6.67±0.17 b

significant increase in the four parameters measured, fresh weight, height, neck root diameter and slender index (height/neck root diameter) with regard to the control. Since Aur 6 has been shown to produce auxins and to mobilize iron, the improved shoot growth could be the final result of improved nutrient uptake and plant nutrition. This strain has also been inoculated, under real conditions, in seedlings of pine and oak, stimulating the growth of these plants (unpublished data).

In conclusion, these results show that Aur 6 has great colonization, adaptive and competitive capacities in the rhizosphere of pepper. These qualities are very important for the use of this bacterium as an inoculum in production conditions. However, the application of inocula in agriculture needs further research to understand better the interactions between plants and microorganisms. Not only is it necessary to provide the right microorganisms, but also the correct techniques to check the fate of the inoculum in order to establish the most suitable way to use the microorganisms in agriculture. The lack of such information has been shown to be the main cause of failure in the use of PGPBs.

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