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SIDEROPHORE PRODUCING *Pseudomonas* AS PATHOGENIC *Rhisoctonia solani* AND B*otrytis cinerea* ANTAGONISTS

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RESUMEN

Pseudomonas aeruginosa, *Pseudomonas putida* biovar B, *Pseudomonas marginalis* y *Burkholderia cepacia*, aisladas de rizosfera y filosfera de plantas de rosa y alstroemeria, identificadas por ensayos bioquímicos y cultivadas en medio King B, mostraron propiedades antagónicas contra los patógenos (se usó medio PDA agar par el cultivo) *Rhizoctonia solani* y *Botrytis cinerea.* Estas propiedades coincidieron con la presencia de un sideróforo, sustancia polar con bandas de absorción en 260 nm y 402 nm. Se observó incremento del crecimiento longitudinal de las plantas, medido sobre el tallo central, por influencia de *P. putida* biovar B, *P. aeruginosa* y *P. marginalis.* El crecimiento de rizomas (a: 0.05) fue notorio bajo la influencia de *P. marginalis*.

Palabras clave: Alstroemeria, Antagonismo, Filosfera, Fito-patógeno, *Pseudomonas sp*, Sideróforo.

ABSTRACT

Pseudomonas aeruginosa, *Pseudomonas putida* biovar B, *Pseudomonas marginalis* and *Burkholderia cepacia*, isolated from the rhizosphere and the phyllosphere of rose and alstroemeria plants, identified by biochemical assays, and cultured in King B medium, showed antagonistic properties, *in vitro*, against the pathogens *Rhizoctonia solani* and *Botrytis cinerea* (PDA agar medium was used for culturing). These properties coincided with the presence of siderophore, a polar substance with bands of absorption at 260 nm and 402 nm. Longitudinal plant growth enhancement, measured on the central stem of alstroemeria, was observed under the influence of *P. putida* biovar B, *P. aeruginosa* and *P. marginalis*. Rhizome growth (a: 0.05) was notable under the influence of *P. marginalis*.

Key words: Alstroemeria, Antagonism, Phyllosphere, Phytopathogenic, *Pseudomonas sp*, Siderophore.

INTRODUCTION

The genus *Pseudomonas*, especially the group of fluorescent *Pseudomonas*, produces secondary metabolites with antagonistic and/or antibiotic activity against various rhizosphere pathogens. Siderophores are themselves growth inhibitors of various phytopathogenic fungi, such as *Phytophthora parasitica*[11, 13, 14], *Phythium ultimum*[4], *Fusarium oxysporum veri dianthi*[3] and *Sclerotinia sclerotiorum*[9]. Siderophores, whose chemical structures depend upon

their producer microorganism, may provide iron (III) to some vegetable cells. These metabolites, due to their antagonistic capability against pathogenic microorganisms, could act as growth factors in plants [1]. The main purposes of the present project were: 1. the evaluation of the vegetative growth promoting action of the fluorescent *Pseudomonas*, isolated from rose and alstroemeria crops; 2. the detection of a possible inhibitory action of fluorescent *Pseudomonas* against phytopathogens; 3. the finding of an easy and rapid detection technique for the siderophore(s); and 4. finding an acceptable and inexpensive culture medium for these microorganisms. It is hoped that this work will contribute to the bio-control of phytopathogens and to the bio-fertilization of rose and alstroemeria crops.

MATERIALS AND METHODS

Cultivation, isolation and identification of microorganisms. Thirty samples, 100 g each, of rhizosphere and phyllosphere from roses and alstroemeria crops, located near the town of Funza, 30 Km from Bogotá, Colombia, with an environmental temperature ranging from 2 °C to 19 °C, were cultured in King B medium, to which sodium G-penicillin 75000 UI and F-Agar were added. Strains showing fluorescence and production of diffusible pigments were selected. Identification of these microorganisms was based on specific biochemical assays for the genus *Pseudomonas*[5].

In vitro* production of siderophores by fluorescent *Pseudomonas. To determine *in vitro* siderophore production, all isolated microorganisms were cultured on King B agar and incubated at room temperature for 48 hours [2].

***In vitro* evaluation of *Pseudomonas* antagonistic activity against *hiroctonia*, R. *solani* and *Botrytis cinerea*.** Among the several *Pseudomonas* strains incubated, four (*Pseudomonas aeruginosa*, *Pseudomonas putida* biovar B, *Pseudomonas marginalis* and *Burkholderia cepacia*) with the greatest production of a water soluble pigment were selected, and seeded at a concentration of 10^6 UFC/mL[8] in PDA medium. Each of the three pathogenic fungi (*hiroctonia*, *solani* and *Botrytis cinerea*) was placed at the center of a 1 cm x 1 cm square area on different plates containing the *Pseudomonas* strains. The plates were incubated for one week at room temperature and the fungal growth zones were measured (in mm) and compared with two controls, here called number 1, containing the assayed bacterium in the medium, and the control number 2, supporting the studied fungus in the same medium. For control number 1, the culturing was done by the stria technique. The growth of the microorganism was classified as good or bad. In control number 2, the invasion of the whole 90 mm plate was taken as necessary to determine the assay confidence (Table 1).

Chromatographic assays on the siderophore. The water soluble pigment produced by *Pseudomonas* was treated with 1.0 mL of the following solvents: methanol, acetone, ethyl acetate, 0.5% hydrochloric acid and ethyl ether, to test its solubility. The substance was dissolved in methanol and developed in a saturated chromatographic chamber. Activated silica gel, non-activated silica gel and alumina were tested as the TLC stationary phase; hexane, dichloromethane, chloroform and ethyl acetate were assayed as eluents. A methanol solution of the dye was examined under the following chromatographic column conditions: Water (pH: 6.0); acid water (pH: 3.0); alkaline water (pH: 10.0); methanol (pH: 7.0); methanol, to which drops of 25% NH_3 were added up to a pH value of 8.0; and methanol, to which drops

TABLE 1. Measurements (mm) of *R. solani* and *B. cinerea* growth zone diameters, against the studied microbial strains.

BACTERIA	FUNGICAL GROWTH DIAMETER (cm)					
	Rhizoctonia solani			*Botrytis cinerea*		
	Mean[1]	Control 1[2]	Control 2[3]	Mean[1]	Control 1[2]	Control 2[3]
P. putida biovar B	21	GBG[4]	90	45	GBG	90
P. marginalis	30	GBG	90	43	GBG	90
P. aeruginosa	53	GBG	90	31	GBG	90
B. cepacia	31	GBG	90	25	GBG	90
P. fluorescens	46	GBG	90	40	GBG	90
P. putida	15	GBG	90	40	GBG	90

1. Mean of the three replicates in the inhibition assay.
2. Control 1: Bacterial growth.
3. Control 2: Fungical growth.
4. GBG: Good bacterial growth in King B medium.

of 99% acetic acid were added up to a pH value of 6.0, were used as eluents in a non-activated silica column. Neutral methanol was used to prepare the columns. The following paper chromatography assays were also carried out: Whatman No.2 paper strips, 7 cm. long and 3 cm. wide, were fed as a stripe with the solution of the pigment in methanol, and the chromatography was then developed in a saturated chamber, using as experimental chromatographic solvents: methanol, distilled water, water-methanol 1:1, water-methanol 3:1, water-methanol 3:2, water- methanol 55:45 and water-methanol–pyridine, 56:47:3. Spots separated by paper chromatography, corresponding to the greenish yellow pigment produced by *Pseudomonas marginalis*, were cut off and extracted with distilled water. The resulting solution was examined under a UV-VIS, Bausch & Lomb, Spectronic 2000 spectrophotometer. The highest absorption band was found at 260 nm.

***In vivo*, fluorescent *Pseudomonas* effects on plant growth and on pathogenic *R. solani*.** Evaluation of antagonistic properties was carried out, in triplicate, by means of a randomized design involving three experimental units. *Pseudomonas* strains, isolated from the rhizosphere, were taken as treatments; with phytopathogenicity, the number of sprouts produced, and rhizome growth considered as variables. The progress of the experiment was followed for one a month.

Experimental culture medium for the isolated siderophore. Molasses, glycerol and glucose were assayed as carbon sources; peptone and soy flour as nitrogen sources. The good behavior of these nutrients was determined by microorganism

growth curves, according to the modified Breed method[10], which includes readings every 2 h, for 24 hours.

RESULTS AND DISCUSSION

Classification of the microorganisms discovered. Fifty one percent of the identified bacteria were of the *Pseudomonas* or *Burkholderia* species; the remaining ones belonged to *Enterobacter*, *Aeromonas*, *Hafnia*, *Cedecia*, *Pantoea*, *Klebsiella* and *Salmonella* spp. The relative abundance of the first two genera can be attributed to their rhizosphere and phyllosphere[3] colonization capacities.

Study of the siderophore. After 24 h of incubation in liquid or solid media, *Pseudomonas aeruginosa*, *Pseudomonas putida biovar B*, *Pseudomonas marginalis and Burkholderia cepacia* showed the presence of siderophores by the appearance of a greenish yellow pigment (Figure 1). Observed siderophore coloring coincides with that described for pioverdines or pseudobactines [6, 7, 12, 14]. The more greenish coloration presented in King B Agar medium by *Pseudomonas aeruginosa* cultures can be attributed, in addition to some pseudobactine, to piocianine-type siderophore[2, 11] production.

The siderophore showed strong water-solubility; it also dissolved well in methanol and acetone; scarcely in a 0.5% solution of HCl and very poorly in ethyl acetate (Table 2). This behavior indicates the polar nature of this siderophore molecule. The compound exhibits some acid character and absence of basic functional groups such as, **:**NH2, **:**NHR, or **:**NR_2; and if it contains one or more of these groups, they may conjugate their pair of non-shared electrons in a delocalized Pi system, or they may have electron acceptor groups as neighbors; so the basic solubility property of the molecule is very low or non-existant. In the event of nitrogen atoms existing in this molecule, the lack of basic properties may be due to the intervention of their non-bonding electron pair into coordination bonds with metals, such as Fe[12]. The low R_f values for siderophore in polar stationary phases (silica gel and alumina) confirm the polar character of the

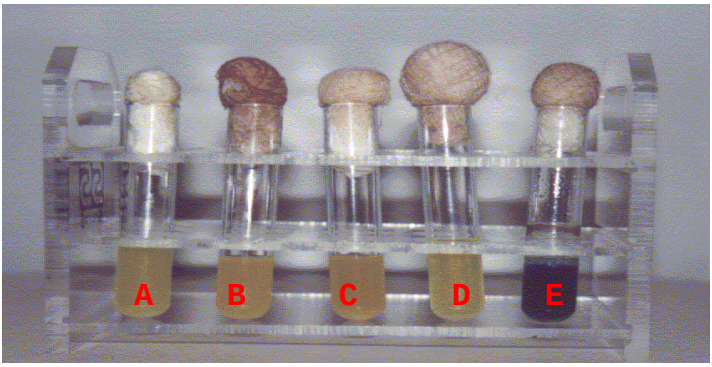

FIGURE 1. Siderophore production by *P. putida biovar B* (A), *P. marginalis* (B), *P. aeruginosa* (C), *Burkholderia cepacia* (D), and *Pseudomonas aeruginosa* control (E) in King B liquid medium.

TABLE 2. Siderophore solubility.

STRAIN	Solvent solubility of siderophores				
	Water	Methanol	Acetone	Ethyl acetate	HCl 0.5%
P. aeruginosa	+	+	+	-	-
B. cepacia	+	+	+	-	-
P. putida biovar B	+	+	+	-	-
P. marginalis	+	+	+	-	-

+ = Soluble.

- = Insoluble.

molecule. Cellulose accompanied by solvents with high polarity (water-methanol, 3:2 or 2:3) produced more adequate R_f values (0.30 to 0.60) for purifying the dye.

The pigment separated from *Pseudomonas aeruginosa* cultures by paper chromatography (water-methanol 3:2) showed, in the 780-200 nm interval, two maximum adsorption peaks (402 nm., A_{max}: 0.17; and 260 nm., A_{max}: 0.79). The siderophore color could mainly be attributed to the 402 nm peak (Figure 2).

Comparative *in vitro* evaluation of the isolated *Pseudomonas* antagonistic activity against the pathogenic fungi *R. solani* and *Botrytis cinerea*. *Pseudomonas putida* showed the highest antagonistic activity against *R. solani*, followed in decreasing order by *Pseudomonas putida biovar* B, *Pseudomonas marginalis*, *Burkholderia cepacia*, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* (Table 1). The first strain showed a good siderophore production, which may suggest that its antagonistic effect on *R. solani* may be attributed mainly to the production of this metabolite. Mechanisms such as colonization of hyphae and occurrence of antibiotics may contribute to the effectiveness of *P. putida* and *P. fluorescens*. The antagonistic property of the assayed strains versus the pathogen *Botrytis cinerea* increased in the following order: *Pseudomonas putida biovar* B, *P. marginalis*, *P. fluorescens*, *P. putida*, *P. aeruginosa*, and *B. cepacia* (Table 1). Although the first bacterium, a high siderophore producer, shows low activity, the latter three, with the largest antagonistic effect on the phyto-pathogenic fungi, are good siderophore-producing strains, an important parameter in designing phyto-sanitary activities aimed at counteracting fungal diseases.

Fluorescent *Pseudomonas* antagonistic effect on *R. solani*. This bacteria's antagonistic influence was evident in the 0.05 significance values for rhizome lengths and plant growth variances. However, the number of sprouts produced did not show revealing changes. Duncan's Multiple Range (DMR) test , used to compare rhizome length, indicated that treatment with *Pseudomonas marginalis* produced superior results (DMR = 1.22) as compared with those obtained from other microorganisms studied. The same results, to a lesser extent, were obtained with

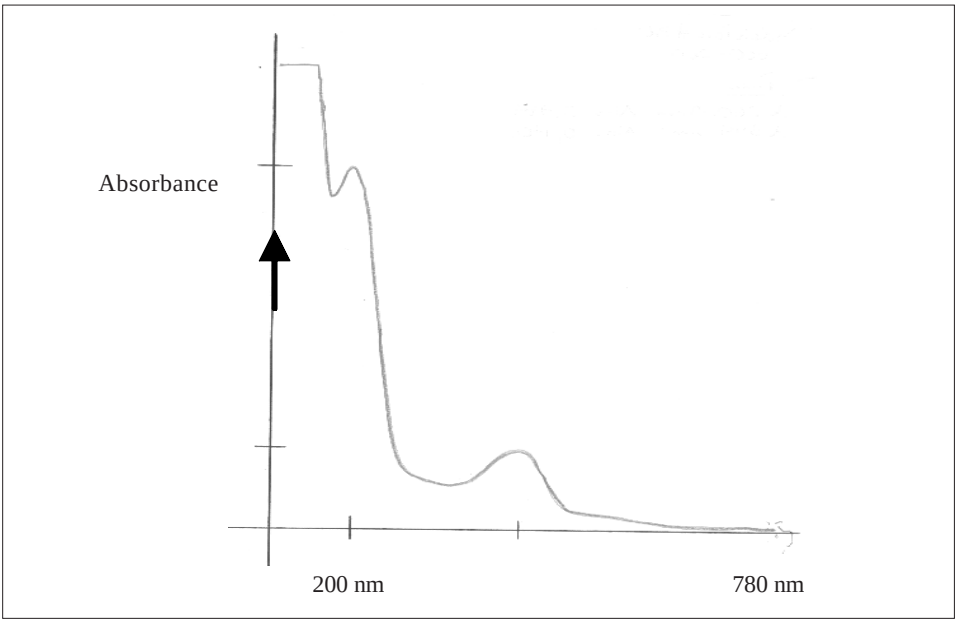

FIGURE 2. Spectral curve (absorbance against wave length) of the pigment produced by *P. marginalis*.

Pseudomonas putida biovar B, *Pseudomonas aeruginosa*, and *Burkholderia cepacia*. The stimulation of the plant growth parameter by *Pseudomonas marginalis*, as compared with the non-inoculated plant control, was two-fold greater (Table 3). In comparison with non-inoculated plants, the treatment of alstroemeria with *Pseudomonas putida* biovar B produced an improvement in the production of sprouts as high as 37.4%. Treatment with other microorganisms did not show changes (Table 3).

The longitudinal growth of plants was enhanced by inoculation with *Pseudomonas putida* biovar B (17.5 cm), *Pseudomonas aeruginosa* (17.3 cm), and *Pseudomonas marginalis* (16.6 cm). However, according to the DMR test, these bacterial treatments did not show significant differences, in comparison with the sterile soil control (control 2), in contrast to the case where *R. solani* (control 1) was involved (Table 3).

The phytopathogenecity of *R. solani* was evaluated by observing the rhizome color, assuming the following severity levels: 0, white coloring, for undamaged rhizomes (disease absence) 1, pink to brownish red, for infected rhizomes; 2, brown, for more severely infected rhizomes; and 3, dark brown and cessation of growth, for dead rhizomes (Figure 3). Pink to red coloring was clearly evident in control 1 rhizomes (having *R. solani),* indicating that the fungal strain used was pathogenic to alstroemeria (Table 4). At the beginning of the treatment with fluorescent *Pseudomonas*, 100% of the rhizomes were healthy (white), while at the end, 64.81 % were still unaffected, 27.78% showed some signs of disease (pink to brown colouring[4]) and the remaining 7.4% corresponded to dead plants.

Duncan's Multiple Range test, at a 0.05 level, showed significant differences between the *R. solani* inoculated alstroemeria seedlings (the control blank exhibiting the greatest average disease symptomatology) and those treated differently, including the non-inoculated ones. The latter showed no differences among themselves (Table 4). The fungus-infected seedlings, which were inoculated with bacteria, and the non-inoculated control displayed low average phyto-pathogenesis (Table 4), indicating the good inhibition properties of the various strains of siderophore-producing *Pseudomonas* against the *R. solani.*

Culture medium for *Pseudomonas* isolated from rose and alstroemeria cultivations. Growth curves showed that *Pseudomonas putida* biovar B, *Pseudomonas marginalis,* and *Pseudomonas aeruginosa* grew more abundantly in King B medium, while *Burkholderia cepacia* grew plentifully in what is here called "medium 2" (peptone, 10 g; molasses, 5 g; asparagines, 1.62 g; K_2HPO_4, 1.08 g; and $MgSO_4$. H_2O, 1.08 g) (Figure 4).

Medium 3 (soy flour, 10 g; molasses, 5 g; asparagine, 1.62 g; K_2HPO_4, 1.08 g; $MgSO_4$. H_2O, 1.08 g), with relatively low

TABLE 3. Effects of the inoculation with siderophore producing *Pseudomonas* on Alstroemeria infected with *R. solani.*

Treatment	PLANT GROWTH PARAMETER		
	Rhizome Length *1	Sprout Production *2	Plant Height *1
P. putida biovar B	0.50 bc	3.67	17.50 a
P. marginalis	1.72 a	3.78	16.6 a
P. aeruginosa	0.94 b	2.56	17.3 a
B.cepacia	0.44 bc	3.56	15.00 a
Control 1 (Only R. s*olani*)	0.06 c	1.00	9.44 b
Control 2 (un-inoculated plants)	0.23 c	2.67	15.67 a

*1. Significance at 0.05 levels

*2. Non significance at 0.05 levels, according to ANOVA

Note. Data represent average of three replicates. Values accompanied by same letters in each column do not differ significantly at 0.05 levels, according to Duncan's multiple range tests.

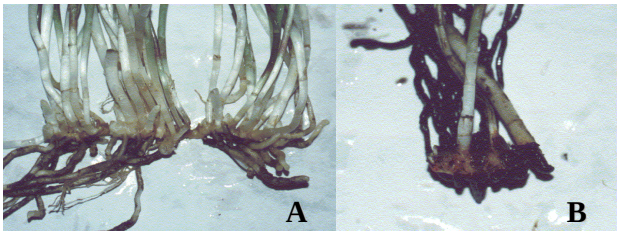

FIGURE 3.
Alstroemeria rhizomes:.
(A) undamaged,
(B) infected.

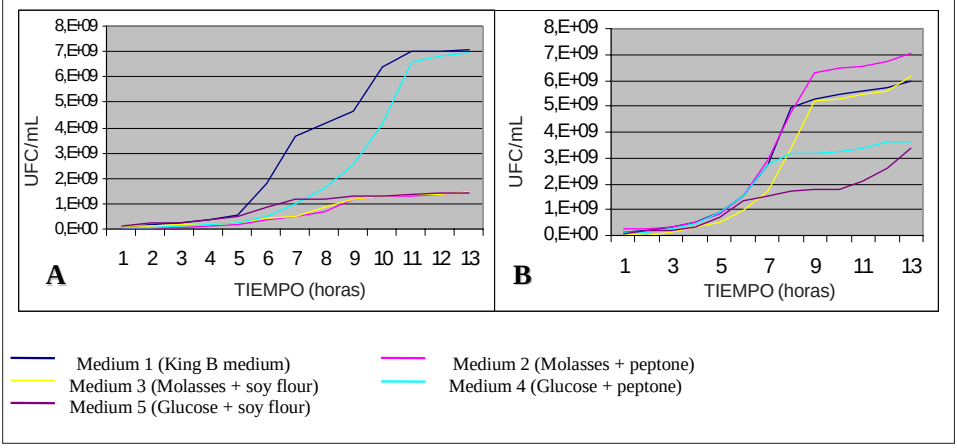

FIGURE 4. Microbial growth curve in different media: (A) *P. putida* biovar B, (B) *B. cepacia*

TABLE 4. Severity of *R. solani* inhibition, on alstroemeria seedlings inoculated with *Pseudomonas* strains.

Treatment	Severity Level *
P. putida biovar B	0,78 (b)
P. marginalis	0,22 (b)
P. aeruginosa	0,22 (b)
B. cepacia	0,22 (b)
Control 1 (Only *R. solani*)	1,33 (a)
Un-inoculated Control 2	0,33 (a)

*1. Significance at 0.05 levels

Note. Data represent means of three replicates. Values accompanied by the same letters in each column do not differ significantly at 0.05 level according to Duncan's multiple range test

cost nitrogen and carbon sources (soy flour and molasses), and easily accessible to tropical farmers, produced good cell counts ($40x10^8$ – $60x10^8$ CFU/mL) after 13 h of culturing with *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Medium 5, also involving a soy product (soy flour, 10 g; glucose, 5 g; asparagines, 1.62 g; K_2HPO_4, 1.08 g; $MgSO_4$. H_2O, 1.08 g) produced acceptable counts ($35x10^8$ – $58x10^8$ CFU/mL) after 13 h of culturing with *Pseudomonas aeruginosa* and *Burkholderia cepacia*. The quoted count values have been considered adequate for root colonization[8, 9, 11], a factor contributing to plant growth promotion and some disease control.

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