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Isolation of plant growth promoting rhizobacteria with inhibitory activity against *Pseudomonas solanacearum* and *Erwinia carotovora*

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Selected Plant Growth Promoting Rhizobacteria (PGPR) were studied in relation to the ***in vitro*** *inhibition toward* ***Pseudomonas solanacearum*** *and* ***Erwinia carotovora****. Biochemical and microbiological studies were conducted to determine the mode of action of pathogen inhibition, purification of the compounds involved in this inhibition, and determination of parameters controlling their expression. Results indicate that the PGPR are mainly strains of the* ***Pseudomonas fluorescens*** *group and produce siderophores like substances that chelate iron. These compounds probably affect the growth of the pathogens studied because they cause* ***in vitro*** *inhibition of* ***P. solanacearum*** *and* ***E. carotovora****. The regulation of the inhibition is dependent on iron concentration in the culture media.*

Key words: ***Erwinia carotovora****, plant growth promoting rhizobacteria, plant pathogen inhibition,* ***Pseudomonas fluorescens****,* ***Pseudomonas solanacearum****, siderophores.*

INTRODUCTION

Under natural conditions a large number of species of soil microorganisms are found in the rhizosphere of cultivated plants. Some of these bacteria have been studied in relation to their capability to colonize the rhizosphere and to protect cultivated plants from the action of plant pathogens (28).

One of the most interesting bacterial groups that have a favorable effect toward growth of cultivated crops are the so called «Plant Growth Promoting Rhizobacteria» (PGPR) which, by the action and production of siderophores like-substances, increase and promote plant growth (18, 19).

Inoculation of PGPR to seeds of cultivated plants has allowed crops to have a better competition when face unwanted soil microorganisms (6, 15). By this process, it is possible to change and direct the root micro-environment toward an easier establishment of economic crops, keeping out root and plant pathogens. This inoculation process has resulted in better growth and yield of plants (6, 10, 20).

Several kinds of mechanisms are used by PGPR to compete and to displace other native soil microorganisms. Among them it can be mentioned: a) the biosynthesis of one or several chemical compounds with detrimental action to other microorganisms (25, 28), and b) the sequestration of important elements for plant pathogens (15, 18, 21, 28).

Studies show that the isolation of green fluorescent bacteria included in the genus *Pseudomonas* may have a positive effect in biocontrolling bacterial wilt of potato caused by *Pseudomonas solanacearum*. These

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isolates and others PGPR's recently isolated can be obtained from soil and rhizosphere of potato and rapeseed plants growing in agricultural soils of Southern Chile (7,9).

Nowadays, the use of bacterial inoculants delivered on seeds is a practice with renewed interest, with the purpose of increasing crop yield and control of plant pathogens. Compared to pesticides, these inoculants have advantages in farming practices: less environmental pollution, no toxicity and easiness to manage. Work conducted with *Rhizobium* and *Azotobacter* spp. has made possible the selection of nitrogen fixing bacteria that produce antibiotic compounds and are very effective as agents of biological control (1, 24).

The purpose of this research was to isolate siderophores like-substances produced by 12 selected PGPR strains and to study how are they related to the inhibition of *Pseudomonas solanacearum* and *Erwinia carotovora*, important pathogens of potato *(Solanum tuberosum* L.), and *P. fluorescens* (biotype II), an opportunistic bacterium.

MATERIAL AND METHODS

Siderophore production by selected green fluorescent strains

Twelve selected isolates (I18, I19, P23B, P21A, P22, P25, P27, P28B, P28C, R5A, R11C and 17B) that showed a notorious effect on promoting plant growth were used to determine their production of Fe^{3+} chelating agents (10). Siderophore production was studied in two culture media: a) King's Medium B (KB) (14) and KB added with 100 μM $FeCl_3$. The inhibitory effect of the selected PGPR was studied against three strains of race 3 of *P. solanacearum*.

The inhibitory effect of several Fe^{+3} concentrations was studied on different plant pathogenic agents by adding increasing amounts of $FeCl_3$ (5, 10, 20, 50, 100 and 150 μM), to KB plates and incubating for 24 h at 28°C. Bacterial cells were killed by adding 1 ml of chloroform to the agar surface and keeping the plate closed for 1 h. After evaporation of the solvent a double soft agar layer at 50°C was added to the following plant pathogens: *E. carotovora* subsp. *atroseptica* strain E10; *P. fluorescens* Biotype II strain D10 and *P. solanacearum* strain B. Cell concentration for all isolates was 1 x 10^8 cfu/ml (O.D. 1.0 at 600 nm). The plant pathogenic isolates were obtained from the Bacterial Culture Collection of the Biotechnology Laboratory, Plant Production and Protection Institute, Austral University of Chile, Valdivia.

Isolation *and purification of iron chelating substances and establishment of their inhibitory effect*

To study how many of the 12 selected PGPR produced Fe^{3+} chelating like substances, the procedures described by Meyer and Abdallah (20) and Cox and Graham (8) were used. The siderophore free medium described as CAA by Carlton and Brown (5) was used as control; this medium was used free of bacteria and assayed along with the cultured samples. Several chromatograms were run with extracts from samples (8, 20).

The inhibitory effect of the isolated compounds was assayed against three strains (A, B, and C) of *P. solanacearum* (race 3). A layer of each one of the isolates of the pathogen was placed on the surface of KB medium plates. The bacterial cell concentration was 1.0 O.D. at 600 nm. On top of this cell layer 100 μl of the isolated compounds were placed, keeping the plates during 24 h at 28°C. Inhibition of the pathogen was established as a clear zone.

Selection of PGPR non-pigmented mutants and inhibitory relation to plant pathogenic agents

To study the relation between loss of fluorescence and change in the inhibitory activity toward the bacterial pathogens, three selected PGPR isolates (P22, P27 and P25) were grown on KB medium and treated with UV light at 254 nm during 24 s, according to the methodology described by Carlton and Brown (5). Non pigmented colonies were selected on the same agar medium plates under UV light. These strains were considered fluorescent$^{(-)}$ mutants. To

establish their inhibitory effect toward the bacterial pathogens, the same tests were performed.

Identification of the selected PGPR isolates

The 12 PGPR isolates used in this study were identified according to their biochemical and growth characteristics. The following tests were performed with these strains: oxidase, potato rot, starch hydrolysis, levan production, use of arginine and trehalose, nitrate reduction, polyhydroxybutyrate production, gelatin liquefaction and growth at 41°C. To the non-fluorescent isolate (17B), the following tests were added: lactose and mannose fermentation, indole formation, use of lysine, ornithine, phenylalanine, glucose, citrate and palatinose, gas formation from glucose, production of H_2S, and alpha-methyl-glucoside utilization. The material and methods used to conduct these tests are described in the Bergey's Manual (23).

RESULTS

Siderophore production by selected green fluorescent strains.

The effects of siderophore production by 12 selected PGPR on the growth of 3 strains of *P. solanacearum* (A,B and C), showed a relation with the iron content in the media (Table I). With the exception of the strain 17B, a non-fluorescent isolate, the inhibition was achieved in all KB plates with no iron added. However, the inhibition is lost when the same experiment was carried out on plates with Fe^{3+} added. An exception was detected with isolate P27 (a non pigmented mutant), which was able to cause pathogen inhibition in both media.

Isolation and purification of iron chelating substances and establishment of their inhibitory effect.

Out of twelve selected PGPR, 11 presented fluorescent activity in both KB and CAA media. Isolation of pigments allowed to obtain crude extracts that after a chromatographic process showed the typical characteristics of an Fe^{3+} chelating agent. They were fluorescent under UV light and showed a reddish-pink color when their chromatogram was developed with Fe^{+3} reagent or a bluish color when developed with potassium ferric cyanide. Eleven of the twelve PGPR studied were able to synthesize siderophore-like compounds. Pigmentation was pink in Fe^{3+} and blue in potassium ferrocianurum chromatograms; products of these bacteria were observed in chromatograms conducted in paper and cellulose acetate. The only non-fluorescent isolate (17B), which was negative in the production of these compounds, showed no chromatographic bands.

Figure 1 shows the fluorescence of the iron chelating agents produced by our 11 PGPR including the non-fluorescent isolate 17B and non-fluorescent controls (1 to 7 in

TABLE I

Inhibitory effect of 12 plant growth promoting rhizobacteria (PGPR) on three strains of *Pseudomonas solanacearum* Race 3 on King's medium B (KB) added with 150 μM $FeCl_3$

	PGPR											
P. solanacearum	I28	P22	17B	P27	R11C	R5A	P21A	I19	P25	P13B	P28B	P28C
Strain C												
KB	+	+	–	+	+	+	+	+	+	+	+	+
KB + Fe	–	–	–	+	–	–	–	–	–	–	–	–
Strain B												
KB	+	+	–	+	+	+	+	+	+	+	+	+
KB + Fe	–	–	–	+	–	–	–	–	–	–	–	–
Strain D												
KB	+	+	–	+	+	+	+	+	+	+	+	+
KB + Fe	–	–	–	+	–	–	–	–	–	–	–	–

\+ = Inhibition observed
\- = Inhibition not observed

Fig 1). The control was a bacteria free medium, treated in the same manner as the bacterial samples, whose chromatogram did not present bands or spots either in the paper nor in cellulose acetate (8 in Fig 1).

FIG 1. Chromatogram of siderophores like-substances partially purified from seven bacterial isolates observed under ultraviolet light (254 nm). From 1 to 8, paper chromatography runs of isolates P22, P27, I28, R5A, R11C, P28C, P21A and control. Siderophores are present as fluorescent bands at the top. Control (8) can be observed as a light circular spot, at the bottom of the right column.

The siderophore-like substances that were isolated were studied in assays using isolates of *P. solanacearum*. The effect of these partially purified compounds was very effective against the three isolates; only isolate B was not inhibited by substances produced by isolates R5A and 17B. Isolate C was not affected by strain 17B and isolate D was not inhibited by siderophores produced by strains P22, R5A and 17B. Substances from control had no inhibitory effects. Strain 17B did not produce these compounds and caused no inhibition on the tested isolates of *P. solanacearum*. The isolated siderophores were suspended in methanol at pH 2.0 and, when assayed in a similar test using different pH's (5.0 and 7.0) and suspended in aqueous solution, the results were erratic in relation to the inhibition of the pathogenic isolates.

The inhibition caused by PGPR strains toward 3 plant pathogenic bacteria on KB medium added with increasing amounts of Fe^{3+} is presented in Table II. It is possible to observe loss of inhibitory activity by PGPR related to increasing amounts of iron. However, isolate P27 caused inhibition of *P. solanacearum* at all concentrations of Fe^{3+} (Fig 2). Isolate P22 caused the same effect, but the inhibition rings at the highest Fe^{3+} concentration were smaller than those observed at lower concentrations. Almost all the selected PGPR cause inhibition in the media up to 50 μM of iron, and only in a few of them the inhibition was not related to the presence of iron in the media, such as strain P 27 that caused inhibitions up to concentrations of 10 μM and isolates P22 and P21A that inhibited growth of isolate D10 of *P. fluorescens* up to 150 μM of iron. Three isolates caused no inhibition and two were effective in absence of Fe^{3+}.

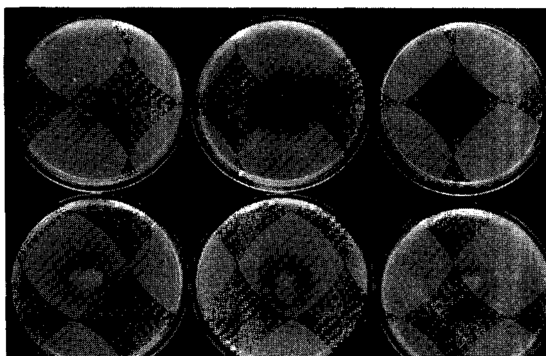

FIG 2. Inhibition of the strain B of *P. solanacearum* Race 3 induced by the strain P27 in King's medium B added with different amounts of $FeCl_3$ (from left to right; bottom: 5, 10 and 20 μM; top: 50, 100 and 150 μM). Plates show inhibition zones around bacterial colonies previously treated with chloroform. This bacterial strain could have a different mechanism to produce inhibition, besides the siderophore production.

None of the isolates caused inhibition to isolate E10 of *E. carotovora* at concentrations of 150 μM $FeCl_3$. However, isolates P27, P25 and P13B could not do it at 100 and 150 μM $FeCl_3$. Three isolates plus the selected mutant 17B were unable to inhibit *Erwinia* and the rest of them caused inhibition up to 50 μM $FeCl_3$. Inhibition of *Erwinia* by strain P27 occurs after 48 h at concentrations of 50 and 100 μM of Fe^{3+}. In Figure 3 it is possible to demonstrate inhibition of strain E10 of *E. carotovora* by the antagonist strain P13B.

Selection of non-pigmented mutants and its relation to inhibition of plant pathogenic agents.

Non-fluorescent mutants selected from strains P22, P27, and P25 were always kept

TABLE II

Effects of different concentrations of $FeCl_3$ added to King's medium B on the inhibition exerted by 12 PGPR strains on the growth of *Pseudomonas solanacearum, P. fluorescens* and *Erwinia carotovora.*

PGPR	Phytopathogenic bacteria	$FeCl_3$ (µM) 0	5	10	20	50	100	150
P27	*P. solanacearum*	+	+	+	+	+	+	+
	P. fluorecens	+	+	+	–	–	–	–
	E. carotovora	+	+	+	+	+*	+*	–
P25	*P. solanacearum*	+	+	+	+	+	–	–
	P. fluorecens	+	+	+	+	+	–	–
	E. carotovora	+	+	+	+	+	–	–
P22	*P. solanacearum*	+	+	+	+	+	+	+**
	P. fluorecens	+	+	+	+	+	+	+
	E. carotovora	+	+	+	+	+	–	–
P21A	*P. solanacearum*	+	+	+	+	+	–	–
	P. fluorecens	+	+	+	+	+	+	+
	E. carotovora	+	+	+	+	–	–	–
R11C	*P. solanacearum*	+	–	–	–	–	–	–
	P. fluorecens	+	+	+	+	+	+*	–
	E. carotovora	–	–	–	–	–	–	–
R5A	*P. solanacearum*	+	–	–	–	–	–	–
	P. fluorecens	–	–	–	–	–	–	–
	E. carotovora	–	–	–	–	–	–	–
I28	*P. solanacearum*	+	+	+	+	+	–	–
	P. fluorecens	+	+	+	+	+	+	–
	E. carotovora	+	+	+	+	+	–	–
I19	*P. solanacearum*	+	+	+	–	–	–	–
	P. fluorecens	+	+	+	+	+	–	–
	E. carotovora	+	+	+	+	+	–	–
P28B	*P. solanacearum*	+	–	–	–	–	–	–
	P. fluorecens	–	–	–	–	–	–	–
	E. carotovora	–	–	–	–	–	–	–
P28C	*P. solanacearum*	+	+	+	+	+	–	–
	P. fluorecens	–	–	–	–	–	–	–
	E. carotovora	+	+	+	+	–	–	–
P13B	*P. solanacearum*	+	+	+	+	+	–	–
	P. fluorecens	–	–	–	–	–	–	–
	E. carotovora	+	+	+	+	+	+	–
17B	*P. solanacearum*	–	–	–	–	–	–	–
	P. fluorecens	–	–	–	–	–	–	–
	E. carotovora	–	–	–	–	–	–	–

+ Inhibition observed
- Inhibition not observed
* Observed after 48 h of incubation
** Inhibition halos of smaller diameter than those observed at 100 µM $FeCl_3$

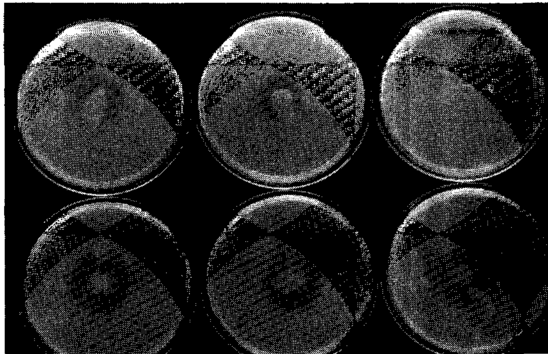

FIG 3. Inhibition of the strain E10 of *E. carotovora* susp. *atroseptica* induced by isolate P13B on King's medium B added with different amounts of $FeCl_3$ (from left to right: bottom: 5, 10 and 20 µM; top: 50, 100 and 150 µM). It is possible to observe the regulatory effects of iron on the inhibition induced by the siderophore of this PGPB. At the highest concentration of $FeCl_3$ (150 µM) the inhibition is lost.

in darkness, to avoid reactivation of fluorescence induced by visible light. Inhibition assays were negative on *P. solanacearum, E. carotovora*, and isolate D10 of *P. fluorescens*. A strain of isolate P27 fluorescent$^{(-)}$, however, developed small inhibition rings on *P. solanacearum*.

Identification of the selected PGPR isolates.

The results of the microbiological tests conducted to identify the 12 PGPR are shown in Table III. Eleven isolates are fluorescent and belong to the genus *Pseudomonas* being three of Biotype I (isolates P22, P25 y P28C); one of Biotype III (isolate P21A) and 5 of Biotype V (isolates P13B, P27, P28B, R5A y R11C). The other 2 isolates (I19 and I28) belong to *P. putida*.

Several tests were made to the only non-fluorescent isolate (17B). The results of these tests have led to establish that this isolate, a fermentative motile Gram(-) bacteria, corresponds to an isolate included in the Enterobacteriaceae family, specifically to the genus *Erwinia* species *raphontici*. The pink colonies in common culture media with added 2% sucrose, plus levan (+), lactose (+), glucose (+), citrate (+), phenylalanine (+), trehalose (+), nitrate reduction (+), tests of indole (-), production of H2S (-), gas from glucose (-), and utilization of lysine (-), ornithine (-) and methylglucoside (-) match the description of this species.

DISCUSSION

The great potential of the PGPR as biological control agents can be demonstrated by their ability to cause *in vitro* inhibition to important pathogens such as *P. solanacearum* and both subspecies of *E. carotovora*. These bacterial agents are responsible for important crop losses in our

TABLE III

Microbiological tests of 12 selected plant growth promoting rhizobacteria (PGPR) active against *Pseudomonas solanacearum, P. fluorescens* and *Erwinia carotovora*

Characteristics of PGPR	P13B	P28B	P21A	P25	I28	P27	P28C	P22	17B	I19	R11C	R11C	R5A
Oxidase	+	+	+	+	–	+	+	+	–	+	–	–	
Fluorescense	+	+	+	+	+	+	+	+	–	+	+	+	
Potato rot	–	?	–	–	–	–	?	?	–	–	–	–	
Arginine dihydrolase	+	+	+	+	+	+	+	+	–	+	+	+	
PHB accumulation	–	–	–	–	–	–	–	–	–	–	–	–	
Levan from sucrose**	–	–	–	+	–	–	+	+	+*	–	–	–	
Starch hydrolysis	–	–	–	–	–	–	–	–	–	–	–	–	
Growth at 41°C	–	–	–	–	–	–	–	–	–	–	–	–	
Reduction NO_3 to NO_2	–	–	+	–	–	–	–	–	+	–	–	–	
Utilization of trehalose	–	–	–	–	–	–	–	–	+	–	+	–	
Gelatin liquefaction	+	+	+	+	–	+	+	+	–	–	+	+	

* = production of pink diffusible pigment
** = pink colonies in 2% sucrose medium
? = variable results

country and in many others of the world, where chemical control has failed (9, 13, 16, 25). This potential can be extended to strains of *P. fluorescens* biotype II, an opportunistic pathogen that can cause tuber rot under inefficient storage conditions, and also to *Rhizoctonia solani* and *Streptomyces scabies*, which were very sensitive to our PGPR in previous experiments (9), and constitute important potato pathogens throughout the world.

It is very critical and important to search, find and select not only one or more isolates having a siderophore mechanism more efficient among pseudomonas, but also having another mechanism of pathogen inhibition (*e.g.* bacteriocin and/or secondary metabolites). This could allow the antagonistic strains to survive and compete more efficiently in the environment. This is an important factor, because around 10% of *Pseudomonas* strains found in the rhizosphere of growing cultivated plants may induce diseases to crops, such as rapeseed, wheat and potato (4). However, isolates of bacteria, included in the genus *Pseudomonas* and some of other genera, have raised the question of how to prove a common mechanism of inhibition. It is known that there are large or small differences between isolates of the same species or other genera (5, 24)

Taking into consideration the previous idea, it was very important to direct our research toward the isolation of PGPR showing broad characteristics, such as some fluorescent strains mainly responsible for chelating iron. This could give us a natural mechanism of biological control, fact commonly cited in recent publications (6, 11, 16, 18, 19, 28).

Siderophore production among our fluorescent isolates seems to have an important inhibition system when tested on the plant pathogenic agents. This was evident with the inhibition of *P. solanacearum*, *P. fluorescens* and *Erwinia*. However, this effect was lost when iron was added to the culture media. Our results are related to and in concordance with other reports (11, 20, 21), which have demonstrated that fluorescent pseudomonas are responsible for the production of substances such as siderophores, that may cause *in vitro* inhibition of many plant pathogenic fungi and bacteria responsible for important losses to many economic crops (17, 18, 25, 28). Furthermore, Kloepper *et al* (13) and others (9, 18, 19) have demonstrated how siderophore producing bacteria play an important role as biological control agents. Our findings confirm previous experiments in which soil bacteria were isolated and proved to be very active against *Rhizoctonia solani*, *Phytium* and *Fusarium*, important plant pathogenic agents (6).

Studies conducted with Chilean soils (25) indicate that the content of iron is considered satisfactory according to international standards. However, the availability of iron for microbial assimilation in environments such as the rhizosphere is extremely limiting (22). Therefore, the importance to biologically control the bacterial plant pathogens such as those used in this study could be related to make this element unavailable to them. The complexity of the relation between siderophores like substances and iron in nature is demonstrated by the common feature that all siderophores form complexes with this element.

Other inhibitory systems unrelated to Fe^{3+} dependance were found. Isolate P27 could maintain inhibition of the pathogens assayed even at higher iron concentrations, and isolate 17B could cause pathogen inhibition without being a fluorescent strain. These facts could be related to the production of antibiotic-like substances or bacteriocin. Tests to determine these compounds were not explored in this opportunity. The important fact is that only those isolates that produce siderophores could increase the spectra effect on pathogen inhibition when delivered together.

In Chile, the use of biological inoculants is restricted to bacteria of the *Rhizobium* and *Bacillus* genera. Research so far has been conducted only toward the construction of efficient and effective nitrogen fixation inoculants. However, this very limited field is having a new direction, in order to construct inoculants to biologically control plant pathogens. This unique experience in Chile is new and in the last years has been concentrated in the Universidad Austral (2, 3, 7).

We must emphasize that this kind of research is very important, because it is known that the use of biological inoculants developed for different edafic and climatic areas may have good, little or no results on improving yield if used in other places. Nowadays, one of the main objectives of our research is to conduct future field experiments to extend *in vitro* results with field success. If this happens, other research areas will be open, especially in the field of biotechnology. The possibilities of taking selected and field proved PGPR's as gene donors to develop transgenic plants arise as one of the most important research areas for the future (16).

We must point out that, due to the low resolution of paper chromatography, our results strongly suggest that the substances involved in the inhibition observed toward *P. solanacearum*, *E. carotovora* and *P. fluorescens* could be siderophore related substances. To further prove this fact, these substances must be fully isolated and purified, to finally describe not only their chemical nature but also their structure. The production of bacteriocin, secondary metabolites and siderophores in bacterial cells are gene mediated, and these characteristics are found in extrachromosomal entities in many cases. Eventually, the gene sequence could be identified, copied and ensembled in a gene vector, to insert these new characteristics in plants (12). Furthermore, the fact that substances such as siderophores are found in plants that are very alike those produced by bacterial cells suggests that expression of these bacterial siderophores could be achieved successfully in eukaryotic cells (18).

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