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Antagonist capacity of Newly Isolated Strains of *Pseudomonas Fluorescens* against Three Important Phytopathogenic Bacteria

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Abstract: Problem statement: Phytopatogenic bacteria cause several damages to plants with important economical consequences. They provoke losses of product quality affecting all commercial chain of crops, for this reason, their control is a priority. **Approach:** We evaluated antagonist capacity of newly isolated *Pseudonomas fluorescens* strains against three important phytopatogenic bacteria (*Clavibacter michiganensis*, *Xanthomonas axonopodis* and *Erwinia carotovora*). Soils from commercial cropping of *Capsicum annum L* of several Mexican regions were used to isolate *P. fluorescens* strains. **Results:** Isolates producing flourescein were purified on King B agar and biochemically identified. Crude extracts with and without cells were produced in King B broths and their antagonist capacities were evaluated by the plate diffusion procedure on nutritive agar. **Conclusion:** Obtained results demonstrated that cell free extracts exhibited a limited antagonist capacity in comparison of those extracts with cells, which showed an excellent capacity to inhibit the growth of *C. michiganensis*, *X. axonopodis* and *E. carotovora*, demonstrating the intracellular nature of the bioactive metabolites associated to bacterial growth inhibition.

Key words: *Pseudonomas fluorescens*, ellular nature, Mexican agriculture, Phytopatogen bacteria, foliar spots, Free Cell crude extracts (FC), fluorescens strains, polypeptone, microorganisms, economical losses

INTRODUCTION

Mexican agriculture present different troubles among which are included: severe changes in temperatures during the growing season, a growing desertification process and dissemination of several plant diseases characterized for a persistent resistance to chemical products. Plant diseases are caused by microorganisms provoking several symptoms like foliar spots, cell wall degradation, deformations, necrosis, rottenness of products. In Mexico, agricultural losses caused by microorganisms vary from 20-100 percent for some crops, these damages are in some cases higher than that calculated for around the world, however, in recent years there is a renewed interest to reduce the economical losses and to improve the quality and yield of crops using more friendly environment technologies under a organic agriculture philosophy.

C. michiganensis, *X. axonopodis* and *E. carotovora* are three phytopatogenic bacteria which cause important negative effects to agricultural commodities. *C. michiganensis* is an aerobic non-sporulating Gram-positive plant pathogenic organism and depending of the subspecie it is the causal agent of bacterial canker of tomato (*Lycopersicon esculentum* Mill.), a disease in potatoes known as 'ring rot', infects maize (*Zea mays*), wheat and alfalfa (Eichenlaub *et al*., 2006; Kleitman *et al*., 2008). *X. axonopodis* is a gram negative and aerobic microorganism with rod shape and polar flagella. It can be found on the leaves, stems and fruit of citrus trees all around the world. This bacterium causes citrus cankers. The leaves, stems and fruits that are infected with *X. axonopodis* display lesions which take on yellow halo or ring shaped appearances (Vidhyasekaran *et al*., 2001; Khodakaramian and Swings, 2011). *E. carotovora* is a rod shaped bacterium

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which infects a variety of vegetables and plants including carrots, potatoes, cucumbers, onions, tomatoes, lettuce and ornamental plants like iris (Laurent *et al*., 2001; Chernyshov *et al*., 2007). These widespread microbes can be found in soil, guts of insects, water and suspended aerosols in air.

Biocontrol of this kind of phytopatogens requires of effective and potent microorganisms in order to reduce damages and economical losses. *Pseudomonas fluorescens* is a bacterium with antagonist properties which has not been used as others due that its mechanisms of action are diverse and still remain confuse. *P. flourescens* is a gram negative rod organism which promotes growth of some plants due its capactity to produce growth-stimulating substances like auxins, giberelins and cytokinins. Physiologically, this microorganism promotes seed germination, increases root formation and accelerate plant growth (Moore *et al*., 2006; Babalola, 2010).

In the present study, the antagonist capacity of newly isolated *Pseudonomas fluorescens* strains against three important phytopatogenic bacteria (*Clavibacter michiganensis, Xanthomonas axonopodis* and *Erwinia carotovora*) was evaluated.

MATERIALS AND METHODS

Isolation of P. fluorescens strains: Nine soil samples from commercial production lots of pepper (*Capsicum annum L*) were used in this study. Soil samples were collected from the Mexican states of Chihuahua, Sinaloa, Veracruz, Nayarit and Guerrero by workers of GreenCorp Bioorganiks de Mexico SA de CV.

Soil samples were immediately processed to *Pseudomonas fluorescens* strains isolation. Series of 7 decimal dilutions with distilled water at room temperature were used. Aliquots of 100 μL from the last three dilutions were inoculated on King B agar plates, which were incubated at 27 ± 2°C for 24h. Presence of fluorescence on UV light was used to select those putative *P. fluorescens* colonies. Each colony was re-inoculated in the same culture medium for its purification. After incubation, each colony was morphologically analyzed using microscopy and the Gram technique. Those Gram negative rods producing fluorescence were selected for this study.

Identification and conservation of *P. fluorescens* strains: After selection of pure strains, these were grown in several culture media for a biochemical characterization and avoid the presence of *P, putida* and *P aureginosa*, which also produce similar pigments. Those colonies indentified as *P fluorescens* were conserved in slants at refrigeration and under cryo-conserved at -20°C.

Phytopatogen bacteria: The phytopatogens used in this study were *Clavibacter michiganensis*, *Xanthomonas axonopodis* and *Erwinia carotovora* and they belong to The Center of Applied Microbiology (CEMAP) collection, GreenCorp Biorganiks de Mexico SA de CV. Saltillo, Coahuila, Mexico. All three bacteria were grown in 250 ml Erlenmeter flasks with 100 ml of nutritive broth enriched with potato infusion at 30°C by 24h.

Culture conditions of *P. fluorescens*: For production of crude extracts with and without cells, two culture media were compared. Both media contained a base formulated (per liter) with glycerol (10 g), dibasic potassium phosphate (1.5 g), magnesium sulfate (1.5 g), but one of them enriched with 20 g of potato starch and the other with 20 g of polypeptone. Cell concentration was evaluated by counting in a Neubauer chamber after 24 h of culture time at 28°C. After culture medium was selected, influence of pH (6.5, 7.0 and 7.5) and temperature (25, 30 and 35°C) were evaluated;

Non Free Cell crude extracts (NFC): Selected *P. fluorescens* strains were grown on 500 ml culture bottles containing 100 mL of King B Broth at 28°C during 24 h in a innova 44® shaker at 200 rpm. An inoculation level of 1×10^6 cells per culture bottle was used. After culture time, all culture bottle content was used to evaluated the antagonist capacity.

Free Cell crude extracts (FC): Selected *P. fluorescens* strains were grown under the same culture conditions described above. After culture time, the content was put into conic tubes and centrifuged at 6000 rpm during 15 min at 4°C. Supernatant was carefully removed and considered as free cell crude extracts. To remove cells and works with a real free cell crude extract, each sample was filtered through 0.45μm sterile Nylon membrane (Millipore®). Obtained filtrates were used to evaluate their antagonist capacity.

Antagonist capacity of crude extracts: Plates of nutritive agar were completely inoculated with each phytopatogenic bacteria. Four little cavities (5mm diameter) were made on the agar surface and then were filled with an aliquot of 100 mL of crude extracts. Plates were incubated at 30°C for 24 h. After that, inhibition halos were measured.

Experimental design and data analysis: Tests for determination of culture conditions and antagonist capacity were established under a completely randomized block design with a factorial fix. ANOVA procedure was used to evaluate each effect and when it was needed a comparison test of mean values (Tukey Test) was used. SAS software was used for data analysis.

RESULTS

In this study, several experimental steps were conducted to evaluate the biological potential of newly isolated strains of *P. fluorescens.*

Isolation of *P. fluorescens* strains: The protocol used for isolation permitted to obtain suspected colonies of *P. fluorescens* from the soil samples. Simple microscopy revealed Gram-negative and short rods. Pigment production was easily revealed when plates were put under UV light. It is important to note that *P. fluorescens* produces pyoverdins (Fig. 1), bioactive molecules of high affinity called siderophores, associated to colonization of rhizosphere and very well studied in the models *P. aeruginosa* and *P. putida* (Redly and Poole, 2003; Molina *et al*., 2005). All morphological characteristics of isolated strains were compared with a control strain (*P. fluorecsens* M f1).

Identification and conservation of *P. fluorescens* strains: Selected strains were identified by biochemical differentiation. Table 1 shows the biochemical behavior of selected strains. All strains had similar biochemical behaivor to control, except sample M 6a, which was immobile. *P. fluorescens* does not ferment glucose, lactose and sucrose, neither produces gas from fermentation. In contrast, it grows descarboxylating ornitine and lysine, with motility and producing oxidase activity.

Culture conditions of *P. fluorescens*: Culture conditions were established using the growth of M 1f strain (control) as criterion of selection. Figure 2 shows the effect of culture media, pH and temperature on bacterial growth. It is important to note that the experimental design used (sequenced mono-factorial fix) permitted a significant increase of number of Colony-Forming Units (CFU). The maximun *P. fluorescens* growth was achieved on a culture medium based on polypeptone, with an initial pH of 7.5 and a temperature of 35°C.

Table 1: Biochemical differentiation of *P. fluorescens* strains

	Glu-Suc -Lac	Gas	Motility	Ornitine Descarb	Lysine Descarb.	oxidase
M 6a	(-)	(-)	(-)	(+)	(+)	(+)
M 6b	(-)	(-)	(+)	(+)	(+)	(+)
M6c	(-)	(-)	(+)	(+)	(+)	(+)
M 4a	(-)	(-)	(+)	(+)	(+)	(+)
M 4b	(-)	(-)	(+)	(+)	(+)	(+)
M 4c	(-)	(-)	(+)	(+)	(+)	(+)
M 4d	(-)	(-)	(+)	(+)	(+)	(+)
M 1f	(-)	(-)	(+)	(+)	(+)	(+)

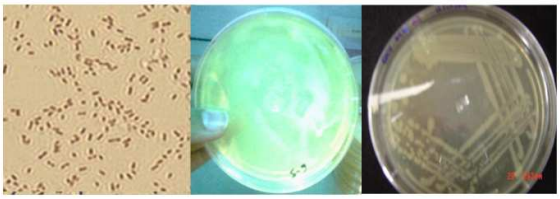

Fig. 1: Pyoverdine production (revealed under UV light) and morphology of *P. fluorescens*

Antagonist capacity of FC extracts. Plates of nutritive agar were completely inoculated with each FC extracts and the antagonist capacity was tested against the three phytopathogenic bacteria. Under culture conditions of *P. fluorescens* strains, none of FC extracts inhibited the growth of the phytopathogen bacteria.

Antagonist capacity of NFC extracts: Same assays conditions were used to evaluate the antagonist capacities of NFC extracts to inhibit the growth of phytopathogen bacteria. Figure 3 shows the inhibition of *C. michiganensis* growth. M6a and M1f strains showed the highest antagonist capacity against *C. michiganensis.* M6c strain also inhibited *C. michiganensis* growth while the remaining *P. fluorescens* strains did not show any antagonist capacity. Four *P. fluorescens* strains showed a strong antagonist capacity against *E. carotovora* (Fig. 4). M6b, M6c and M1f strains showed the highest antagonist capacity against this phytopathogenic bacteria. M6a strain also inhibited *E. carotovora* growth while the remaining *P. fluorescens* strains did not show any antagonist capacity. *X. axonopodis* growth was inhibited by the *P. fluorescens* strains M6c, M1f and M6a (Fig. 5).

The antagonist capacity of the new isolated *P. fluorescens* strains was evaluated under a completely randomized block design with 4 replicates. Analysis of variance is shown in Table 2, while the Tukey's range tests are presented in Table 3-4.

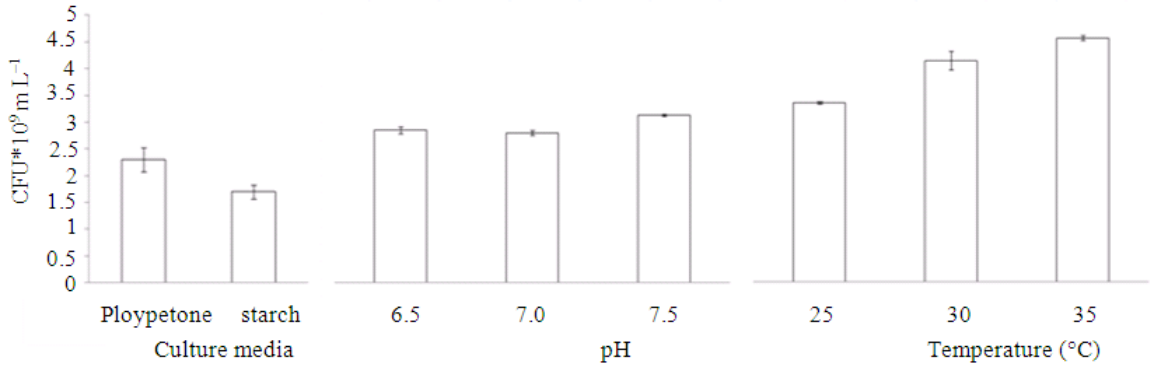

Fig. 2: Bacterial growth of *P. fluorescens* as affected by the culture media, pH and temperature level

Table 2: Analysis of variance for phytopathogenic bacteria growth inhibition by different *P. fluorescens* strains

Source	df	Sum of squares	Mean square	F value	F. value Prob
Replicate	3	0.018	0.006	0.38	0.766
Antagonist strain (CA)	3	15.409	5.136	311.18	<0.001
Pathogenic strain (CP)	2	5.887	2.943	178.34	<0.001
Interaction CA-CP	6	10.761	1.793	108.66	<0.001
Error	33	0.544	0.016		
Total	47	32.602			

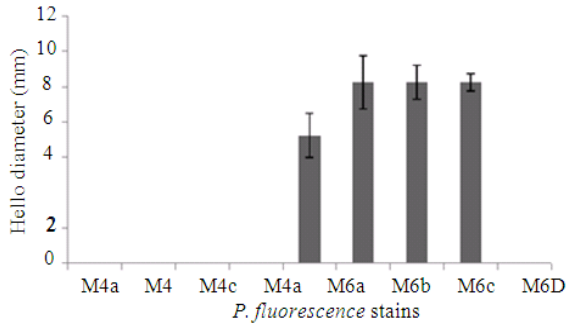

Fig. 3: Inhibition of *C. michiganensis* growth by *P. fluorescens* strains

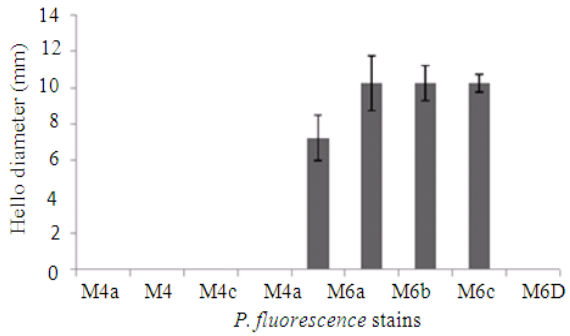

Fig. 4: Inhibition of *E. carotovora* growth by *P. fluorescens* strains

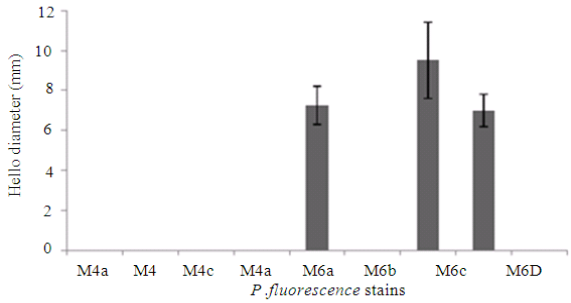

Fig. 5: Inhibition of *X. axonopodis* growth by *P. fluorescens* strains

DISCUSSION

Independent variables (CA and CP) as their interaction (CA-CP) shown a significant effect on the inhibition of phytopathogenic bacteria growth, for this reason a comparative analysis of mean values was made between antagonist strains, demonstrating that M6b strain showed the lowest capacity of inhibition against the phytopathogen bacteria. The rest of antagonist strains were not significantly different (Table 3). While the results presented in Table 4 demonstrated that *E. carotovora* was the phytopathogen more inhibited by the *P. fluorescens* strains.

The significant difference found among the interaction antagonist-phytophatogen suggests a specific combination of *P. fluorescens* strains for the pathogenic bacteria inhibition, probably due to the pool of metabolites related to antagonist capacity.

Table 3: *P. fluorescens* strains effect on Phytopathogenic bacteria growth inhibition

P. fluorescens strains	Halo diameter (cm)
1f	2.21 a
6a	2.20 a
6c	2.10 a
6b	0.86 b

Treatment means followed by the same letter are not significant different according to the Tukey's range test ($p \leq 0.05$). Analysis was done using values were transformed to log (x + 1)

Table 4: Phytopathogenic bacteria growth inhibition by different *P. fluorescens* strains

Phytopathogen	Mean value
E. carotovora	2.33 a
X. axonopodis	1.65 b
C. michiganensis	1.54 b

Treatment means followed by the same letter are not significant different according to the Tukey's range test ($p \leq 0.05$). Analysis was done using values were transformed to log (x + 1)

M6a, M6c and M1f were the best antagonist bacteria, while *E. carotovora* was the phytopathogens more sensible to these new isolates of *P. fluorescens*.

Null formation of inhibition halos with FC extracts demonstrated the poor role of secreted metabolites to the culture broth to inhibite these pathogenic bacteria. In contrast, a wide range of possibilities can be considered to explain the antagonist capacity of isolates, including direct and indirect effects. Among the first, the diffusion phenomenon of soluble nutrients followed of an improved absorption of them by the plants was reported by Lifshitz *et al*. (1987) and Zhang *et al*. (2010). Antibiotic production by fungi and bacteria was mentioned by Dianez *et al*. (2007) and the release of phytohormones (auxines, gibberellins, cytocinnins and ethylene) was reported by Kulaeva and Prokoptseva (2004). Indirect effects include the increment in nitrogen fixation, which improve the number of root nodules and the increase of nitrogenase which also induce systemic resistance to the plant against the phytopathogens activity (Zhang *et al*., 1996; Intra *et al*., 2011).

CONCLUSION

In this study, the antagonist capacity of Newly Isolated Strains of *Pseudomonas Fluorescens* against Three Important Phytopathogenic Bacteria was clearly demonstrated, Cell free extracts exhibited a limited antagonist capacity in comparison of those extracts with cells, which showed an excellent capacity to inhibit the growth of *C. michiganensis*, *X. axonopodis* and *E. carotovora*, demonstrating the intracellular nature of the bioactive metabolites associated to bacterial growth inhibition.

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