Inhibitory compound of the soil bacteria *Pseudomonas fluorescens* against the fungus *Aspergillus flavus* L.

Isaac Luna-Romero [1], Magda Carvajal [2], Guillermo Carrillo-Castañeda [3], Carlos Flores[4]

[1] Departamento de Microbiología, ENCB, Instituto Politécnico Nacional, México, D.F. [2] Departamento de Botánica, Instituto de Biología, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510 México, D.F. [3] Instituto de Genética, Colegio de Posgraduados, Montecillo, Estado de México, México. [1] Departamento de Ecología, ENCB, Instituto Politécnico Nacional, México, D.F.

Compuesto inhibidor de las bacterias del suelo *Pseudomonas fluorescens* contra el hongo *Aspergillus flavus* L.

Resumen. El suelo de rizosfera, estigmas y brácteas de maíz fueron muestreadas de México: Cocotitlán y Tezoyucan en el Estado de México; y el Bajío en el Estado de Guanajuato con la técnica de dilución en placa se obtuvieron 3425 aislamientos bacterianos; y se probó su capacidad antagónica. Los aislamientos de estigmas y brácteas no tuvieron actividad antagónica. Los aislamientos de la rizosfera Co-47, Co-81, Tez-118, Tez-226 y Ba-72 con actividad antagónica se caracterizaron mediante pruebas morfológicas y bioquímicas y fueron identificados como *Pseudomonas fluorescens*. Con el aislamiento C0-47 hubo 100% de inhibición sobre conidios y esclerocios de *A. flavus* en PDA, con 72 h de incubación y en la siembra posterior del hongo se mantuvo el efecto antagónico, pero sin cambios en la actividad antagónica de las bacterias. En invernadero y campo, Co-47 y Co-81 mostraron efecto protector contra *A. flavus*. El componente activo involucrado está relacionado con la presencia de sideróforos en Co-47, Co-81 y Tez-118; pero no para el Tez-226. El componente activo de Co-47 fue proteico y de carácter termolábil. Los aislamientos bacterianos de la rizosfera del maíz son saprófitos, con potencial antagónico para el control biológico de *A. flavus*.

Palabras clave: Control biológico, *Pseudomonas*, *Aspergillus*, antagónico, antagonismo.

Abstract. Maize silks, bracts and rhizosphere soil, were sampled from Mexico: Cocotitlan and Tezoyucan (State of Mexico), and from El Bajio (State of Guanajuato). A dilution plate method was used to obtain 3425 antagonistic bacteria isolates, and their activity was tested. Isolates from maize silks and bracts had no activity against *Aspergillus flavus*. Five rhizosphere isolates Co-47,Co-81, Tez-118, Tez-226 and Ba-72 with antagonistic activity against this fungus were identified as *Pseudomonas fluorescens* by morphological and biochemical tests. A total inhibitory effect on conidia and sclerotia from *A. flavus* was observed with isolate Co-47 with an incubation period of 72 hours on PDA medium, with fungal inoculation afterwards; after this time period there were no changes in the bacterial antagonistic activity. At greenhouse and field, isolates Co-47 and Co-81 showed a protective effect against *A. flavus* infection. Siderophores are the active component of isolates Co-47, Co-81 and Tez-118 but not of Tez-226. The active component of isolate Co-47 was a protein with thermolabile properties. Bacteria isolated from maize soil rhizosphere are considered saprophytic and potentially antagonistic to control *A. flavus*.

Keywords: Biological control, *Pseudomonas*, *Aspergillus*, antagonistic, antagonism.

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Autor para correspondencia: Magda Carvajal
magdac@servidor.unam.mx

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Introduction

Antagonism is the relation between two species of opposite organisms, in which one affects the life of the other, inhibiting partially or totally its growth or even killing it [57]. The mechanisms of antagonism observed in nature are competition, antibiosis, predation and hyperparasitism. Antibiosis is the inhibitory effect of one microorganism against the physiological processes of another [58].

Phyllosphere and rhyzosphere are areas of strong microbial activity, and many microorganisms on these ecological areas, as bacteria, are useful for biocontrol of fungi that cause plant diseases in nature. The biological control of pathogens is more successful in rhizosphere than in phyllosphere [3]. Microorganisms that grow on the rhizosphere are ideal as biocontrol agents, since this region provides the front line defense. The success of bacteria as biocontrol agents depends on their ability to compete *in situ* with plant microflora for roots against pathogen attack and to express genes essential for control. Indeed the great majority of commercial products for biological control are directed against root pathogens. The diversity of rhizosphere soil samples from maize plants, the full spectrum of potentially effective strains has barely been explored [59].

These antagonistic microorganisms are present in suppressive soils which inhibit the growth of pathogens. There are many genera among which *Pseudomonas* [34] and other endophytic bacteria have been identified as useful to control many plant pathogenic fungi. Several species of *Pseudomonas* such as *P. fluorescens* S3, *P. tolaasii* S10, *P. veronii* S21 and *P. cepacia*, are used to control plant pathogens [1, 11, 59].

Pseudomonas cepacia (Burkholderia cepacia), isolated from conidia of *Bipolaris maydis* infecting maize leaves, was useful for the biocontrol of *Aspergillus flavus* [41]. Rhyzospheric *P. fluorescens* were reported as rejuvenating and root proliferating agents in black pepper [16]. In fact the genus *Pseudomonas* (*sensu stricto*) represents a group of microorganisms directly involved in functions conferring plant health, and the identification and specific detection of a novel Pseudomonadaceae cluster associated with soils, from winter wheat plots, was reported [48]. *P. fluorescens* showed high attachment to vital hyphae of arbuscular mycorrhizhal fungi, helping for the good nutrient supply and plant health [56]. *P. fluorescens* is used for efficient biodegradation of benzene [31] and 2-4-dinitrotoluene [44]. Even the use of non toxigenic strains of the same *A. flavus* in several cultivars such as peanuts [17, 28], wheat [6], maize and peanut [18,19], rice [12,33], and cotton [22] were reported to control toxigenic *A. flavus* and *A. parasiticus*, and their aflatoxins; also the encapsulation of the non toxigenic fungus in pellets is reported for that purpose [13-15].

The aims of the present study were to isolate the active principle of the antagonism of *Pseudomonas fluorescens* from rhizosphere soil samples from maize plants and evaluate its antagonistic activity against *Aspergillus flavus.*

Materials and methods

Fungus isolation

Aspergillus flavus was isolated from damaged maize grains collected in the warehouse "Las Yescas" at Valle Hermoso, State of Tamaulipas, Mexico, and the fungus was identified following Raper and Fennell's (1965) key [51]. Total aflatoxins in that maize, varied from 15 to 1999 µg/kg, confirmed by Carvajal and Arroyo [9]. Five maize grains were desinfected in 2% chloride for two minutes, rinsed twice with distilled water and placed on potato dextrose agar (PDA) medium, the Petri dishes were kept at 28 °C until the growth of *A. flavus* was observed.

Sampling and isolation of antagonistic bacteria

Soil samples from the rhizosphere of maize plants were collected in 1995, at three places: Cocotitlan and Tezoyucan, State of Mexico and El Bajio, State of Guanajuato. Old plants were removed from the soil, roots with enclosed soil, placed in plastic bags, were carried to the lab and kept at 4º C.

To isolate bacteria from the rhizosphere, 10 g of soil were mixed in 90 mL of distilled and sterilized water and the suspension was diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) several times and 0.1 mL from each dilution, was grown on B King media [32] and kept at 28ºC until the bacterial colonies were formed, and their antagonistic activity against *A. flavus* on PDA plates was tested. The antagonistic isolates were named Co from Cocotitlan (eg. Co-47, Co-81), Tez from Tezoyucan (Tez-118, Tez-226), and Ba from El Bajío (Ba-53, Ba-72, Ba-205 and Ba-252).

Antagonism tests

All the 3425 bacterial isolates were tested, in a Petri dish with PDA medium, eight different bacterial colonies, isolated from rhizosphere, were sown equidistantly on PDA and one µL of fungal conidial suspension, 3.5 x 10^5 conidia/mL, was applied at the center, to know the effect of each bacterial isolate, from the outside circle, against the fungus sown in the middle of the plate. Later the plates were incubated in the darkness for eight days at 28º C.

Each bacterial isolate that inhibited the growth of *A. flavus* was selected and tested individually. A control plate of fungal growth, without bacterial isolates, was measured daily during eight days. The stability or loss of the antagonistic activity of the isolates was observed for two months by measuring the growth of the fungal colony diameter at the center of the plate, and the growth of the colony diameter of the isolate(s). The cultures were transferred five times per month. A non parametric Kruskall-Wallis variance analysis [62] was applied to evaluate the differences between the inhibitory capacities of tested isolates.

Identification of antagonistic bacterial strains

The bacterial isolates with best antagonistic activity against *A. flavus* were identified up to species using the following criteria: Colonial and cellular morphology, pigmentation, production of diffusible fluorescent pigment in B King media, Gram stain, oxidative/fermentative metabolism of glucose, oxidase production, reduction of nitrates to nitrites, denitrification, catalase production, growth in FLO medium (peptone 10 g, meat peptone 10 g, $MgSO_4$ 1.5 g, K_2HPO_4 1.5 g, agar 12 g, glycerol 10 mL), and nitrogen fixation on FW79 medium (yeast extract 4 g , manitol 10 g , $CaCO_3$ 3 g , K_2HPO_4 0.5 g, $MgSO_4$ 7 H_2O 0.2 g , NaCl 0.1, Agar (noble) = 15 g and distilled water 1 L), number and position of flagella were observed under electronic microscope (Figure 1); other biochemical tests applied to identify the isolates were: starch hydrolysis, arginine, dihydrolase, gelatin hydrolysis, nitrate reduction, oxidase, acid production from different sources such as cellobiose, manitol, adonitol, trehalose, xilose, sorbitol, maltose, ethanol, sucrose, arabinose, manose, inositol, and dulcitol, also the bacteria growth at 41°C and 5°C; and their tolerance to sodium chloride [38].

The identification of the bacterial isolates (Co-47, Co-81, Tez-118, Tez-226 and Ba-72) was done following Bergey´s Manual [27]. Isolate Co-47 was identified following API 20 E and 20 NE [2, 5].

Other identification tests applied to the inhibitory bacteria were to know their effects on mycelia, conidia and sclerotia of *Aspergillus flavus,* the hypersensitive test on tobacco plant, potato rot test and the effect on other vegetables, but this studies were reported elsewere[38].

Qualitative antagonism tests is only when the inhibition halo is seen, without any measurements, and quantitative antagonism tests is when the diameter of the bacterial or fungal colonies are measured.

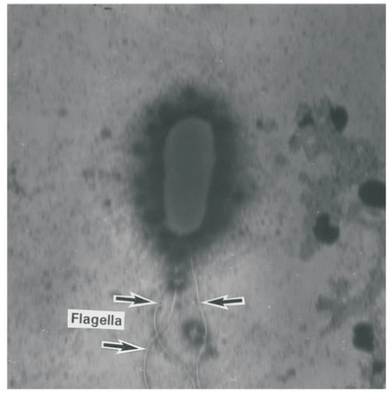

Figure 1. Electron microscopy (13,000 x) of bacteria Co-47 with three polar flagella, identified as *Pseudomonas fluorescens*.

Antagonism in greenhouse and in field

Several assays of antagonism in greenhouse and in open field were done to established the degree of protection of the different bacterial isolates and the results were published elsewere [38].

Characterization of the inhibitory compound

The inhibitory principle of the isolates Co-47, Tez-118, Tez-226 and Co-81 were directed towards the identification of siderophores, compounds of low molecular weight and high affinity for the iron ion, excreted by the bacteria that have been reported as responsible of the inhibition of a great number of plant pathogenic microorganisms. The ability of the antagonistic bacterial isolates to produce siderophores as a mechanism to inhibit *A. flavus* was tested in two ways:

1) Use of a minimum medium

A. Media deficient in iron are used to determine if a microorganism produces siderophores, because under these conditions the bacteria is stimulated to produce them. A drop of conidial suspension of *A. flavus* was distributed, with a glass triangle, in the surface of the solid medium deficient in iron [38].

B. Minimum medium for siderophorus detection

Stock solutions: L-glutamic acid at a concentration of 10% dissolved with a KOH 5M solution and adjusted to pH 6.5 with deionized water.

Manitol (10%); $KH_2PO_4 + Na_2HPO_4$ (3 g) in 100 mL H_2O; $MgSO_4 \cdot 7H_2O$ (1%); $CaCl_2 \cdot 2H_2O$ at 5%; solution of trace elements: H_3BO_3, $ZnSO_4.7H_2O$, $CuSO_4.5H_20$, $CuSO_4.5H_2O$, $MnCl_2.4H_2O$, $NaMo0_4.2H_2O$. One g of each reactive was dissolved independently in 100 mL of deionized water.

C. Final solution of trace elements

The addition of trace elements to 88 mL of deionized sterilized water was done using 10 mL of solution H_3BO_3; $ZnSO_4 \cdot 7\ H_2O$ (1 mL), $CuSO_4 \cdot 5\ H_2O$ (0.5 mL); $MnCl_2 \cdot 4H_2O$ (0.5 mL) and $Na_2MoO_4 \cdot 2\ H_2O$ (0.1 mL).

D. Final minimum medium

The addition of 900 mL of dionized and sterilized water to 10 mL of each one of the stock solutions, and 10 mL of final trace elements was done. Later a drop of bacterial suspension, grown on the base medium with glutamic acid, was placed. Controls had the fungi sown, and later the bacteria in a medium with iron ion. All the treatments had four replicates and the inhibition halo between the bacteria and the fungi was measured, after an incubation of 48 hours at 28ºC. The presence of an inhibition halo around the bacterial strain grown near *A. flavus* on a deficient medium in iron ion would indicate the production of siderophores, in comparison with the control without the halo.

2) Development of the bacterial isolate in a universal medium based on chromium azurol (CAS) for the detection of siderophores.

2 a. Chromo azurol (CAS) the universal medium for the detection of siderophores. Schwyn and Neilands [53] developed a highly sensitive medium to detect siderophores. Chromo azurol is a weak quelant of the ferric ion, accepted as a qualitative reliable test to determine the production of siderophores by microorganisms. A drop of a bacterial dilution (3 x 10^8 cells/mL) of the isolates Co-47, Co-81, Tez-118 and Tez-226, were sown in CAS universal medium; in the same Petri dish the strain *Rhizobium melliloti* CP 98 was sown as control and incubated at 28C for 48 h. The presence of a yellow or orange halo around the bacterial isolates indicated the presence of siderophores by the isolates able to produce them.

Proteinic nature of the active compound from the isolate Co-47.

The best bacterial isolate to produce the inhibitory compound in culture medium was Co-47, and it was extracted, to determine its chemical nature, the effect of temperature and the production of siderophores were analyzed.

The best extract for the production of the inhibitory compound in culture medium.

The behaviour of the several bacterial isolates on different extracts was tested.The bacterial isolate Co-47 was the best to produce the inhibitory compound and it was used to obtain the culture medium for the production of the inhibitory compound. Although the other antagonist bacterial isolates, with positive siderophore test, were tested too. The behaviour of several media (yeast, meat and potato) was tested, at concentrations of 0.25%, 0.50%, 1%, 1.5% and 2%. Each bacterial isolate was sown around a circle of 5.5.cm of diameter at eight points, and the fungus was sown at the center. As control, the fungus was sown alone in each extract and concentration, the Petri dishes were incubated at 28C for 8 days, time required for the fungus to grow and cover 100 % of the Petri dish surface.

Extraction of the raw inhibitory compound

The bacterial isolate Co-47, with the highest antagonistic activity over the fungus, was sown in culture medium. Shannahan *et al*. [54] method was applied.

Effect of temperature on the inhibitory compound

To know if the active compound of the raw extract was heat resistant, eight flasks, each with 30 mL of the raw extract, were heated in a calibrated stove at evaporation temperatures of 30, 35, 40, 45, 50, 55, 60, 65 and 70°C. The active compound, from each temperature treatment, was centrifugated at 8000 rpm and the supernatants were filtrated with Millipore (0.25 µ) sterilized filters. The filtrate was used in a Petri dish bioassay, to measure the antagonistic activity of the active compound or the lost of its effect due to the thermal treatment.

Determination of the proteinic nature of the active compound

Lowry *et al*. [37] colorimetric method was used because it is highly sensitive to quantify the proteins present in the raw extract. A type curve with albumin at concentrations of 0, 100, 200, 300, 400 and 500 µ/mL was done. To avoid the additive effect of the proteinic content of the bacteria, once the active antagonistic component was produced, the cells were eliminated from the medium surface with three washes of H_2O. The raw extract and the albumin solutions, at different concentrations, were treated with the same Lowry method to quantify proteins. The raw extract and the albumin solutions, at different concentrations, were read in a spectrophotometer Spectronic^R 20 Genesys^{TM} in 600 nm absorbance. To obtain the protein concentration of the raw extract grown in solid PDA medium, the proteins of the control medium, without bacterial growth, were substracted to have the accurate value.

Results

Fungus isolation. A greenish-yellow, slow growing fungus was isolated from damaged maize grains of `Las Yescas´, Valle Hermoso, State of Tamaulipas. The microscopic features showed conidiophores characteristic of genus *Aspergillus* with biseriated phyalides, and after eight days, brown sclerotia of 1-2 mm were observed. Following Raper and Fennell's classification key [51], the fungus was identified as *Aspergillus flavus* Link.

Isolation of antagonistic bacteria. A total of 3425 bacterial isolates were obtained: 2168 from rhizosphere maize soil, 686 from maize bracts, and 571 from maize silks. All bacteria colonies were tested in two replications, in a qualitative antagonistic test. The 1257 bacterial isolates obtained from maize phyllosphere (silks and bracts) had no potential to control *A. flavus*.

Qualitative antagonism test. From 2168 bacterial isolates from maize rhizosphere, only 9 isolates (Co-47 as Biovar IV, Co-81 as Biovar III, Tez-118, Tez-226, Tez-227, Ba-53, Ba-72, Ba-205 and Ba-72 as Biovar II) inhibited the growth of *A. flavus* producing different inhibition halos, depending on the ability to produce toxic metabolites against the fungus. Isolates Co-47, Co-81 were the most efficient ones and had potentiality to control *A. flavus* based on tests in laboratory, greenhouse and field [38]. Isolates Co-47, Co-81, Tez-118, Tez-226 and Ba-72 destroyed mycelia, sclerotia and conidia of *A. flavus*. The test finished after eight days when the fungus of the control plate grew entirely on the PDA medium, and the zones of inhibition produced by different bacterial colonies can be observed. All bacterial isolates that inhibited the fungal growth were considered for the quantitative antagonism test. Although some bacterial isolates didn´t produce inhibition zones, they were considered in the quantitative test.

Quantitative antagonism test. The bacterial isolates designed as Co-47 (Figure 2), Co-81, Tez-118 had the highest inhibitory activity on the growth of *A. flavus,* other isolates: Ba-53, Ba-72, Ba205, Ba,252, Tez-227 and Co-165 had antagonistic activity too, but loss their inhibitoty activity after three weeks of handling in Petri dishes. The isolates Co-47, Co-81, Tez-118 and Tez-226 retained their antagonistic activity for long time when conserved in distilled water. All bacterial isolates preserved on distilled water after two months developed two kinds of colonies one translucent and the other with white color. The white colonies show the best antagonistic activity. The isolates from silks and bracts selected for the quantitative test did not inhibit the fungus in comparison with isolate Co-47. There were significant statistical differences between the effectiveness of the isolates to inhibit the fungus ($H=85.16$, $p < 0.0001$). Isolates Co-47, Co-81, Ba-53, Ba-72, Ba-205, Ba-252, Tez-118 and Tez-226 were the most effective, reducing by > 50 % the average diameter of the fungal colony. The isolates Tez-227 and Co-165 from rhizosphere and all from phyllosphere were not considered because of their low inhibitory capacity. The bacterial inhibitory activity limit up to 0.23% against *A. flavus* was considered antagonistic. There were very few bacterial isolates with antagonistic capacity to protect maize against *A. flavus* infection (Table 1).

Identification of antagonistic bacterial strains. Four out of five antagonistic isolates were Gram negative, oxidative and non-fermentative, and produced fluorescent green pigment in B King media and in slant media. Co-47 was also Gram-negative, oxidative and non-fermentative, but was not fluorescent and the bacteria had three polar flagella (Figure 1). Results of biochemical tests are presented elsewere [38]. Following Bergey´s Manual [27], isolates Co-47, Co-81, Tez-118, Tez-226 and Ba-72 were identified as *Pseudomonas fluorescens*; isolate Co-47 following API 20E and 20NE was also identified as *P. fluorescens* non fluorescent.

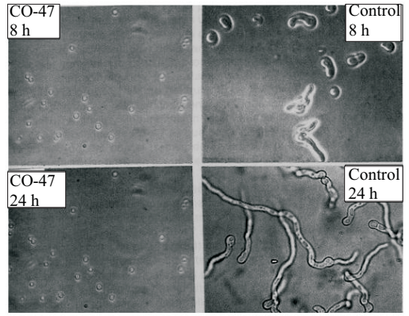

Figure 2. Effect of the isolate Co-47 on the germination of conidia of *Aspergillus flavus* at different incubation times.

Antagonism tests in maize plants from greenhouse and field. The isolates that show more antagonisms in the greenhouse and in field assays were Co-47 and Co-81 which had statistically meaningful effect over the infection of *A. flavus* ($X_2 = 184.8$, $P< 0.0001$). There were no statistically meaningful differences between the control and effect of the isolate Ba-72 against *A. flavus,* therefore it was the less effective for biocontrol of the fungus.

Characterization of the active principle of the strain Co-47

a) Minimum medium. The antagonistic bacterial isolates Co-47, Co-81 and Tez-118 produced a halo of inhibition of the growth of *A. flavus* when the fungus was grown in a medium added with iron ion ; the same result result was obtained when the bacterial isolates and the fungus were cultured in a medium without iron (Table 2). As the test resulted positive for siderophores, to verify and assure its production a more specific and sensitive test in Universal chrome azurol medium was done.

b) Universal Chrome azurol (CAS) medium for the detection of siderophores. After 48 hours of incubation a yellow-amber halo was seen around the bacterial isolates Co-47, Co-81and Tez-118, this means that they able to produce siderophores, while the control *Rhizobium*

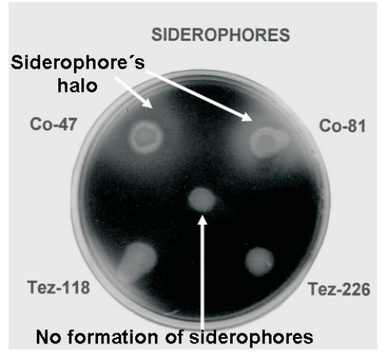

Figure 3. Detection of siderophores in chromo zurol medium. The yellow halo around the bacterial colonies indicates a positive rection. At the center is *Rhizobium melliloti* as control.

melliloti CP Mex-85 did not form halo (Figure 3). The yellow-amber halo is formed because the siderophores capture the iron ion link to the chrome azurol that is a weak quelant of the iron ion, this reaction originates a change in the medium coloration from blue to yellow-amber. This test has been universally adopted because of its high sensitivity for the detection of siderophores produced by microorganisms as bacteria and fungi (Figure 3). This test confirms that the siderophores are related to the process of inhibition of isolates Co-47, Co-81 and Tez-118 over *A. flavus.*

Proteinic conformation of the inhibitory compound from isolate Co-47

a) Selection of the medium extract and its concentration for the production of the inhibitory compound. The inhibition values of the fungi with each extract are in Table 1. The control fungus developed well.

The Kruskall-Wallis variance analysis, showed meaningful differences among media ($F_{(2,30)} = 502.46$, $P < 0.0001$), concentrations ($F_{(4,30)} = 130.77$, $P < 0.0001$) as well as media and concentration ($F_{(8,30)} = P < 0.0001$), the best inductor medium that produces the highest amount of active compound is PDA at a concentration of 2% with the highest inhibition in growth of *A. flavus* (Table 3). The other two

Table 1. Inhibition of *A.flavus* sown at different times, due to the antagonistic bacteria

Antagonistic Bacteria	Incubation time of the antagonistic bacteria in relation to the diameter (cm) of the colony of *A. flavus*			
	0 h	24 h	48 h	72 h
Control	8.5	8.5	8.5	8.5
	8.5	8.5	8.5	8.5
	8.5	8.5	8.5	8.5
Average	8.5	8.5	8.5	8.5
Co-47	1.3	0.2	0.0	0.0
	1.6	0.0	0.0	0.0
	1.6	0.0	0.0	0.0
Average	1.5	0.6	0.0	0.0
Co-81	1.6	1.2	0.3	0.9
	1.6	1.2	1.2	0.0
	1.6	1.1	1.2	0.0
Average	1.6	1.2	0.9	0.3
Tez-118	1.7	1.4	0.0	0.0
	1.7	0.2	0.0	0.0
	1.6	0.9	0.0	0.0
Average	1.7	0.8	0.0	0.0
Tez-226	1.7	1.6	0.0	0.0
	1.8	1.6	0.0	0.0
	1.9	1.1	0.0	0.0
Average	1.8	1.4	0.0	0.0
Ba-72	2.1	1.5	1.4	0.4
	2.3	1.8	0.6	0.4
	2.4	1.6	0.6	0.4
Average	2.3	1.6	0.9	0.4
Ba-252	7.2	5.5	3.5	3.2
	7.3	5.8	3.6	3.1
	7.2	5.5	3.7	3.2
Average	7.2	5.6	3.6	3.2

extracts were less effective, although they showed a good behavior at a concentration of 1.5%.

b) Extraction of the active compound. In accordance to the extraction method proposed by Shannahan *et al*. [54], a yellow-amber extract of acetone/water was obtained and used in the bioassays to prove and assure the inhibitory effect over *A. flavus*. These inhibition assays resulted positive, so the extract was stored in 200 mL bottles at 4 °C.

C) Effect of temperature on the active compound. The active compound present in the raw extract had important changes in its inhibitory activity over *A. flavus* when it was submitted to different temperatures, showing a total lost of inhibitory activity at 45, 50, 55, 60, 65 and 70 °C; but this activity was present at temperatures of 35 and 40 °C, which means they were able to produce the active compound that produces the isolate Co-47 changes with temperature due to its low inactivation temperature. This behavior with temperature was responsible of the failure of the assays with several aminoacids in liquid media to try to obtain the active compound, because the minimal temperature of evaporation applied to concentrate the extract was 50 °C, in which the inhibitory compound lost its activity against *A. flavus*.

d) Determination of the proteic nature of the inhibitory compound. In agreement with Lowry´s [37] method the proteic nature of the inhibitory compound in the raw extract was obtained, in a concentration of 1073.33 mg/mL. This value was obtained substracting the value of the proteinic content of the extract in PDA medium, without culturing the isolate Co-47. The proteins present in potato were substrated from the protein produced by the bacterial strain. Calibration of albumin is presented in Table 4.

Discussion

Antagonistic isolates from maize rhizosphere could be important in preventing fungal infections, and this protective effect, in the case of *A. flavus* control, will depend on their capacity to adapt effectively to the phyllosphere system which has different conditions of humidity and temperature. Microbial action cannot be restricted to one niche; so it is necessary to test their adaptation to other sites especially when bacteria have expressed a good antagonistic activity against specific phytopathogenic fungi. Antibiosis *in vitro*

Table 2. Production of siderophores by bacterial isolates in a deficient iron ion medium and in another enriched with iron

Medio	Halo diameter (cm) of the inhibition produced by the bacterial isolates over *A. flavus*			
	Co-47	Co-81	Tez-118	Tez-226
M – Fe	1.6	1.4	1.5	1.6
	1.6	1.5	1.5	1.5
	1.6	1.5	1.5	1.5
	1.7	1.4	1.6	1.5
Average	1.6	1.4	1.5	1.5
M + Fe	1.6	1.6	1.6	1.6
	1.5	1.5	1.5	1.6
	1.6	1.5	1.5	1.5
	1.6	1.5	1.6	1.5
Average	1.6	1.5	1.5	1.5

M - Fe= Medium without iron ion; M + Fe= medium with iron ion.

showed that isolates Co- 47 and Co-81 had the highest antagonistic activity, so it is important to determine that their behavior in phyllosphere is as useful as the one shown in milky ear bioassay done previously [39]. Because many spores of *Aspergillus flavus* stay in the maize leaves as a potential pathological inoculum and if these bacteria can destroy them will be helpful to diminish the inoculum in the field.

On respect to the stability of isolates, after two months Co-47 remained stable. Other isolates (Ba-53, Ba-205, and Ba-252) soon lost their stability, producing two kinds of colony types, a mucous white colony, that kept the antagonistic properties, and a translucent one. This indicates that some isolates with stable inhibition had an advantage over others and can be used or adapted to the phyllosphere system.

The method employed to quantify the antagonistic capacity of the bacteria isolates in PDA solid media, where *A. flavus* with no bacterial isolates was included, is more precise than the one used by Jiménez [30], who measured the antagonism of *Bacillus* sp. against *Rhizoctonia solani* by the biomass determination produced in liquid media.The problem is that the liquid condition is not the best for fungal development, the fungus would be in disadvantage in relation to the antagonistic bacteria. Solid media facilitates the accurate measurement of both fungal and antagonistic bacteria growth diameters, and it is closer to their natural environmental conditions. This method quantified antibiosis measuring the inhibition halos such as others have suggested [47]

Askew and Laing [4] proposed a method also in solid media in which four levels of inhibition were established depending on the growth of *Trichoderma* grown with *Rhizoctonia*, this method is semiquantitative and does not determine the antagonistic capacity of a microorganism. This test has been worlwide adopted is more accurate, but is not applicable to antagonists that produce volatile compounds.

Pseudomonas are the bacteria responsible of antagonism by siderophores. Siderophores are low molecular weight compounds with high affinity for iron ion and they are responsible of the inhibition that bacteria cause to many plant pathogens. The genus *Pseudomonas* has been reported as responsible of the antagonistic processes by siderophores [35, 50, 53]. There are reports about the highest siderophores concentration obtained in succinate medium, ferric iron increases the growth yield and completely repressed siderophores production above 200 g/L, but had a positive effect below 160 g/L [50]. *Pseudomonas* strains promoted the uptake of both Cu and Fe in *Phaseolus vulgaris* seedlings [8].

P. fluorescens are well known bacteria useful for biological control in seedling disease of rice [1,33]; fireblight caused by *Erwinia amylovora* in pear [40]; and there are studies about *P. fluorescens* used for biocontrol and obtained from rhizosphere of maize, bean, cucumber, wheat [46,49], and sugarbeets [43]. *P. fluorescens* was obtained also from wood frog and their enhancement of ice-nucleating activity has potential as biological control agents for overwintering adults of Colorado potato beetles [10]. This bacteria has been reported also for phyto-remediation strategy because it can

Table 3. Effect of different extracts on the production of the active compound of the isolate Co-47

Extract	Final diameter of the colony of *A. flavus* at different concentrations of extracts				
	0.25 %	0.5 %	1.0 %	1.5 %	2.0 %
Meat	4.3	4.0	3.8	3.0	3.8
	4.3	4.0	3.7	3.0	3.9
	4.2	4.1	3.7	3.2	3.6
Average	4.3	4.0	3.7	3.0	3.8
Control	8.5	8.5	8.5	8.5	8.5
	8.5	8.5	8.5	8.5	8.5
	8.5	8.5	8.5	8.5	8.5
Average	8.5	8.5	8.5	8.5	8.5
Yeast	4.4	3.5	2.6	2.9	3.2
	4.2	3.5	2.6	2.8	3.0
	4.2	3.2	2.6	3.0	3.3
Average	4.3	3.4	2.6	2.9	3.2
Control	8.5	8.5	8.5	8.5	8.5
	8.5	8.5	8.5	8.5	8.5
	8.5	8.5	8.5	8.5	8.5
Average	8.5	8.5	8.5	8.5	8.5
Potato	2.8	2.6	2.5	2.3	2.2
	3.0	3.8	2.5	2.3	2.2
	3.0	2.4	2.4	2.4	2.3
Average	2.9	2.6	2.5	2.3	2.2
Control	8.5	8.5	8.5	8.5	8.5
	8.5	8.5	8.5	8.5	8.5
	8.5	8.5	8.5	8.5	8.5
Average	8.5	8.5	8.5	8.5	8.5

Table 4. Values for the calibration of albumin and the protein determination of the raw extract

Albumin 100 µ/mL (mL)	Absorbance (nm)			
	Observed	Expected	Raw extract	Potato extract
0.0	0.0	0.0		
0.1	0.167	0.1477	0.262	0.121
0.2	0.268	0.2697	0.479	0.253
0.3	0.427	0.3917		
0.4	0.506	0.5137		
0.5	0.621	0.6357		

catabolize herbicides such as chloroacetamides (benoxacor) in corn [26].

Isolate Co-47 was identified as *P. fluorescens*, and showed a loss of the capacity to produce fluorescent pigment on B King medium, but kept its antagonistic activity. This phenomenon has been observed in some bacteria of the fluorescent group [55]. This change in the physiology of the bacteria could be the result of mutations due to environmental pressure, or the chemical effect caused by the use of pesticides.

The genus *Burkholderia,* proposed by Yabuuchi *et al*. [60], or *Ralstonia* [61], are new genera with species related phyllogenetically with *P. solanacearum* [7], but the isolate Co-47 differs from them by the biochemical and physiological characteristics. With the exception of *Burkholderia cepacia*, no species are able to grow in 5% sodium chloride, while isolate Co-47 could do so. Besides, the use of carbohydrates separates Co-47 from *Ralstonia* and the production of blue and yellow pigment by isolate Co-47, separates it from the genus *Burkholderia* that do not produce these pigments.

Although, the behaviour of these three genera does not differ much, the genera *Burkholderia* and *Ralstonia* have plant, animal and human pathogenic species, and *P. fluorescens* was reported both as spoilage microorganism of vegetables [29] or present in fresh broiler chicken carcasses producing off odors [52].

The antagonistic bacterial isolates from maize rhizosphere were innocuous on germination of seeds and behaved as saprophytic in the phytopathogenic tests of potato rot and tobacco leaf hypersensitivity [38]. The increase in germination of some seeds and their inhibiting effect against *A. flavus* made these antagonistic bacteria an excellent prospect for biological control. Many *P. fluorescens* isolated from rhizosphere of maize are considered as Promoting Growth Plant Rhizobacteria, this could be the case of isolates Co-47, Co-81, Tez-226, Tez-118 and Ba-72 due to their positive effect on germination of seed crops, their origin from maize rhizosphere and saprophytic behavior. Isolate Tez-227 had an exponential growth control; while isolate Co-165 at the beginning just inhibited the fungus, nevertheless *A. flavus* finally grew completely on PDA medium. The other tested isolates from rhizosphere had good inhibition effect.

P. fluorescens have been thoroughly used in molecular engineering from Novartis [36]; for the control of plant pathogens, even human parthogens [20, 21, 24, 25, 42]. Some isolates of *P. fluorescens* increased plant growth, and inhibited the development of phytopathogenic microorganisms [35], such as fungal pathogens (*Pythium* spp.) of cucumber [23], and *Pythium ultimum* from pea rhizosphere [45] protecting these vegetables. All phytopathogenic tests done in plant tissues and its effectiveness as *A. flavus* inhibitors are valuable features to consider these isolates as potentially effective biofungicides in competition with fungicides of traditional use.

The present work includes the isolation of 3425 bacterial colonies which were tested against *A.flavus*, where the useful isolates were identified and their biochemistry, antibiosis and chemical analysis were done. Futhermore, no bacterial isolates from bracts and silks, were antagonistic, meaning that that vulnerable areas are not protected by these isolates.

The conclusion of the present study is that *P. fluorescens* isolate Co-47 can be a good biological control agent against *A. flavus* for its behavior as inhibitor of this fungus for two months and as a promoter of seed germination.This work is part of a more complete research study from the sampling of bacteria from maize crop in different geographical places, to their identification, biochemistry, antagonistic behavior and characterization of the inhibitory compound against *A. flavus.* The data presented here can be useful for the industrial production of biocontrol bacteria or their metabolites. This is an effort to substitute the employment of fungicides to reduce the chemical contamination.

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