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Characterization of *Paenibacillus* spp. CBRM17 as antagonist of phytopathogenic fungi and growth promoter of *Capsicum chinense* Jacq.

Caracterización de *Paenibacillus* spp. CBRM17 como antagonista de hongos fitopatógenos y promotor de crecimiento de *Capsicum chinense* Jacq.

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Abstract

Studies of the plant-microorganism relationship have made it posible to explore the potential of rhizospheric bacteria to improve the health and quality of plants. In the present study, *Paenibacillus* sp. CBRM17 was characterized *in vitro* for its ability to inhibit the growth of phytopathogenic fungi that cause diseases in horticultural crops in the tropics, finding a reduction in the mycelial growth of *Alternaria*, *Fusarium* and *Helmintosphorium* strains in the range of 50 to 70 %, additionally its biochemical properties related to the promotion of plant growth were characterized, registering the production of 0.36 µg/mL of indole acetic acid (IAA), the solubilization of tricalcium phosphate with solubilization index (SI) of 2.41 mm and solubilization efficiency (SE) of 140.6 %, producing in the supernatant 63.5 mg/L of soluble phosphorus, in addition to being positive for ACC deaminase activity. Inoculations trials of habanero chili (*Capsicum chinense* Jacq.) seeds with *Paenibacillus* sp. CBRM17 showed its potential to be used as an inoculant in the growth promotion of this crop, since it increased all growth variables; increasing the total fresh and dry biomass by 93.3 and 96.4 %, respectively.

Microbial antagonism, Indole acetic acid, Phosphate solubilization

Resumen

Los estudios de la relación planta-microorganismo han permitido explorar el potencial de las bacterias rizósfericas para mejorar la sanidad y calidad de las plantas. En el presente estudio se caracterizó *in vitro* la cepa *Paenibacillus* sp. CBRM17 por su capacidad para inhibir el crecimiento de hongos fitopatógenos causantes de enfermedades en los cultivos hortícolas del trópico, encontrando reducción del crecimiento micelial de cepas de *Alternaria*, *Fusarium* y *Helminthosporium* en el orden 50 al 70 %, adicionalmente se caracterizaron sus propiedades bioquímicas relacionadas con la promoción del crecimiento vegetal, registrando la producción de ácido indol acético (AIA) con 0.36 µg/mL, la solubilización de fosfato tricálcico con índices solubilización (IS) de 2.41 mm y eficiencia de solubilización (ES) del 140.6 %, produciendo en el sobrenadante 63.5 mg/L de fósforo soluble, además de resultar positiva para la actividad ACC desaminasa. Ensayos de inoculación de semillas de chile habanero (*Capsicum chinense* Jacq.) con *Paenibacillus* sp. CBRM17 mostraron su potencial para ser usado como inoculante en la promoción de crecimiento de este cultivo, ya que incrementó todas las variables de crecimiento; aumentando la biomasa fresca y seca total en un 93.3 y 96.4 %, respectivamente.

Antagonismo microbiano, Ácido indol acético, Solubilización de fosfatos

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Introduction

Plant pathogenic fungi are a group of microorganisms that cause diseases in tropical crops, mainly *Fusarium* spp., *Alternaria* spp., *Cercospora* spp., *Curvularia* spp. and *Helminthosporium* spp. highly damaging genera that cause different crop pathologies (Vásquez-López *et al*., 2009; Badía *et al*., 2011). In order to control them, organosynthetic fungicides are applied more frequently, which continuously contaminate the environment, affect human health and generate new strains of fungicide-resistant phytopathogens. Therefore, there is an urgent need to search for more agro-ecological alternatives, including the use of antagonistic microorganisms such as *Paenibaillus* spp., which is a rhizospheric bacterium capable of exerting a biocontrol effect on phytopathogenic fungi; due to the production of molecules that exert antifungal activity, this, together with the fact that the genus *Paenibaillus* is capable of producing endospores that ensure its survival in the environment, makes it a viable alternative (Annapurna *et al*., 2013). Some species such as *P. polymyxa* also show growth promoting effect on crops such as pepper (*Capsicum annuum*), soybean (*Glycine max* L.) and tomato (Solanum lycopersicum) (Lamsal *et al*., 2012; Annapurna *et al*., 2013, Mei *et al*., 2014). This is attributed to different microbial mechanisms such as: production of indole acetic acid (IAA), a hormone that induces root and vegetative growth, solubilisation of mineral elements such as phosphorus, atmospheric nitrogen fixation, siderophore production and ACC deaminase activity have also been documented (Çakmakçi *et al*., 2007; Luna *et al*., 2013). In this study, the antagonistic potential of Paenibacillus sp. CBRM17 towards different plant pathogenic fungi affecting tropical crops was determined, as well as its plant growth promoting capacity on habanero pepper (*C. chinense* Jacq.) plants.

Methodology to be developed

Biological material

Paenibacillus spp. CBRM17 belongs to the collection of the Microbiology Laboratory of the Technological Institute of Conkal, previously identified by Mejía *et al*., (2016), and nutrient agar medium (NA) was used for its cultivation. Meanwhile, the fungi *Alternaria* spp. isolated from tomato (*Solanum lycopersicum* L.), *Fusarium* spp. from sugar cane (*Saccharum officinarum*), *Corynespora cassicola*, *Curvularia* spp. *F. equiseti* and *F. solani* from habanero chili (*C. chinense*) and *Helminthosporium* spp. from kerpis palm (*Veitchia merrili*) were provided by the Phytopathology Laboratory of the same institute and were grown on potato dextrose agar medium (PDA) for cultivation and assays.

Antagonism tests

In vitro biocontrol tests of *Paenibacillus* spp. against tropical plant pathogenic fungi were carried out in triplicate on PDA medium. First, a 0.5 cm diameter disc of active mycelium of the plant pathogen was seeded in the centre of the Petri dish, then 6 µL of a bacterial suspension ($1x10^7$ cells/mL) was inoculated at four points at 2 cm equidistant around the fungal disc. Finally, the plates were incubated at 28 °C, as negative control plates were used without the bacterial inoculation only growing the fungus, the assay was terminated when the mycelium covered the entire surface of the medium on the control plates, determining the percentage of inhibition of radial growth (ICR) of the fungus and the inhibition halos generated by the bacterial inoculum (Ezziyyani *et al*., 2004).

Plant growth promoting activities

The activity of ACC deaminase in *Paenibacillus* spp. was first performed by seeding it on Dworkin and Foster (DF) mineral minimal medium containing $(NH_4)2SO_4$ as nitrogen (N) source. After 48 h at 30 °C, a colony was reseeded on the same medium containing 1-aminocyclopropane 1-carboxylate (ACC) (Sigma-Aldrich®) as N source replacing $(NH_4)2SO_4$. The plate was incubated for 72 h at 30 °C to observe the growth (Penrose and Glick 2003).

For the determination of AIA production by *Paenibacillus* spp., 5 mL of a bacterial suspension containing 1x108 cells/mL were inoculated in triplicate into flasks containing 50 mL of nutrient broth supplemented with L-tryptophan (1 g/L), the flasks were shaken at 180 rpm for 72 h at 30 °C. The supernatant was then recovered by centrifugation at 3,000 xg for 15 min, and 1 mL of Salkowski reagent was added to 1 mL of the supernatant, and the mixture was kept in the dark at room temperature for 30 min for complex development (Almoneafy *et al*., 2012).

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Upon completion, the samples were read at 535 nm on a Genesys 10UV spectrometer to determine the concentration of AIA produced (Badía *et al*., 2011).Siderophore production was determined by inoculating the bacterial strain in triplicate into tubes containing 7 mL of M9 liquid minimal salts medium, after 16 h of growth, a subculture was performed by taking an aliquot of 70 µL which was transferred to new tubes containing the same medium, which were placed in agitation at 120 rpm and 30 °C for 24 h. The supernatant was then recovered by centrifugation at 8,000 Xg for 10 min, and 1 mL of this was taken and mixed with 1 mL of chromium azurol-S (CAS), the change in colour of the CAS reagent from blue to orange was considered positive (Alexander and Zuberer 1991).

Phosphate solubilising capacity was tested by quadruplicate inoculation of 0.8 µL of a 1x108 cell/mL suspension of *Paenibacillus* spp. in Petri dishes with Pikovskaya solid medium (Pradhan and Sukla, 2005), the plates were incubated at 30 °C, recording phosphate solubility daily after three days up to 15 days, subsequently calculating the solubility index (SI) and solubility efficiency (SE) of each strain according to Qureshi *et al*. (2012). For the quantitative phosphate solubilization method, bacteria were grown in triplicate on NBRIP (National Botanical Research Institute's Phosphate growth medium) (Mehta and Nautiyal 2001) and at the end of the culture, pH was recorded and soluble phosphorus was determined in the supernatant by molybdenum blue method (Mussa *et al*., 2009).

Growth promotion of habanero pepper (Capsicum chinense Jacq.)

For the plant growth promotion trials, habanero chili (C. chinense Jacq.) was used as a model plant. Seeds of chili cv. naranjo criollo were inoculated with a bacterial suspension of Paenibacillus sp. (1x108 cells/mL) and then sown in trays with commercial substrate Cosmopeat®. Once germinated, they were reinoculated at 15 and 28 days after germination (DDG) with 1 mL of Paenibacillus sp. per plant at a concentration of 1x108 cells/mL (Luna *et al*., 2013). After 28 DDG, transplanting was carried out in 32 oz (0.95 L) unicel cups containing a 1:1 mixture of Luvisol-type soil with bobinaza, taking the plants to the greenhouse.

Subsequently, eight days after transplanting, they were re-inoculated with the same dose (Canto-Martín *et al*., 2004), fertilisation was carried out based on the regional recommendations of the crop (Soria *et al*., 2002) and at the end of 60 DDG the experiment was completed. Each plant was evaluated for height, stem diameter, number of leaves, leaf area, root volume, root length, fresh and dry biomass of the aerial part and root, as growth variables. A completely randomised design with ten replicates was applied, leaving a control lot without inoculation. The data obtained were subjected to analysis of variance (ANDEVA) and mean comparison test (Tukey, $p \leq 0.05$), using SAS software version 9.3 for Windows (SAS Institute Inc. 2010).

Results

Antagonism of Paenibacillus spp. towards tropical plant pathogenic fungi

The *in vitro* antagonism assays show that Paenibacillus sp. CBRM17 inhibits five of the seven fungi tested, presenting ICR ranges from 50.6 to 70.0 % (Table 1), with Fusarium equiseti being the most inhibited. The antagonistic and antifungal activity of Paenibacillus genus towards fungi of agricultural interest has been reported previously, Zhai et al. (2021) show inhibitions from 61.8 to 79.2 % of P. polymyxa HX-140 towards Fusarium, Colletotrichum, Sclerotinia, Phytophthora and Rhizoctonia strains, while Zhang et al. (2018), achieve 69.8 % inhibition of Botrytis cinerea using P. polymyxa ShX301. The inhibitory activity of Paenibacillus also extends to bacterial pathogens such as Clavibacter michiganensis (Chávez et al., 2020), although the inhibition percentages reported are lower (21.7 %).

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The production of diffusible antagonist molecules in the culture medium delimits the mycelial growth of the pathogen. In the present study, inhibition halos were recorded for three of the seven fungi evaluated (Figure 1), with values of 2.77, 1.87 and 6.67 mm for Alternaria sp., *Fusarium* spp. and *F. equiseti*, respectively (Table 1). The nature of these molecules can be diverse, Ran *et al*., (2020), report from *P. polymyxa* 7F1, the production of a 36 kDa enzyme with glycosyl hydrolase domain that inhibits the growth of *Fusarium species*; *F. equiseti*, *F. verticillioide*, *F. semitectum*, *F. graminearum*, *F. proliferatum*, *F. oxysporum*, *F. nivale*, *F. oxysporum* and *Colletotrium nivale* and *Colletotrichum gloeosporioides*, while Soo et al. (2016), found in *Paenibacillus polymyxa*, an increase in protease and amylase activity, which could be related to the biocontrol activity of anthracnose in apple caused by *Colletotrichum gloeosporioides* and *C. acutatum*. Peptide production is another biocontrol strategy identified, Beatty and Jensen (2002) report the production of fusaricidin in Paenibacillus polymyxa PKB1, a cyclic depsipeptide of 882 kDA that inhibits *in vitro* the growth of Leptosphaeria maculans, the fungus causing blackleg in canola.

Mushrooms	Halos (mm)	ICR (%)
Altenaria spp.	2.77 b	60.41 b
Corynespora spp.	0.00 d	0.00 e
Curvularia spp.	0.00 d	0.00 e
Fusarium spp.	1.87 c	50.67 d
F. equiseti	6.67 a	70.04 a
F. solani	0.00 d	54.19 c
Helminthosporium spp.	0.00 d	52.12 dc
DMS	0.56	2.92
ICR= Radial growth inhibition, LSD = Least Significant Difference.		

Table 1 Antagonism of *Paenibacillus* spp. CBRM17 against different tropical phytopathogenic fungi

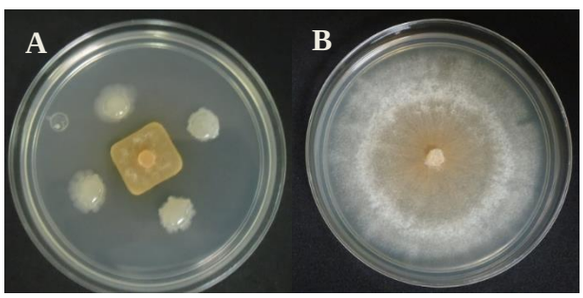

Figure 1 Mycelial growth inhibition in *F. equiseti* and the presence of inhibition halos by *Paenibacillus* spp. CBRM17 A) and control B)

Mechanisms related to the promotion of plant growth

In the test for ACC deaminase enzyme activity, *Paenibacillus* spp. strain CBRM17 was positive when grown on DF medium and used 1-aminocyclopropane-1-carboxylic acid (ACC) as the sole source of nitrogen. Various PGPR such as *Enterobacter cloacae*, Pseudomonas putida and *P. fluorescens* also have the ability to synthesise this enzyme, having effects on the growth promotion of Oryza sativa and Glycine max L. seedlings (Penrose and Glick 2003). The importance of the activity of this enzyme is because it reduces the production of ethylene in plants, due to the degradation of its precursor (ACC), thus avoiding the deleterious effects of ethylene, such as the suppression of root growth, among others; the synthesis of this enzyme is encoded in the acdS gene (Mayak *et al*., 2004; Hernández-Forte *et al*., 2015). Regarding the ability of *Paenibacillus* spp. CBRM17 to solubilise phosphates, after seven days, in the plates with Pikovskaya medium, the formation of solubilisation halos was observed with IS values of 2.41 mm and ES of 140.6 %, while in the tests in liquid medium a concentration of 63.5 mg/L of soluble phosphorus was found and a decrease in the pH of the filtrate to 4.75. Almoneafy *et al*., (2012) and, Xu and Kim (2014), in evaluations of phosphate-solubilising *Paenibacillus* spp. *Bacillus* spp. and *Pseudomonas* spp. also observed the formation of clear zones around bacterial colonies on plates after seven days of incubation. The IS reported in our research is lower than those of Bacillus spp. with values of 3.3 mm and efficiencies higher than 233 % (Qureshi et al., 2012). However, this evidence needs to be corroborated with quantitative analysis, analysis of supernatants of *Paenibacillus kribbensis* UFLA 03-46 and *Paenibacillu* spp. UFLA 03-12 yield values of 25 mg/L soluble phosphorus and a decrease in pH to 5.3 and 5.8, respectively (Marra *et al*., 2012). These values are lower than those obtained in the present study, while Luna et al. (2013) report the concentration of 56.0 mg/L soluble phosphorus in B. megaterium MA06, values similar to those obtained in our trials. The decrease of pH in the media is due to the production of organic acids, responsible for solubilising the mineral phosphate, such as: lactic, isovaleric, isobutyric and acetic acid (Mehta and Nautiyal, 2001).

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In the quantification of AIA and indoles production, *Paenibacillus* spp. at 72 h had a production of 0.36 µg/mL AIA; while in the CAS assay, siderophore production was not detected (Table 2). Studies on Paenibacillus sp., P. polymyxa CF05 and P. polymyxa RC05, found AIA concentrations in the range of 5.0 to 25 µg/mL (Çakmakçi *et al*., 2007, Xu and Kim 2014, Mei *et al*., 2014) higher than those obtained in our study.

Caracter de *Paenibacillus* sp. CBRM17	
IS (mm)	2.41 ± 0.04○
ES (%)	140.6 ± 4.20
P ($mg.L^{-1}$)	63.50 ± 4.29
pH	4.75 ± 0.09
AIA ($\mu g\ mL^{-1}$)	0.36 ± 0.15
ACC deaminase	+
Siderophores	-
IS: solubilisation index; ES: solubilisation efficiency; P: phosphorus (solubilised); IAA: indole acetic acid and +/-: positive or negative to these tests. ○Means and standard error.	

Table 2 Properties of *Paenibacillus* spp. CBRM17 determined for growth promotion in habanero peppers (*Capsicum chinense* Jacq.)

Growth promotion of habanero peppers (*Capsicum chinense* Jacq.)

The results of the growth promotion trial show that the inoculation of *Paenibacillus* spp. CBRM1 in habanero peppers had beneficial effects at 60 DDG, increasing stem diameter, leaf area, root length and volume, as well as biomass through total fresh weight, root and aerial part, and dry weight, respectively, with respect to the control (Table 3 and 4). For fresh and dry biomass, these increases were 93.3 % and 96.4 %, respectively. These increases are dependent on the plant species and crop conditions, Annapurna *et al*., (2013) report that inoculation of soybean with *P. polymyxa* HKA-15 in G. max L. increased the dry weight of root and aerial part by 29.2 and 49. 2 % respectively, higher values found in our study, while inoculation of tomato (*S. lycopersicum*) with indole acetic acid producing *P. polymyxa* CF05 significantly increased root fresh and dry weight and total fresh and dry weight by 21.9, 60.0, 29.4 and 38.3 % respectively (Çakmakçi *et al*., 2007, Mei *et al*., 2014), values lower than those obtained in this study.

Other microorganisms such as Azospirillum sp and Bacillus are also able to promote the growth of *C. chinense* Jacq. *and C. annuum* L. plants, increasing plant height, leaf area, aerial and root dry weight as reported by Canto-Martin *et al*., (2004) and Lim and Kim (2009). The growth-promoting characteristics are mainly attributed to AIA acid production and phosphate solubilisation according to Sanchez-Lopez *et al*., (2012).

Treatment	AL (cm)	DT (mm)	AF (cm^2)	LR (cm)	VR (m^3)
CBRM17	13.9 a○	3.2 a	223.7 a	23.9 a	2.7 a
Witness	10.1 b	2.7 b	128.8 b	20.9 b	1.3 b
DMS	2.4	0.38	82.6	3.0	0.81
AL = height; SD = stem diameter; FA = leaf area; RL = root length; RL = root volume. LSD = Least Significant Difference; ○Means with different letters in each column are statistically different. (Tukey, 0.05).					

Table 3 Effect of Paenibacillus sp. CBRM17 on the growth variables of habanero pepper plants. (*Capsicum chinense* Jacq.)

Treatment	weight (g)					
	PFR	PFPA	PFTo	PSR	PSPA	PSTo
CBRM17	2.9 a○	5.7 a	8.7 a	0.27 a	0.86 a	1.1 a
Control	1.3 b	3.8 b	4.5 b	0.11 b	0.44 b	0.56 b
DMS	0.95	1.9	2.7	0.08	0.26	0.29
PFTo = total fresh weight; RWW = root dry weight; AWW = aerial part dry weight; TWD = total dry weight. LSD = Least Significant Difference; ○Means with different letters in each column are statistically different (Tukey, 0.05).						

Table 4 Biomass found in habanero pepper (*Capsicum chinense* Jacq.) plants inoculated with *Paenibacillus* spp. CBRM17

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Conclusions

Paenibacillus spp. CBRM17 has antagonistic activity to reduce *in vitro* mycelial growth of phytopathogenic fungi, probably due to the production of diffusible metabolites that generate inhibition halos during dual confrontation. In addition, it exhibits biochemical properties related to plant growth promotion, which was proven to generally increase the biomass of *Capsicum chinense* plants, an activity that can be exploited to improve crop productivity.

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