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In vitro antagonistic activity of *Bacillus subtilis* strains isolated from soils of the Yucatan Peninsula against *Macrophomina phaseolina* and *Meloidogyne incognita*

Actividad antagónica *in vitro* de cepas de *Bacillus subtilis* aisladas de suelos de la Península de Yucatán contra *Macrophomina phaseolina* y *Meloidogyne incognita*

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Abstract. The antagonistic activity of native *Bacillus subtilis* strains from Yucatán peninsula soils were evaluated on two soil-borne pathogens. *Bacillus subtilis* cbck36 and cbrf24 caused more than 60% inhibition of colony growth in *Machophomina phaseolina*. Cell-free culture filtrate of *B. subtilis* cbr24 were active against second-stage juveniles (J2) of *Meloidogyne incognita* (LC_{50} 25.8% v/v).

Keywords: Soil-borne pathogens; Cell-free filtrate; Rhizobacteria.

Resumen. La actividad antagónica de las cepas de *Bacillus subtilis* nativas de los suelos de la península de Yucatán fueron evaluadas en dos patógenos de suelo. *Bacillus subtilis* cbck36 y cbrf24 causaron la inhibición de más de 60% de crecimiento en la colonia de *Macho-phomina phaseolina*. El filtrado libre de células de *B. subtilis* cbr24 fue activo contra el segundo estado juvenil (J2) de *Meloidogyne incognita* (CL_{50} 25,8% v/v).

Palabras clave: Patógenos de suelo; Filtrado libre de células; Rizobacteria.

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INTRODUCTION

Soil-borne pathogens such as fungi and nematodes reduce significantly fruit yield in a wide variety of economically important crops; yield losses range from 7 to 15% per year, equivalent worldwide to $100 billion approximately (Raaijmakers et al., 2009). In this context, the fungus *Macrophomina phaseolina* causes dry root rot, stem canker and stalk rot in approximately 500 plant species (Muñoz et al., 2005). The nematode *Meloidogyne incognita* causes root damage by direct feeding and establishment, which in turn leads to plant wilting and chlorosis in approximately 3000 plant species (Castagnone, 2002; Rahman, 2005). Chemical control using synthetic fungicides or nematicides is the most used control strategy (Parra & Ristaino, 2001); however, use of these synthetic products has caused various problems due to environmental pollution, human toxicity, as well as resistance of certain pathogens (Hernández et al., 2005). An alternative to reduce the effect of these plant pathogens is the use of antagonistic microorganisms, such as species of the genus *Bacillus* (Sid et al., 2003; Schisler et al., 2004). *Bacillus subtilis* is a bacterium that has been widely used as antagonistic for soil-borne pathogens. This bacterium produces hydrolytic enzymes such as glucanases or proteases and the antibiotic lipopeptides surfactin, fengycin, and/or iturin A, capable of acting against fungi and nematodes (Cazorla et al., 2007; Knaak et al., 2007; Snook et al., 2009). The present study was carried out to evaluate the *in vitro* effects of native *B. subtilis* on *M. phaseolina* and *M. incognita*.

MATERIALS AND METHODS

Bacillus subtilis strains were isolated from soil samples from the Yucatan peninsula. This region has calcareous soils, and a sub-humid climate with summer rains. Temperatures range from 21 °C to 34 °C throughout the year, and relative humidity ranges from 61% to 83%. Rainfall varies from 1037 mm to 1213 mm per year. From January to December 2012, twenty five soil samples were taken at 10 cm depth, near plants´ rhizospheres in different sites of the Yucatan Peninsula (Conkal, Cansahcab, Mesatunich, Muna and Calakmul). One gram of soil samples was mixed with 10 mL of sterile water under intensively stirring for 1 min at 25 °C. The samples were pasteurized (80 °C/30 min). Twenty µL of pasteurized samples were diluted in sterile water (1:10, v/v) (Escobar et al., 2004). The bacteria isolated in solution were carried off on nutrient agar. A preliminary screening using the direct confrontation technique allowed to evaluate the antagonistic activity of 25 *B. subtilis* strains against *M. phaseolina*. Those strains which caused mycelial growth inhibition above 30% were selected. This yielded a total of seven active strains: cbcc2, cbrf24, cbmn22, cbck47, cbck36, cbmt2 and cbmt51. The molecular identification of the isolates was performed by amplification and sequencing of the 16S rRNA genes by PCR (Geetha et al., 2007). *Macrophomina phaseolina* (Tassi) was donated by Dr. Mario Ramírez-Lepe from the Veracruz Technological Institute, located in Veracruz, México. The antifungal activity on *M. phaseolina* was performed by direct confrontation on Potato Dextose Agar (PDA), using a 4-mm diameter PDA disc of fungal growth and 1 x 10^7 CFU/mL bacterial suspensions. Six days after inoculation, the growth inhibition zone and the percent inhibition of fungal colony growth were measured (Rodas et al., 2009). *Meloidogyne incognita* was obtained from a stock colony maintained in tomato (*Lycopersicon esculentum* Mill.) in a greenhouse, at the Conkal Technological Institute, located in Yucatan, México. The nematicidal activity of the cell-free culture filtrates on second-stage juveniles (J2) of *M. incognita* was evaluated as described by Cristobal et al. (2006). Cell-free filtrate*s* from bacteria cultivated in Nutrient Broth (72 h, 29 °C, and 200 rpm) were obtained by centrifugation (3000 rpm, 20 minutes) and filtering through a Millipore filter of 0.22 µm. Bacterial filtrates were diluted in distillated water (25%, 50% and 75%) and poured in Syracuse dishes, where J2 were placed (100 J2 per mL filtrate) to calculate the median lethal concentration (LC50) after 9 h exposure. Mortality of nematodes was recorded after 3, 6, 9 and 24 h of exposure to calculate the median lethal time (LT50) at a concentration of 50% (v/v). For analysis of variance, data were transformed to arcsin [y = arcsin (sqrt y/100)]. Multiple mean comparisons (Tukey, p=0.05) and calculation of LC_{50} and LT_{50} by probit analysis were performed using the Statgraphics Centurion Software (15.2.06 version).

RESULTS

Gene sequences of 16S rRNA showed that all strains belonged to the *B. subtilis* group (GenBank accessions kc223570, kc22357, kc223572, kc223575, kc223574, kc223571 and kc223576). The most active strains against *M. phaseolina* were cbck36, cbrf24, cbmt51, cbmn22 that caused more than 60% inhibition of fungal colony growth (Table 1). The highest growth inhibition zone (5 mm) was caused by the strain cbck36.

Cell-free culture filtrates of *B. subtilis* showed nematicidal activity on J2 *M. incognita*. The LC_{50} recorded for filtrate of strain cbrf24 (LC_{50}: 25.8%; Confidence Interval, CI: 8.0-36.0%) was significantly ($p<0.001$) lower than those of cbcc2 (LC_{50}: 66.6%; CI: 59.7-73.8%) and cbmn22 (LC_{50}: 53.1%; CI: 48.9-57.4%). Culture filtrates of strains cbck36, cbck47, cbmt2 and cbmt51 showed no nematicidal activity. From strains that showed activity, LT_{50} recorded for cbrf24 (LT_{50}:7.4 h; CI: 6.8-7.9 h) was significantly lower ($p<0.001$) than those for cbcc2 (LT_{50} 9.8 h; CI: 9.2-10.4 h) and cbmn22 (LT_{50} 9.5 h; CI: 9.1-9.9 h).

Table 1. Mean values (± standard error) of inhibition of fungal colony growth and growth inhibition zone caused by *B. subtilis* strains on *M. phaseolina*.
Tabla 1. Valores medios (± error estándar) de inhibición fúngica del crecimiento de colonias y zonas de inhibición causadas por las cepas de *B. subtilis* sobre *M. phaseolina*.

Strains	Inhibition of fungal colony growth (%)	Growth inhibition zone (mm)
cbck36	71.6 ± 2.6 a	5.0 ± 0.4 a
cbrf24	64.4 ± 4.3 a	1.8 ± 0.4 b
cbmt51	62.7 ± 0.4 a	1.6 ± 0.7
cbmn22	60.9 ± 3.2 a	0.4 ± 0.4 bc
cbck47	58.7 ± 1.1 ab	0.4 ± 0.4 bc
cbcc2	56.4 ± 3.3 ab	0.0 ± 0.0 c
cbmt2	38.7 ± 9.8 b	0.0 ± 0.0 c

Means with different letters within a column are significantly different ($p<0.05$).
Las medias con letras diferentes dentro de una columna son significativamente diferentes ($p<0,05$).

DISCUSSION

The *B. subtilis* strains cbck36 and cbrf24 (evaluated in this study) showed the highest antagonistic activity on *M. phaseolina* and *M. incognita*, respectively. The presence of growth inhibition zones in the antifungal bioassays, and the activity of the cell-free culture filtrates on *M. incognita*, suggest that the antagonistic activity of these strains might be in part due to the production of antimicrobial metabolites. Previous works have shown that *B. subtilis* produces at least five different antimicrobial compounds: subtillin, bacitracin, bacillin, subtenolin, and bacilonycin (Killani et al. 2011). We also observed that some strains, like cbcc2 and cbmt2 inhibited mycelial growth of *M. phaseolina* by direct competition, as the bacterial colonies were able to growth radially on the PDA.

The activity of the cell-free culture filtrate of cbrf24 on J2 *M. incognita* suggests that this strain might be able to produce toxic metabolites against this nematode. The presence of toxic components in cell-free culture filtrates of *Bacillus* spp. on nematode immobility and subsequent mortality has been previously reported (Caneiro et al., 1998).

We found that the cbrf24 strain of *B. subtilis* showed a wider antagonistic activity than the cbck36 strain of the same species on two soil-borne fungi pathogens, *M. phaseolina* and *M. incognita*. This strain might be a good candidate to develop a microbial based biopesticide. Additional research is required to identify the active compounds excreted by this bacterium during growth.

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