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Full Length Research Paper

Antagonistic activity of selected strains of *Bacillus thuringiensis* against *Rhizoctonia solani* of chili pepper

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The aim of this work was to determine, *in vitro*, the antagonistic effectiveness of 60 strains of *Bacillus thuringiensis* against damping-off and root and stem rot caused by *Rhizoctonia solani*. The strains were obtained from the International Collection of Entomopathogenic Bacillus at the FCB-UANL. During the *in vitro* dual culture assay only 16 of the strains displayed an inhibitory effect. Six strains were chosen to be screened simultaneously by volatile antibiotics, thermostability and seedling assay. In the volatile antibiotics assay, the strains GM-11 and GM-121 showed the best inhibitory effect over *R. solani* growth. None of the strains showed an efficient antagonistic effect during the thermoestability assay. In seedling assay, majority of the antagonistic isolates, GM-23, GM-11and GM-121, were effective in the reduction of *R. solani* infection. In addition, GM-23 increased the length of pepper seedlings. These results suggest that the *B. thuringiensis* strains studied have an excellent potential to be used as bio-control agents of *R. solani* in chili pepper.

Key words: Antagonist, biological control, damping-off, *Rhizoctonia solani*.

INTRODUCTION

In Mexico, chili pepper (*Capsicum annuum* L.) production is the most important agricultural crop in terms of the land area occupied (FOASTAT, 2005). It is an important employment generating activity (Pozo, 2004). *Rhizoctonia solani* is the main causal agent of damping-off disease of seedlings as well as root and stem rot in young transplants of several plant species (Kucharek et al., 2003). It is considered main soil-borne pathogen of chili pepper (Velásquez et al., 2001). In Mexico, 75% of chili pepper producers point to "damping-off" or root rot as the most important disease during seedling phase (Velásquez, 1991) and it is present in 88 of each 100 seedling plots causing losses from 1 to 15% (Velásquez and Victoriano, 2007). As a result, producers tend to use more seeds than they normally would, as a means of compensating the losses caused by this disease. Another problem is that the disease is generally treated by chemical applications (Pérez et al., 2004), and crop managers tend to overuse them, causing environmental and human health risks, and pest resistance can become a problem.

Biological control is an alternative to the management of diseases caused by soil-borne microorganisms (Zavaleta, 2000). From the plant growth promoting rhizo-

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bacteria (PGPR) bacteria, some species of the genus Bacillus have shown promising results for the biological control of various plant pathogens as well as growth promoters of some crops (Weller, 1988; Podile and Laxmi, 1998). Some species from the Bacillus genus are particularly effective due to their capacity to form, or produce, spores that survive and remain metabolically active under harsh environmental conditions (Rodgers, 1989). *Bacillus* spp. are nonpathogenic, easy to cultivate, and protein and metabolite secretors. These characteristics make them appropriate for the formulation of stable and viable biological products that could be used for soil-borne disease management (Kloepper, 1997). Currently available commercial products includes in its formulae enzymes, antibiotics and insecticide ingredients produced by *Bacillus thuringiensis* which is the most successfully distributed species from this genus (Valadares et al., 1998). *B. thuringiensis* produces a parasporal body named endotoxin-δ, which is toxic to protozoa, nematodes, and insects of several orders (Smith and Couche, 1991). Although it is unknown, its antifungal potential besides its specificity, virulence and safety (Gelernter and Scwab, 1992; Rowe and Margaritis, 1987). And it is also unknown how *B. thuringiensis* metabolites act in the suppression of plant pathogens. In general, *B. thuringiensis* research has led to evaluate the presence and persistence of its spores in the ground that is distant from the root but little has been investigated about the persistence of vegetative cells on plant rhizosphere (West et al., 1984, 1985). It has been reported that exudates from the rhizosphere of soybean is a promoter of the spore germination and growth of *Bacillus* spp. vegetative cells. This allows *Bacillus* spp to compete with root phytopathogens (Liu and Sinclair, 1988; Handelsman et al., 1988, 1990; Handelsman, 1991).

The aim of this work was to determine, *in vitro*, the antagonistic effectiveness of *B. thuringiensis* strains against damping-off and root and stem rot caused by *R. solani*.

MATERIAL AND METHODS

Selection of *B. thuringiensis* strains

The 60 strains of *B. thuringiensis* were obtained from the International Collection of Entomopathogenic Bacillus at the Facultad de Ciencias Biológicas-UANL. These remained lyophilized until they were activated for use in test tubes containing 2.5 ml of nutritive broth at a pH of 7. Then they were incubated to a temperature of 30°C for 24 h in rotatory agitation at 150 rpm. Later they were re-cultured in inclined nutritive agar to pH 7 and maintained in incubation at 28°C for 48 h.

Isolation of *R. solani*

The damping-off pathogen was isolated from diseased pepper seedlings by direct plating method, maintained as pure culture on potato dextrose agar (PDA) at 25°C for a week, and later stored at -20°C in glycerol.

Dual cultures

The methodology of Montealegre et al. (2003), with modifications, was used to determine fungal growth inhibition capacity of *B. thuringiensis* strains. One 5 mm disk of a pure culture of *R. solani* was placed at the center of a Petri dish containing PDA. A circular line, made with a six cm diameter Petri dish dipped in a suspension of *B. thuringiensis* 5×10^9 cfu mL^{-1}, was placed, surrounding the fungal inoculums. Plates were cultured for 72 h, at 25°C, and growth diameter of the pathogen was measured and compared to control growth, where the bacterial suspension was replaced by sterile distilled water. Each experiment using a single *R. solani* isolate was run in triplicate. Results are expressed as the means of the percentage of inhibition of growth of the corresponding *R. solani* isolate in the presence of any of the strain of *B. thuringiensis*. Percent inhibition was calculated using the following formula:

% inhibition = [1 - (Fungal growth / Control growth)] x 100.

Production of volatile antibiotics

The ability of the bacterial isolate to produce volatile antibiotics was evaluated using the procedure described by Montealegre et al. (2003). Results were expressed as means of the percentage of inhibition of growth of *R. solani* in the presence and absence of the bacterial isolate.

Thermostability antibiotics

Colonies of bacteria were transferred to Erlenmeyer flasks, containing 100 ml of liquid culture of dextrose potato medium, and incubating in darkness, under rotatory agitation at 150 rpm for seven days. Samples of 10 ml of the fermented broth were transferred to Erlenmeyer flasks, containing 90 ml of PDA, and were later autoclaved for 20 min to a temperature of 120°C. The suspension was homogenized, and 20 ml were deposited in Petri plates. Once the medium solidified a 5 mm disc of *R. solani* was placed in the center of the Petri plates. These were incubated for 72 h, at 25°C; at the end of this time lapse the pathogen growth was assessed. In the control, the bacterial suspension was replaced by liquid culture of dextrose-potato.

In vitro seedling assay

Pepper seeds were surface sterilized in a 2% sodium hypochlorite solution for 5 min and rinsed several times in sterile water. The seeds were then inoculated by soaking in a bacterial suspension containing 10^8 cfu mL^{-1} for 1 h and placed in Petri plates containing 1% water-agar, previously spread with 200 µl of 10^7 propagules suspension of *R. solani*. The plates were incubated for 20 days in a growth chamber (12 h light at 25°C and 12 h of darkness at 22°C). Disease severity was assessed using a scale of 0 – 5: 0 = no visible disease symptoms; 1 = leaves slightly wilted with brownish lesions beginning to appear on stems; 2 = 30 - 50% of entire plant diseased; 3 = 50–70% of entire plant diseased; 4 = 70–90% of entire plant diseased; 5 = plant dead. The plant growth promoting potential of the antagonists was also monitored by mea-suring the shoot and root length and the germination of the seedlings. Each treatment consisted of five seeds in four replications.

Statistical analysis

Data were subjected to one way analysis of variance (ANOVA) and the means separated by using the Tukey Test (p=0.05).

Table 1. Growth inhibition of *R. solani* in dual cultures against *B. thuringiensis.*

B. thuringiensis strain Nº	*R. solani* radial growth[1] (mm)	% Inhibition of *R. solani* radial growth
GM-64	12.5	66.66 a[2]
HD-203	12.75	65.99 a
GM-23	13.5	63.99 ab
GM-6	13.75	63.33 ab
GM-121	14.08	62.44 ab
GM-11	14.16	62.21 ab
HD-974	14.41	61.55 abc
GM-24	14.75	60.66 abc
GM-52	15.00	59.99 abc
GM-63	15.91	57.55 abc
HD-652	16.41	56.22 abc
HD-263	16.5	55.99 abc
GM-162	17.75	54.44 abc
HD-571	18.66	50.22 bc
GM-116	19.58	47.77 cd
HD-597	24.58	34.44 d
Control	37.5	0.00 e

[1]Growth of *R. solani* in PDA for 72 h at 25°C.
[2]Different letters represent significant differences in the Tukey Multiple Range Test (p=0.05).

RESULTS

Dual cultures

From the 60 strains of B. thuringiensis initially selected (Table 1), only 16 of them reduced R. solani growth (p=0.05). The strains GM-64 and HD-203 displayed the highest percentage of inhibition (66.66 and 65.99%, respectively), whereas strain HD-597 displayed the lowest percentage of inhibition (34.44%). Of these 16 stains, six were chosen to be evaluated in the rest of the tests.

Volatile antibiotics

The six strains evaluated were significantly different in relation to the control (p=0.05). *B. thuringiensis* strains GM-11 and GM-121 were the antagonistic bacteria that showed the best inhibitory effect (at 72 h culture age) on *R. solani* growth with a percentage of inhibition of 76.88 and 74.66, respectively (Table 2). All the strains showed inhibitory effect in comparison with the control.

Thermostability antibiotics

Thermostability assay showed no evidence of antagonistic activity of *B. thuringiensis* on the control of *R. solani* (Table 3).

Table 2. Effect of volatile antibiotics secreted by *B. thuringiensis* on radial growth of *R. solani*.

B. thuringiensis strain Nº	*R. solani* radial growth[1] (mm)	% Inhibition of *R. solani* radial growth
GM-11	8.66	76.88 a[2]
GM-121	9.5	74.66 a
GM-64	10.33	72.44 ab
HD-203	11.16	70.21 ab
GM-23	12.91	65.55 bc
GM-6	14.58	61.10 c
Control	37.5	0.00 d

[1]Growth of *R. solani* in PDA for 72 h at 25°C
[2]Different letters represent significant differences in the Tukey Multiple Range Test (p=0.05).

In vitro seedling assay

The efficiency of antagonists on the control of *R. solani* of chili pepper was evaluated in seedling assay (Table 4). In the *R. solani* inoculated control, the brownish lesions occurred on the stem after four days of inoculation and extended into the upper part of seedlings, whereas the seedlings treated with antagonistic strain showed reduced appearance of brownish lesions. Among all the strains, GM-23, GM-11, and GM-121 showed maximum reduction of disease severity on chili pepper seedlings. Seedlings treated with antagonistic strain showed improved root and shoot growth compared with non-treated control. The major effect was found for the strain GM-23, which was associated with an enhancement of the length of shoot and root by 18.9 and 25.5%, respectively, compared with the *R. solani* inoculated control. The majority of the strains enhanced the growth of both the shoot and root of seedlings, compared with controls. However, *B. thuringiensis* strains, GM-121 and GM-6 had no effect on seedling growth.

DISCUSSION

Bacillus spp. is one of the biological control agents that has shown inhibitory effects against a considerable number of plant pathogens, and the antibiotics that it produces are generally assumed to be responsible for the control activity (Helbig et al., 1998; Krebs et al., 1998). In this investigation, *B. thuringiensis* strains with *in vitro* antifungal activity were used with the objective of selecting efficient antagonists against soil borne infection of *R. solani*. Some authors have suggested that the use of antimicrobially active species and strains of the genus *Bacillus*, or the use of their metabolites, may be an alternative or supplementary method to chemical plant protection (Handelsman et al., 1990; Klich et al., 1994; Berger et al., 1996; Sharga and Lyon, 1998). Many of these bacilli are generally soil-inhabiting bacteria or exist

Table 3. Effect of thermostable antibiotics of *B. thuringiensis on* radial *growth of R. solani*.

B. thuringiensis **Strain Nº**	***R. solani*** **Radial Growth[1] (mm)**	**% Inhibition of *R. solani* Radial Growth**
GM-11	37.5	0.00 a[2]
GM-121	37.5	0.00 a
GM-64	37.5	0.00 a
HD-203	37.5	0.00 a
GM-23	37.5	0.00 a
GM-6	37.5	0.00 a
Control	37.5	0.00 a

[1]Growth of *R. solani* in PDA for 72 hours at 25°C.
[2]Different letters represent significant differences in the Tukey Multiple Range Test (p=0.05).

Table 4. Effect of antagonistic *B. thuringiensis* on *R. solani* disease development and pepper seedling growth.

B. thuringiensis **strain Nº**	**Disease severity**	**Germination**	**Plant growth**	
			Shoot length (mm)	**Root length (mm)**
GM-11	3.0±0.00	95.0±5.0	10.2±1.09	14.9±2.35
GM-121	3.0±0.00	90.0±5.77	12.0±0.66	25.3±2.01
GM-64	4.0±0.00	90.0±5.77	10.8±2.08	11.2±1.68
HD-203	4.0±0.00	95.0±5.00	13.8±1.39	17.8±1.81
GM-23	2.0±0.00	95.0±5.00	18.9±0.36	25.5±3.83
GM-6	4.0±0.00	90.0±5.77	12.2±2.50	23.2±6.19
R. solani	5.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00
Control	-	95.0±5.00	27.42±0.53	25.2±0.35

Disease severity was assessed on a 0 to 5 scale from 0 = no visible disease symptoms to 5 = plant dead
Results are an average of four replicates.
Values are mean ± standard error.

as epiphytes and endophytes in the spermosphere (Walker et al., 1998) and rhizosphere (McKeen et al., 1986; Handelsman et al., 1990; Kajimura et al., 1995). For this reason, *Bacillus* species are ideal candidates for use as biocontrol agents in seed treatment programs against soil-borne pathogens (Walker et al., 1998). The inhibitory effect of *B. thuringiensis* strains on phytopathogenic fungus can be associated with enzyme production that can act against the fungal cell wall (Asaka and Shoda, 1996; Mavingui and Heulin, 1994). In this context, in Mexico Barboza et al. (1999) selected and characterized *B. thuringiensis* enzymes (chitinases) and arrived at the conclusion that the synergistic action between chitinases and Cry proteins can be applied to phytopathogenic biological control. The secondary metabolites produced by certain species and strains of the genus *Bacillus* have been found to show antibacterial and/or antifungal activity against phytopathogenic and food-borne pathogenic microorganisms (Shoji 1978; Smirnov et al., 1986).

We used different screening methods to select a suitable strain for the control of *R. solani* on chili pepper. First, the traditional *in vitro* dual culture assay on PDA culture media was used to measure the antagonistic potential of the strains. From sixty tested strains, only 16 of them displayed an inhibitory effect. Of these 16 strains, six were chosen to be screened simultaneously for volatile antibiotics, thermostability, and seedling assay on the basis of the interaction among the pathogen, antagonists, and host plant. In thermostability assays *B. thuringiensis* showed no effectiveness in the control of *R. solani* of chili pepper. A possible explanation is that most of antibiotics and some toxins are not thermostable, so that when the filtrate is sterilized with heat, the metabolites produced during the bacterial growth may have been inactivated (Dhingra and Sinclair, 1987). In seedling assay, the majority of the antagonistics (GM-23, GM-11, and GM-121) were effective in reduction of *R. solani* chili pepper infections. In addition, the strain GM-23 was associated with increased the length of chili pepper seedlings. The increased growth responses of the plant caused by antagonistic strains are likely related to the capacity of the organisms to survive and develop in the rhizosphere or root. As certain strains benefit the host,

causing plant growth promotion and/or biological control, these strains are collectively called plant growth promoting rhizobacteria (PGPR) (Kloepper et al., 1980).

In conclusion, the strains of B. thuringensis have proved to be efficient in the control of *R. solani* of chili pepper in *in vitro* assays, providing results similar to those obtained by other authors using mainly bacteria and fungi (Ahmad et al., 1999, Berger et al., 1996, Silo-Suh et al., 1994). Antibiosis seems to be the principal mode of action, and some lines of evidence point in this direction (Lee et al., 2003, Ligon et al., 2000). Additional experiments need to be carried out in order to determine the biochemical and microbial activities involved in the biocontrol capability of the B. thuringensis antagonistic strains.

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