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Growth inhibition of *Beauveria bassiana* by bacteria isolated from the cuticular surface of the corn leafhopper, *Dalbulus maidis* and the planthopper, *Delphacodes kuscheli*, two important vectors of maize pathogens

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Abstract

The phytosanitary importance of the corn leafhopper, *Dalbulus maidis* (De Long and Wolcott) (Hemiptera: Cicadellidae) and the planthopper, *Delphacodes kuscheli* Fennah (Hemiptera: Delphacidae) lies in their ability to transmit phloem-associated plant pathogens, mainly viruses and mollicutes, and to cause considerable mechanical damage to corn plants during feeding and oviposition. Fungi, particularly some members of the Ascomycota, are likely candidates for biocontrol agents against these insect pests, but several studies revealed their failure to invade the insect cuticle possibly because of the presence of inhibitory compounds such as phenols, quinones, and lipids and also by the antibiosis effect of the microbiota living on the cuticular surface of the host. The present work aims to understand interactions between the entomopathogenic fungus *Beauveria bassiana* (Balsamao-Crivelli) Vuillemin (Hypocreales: Cordycipitaceae) and bacterial antagonists isolated from the cuticular surface of *D. maidis* and *D. kuscheli*. A total of 155 bacterial isolates were recovered from the insect's cuticle and tested against *B. bassiana*. Ninety-one out of 155 strains inhibited the growth of *B. bassiana*. Bacterial strains isolated from *D. maidis* were significantly more antagonistic against *B. bassiana* than those isolates from *D. kuscheli*. Among the most effective antagonistic strains, six isolates of *Bacillus thuringiensis* Berliner (Bacillales: Bacillaeae (after *B. subtilis*)), one isolate of *B. mycoides* Flügge, eight isolates of *B. megaterium* de Bary, five isolates of *B. pumilus* Meyer and Gottheil, one isolate of *B. licheniformis* (Weigmann) Chester, and four isolates of *B. subtilis* (Ehrenberg) Cohn were identified.

Keywords: *Bacillus licheniformis, Bacillus megaterium, Bacillus mycoides, Bacillus pumilus, Bacillus subtilis, Bacillus thuringiensis,* bacterial antagonists, cicedellids, delphacids, entomopathogenic fungus
Abbreviations: LSD, least significant difference**, MGI,** mycelial growth inhibition**, TSA,** tryptic soy agar
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Introduction

Argentina is a leading maize-producing country, with an annual production of 15,500,000 tons. Several auchenorrynchan (Hemiptera: Auchenorryncha) species belonging to the Cicadellidae (leafhoppers) or the Delphacidae (planthoppers) families can reduce the yield and quality of maize grains because they transmit different plant pathogens, mainly viruses and mollicutes, and cause considerable mechanical damages during feeding and oviposition (Nault and Ammar 1989). The mentioned families include the largest number of vector species, with worldwide representatives (Nault and Ammar 1989). The corn leafhopper *Dalbulus maidis* (De Long and Wolcott) (Hemiptera: Cicadellidae) is widely distributed in tropical areas of the Americas, from southern USA to temperate zones of Argentina (Virla et al. 1990/1991; Giménez Pecci et al. 2002), and it is considered one of the most damaging species to corn due to its role as a vector of maize rayado fino virus, *Spiroplasma kunkelii*, and maize bushy stunt mycoplasm. These three pathogens, alone or in combination, are the ethiological agents of corn stunt, a disease that causes economic losses to corn crops in Mexico and Central and South America, and has been detected in restricted areas in the north of Argentina in 1990 (Giménez Pecci et al. 2000). Among the delphacid pests of maize is the planthopper, *Delphacodes kuscheli* Fennah (Hemiptera: Delphacidae) a native species of Argentina that has been reported as a vector of Mal de Río Cuarto virus (Remes Lenicov et al. 1985; Remes Lenicov and Virla 1999). Due to high incidence and severity of damages, the Mal de Río Cuarto is the most important disease of corn crops in Argentina (Laguna et al. 2000; 2002).

Entomopathogenic fungi are widespread in agroecosystems and belong to a group of microorganisms extensively studied with more than 700 species within 100 genera (Lecuona 1996). These fungi infect a great number of arthropods and hence can be used as pest control agents in an Integrated Pest Management approach (Lecuona 1996). Fungi, particularly some members of Ascomycota, are attractive candidates as biocontrol agents against leafhoppers and planthoppers (Rice and Choo 2000; Toledo et al. 2007), but several studies revealed its failure to invade the insect cuticle, possibly due to the presence of inhibitory compounds such as phenols, quinones, and lipids (Smith and Grula 1981; Szafranek et al. 2001; Howard and Lord 2003; James et al. 2003; Lord and Howard 2004) and also by antibiotic effect of the microbiota living on the cuticular surface of the host (Hubner 1958; Walstad et al. 1970; Schabel 1978). Several works reported antagonistic interactions among microorganisms, such as fungi and bacteria (Currie et al. 1999; Ansari et al. 2005; Alippi and Reynaldi 2006), but there are no studies about the interactions between the microbiota found on the cuticle of hemipterous species and entomopathogenic fungi.

The purpose of the present work was to investigate the interactions among the entomopathogenic fungus *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Hypocreales: Cordycipitaceae) and several bacterial antagonists isolated from the cuticular surfaces of *D. maidis* and *D. kuscheli*.

Materials and Methods

Insect culture

Dalbulus maidis and *Delphacodes kuscheli* were obtained from colonies reared on corn

(*Zea mays* L.) and oat (*Avena sativa* L.) respectively in 24 x 9 cm polyethylene terephthalate plastic cages, in a greenhouse at the Facultad de Ciencias Agrarias y Forestales, UNLP (35º S -57º W), La Plata, Buenos Aires, Argentina.

Fungal isolate and preservation

The *B. bassiana* strain used in this study was isolated from one adult of *Cycloneda sanguinea* L. (Coleoptera: Coccinellidae) associated from corn at El Manantial, Tucumán, Argentina (26º 49' 50.2'' S - 65º 16' 59.4'' W). This isolate has been previously reported as pathogenic to planthoppers and leafhoppers (Toledo et al. 2007), and was deposited as ARSEF 8372 in the Collection of Entomopathogenic Fungal Cultures, Agricultural Research Service, Ithaca, New York, USA, and as CEP 147 in the Collection at Centro de Estudios Parasitológicos y de Vectores, La Plata, Buenos Aires, Argentina.

Isolation and preservation of bacterial strains

Ten adults of each *D. maidis* and *D. kuscheli* (approximately 10 d old) were collected from April 2007 to April 2008 at monthly intervals. Using mouth aspirators, insects were placed into glass vials and transported to the laboratory. A total of 120 living insects were evaluated by placing them individually into sterile vials containing 300 µl of sterile distilled water. Each vial was vortex-mixed for 1 min, from which 25 µl were pippeted out and streaked on plates of tryptic soy agar (TSA) (Britania) using a Drigalski spatula. Plates were incubated at 30º C in aerobiosis and examined for bacterial growth every 24 h for and up to 10 days. Potential bacterial antagonists were primarily identified on the basis of Gram reaction and colony morphology. Microscopic examination of bacterial smears stained using the Schaeffer-Fulton technique was made to determine presence and location of spores within cells as well as the size and shape of vegetative cells. The isolates were maintained on sterile mineral water (Glaciar, www.glacierwater.com) at 4º C and stored in tryptic soy broth plus 20 % glycerol (v/v) at -80º C.

Preliminary screening for bacterial strains with antagonistic activity and statistical analysis

A total of 155 bacterial isolates were recovered from the cuticular surface throughout the sampling period, and tested for antagonistic effect against *B. bassiana*. A first screening to evaluate the effect of potential antagonists on the fungal growth was carried out by a central disk test assay (Reynaldi et al. 2004). Briefly, the fungal strain was cultured on malt extract agar for 7 days at 25º C in darkness and a 7 mm mycelium disk from the sporulating area was cut and transferred to the centre of a TSA plate. At the same time, three 7 mm disks containing each bacterial strain from a 48 h culture on TSA were transferred to each plate in the same way and placed at three equidistant points from the central disk. For controls, only a central disk of fungal growth was used. There were 3 replicate plates for each bacterial strain and for each control group (making a total of 65 controls). Treated and control plates were incubated at 30º C and the evaluation was performed by measuring the diameter of the fungal colony at 7 and 10 days, respectively. The percentage of mycelial growth inhibition (MGI) was calculated according to the formula proposed by Michereff et al. (1994).

Only those treatments in which the fungal growth in the presence of bacteria was smaller than that of the controls were included in the statistical analysis. Differences in inhibition

growth levels among treatments were analyzed by Kruskal–Wallis test, and means were compared by Fisher's least significant difference (LSD) multiple range test option ($P \leq 0.05$) using Statgraphics statistical software (STSC, 1994-2001). Bacterial strains isolated from *D. maidis* and *D. kuscheli* were analyzed separately. Differences in biological activity

Table 1. Lifetable, fecundity and rate of natural increase of Commom Hoopae louse (*Upupicola upupae*).

Bacterial strain	MGI (%)		Bacterial strain	MGI (%)	
	7 days	*10 days*		*7 days*	*10 days*
Dk-B25	68.2 ± 7.9 i	75.9 ± 6.0 q	Dm-B3	79.1 ± 0.0 o	82.9 ± 0.0 n
Dk-B12	67.7 ± 5.5 hi	61.5 ± 16.9 mnopq	Dm-B47	79.1 ± 0.0 o	82.9 ± 0.0 n
Dk-B3	59.7 ± 2.3 ghi	69.1 ± 1.9 pq	Dm-B23	74.8 ± 4.4 no	79.4 ± 3.6 mn
Dk-B57	58.2 ± 0.0 fghi	40.4 ± 2.7 ghijklm	Dm-B4	72.6 ± 3.5 no	76.9 ± 3.0 lmn
Dk-B44	55.2 ± 8.9 fghi	29.1 ± 3.8 bcdefghi	Dm-B22	72.2 ± 3.5 mno	76.5 ± 3.3 lmn
Dk-B23	52.2 ± 15.3 fghi	65.3 ± 10.2 opq	Dm-B10	70.4 ± 6.3 lmno	75.1 ± 5.8 lmn
Dk-B11	51.2 ± 4.3 fghi	62.3 ± 2.7 nopq	Dm-B55	68.7 ± 2.6 klmno	74.4 ± 2.1 klmn
Dk-B1	49.8 ± 5.9 efghi	61.5 ± 4.7 mnopq	Dm-B17	67.8 ± 0.9klmno	73.7 ± 0.7 klmn
Dk-B4	48.8 ± 13.3 efgh	49.5 ± 19.9 ijklmnop	Dm-B59	62.6 ± 2.3 jklmn	69.4 ± 1.9 jklmn
Dk-B6	48.3 ± 15.6 efg	52.5 ± 18.5 jklmnop	Dm-B73	61.7 ± 2.3 ijklmn	68.7 ± 1.9 jklmn
Dk-B47	44.3 ± 10.4 efg	61.5 ± 5.7 mnopq	Dm-B56	59.9 ± 6.2 ijklmn	67.3 ± 4.9 ijklm
Dk-B40	42.3 ± 13.2 efg	29.9 ± 9.4 cdefghi	Dm-B63	59.9 ± 3.5 ijklmn	67.3 ± 2.8 ijklm
Dk-B5	41.8 ± 7.5 efg	42.3 ± 12.7 ghijklmn	Dm-B33	59.1 ± 20.0 hijklmn	68.0 ± 14.9 jklmn
Dk-B49	39.3 ± 3.6 def	40.4 ± 0.8 ghijklm	Dm-B46	59.1 ± 2.3 hijklmn	65.9 ± 2.1 ijklm
Dk-B66	38.3 ± 2.6 cdef	08.8 ± 0.8 abc	Dm-B24	56.5 ± 0.9 ghijklm	64.5 ± 0.7 ijklm
Dk-B64	31.3 ± 3.5 bcde	35.1 ± 4.6 fghijk	Dm-B60	56.5 ± 2.3 ghijklm	64.5 ± 1.9 ijklm
Dk-B48	21.4 ± 0.9 abcd	57.8 ± 7.9 lmnopq	Dm-B64	54.7 ± 2.3 ghijkl	61.6 ± 2.5 ghijkl
Dk-B55	21.4 ± 3.6 abcd	44.2 ± 13.1 ghijklmno	Dm-B61	53.9 ± 6.3 ghijk	62.3 ± 1.9 hijkl
Dk-B32	20.4 ± 8.5 abc	39.9 ± 6.9 ghijkl	Dm-B62	53.9 ± 6.3 ghijk	62.3 ± 5.1 hijkl
Dk-B13	18.4 ± 2.2 ab	NV	Dm-B5	53.9 ± 13.2 ghijk	56.6 ± 13.3 efghij
Dk-B2	17.9 ± 5.4 ab	NV	Dm-B58	50.4 ± 7.9 fghij	59.5 ± 6.5 fghijk
Dk-B75	17.4 ± 0.9 ab	32.9 ± 1.9 efghij	Dm-B39	47.8 ± 10.6 fghij	54.9 ± 6.8 efghij
Dk-B39	17.4 ± 4.3 ab	NV	Dm-B9	46.5 ± 5.3 fghi	59.1 ± 8.1 fghijk
Dk-B8	15.4 ± 0.9 ab	13.3 ± 3.8 abcde	Dm-B54	43.4 ± 3.5 efgh	52.4 ± 2.6 efghi
Dk-B14	14.9 ± 4.5 ab	NV	Dm-B74	42.6 ± 1.5 efg	52.4 ± 0.7 efghi
Dk-B73	14.4 ± 3.6 ab	35.1 ± 2.7 fghijk	Dm-B75	41.7 ± 4.4 efg	45.3 ± 3.6 def
Dk-B61	13.4 ± 5.2 ab	68.3 ± 0.0 pq	Dm-B87	40.8 ± 2.8 efg	48.1 ± 6.8 efgh
Dk-B42	13.4 ± 3.5 ab	55.5 ± 9.3 klmnopq	Dm-B67	36.5 ± 5.3 def	46.7 ± 4.4 defg
Dk-B9	12.9 ± 6.9 ab	15.5 ± 7.2 abcdef	Dm-B7	35.2 ± 12.4 def	44.2 ± 12.0 def
Dk-B7	11.9 ± 1.5 a	NV	Dm-B57	34.7 ± 5.4 def	45.9 ± 4.9 def
Dk-B76	9.5 ± 0.9 a	24.6 ± 1.9 bcdefg	Dm-B38	30.4 ± 0.9 cde	43.1 ± 0.7 de
Dk-B45	9.5 ± 0.9 a	NV	Dm-B52	30.4 ± 3.8 cde	43.1 ± 3.1 de
Dk-B77	8.5 ± 1.9 a	30.6 ± 1.5 defghi	Dm-B6	23.4 ± 4.8 bcd	31.8 ± 4.3 cd
Dk-B16	7.5 ± 1.7 a	23.8 ± 0.8 abcdefg	Dm-B8	18.2 ± 2.3abc	27.5 ± 4.3 c
Dk-B51	NV	54.0 ± 2.7 jklmnop	Dm-B1	17.8 ± 2.3 abc	23.9 ± 7.4 bc
Dk-B67	NV	53.2 ± 1.9 jklmnop	Dm-B41	17.3 ± 4.6 abc	31.8 ± 4.4 cd
Dk-B65	NV	47.9 ± 2.6 hijklmnop	Dm-B13	17.3 ± 4.4 abc	NV
Dk-B72	NV	26.9 ± 12.9 bcdefgh	Dm-B49	17.3 ± 3.5 abc	NV
Dk-B15	NV	10.3 ± 5.3 abcd	Dm-B2	16.5 ± 5.2 abc	23.9 ± 10.0 bc
Dk-B36	NV	9.5 ± 5.2 abcd	Dm-B44	12.1 ± 4.8 ab	9.0 ± 3.9 ab
Dk-B38	NV	7.9 ± 2.7 ab	Dm-B48	12.1 ± 0.9 ab	NV
Dk-B71	NV	7.9 ± 2.7 ab	Dm-B50	7.8 ± 0.9 ab	NV
Dk-B68	NV	2.7 ± 1.3 a	Dm-B51	6.9 ± 3.5 a	11.9 ± 6.9 ab
Dk-B70	NV	2.7 ± 0.0 a	Dm-B45	3.4 ± 1.5 a	NV
			Dm-B53	2.5± 1.7 a	9.7 ± 0.7 ab
			Dm-B40	NV	11.9 ± 4.9 ab
			Dm-B16	NV	6.9 ± 3.1 a

Values followed by the same letters do not differ significantly according to LSD test ($P \leq 0.05$).
NV= Negative values, where fungal growth in the presence of bacteria was greater than that of the controls, were not included in the statistical analysis. Table only shown the 91 strains that inhibited the growth of *B. bassiana*

between bacteria isolated from *D. maidis* and those isolated from *D. kuscheli* were analyzed by analysis of variance (ANOVA), and their means were compared by LSD test ($P \leq 0.05$) using the Statgraphics software.

Identification of selected bacterial antagonists

Twenty-four bacterial isolates that showed the most effective antagonist effect against *B. bassiana* (MGI values between 40% and 83%) were further characterized to identify them at species level. Tests performed include catalase reaction, oxidase activity, motility, lipid globule staining, production of lecithinase, haemolytic activity, reduction of nitrate, anaerobic utilization of glucose, mannitol and arabinose utilization, and starch and gelatin hydrolysis according to standard protocols (Gordon et al. 1973). When necessary, API 20E and API 50CH strips plus API 50CHB medium and data base Apiweb (Biomerieux, www.biomerieux.com) were used.

Antagonistic activity against conidial germination of *B. bassiana*

According to the results obtained in the

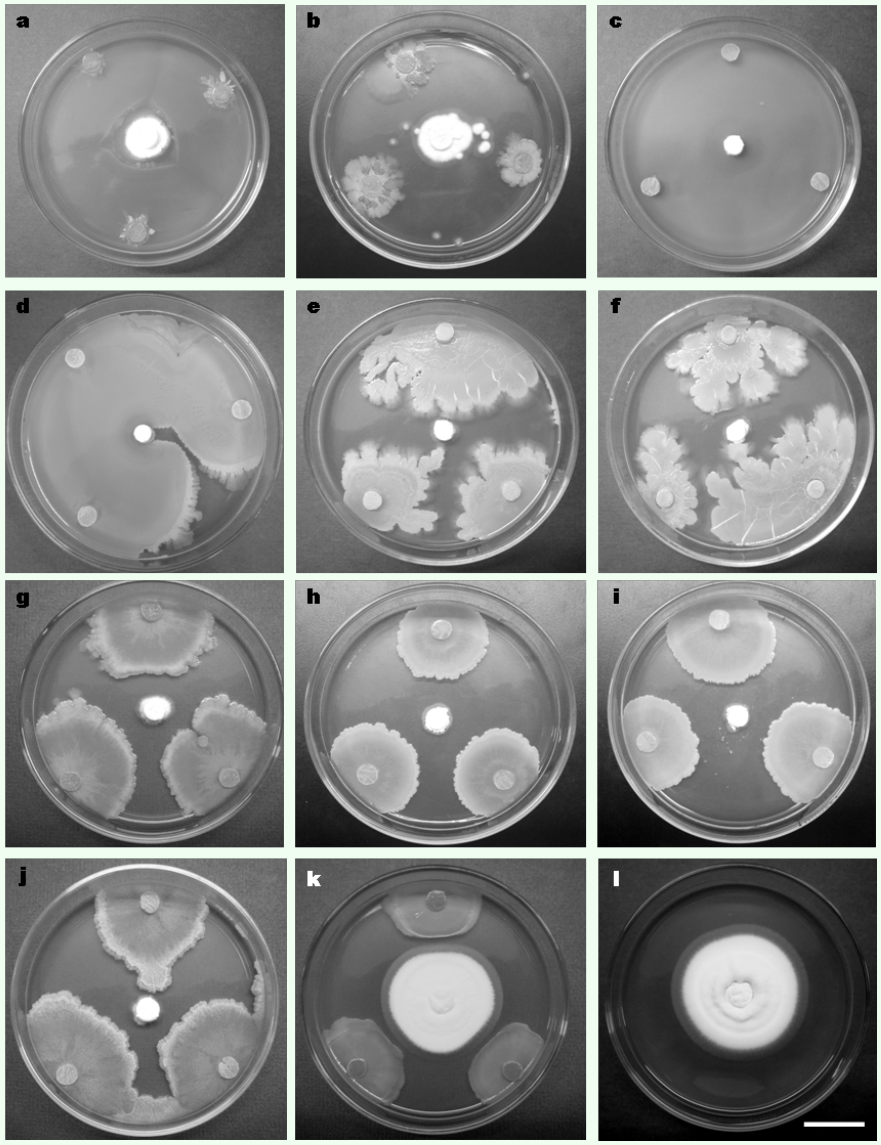

Figure 1. Antifungal activity of *Bacillus licheniformis* Dk-B23 (plate a), *B. pumilus* Dk-B12, Dk-B25, Dm-B3, Dm-B22, Dm-B23 (plates b, c, d, e and f), and *B. subtilis* Dk-B57, Dm-B4, Dm-B17, Dm-B55 (plates g, h, i and j) against *B. bassiana* on TSA plates after 7 days post-incubation. Controls: bacterial strain that did not exhibit antifungal activity (plate k) and fungal growth without bacteria after 7 d of incubation (plate l). Scale bar: 1.8 cm. High quality figures are available online.

preliminary screening, ten bacterial strains were selected for testing their antagonistic activity on the conidial germination of *B. bassiana* ARSEF 8372 by means of a paired suspension assay. *B. bassiana* was cultured on malt extract agar and incubated for 8 days at 26º C in darkness. Conidia were harvested with a sterile loop and placed into test tubes containing 5 ml of Tween 80 (0.1 % v/v) (Sigma, www.sigmaaldrich.com). The suspensions were vortex-mixed for 1 min, filtered through a sterile muslin layer, and adjusted to a concentration of 1 x 10^8 conidia/ml after determination of conidial concentration using a Neubauer hemacytometer. Each test bacterium suspension (vegetative cells after 24 h incubation or spores after 7 days incubation on TSA) was adjusted to a concentration of 0.5 Mc Farland. Bacterial suspensions were prepared in Tween 80 (0.1 % v/v).

Five µl of each conidial suspension and 5 µl of each bacterial suspension (vegetative cells or spores) were deposited on the surface of a microscopic slide containing 100 µl of water agar medium as a substrate. The slides were placed over moist filter paper inside sterile 90 mm-diameter Petri dishes and incubated in darkness at 30º C. After 24 h germinated conidia were counted under a light microscope (400 X) by counting 3 times 100 conidia for each fungus-bacterial combination and each control taking into account that germinated conidia are those exhibiting a germ tube greater than the conidial diameter (usually once or twice). There were 3 replicates and one control per treatment. The whole assay was run twice over time in the same conditions mentioned above. The differences in inhibition growth levels among treatments were analyzed by Kruskal–Wallis analysis, and means were separated by LSD test ($P \leq 0.05$) using Statgraphics statistical software. In addition, the abnormalities of the conidial germ tubes, if any, were registered.

Results

Inhibition of mycelial growth

A total of 155 bacterial isolates were obtained from the cuticular surface of *D. maidis* and *D. kuscheli*. Eighty-three isolates collected from *D. maidis* were represented by 52% Gram-positive spore-forming bacilli, 37% Gram-positive non-spore-forming bacilli, 4% Gram-negative bacilli, and 7% Gram-positive cocci, whereas the 72 isolates from *D. kuscheli* were represented by 46% Gram-positive spore-forming bacilli, 22% Gram-positive non-spore-forming bacilli, 6% Gram-negative bacilli, and 26% Gram-positive cocci. As shown in Table 1, 91 out of 155 strains tested inhibited the growth of *B. bassiana.* After 7 days of incubation significant differences among treatments were recorded for *D. kuscheli* (K= 81.9; P= 0.00). Strains Dk-B3, Dk-B11, Dk-B12, Dk-B23, Dk-B25, Dk-B44, and Dk-B57 showed the most effective antagonistic effect against *B. bassiana*, with percentages of MGI of 50% or more. After 10 days of incubation significant differences were also observed among treatments (K= 89.3; P= 0.00), the most effective antagonists were Dk-B1, Dk-B3, Dk-B6, Dk-B11, Dk-B12, Dk-B23, Dk-B25, Dk-B42, Dk-B47, Dk-B48, Dk-B51, Dk-B61, and Dk-B67. In relation to *D. maidis,* significant differences were also recorded among the 83 strains isolates tested after 7 d (K = 118.2; P = 0.00) and 10 d (K = 108.0; P = 0.00) after incubation. Strains Dm-B3, Dm-B4, Dm-B5, Dm-B10, Dm-B17, Dm-B22, Dm-B23, Dm-B24, Dm-B33, Dm-B46, Dm-B47, Dm-B55, Dm-B56, Dm-B58, Dm-B59, Dm-B60, Dm-B61, Dm-B62, Dm-B63, Dm-B64, and Dm-B73 were the most effective antagonists (Table 1 and Figure 1).

Table 2. Inhibition of *B. bassiana* conidial germination (% mean ± SE) by selected bacterial antagonists preparations obtained from vegetative cells (cutures of 24 hours of incubation) or spores (cultures of 7 days of incubation), respectively.

	Conidial germination	
Treatment	***Bacterial vegetative cells***	***Bacterial spores***
Control	85.6 ± 6.1 b	91.8 ± 0.9 c
B. subtilis Dk-B57	72.8 ± 9.5 ab	54.5 ± 17.4 ab
B. subtilis Dm-B17	68.7 ± 13.3 ab	62.2 ± 11.4 abc
B. pumilus Dk-B12	66.7 ± 13.1 ab	76.9 ± 9.8 bc
B. subtilis Dm-B55	61.9 ± 11.9 ab	58.6 ± 10.7 ab
B. pumilus Dk-B25	60.5 ± 9.5 ab	59.8 ± 10.8 ab
B. pumilus Dm-B3	59.4 ± 15.2 ab	63.7 ± 13.4 abc
B. pumilus Dm-B22	58.7 ± 13.9 ab	50.3 ± 8.9 ab
B. subtilis Dm-B4	53.6 ± 10.3 ab	55.0 ± 6.2 ab
B. licheniformis Dk-B23	44.5 ± 13.3 a	71.0 ± 10.9 abc
B. pumilus Dm-B23	42.6 ± 11.9 a	45.7 ± 6.5 a

Values followed by the same letters do not differ significantly according to LSD test ($P \leq 0.05$).

More bacterial strains isolated from *D. maidis* were more antagonistic to *B. bassiana* than those from *D. kuscheli* at 7 days ($F = 5.76$; df = 1; 77; $P = 0.018$) and 10 days ($F = 8.16$; df = 1; 78; $P = 0.0055$) of incubation, respectively. Twenty-five isolates from *D. maidis* and 13 isolates from *D. kuscheli* showed values of MGI of 50% or more, respectively (Table 1 and Figure 1).

Among the most effective antagonistic strains, Dk-B1, Dk-B11, Dm-B9, Dm-B24, Dm-B54, and Dm-B58 were identified as *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae), Dk-B3 as *Bacillus mycoides* Flügge, Dm-B5, Dm-B10, Dm-B47, Dm-B59, Dm-B60, Dm-B61, Dm-B62, and Dm-B63 as *Bacillus megaterium* de Bary. Using the API 20E and API 50CH strips and data base

Table 3. Effects of bacterial antagonist preparations obtained from vegetative cells or spores, upon the growth of germinative tubes of *B. bassiana* (µm mean ± SE).

	Length of germinative tubes	
Treatment	***Bacterial vegetative cells***	***Bacterial spores***
Control	40.7 ± 3.6 f	36.8 ± 5.4 e
B. subtilis Dk-B57	39.8 ± 3.7 ef	31.6 ± 3.7 de
B. subtilis Dm-B17	34.3 ± 3.2 def	29.5 ± 5.4 cde
B. pumilus Dk-B12	32.2 ± 3.7 cde	27.9 ± 3.7 cde
B. subtilis Dm-B55	30.4 ± 3.3 bcd	26.4 ± 4.4 bcd
B. pumilus Dk-B25	24.3 ± 2.4 abc	19.6 ± 3.1 abc
B. pumilus Dm-B22	23.9 ± 3.2 ab	17.2 ± 1.3 ab
B. licheniformis Dk-B23	19.9 ± 2.2 a	28.4 ± 3.2 cde
B. subtilis Dm-B4	19.8 ± 2.6 a	16.9 ± 2.6 ab
B. pumilus Dm-B3	18.6 ± 1.7 a	21.6 ± 2.7 bcd
B. pumilus Dm-B23	17.6 ± 3.1 a	10.8 ± 1.1 a

Values followed by the same letters do not differ significantly according to LSD test ($P \leq 0.05$).

Api*web* isolates Dk-B12, Dk-B25, Dm-B3, Dm-B22, and Dm-B23 matched as *Bacillus pumilus* Meyer and Gottheil (99.9%, 99.4%, 99.9%, 99.9%, and 99.5% ID, respectively). Strain Dk-B23 matched as *Bacillus licheniformis* (Weigmann) Chester (97.3% ID) and Dk-B57, Dm-B4, Dm-B17, and Dm-B55 matched as *Bacillus subtilis* (Ehrenberg) Cohn (85.3%, 98.1%, 99,1%, and 70.9% ID, respectively).

Inhibition of conidial germination

After 24 h of incubation no significant differences were found among treatments for vegetative cells (K= 13.4; P= 0.2) or spores (K= 16.3; P= 0.1), although *B. pumilus* Dm-B23 showed a higher inhibitory activity in both cases (Table 2). In addition, significant differences in conidial germ tube lengths were found for both vegetative cells (K= 88.3; P= 0.00) and spores (K= 45.4; P= 0.00), with *B. pumilus* Dm-B23 found to be the most effective antagonist (Table 3). No abnormalities of the conidial germ tubes were found in any of the bacterial-fungus combinations tested.

Discussion

According to the results presented here, Gram-positive aerobic spore forming bacteria belonging to the species *B. megaterium*, *B. mycoides*, *B. pumilus*, *B. licheniformis, B. subtilis*, and *B. thuringiensis* showed the most effective antagonistic effect on *B. bassiana* mycelial growth and conidial germination.

Bacillus pumilus, *B. licheniformis*, and *B. subtilis* along with *Bacillus atrophaeus* and *B. amyloliquefaciens* are closely related species that comprise the *Bacillus subtilis* group (Wattiau et al. 2001). The ability of this group of bacteria to inhibit fungal and bacterial growth by secreting antibiotics, antibiotic-like compounds, bacteriocins, or antifungal compounds has been well documented (Thimon et al. 1992; Feignier et al. 1995; Leifert et al. 1995; Gálvez et al. 1993; Martinari et al. 2002). These substances could play an important role in antagonistic interactions between microorganisms, which may be based on parasitism, direct competition, or antibiosis (Singh and Faull 1988).

Bacterial strains isolated from *D. maidis* were significantly more antagonistic, or at least produced large amounts of antagonistic compounds against *B. bassiana* than those isolates from *D. kuscheli.* The failure of *B. bassiana* to invade the *D. maidis* cuticle and the greater mortality rates previously observed in *D. kuscheli* could be related to a less antagonistic activity of the bacteria living in the same ecological niche. This hypothesis might explain those results observed in previous studies (Toledo et al. 2007) reporting that *D. maidis* mortality caused by *B. bassiana* was 23% less than that of *D. kuscheli* after 14 d post-inoculation. Although, further bioassays will be necessary to clarify this hypothesis by testing insects previously treated with antibiotics and inoculated separately with each bacterial strain and then with the pathogenic fungus.

This is the first report of bacterial isolates obtained from cuticular surfaces of Cicadellids and Delphacids able to inhibit the growth of the entomopathogenic fungus *B. bassiana*. Other examples of bacterial strains having antifungal activity include strains of *B. pumilus* against Mucoraceae, *Aspergillus flavus*, and *A. parasiticus* (Eurotiales: Trichocomaceae) species (Bottone and Peluso 2003; Cho et al. 2009) and also against *Bipolaris sorokiniana* (Pleosporales: Pleosporaceae) and *Septoria tritici*

(Capnodiales: Mycosphaerelaceae) (Alippi et al. 2000) have been reported. In addition, antagonistic effects of *B. subtilis* strains against different phytopathogenic fungi like *Colletotrichum trifolii* (Phyllachorales: Phyllachoraceae) (Douville and Boland 1992), *Exserohilum turcicum* (Pleosporales: Pleosporaceae) (Reis et al. 1994), *A. flavus* (Moyne et al. 2001), *Pythium aphanidermatum* (Pythiales: Pythiaceae) (Leclere et al. 2005), *B. sorokiniana, S. tritici*, and *Alternaria triticimaculans* (Dothideales: Pleosporaceae) (Alippi et al. 2000) and also against entomopathogenic fungi like *Ascophaera apis* (Eurotiales: Ascophaeraceae), the causative agent of chalkbrood disease in honeybee larvae (Basim and Gürel 1999; Reynaldi et al. 2004) have been established. Additionally *B. licheniformis* strains with antifungal compounds against a wide variety of plant pathogenic fungi have been isolated (Galvez et al. 1993; Lebbadi et al. 1994; Alippi et al. 2000). Similar results have been reported for *B. megaterium* on *A. apis* (Reynaldi et al. 2004; Gilliam 1993), on *S. tritici* (Kildea et al. 2008), and on *Phytophtora capsici* (Peronosporales: Pythiaceae) (Akgul and Mirik 2008). It is interesting to point out that previous reports showed that symbiotic bacteria of entomopathogenic nematodes as *Xenorhabdus nematophilus*, *X. bovienii*, and *Photorhabdus luminescens* were antagonistic to the entomopathogenic fungi *B. bassiana* and *M. anisopliae* (Hypocreales: Clavicipitaceae) by inhibiting their growth and conidial production (Barbercheck and Kaya 1990; Chen et al. 1994; Ansari et al. 2005).

Our findings suggest the existence of a kind of antimicrobial activity possibly due to antibiosis effect and/or direct competition of spore-forming bacteria associated with Cicadellidae and Delphacidae that can reduce or inhibit the growth of *B. bassiana*. The presence of bacteria belonging to *Bacillus cereus* sensu lato group, *B. megaterium, B. subtilis* and closely related species in the cuticle of hemipterous insects could be an obstacle for the optimization and promotion of the use of entomopathogenic fungi in an integrated pest management approach in corn crops. Further studies are needed in order to clarify these microbial interactions and to characterize the chemical nature of the compounds involved in the inhibitory activities.

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