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Antagonism of entomopathogenic fungi by *Bacillus* spp. associated with the integument of cicadellids and delphacids

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Summary. Entomopathogenic fungi are potential tools to biocontrol cicadellids and delphacids, two groups of insects that cause extensive damage to agricultural crops. However, bacteria living on the host cuticle may inhibit fungal growth. In the present work, following the molecular characterization of 10 strains of *Bacillus* isolated from the integument of cicadellids and delphacids, we selected isolates of the fungi *Beauveria bassiana* and *Metarhizium anisopliae* that are resistant to the antimicrobials secreted by these bacterial strains. The antagonistic activity of the 10 bacterial isolates belonging to the genus *Bacillus* (i.e., *B. amyloliquefaciens*, *B. pumilus*, and *B. subtilis*) against 41 isolates of *Bea. bassiana* and 20 isolates of *M. anisopliae* was investigated in vitro on tryptic soy agar using the central disk test. With this approach, isolates of *Bea. bassiana* and *M. anisopliae* resistant to antagonistic bacteria were identified that can be further developed as biological control agents. [**Int Microbiol** 2015; 18(2):91-97]

Keywords: *Bacillus* spp. · antagonism · entomopathogenic fungi · Cicadellidae · Delphacidae

Introduction

Cicadellids and delphacids (Hemiptera: Auchenorrhyncha) include a large number of species, many of which cause extensive damage to agricultural crops. These insects are widely distributed and can be found anywhere between the southern United States and temperate areas of Argentina [29,48]. They not only cause mechanical damage to crop plants during feeding and oviposition, but are also vectors of phloem-associated plant pathogens, mainly viruses and bacterial phytoplasmas [21].

Within the cicadellids, *Dalbulus maidis* (DeLong & Wolcott, 1923) is the main vector of maize pathogens on the American continent, mostly in tropical areas of South and Central America but also in those of the Caribbean. In tropical America, *D. maidis* is a vector of Maize Rayado Fino Virus (MRFV), Corn Stunt Spiroplasma (CSS), and Maize

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Bushy Stunt Mycoplasma (MBSM). Corn stunt is the most important disease of maize in USA, Mexico, and South and Central America. It was first identified in Argentina in the early 1990s [4]. Among the delphacids, *Delphacodes kuscheli* Fennah 1955 is the main vector of Mal de Río Cuarto virus, an important endemic disease in the central region of Argentina [22] that has had a considerable impact along the country's corn belt [24].

Among the many different strategies developed to control corn diseases, the use of maize genotypes tolerant to infection has gained the most attention [23,46]. However, biological control agents, including fungi that parasitize these insects, offer an interesting alternative [10,12,15]. Entomopathogenic fungi were the first organisms considered as control agents at the end of the 19th century. Since then, their value in insect control has been widely demonstrated, mainly within Integrated Pest Management programs [7,14]. Generally, the application of entomopathogenic fungi requires high specificity and the absence of resistance in the target organisms. As long as no secondary pest outbreaks occur, long-term control is feasible. Moreover, the use of entomopathogenic fungal strains is frequently compatible with that of other biological control agents, certain fungicides, and many other types of pesticides. A further advantage is that no pre-harvest interval is required [5,6,42,50].

The commercialization of entomopathogenic fungi is usually restricted to those species that are amenable to mass production in vitro on economical substrates. Among the commercial products developed to date are several that are based on species within the Hypocreales, such as *Beauveria bassiana* (Bals.-Criv.) Vuill., *Bea. brongniartii* (Sacc.) Petch, *Isaria fumosorosea* Wize, *Lecanicillium* spp. (Cordycipitaceae), *Metarhizium anisopliae* (Metchn.) Sorokin, and *Nomuraea rileyi* (Farl.) Samson (Clavicipitaceae) [7,49]. In the control of cicadellids and delphacids, entomopathogenic fungi have considerable potential because they invade their hosts through the integument [38]. However, fungal invasion of the host occasionally fails, not only due to the presence of antimicrobial substances associated with the insect cuticle, such as phenol groups, quinones, aldehydes, poisonous alkaloids, short-chain fatty acids, and cationic peptides [8,11,17,30,34], but also because of the presence of other fungi and bacteria on the insect surface that, by producing antimicrobial substances, inhibit germination of the conidia of entomopathogenic fungi [9,18,29,45].

According to Steinhaus [33], the bacterial populations found on the external surface of the insects are predominantly gram-positive, aerobic, spore-forming bacilli. Toledo et al. [40] recently isolated different *Bacillus* species, including *B. subtilis*, *B. pumilus*, and *B. amyloliquefaciens*, from the integument of *D. maidis* and *D. kuscheli*. The bacteria were found to be antagonistic to entomopathogenic *Bea. bassiana*, inhibiting germination as well as growth of conidia. Indeed, the ability of *Bacillus* to produce antibiotic-like compounds, antifungal compounds, and/or bacteriocins, such as surfactin, bacylisin, fengycin, bacyllomicin, subtilin and iturin, has led to the use of these bacteria throughout the world to control phytopathogens [2,3,16,20,36].

The development of novel formulations of biocides for use in the sustainable management of maize agroecosystems requires an understanding of the interactions between entomopathogenic fungi and the microbial populations living on the cuticle of insects. Thus, in the present work we characterized 10 strains of *Bacillus* by means of molecular techniques and then selected isolates of *Bea. bassiana* and *M. anisopliae* that were resistant to the antimicrobial compounds secreted by these bacteria.

Materials and methods

Bacterial strains. All bacterial strains used in this study were isolated from the integument of *D. maidis* and *D. kuscheli* [40]. Genomic DNA was extracted from these strains using the Wizard Genomic DNA purification kit (Promega). The 16S rDNA of strains Dm-B3, Dm-B4, Dm-B10, Dm-B17, Dm-B22, Dm-B23, Dm-B47, Dm-B55, Dm-B59, and Dk-B25 was amplified in a thermocycler (Minicycler, MJ Research) and sequenced according to Sanger et al. [27]. The sequences were deposited in the GenBank database of the National Center for Biotechnology Information (NCBI). From an analysis of the sequences using the Basic Local Alignment Search Tool (BLAST), 10 sequences were obtained and then aligned with those of reference strains *B. amyloliquefaciens*, *B. pumilus*, *B. megaterium*, *B. cereus*, and *B. thuringiensis* by means of the multiple sequence alignment program Clustal W. A UPGMA phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis version 5 (MEGA5) [35].

Fungal isolates. Forty-one isolates of *Bea. bassiana* and 20 isolates of *M. anisopliae* were used in this study. Fungal isolates were obtained from their insect hosts, which belonged to the orders Hemiptera, Coleoptera, and Dermaptera, and from soil samples collected from sorghum and corn crops. All of the isolates were obtained in Buenos Aires, Corrientes, and Tucumán provinces of northern Argentina. They were stored in the Mycological Collections of Centro de Estudios Parasitológicos y de Vectores (CEPAVE, La Plata, Buenos Aires, Argentina), in the Agricultural Research Service, Collection of Entomopathogenic Fungi (ARSEF, Ithaca, New York, USA), and in the collection of the Centro de Investigaciones de Fitopatología (CIDEFI, La Plata, Buenos Aires, Argentina). The isolates were characterized according to both their morphology [39] and their virulence against cicadellids and delphacids [38].

Inhibition of fungal growth by bacteria. The antagonistic activity of 10 *Bacillus* strains against 41 isolates of *Bea. bassiana* and 20 isolates of *M. anisopliae* was tested using the central disk test [26]. Fungal isolates were cultured on malt extract agar (MEA 2%) at 25°C in the dark for 7 days. A 7-mm mycelium disk was cut and transferred to the center of a tryptic soy agar (TSA; Britania) plate and cultured at 30°C for 48 h. Three such disks were transferred to each TSA plate and placed at equidistant points from the central disk. Each treatment consisted of six replicates and one control (plates containing only a central disk of the fungus). The plates were incubated at 30ºC in darkness. Mycelial growth was estimated based on the radial increase in colony size, which was measured between two orthogonal diameters drawn 10 days after the incubation. Antagonism was estimated based on the percentage of mycelial growth inhibition (MGI), which was calculated as suggested by Michereff et al. [19].

Statistical analysis. The effects of treatments were determined by the factorial analysis of variance (ANOVA). The mean values were separated using Tukey's honestly significant difference (HSD) test ($P < 0.05$) [31].

Results and Discussion

Bacterial isolates. The 16S rDNA sequences confirmed that all of the strains belonged to the genus *Bacillus* and suggested that strain Dm-B3 was *Bacillus amyloliquefaciens* (Gen Bank accession number: HQ339952), strains Dm-B22, Dm-B23, and Dk-B25 were *B. pumilus* (KC460218, KC460219, and KC460215, respectively), and that strains Dm-B4, Dm-B17, Dm-B47, and Dm-B55 were *B. subtilis* (HQ111352, KC460217, HQ111353, and HQ111354, respectively). However, strains Dm-B10 and Dm-B59 (KC460216 and KC460220), initially identified by Toledo et al. [40] by means of biochemical reactions as *B. megaterium*, had a

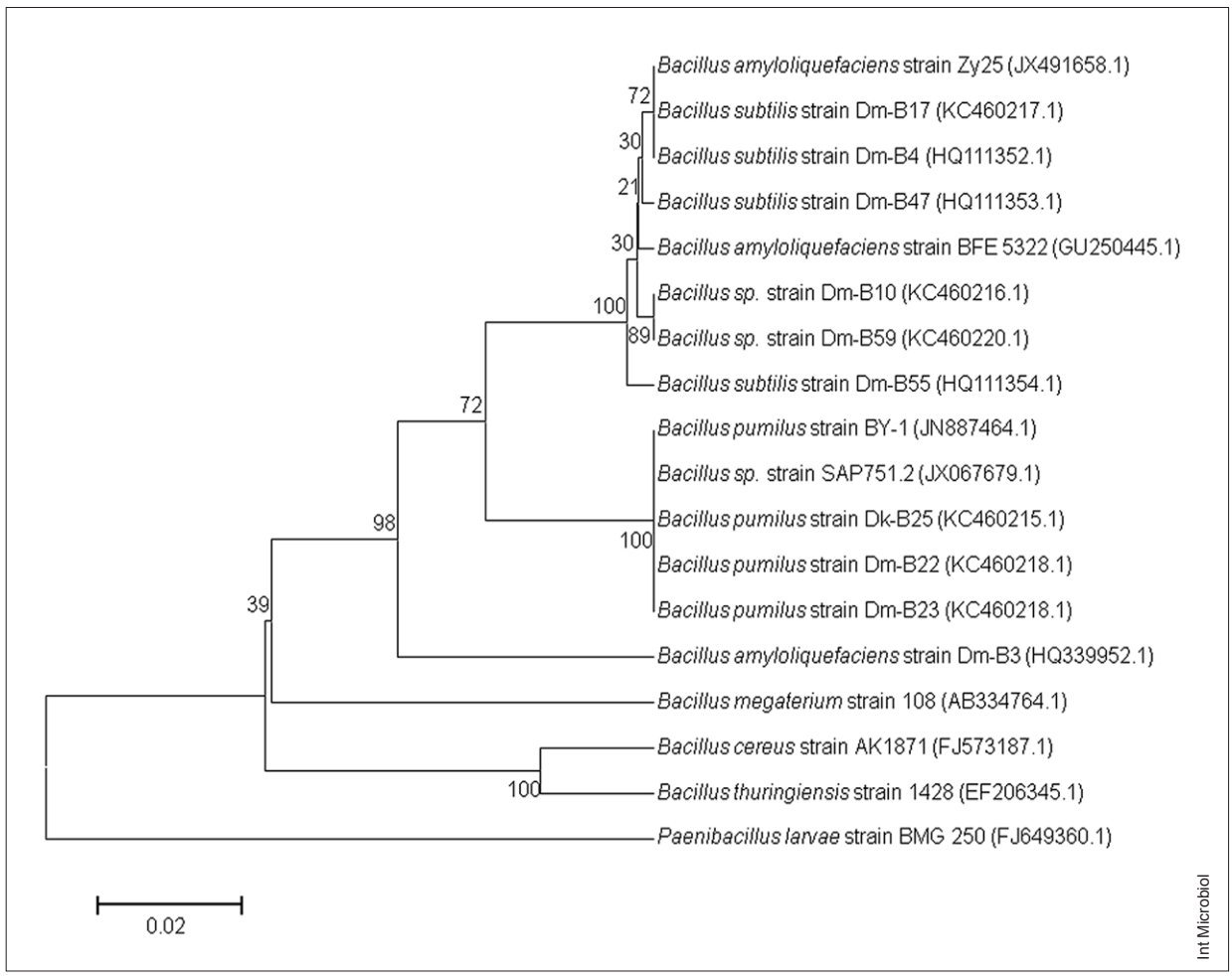

Fig. 1. Dendrogram showing the identity and relationship of the major antagonistic bacteria isolated from the cuticular surfaces of *Delphacodes kuscheli* and *Dalbulus maidis*. Numbers on the branches represent bootstrap values obtained from 1000 replicates. The bar indicates 0.02 substitutions per site. Species names are followed, in parentheses, by the National Center for Biotechnology Information (NCBI) GenBank database accession numbers.

16S rDNA sequence 99% homologous to the full sequence of *B. subtilis* and *B. amyloliquefaciens*. Therefore, pending additional molecular data, both strains were reclassified as *Bacillus* sp. (Dm-B10 and Dm-B59, respectively).

The bacterial strains were grouped into four clusters (Fig. 1). The first one comprised six strains and two reference sequences of *B. amyloliquefaciens*. It was supported by a bootstrap value of 100%. Within the cluster, there were four representatives of *B. subtilis* (Dm-B17, Dm-B4, Dm-B47, and Dm-B55) and two of *Bacillus* sp. (Dm-B59 and Dm-B10). The isolates of *B. subtilis* were clustered in separate groups; thus, Dm-B4, Dm-B17, and Dm-B47 formed a cluster (30% bootstrap) that was clearly distinct from that formed by strain Dm-B55 (*B. subtilis*). The second cluster was also supported by a bootstrap value of 100% and was made up of three isolates of *B. pumilus* (Dm-B22, Dk-B25, and Dm-B23) and the reference sequences of *B. pumilus* (BY-1) and *Bacillus* sp. (SAP751.2). The third cluster contained a single isolate, Dm-B3, identified as *B. amyloliquefaciens*. It merits further study to confirm its identity and to identify the nucleotides that render it distinct—including, perhaps, phenotypically—from the other isolates of the same species. Nonetheless, all of the studied strains belong to a monophyletic cluster comprising closely related organisms with strong similarity at the 16S rDNA sequence level and clustering separately from other *Bacillus* species, among them *B. cereus*, *B. thuringiensis*, and *B. megaterium*.

Inhibition of fungal growth by bacteria. The MGI of *M. anisopliae* was dependent on the bacterial strain ($F = 9.5$; df = 9, 1171; $P < 0.0001$) and on the targeted fungal isolate ($F = 35.8$; df = 19, 1171; $P < 0.0001$). The 10 bacterial strains differed in their antifungal activity ($P < 0.05$) and could be separated into four homogeneous groups. *Bacillus subtilis* Dm-B47 (68.4%), *B. amyloliquefaciens* Dm-B3 (64.4%), and *B. pumilus* Dk-B25 (64.3%) differed significantly from the other strains and showed the greatest antagonism against *M. anisopliae*, whereas *B. subtilis* Dm-B17 (52.8%) and *Bacillus* sp. Dm-B10 (52.6%), were the least antagonistic against the fungus. Similar results were obtained with *Bea. bassiana*. Both the bacterial strains ($F = 10.8$; df = 9, 2350; $P < 0.0001$) and the fungal isolates ($F = 91.2$; df = 40, 2350; $P < 0.0001$) had a significant effect on MGI. In this case, the bacterial strains could be separated according to their antagonistic activity into five statistically different groups ($P < 0.05$). *Bacillus pumilus* Dm-B22 (64.6%), Dm-B23 (62.8%), and Dk-B25 (63.2%) differed significantly from other strains and were the most antagonistic strains when tested against *Bea. bassiana*, whereas *B. subtilis* Dm-B55 (57.5%) and Dm-B4 (57.3%) were the least antagonistic (Table 1). Some bacterial strains differed in their behavior against the two fungal species. For example, *B. subtilis* Dm-B4 was one of the least antagonistic strains against *Bea. bassiana* but it was one of the most antagonistic ones against *M. anisopliae* isolates.

Table 1. Mycelial growth inhibition (MGI) of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* by *Bacillus* strains isolated from the cuticular surfaces of cicadellids and delphacids

Bacterial strain	MGI (%)*	
	M. anisopliae	*Bea. bassiana*
Dm-B10	52.6 ± 1.7 a	61.4 ± 0.8 cde
Dm-B17	52.8 ± 1.7 a	58.3 ± 0.8 abc
Dm-B55	56.2 ± 1.7 ab	57.5 ± 0.8 ab
Dm-B23	57.7 ± 1.7 abc	62.8 ± 0.8 de
Dm-B22	58.3 ± 1.7 abc	64.6 ± 0.8 e
Dm-B59	60.7 ± 1.7 bc	57.9 ± 0.8 abc
Dm-B4	63.3 ± 1.7 bcd	57.3 ± 0.8 a
Dk-B25	64.3 ± 1.7 cd	63.2 ± 0.8 de
Dm-B3	64.4 ± 1.7 cd	61.3 ± 0.8 cde
Dm-B47	68.4 ± 1.7 d	60.9 ± 0.8 bcd

*Mean ± standard error. Values with the same letters are not significantly different according to Tukey's HSD test ($P < 0.05$).

The most antagonistic bacterial strains in our study belonged to the *B. subtilis* group, which includes *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus* and other close relatives of *B. subtilis*. In previous reports, a number of species of bacteria belonging to the *B. subtilis* group, such as *B. pumilus, B. licheniformis, B subtilis, B. atrophaeus*, and *B. amyloliquefaciens*, were shown to secrete inhibitors of bacterial and fungal growth. These compounds are thought to play a crucial role in competition or microbial interactions [2,3,16,36,47].

Fungal susceptibility to antagonistic bacteria seems to be a variable trait. In this study, the susceptibility of *Bea. bassiana* was much more variable than that of *M. anisopliae*. Thus, *Bea. bassiana* isolates 099 (5.9%) and 111 (12.4%) were the least inhibited, and isolates Bb075 (78.1%) and Bb189 (76.5%) the most inhibited ones (Table 2). By contrast, representatives of *M. anisopliae* exhibited less variability in terms of their susceptibility to bacteria. For these species, bacterial inhibition was strongest for isolates Ma120 (77.9%), Ma35

Table 2. Isolates of *Beauveria bassiana* and *Metarhizium anisopliae* and the inhibition (expressed as the percent of the control) of mycelial growth (MGI) by *Bacillus* strains

Isolate	MGI (%)*	Isolate	MGI (%)	Isolate	MGI (%)
Ma079	31.8 ± 2.4 a	Bb099	5.9 ± 3.6 a	Bb147	69.1 ± 1.6 jklmnop
Ma003	36.9 ± 2.4 ab	Bb111	12.4 ± 1.8 a	Bb112	69.1 ± 1.6 jklmnop
Ma34	44.1 ± 2.4 bc	Bb001	31.8 ± 1.6 b	Bb137	69.6 ± 1.6 jklmnopq
Ma31	44.3 ± 2.4 bc	Bb061	33.3 ± 1.6 b	Bb081	69.9 ± 1.6 jklmnopq
Ma33	45.6 ± 2.4 bc	Bb074	33.3 ± 1.6 b	Bb175	70.1 ± 1.6 jklmnopq
Ma076	51.0 ± 2.4 cd	Bb072	33.3 ± 1.6 b	Bb148	70.5 ± 1.6 jklmnopq
Ma36	53.6 ± 2.4 cd	Bb092	44.1 ± 1.6 c	Bb114	71.0 ± 1.6 klmnopq
Ma37	53.7 ± 2.4 cd	Bb249	46.7 ± 1.6 cd	Bb145	71.1 ± 1.6 klmnopq
Ma30	55.5 ± 2.4 cde	Bb140	48.4 ± 1.6 cde	Bb143	71.1 ± 1.6 klmnopq
Ma078	58.9 ± 2.4 def	Bb117	53.9 ± 1.6 def	Bb146	71.5 ± 1.6 lmnopq
Ma178	61.1 ± 2.4 defg	Bb136	57.1 ± 1.6 efg	Bb176	72.2 ± 1.6 lmnopq
Ma095	67.3 ± 2.4 efgh	Bb149	57.4 ± 1.6 fg	Bb142	72.9 ± 1.6 mnopq
Ma39	68.0 ± 2.4 fgh	Bb080	58.3 ± 1.6 fgh	Bb54	73.2 ± 1.6 mnopq
Ma086	71.6 ± 2.4 gh	Bb083	59.9 ± 1.6 fghi	Bb153	73.6 ± 1.6 mnopq
Ma32	73.5 ± 2.4 h	Bb118	62.2 ± 1.6 fghij	Bb069	73.7 ± 1.6 mnopq
Ma29	74.2 ± 2.4 h	Bb119	62.6 ± 1.6 fghijk	Bb116	75.1 ± 1.6 nopq
Ma160	74.9 ± 2.4 h	Bb077	63.7 ± 1.6 ghijkl	Bb150	75.3 ± 1.6 opq
Ma38	76.3 ± 2.4 h	Bb113	66.2 ± 1.6 hijklm	Bb141	75.5 ± 1.6 pq
Ma35	76.7 ± 2.4 h	Bb138	66.5 ± 1.6 hijklmn	Bb189	76.5 ± 1.6 pq
Ma120	77.9 ± 2.4 h	Bb002	66.6 ± 1.6 hijklmno	Bb075	78.1 ± 1.6 q
		Bb151	66,7 ± 1.6 ijklmnop		

*Mean ± standard error. Values with the same letters are not significantly different according to Tukey's HSD test ($P < 0.05$).

(76.7%), Ma38 (76.3%), and Ma160 (74.9%) and weakest for isolates Ma003 (36.9%) and Ma079 (31.8%) (Table 2).

Therefore, in this study we identified two isolates of *Bea. bassiana* (Bb099 and Bb111) and two of *M. anisopliae* (Ma003 and Ma079) as the most resistant to antagonism by the ten *Bacillus* strains tested. Figure 2 shows the results of the disk tests for the most and the lest inhibited fungal species.

The differences in the responses of the fungal isolates to bacterial attack might be due to their different abilities to detoxify bacterial growth inhibitors, for example, by producing secondary metabolites with antibacterial activity. Diverse toxic metabolites have been described in several fungal biological control agents, including species of *Beauveria, Metarhizium*, and *Isaria* [43]. Some of these metabolites have antibiotic, fungicidal, or insecticidal properties [13,43]. Recently, Sahab [28] characterized a crude ethyl acetate extract of *Bea. bassiana* with antibacterial and antifungal activities. The antibacterial activity was effective at any of the concentrations tested when used against different strains of gram-positive and gram-negative bacteria.

Several studies have shown that the insect cuticle is an ecological niche for microbes, where fungi and bacteria co-exist and interact [9,18,29,45]. Among the mechanisms proposed for the biocontrol activity of *Bacillus* spp., competition, the induction of systemic resistance, and antibiotic production appear to be the most important one [1,32,37].

A better understanding of fungal-bacterial interactions may lead to the development of potent formulations of *Bea. bassiana* and *M. anisopliae* for their use in insect control. Further studies on the diversity of microorganisms that colonize the insect cuticle, their role, and their impact in nature are needed in order to develop biological control agents

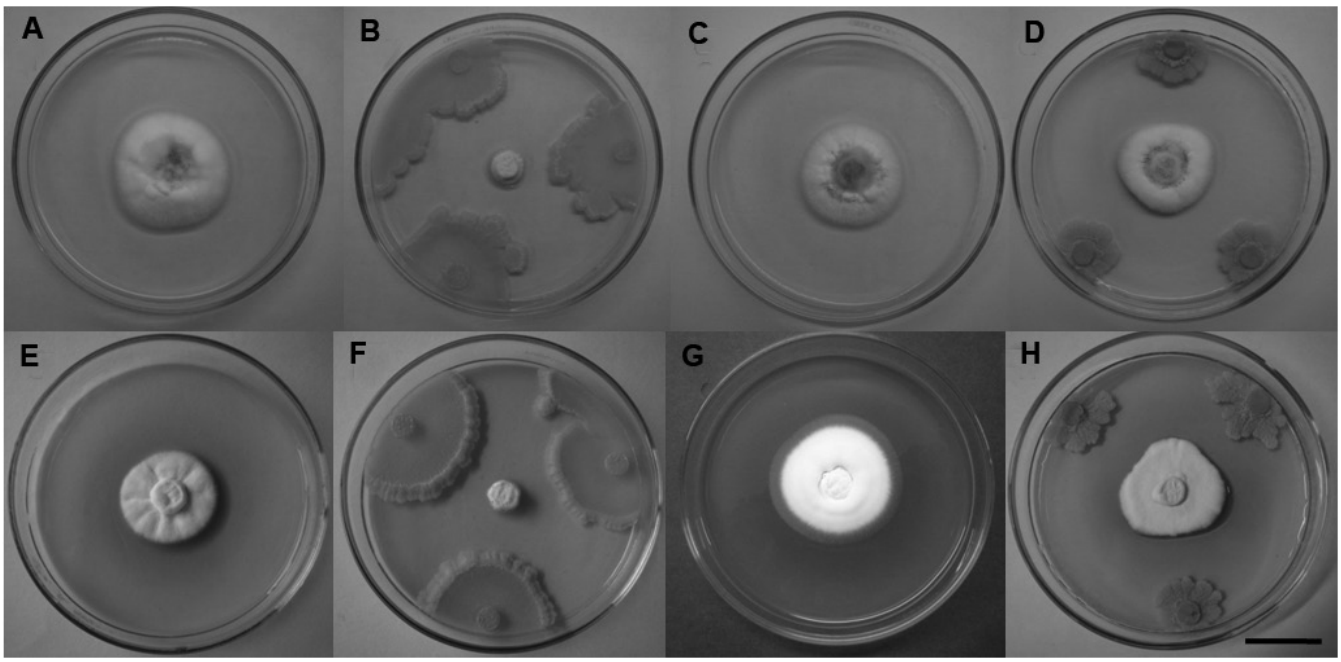

Fig. 2. Antifungal activity of *Bacillus* strains against *Metarhizium anisopliae* and *Beauveria bassiana* (Bb) on tryptic soy agar plates after 10 days at 30°C in the dark. From left to right, fungal isolates more and less inhibited by bacteria. (A–D) *M. anisopliae* (Ma); (E–H**)** *Bea. bassiana* (Bb). (**A**) Ma35 control. (**B).** Ma35 in the presence of *B. subtilis* DM-B17. (**C**)**.** Ma079 control. (**D**)**.** Ma079 in the presence of *B. subtilis* Dm-B4. (**E**) Bb075 control**.** (**F**) Bb075 in the presence of *B. subtilis* Dm-B55. (**G**) Bb099 control. (**H**) Bb099 in the presence of *B. subtilis* Dm-B4. Scale bar = 2.5 cm.

that are effective against insect pests such as cicadellids and delphacids.

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Competing interests. None declared.

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