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Inhibition of the growth of *Ascosphaera apis* by *Bacillus* and *Paenibacillus* strains isolated from honey

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SUMMARY

The fungus *Ascosphaera apis*, the causative agent of chalkbrood disease in honeybee larvae, occurs throughout the world and is found in many beekeeping areas of Argentina. The potential as biocontrol agents of 249 aerobic spore-forming bacterial antagonists isolated from honey samples was evaluated. Each isolate was screened against *A. apis* by a central disk test assay. Ten bacterial strains that showed the best antagonistic effect to *A. apis* were selected for further study and identified as *Bacillus cereus* (m363, mv86, mv81, mv75), *Bacillus circulans* (Fr231, m448b), *Bacillus megaterium* (m435), *Bacillus pumilus* (m354), *Bacillus subtilis* (m329), and *Paenibacillus alvei* (m321). For testing the efficiency of the selected strains, a paired culture test was used with 5 replicates of each combination bacterial antagonist / *A. apis* strain, and 5 replications for each control on 4 different culture media. The analysis of variance and posterior comparison of means according to LSD method showed that the best antagonists when using YGPSA medium were *B. subtilis* (m329) and *B. megaterium* (m435), and in the case of MYPGP medium the most efficient were *B. circulans* strains Fr 231 and m448b.

Key words: *Ascosphaera apis,* chalkbrood, biocontrol, bacterial antagonists, *Bacillus* spp., *Paenibacillus* spp., honeybees, *Apis mellifera.*

RESUMEN

Inhibición del crecimiento de *Ascosphaera apis* mediante cepas de *Bacillus* y *Paenibacillus* aisladas de miel. La cría yesificada es una micosis invasiva ocasionada por el hongo heterotálico *Ascosphaera apis* que afecta exclusivamente a las larvas de las abejas. La enfermedad tiene difusión mundial y en la Argentina se halla diseminada en todas las áreas donde se realiza apicultura. Se estudió la potencialidad de 249 cepas de bacterias esporuladas aeróbicas aisladas de miel como agentes biocontroladores del hongo mediante un ensayo en disco central en condiciones de laboratorio. Se seleccionaron como mejores antagonistas 10 cepas bacterianas identificadas como *Bacillus cereus* (m363, mv86, mv81, mv75), *Bacillus circulans* (Fr231, m448b), *Bacillus megaterium* (m435), *Bacillus pumilus* (m354), *Bacillus subtilis* (m329), y *Paenibacillus alvei* (m321). Para probar la eficiencia de las cepas seleccionadas, se empleó la técnica de cultivo dual con 5 repeticiones de cada combinación antagonista bacteriano / cepa de *A. apis,* 5 para cada control y 4 medios de cultivo diferentes empleando 10 cepas del hongo de distintos orígenes geográficos. El análisis de la variancia y posterior comparación de medias LSD (Least Square Dfferences) mostró que los mejores antagonistas fueron *B. subtilis* (m329) y *B. megaterium* (m435) para el caso del medio YGPSA, mientras que para MYPGP las más eficientes fueron las cepas de *B. circulans* Fr 231 y m448b.

Palabras clave: *Ascosphaera apis,* cría yesificada, control biológico, antagonistas bacterianos, *Bacillus* spp., *Paenibacillus* spp., abejas, *Apis mellifera.*

Ascosphaera apis (Maassen ex Clausen) Olive & Spiltoir, the causative agent of chalkbrood disease in honeybee larvae (*Apis mellifera* L.) occurs throughout the world and is found in most beekeeping areas of Argentina. *A. apis* is a heterotallic fungus that sporulates only when mycelia of the opposite sex (plus and minus) come in contact and, after interaction of both mycelia, spores form within dark green fruiting bodies called ascocarps. Diseased larvae are covered by a fluffy white mould, becoming dark grey or black mummies if fruiting bodies are formed (5, 13). Fungal spores are highly resistant to environmental extremes and can survive for years. This situation, together with the need for predisposing conditions, which are not well defined, implies that outbreaks of the disease can readily appear unexpectedly. There is no efficacious chemotherapeutic agent available for use against chalkbrood in honeybee colonies, although there are several commercial products in use (5). In addition, research on selecting and breeding honeybees that show elevated hygienic behaviour for chalkbrood control is still in progress (14, 15).

The objective of this study was to isolate spore-forming bacteria from honey samples that inhibit the growth and or sporulation of *A. apis* as potential biocontrol agents. Emphasis was placed on the isolation of *Bacillus*

spp. because members of this genus have been frequently isolated from apiarian sources and have been reported to be effective biocontrol agents by producing antibiotics, antibiotic-like compounds and antifungal metabolites (9). Furthermore, honeybees may use *Bacillus* spp. to process and conserve stored food in order to prevent spoilage, particularly by fungi (5).

Ten strains of *A. apis* (sporulated and non-sporulated) isolated from larvae with chalkbrood symptoms and honey samples, located in different areas of Argentina and Chile, and also a reference culture (DSM 3116, mating type + from Japan) were used in this study. The isolations and identification of fungal strains were done as previously described (2, 12). All the isolates were maintained in 20% glycerol at –80 °C; for short-term storage, the strains were maintained at 4 °C on Saboureaud dextrose agar slant cultures (3).

A total of 249 potential bacterial antagonists were isolated from honey samples from different geographical areas. Honeys were homogenized in a water bath at 40 °C and 10 ml were mixed (1:1 v/v) with 0.01 M phosphate buffered saline pH 7.2 and centrifuged at 3,500 g for 40 min at 5 °C to concentrate bacterial spores. The supernatant fluid was discarded living approximately 3 ml per tube that was vortex-mixed to resuspend the pellet during 1 min and heated at 80 °C for 10 min. The samples were high speed vortex-mixed again for 2 min and 50 µl was streaked on plates of triptic soy agar (Britania®) or MYPGP agar (4) and incubated at 30 °C in aerobiosis. MYPGP agar contains, in g per l: Muller-Hinton broth (Britania®) 10 g, yeast extract 15 g, glucose (Merck®) 2 g, K_2HPO_4 3 g, sodium pyruvate (Sigma®) 1 g, agar 20 g; pH 7.3; the glucose stock solution is autoclaved separately (50% wt/v) and added to the culture medium cooled at 50 °C before plating. All the strains were stored as spore suspensions in 20% glycerol in MYPGP broth at - 80 °C until used.

Each isolate was screened against *A. apis* Aa3 by a central disk test assay (Figure 1). Briefly, the fungus was cultured on YGPSA (2) for 7 days at 30 °C in darkness. YGPSA contains in g/l: yeast extract 10 g, glucose 10 g, KH_2PO_4 13.5 g, soluble starch 10 g, and agar 20 g; pH 6.6. A 7 mm mycelium disk from the sporulating area was cut by using as a punch the opposite side of a P-1000 sterile plastic tip and transferred to the centre of a new plate of YGPSA. Three 7 mm disks containing each bacterial strain of a 48 h culture on triptic soy agar or MYPGP agar were transferred to the plate in the same way and place at 3 equidistant points from the central disk of fungal growth. For controls, only a central disk of fungal growth was used. After 7 days, plates were evaluated by measuring the diameter of the fungal colony in relation to controls and, at the same time, the mycelia were observed microscopically to determine abnormal characteristics of hyphae.

Ten bacterial strains that showed the best antagonistic effect to *A. apis* were selected for further study, and identified on the basis of Gram reaction, colony morphology, and microscopic examination of bacterial smears by lipid globule staining procedure (7). Presence of spores, determining size, shape, and location and size of vegetative cells were also evaluated. Bacterial cultures were tested by catalase reaction, production of lecithinase, tyrosinase activity, anaerobic utilization of glucose, haemolysis, and starch hydrolysis (6, 10). A more complete identification was performed by the analytical profile index (API) system by using API 20E and API 50CH strips plus API 50 CHB medium (Biomerieuxâ). Bacteria were identified as: *Bacillus cereus* (m363), (mv86), (mv81), and (mv75); *Bacillus circulans* (Fr231), and (m448b); *Bacillus megaterium* (m435); *Bacillus pumilus* (m354); *Bacillus subtilis* (m329); and *Paenibacillus alvei* (m321).

For testing the potential of biocontrol efficiency of the selected bacterial strains, a paired culture test was performed. In short, a 7 mm-diameter disk of each *A. apis* isolate was placed at a distance of 70 mm apart from a 7-mm diameter disk of each bacterial antagonist from a 24 h old culture on Tryptic soy agar (TSA) or MYPGP according to the strain tested. There were five replicates for each combination *A. apis* strain / bacterial antagonist and five replications for each control. In the case of *B. circulans* strains, these combinations were evaluated on MYPGP agar and V8 agar (3), because this species do not grow on most common media, while the rest of *Bacillus* and *Paenibacillus* species/*A. apis* combinations were evaluated on YGPSA and MY20 media. MY 20 medium contains peptone 5 g, yeast extract 3 g, malt extract 3 g, glucose 200 g, agar 20 g, distilled water 1,000 ml (11); while V8 medium contains V8 juice 200 ml (Swift-Armour®), Co_3Ca 4 g, agar 20 g, distilled water 800 ml, pH 7.3 (3). YGPSA and MYPGP showed lower values of standard deviation and better residual distribution than MY20 and V8, respectively, so the first ones were selected for further analysis. The analysis of variance and posterior comparison of means according to LSD method (Least Square Differences) showed significant differences ($p < 0.0005$) between the growth of controls and the growth of fungi in the presence of bacterial antagonists (Figure 2A), being the best antagonists *B. subtilis* (m329) and *B. megaterium* (m435). In the case of medium MYPGP, significant differences ($p<0.0005$) between all *A. apis* isolates in the presence of *B. circulans* (Fr 231) and (m448b) and controls were observed, showing that both bacterial strains were highly effective in the inhibition of *A. apis* (Figure 2B).

B. subtilis, B. megaterium and *B. circulans* have been reported as normal microorganisms associated with honey bees and also, to inhibit *A. apis* growth (5). The ability of these spore-forming species to inhibit different species of fungi by secreting antibiotics with antifungal properties like iturins, subtilins, mycosubtilins, megacins, and circulins, has been well documented (1, 8, 9). Further studies are needed in order to determine not only the toxic antifungal metabolites produced by these strains but also their potential use in future control strategies in honeybee colonies involving direct application of a biocontrol agent combined with breeding for hygienic behaviour.

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Figure 2. A. Inhibition of mycelial growth of ten *Ascosphaera apis* strains induced by eight bacterial antagonists in YGPSA medium after 7 days of growth at 30 ± 2 °C. Each value (solid point) is the mean of five replicates for the diameter (in mm) of mycelial growth and horizontal lines indicate standard deviations. **B.** Inhibition of mycelial growth of ten *Ascosphaera apis* strains induced by two bacterial antagonists in MYPGP medium after 7 days of incubation at 30 ± 2 °C. Each value (solid point) is the mean of five replicates for the diameter (in mm) of mycelial growth and horizontal lines indicate standard deviations.

Figure 1. Central test disk assay for the preliminary screening of bacterial antagonists against *Ascosphaera apis.*

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