Short communication. Viability and pathogenicity of *Ascosphaera apis* preserved in integral rice cultures

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Abstract

Inoculation of *Apis mellifera* colonies with chalkbrood is a good way to test for hygienic behavior. The use of compatible pure strains of *Ascosphaera apis,* the causative agent of chalkbrood, could maintain homogeneity among experiments. Mycelium aging, viability, purity and capacity to produce spore-cysts were evaluated in MY20 and integral rice kernels (IRK) medium. *Ascosphaera apis* strains on MY20 developed aging symptoms by the 30th day and developed pure colonies until the 77th day. The same strains on IRK showed aging symptoms by the 30th day of growing, but they produced pure colonies for 360 days. Spore-cysts obtained from pure strains preserved in IRK and spore-cysts from wild black mummies obtained from honeybee hives were used to inoculate fifth instar *A. mellifera* larvae. Ascospores from black wild mummies or spore-cysts obtained from pure strains preserved in IRK were equally effective in causing clinical symptoms of the disease, which has main advantages: permanent inoculum availability and genetic homogeneity.

Additional key words: *Apis mellifera*, chalkbrood, culture media, honey bees, hygienic behavior, larvae inoculation, preservation.

Resumen

Comunicación corta. Viabilidad y patogenicidad de *Ascosphaera apis* preservado en cultivos de arroz integral

La cría yesificada es un buen modelo para evaluar el comportamiento higiénico de la abeja melífera, pero la limitada viabilidad de *Ascosphaera apis* en medios sintéticos es una limitación. El envejecimiento del micelio, la viabilidad, pureza y capacidad de producir esporas fueron evaluadas en cepas de *A. apis* mantenidas en MY20 y en arroz integral (IRK). Las cepas de *A. apis* en MY20 mostraron síntomas de envejecimiento a los 30 días y desarrollaron colonias puras hasta el día 77. Las cepas mantenidas en IRK mostraron síntomas de envejecimiento a los 30 días, pero produjeron colonias puras durante 360 días. Las esporas obtenidas de cepas puras preservadas en IRK o de momias negras obtenidas de colmenas fueron usadas para inocular larvas L5 de *Apis mellifera*. Las ascosporas obtenidas de momias naturales o a partir de cepas preservadas en IRK fueron igualmente efectivas para reproducir la enfermedad y esta última permite disponer de inóculo en forma permanente y con homogeneidad genética.

Palabras clave adicionales: abejas, *Apis mellifera,* comportamiento higiénico, cría yesificada, inoculación de larvas, medios de cultivo, preservación.

Chalkbrood is a fungal disease of honeybees (*Apis mellifera* L., Hymenoptera:Apidae) caused by *Ascosphaera apis* Maassen ex Claussen (Olive et Spiltoir). *Ascosphaera apis* is a heterothallic fungus that affects larvae of the honeybee. Diseased brood becomes mummi-fied and mummies are white, gray, or black depending on the predominance of the sexual stage of the fungus. If spore-cysts are formed as a result of mating, infected larvae become gray or black in color. Larvae infected only by mycelia, without spore-cysts, are white (Gilliam *et al*., 1988; Gilliam and Lorenz, 1993).

Larvae ingest the fungal spores when they are fed by adult bees that carry the spores. The spores are not

infectious until they break through gut wall when larvae are in the 5th instar. In addition, the development of the disease requires a predisposing condition in the susceptible larva (Heath, 1982). Larvae in the 5th instar, prior to and some hours after cell capping, are more susceptible to the stress factors that can trigger disease expression (Bayley, 1967; Puerta *et al.,* 1994; Flores *et al.,* 1996). Many factors have been suggested as causes of stress such as excessive moisture, weak colonies, presence of other diseases, inadequate nutrition, excessive colony manipulation (Gilliam, 1990) and chilling of the brood (Bailey, 1967; Flores *et al*., 1996).

Chalkbrood is distributed worldwide. In Argentina it was first detected in 1978 by Rossi and Carranza (1980). At first symptoms were seen primarily during spring and colonies recovered without treatment as the summer progressed (Albo *et al*., 1994).

Since 1988, the incidence of chalkbrood has increased and it is noted more frequently in apiaries (Flores *et al*., 1999). The disease does not normally lead to colony losses but it does weaken colonies and lead to decreased honey production. Efforts to control this disease with chemicals have not been successful and sometimes the treatments have negative secondary effects on the brood, adult bees and honey (Gilliam and Vandenberg, 1990; Flores *et al.,* 2001). Periodic inspection of colonies and disinfection of hive equipment, as is recommended for American Foulbrood control, allows for earlier detection of the disease and the elimination of *A. apis* spores.

One mechanism of resistance to chalkbrood involves naturally occurring antimycotic substances that are antagonist to chalkbrood fungus (Gilliam *et al.,* 1988; Spivak and Gilliam, 1998). However, the main mechanism of resistance to this disease is hygienic behavior of adult bees. Hygienic behavior is defined as the ability of worker honey bees to detect cells containing dead or diseased brood, uncap the wax capping over the cells, and remove the abnormal brood from the cell and nest. The behavior was first described by Rothenbuhler (1964). Early removal of affected larvae by adult bees prevents dissemination of disease through the colony because the bees remove the diseased brood before the pathogen reaches the infectious spore stage.

The most common assays to test colonies for hygienic behavior are indirect: brood is killed either by freezing (Gonçalves and Kerr, 1970; Reuter and Spivak, 1998; Spivak and Reuter, 1998; Palacio *et al.,* 2000) or by piercing (Cosenza and Silva, 1972; Newton *et al*., 1975; Gilliam *et al.,* 1978; Message, 1979; Taber, 1982; Milne, 1983; Spivak and Gilliam, 1991). Colonies that quickly remove the dead brood also tend to remove diseased brood rapidly. However, to conduct behavioral studies of the hygienic trait it would be better to challenge colonies directly with a pathogen to test for their ability to remove diseased brood, and subsequently compare the rate of removal with the other assays. In order to challenge colonies with the chalkbrood pathogen, it is important to minimize variables to ensure similar conditions for the tests but there are problems in obtaining homogeneous cultures of *A. apis*.

When hygienic behavior has been studied in relation to chalkbrood resistance, inoculation with pathogen has been done using black and white homogenized chalkbrood mummies in pollen patties (Spivak and Gilliam, 1993, 1998; Invernizzi, 2001). This natural inoculum comes from a mix of compatible genotypes and does not allow the maintenance of genetic homogeneity of the pathogen among experiments. The use of compatible pure strains could overcome these limitations.

The short viability of *A. apis* in synthetic media has been a persistent problem. MY20 agar (Raper and Fenel, 1965, cited by Bissett, 1988) is the most frequently used medium (Takatori and Tanaka, 1982; Bissett, 1988; Lee *et al.,* 1989; Alonso, 1991; Alonso *et al.,* 1993; Puerta *et al.,* 1994), but *A. apis* colonies start to show aging signs after 30 days of growth (Ruffinengo *et al.,* 2000). Because *A. apis* cultivated on integral (or brown) rice did not show signs of aging after 60 days of incubation (Ruffinengo *et al.,* 2000), this medium may be promising for culturing and maintaining *A. apis*.

The objective of this research was to determine the viability and pathogenicity of *A. apis* preserved in integral rice cultures with the goal of using the fungal culture as an inoculum to challenge colonies to facilitate studies of honey bees hygienic behaviour.

MY20 agar (MY20, Raper and Fenel, 1965, cited by Bissett, 1988) and integral rice kernels (IRK, Ruffinengo *et al.,* 2000) were used throughout the experiments, unless otherwise stated. MY20 was placed in 9 cm Petri dishes (20 mL dish[-1]). IRK was placed in Petri dishes (20 g moistened kernels dish[-1]) or in 500 cm[3] transparent glass bottles (95 g moistened kernels bottle[-1]) unless otherwise stated. All media were autoclaved at 1 atmosphere and 121°C during 15 min.

A. apis strains, maintained as mycelium on MY20 or IRK, were tested. A randomized complete block design with three replications was used. Treatments were arranged in a factorial of two strains (D and G)

from Balcarce Fungal Collection (BFC) and two culture media (MY20 and IRK). The experimental unit was a Petri dish culture for MY20 and a bottle culture for IRK.

Inoculum for starting cultures was produced on MY20. The strains were grown separately at $30 \pm 2°C$, darkness and aerobic conditions until the mycelium reached the border of the Petri dish (approx. 7 d). One or eight 4-mm diameter plugs from the border of a growing agar colony, were used to inoculate Petri dishes containing MY20 or IRK, respectively. Immediately after inoculation, IRK cultures were hand-shaked vigorously to distribute the inoculum. IRK cultures were stored horizontally to avoid condensation of water on the bottle plug interior. Cultures were incubated under dark and aerobic conditions at $30 \pm 2°C$ for 15 d and stored at $19 \pm 4°C$ until use. Data were analyzed by a General Linear Model (SAS Institute, Cary, NC, USA).

The effect of repeated openings of the same IRK bottle culture was studied. Materials and methods were as described before. An incomplete block design with three replications was used. The same bottles opened at the first evaluation were used throughout the experiment. Unopened cultures were compared with the previously opened ones at each evaluation time.

Mycelium aging was evaluated according to colony appearance. Cultures were monitored daily until the 360th day. Development of yellow-brown pigments, exudates, oily-wool like and collapse of mycelium were indicators of aging, according to Alonso *et al*. (1993). The date when these signs were evident in each culture was registered. The length of the period without mycelium aging (number of days) was analyzed. An F test was used to detect significant differences between two means.

On the 30th, 90th, 180th, 270th and 360th day of preservation, the viability of preserved cultures was tested. From each experimental unit, two 4-mm agar plugs from the border of growing colonies on MY20 or 16 rice kernels from IRK were transferred onto MY20. Transfers from a 7-d old colony on MY20 were used as a non-preserved control. Cultures were incubated as indicated before. In 5-d cultures, growth and presence of contaminants coming from the preserved propagules was registered. Three replicates were performed for each evaluation time. A regression of percentage of pure colonies obtained throughout the preservation period was performed.

Only cultures preserved in IRK were used. On the 30th, 90th, 180th, 270th and 360th day of preservation, the capacity of pure strains to produce spore-cysts was evaluated. Dual cultures of compatible strains D and G were set up on MY20 and IRK. One rice kernel colonized with mycelium of each sexual compatibility group was plated 4-cm apart from the other and incubated as indicated before until spore-cyst production (approx. 15 d). Four replicates were performed. The presence or absence of spore-cyst was registered.

It was tested if it would be possible to use compatible pure strains preserved as mycelium in IRK to obtain mummies in a controlled way. One experiment repeated three times was run. Strains D and G were matched on IRK Petri dishes 15 d before inoculation to obtain spore-cysts ($D \times G$ spore-cysts). As controls, spore-cysts from wild black mummies obtained from honeybee hives were included and a control treatment without the pathogen (a sterile solution of honey and water 1:1, v:v). Immediately before inoculation, $D \times G$-rice kernels or wild black mummies were placed in a test tube containing 2 mL sterile distilled water. Test tubes were stirred for 60 s. To release spores from the spore-cysts, the suspension was homogenized in a test tube with a ground-glass pestle (Cole-Parmer, Buenos Aires, Argentina). Spore concentration was measured with a haemocytometer and adjusted to 2×10^8 ascospores mL^{-1}. The number of ascospores was counted at $500 \times$ in a light microscope. This suspension was mixed with liquid honey (1:1, v:v).

Pathogenicity was tested according to the methodology of Flores *et al*. (1996) with minimal modifications. Each experimental unit had a piece of honeybee-comb (40 cm^2) containing 40 to 60 unsealed fifth instar larvae. These comb pieces containing larvae were collected from commercial hives. Each larva received a single dose of 5×10^5 spores in 5 µL food, placed near the mouthpart. Extreme care was taken to avoid touching the larvae. Consumption was confirmed by direct observation. Immediately, the comb pieces with larvae were cooled ($18 \pm 2°C$) for 24 h and reinstalled into healthy and strong hives until the bees sealed the comb cells (approximately 24 h). Comb pieces containing the treated larvae were taken from the hives, incubated at $25° \pm 2°C$ for 7 days and unsealed to count the mummified larvae. An incomplete block design with three replications was used. Every hive used constituted one block. Data of each experiment were analyzed by a General Linear Model (SAS Institute, Cary, NC, USA) *F* test of homogeneity of variance and a combined analysis of pathogenicity experiments were performed. A multiple comparison test by Duncan was used.

A. apis colonized MY20 or IRK Petri dishes in 7 and 15 d, respectively.

A. apis mycelium growing on MY20 agar showed all aging signs indicated by Alonso *et al.* (1993). Thirty days after inoculation, a brown-yellow pigmentation was observed. Fifteen days later, exudates and oily, wool-like mycelium were detected. Cultures on IRK did not have these signs. On the 30th day, some hyphae collapsed on IRK.

In MY20 agar, all cultures preserved for 42 d produced pure colonies. Transfers on the 49th and 56th day gave few contaminated colonies. At the 77th day of preservation, only 25 and 13% pure colonies were obtained by D and G, respectively. On the 84th day, all transfers resulted in contaminated colonies.

In IRK, most cultures produced pure colonies; from 1096 transfers, 94% produced pure colonies. Failures were caused by no growth or contamination. The first contamination was detected on the 180th day evaluation. Most of the failures (26%) were registered in the last evaluation ($P = 0.0001$; $R^2 = 0.77$, Fig. 1).

No difference was detected for percentage of pure and developed colonies for both strains ($0.97 < P < 1.00$; $0.77 < R^2 < 0.79$). No difference was detected between cultures obtained from bottles not previously opened and bottles opened at each evaluation date ($P = 0.11$; $R^2 = 0.77$).

Mycelium of D or G strains, preserved for 360 days in IRK, remained suitable to produce spore-cysts.

Variances of the three experiments were homogeneous ($0.29 < P < 0.41$). No effect of experiment ($P = 0.09$) or the interaction between experiment and inoculation treatment ($P = 0.90$) was detected. Ascospores from black wild mummies or D×G spore-cysts were equally
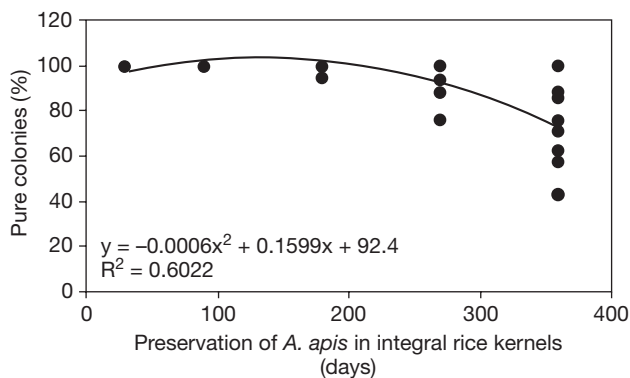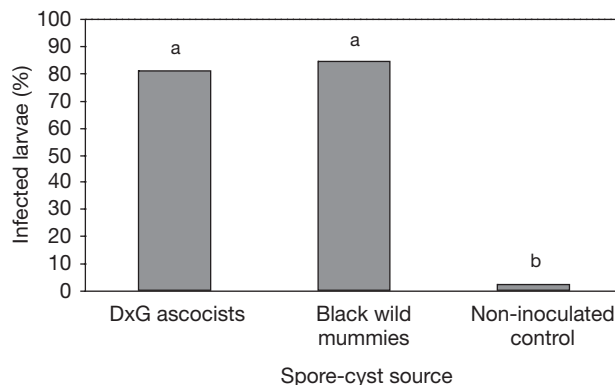
Figure 2. Incidence of chalkbrood obtained by inoculating honeybee larvae with *A. apis* ascospores from cultures maintained and matched 15 days before inoculation in IRK (D×G spore-cysts) and black wild mummies as compared with a non inoculated control. D×G are spore-cysts produced by compatible strains D and G preserved as mycelium on IRK and matched on IRK 15 days before inoculation. Different letters indicate significant differences among treatments ($P = 0.0001$; $R^2 = 0.99$). Values are the mean of three experiments and three replications.

effective to reproduce the disease. Both treatments differed significantly from the non-inoculated control ($P = 0.0001$). Pathogen inoculated treatments resulted in more than 82% infected larvae (Fig. 2).

After 18 months of preservation as mycelium in IRK, *A. apis* strains remained pathogenic. Inoculum preservation would solve the lack of availability and genetic homogeneity of *A. apis* isolates. MY20 is the most popular medium to maintain *A. apis* (Takatori and Tanaka, 1982; Bissett, 1988; Lee *et al.*, 1989; Alonso, 1991; Alonso *et al.*, 1993; Puerta *et al.*,1994). In the experiments, the strains D and G on MY20, developed aging symptoms on the 30th and 45th day of growing, and developed pure colonies until the 77th day of growing (data not shown). These strains on IRK showed aging symptoms at the 30th day of growing, but they produced pure colonies throughout the 360 d of preservation. Thus, it would be better to preserve *A. apis* on IRK than on MY20.

In pathogenicity tests, it was obtained more than 82% infected larvae with ascospores from D×G. The inoculum efficacy was comparable with that from wild mummies in this research (85%) and in the experiments of Puerta *et al.* (1994) and Flores *et al.* (1996) (48 to 82% and 90 to 100% infected larvae, respectively). The minimal percentage of chalkbrood in the non-inoculated control could be attributed to natural inoculum in the hives favored by the thermal stress imposed to the comb pieces (Koenig *et al.,* 1987; Flores *et al.,* 1996). The non-significant interaction between

Figure 1. Pure colonies of *A. apis* obtained from strains D and G preserved as mycelium on integral rice kernels (IRK). Values are the mean of three or six replications.

experiments and inoculation treatments accounts for the repeatability of results, the efficacy of the inoculum sources and the reliability of the method.

The inoculum from mycelia preserved in IRK as compared with the one from wild black mummies has two main advantages: permanent inoculum availability and genetic homogeneity. Also this inoculum from IRK is simple to produce and preserve and it has high efficacy. These characteristics made the use of ascospores from IRK grown ascocysts an interesting tool to study predisposition factors for chalkbrood or hygienic behavior of honeybees.

Acknowledgements

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