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A MODIFIED METHOD TO VISUALIZE INFECTION SITES OF SPORES OF THE ENTOMOPATHOGEN *BEAUVERIA BASSIANA* (DEUTEROMYCOTINA: HYPHOMYCETES) ON THE EXOSKELETON OF CITRUS ROOT WEEVIL *DIAPREPES ABBREVIATUS* (COLEOPTERA: CURCULIONIDAE) ADULTS

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ABSTRACT

Beauveria bassiana is a widespread entomopathogen which is infectious to a great variety of insects. A commercial preparation of this fungus was used to study its potential as a biocontrol agent of the citrus root weevil, *Diaprepes abbreviatus.* Laboratory reared adult weevils were placed in clean plastic bags with 0.05 g of powdered inoculum per bag, shaken for thirty seconds, and placed in holding cages. In order to determine the spore concentration, germination, and position of ingress, inoculated weevils were dipped in a collodion solution after 30 min, 6 h, 12 h, 18 h, and 30 h. Collodion peels from various areas of the exoskeleton were removed, stained with lactophenol cotton blue, and observed microscopically. At 12 h post-inoculation, spores began to swell, and at 18 h close to 25% germinated on all body parts except on the elytra. After 30 h, between 45% and 75% of the spores germinated, depending on the body part. Most active spore germination occurred around the eyes (75%), followed by the ventral abdomen (65%), the dorsal pronotum (60%), scales (45%), and dorsal surface of the elytra (7%). Spores were washed from the surface of inoculated weevils with distilled water and plating onto potato dextrose agar. Spore density on the weevils' surface was determined to be 15,815 ± 479 spores initially after inoculation, and was reduced to 5,782 ± 136 spores after 3 h.

Key Words: biocontrol, conidia, entomopathogenic fungus, infection sites, spore germination

RESUMEN

Beauveria bassiana es un entomopatógeno cosmopolita que es infeccioso para un gran variedad de insectos. Una preparación comercial de este hongo fue usada para estudiar su potencial como un agente de control biológico para el picudo de la raíz de cítricos, *Diaprepes abbreviatus.* Adultos del picudo criados en el laboratorio fueron puestos en bolsas plásticas y limpias con 0.05 g de inoculo en polvo en cada bolsa, agitado por treinta segundos, y puestos en jaulas de espera. Para determinar la concentración de esporas, germinación y la posición de su entrada, picudos inoculados fueron metidos en una solución adhesiva después de 30 minutos, 6 h (horas), 18 h y 30 h. Las cáscaras de colodio de varias áreas del exoesqueleto fueron quitadas, tintadas con el algodón azul de lactofenol, y observadas bajo el microscopio. A los 12 h después del inoculo, las esporas empezaron a hincharse, y a los 18 h aproximadamente 25% germinaron sobre todas las partes del cuerpo menos los elitros. Después de 30 h, entre 45% y 75% de las esporas germinaron, según la parte del cuerpo. La mayor parte de la germinación de las esporas sucedió alrededor de los ojos (75%), seguida por la parte ventral del abdomen (65%), el dorso de pronoto (60%), escamas (45%) y la superficie dorsal de los elitras (7%). Las esporas fueron lavadas de la superficie de los picudos inoculados con agua destilada y puestas en placas de agar de dextrosa de papa. La densidad de las esporas sobre la superficie de los picudos fue determinada como 15,815 ± 479 esporas inicialmente después del inoculo, y fue reducida a 5,782 ± 136 esporas después de 3 horas.

Beauveria bassiana (Balsamo) Vuillemin (Deuteromycotina: Hyphomycetes) is a disease-causing agent in insects (Steinhaus 1949). De Hoog (1972) has done the most definitive work on the genus *Beauveria* in which he was able to delimitate *Beauveria* from the genera *Isaria, Trititachium,* and *Acrodontium*. Scrutinized as a biological control agent of hypogeous insects, *B. bassiana* has been found around the world and in many habitats (Ferron 1981). The fungus regulates populations of coleopterous insects of the genera *Diabrotica, Colaspis,* and *Maecolaspis* in soybeans grown in Argentina and Brazil. In Brazil, the fungus achieves high prevalence in populations of *Aracanthus*, an important pest of beans (Sosa-Gomez et al. 1994), and in France, England, and Morocco it can be found on *Sitonia* weevils, a major pest of cultivated Fabacae (Poprawski et al. 1988).

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Isolates from the United States, Canada, and China have been collected and used in immunochemical characterization to identify a quality control program to ensure virulence of a strain (Tan & Ekramoddoullah 1990). Although most *B. bassiana* isolates are morphologically indistinguishable, they vary in virulence (Safavi et al. 2007). Biochemical characterization and large-scale production of these virulent isolates are important for successful biological control (Zhang & Tan 1987). In 2003 *B. bassiana* (strain GHA) produced by the MycoTech Corporation (USA, Mycotrol ES, Emerald BioAgriculture, Butte, MT, 59702) was in commercial production as a mycoinsecticide and this product was used in all of the experiments reported herein.

The *Diaprepes* root weevil (*Diaprepes abbreviatus* L.) (Coleoptera: Curculionidae) is a pest in Florida on citrus and other crops. It was first reported in the United States in a Florida nursery (Woodruff 1964); now the weevil infests more than 50,000 ha of the 300,000 ha of citrus in production (Duncan & McCoy 2001). The weevil larvae feed on roots of trees creating an entry for fungal and other plant pathogens. It has been shown that species of *Phytophthora* can gain ingress into citrus root systems where root integrity has been compromised by weevil damage (Rogers et al. 1996; Graham et al. 1997). Graham et al. (1997) found that in some soil types *Phytophthora palmivora* caused more damage to structural roots than the weevil larvae. Current control of the *Diaprepes* root weevil relies on timed applications of chemical pesticides, but these control measures have yet to suppress weevil populations (Bullock et al. 1988). With growing concerns in the use of chemical pesticides, costs, and deleterious effects to the environment, it is important that we explore the potential of enhancing biological control.

This study was initiated to examine the propensity of a formulated strain of *B. bassiana* to germinate on the exoskeleton of adult *D. abbreviatus*. It is known that *B. bassiana* will infect adult and larval *D. abbreviatus* in nature (Futch & McCoy 1992). However, fungal modes of ingress and germination rates on different areas of adult *Diaprepes* exoskeleton have yet to be established. Humidity and temperature are necessary factors for the germination of *B. bassiana* (Thomas & Blanford 2003); however, neither humidity nor water alone is enough to stimulate germination of conidia (Hunt et al. 1984). The insect exoskeleton is known to have chemical compounds that affect the germination of *B. bassiana* (Woods & Grula 1984). A lack of nutrients on sclerotized beetle cuticle can be a limiting factor in fungal growth and development, including conidial germination (Hunt et al. 1984). Amino acids and glucosamine have been found on the larval cuticle of *Heliothis zea*. These fluctuate in levels during larval development but are always sufficient to trigger the germination of *B. bassiana* (Woods & Grula 1984). Amines and peptides on the larval exoskeleton of *H. zea* do not inhibit the germination of *B. bassiana* (Woods & Grula 1984). Information gathered on the most vulnerable areas of the insect exoskeleton will be considered in the future production of a spore dissemination method for the use of *Beauveria* in an integrated pest management system to control adult population levels of *D. abbreviatus*.

Materials and Methods

Conidial Delivery

Cages (Sho-Bowl, 64 oz. tub, ribbed dome lid, Ultra Pac, Rogers, Mn. 55374) with pin holes in the lid for ventilation were prepared that contained ~100g of dry play sand. The sand and cages were sterilized in a UV hood for 1 h. Work surfaces, including the balance used, were cleaned with a 10% Clorox® solution before and after treatment to avoid contamination of control insects.

Diaprepes abbreviatus were reared at the Florida Department of Agriculture facility in Gainesville, Florida, by Beavers' (1982) methods for rearing on an artificial diet. A dry commercially produced wettable powder formulation of *B. bassiana* conidia produced by the MycoTech Corporation (strain GHA, Mycotrol ES, Emerald BioAgriculture, Butte, MT, 59702) was used as an inoculum. This strain was previously used by Furlong & Groden (2003) in their study of larvae of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). Citrus root weevils were separated by gender as described by Harari & Landolt (1997) and placed in batches into clean plastic sandwich bags containing 0.05 g of powder per insect. Bags with insects and powder were shaken for 30 s. This method of conidial delivery was similar to that used by Hedlund & Pass (1968) on the alfalfa weevil. Insects were transferred to holding cages, based on gender, with 2 organically grown baby carrots and a cotton ball saturated with sterilized water. Control groups were agitated in a clean plastic bag to simulate the possibility of mechanical damage that the insects might incur during treatment. Insects were maintained in an incubator with a 14 h:10 h light:dark cycle, a relative humidity of 85-90%, and a temperature range between 22°C and 24°C.

Modified Collodion Method

Thirty min after treatment, 5 insects of each gender were removed from the holding containers and the live insects were pinned through the right elytra with a number 2 nylon headed insect pin. Insects were dipped into a collodion solution (Collodion Flexible USP, Alcohol 22%, Ether 67%, Pyroxylin 4.75%, Camphor 2% and Castor Oil 3%, HUMCO, Texarkana, TX 75501), and allowed to stand at room temperature until the layer was so-

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lidified but not hardened (approximately 45 min) (Delp 1954; Kimbrough 1963). The solidified collodion film was removed from the insect cuticle with jeweler's forceps. This process removes loose and attached conidia and some insect cuticular components. Peels were made from the ventral abdominal sterna and related inter-segmental membranes, and from the dorsal elytra to the beak including the dorsal pronotum, eyes, and antennae.

Each peel was placed with the exoskeleton contact side facing up to reduce interference from the collodion when visualizing the peel with the compound light microscope. A drop of lactophenol cotton blue stain was placed on the peels of collodion on glass slides (Shurtleff & Averre 1997). After 5 min excess stain was wicked off of the peels with absorbent tissue. The slides were sealed with a few drops of Cytoseal™ 60 (Stephens Scientific, Division of Richard-Allan Scientific, Kalamazoo, MI 49007-3538) around the edges of a 50 × 24-mm cover glass. To ensure thin slides and remove air bubbles, slides were placed on a slide warmer for 6 h at 50°C with a 15-g lead weight on the cover glass. Slides were removed from the slide warmer and allowed to cool for 6 h before the weight was removed from the cover glass. Collodion dipping and the preparation of epidermal peels were repeated every 6 h with 5 fresh insects of each gender from the treatment cohort until 30 h past the initial inoculations. Conidial density, germination rate, and growth including the formation of germ tubes and points of fungal ingress were observed and photographed with a Nikon 990 digital camera attached to a light microscope. Dorsal and ventral photographs of weevils were labeled for rapid identification of areas.

Conidial Viability and Weevil Contact

A simple procedure was used to determine the number of viable conidia on each treated insect. Twenty insects were treated with 0.05 g of dry commercially produced wettable powder of *B. bassiana* per insect using the previously described plastic bag procedure. The weevils were then placed in a holding container for 3 h. The weevils were then removed from the holding container and placed individually in 100-mL glass vials. One milliliter of deionized water was added to the vial containing the weevil. The vial was shaken vigorously for 1 min. The water from each weevil rinse was poured onto potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI, 48232-7058) and the Petri dishes were sealed with Parafilm M Lab Wrapping Film and placed inverted in an incubator at room temperature for 48 h. Twenty control insects were treated with the powder and immediately washed as described above. After incubation, the plates were examined for signs of fungal growth. Colonies forming on the plates were counted on a grid system under a dissecting microscope at 40×. Data were analyzed with Excel (Microsoft Corp 1997).

Results

Spores on the surface of the weevil were not evenly distributed at 30 min post inoculation. Total spore density on weevil surfaces could not be determined with the collodion method. It could be determined, however, by washing spores from the surface of the weevils with distilled water and plating onto PDA. Spore density on the weevils' surface was determined to be 15,815 ± 479 spores initially after inoculation. Spore load on the surface of the weevils was reduced to 5,782 ± 136 spores after 3 h. During this 3 h time frame, weevil activities such as contact with inanimate objects and other weevils or self-grooming were observed in the holding cage. Thirty min post inoculation, conidia were found in clusters around ommatidia on the eyes, on setae, in depressions in the integument, on the ventral pronotum, and in the grooves of scales located along the ventral surfaces. At 6 h, weevils were checked for signs of spore enlargement or germination. No evidence was found to suggest spore growth at that time. At 12 h, peels were similar to those taken at 30 min and 6 h post inoculation. Spores were found on plumose setae, around the base of setae, and in clusters around ommatidia. Spores were abundant in pits of the pronotum and in the grooves between the ommatidia of both eyes. Debris from insect rearing medium collected on some of the peels could account for spores clustering in particular areas. Clusters of spores associated with rearing medium were not tabulated with the results.

Initially, spores measured 1.3 µm in length, and began to enlarge at 12 h to 2.9 µm. Measurements were taken for spore length not width. Germination observed at 18 h was 24 ± 3% on abdominal sections, 10 ± 3% on the pronotum and beak, 35 ± 3% on the eyes, and 9 ± 2% on scales on ventral abdominal sections (Fig. 1). Enlarged conidia were present on the elytra but had not begun to germinate at 18 h. Germination observed at 30 h

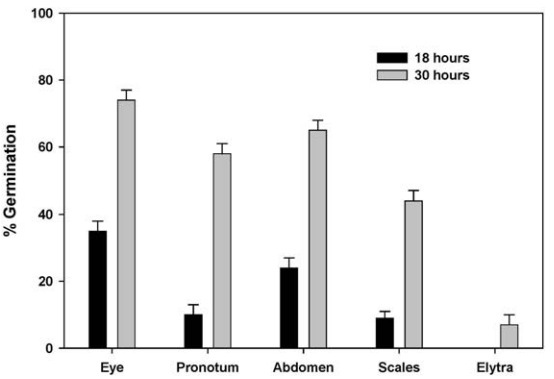
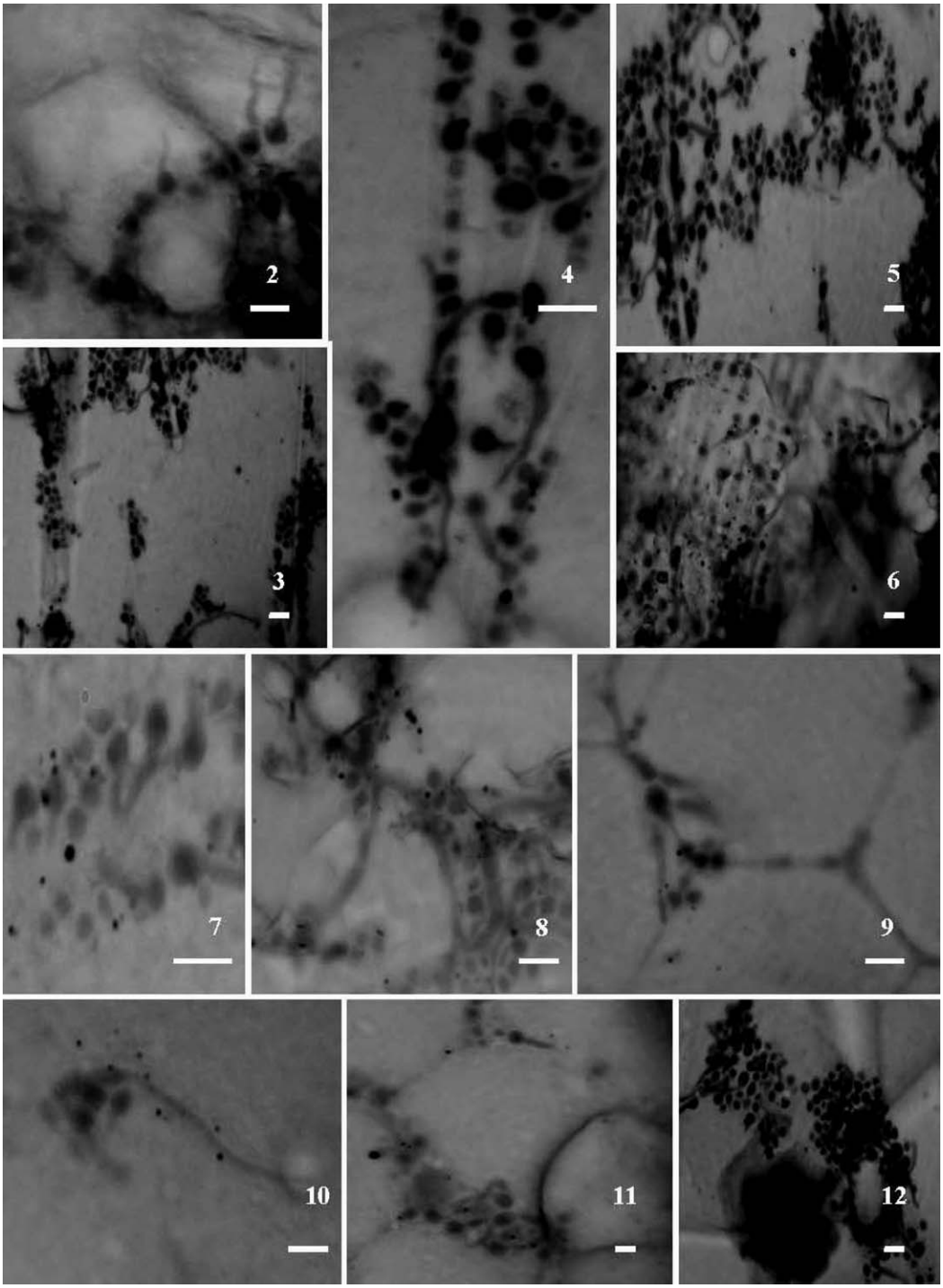


Fig. 1. Percentage of *Beauveria bassiana* conidia germinating on different areas of adult *Diaprepes abbreviatus* exoskeleton at 18 and 30 h post inoculation.

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Figs. 2-12. *Beauveria bassiana* conidia on adult *Diaprepes abbreviatus* exoskeleton 30 h post inoculation. 2. Conidia germinating on ovipositor. 3. Conidia germinating in cluster on edge of sterna. 4. Conidia germinating on edge of sterna. 5. Conidia germinating on ventral metathorax. 6. Conidia in clusters germinating among scales of sterna. 7. Conidia germinating on pronotum. 8. Conidia germinating around hair on sterna. 9. Conidia germinating on ommatidia of left eye. 10. Conidia germinating on beak near eye. 11. Conidia germinating on ommatidia of right eye. 12. Conidia germinating on edge of eye. Scale bars: 5 µm.

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was 58 ± 3% on the pronotum, 74 ± 3% on the eyes, 65 ± 3% on abdominal sections, and 44 ± 3% on scales on abdominal sections (Fig. 1). At 30 h post inoculation conidia were germinating on the ovipositor (Fig. 2), in clusters on the edge of sternal segments (Fig. 3-4), on the ventral metasternum (Fig. 5), and among scales of sterna (Fig. 6). At 30 h, germination was observed on the dorsal surface of both of the elytra at a rate of 7 ± 3%. Conidia were also found germinating on the dorsal surface of the pronotum (Fig. 7), around hairs on the sterna (Fig. 8), on the ommatidia of the left eye (Fig. 9), on the beak near the left eye (Fig. 10), on the ommatidia of the right eye (Fig. 11), and on the edge of the right eye (Fig. 12).

Discussion

Diaprepes weevils were infected with a dry, commercially produced wettable powder of *B. bassiana* conidia, strain GHA, a strain previously used in studies of Colorado potato beetle larvae by Furlong & Groden (2003). Conidial lengths of *B. bassiana* isolates tested by Sosa-Gomez et al. (1994) showed lengths varying among isolates from 0.95 to 3.41 µm. These dimensions agree with those measured in the present study at the time of inoculation. The development of *B. bassiana* on adults and larvae of *D. abbreviatus* is similar to other entomopathogenic deuteromycetes, including *Nomuraea* (Boucias & Pendland 1982).

Essentially nothing was known as to how spores of *B. bassiana* would attach to and aggregate on the surface of *Diaprepes* root weevil adults after inoculation. Our results demonstrated that spore concentrations on the weevils surface was reduced approximately 3-fold 3 h post inoculation. This reduction may be explained by weevil activity, contact, or self grooming observed during this time. This research showed that conidia tend to condense in intersegmental areas and around hairs and the base of appendages. Natural grooves in scales also filled with spores. There has been little definitive data as to the timing of spore germination once spores make contact with adult *D. abbreviatus* integument. We have shown that spores germinate at different rates on different areas of the weevils' exoskeleton. Several authors have studied why *B. bassiana* spores germinate when they come in contact with insect cuticle. Factors that may inhibit or enhance germination and penetration include cuticle density or compounds on insect integument (Woods & Grula 1984; Soza-Gomez et al. 1997). In addition, the lack of nutrients on sclerotized beetle cuticle is a limiting factor in fungal growth and development (Hunt et al. 1984). Results obtained from this study indicate that germination is limited on the heavily sclerotized elytra. By observing germination rates on various regions we could document (1) where the largest numbers of spores will germinate most readily on the *Diaprepes* exoskeleton, and (2) the viability of conidia in a dry commercially available wettable powder. Data generated in this phase of research will enable one to project the likely source of direct fungal entry and provide researchers with the information they need to produce an effective conidial delivery system for *Diaprepes* root weevil control.

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