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Pathogenicity of Microencapsulated Insecticide from *Beauveria bassiana* and *Metarhizium anisopliae* against Tobacco Budworm, *Heliothis virescens* (Fabricius)

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Abstract. Pathogenicity of microencapsulated spores of *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metsch) Sorokin at a dose of 1 x 10^8 spores per milliliter were used against tobacco budworm, *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae). Toxicity of non-microencapsulated spores at the same dose was also assessed. Bovine gelatin was used as a matrix to produce microcapsules by the spray-drying technique with an inlet temperature of 85°C and an outlet temperature of 33°C in the spray dryer process. Scanning electron microcopy was used to assist with measuring the microcapsule size and determining spore shape. Spore viability and moisture were also evaluated. Both fungal formulations were applied by immersing first-instar tobacco budworm larvae for 30 seconds. The number of larvae that died was recorded 48 hours post inoculation. Microcapsules were <20 µm. Spore viability was 16.0 ± 0.7% for *B. bassiana* and 13.2 ± 0.8% for *M. anisopliae*. Moisture contents of the fungi were 9.6 ± 3.9 and 9.3 ± 3.8%. Percentages of larvae killed were 33.3 ± 5.7 and 56.8 ± 5.8% for *B. bassiana* and *M. anisopliae*, respectively. The median lethal times were 60.0 ± 16.8 hours post inoculation for *M. anisopliae* and 85.9 ± 15.4 for *B. bassiana* non-microencapsulated spores, while the same microencapsulated fungi were 67.8 ± 15.3 and 72.0 ± 16.0 hours post inoculation. Spores of both fungi survived the spray-drying procedure (at 85 and 33°C) and were able to infect first-instar tobacco budworm larvae under laboratory conditions.

Resumen. Se evaluó la patogenicidad de esporas microencapsuladas de *B. bassiana* (Balsamo) Vuillemin y *Metarhizium anisopliae* (Metsch) Sorokin a una dosis de 1 x 10^8 esporas/ml sobre el gusano del fruto de tomate, *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae). La toxicidad de las esporas no microencapsuladas fue medida también a la misma dosis. Las microcápsulas fueron producidas empleando gelatina bovina como matriz, usando la técnica de secado por aspersión a 85 y 33°C como temperaturas de entrada y salida en el proces. Las microcápsulas fueron analizadas en un microscopio electrónico de barrido para medir el tamaño de la partícula. Se evaluó también la viabilidad de las esporas y su contenido de humedad. Para la prueba de patogenicidad de ambas

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formulaciones de hongos se aplicó la técnica de inmersión de larvas por 30 s usando larvas del primer ínstar de *H. virescens* y la mortalidad de larvas fue registrada 48 h después de la inoculación. Se obtuvieron microencapsulados de los hongos con un tamaño de partícula <20 µm, una viabilidad de esporas de 16.0 ± 0.7% para *B. bassiana* y 13.2 ± 0.8% para *M. anisopliae*, y un contenido de humedad de 9.6 ± 3.9 y 9.3 ± 3.8% para cada uno de estos hongos. Se registró una mortalidad de larvas de 33.3 ± 5.7% para *B. bassiana* y 56.8 ± 5.8% para *M. anisopliae* a las 48 h. El tiempo letal promedio para las esporas sin formular de *M. anisopliae* fue de 60.0 ± 16.8 y *B. bassiana* de 85.9 ± 15.4 (hpi), mientras que para las esporas microencapsuladas de estos mismos hongos fue de 67.8 ± 15.3 y 72.0 ± 16.0, respectivamente. Estos resultados demuestran que las esporas de estos hongos pueden sobrevivir al procedimiento secado por aspersión (a 85 y 33°C) conservando su capacidad para infectar larvas del primer ínstar de *H. virescens* en laboratorio.

Introduction

The tomato, *Solanum lycopersicum* L., is among the five most important agricultural products in Mexico. During 2013, 48,234 ha in Mexico produced 2,694,358 tons of tomatoes (SAGARPA 2014). Each growing season, the crop is attacked by several phytophagous insects, including tobacco budworm, *Heliothis virescens* (Fabricius). The larvae feed on leaves and fruit, and make cavities, that become infected by fungi or bacteria and cause fruit rot.

Insecticides such as diflubenzuron (Dimilín®), chlorpyrifos (Lorsban), emamectin benzoate (Proclaim®), methomyl (Lanate®), chlorfenapyr (Sunfire®), and methamidophos (Tamaron®) are used to control the pest (Ruiz et al. 2011). Injudicious use of insecticide negatively impacts the environment and human health in agricultural areas of Mexico (Leyva et al. 2014). Fewer organic than synthetic insecticides are used on tomato crops (INEGI 2007); therefore, use of bioinsecticide should be promoted. Microencapsulated insecticides based on entomopathogenic bacteria or fungi are alternatives to produce biological control products (Rosas and De Luna 2006, De Luna et al. 2011).

B. bassiana and *M. anisopliae* are two of the most promising fungal species for biological control potentially effective against a wide range of pests (Legaspi et al. 2000, Balanchander et al. 2012, Zibaee and Sendi 2013, Contreras et al. 2014). Effectiveness of fungi in the field can be affected by environmental factors, so microencapsulated formulations could protect active ingredients and increase spore viability (Winder et al. 2003). Microencapsulated formulations consist of a bioactive substance (mycelia and spores) encapsulated into a matrix or wall system (Poshadri and Kuna 2010). The matrix may include feeding stimulants, ultraviolet protectors, and adherents to protect the fungus from environmental conditions such as rain, wind, and ultraviolet radiation (Horaczek and Viernestein 2004, Adjallé et al. 2011, Masuda 2011). Jin and Custis (2011) reported that microencapsulation of *Trichoderma harzianum* (Rifai) using 2% sucrose, and inlet and outlet temperatures of 60 and 30°C, respectively, increased survival rate compared to that by non-microencapsulated spores. Muñoz-Celaya et al. (2012) used gum arabic and maltodextrin as matrices to increase the percentage of viability of the fungal spores during storage.

Other studies compared different drying techniques on longevity of entomopathogenic fungal spores. Horaczek and Viernestein (2004) found that 100°C as an inlet temperature reduced to 3 and 3.8% spore germination of *M.*

anisopliae and *B. brongniartii*, respectively. García-Gutiérrez et al. (2002) reported that 120°C as an inlet temperature was acceptable to obtain spores able to kill 44.2 to 72.2% in two independent bioassays against first-instar larvae of Mexican bean beetle, *Epilachna varivestis* (Mulsant).

In the present study, two microencapsulated formulations of *B. bassiana* and *M. anisopliae* were used. A biodegradable matrix and red dye as an ultraviolet light protector were used in the formulation. Viability of spores in the formulations was evaluated after a spray-drying process, as was pathogenicity on tobacco budworm larvae under laboratory conditions.

Materials and Methods

Pathogenicity of native Sinaloa strains of *B. bassiana* (Balsamo) Vuillemin code B9 and *M. anisopliae* (Metsch) Sorokin code M20 from soil samples were evaluated using honeycomb *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) to maintain the fungi on potato dextrose agar medium (Ruelas-Ayala et al. 2013). The strains were deposited in the collection of entomopathogenic fungi in the laboratory of bioinsecticides of CIIDIR Sinaloa-IPN.

Tobacco budworm first-instar larvae were placed individually into plastic containers with artificial diet (cubes 3 cm^3). The containers were kept at 25 ± 2°C and 50% relative humidity using a Bio-Rad humidifier. The adults were placed into paper bags with a piece of cotton soaked in 1% glucose solution, then into a 30-ml plastic container to feed during copulation and subsequent production of eggs from which to obtain neonate larvae.

Strains of *B. bassiana* B9 and *M. anisopliae* M20 were selected based on ability to growth on potato dextrose agar, and pathogenicity assessed through preliminary bioassays against the insect. Strains were propagated by fermentation using rice as a substrate (Acuña et al. 2015). Suspensions of spores were prepared by using an inoculation loupe to scrape the surface of solid cultures of 20-day-old fungi and resuspending them in 5 ml of 0.1% Tween 80.

A Neubauer chamber (Hausser Scientific) was used to determine spore concentration and the suspension adjusted to 2.3 x 10^8 spores per milliliter. Five milliliters of suspension inoculum of a spore was placed into high-density plastic bags with 300 g of rice and 200 ml of sterile distilled water at 28 ± 1°C for about 20 days until sporulation. The substrate was mixed every 4 days to increase contact with the spores. Viability was determined by counting the spores on a 1 mm^2 surface four times and recorded as number per milliliter (Acuña et al. 2015).

The spores were prepared in liquid containing 1.25 x 10^{11} per liter, 32.5 g of bovine gelatin, 25 ml of corn oil, and 5 g of red dye. The active ingredient (mycelia and spores) was obtained by homogenizing 200 g of rice with fungus in 1 liter of 0.1% Tween 80 solution, and filtered through sterile gauze. Each liquid formula was dried to a powder in the spray dryer. A Mini Spray Dryer Büchi B-290 (BÜCHI Labortechnik AG, Flawil, Switzerland) was used, and the operating conditions for drying air were: 85°C inlet temperature and 33°C outlet temperature in the dryer process and 3.34 ml per minute flow rate. Percentage of spore viability was determined at the end of the drying process. Moisture content was measured using the steaming method according to Mexican protocol NMX-F-083 (1986). A scanning electron microscope (Dual Beam FEI model Quanta FEG 3D) was used to determine the particle size of the microcapsules and compare the shape and structure of the fungal spores. A completely randomized design was used to

compare germination percentage between microencapsulated and non-microencapsulated spores. Spore viability data for each fungus were analyzed by the Student´s t test ($P < 0.05$).

Pathogenicity of *B. bassiana* and *M. anisopliae* strains were evaluated against first-instar tobacco budworm larvae. In the bioassays, the larvae were immersed for 30 seconds in 5 ml of spore suspension (1 x 10^8 spores per milliliters), and individually placed into 30-ml containers with artificial diet. The treatments were spores of *B. bassiana* and *M. anisopliae*, microencapsulated formulation of *B. bassiana* and *M. anisopliae,* and 0.1% Tween 80 as a check. Three replications for each treatment were used; each replication consisted of 10 larvae. The larvae were placed at 25 ± 2°C and 30-50% relative humidity, and total insect mortality was recorded after 7 days. A completely randomized design was used to compare pathogenicity between microencapsulated and non-microencapsulated spores. The numbers of insects killed by the fungi were analyzed by Student´s t test ($P < 0.05$).

Results and Discussion

As a result of the microencapsulation process, dry formulations in the form of a red powder were obtained. The particle size was less than 20 µm for both fungi, and the humidity was 9.3 ± 3.8 and 9.6 ± 3.9% for *M. anisopliae* and *B. bassiana* formulations, respectively. The average size of microcapsules by spray-drying was between 15 and 20 µm (Muñoz-Celaya 2012). Horaczeck and Viernstein (2004) found 5.1 to 5.7% moisture for *M. anisopliae* and *B. brongniartii* (Sacc.) Petch, respectively. Rosas-García et al. (2001) reported moisture of 3.8 to 10.9% in *B. bassiana* formulations.

After the microencapsulation process, spore germination was determined in both species. In *B. bassiana*, germination was 16.0 ± 0.7% whereas in *M. anisopliae* it was 13.2 ± 0.8% (Table 1). The fungi survived the process of spray drying. Fungal survival is attributed to the ability of the cell wall to retain water because of its hydrophobic properties. Exposure to 85/33°C (inlet and outlet) temperatures during spray drying caused a decrease from 88.7 and 87.7% to 13.2 and 16.0% for germination of *M. anisopliae* and *B. bassiana*. The drying process damaged spores of the two fungal species.

In a previous study by Rosas-García et al. (2001), germination of spores of *B. bassiana* decreased to 11.6% after the spray drying process. Horaczeck and Viernstein (2004) reported a decrease in germination percentage from 96.4 to 3% for spores of *B. brongniartii* and from 96.1 to 3.8% for *M. anisopliae* when exposed to 100°C inlet temperature.

Table 1. Viability of Spores of *B. bassiana* and *M. anisopliae* before and after a Spray-Drying Process
Cuadro 1. Viabilidad de Esporas de *B. bassiana* y *M. anisopliae* antes y después del Proceso de Secado por Aspersión

	Germination (%)	
Fungus	Initial	Final
Beauveria bassiana	87.7 ± 0.6 a	16.0 ± 0.7 b
Metarhizium anisopliae	88.7 ± 0.6 a	13.2 ± 0.8 a

Germination percentages were determined at 24 hours after incubation. The results represent the mean ± SD of three replications.

The decreasing spore viability was possibly caused by dehydration during the drying process because changes in the appearance and shape of the microencapsulated spores were observed by electron microscopy for *M. anisopliae* (Fig. 1). The *M. anisopliae* and *B. bassiana* spores maintained their pathogenicity after the drying process because 33.3 ± 5.7 and 56.7 ± 5.8%, respectively, of insects were killed. This demonstrates that spores were able to survive the drying process without complete loss of infective capacity. These results are in agreement with García-Gutiérrez et al. (2002) who reported that despite using an inlet temperature of 120°C, they obtained viable spores of *B. bassiana* after drying (296-444 spores germinated per square millimeter) which killed 44.2 to 72.2% in bioassays against first-instar larvae of the Mexican bean beetle.

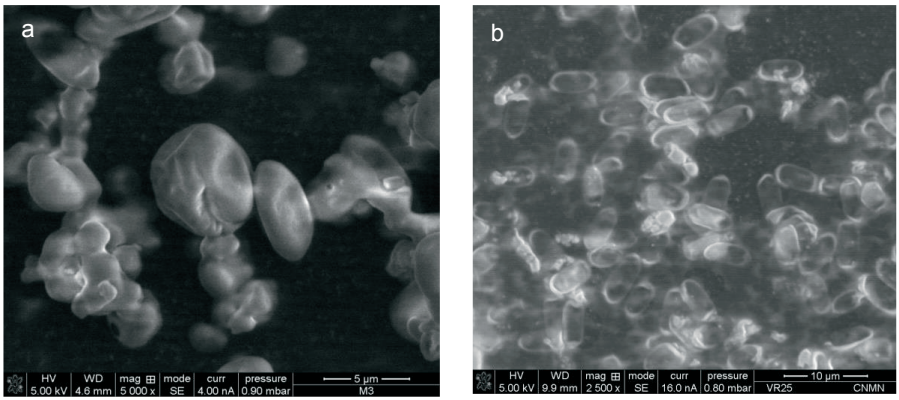

Fig. 1. a) *M. anisopliae* microencapsulated with bovine gelatin, UV protector, and corn oil, dried inlet/outlet temperatures of 85/33°C, in a Buchi Mini Spray Dryer B - 290. b) *M. anisopliae* spores with size of 7 µm.
Fig. 1. a) Microencapsulado de *M. anisopliae* elaborado con gelatina bovina, protector de luz UV y aceite de maíz, secadas a temperaturas de entrada/salida de 85/33°C con un BUCHI Mini Spray Dryer B-290. b) Esporas de *M. anisopliae* con tamaño de 7 µm.

The effect of entomopathogenic fungi on insects was recorded 48 hours after application. Fig. 2 shows an average lethal time for *M. anisopliae* spores of 60 ± 16.8 hours post inoculum and 85.9 ± 15.4 for *B. bassiana.* With microencapsulated formulations, average lethal times where longer, 67.8 ± 15.3 and 72.0 ± 16.0 for *M. anisopliae* and *B. bassiana*, respectively.

B. bassiana microencapsulated formulation killed fewer larvae (33.3 ± 5.7%) than non-microencapsulated spores (86.6 ± 5.7%) (Fig. 3). Microencapsulated spores of *M. anisopliae* killed 56.6 ± 5.7% compared to 86.6 ± 5.7% by non-microencapsulated spores. The microencapsulation process killed fewer larvae but the microencapsulated spores maintained an acceptable amount of effectiveness against the insect.

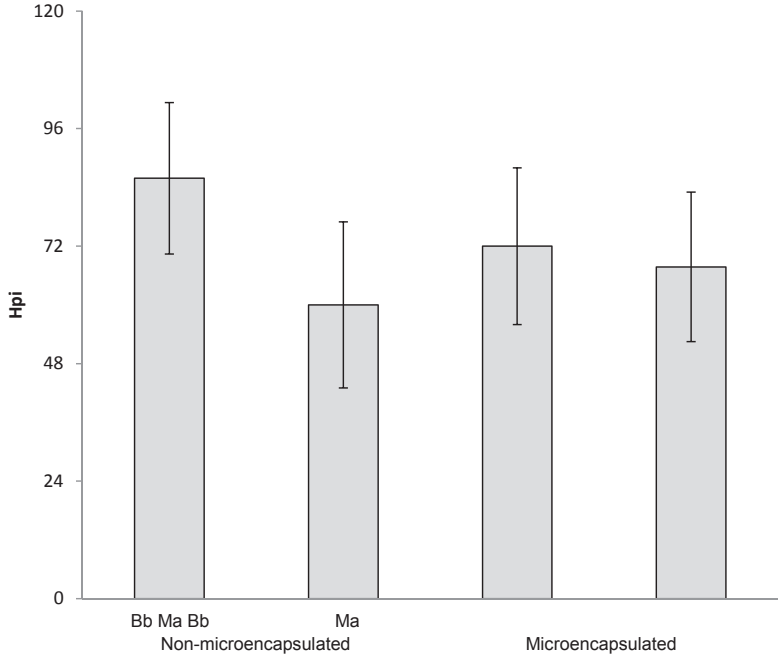

Fig. 2. Mean lethal time after inoculation of first-instar tobacco budworm larvae with non-microencapsulated and microencapsulated *B. Bassiana* and M. *anisopliae*.
Fig. 2. Tiempo letal medio de larvas después de la inoculación con *B. bassiana* y *M. anisopliae* microencapsulado y sin formular sobre larvas del primer instar de *H. virescens*.

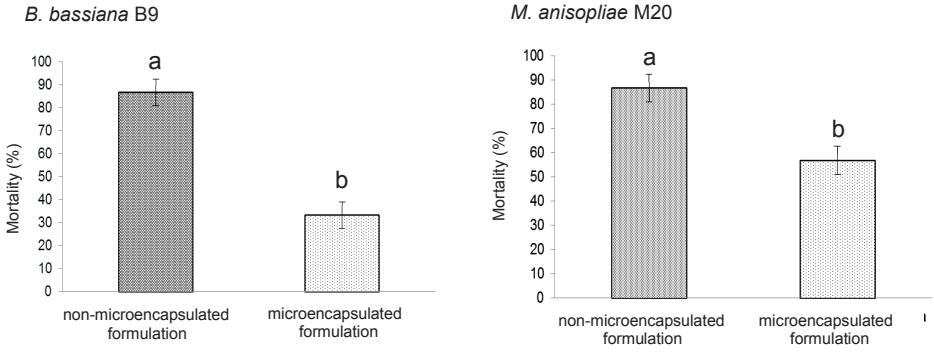

Fig. 3. Number of first-instar tobacco budworm larvae killed by native strains and microencapsulated *B. bassiana* and *M. anisopliae* ($p < 0.05$).
Fig. 3. Mortalidad de larvas del primer instar de *H. virescens* con *B. bassiana* y *M. anisopliae* sin formular y en formulaciones microencapsuladas ($p < 0.05$).

Conclusion

Microencapsulated formulations of *B. bassiana* and *M. anisopliae* had particle sizes less than 20 µm, spore viability of 13 to 16%, and moisture of 9.31 to 9.55%, respectively. The microcapsules of *B. bassiana* and *M. anisopliae* killed 33.3 and 56.6% of first-instar tobacco budworm larvae.

Although microencapsulates were less effective than non-treated spores, they were still able to kill an acceptable amount of insect larvae. This, along with the fact that spore viability was maintained after drying, make microencapsulates good candidates to be tested for control of tobacco budworm in the field.

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