Pathogenicity and Infection Events of a *Beauveria bassiana* Native Strain in Tobacco Budworm, *Heliothis virescens*

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Pathogenicity and Infection Events of a *Beauveria bassiana* Native Strain in Tobacco Budworm, *Heliothis virescens*[1]

Patogenicidad y Eventos de Infección de una Cepa Nativa de *Beauveria bassiana* en el Gusano Tabacalero, *Heliothis virescens*[1]

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Abstract. Pathogenicity of a native strain of *Beauveria bassiana* Vuill. (Ascomycota: Hypocreales) was evaluated on third-and fifth-instar larvae of tobacco budworm, *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae). Larvae were immersed in $1x10^8$ conidia ml^{-1}. After 72 hours, the percentage of each treated larval instar that died was 71 and 55%, respectively, while mortality of the nontreated check was 8 and 11%. The fungus was found across the cuticle and grew in the hemocoel 48 to 60 hours after inoculation of third-instar larvae and 60 to 72 hours for fifth instars. Fungal infection events also were studied in tobacco budworm larvae. The microscope and staining procedure showed mycelia grew as filamentous hyphae on the surface and penetrated the cuticle and spiracles; this phenomenon killed larvae. Inside the larvae, fungus grew as filamentous hyphae, but blastospores were not noticed. Results showed many young larval stages died and specific infection events in tobacco budworm compared to other data reported. Results suggested this native strain has potential as a bioinsecticide for tobacco budworm.

Resumen. Se evaluó la patogenicidad de un aislamiento nativo de *Beauveria bassiana* Vuill. (Ascomycota: Hypocreales) sobre larvas del tercero y quinto instar del gusano tabacalero, *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae). Las larvas fueron sumergidas en una solución de $1x10^8$ conidios ml^{-1}. Después de 72 horas la mortalidad de larvas tratadas de cada instar fue 71 y 55%, respectivamente, mientras que para las no tratadas o testigo fue de 8 y 11%. En el tercer instar larval, el hongo penetró la cutícula y creció en el homocele a las 48 y 60 horas después de la inoculación (hdi), mientras que en el quinto instar esto ocurrió a las 60 y 72 horas. También se llevó a cabo un estudio de los eventos de infección del hongo en larvas del gusano tabacalero. Las observaciones al microscopio y la técnica de tinción utilizada permitieron observar que el micelio creció como filamentos hifales sobre la superficie del insecto, penetrando a través de la cutícula y espiráculos; este fenómeno fue suficiente para causar la mortalidad

[1]Lepidoptera: Noctuidae

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de los insectos. Dentro de las larvas el hongo creció formando filamentos hifales; sin embargo, no se observaron blastosporas. Estos resultados demostraron una alta mortalidad de larvas jóvenes en corto tiempo y una forma específica de los eventos de infección del hongo en larvas de gusano tabacalero, en comparación con otros reportes. Estos resultados sugieren que este aislamiento nativo tiene potencial como bioinsecticida para el control del gusano tabacalero *H. virescens*.

Introduction

At Sinaloa, México, tobacco budworm, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), is a recurrent pest of tomato *Solanum lycopersicum* (L.), and this cause severely damages to garbanzo bean *Cicer arietinum* (L.) crops (Acuña-Jiménez et al. 2015a). *Beauveria bassiana* Vuill. (Ascomycota: Hypocreales) is one of the entomopathogenic fungi most used to control the pest in agriculture. However, effectiveness depends on pathogenicity of the strain (Pandey and Varshneya 2010).

In corn earworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), conidia from very pathogenic *B. bassiana* germinate on the epicuticle and penetrate the surface of the larva, except the head, within 18 hours. The fungus grows extensively as hyphae in the hemocoel, and larvae die with minimal damage to the internal tissues (Pekrul and Grula 1979). Growing tips of hyphae secrete lipases, proteases, and chitinases (Smith et al. 1981, Khan et al. 2012, Ruiz-Herrera 2012, de Carolina-Sanchez-Perez et al. 2014), enzymes used for host penetration and with activity positively correlated with virulence of the fungal strain (Lu et al. 2015). Conidia of *B. bassiana* injected into the hemocoel of corn earworm larvae germinated within 24 hours, with abundant free blastospores and short hyphal bodies in the hemocoel (Pekrul and Grula 1979). Alves et al. (2002) mentioned that *B. bassiana* penetrated the host with a germ tube and colonized it through a yeast phase, considered an obligatory parasitic phase for most entomogenous fungi, as is cited for *Metarhizium anisopliae* (Metschn.) Sorokin (Hajek and St. Leger 1994). Alves et al. (2002) demonstrated that *B. bassiana* grow as yeast-like cells in MacConkey's agar. Lohse et al. (2014) found that *B. bassiana* produced mainly yeast-like hyphal bodies (blastospores) and a few submerged conidiospores depending on the culture media in liquid shaker flasks. This form of growth could favor dispersion and colonization of the hemocoel, optimize nutrient acquisition, and help evade the immune system of an insect (Clarkson and Charnley 1996). Because the fungal genome is especially variable because of the nucleus movement along the hyphae through the incomplete septa (Webster and Weber 2007), there is considerable intraspecific capacity of entomogenous fungi to produce blastospores (Butt and Goettel 2000). However, representative species of Onigenales and Hypocreales (Ascomycota) proliferate as septate hyphae in contrast to many of entomophthoraleans that proliferate as yeast-like hyphal bodies in an insect body (Boomsma et al. 2014). Nevertheless, *B. bassiana* is considered a complex of strains (Hajek and St. Leger 1994, Rehner and Buckley 2003, Rehner et al. 2011) that could vary in infection depending on the fungal strain, target host, and environmental factors (Pandey and Varshneya 2010, Krull et al. 2013). Thus, pathogenicity and virulence of *B. bassiana* to insects depend on intrinsic characters of the fungus, host susceptibility, and environmental factors (Pekrul and Grula 1979, Hajek and St. Leger 1994, Charnley and Collins 2007, Motta-Delgado and Murcia-Ordóñez 2011).

In the Laboratory of Bioinsecticides at CIIDIR, Sinaloa, Mexico, native strains of entomopathogenous fungi are researched to control important agricultural pests. *B. bassiana* coded CIDSB03, with GenBank accession KR998505, was very pathogenic in Lepidoptera larvae (Acuña-Jiménez et al. 2015a). However, details of pathogenicity and development events of the strain in tobacco budworm are not known, so the aim was to study pathogenicity infection events to improve biocontrol action for pest management (Ortiz-Urquiza and Keyhani 2013, Lu et al. 2015).

Materials and Methods

Beauveria bassiana CIDSB03, GenBank accession KR998505, was grown on potato dextrose agar yeast. When white mycelia filled the Petri dish and showed dense yellowish sporulation after 15 days, conidia were harvested by scraping and suspended in 0.05% Tween 80 in distilled water (Butt and Goettel 2000). The conidia concentration was assessed by an improved Neubauer chamber (Bright-Line Catalog #3110, Hausser Scientific, Horsham, PA). Viability of conidia was assessed as a percentage of spore germination at 18-24 hours in potato dextrose agar, 24 hours before initiation of bioassays. The suspension was adjusted to 1 x 10^8 conidia ml^{-1} (Véjar-Cota and García-Gutiérrez 2014).

Tobacco budworm larvae were collected in an infested fruiting chickpeas (garbanzo bean) crop with no insecticide applied in an experimental field (25.547 N, 108.482 W) of CIIDIR Guasave, Sinaloa, Mexico on February 2014. Chickpea is one of the main hosts of the pest in the region (Blanco et al. 2009); larvae were found in damaged fruit in a tomato crop but not in an adjacent crop of maize, *Zea mays* L., during kernel development. All larvae and adults reared presented characteristics described by Capinera (2012) and Méndez-Barceló (2003) for tobacco budworm. The larvae were individually reared until pupation in 1 cm^3 artificial diet in 35-ml plastic PL1 containers (Acuña-Jiménez et al. 2015b). Pupae were put into an open Petri dish containing two layers of filter paper moistened with distilled water to maintain relative humidity more than 70% in an emergence chamber at room temperature (25 to 30°C). After emergence, 10 to 15 adults were placed into each of 3-liter paper bags containing a Petri dish lid with a cotton bed moistened with 5% honey in water for feeding. The adults laid eggs dispersed on the inside walls of the bag. Each bag was daily substituted for a new one, transferring the moths after anesthetizing by freezing for 2 minutes. Sections of bags with eggs were moistened with distilled water and put into capped plastic containers (2 liters) at room temperature to obtain larvae. Larvae were transferred with a 00 paintbrush to square, 2-liter plastic containers having a 0.5-cm-deep layer of diet formulated as described by Acuña-Jiménez et al. (2015b). A fork was used to make small furrows in the diet to shelter the larvae. F_1 larvae 0.5 cm long were placed individually into capped PL1 35-ml plastic containers having 1 cm^3 of diet. The larvae of third and fifth instars (10 and 25 mm) reared at relative humidity more than 70% and room temperature (25 to 30°C) were used in bioassays for pathogenicity (Méndez-Barceló 2003).

Tobacco budworm larvae were inoculated with a suspension of CIDSB03 *B. bassiana.* Before treatment, 10 lots of 24 larvae were disinfected by immersion for 30 seconds in a 0.5% sodium hypochlorite solution, washed with distilled sterilized water for 5 seconds three times. After 5-24-larva lots were inoculated by immersion for 30 seconds in a suspension of 1 x 10^8 conidia ml^{-1} (Véjar-Cota and García-Gutiérrez 2014, Acuña-Jiménez et al. 2015a), the remaining 5-24-larva lots were

not inoculated but used as a check and immersed in 0.05% Tween 80 in distilled water (Butt and Goettel 2000, Inglis et al. 2012) for 30 seconds. The check and inoculated larvae individually grew in a disposable 35-ml plastic pL1 container with a sterilized circle of filter paper moistened with 200 µl of sterilized distilled water. The dead larvae from each 24-larvae lots was counted one time at 24, 36, 48, 60, and 72 hours after inoculation. The experiment was repeated 5 days later with third-instar larvae.

The immersed and check larvae were fixed for 12 to 24 hours in Carnoy's B solution (Jensen 1962, Humason 1979, Barbosa et al. 2015). After fixation, the larvae were washed three times in ethyl alcohol and kept in the alcohol overnight. Tissue was embedded by two changes of ethyl alcohol, xilol, and paraffin for 2 hours each in a tissue processor (Thermo Scientific Microm STP 20 GmbH, Walldorf, Germany). Inclusion in paraffin was in a tissue includer (Thermo Scientific HistoStar, Runcorn, Cheshire, UK). Sagittal and transverse sections 6 µm thick were cut by using a Thermo Scientific HM340E microtome (International Gmbh, Walldorf, Germany). Tissue sections were prepared on clean slides containing a film of Haupt's solution (Jensen 1962). Three staining methods for fungi were used: 1) toluidine blue O, 2) Delafield's hematoxylin, and 3) safranin and Delafield's hematoxylin counterstaining. Staining with 0.05% toluidine blue O in phosphate buffer pH 5.5 (KH_2PO_4 9.08 gl^{-1}) (Ruzin 1999) was on paraffin-embedded tissue sections affixed to glass slides; the tissues were stained for 10 minutes, washed in tap water 30 seconds, air dried 5 minutes, deparaffinized by two changes for 2 minutes in xilol, air dried for 5 minutes, mounted in Entellan resin (Merk Damstadt, Germany), covered with a clean glass coverslip, air dried for 48 hours, and observed with the aid of a light microscope. Staining by Delafield's hematoxylin and safranin-Delafield's hematoxylin was done as described by Jensen (1962). Two light microscopes were used: Primo Star Carl Zeiss Micro Imaging Gmbh (Gottingen, Germany) with photomicrographs taken with a manual 16.1-megapixel camera (Sony Cyber-shot DSC-W650, China), and Leika DM 4000 B LED microscope (Germany) equipped with a Leika DFC 450C camera (Germany).

Results and Discussion

More third- than fifth-instar tobacco budworm larvae died after inoculation with $1x10^8$ conidia/ml^{-1} of *B. bassiana.* Third-instar larvae were found dead beginning 24 hours after inoculation. At 72 hours, 71 and 55% of fourth and fifth instars were dead, respectively. In each check, 8 and 11% of the same instars were also dead. After 72 hours, inoculated tobacco budworm had mycelia on the body, mainly on the dorsal area and last abdominal segment.

The process of infection of tobacco budworm larvae by fungi is shown in Fig. 1; conidia germination and cuticle penetration by hyphae began at 24 hours (1A); at 48 hours, mycelia were growing on the larval cuticle (1B), followed by extensive growth of hyphae on and in the larva and cuticle 60 hours after inoculation (1C). The time for fungi to reach the hemocoel apparently depended on cuticle thickness; in a thick ventral cuticle (1D), more than 72 hours were required, while in thinner cuticle as in the dorsal area (1E) and trachea (1F) *B. bassiana* was through the cuticle, hemolymph, and fat bodies. Most of the time, the hyphae grew in the hemocoel and on the side of the tracheal system, crossing through fat bodies and muscles, but no mycelia were found obstructing the tracheal lumen.

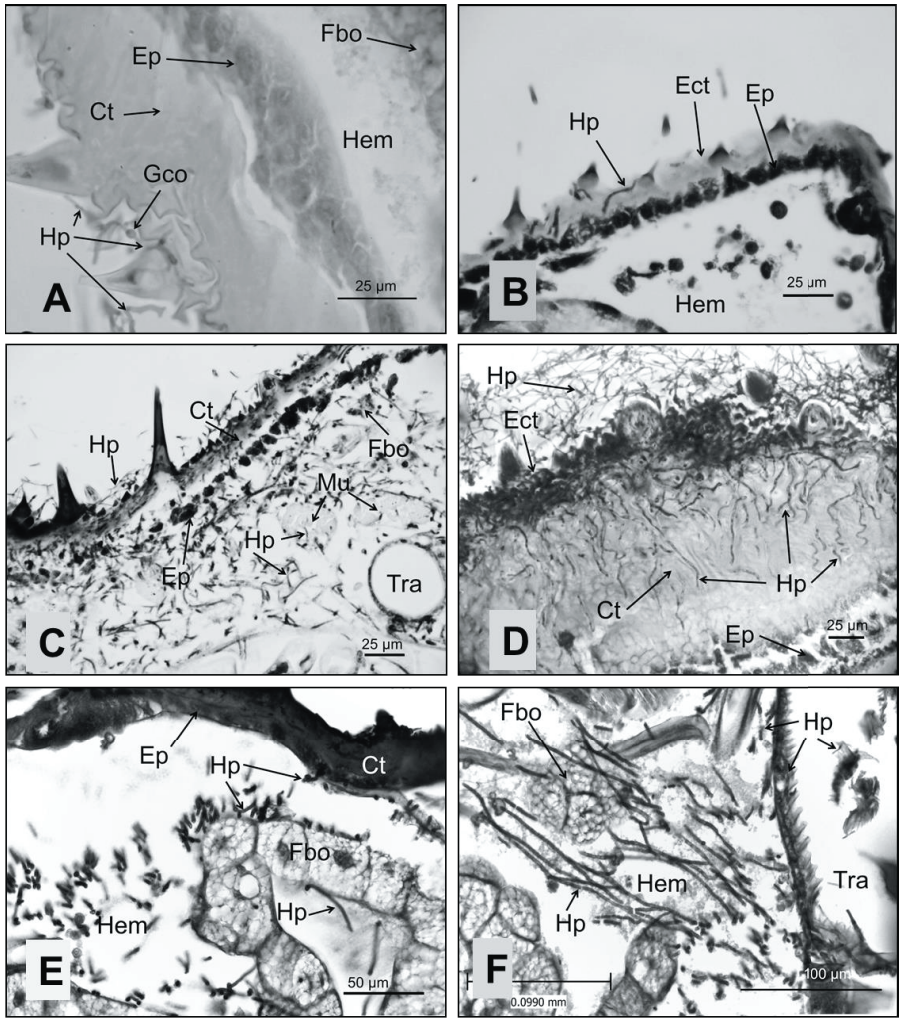

Fig. 1. Infection events by *Beauveria bassiana* in *Heliothis virescens* third instar (B, C), fifth instar (A, D, E, F), stained with safranin and Delafield's hematoxylin (A = 24 hai, D = 72hai, E = 72 hai), toluidine blue O (B = 48 hai, C = 60hai), and Delafield's hematoxilin (E, F = 72 hai). Ct = procuticle, Ect = epicuticle, Ep = epidermal cells, Fbo = fat bodies, Gco = germinating conidium, Hem = hemocoel, Hp = hyphae.

We noted that fungal growth was filamentous even when crossing the cuticle. Inside the larvae, the mycelia grew in the hemolymph and crossed the fat bodies and muscles as hyphae. The cut section showed hyphae longer than 100 µm growing from the trachea. We did not note blastospores in tissues within 72 hours

after inoculation. Alves et al. (2002) said *B. bassiana* colonizes insect hosts initially as a yeast phase, while formation of blastospores was mentioned in the model proposed by Hajek and St. Leger (1994) for *M. anisopliae*.

Fixation with Carnoy solution (Humason 1979) rendered larval tissues manageable after paraffin infiltration and embedding. It was possible to make microtome sections of 6 µm. Three staining methods: 1) toluidine blue O, 2) Delafield's hematoxylin, and 3) safranin and Delafield's hematoxylin, enabled finding fungal structures on and inside infected larvae. Toluidine blue O is a metachromatic staining method that differentiates cuticle layers and internal organs in larvae; epicuticle is stained yellow to brown, cuticle blue, and epidermal cell layers purple, as do the fungal hyphae; it is a fast and easy staining method for infected larvae. Delafield's hematoxylin stains the larvae and fungal structure blue. It allows observation of filamentous fungi in an infected larva. Safranin Delafield's hematoxylin gives good contrast of the fungi in infected larvae; it stains the cuticle pink, epicuticle brown, hyphae purple, and epidermal cells purple-blue. Each staining method indicated fungi in the infected larvae.

B. bassiana strain CIDSB03 had 100% conidia viability completely germinating on potato dextrose agar before 18 hours. This strain at concentration of 1 x 10^8 conidia ml^{-1} was very pathogenic to third- and fifth-instar tobacco budworms. At 24 hours after inoculation, third-instar larvae began to die, while fifth instars began dying 36 hours after inoculation. Seventy-one and 55% of third and fifth instars were dead at 72 hours after inoculation. Third- and fifth-instar larvae began to die 36 and 48 hours after inoculation. Seventy-two hours after treatment, 11 and 8% of third and fifth instars had died, respectively (Inglis et al. 2012). At 72 hours, we noticed check larvae were still mobile, while treated larvae responded only to probing. Sluggishness has been linked with cessation of feeding and loss of larval weight (Cheung and Grula 1982). Growers with tobacco budworms in their fields often do not recognize signs of sickness, and it is difficult for pest control advisors to convey the fact that infected larvae will not continue damaging crops. Pekrul and Grula (1979) inoculated corn earworm larvae with mutant strains of *B. bassiana* considered very pathogenic. The conidia germinated in 18 hours and immediately penetrated the larval cuticle, while less- or non-pathogenic strains delayed cuticle penetration and errantly grew over the cuticle of the larvae. Larvae inoculated with CIDSB03 had mycelia visible mainly in the dorsal area and last abdominal segment 60 hours after inoculation, but when observed with the aid of a microscope, mycelia were growing on the surface, mainly in the cuticle folds and intersegmental areas of the larvae.

Fig. 1 shows that in third and fifth instars, infection began by 24 hours post inoculation, because conidia germinated and began cuticle penetration. The infection proceeded in a similar way in the two larval stages but was faster in the third instar. In the third instar, the hyphae invaded the cuticle in 48 hours and grew in the hemocoel by 60 hours after inoculation, while those of the fifth instar needed 72 or more hours to invade the hemocoel, apparently depending on cuticle thickness, as observed in Figs. 1C and D. The fungus probably entered the larva through the spiracle but did not clog the tracheae; instead it grew to the hemocoel side as seen in Fig. 1F. In our research we saw hyphae filling only the larval tissues and hemocoel but did not see blastospores or hyphal bodies. At least in the fifth-instar larvae, the hyphae did not reach the gut before invading and collapsing peripheral muscles and fat bodies. It is possible that damaged muscles impaired larval movement and the damaged fat bodies could not coordinate general

metabolic functions and activity (Chapman et al. 2013). Strain CIDSB03 was very effective against a serious pest of tomato and garbanzo bean at Sinaloa. The development of commercial formulations of the strain might benefit local growers by controlling tobacco budworm and other species of Lepidoptera. Entomopathogenic fungi in integrated pest management are scarcely used at Sinaloa; there is limited use mainly in tomato production in the Culiacan Valley. The biggest obstacles for growers to use the CIDSB03 strain are 1) lack of availability in the market of the strain and others we have isolated and characterized in our laboratory, 2) speed at which the organisms act, as compared with synthetic insecticide, and 3) growers accustomed to seeing results within hours. Pest control operators at Sinaloa are aware of slow-acting insecticides such as insect growth regulators. Therefore, we believe the fast infection action of CIDSB03 strain (≤48 hours) would not be an obstacle to adoption in cropping conditions at Sinaloa.

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