Evaluation of the Pathogenicity of Isolates of *Beauveria bassiana* Against *Rhyssomatus nigerrimus*

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Evaluation of the Pathogenicity of Isolates of *Beauveria bassiana*\(^1\) against *Rhyssomatus nigerrimus*\(^2\)

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Abstract. The soybean weevil, *Rhyssomatus nigerrimus* Fahraeus, seriously damages vegetative and reproductive stages of soybean, *Glycine max* (L.) Merr., in Mexico. The weevil is currently controlled with conventional insecticide, and it is necessary to generate control measures to reduce the negative impact of this new pest. The fungus *Beauveria bassiana* (Balsamo) Vuillemin has shown potential for management of some coleopteran pest species. The objective of this study was to assess the virulence of eight strains of *B. bassiana* against *R. nigerrimus* under laboratory conditions. Results showed that *B. bassiana* is a microbial agent that can potentially be incorporated into a program to control *R. nigerrimus*. All of the *B. bassiana* strains reduced survival of adult weevils, relative to the check. The most aggressive strains were Bb-Hy, Bb-Rhy, and Bb-13, with LC\(_{50}\) of \(1.07 \times 10^7\), \(1.55 \times 10^7\), and \(1.31 \times 10^{10}\) conidia per milliliter of suspension, respectively. Lethal time means (LT\(_{50}\)) were between 7.5 and 14.5 days. We discuss implications of the results obtained with the evaluated strains.

Resumen. El picudo de la soya, *Rhyssomatus nigerrimus* Fahraeus, es una plaga que daña seriamente el estado vegetativo y reproductivo de cultivos de soya en México. Actualmente, el picudo se controla por medio de insecticidas convencionales, por lo que resulta necesario generar medidas de control eficaces y sustentables que reduzcan el impacto negativo de esta nueva plaga. El hongo, *Beauveria bassiana* (Balsamo) Vuillemin, ha mostrado un gran potencial de manejo para algunas especies de insectos plaga del orden Coleoptera. El objetivo de este trabajo fue evaluar la virulencia de ocho cepas del hongo *B. bassiana* contra el picudo de la soya, *R. nigerrimus*, en condiciones de laboratorio. Los resultados muestran que *B. bassiana* es un agente potencial microbiano para incorporar en un programa de control dirigido contra *R. nigerrimus*. Todas las cepas *B. bassiana* redujeron la sobrevivencia de los adultos del picudo con respecto al control. Las cepas más agresivas fueron Bb-Hy, Bb-Rhy, Bb-13, con LC\(_{50}\) de \(1.07 \times 10^7\), \(1.55 \times 10^7\), y \(1.31 \times 10^{10}\) conidios por mL de suspensión, respectivamente. Las medias del tiempo letal (TL\(_{50}\)) tuvieron valores entre 7.5 y 14.5 días. Se discuten las implicaciones de los resultados obtenidos con las cepas evaluadas.

\(^{1}\)(Hypocreales: Clavicipitaceae)  
\(^{2}\)(Coleoptera: Curculionidae)  
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Introduction

The soybean weevil, *Rhyssomatus nigerrimus* Fahraeus (Coleoptera: Curculionidae), causes considerable damage to soybean [*Glycine max (L.) Merr.*] crops in Mexico (Terán-Vargas et al. 2011 López-Guillén et al. 2012). Adult weevils feed on vegetative and reproductive parts of the soybean plant. The larva, however, causes the most damage because it feeds on and develops inside a pod, reducing soybean yield and quality (López-Guillén et al. 2012, Terán-Vargas and López-Guillén 2014). The weevil is geographically distributed in the largest soybean-growing regions in the states of Chiapas, Tamaulipas, San Luis Potosí, and Veracruz in northern and southern Mexico, and has potential to disperse into other states (Terán-Vargas et al. 2011, Terán-Vargas and López-Guillén 2014).

Systemic, contact, and ingested insecticides have been used to control the *Rhyssomatus* soybean weevil. Cazado et al. (2014) proposed the use of seed and foliar insecticides to control *R. subtilis* Fiedler in Argentina. *R. nigerrimus* in Mexico is controlled by foliar insecticide and seed treatments (Terán-Vargas 2013, Terán-Vargas and López-Guillén 2014). Chemical pest control in soybeans usually has not been satisfactory for sustainable production because of high cost, risk of resistance to insecticide, and adverse effects on beneficial organisms and on the environment (Zunino et al. 2012, Panizi 2013). A similar panorama can be expected with insecticides used to control *R. nigerrimus*. An alternative to chemical control is use of microbial agents of biological control, particularly entomopathogenic fungi. This alternative is increasing in demand for sustainable production with low or no risk to human or environmental health (Zimmermann 2007, Roy et al. 2010, Erler and Ozgur Ates 2015).

Study of microbial agents of biological control is essential to understand their potential to control a pest (Humber 1992, Lopes et al. 2012). *Beauveria bassiana* (Balsamo) Vuillemin is an anamorphic entomopathogenic cosmopolitan fungus that attacks and causes acute mycosis to several arthropods. It is virulent with a broad range of hosts and is easy to reproduce massively, store, and apply (Hajek and St Leger 1994, Li et al. 2014). *B. bassiana* has been assessed and the results have been encouraging for microbial control of certain coleopteran pests of economic importance (Neves and Hirose 2005, Güerri-Agulló et al. 2011, Reay et al. 2012). Adult soybean weevils infected by *B. bassiana* have been registered in soybean crops in Chiapas (López-Guillén, unpublished observation). However, there is no information on the virulence of native and other strains of *B. bassiana* against soybean weevil adults. Determining virulence in a laboratory is necessary before recommending use to control soybean weevil in the field. The objective of this study was to determine virulence against soybean weevil adults by *B. bassiana* strains isolated from different insect hosts from different geographical origin.

Materials and Methods

Adult soybean weevils were collected in field cages (40 x 100 x 100 cm) in soybean crops at Tapachula, Chiapas, Mexico (14° 44’ 53” N; 92° 21’ 35” W). Adults were reared in the field by releasing pairs of weevils into cages containing healthy stage-R4 soybean plants to assure sufficient offspring with similar ages the following crop cycle. Infestation with adult weevils in field cages with soybean plants was done in October 2013. Offspring from emerged adult weevils in field
cages were collected in August 2014 and placed into 1-liter plastic containers previously sterilized with sodium hypochlorite (0.5% v/v) and covered with organdy fabric. In a laboratory, the weevils were fed soybean pods sterilized with sodium hypochlorite and placed individually into a container; pods were replaced daily. The weevils were kept at 27 ± 1°C, 70 ± 5% relative humidity, and a photoperiod of 12:12 light:dark hours.

Conidia of eight strains of the entomopathogenic *B. bassiana* fungus from different geographical regions were assessed by pathogenicity tests against soybean weevil adults. The strains were isolated from insects of the orders Coleoptera and Lepidoptera and a commercial product manufactured by the Ukrainian Institute for Plant Protection, Kiev, Ukraine. All strains had been stored in the collection of entomopathogenic fungi in the insect pathology laboratory of El Colegio de la Frontera Sur (ECOSUR) at Tapachula, Chiapas, Mexico (Table 1).

Table 1. *Beauveria bassiana* Fungus Strains Evaluated against *Rhyssomatus nigerrimus*, Original Insect Host, Geographical Origin, and Strain Code Followed by Identification Number in the Collection of Entomopathogenic Fungi at ECOSUR

<table>
<thead>
<tr>
<th>Strain code</th>
<th>Original insect host</th>
<th>Geographical origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bb-4</td>
<td>Hypotenemus hampei Ferrari (Coleoptera: Curculionidae)</td>
<td>Ecuador</td>
</tr>
<tr>
<td>Bb-8</td>
<td>Leptinotarsa sp. (Coleoptera: Chrysomelidae)</td>
<td>Texcoco, Edo. de México, México</td>
</tr>
<tr>
<td>Bb-13</td>
<td>Commercial formulation (Boverin)</td>
<td>Kiev, Ukraine</td>
</tr>
<tr>
<td>Bb-15</td>
<td>Chalcodermus aeneus (Boheman) (Coleoptera: Curculionidae)</td>
<td>Goias, Brazil</td>
</tr>
<tr>
<td>Bb-18</td>
<td>Lepidopteran larva</td>
<td>Papantla, Veracruz, México</td>
</tr>
<tr>
<td>Bb-19</td>
<td>Lepidopteran larva</td>
<td>Gregorio Méndez, Tabasco, México</td>
</tr>
<tr>
<td>Bb-Rhy</td>
<td>Rhyssomatus nigerrimus</td>
<td>Tapachula, Chiapas, México</td>
</tr>
<tr>
<td>Bb-Hy</td>
<td>Hypothenenemus hampei</td>
<td>Finca Alianza, Municipio de Cacahuatán, Chiapas, México</td>
</tr>
</tbody>
</table>

Conidia of the strains were transferred to tilted test tubes (2 cm diameter x 20 cm long) containing Sabouraud dextrose agar solid culture media (40 g dextrose, 10 g pepsin, 15 g agar, 10 g yeast extract, and 40 mg tetracycline), and 1,000 ml of sterile distilled water was added. The medium was transferred immediately to sterile test tubes (22 x 175 mm) inoculated with conidia by streaking in a laminar flow cabinet. The inoculated media were incubated at 27 ± 2°C, 80 ± 5% relative humidity, and a photoperiod of 12:12 light:dark hours to induce growth and sporulation of fungus. Conidia produced after a period of 20 days were harvested by scraping the media in the test tubes with a sterile bacteriological scraper and were stored at 4°C in sterile 50-ml precipitation flasks covered with aluminum foil.

Conidium viability was verified before the bioassays by using the method of microcultures proposed by Jiménez (1989). The microcultures were prepared by placing a thin layer of Sabouraud dextrose agar on the surface of a slide and...
inoculating it with fungus. The inoculum was used to prepare suspensions of 0.3 mg conidia in 5 ml of sterile distilled water plus Tween 80® (0.01% v/v) in 10-ml sterile precipitation flasks covered with aluminum foil. The suspension was homogenized with a stirrer/hot plate (PC-320, Corning, NY) for 30 minutes. From the resulting suspension, 0.3 ml (1 x 10^6 conidia per milliliter) was taken with a sterile pipette to inoculate the Sabouraud dextrose agar. The inoculated slides were placed in on moist filter paper in Petri dishes (9 cm diameter) and incubated for 36 hours at 27 ± 2°C and 80 ± 5% relative humidity. The microcultures were observed with the aid of a compound microscope, and viability was determined by the presence of 100-200 conidia per slide. A spore was considered viable when growth of the germination tube was observed.

Conidia produced during a period of 20 days in the test tubes containing Sabouraud dextrose agar were scraped off and used to prepare suspensions in sterile distilled water and Tween 80® (0.01% v/v). The suspensions were homogenized by shaking for 30 minutes. The concentration was adjusted to 1 x 10^8 conidia per milliliter by using a Neubauer chamber to assure infection and disease symptoms. A group of soybean weevil adults was submerged in fungal suspension for 20 seconds, while a check group was immersed in only sterile distilled water with Tween 80®. All weevils treated or not treated with B. bassiana were transferred to 1-liter containers with food as described previously. The containers with insects were stored at 27 ± 2°C, 70 ± 5% relative humidity, and a photoperiod of 12:12 light:dark hours. The containers with weevils were checked daily to record the number of dead weevils. The dead insects were sterilized superficially with a solution of sodium hypochlorite (1% v/v) for 1 minute and washed once in ethanol (70% v/v) and three times in sterile distilled water for 10 seconds. Each dead processed insect was placed on a moist filter paper disk in a sterile Petri dish to determine the cause of death. The insects placed into a moist chamber were incubated by using the environmental conditions described previously. The percentage that died in 20 days was used to determine pathogenicity, while daily observations were used to determine LT_{50}. Groups of 50 soybean weevil adults were submerged in suspensions of each strain or the check solution.

The three strains that killed the most and had the shortest LT_{50} were used in the bioassays. Fungal suspensions were prepared at concentrations of 1 x 10^9, 1 x 10^8, 1 x 10^7, 1 x 10^6, 1 x 10^5, and 1 x 10^4 conidia per milliliter in sterile distilled water and Tween 80®. A group of soybean weevil adults was submerged in a fungal suspension for 20 seconds, while the check group was submerged in sterile distilled water with Tween 80®. Fifty soybean weevil adults of each treatment and the check were kept under the controlled conditions described previously. Adults that died were recorded once each 24 hours for 20 days and placed into moist chambers to incubate for fungus under the conditions described previously.

Adult soybean weevil survival was analyzed with the Cox proportional model (Zwiener et al. 2011). Treatments were compared using orthogonal contrast with a 5% level of significance, which was adjusted with the Bonferroni test. LT_{50} of each of the evaluated treatments was also estimated with the Cox proportional model, with their respective 95% fiducial limits. The data corresponding to mortality-dosage were analyzed with a probit model, with which LC_{50} was determined at 95% fiducial limits. LT_{50} and LC_{50} were compared by means of fiducial limits, which were considered significantly different when they did not overlap. The data were analyzed with R software (R Development Core Team 2009).
Results

Few weevils in the check group died, and growth of mycelia was not observed in the dead check insects. The Cox proportional model revealed significant differences between the survival curves of the eight B. bassiana strains and that of the check ($\chi^2 = 44.87$, df = 8, $P \leq 0.05$) after immersion. The Bonferroni fit for the test of multiple comparisons showed significant differences between the survival curves of some pairs ($P < 0.001$). Survival of soybean weevils was significantly different between strains Bb-13 and Bb-15 ($Z = 3.95$, df = 1, $P < 0.001$), Bb-13 and Bb-18 ($Z = 4.03$, df = 1, $P < 0.001$), Bb-13 and Bb-4 ($Z = 4.79$, df = 1, $P < 0.001$), Bb-13 and Bb-8 ($Z = 4.70$, df = 1, $P < 0.001$), Bb-4 and Bb-Hy ($Z = -3.92$, df = 1, $P < 0.001$), and Bb-8 and Bb-Hy ($Z = -3.77$, df = 1, $P < 0.001$). There were no significant differences between the other pairs shown by the survival curves of the B. bassiana strains (Fig. 1). Percentages of adult soybean weevils that died from

![Figure 1](image.png)

**Fig. 1.** Survival of Rhyssomatus nigerrimus adults after immersion in suspensions of Beauveria bassiana strains at a concentration of $1 \times 10^8$ conidia per milliliter. The survival curves were compared with the Cox proportional model with orthogonal contrasts for the function of survival.

**Fig. 1.** Sobrevivencia de adultos de Rhyssomatis nigerrimus después de la inmersión en cepas de Beauveria bassiana a concentración de $1 \times 10^8$ conidios/ml. La comparación de las curvas de sobrevivencia fue llevada a cabo aplicando el modelo de proporcional Cox con contrastes ortogonales para la función de sobrevivencia.
the eight strains of \textit{B. bassiana} were: 96 Bb-Hy, 94 Bb-15, 92 Bb-13, 90 Bb-19, 86 Bb-Rhy, 78 Bb-18, 76 Bb-8, and 76% Bb-4.

Analysis of survival showed that eight of the \textit{B. bassiana} strains killed more than 50% of the soybean weevil adults 20 days after immersion (Table 2). Significant differences in LT\textsubscript{50} were found among the eight strains, according to the confidence interval of each strain. The shortest LT\textsubscript{50} was observed with strains Bb-Rhy, Bb-Hy, Bb-13, and Bb-19, while Bb-15 and Bb-18 were intermediate. The significantly longest LT\textsubscript{50} values were observed with strains Bb-4 and Bb-8.

Table 3 shows that LC\textsubscript{50} 20 days after immersion of soybean weevil adults did not differ significantly among the three \textit{B. bassiana} strains compared by the interval of each confidence limit; the three strains killed more than 50% of the weevils. The estimated LC\textsubscript{50} values for Bb-Hy, Bb-13, and Bb-Rhy were \(1.07 \times 10^7\), \(1.55 \times 10^7\), and \(1.31 \times 10^{10}\) conidia per milliliter, respectively.

### Table 2. Lethal Time (LT\textsubscript{50}) with 95% Confidence Limit for Each \textit{Beauveria bassiana} Strain at Concentration of \(1 \times 10^8\) Conidia per Milliliter Tested against Adults \textit{Rhyssomatus nigerrimus}

<table>
<thead>
<tr>
<th>Strain</th>
<th>LT\textsubscript{50} (days) + SE</th>
<th>Fiducial limit (95%) (conidia/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower limit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Upper limit</td>
</tr>
<tr>
<td>Bb-Rhy</td>
<td>8.5 ± 1.18ab</td>
<td>6.2</td>
</tr>
<tr>
<td>Bb-Hy</td>
<td>8.5 ± 0.79ab</td>
<td>7.0</td>
</tr>
<tr>
<td>Bb-13</td>
<td>7.5 ± 0.64a</td>
<td>6.3</td>
</tr>
<tr>
<td>Bb-15</td>
<td>11.5 ± 1.18abc</td>
<td>9.2</td>
</tr>
<tr>
<td>Bb-18</td>
<td>12.5 ± 1.51abc</td>
<td>9.5</td>
</tr>
<tr>
<td>Bb-19</td>
<td>9.5 ± 0.71ab</td>
<td>8.0</td>
</tr>
<tr>
<td>Bb-4</td>
<td>14.5 ± 1.75cd</td>
<td>11.1</td>
</tr>
<tr>
<td>Bb-8</td>
<td>14.5 ± 1.00cd</td>
<td>12.5</td>
</tr>
</tbody>
</table>

### Table 3. Estimation of Lethal Concentration (LC\textsubscript{50}) with Confidence Limit of 95% for Three \textit{Beauveria bassiana} Strains Evaluated against \textit{Rhyssomatus nigerrimus} Adults

<table>
<thead>
<tr>
<th>Strain</th>
<th>LC\textsubscript{50} (conidia/ml)</th>
<th>Fiducial limits (95%) (conidia/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lower limit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Upper limit</td>
</tr>
<tr>
<td>Bb-Hy</td>
<td>(1.1 \times 10^7)\textsubscript{a}</td>
<td>5.9 \times 10^5</td>
</tr>
<tr>
<td>Bb-13</td>
<td>(1.6 \times 10^7)\textsubscript{a}</td>
<td>8.3 \times 10^5</td>
</tr>
<tr>
<td>Bb-Rhy</td>
<td>(1.3 \times 10^{10})\textsubscript{a}</td>
<td>2.6 \times 10^7</td>
</tr>
</tbody>
</table>

### Discussion

This is the first study of the potential of eight \textit{B. bassiana} strains as bioinsecticides to control soybean weevil adults. Results indicated that all strains
assessed were virulent; they killed more than 50% of the adult soybean weevils. Trudel et al. (2007) and El-sufty et al. (2009) reported similar results; they found that \textit{B. bassiana} killed more than 50% of \textit{Pissodes strobi} (Peck) (Coleoptera: Curculionidae) and \textit{Rhynchophorus ferrugineus} (Olivier) (Coleoptera: Curculionidae), respectively. We also found strains Bb-Hy, Bb-Rhy, Bb-4, Bb-13, Bb-15, Bb-18, and Bb-19 most virulent, killing 76 to 96% of soybean weevils. De la Rosa et al. (1997) reported that the same \textit{B. bassiana} strains killed 79 to 91% of \textit{Hypothenemus hampei} (Ferrari) (Coleoptera: Curculionidae) adults, but the strains tested against \textit{H. hampei} and \textit{R. nigerrimus} had different LT$_{50}$: with \textit{H. hampei}, LT$_{50}$ was 7 or fewer days, while with \textit{R. nigerrimus} it was longer than 7.5 days. Variation in LT$_{50}$ of the same strains in both insect species probably was because of production and activity of hydrolytic and proteolytic enzymes such as protease, lipase, urease, and chitinase. The fungus uses enzymes to penetrate the cuticle of the host insect (Zimmermann 2007, Zhang et al. 2008, Zibaee and Bandani 2009).

In general, \textit{B. bassiana} strains were more virulent in the host from which they were isolated or in species in the same order as the original host (Bugeme et al. 2008). Our results showed that strains Bb-Rhy, Bb-Hy, and Bb-15, isolated from adult soybean weevils and species of the order Coleoptera, respectively, were most virulent, killing adult soybean weevils in a shorter LT$_{50}$. Ricaño et al. (2013) reported similar results with \textit{B. bassiana} strains 203 and 193 isolated from \textit{R. ferrugineus} and strains 53 and 19 from coleopteran insects. The authors found most \textit{R. ferrugineus} adults died between 10 and 30 days after applying the strains. The three \textit{B. bassiana} strains tested against \textit{R. nigerrimus} had similar values for LC$_{50}$; strain Bb-4 had LC$_{50}$ similar to that reported by De la Rosa et al. (1997) against \textit{H. hampei} who also found that strains from \textit{H. hampei} had better LC$_{50}$, as found in our study with Bb-Hy isolated from \textit{R. nigerrimus}. Native strains from host insects of the same order may be more virulent, probably because of genetic adaptations to environmental conditions, as suggested by Meyling and Eilenberg (2007), but some reports indicate the opposite. For example, few \textit{Tribolium castaneum} (Herbst) (Coleoptera: Tenebrionidae) died when infected with \textit{B. bassiana} strains isolated from coleopteran insects, and virulence groups of \textit{B. bassiana} strains and diversity clusters were not entirely related (Golshan et al. 2014). In contrast, Sevim et al. (2012) found that diversity of \textit{B. bassiana} strains and their geographical distribution were associated with virulence.

In conclusion, the fungus \textit{B. bassiana} has potential for microbial biological control and is available for incorporation into an integrated \textit{R. nigerrimus} management program. The most virulent strains should be evaluated in the field to determine impact on \textit{R. nigerrimus} populations in natural conditions. Field assessment of different formulations and the most suitable times for application according to soybean crop phenology and economic damage by the weevil is recommended for \textit{B. bassiana} fungal suspensions.

Acknowledgment

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