N O T E

Effect of Spinosad Combined with *Beauveria bassiana* (Hypocreales: Clavicipitaceae) on *Hypothenemus hampei* (Coleoptera: Curculionidae) under Laboratory Conditions[1]

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The coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae), is a major worldwide pest of coffee. In 1978, it entered Mexico via the southern border of Chiapas with Guatemala. Currently, it is found in most of the coffee-producing states of the country (Barrera 2008. pp. 961–998, *In* Encyclopedia of Entomology, Vol. 4. Springer International). In Mexico, it currently affects 56.2% of the total area of coffee cultivation, with estimated losses of just over 157 million pesos per cultivation cycle (Ramírez et al. 2007, Pp. 73–81, *In* La broca del café en América Tropical: hallazgos y enfoques. Sociedad Mexicana de Entomología y El Colegio de la Frontera Sur.).

Synthetic pesticides have been used to control this pest. However, the misuse of insecticides may cause ecological and environmental damage or lead to resistance in the target pest. For this reason, safer alternatives for its management have been investigated, including the use of the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) (Benavides et al. 2012, 511–540, Insecticides—Advances in Integrated Pest Management. IntechOpen 2012: 511–540 https://www.intechopen.com/books/insecticides-advances-in-integrated-pest-management; De la Rosa et al. 1997, J. Econ. Entomol. 90: 1534–1538; De la Rosa et al. 2000, J. Econ. Entomol. 93: 1409–1414).

In recent years, with increased interest in organically grown coffee, more attention has been directed to "green" insecticides. Spinosad is one of these insecticides; besides being accepted by diverse organic crops, it has demonstrated insecticidal activity against a large number of agricultural pests (USDA 2000, Agricultural Magazine 48: 10–12). Recent studies also have suggested methods to

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use insect pathogens in combination with other pest management strategies including mixtures of entomopathogens and chemical insecticides (Ericson et al. 2007, J. Econ. Entomol. 100: 31–38; Gosselin et al. 2009, Biocontrol Sci. Techn. 19: 201–217; Sharififard et al. 2011, Iran J. Arthropod Borne Dis. 5: 28–36). Combined application or use of spinosad with *B. bassiana* does not have significant effects on viability, growth, or sporulation of the fungus (Amutha et al. 2010, J. Biopest. 3: 143–146; Faraji et al. 2016, J. Entomol. Soc. Iran 36: 137–146; Rajanikanth et al. 2010, Biol. Control 24: 238–243). Additional advantages in using multiple mortality factors include additive or synergetic effects (Rajanikanth et al. 2010) and use of a less-active ingredient (Ericson et al. 2007). These assessments have not been conducted against the coffee berry borer; thus, these laboratory bioassays reported herein were conducted to initially assess the potential of using spinosad and *B. bassiana* against the borer pest.

Laboratory assays were conducted in the Biological Control Laboratory of the Arthropod Ecology and Pest Management research group of El Colegio de la Frontera Sur (ECOSUR), located in Tapachula, Chiapas, Mexico in 28 $\pm$ 2°C, 80 $\pm$ 2% relative humidity, and on a 12 $\pm$ 2 h light:dark photoperiod. Coffee berry borer adults were obtained from ripe Arabic coffee fruits collected in the field 1 week before the bioassays. Perforated fruits from the field were transported to the laboratory where they were placed on absorbent paper inside a container with a ventilated bottom. One day before the bioassays, adult borers were extracted from infested fruit. A dark-colored powder coming from the borer gallery entrance indicated that the fruits housed mainly *H. hampei* adults. The fruits were disinfected with 0.5% sodium hypochlorite (Bustillo and Marín 2002, Manejo Integrado de Plagas 63: 1–4) and dried. The adult female borers were then extracted and placed in glass tubes containing a meridic diet described by Villacorta and Barrera (1993, An. Soc. Ent. Bras. 14: 316–319), where they remained until used in the bioassays.

The strain of *B. bassiana* was the native strain Bb-Hy, isolated from coffee berry borer collected on a coffee farm in the Soconusco Region 2 yr prior to this study. The strain was preserved in silica gel as part of the ECOSUR ceparium. The culture was reactivated 2 mo prior to initiating the bioassays and grown on Sabouraud dextrose agar. Aerial conidia were harvested 30 d later and used to prepare a conidial suspension in sterile distilled water with Tween 80 (0.05%, v/v), which was used to prepare the concentrations for the bioassays. Viability of the conidia was determined before the bioassays, observing their germination following the methodology described by Vélez et al. (1997, Boletín Técnico No. 17. http://biblioteca.cenicafe.org/handle/10778/709). Germination was more than 95%. The commercial spinosad product used for the bioassays was SpinTor 125 SC™ (120 g a.i./L active ingredient; Dow AgroSciences, Indianapolis, IN, USA). The different dilutions were prepared with sterile distilled water.

Concentration–mortality response of coffee berry borer to spinosad and *B. bassiana* were determined separately. We used concentrations of 200, 300, and 400 parts per million (ppm) of spinosad, frequently used in bioassays with coleopterans (Khashaveh et al. 2009, Turk. J. Agric. For. 33: 203–209; Khashaveh et al. 2011, J. Plant Prot. Res. 51: 77–81). Concentrations of 1×10^5, 1×10^6, 1×10^7, and 1×10^8 conidia/ml of *B. bassiana* were used as per several previous studies (Cruz et al. 2006, Appl. Microbiol. Biotechnol. 71: 918–926; De la Rosa et al. 1997; Gerónimo-Torres et al. 2016, Rev. Colomb. Entomol. 42: 28–35; Morales

et al. 2014, Fitosanidad 18: 5–14; Samuels et al. 2002, Biocontrol Sci. Technol. 12: 631–635; Santoro et al. 2007, 5 Simpósio de Pesquisa dos Cafés do Brasil, http://www.sbicafe.ufv.br/handle/123456789/2255; Varela and Morales 1996, J. Invertebr. Pathol. 67: 147–152). For each agent, 20 adult *H. hampei* females were used per replicate with five independent replicates per treatment. Treatment suspensions were sprayed using 20-ml atomizers. Two milliliters of each suspension was sprayed over the adult borers in each replicate as they walked over a surface covered with white paper, after which they were placed individually in glass vials (17.5 cm H $\times$ 1.5 cm D) containing the meridic diet (Villacorta and Barrera 1993). The control treatment consisted of applications of sterile distilled water with Tween 80 (0.05%, v/v). The vials were checked daily for 12 d, and dead borers were removed and placed into moist chambers to stimulate mycelial growth. Survival was analyzed with R software (R Core Team 2015, R Foundation for Statistical Computing, Vienna, Austria) to determine median lethal time (LT_{50}) and with probit analysis to determine median lethal concentration (LC_{50}) for spinosad and *B. bassiana* individually.

The LC_{50} of each agent was then used in assays of the response of coffee berry borer to the combined action of spinosad with *B. bassiana*. This assay was conducted as previously described with treatments of (a) mixtures of spinosad (200 ppm) and *B. bassiana* (1×10^6 conidia/ml), (b) spinosad alone at 200 ppm, (c) *B. bassiana* alone at 1×10^6 conidia/ml, and (d) a control of sterile distilled water plus Tween 80. Vials containing treated borers were checked daily for 5 d, based on the LT_{50} established in the previous bioassay. Dead borers were placed in moist chambers to verify the presence of mycelia. Data were analyzed with a generalized linear model with binomial response. An analysis of variance (ANOVA) was also performed, and treatment means were compared using the Tukey test ($P < 0.05$).

A final assay was conducted to observe if the combination of spinosad with *B. bassiana* inhibited growth and sporulation of the fungus. For this, an aliquot of *B. bassiana* at a concentration of 1×10^6 conidia/ml was placed on a Petri dish containing potato dextrose agar. In another Petri dish with the same growth medium, an aliquot of the spinosad (200 ppm) mixture with *B. bassiana* (1×10^6 conidia/ml) was added. After 8 d, growth of the fungus was observed and a small sample (0.5-ml diameter) was taken with a punch graft, which was placed in a tube containing 10 ml of distilled, sterile water plus Tween 80 (0.05%), and shaken on a vortex for 5 min. The concentration of conidia in each suspension was determined using a hemocytometer. This experiment was repeated four times. Chi-squared tests were performed to determine the type of interaction (additive, synergistic, or antagonistic) (Sharififard et al. 2011). Expected mortality (E) was generated from the following formula (Ericson et al. 2007): $E = Ospin + Obeau\ (1 - Ospin)$, where E is the expected mortality, and Ospin + Obeau represent the proportional mortality due to treatments of pure spinosad and pure *B. bassiana*, respectively. The predicted effects of spinosad and *B. bassiana* treatments (E) were compared with the observed mortality of the binary treatments (O) applying the following formula, $X2 = \{(O?\ E)\ 2\}$ / E (Ericson et al. 2007).

This is the first study in which a native strain of the fungus combined with the bioinsecticide has been tested against *H. hampei*. The results showed that the mixture of the fungus *B. bassiana* and the natural insecticide spinosad achieved

higher mortality of adult coffee borers than did their individual application under laboratory conditions.

These bioassays found that the insecticide spinosad caused 61, 76, and 79% mortality of *H. hampei* females at concentrations of 200, 300, and 400 ppm, respectively, at day 12 of observation. The LT_{50} for the same concentrations was 8.5, 3.5, and 2.5 days, respectively. The LC_{50} obtained was 60.4 ppm with lower and upper fiducial limits of 29.9 and 75.8 ppm, respectively. The native strain Bb-Hy of *B. bassiana* caused mortalities of 28, 50, 71, and 86% at concentrations of 1×10^5, 1×10^6, 1×10^7, and 1×10^8 conidia/ml, respectively. The LT_{50} for concentrations of 1×10^7 and 1×10^8 were 4 and 1.5 days while at concentrations of 1×10^5 and 1×10^6, the LT_{50} was not possible to determine because insect mortality did not surpass 50%. The LC_{50} for *B. bassiana* was 2.47×10^6, with lower and upper fiducial limits of 4.38×10^5 and 4.34×10^6 conidia/ml, respectively. The presence of mycelia on the dead borers treated with the fungus was 0, 23, 49, 67, and 83% for the control and the concentrations 1×10^5, 1×10^6, 1×10^7, and 1×10^8, respectively.

The mixture of the insecticide spinosad and the fungus *B. bassiana* at the lowest concentrations caused 94% mortality of *H. hampei* at day 5. In contrast, treatments with spinosad and *B. bassiana* alone caused 61 and 49% mortality, respectively, while control mortality was only 1%. The statistical analysis showed differences in response to the mixture compared to the treatments applied separately. The insecticide and *B. bassiana* alone were statistically equal, but different from the mixture and the control ($\chi^2 = 225.34$; df $= 3$; $P < 0.001$). Chi-squared estimation showed additive effect in the combination treatments of spinosad and *B. bassiana*, and there was no significant interaction ($\chi^2 = 0.02408$; df $= 1$; $P = 0.2467$).

The results observed in the development of *B. bassiana* placed in potato dextrose agar individually or combined with spinosad show that the insecticide has no effect on the growth and sporulation of the fungus. The *B. bassiana* fungus grew successfully both individually and in the mixture with spinosad. The average quantity of conidia counted after 8 d of growth was of 5.8125×10^6 ($\pm 6.12883 \times 10^5$) and 5.775×10^6 ($\pm 2.84312 \times 10^5$) conidia/ml, respectively.

Our results show that for the combination of *B. bassiana* 1×10^6 conidia/ml with spinosad 200 ppm, there was no significant interaction between the insecticide and the fungus. The increased mortality was the result of an additive effect, resulting in higher mortality of *H. hampei* adults when the two control agents were combined than when each was applied alone. These results suggest that spinosad is an excellent candidate for controlling the coffee berry borer, *H. hampei*, but combining it with the fungus *B. bassiana* may yield additive mortality effects. Its strength increases, causing high levels of mortality among adult coffee bean borers. Further studies should be conducted in field conditions to assess this possible use in an integrated pest management program for *H. hampei*.

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