SELECTION OF *BACILLUS THURINGIENSIS* STRAINS NATIVE TO MEXICO ACTIVE AGAINST THE COFFEE BERRY BORER *HYPOTHENEMUS HAMPEI* (FERRARI) (COLEOPTERA: CURCULIONIDAE: SCOLYTINAE)

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ABSTRACT The effect of 61 strains of *Bacillus thuringiensis* native to Mexico was determined under laboratory conditions, on the coffee berry borer *Hypothenemus hampei*, through bioassays carried out under controlled conditions (27±2ºC; 75±5% RH; photoperiod 12:12 h L:D). As expected, the most susceptible life stage of *H. hampei* proved to be the first instar larva, with an average mean lethal time of 6.4±1.8 days. The most virulent strains to the coffee berry borer were LBIT-129, LBIT-30 and LBIT-130 with estimated mean lethal concentration values of 21.04, 21.26 and 29.07 µg/g of diet, respectively.

KEYWORDS: *Bacillus thuringiensis*, *Hypothenemus hampei*, bioassays, native strains, virulence, Mexico.

RESUMEN Se determinó el efecto de 61 cepas nativas de *Bacillus thuringiensis* sobre la broca del café, *Hypothenemus hampei* mediante bioensayos llevados bajo condiciones de laboratorio (27±2ºC; 75±5% HR; fotoperíodo 12:12 h L:O). De acuerdo a lo esperado, el estadio más susceptible de *H. hampei* fue su primer instar larvario, con un promedio en su tiempo letal medio de 6.4±1.8 días. Las cepas de *B. thuringiensis* que mostraron su mayor actividad contra la broca del café fueron: LBIT-129, LBIT-30 y LBIT-130, con concentraciones letales medias estimadas en 21.04, 21.23 y 29.07 µg/g de dieta, respectivamente.

DESCRIPTORES: *Bacillus thuringiensis*, *Hypothenemus hampei*, bioensayos, cepas nativas, virulencia, México.

INTRODUCTION

The coffee berry borer (CBB) *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae: Scolytinae) is the most important pest of coffee in Mexico (Damon 2000). The pest feeds and reproduces inside the coffee beans, reducing their weight and quality, and causing losses of up to 50% of the harvest (Murphy & Moore 1990). Several biological control methods have been employed for the control of *H. hampei*, and advances have been obtained with the parasitoids *Cephalonomia stephanoderis* Betrem, *Prorops nasuta* Waterston (Hymenoptera: Bethylidae) and *Phymastichus coffea* LaSalle (Hymenoptera: Eulophidae) and the entomopathogenic fungi *Beauveria bassiana* (Bals.) Vuill. and *Metarhizium anisopliae*, (Metsch.) Sorok. (Ascomycotina) (Barrera 1994; de la Rosa et al. 1995, 1997, 2000; Hawksworth 1998; Infante et al. 1994). Furthermore, the CBB was found to be susceptible to nematodes of the genera *Steinernema* Travassos and *Heterorhabditis* Poinar under controlled conditions

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(Allard & Moore 1989; Castillo & Marbán 1996). Recently, other micro-organisms have been found associated with the CBB, such as protozoa, bacteria and fungi, which could have a potential application as biological control agents (Murphy & Moore 1990; Damon 2000).

Another potential agent is *Bacillus thuringiensis* Berliner, which produces crystalline parasporal inclusions during sporulation. These inclusions contain proteins with insecticidal characteristics (Ibarra 1993; DeMaagd et al. 2003) which are effective towards species of the orders Lepidoptera, Diptera and Coleoptera (Borbolla 1984; Beegle & Yamamoto 1992; Ibarra 1993; McGuire et al. 1994). Amongst the order Coleoptera, *B. thuringiensis* subsp. *kurstaki*, have been reported to be moderately toxic to two species of scolytids, namely *Scolytus scolytus* (F.) and *S. multistriatus* (Marsham) (Jassim et al. 1990). Nevertheless, to date, no study has been carried out to test the toxicity of this bacterium towards *H. hampei*. The objective of the present study, therefore, is to determine the effect of several Mexican native strains of *B. thuringiensis* against CBB, under laboratory conditions.

MATERIALS AND METHODS

Source of CBB. All biological stages of *Hypothenemus hampei*, except pupae, were tested in this work and were produced in the laboratory, using a meridic diet developed at El Colegio de la Frontera Sur (Villacorta & Barrera 1993). Female adults, eggs and first and second instar larvae (L1 and L2) (Ruiz et al. 1996) were collected from test tubes containing the diet, 30, 60 and 90 days after infestation. Adult females were treated with 2% formaldehyde for 30 seconds and rinsed with sterile water for 15 seconds, to eliminate possible contaminants (Villacorta & Barrera 1993).

Standardization of the Bioassay. Eggs, larvae (L1 and L2) and adult females of *H. hampei* were tested to select the most manageable life stage for the bioassay procedure. A total of 50, 60, 75 and 90 individuals of each stage per replicate were reared in minicultures, constituting a total of four densities with four replicates per density. Minicultures of all CBB stages (eggs, L1, L2 and adults) were set up on thin (1 mm) layers of diet, spread onto glass microscope slides (Jiménez 1992) and then placed into sterile Petri dishes. Mortality was recorded 5, 10 and 15 days after infestation, for each treatment. Individuals showing no movement and brown color were considered dead.

Selection of *Bacillus thuringiensis* strains. The 61 isolates of *B. thuringiensis* bioassayed against CBB belong to the Cinvestav-Irapuato strain collection of entomopathogens. Spore-crystal complexes of each strain were mixed to homogeneity with meridic diet, and then spread on microscopic slides before solidification. Once the first instar larva was established as the most susceptible stage, 15 L1's were placed on each slide and then into Petri dishes, to be incubated under rearing conditions (see above) for 10 days. Each strain was subjected to a series of 4 replicates, and each replicate included 4 concentrations: 0, 1, 10 and 100 µg spore-crystal complex/g diet. Mortality was recorded after 10 days of incubation.

Estimation of Mean Lethal Time (LT_{50}). From the strain selection bioassays, eight strains of *B. thuringiensis* were chosen in order to estimate the mean lethal time (LT_{50}) of the treated larvae. Four replicates of 15 larvae (L1) each were used per *B. thuringiensis* strain. Larvae were added to diet dispersed on microscope slides at a concentration of 100 µg/g de diet of the spore-crystal complex. Minicultures were incubated as mentioned above. Larval mortality was recorded daily.

Estimation of Mean Lethal Concentrations (LC_{50}s). From the strain selection bioassays, the five most active strains were selected to estimate with precision the dose-mortality relationship of *B. thuringiensis* on *H. hampei*. A total of eight concentrations were tested per replicate, as follows: 0.1, 0.5, 1.0, 5.0, 10, 50, 100 and 500 µg of the spore-crystal complex/g of diet and a negative control (without spore-crystal complex). Each concentration was tested with 60 larvae (L1) per replicate and per dose. Bioassays were incubated as mentioned above for six days, and mortality was recorded.

Statistical Analysis. For all experiments, each test was repeated four times to ensure the reliability of data. Mortality data from the bioassays were adjusted with Abbot's formula (Abbot 1925) for apparent mortality. Analysis was carried out using the statistical package PcPROBIT (Camacho 1990), to estimate each strain's LC_{50} and to plot the log dose-Probit mortality response line. Mean lethal times (LC_{50}) were compared using their fiducial limits at P=0.95.

RESULTS AND DISCUSSION

Selection of *H. hampei* most manageable life stage. Tests were performed to select the most appropriate developmental stage to be used in the bioassays, and it was obvious that L1 larvae were the most suitable. They were easy to handle and also they actively feed on the diet. In contrast, eggs were extremely fragile to handle and hatching rate was as low as 14%. Therefore, neonate larvae were not further considered for the bioassays. L2 larvae were more manageable, even than L1 larvae; however, after five days of incubation (and more markedly after 10 and 15 days of incubation) pre-pupae and pupae (non-feeding stages) were more frequent and should be discarded as bioassay individuals. Adult females were soon discarded as the optimal bioassay stage as they crawled all over the Petri dish, without staying on the diet, during the whole bioassay period. Bioassaying *B. thuringiensis* strains is based on the ability of test individuals to feed on the contaminated diet, because of the mode of action of this bacterium (Ibarra 1993). L1 larvae fed so actively on the test diet, that most of them were L2 larvae at the end of the test. Similar results were observed by Penados (1979), who mentioned that L1 larvae lasted from 10 to 26 days at temperatures ranging from 27 to 18.7ºC, respectively.

Selection of *B. thuringiensis* native strains. Of the 61 strains evaluated, all showed some degree of toxicity towards the L1 larvae of *H. hampei*, with mortality values ranging from 8.3 to 83%. Fifteen of the strains evaluated displayed relatively high toxicity at the highest dose tested (100 µg/g of diet). Strain LBIT-94 (svar. *entomocidus*) caused the highest mortality (83%), followed by strains LBIT-156 (unknown svar.), LBIT-269 (svar. *kurstaki*), LBIT-129 (svar. *thuringiensis*), and the standard strain HD-1 of *B. thuringiensis* svar. *kurstaki*, with 72, 72, 70 and 66%, respectively (Table 1). However, at the lowest dose tested (1 µg/g of diet) strain LBIT-281 (svar. *kurstaki*) was the most toxic causing 63% mortality (Table 1). Mortality in the control experiments averaged 14% for all the bioassays. These results are comparable to those reported by Jassim et al. (1990) for other economically important scolytids, who observed mortalities between 20 and 80% on fifth instar larvae of *S. scolytus* and *S. multistriatus* with *B. thuringiensis* svar. *kurstaki*, although they used lower concentrations (1.43, 9.1, 15.1, 30.1 and 60.3 µg/ml). Suzuki et al. (1993) observed mortalities of 92 to 84% in L1 and L2 larvae of *Anomala cuprea* Hope using *B. thuringiensis* svar. *japonensis* at a concentration of 2 mg/g of the diet. Ferro & Gelernter (1989) reported mortalities of 40 to

98% in L1 larvae of *Leptinotarsa decemlineata* (Say), using the strain *san diego* of *B. thuringiensis*. Later, Ferro & Lyon (1991), Ferro et al. (1993) and Giroux et al. (1994), using different commercial strains of *B. thuringiensis*, reported 100, 85 and 100% mortality, respectively, for L1 larvae of *L. decemlineata*.

Table 1. Mortality caused by the most active strains of *B. thuringiensis* tested against L1 larvae of *H. hampei*, at three different concentrations.

Strain	Mortality (%)[1]		
	1 µg/g	10 µg/g	100 µg/g
LBIT-18 svar. *morrisoni*	30	13	30
LBIT-30 svar. *thuringiensis*	45	60	52
LBIT-73 svar. *morrisoni*	31	39	47
LBIT-74 (auto agglutinated strain)	20	23	32
LBIT-94 svar. *entomocidus*	55	72	83
LBIT-129 svar. *morrisoni*	42	60	70
LBIT-130 svar. *thuringiensis*	53	55	58
LBIT-156 (unknown svar.)	50	32	72
LBIT-269 svar. *thuringiensis*	37	33	72
LBIT-281 svar. *kurstaki*	63	63	67
LBIT-358 svar. *morrisoni*	38	22	32
LBIT-419 svar. *morrisoni*	50	47	42
Btk svar. *kurstaki* [2]	22	42	66
Bti svar. *israelensis* [3]	25	23	24
Btt svar. *morrisoni* (*tenebrionis*) [4]	32	30	26

1, Percent mortality corrected by Abbott's formula (Abbott 1925); 2, Standard strain HD-1 of *B. thuringiensis*; 3, Standard strain IPS-82 of *B. thuringiensis*; 4, Standard strain DSM-2803 of *B. thuringiensis*.

Estimation of LT_{50}s. From the fifteen strains of *B. thuringiensis* that showed the highest activity against CBB, eight were selected to estimate their LT_{50}s. Using L1 larvae of *H. hampei*, the standard strain HD-1 of *B. thuringiensis* svar. *kurstaki* showed the lowest mean lethal time at 4.5 days (Fiducial Limits, FL_{95}=3.7 to 5.2). The highest LT_{50} was estimated for the strain LBIT-74 (auto agglutinated strain) with a value of 10.3 days (FL_{95}=7.0 to 21.9). The eight strains presented a mean LT_{50} of 6.4±1.8 days. The range between the lowest and highest LT_{50} values was 5.8 days.

As a result of the statistical analysis, the strains were categorized into three groups with a statistical difference between the groups (FL=95%), as shown in Table 2. These results are similar to those reported by Jassim et al. (1990) who estimated an LT_{50} of four days for *S. scolytus* using *B. thuringiensis* svar. *kurstaki*. Ohba et al. (1992) reported LT_{50} values of five days in larvae of *Chrysomela scripta* F. treated with *B. thuringiensis* svar. *japonensis*, and 2.8 days with *B. thuringiensis* strain *tenebrionis*.

Estimation of LC_{50}s. Bioassays conducted with eight different concentrations of spore-crystal complexes of *B. thuringiensis* tested against L1 larvae of *H. hampei*, were used to estimate the LC_{50}s of each tested strain. The most toxic strains, showing the lowest LC_{50}s values, were LBIT-129, LBIT-30 and LBIT-130, with estimated values of 21.04, 21.26 and 29.07 µg/g diet, respectively (Table 3). Because of the wide variation amongst the estimated LC_{50}s, values from 21.04 to 65.83 µg/g were statistically equal, while only that of strain LBIT-281 (256 µg/g) was significantly different to LBIT-129 and LBIT-30, according to their fiducial limits (P=0.95). These LC_{50} values are similar to that reported by Suzuki et al. (1993), who estimated 20 µg/g of diet, using *B. thuringiensis* svar. *japonensis* against

Table 2. Mean lethal time (LT_{50}) estimated for eight selected strains of *B. thuringiensis* tested on L1 larvae of *H. hampei*.

Strain[1]	LT_{50} (days)	Fiducial Limits (95%)[2]	Regression line equation	χ^2
LBIT-18	5.7	4.2 - 7.9 b	y = 2.06 + 3.18x	0.19
LBIT-73	6.4	5.8 - 10.0 b	y = 3.18 + 2.03x	1.22
LBIT-74	10.3	7.0 - 21.9 c	y = 3.16 + 1.63x	0.49
LBIT-358	6.3	5.3 - 11.7 b	y = 3.39 + 1.80x	0.18
LBIT-419	5.1	4.5 - 6.8 b	y= 1.43 + 4.54x	1.99
Bti	6.1	5.0 - 12.7 b	y = 2.62 + 2.40x	2.45
Btk	4.5	3.7 - 5.2 a	y = 2.95 + 3.13x	0.11
Btt	7.2	6.2 - 11.6 b	y = 2.38 + 2.79x	0.01

1, See Table 1 for strain identification; 2, Values followed by the same letter are not significantly different, according to FL 95%.

Table 3. Mean lethal concentration (LC_{50}) estimated for five selected strains of *Bacillus thuringiensis* tested on L1 larvae of *H. hampei*.

Strain[1]	n	LC_{50} (μg/g)	Fiducial Limits (95%)[2]	Regression line equation	χ^2
LBIT-129	960	21.04	10.69 - 47.03 a	y = 4.33 + 0.50x	6.15
LBIT-30	960	21.26	9.96 - 53.76 a	y = 4.41 + 0.44x	9.00
LBIT-130	960	29.07	12.67 - 87.01 ab	y = 4.40 + 0.40x	1.97
LBIT-94	960	65.83	31.32 - 183.05 ab	y = 4.08 + 0.50x	4.39
LBIT-281	960	256.00	59.14 - 694.92 b	y = 4.33 + 0.27x	1.19

1, See Table 1 for strain identification; 2, Values followed by the same letter are not significantly different, according to FL 95%.

L3 larvae of *A. cuprea*. Other authors, such as Ramachandran et al. (1993), reported similar values for the strain HD-290-1 of svar. *thuringiensis*, tested on L1 and L3 larvae of the chrysomelid *C. scripta*, with LC_{50} values of 5.3 and 21.9 μg/g, respectively.

The preset report clearly indicates that *B. thuringiensis* is toxic to *H. hampei*, and further studies are required to continue the selection of strains. It must be acknowledged that toxic levels found in this report are still moderate, when compared to other toxic levels reported for other *B. thuringiensis* strains when tested against more susceptible insect species. Still, these results show that CBB is susceptible to *B. thuringiensis*, and that further strain

selection may render in higher levels of toxicity which may allow the practical use of this bacterium's toxins in IPM programs against this serious coffee pest.

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