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Biological Control and Crop Protection

Isolation, morphological and molecular characterization of *Bacillus thuringiensis* strains against *Hypothenemus hampei* Ferrari (Coleoptera: Curculionidae: Scolytinae)☆

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ABSTRACT

The coffee berry borer *Hypothenemus hampei* Ferrari, 1876 (Coleoptera: Curculionidae: Scolytinae) is considered the most serious pest of the coffee crop and is controlled primarily with the use of chemical insecticides. An alternative to this control method is the use of the entomopathogenic bacterium, *Bacillus thuringiensis* Berliner, 1911. Therefore, the objective of this work was to select strains of *B. thuringiensis* virulent against *H. hampei* and characterize them by morphological and molecular methods to identify possible genes for the production of genetically modified plants. To achieve this objective, 34 strains of *B. thuringiensis* underwent a selective bioassay to evaluate their toxicity to *H. hampei* first-instar larvae. Among the strains tested, 11 and the standard *B. thuringiensis* subspecies *israelensis* (IPS-82) caused mortality above 90%. Then, the median lethal concentration (LC_{50}) was estimated for these strains followed by characterization using morphological, biochemical and molecular methods. The lowest LC_{50} was obtained for strain BR58, although this concentration did not differ significantly from that of the standard strain IPS-82 or from that of strains BR137, BR80 and BR67. The molecular characterization detected *cry4A*, *cry4B*, *cry10*, *cry11* and *cyt1* genes in 10 of the most virulent strains (BR58, BR137, BR80, BR81, BR147, BR135, BR146, BR138, BR139, BR140). Strain BR67 differed completely from the others and amplified only the *cry3* gene. This strain was more virulent than BR135, BR146, BR138, BR139 and BR140, but it did not differ from BR58, BR137, BR80, BR81 and BR147. The protein profile revealed proteins of 28, 65, 70 and 130 kDa, and the morphological analysis identified spherical crystalline inclusions in all strains. The results showed that the 11 strains studied have potential for use as a gene source for insertion into coffee plants for the control *H. hampei*, especially the *cry3*, *cry4A*, *cry4B*, *cry10*, *cry11* and *cyt1* genes, that were repeated in the most virulent isolates.

Introduction

The role of the coffee crop is extremely significant in the wealth and financial resources of producing and consuming countries and is also of crucial importance in the economy and politics of many developing countries (USDA, 2014). However, coffee crops are faced with several phytosanitary problems, which include the coffee berry borer *Hypothenemus hampei* Ferrari, 1867 (Coleoptera: Curculionidae: Scolytinae), considered the major pest that attacks the coffee crop (Vega et al., 2012a).

H. hampei occurs in almost all coffee-producing countries (Vega et al., 2012b), causing damage to crops and severe economic losses. Adult females produce internal galleries in the coffee berry to lay their eggs, and the larval feeding causes damages that reduce yields and the quality of the seed and can also result in the abscission of the berry (Vega et al., 2009). Research investigating different control methods is underway, but the internal feeding habit of remaining most of the life cycle inside the coffee fruit hampers management.

Chemical control is the widest method used for control of the coffee berry borer, but in addition to high cost, health and environmental damage, few efficient and safe active ingredients are registered for managing this pest (Reis, 2007; Souza et al., 2013).

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Endosulfan (C9H6Cl6O3S) is a broad-spectrum insecticide and is the most efficient product against *H. hampei*. However, due to human and environmental risks related to its use, endosulfan has been banned, which increases the importance of research on new methods of control (Janssen, 2011; Lubick, 2010).

Among the control alternatives causing less environmental damage is the use of the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuill. that is considered one potential biological control agent for coffee berry borer and has been used as a safe bioinsecticide due to properties like nontoxicity to workers and low impact on non-target organisms including coffee berry borer natural enemies. However, there are some disadvantages of using *B. bassiana* under field conditions since the fungus effectiveness depends on good weather conditions like high humidity and mild temperatures and other factors, including the strain, concentration, virulence and application efficiency (Vega et al., 2012a).

The use of bacterium *Bacillus thuringiensis* (Berliner, 1911) is also one of the alternatives to reduce the use of chemical insecticides for the coffee berry borer control. The entomopathogenic action of this species is produced by crystal proteins, which are formed by Cry proteins encoded by different cry genes (Angelo et al., 2010; Vidal-Quist et al., 2009; Vilas-Bôas et al., 2007), which can be inserted into the plant genome by genetic manipulation of coffee plants, which is considered an important strategy of control, since the pest behavior makes it more difficult. Besides this, a genetically modified plant can reduce the influence of abiotic factors on entomopathogen agents. Considering that many potential new strains and their genes remain unknown, their contents and potential for use as an alternative strategy in insect control must be identified (Sun et al., 2008).

B. thuringiensis subsp. *israelensis* IPS-82strain contains the genes *cry4*, *cry10*, *cry11* and *cyt* (Schnepf et al., 1998), and although active against Diptera, were also toxic to *H. hampei* (Méndez-López et al., 2003). More recently, strains with *cry1Ba* and *cry3Aa* genes were also identified because of their insecticidal activity against this beetle pest (López-Pazos et al., 2009).

The results obtained by various authors highlight the importance of mosquitocidal strains in the control of coleopterans, such as *Anthonomus grandis* Boheman, 1843 (Coleoptera: Curculionidae) and *H. hampei*, and have stimulated research on the effect of genes active against dipterans for the control of Coleoptera (Méndez-López et al., 2003; Monnerat et al., 2012).

Therefore, in addition to selecting strains of *B. thuringiensis* toxic to the insect, the genetic profile of these strains must also be identified for a thorough study of their *cry* gene contents. Thus, the purpose of this work was to select strains of *B. thuringiensis* virulent against *H. hampei* and then characterize them by morphological and molecular methods for possible use as a source of genes to produce genetically modified plants.

Materials and methods

Rearing of H. hampei *stock on an artificial diet*

H. hampei used in the bioassays were reared and maintained in Petri plates containing artificial diet (Villacorta and Barrera, 1993). The plates were kept in a dark climate chamber (25 ± 2 °C, 60 ± 10% RH) at the Laboratory of Ecological Pest Management of the Agronomic Institute of Paraná-IAPAR. After 30 days, the Petri plates were opened to collect and transfer eggs to new plates with artificial diet in which they remained until sufficient numbers of first instar larvae were available for use in the bioassays.

Production of the spore/crystal suspension

Strains of *B. thuringiensis* obtained from soil samples, stored in a filter paper in the form of spores and crystals, were recovered on Petri plates with nutrient agar (NA) and kept under controlled conditions (30 °C) for 96 h to complete sporulation. Then, the cultures of each strain were scraped from the culture media with the help of a Drigalski spatula and transferred into microcentrifuge tubes. These mixtures of spores and crystals, quantified using a Neubauer as 1×10^9 spores/mL for all strains, were used to perform the bioassays.

Selective bioassay of strains of B. thuringiensis *active against* H. hampei

This bioassay was conducted with 34 strains of *B. thuringiensis* from the Collection of Entomopathogenic Microorganisms (Laboratory of Genetics and Taxonomy of Microorganisms) of the State University of Londrina (Universidade Estadual de Londrina–UEL) that were identified by the presence of Cry proteins toxic to the orders Coleoptera and Diptera. *B. thuringiensis* subsp. *israelensis* IPS-82 strain and *B. thuringiensis* subsp. *kurstaki* HD-1 were provided as a courtesy of the Pasteur Institute, Paris, and were used as positive and negative controls, respectively (Méndez-López et al., 2003). For the bioassays, artificial diet (Villacorta and Barrera, 1993) was distributed (3 mL/well) in a square polymethylmethacrylate plates (10 cm × 10 cm) consisting of 16 wells (1.8 cm diameter by 1.5 cm depth). After diet solidification, 50 μL of the mixture of spores and crystals was added to each well. After complete drying of the suspension, five first instar larvae were placed in each well. The insects were maintained in a dark climate chamber (25 ± 2 °C, 60 ± 10% RH) for 5 days and then mortality was quantified. The bioassay was performed in duplicate and consisted of 12 repetitions with five larvae each. All 12 repetitions were performed in the same dish with 16 wells, with the remaining 4 wells used as negative and positive controls. The strains that caused mortality above 90% were selected for further studies. Mortality data were submitted to the Scott–Knott test ($p < 0.05$) using SASM-Agri software (Canteri et al., 2001).

Estimation of the median lethal concentration (LC_{50}) of B. thuringiensis *strains*

The dose bioassays involved the 11 most virulent strains and IPS-82 as the standard strain. Seven concentrations (100, 30, 10, 3, 1, 0.3 and 0.1%) of each strain were prepared from the initial suspension of spores and crystals (100%) used in the selective bioassays (1×10^9 spores/mL for all strains). These suspensions were diluted in sterile water with Tween at 0.01%. For each concentration, 12 repetitions were performed divided among three square polymethylmethacrylate plates. In each plate, four lines with four wells each were used for different concentrations. Thus, in the three plates, 12 repetitions of four different concentrations were performed. Each well containing five larvae ($n = 60$) was used as one repetition, as in the selective bioassay, and received 50 μL of a suspension. The insects were maintained in a dark climate chamber (25 ± 2 °C, 60 ± 10% RH) for 5 days, followed by quantification of mortality. The bioassays were repeated twice, and the mortality data were subjected to probit analysis (Finney, 1971) using the Polo-PC program (LeOra Software, 1987) to determine the lethal concentration. The LC_{50} bioassay results were analyzed by checking for overlap of the 95% confidence intervals according to probit analysis.

Molecular characterization of B. thuringiensis *strains toxic to* H. hampei

Total DNA samples from strains of *B. thuringiensis* were extracted according to the method described by Ricieto et al. (2013). The strains were grown at 30 °C for 15 h on plates containing Luria–Bertani broth (LB) (Bertani, 1951). A colony of approximately 2 mm in diameter was transferred to a microtube containing 200 μL of TE buffer (10 mM Tris; 1 mM EDTA; pH 8.0) using a sterile toothpick. The suspension was homogenized and incubated for 10 min in a boiling water bath. Then, the suspension was centrifuged at 12 000*g* for 3 min. The supernatant was transferred to a new microcentrifuge tube and used as a DNA sample for PCR amplification reactions (polymerase chain reaction). The presence of *cry*1, *cry*2, *cry*3, *cry4A*, *cry4B*, *cry10*, *cry11* and *cyt1* was evaluated using primers and specific amplification conditions. All reactions underwent an initial denaturation step at 94 °C for 2 min and a final extension step at 72 °C for 5 min performed as described by the authors (Bravo et al., 1998; Céron et al., 1995; Ibarra et al., 2003; Vidal-Quist et al., 2009). DNA amplification was performed using a Techne® Endurance TC-412 Thermal Cycler (Techne Limited, Staffordshire, UK). Each amplification reaction was mixed in a total volume of 20 μL containing 1 U Taq DNA polymerase (Invitrogen, Brazil), buffer (20 mM Tris–HCl, pH 8.0, 50 mM KCl), 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 μM each primer, 2 μL of extracted DNA and sterilized Milli-Q water). The same reaction was used for all primers described. The amplified products were visualized by electrophoresis on 1.2% agarose gel in TBE buffer (89 mM Tris–borate, 2 mM EDTA, pH 8.0) stained with SYBR® Safe (Invitrogen, UK) using a 100 bp marker DNA Ladder (Invitrogen, UK). After electrophoresis, the gel images were captured using a Sony Cyber-shot 8.1 digital camera.

Characterization of Cry proteins through SDS–PAGE

The protein profiles of the crystals of *B. thuringiensis* strains were characterized by protein electrophoresis on 10% polyacrylamide gel (SDS–PAGE). Initially, the crystals were obtained according to the protocol described by Lecadet et al. (1992). Each strain was cultivated in Nutrient Broth medium (NB) (Downes and Ito, 2001) at 30 °C for 72 h at 200 rpm, until complete sporulation. The preparations of the strain spores/crystals were analyzed by SDS–PAGE according to the procedure outlined by Laemmli (1970). For electrophoresis in Tris–glycine buffer at a constant voltage of 30 mA for 3 h, 15 μL of solubilized spore-crystal preparations was used. *B. thuringiensis* subsp. *israelensis* strain IPS-82, with a known protein profile, was used as a reference.

Morphological characterization of B. thuringiensis *strains*

The morphological characterization of the strains was initially performed using an optical microscope (CHS Model; Olympus Optical Co. Ltd., Tokyo, Japan) with a 100× phase contrast lens. For electron microscopy, the lyophilized material of the strains used in the bioassays was deposited directly on metal supports coated with gold for 180 s undervacuum (10-1 mbar) at a current intensity of 40 mA in a BAL-TEC sputter Coater Model SCD-050 and analyzed on a Philips FEI Quanta 200 scanning electron microscope QUANTA 200 (EIF) under high vacuum at a voltage of 20 kV with a 10.2 mm working distance.

Table 1
Mortality caused by *B. thuringiensis* against first instar larvae of *H. hampei* on the five days of the selective bioassay (dark chamber, 25 ± 2 °C, 60 ± 10% RH).

Strain	Mortality (%)[a]	Strain	Mortality (%)[a]
IPS-82[b]	100.00a	BR127	10.00c
BR80	100.00a	BR145	10.00c
BR81	100.00a	BR78	8.75c
BR140	98.80a	BR164	8.75c
BR58	98.75a	BR07	7.50c
BR67	98.75a	BR83	6.25c
BR135	98.75a	BR141	6.25c
BR137	98.75a	BR143	6.25c
BR139	98.75a	S1269	5.00c
BR147	98.75a	BR03	5.00c
BR146	97.50a	BR05	3.75c
BR138	96.25a	BR87	3.75c
BR37	21.25b	BR43	1.25c
BR79	21.25b	BR148	1.25c
BR18	20.00b	BR09	0.00c
BR131	17.50b	BR187	0.00c
BR149	16.25b	HD1[c]	0.00c
BR105	15.00b		
BR38	15.00b	Control sample	1.25c

[a] Means followed by the same letter in the columns do not differ from each other according to the Scott–Knott test ($p < 0.05$).
[b] *B. thuringiensis* subsp. *israelensis* standard IPS-82 strain-positive control.
[c] *B. thuringiensis* subsp. *kurstaki* standard HD-1 strain-negative control.

Results

Selective bioassay of B. thuringiensis *strains against* H. hampei

Thirty-four strains of *B. thuringiensis* were included in the selective bioassay. Among these strains, 11 (32.3%) caused mortality to *H. hampei* larvae that exceeded 96%, which did not differ significantly among the 11 strains or from the standard IPS-82 strain. These 11 strains underwent quantitative bioassays to determine the median lethal concentration (LC_{50}). The remaining 23 strains (67.6%) caused mortality rates below 21.2%. Among these strains, BR09 and BR187 and the standard strain *B. thuringiensis* subsp. *kurstaki* HD-1 were non-pathogenic to *H. hampei* (Table 1).

Estimation of median lethal concentration (LC_{50}) of B. thuringiensis *strains*

The LC_{50} for the *H. hampei* larvae ranged from 0.037×10^9 to 0.956×10^9 spores/mL. The lowest LC_{50} was for the BR58 strain, which did not differ significantly from the standard IPS-82. No significant differences were detected among the LC_{50} values of BR58, BR137, IPS82, BR80 and BR67strains, which showed the best results and were the most effective against *H. hampei* (Table 2).

In a second group classified by the LC_{50} values, the strains (BR147, BR135 and BR146) differed from the standard IPS-82 strain and from the strains that had the lowest LC_{50} values (BR58 and BR137). The exception was the BR81strain, which did not differ significantly from the IPS-82 strain but was also inserted in this group.

The third group comprised the BR138 and BR139 strains, which showed an LC_{50} value higher than that of the five most toxic strains. Finally, the BR140 strain, with the lowest toxicity, differed from all other strains and had the highest LC_{50} value. As the exception, the BR81 strain LC_{50} value was approximately threefold higher than that of the BR58 and BR137 strains but was not different from those of the BR80 and BR67 strains and from that of the standard IPS-82 strain.

The BR58 strain was approximately 25-fold more toxic than the BR140 strain, which showed the highest LC_{50} value and differed from all other strains (Table 2). These isolates for which median

Table 2
Median lethal concentration (LC_{50}) of *B. thuringiensis* strains against first instar larvae of *H. hampei* ($n = 60$) on the 5 days of the dose–response bioassay (dark chamber, 25 ± 2 °C, 60 ± 10% RH).

Strain	LC_{50} (Spores/mL)	95% CI		Slope ± SE
		Lower	Upper	
BR58	0.037×10^9a*	0.017×10^9	0.070×10^9	1.410 ± 0.124
BR137	0.039×10^9a	0.023×10^9	0.067×10^9	1.227 ± 0.108
IPS-82	0.040×10^9ab	0.017×10^9	0.080×10^9	1.519 ± 0.137
BR80	0.049×10^9abc	0.024×10^9	0.089×10^9	1.402 ± 0.122
BR67	0.113×10^9abc	0.050×10^9	0.104×10^9	1.543 ± 0.163
BR81	0.114×10^9bcd	0.074×10^9	0.169×10^9	0.998 ± 0.097
BR147	0.148×10^9cde	0.089×10^9	0.243×10^9	1.292 ± 0.107
BR135	0.164×10^9de	0.122×10^9	0.217×10^9	1.430 ± 0.128
BR146	0.196×10^9de	0.134×10^9	0.273×10^9	1.232 ± 0.137
BR138	0.251×10^9e	0.180×10^9	0.344×10^9	1.348 ± 0.159
BR139	0.297×10^9e	0.203×10^9	0.424×10^9	1.159 ± 0.140
BR140	0.956×10^9f	0.624×10^9	1.60×10^9	1.049 ± 0.183

* Means followed by the same letter in the column do not differ from each other by overlapping of 95% confidence intervals, according to probit analysis.

Table 3
Genetic and protein profiles of *B. thuringiensis* strains selected in a bioassay against *H. hampei*.

Strain	Genetic profile	Protein profile (kDa)
BR58	*cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*	130/70
BR137	*cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*	130/65
IPS-82	*cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*	130/70/28
BR80	*cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*	130/70
BR67	*cry3*	65/70
BR81	*cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*	130/70
BR147	*cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*	130/65
BR135	*cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*	130
BR146	*cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*	130
BR138	*cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*	130/65
BR139	*cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*	130
BR140	*cry4A*, *cry4B*, *cry10*, *cry11*, *cyt1*	130/65

lethal concentration (LC_{50}) values were determined were selected for further molecular, morphological and protein characterization.

Molecular characterization of B. thuringiensis *strains against* H. hampei

In the molecular characterization, different *cry* gene groups were detected in the *B. thuringiensis* strains. The combination *cry4A*, *cry4B*, *cry10*, *cry11* and *cyt1* was observed in 10 strains, which exhibited an amplification product similar to that expected for *B. thuringiensis* subsp. *israelensis* (*cry4Aa*, *cry4Ba*, *cry10Aa*, *cry11Aa*, *cyt1Aa*, *cyt1Ca* and *cyt2Ba*, located on a single plasmid of 72 MDa) (Berry et al., 2002; Guerchicoff et al., 1997; Ibarra et al., 2003). BR67 was the only strain that did not show any amplification product for the above genes and amplified only for the *cry3* gene, which has insecticidal activity against coleopterans (Schnepf et al., 1998) (Table 3).

Morphological and protein characterization

The analysis of the protein profiles of spore and crystal mixtures by electrophoresis on 10% polyacrylamide gel (SDS–PAGE) of the strains more toxic to *H. hampei* revealed bands of 28, 65, 70 and 130 kDa (Table 3). The protein profile of the strain used as the standard, *B. thuringiensis* subsp. *israelensis* (IPS-82), was similar to that previously reported, with bands at 27, 65, 128 and 135 kDa (Becker and Margalit, 1993).

All profiles analyzed, except that for BR67, had a molecular mass of 130 kDa, which is often related to the crystal proteins of Cry4A and Cry4B classes with a molecular weight range 128–135 kDa (Lereclus et al., 1989). BR58 strain presented peptides of 130 kDa, which might be correlated with Cry4 proteins. The BR58 strain also had peptides of 70 kDa, which could be associated with both the Cry10 (~78 kDa) (Thorne et al., 1986) and Cry11 proteins (~72 kDa) (Delécluse et al., 1995). Almost all strains revealed molecular masses from 65 to 70 kDa, confirming the presence of the expected genes (*cry10* and *cry11*).

The morphological analysis by optical and scanning electron microscopy confirmed the typical characteristics of strains of the species *B. thuringiensis*, i.e., spores, crystals and rod-shaped vegetative cells. The images revealed that all strains had spherical crystals, very similar to those found in *B. thuringiensis* subsp. *israelensis*.

Discussion

Among the 34 strains tested against *H. hampei* in the selective bioassay, 11 were the most toxic and presented morphological (spherical crystals) and molecular (*cry4A*, *cry4B*, *cry10*, *cry11* and *cyt1*) characteristics and protein profiles (130/70–65 kDa) similar to one another and to the *B. thuringiensis* subsp. *israelensis* standard IPS-82 (Table 3). This molecular profile of the strains is responsible for the high toxicity to Diptera. Nevertheless, this combination of morphological, molecular and protein profiles has previously been cited as a control agent against Coleoptera, such as *H. hampei* and *A. grandis* (Méndez-López et al., 2003; Monnerat et al., 2012). The strains containing *cry1*, *cry2* and *cry3*, such as strain BR145 (Ricieto et al., 2013), which has active genes against Coleoptera, showed low toxicity to *H. hampei* causing only 10% mortality (Table 1).

Méndez-López et al. (2003) achieved similar results. After evaluating 170 *B. thuringiensis* strains for the control of *H. hampei*, these authors found that only 32 caused mortality between 90 and 100%. They also observed that the Diptera-specific strains had similar molecular, morphological and protein characterization and were active against *Aedes aegypti* Linnaeus, 1762 (Diptera: Culicidae). The authors also tested nine strains with mosquitocidal activity against *H. hampei* provided by the Pasteur Institute. They observed that *B. thuringiensis* subspecies *israelensis* and *thompsoni* obtained 100% mortality and the *morrisoni* (PG14) and *malaysiensis* subspecies caused a mortality rate above 80% and did not differ significantly from the most toxic strains.

The BR58 and BR137 strains, which had the lowest LC_{50} values, amplified the *cry4A*, *cry4B*, *cry10*, *cry11* and *cyt1* genes. These strains also exhibited bands of 130 and 65–70 kDa, which might be related to the *cry4A* and *cry4B* genes and to the *cry10* and *cry11* genes, respectively (Monnerat et al., 2012). The BR135, BR146 and BR139 strains showed the same gene pool as the most virulent strains but presented only one band of 130 kDa. These three strains resulted in higher LC_{50} values, suggesting that the low toxicity was either due to low levels of or to the lack of expression of the *cry10*, *cry11* and *cyt1* genes.

The PCR technique provides a rapid and superficial molecular characterization based on the primers used. However, the technique cannot identify all genes in a strain or detect whether a gene is expressed. For example, the *cry10*, *cry11* and *cyt1* amplified genes of BR135, BR146 and BR139 strains, respectively, might be present at very low levels or be disrupted, mutated or under the control of a defective promoter. Therefore, the contribution to the lethal effect against the pest was low, or the expression of these genes was insufficient to form detectable protein levels in the protein characterization.

The genes *cry4* and *cyt1* were found in almost all strains in this study, which are also the genes responsible for the high mortality of *A. grandis* (Monnerat et al., 2012). These genes were also identified in the strains *B. thuringiensis* subsp. *israelensis* and subsp. *morrisoni* (PG14) (Choi et al., 2004; Waalwijk et al., 1985), which are considered toxic to *H. hampei* (Méndez-López et al., 2003). Therefore,

these studies provide evidence of the important role of these toxins in activity against the order Coleoptera.

Thus, among other hypotheses, the toxic effect of the strains against *H. hampei* might be due to the action of Cry4 and Cyt1 proteins. Although only toxic to Diptera *in vitro* (Höfte and Whiteley, 1989), the Cyt1 proteins have also shown their potential against *H. hampei*, *A. grandis* and *Chrysomela scripta* Fabricius, 1801 (Coleoptera: Chrysomelidae), when individually tested against these beetles (Federici and Bauer, 1998).

In addition to their insecticidal activity, the Cyt1 proteins also suppress high selection levels of *C. scripta* populations resistant to the Cry3A protein (Federici and Bauer, 1998). This action may be due to the structure and differentiated mode of action of Cyt proteins, which make them powerful allies in the management of cross-resistance of insect pests both in the composition of biological insecticides and the construction of genetically modified plants.

Delécluse et al. (1991) showed that the Cyt1A protein was not essential for the entomopathogenic activity of *B. thuringiensis* subsp. *israelensis* against *A. aegypti* and *Culex pipiens* Linnaeus, 1758 (Diptera: Culicidae), because the suppression of the *cyt1A* gene resulted in a strain similar to the wild entomopathogenic strains. However, the frequent occurrence of Cyt1A protein crystals suggests that it may be a significant component of the entomopathogenic feature of *B. thuringiensis* subsp. *israelensis* strains; for example, in synergistic action in conjunction with other proteins or other virulence factors capable of increasing the toxicity of the strain (Chilcott and Ellar, 1988).

The synergism between the toxins produced by *B. thuringiensis* subsp. *israelensis* was also observed between the Cry4 and Cyt1 proteins by Tabashnik (1992) who estimated the LC_{50} values of Cry4 and Cyt1 proteins against *A. aegypti* larvae. The values were 10-fold lower when these proteins were tested in combination than when each was used separately.

Given the functions and role of the Cyt1 protein, the protein is likely an important component of the protein assembly of the toxic strains against *H. hampei*. The Cyt1 protein should be tested separately and combined with other proteins in future research. Similarly, in addition to assessing strain toxicity to a pest, the synergistic relationships of the Cyt1 protein with other proteins and the effect on suppression or delay in cases of resistance of insects to other proteins of *B. thuringiensis* inserted into transgenic plants require further investigation.

This observation may assist researchers in deciding whether the insertion of one or more *cry* and *cyt* genes into coffee plants will yield plants more resistant to *H. hampei*, because in addition to the synergism between the Cry and Cyt proteins, some authors show that expression of multiple *B. thuringiensis* toxins can reduce instances of selection of insect populations resistant to Cry proteins (Li et al., 2014; Moar et al., 1990). Faced with the necessity to study the gene pool found in more strains, the knowledge of each *cry* gene function published in previous papers is of utmost importance for the selection of genes for the control of *H. hampei*, which shall initially be submitted to bioassays, together and individually.

The strains most toxic to *H. hampei* selected in this study, except for strain BR67, amplified the *cry4A* and *cry4B* genes. As shown in previous works, these genes show toxicity not only to Diptera but also to Coleoptera, which is significant, because in addition to the important individual toxicity of Cry4A and Cry4B proteins, reports indicate synergistic activity against mosquito larvae (Bravo et al., 2007).

The *cry11* gene that was amplified in almost all evaluated strain, encodes the Cry11 protein which is one of the most active proteins against *A. aegypti* (Crickmore et al., 1995). However, when individually tested against the coleopteran *A. grandis*, the protein showed low toxicity (Monnerat et al., 2012). Additionally, studies conducted by Méndez-López et al. (2003) revealed that strains containing Cry11 proteins, i.e., *B. thuringiensis* subsp. *fukuokaensis* (strain T03C-001), *jegathesan* (strain T28A-001) and *medellin* (strain T30-001) (Crickmore et al., 1998), were not toxic to *H. hampei*. Thus, we inferred that the *cry11* gene alone might not be responsible for the toxicity to *H. hampei* in the present study.

The Cry3 protein, which has known biological activity against Coleoptera (Van Frankenhuyzen, 2009), is also toxic to *H. hampei* (López-Pazos et al., 2009). Furthermore, this protein was identified in a *B. thuringiensis* strain in coffee plantations in Costa Rica (Arrieta et al., 2004). Strain BR67 was the only one to obtain an amplification product for the *cry3* gene alone. However, the LC_{50} value of this strain did not differ significantly from those of the standard IPS-82 strain and the other strains in the group of greater toxicity. Strain BR67 also showed a single band between 65 and 70 kDa in the protein profile, which corresponds to the *cry3* gene.

In contrast to the results reported by Méndez-López et al. (2003) and those in the present study, López-Pazos et al. (2009) found no mortality of *H. hampei* caused by *B. thuringiensis* subsp. *israelelensis* IPS-82. The latter authors explained that the absence of death was most likely due to differences in the IPS-82 strain caused by changes in the colonies of *B. thuringiensis.* Such divergences can result from different conditions of crop cultivation, such as nutrition and incubation temperature, which lead to a loss of multicellular attributes. These dissimilarities may also be attributable to any mutation occurring in the gene promoters essential for the toxicity of the bacteria.

Other methodological divergences might also have influenced the results; for example, the bioassays conducted by López-Pazos et al. (2009) were performed only with Cry recombinant proteins. Nevertheless, the work of Méndez-López et al. (2003) and this study, with the initial objective to analyze the entire contents of the strains, used spore and crystal mixtures. Thus, toxicity to the insect could be related not only to Cry proteins, but also to the virulence factors, such as proteases, chitinases, proteins in the vegetative phase and Cyt proteins. The sample purification protocol can also result in differences because the toxin content can vary between 20 and 90% in unpurified preparations. Additionally, the purification method can influence the outcomes in cases of comparison of toxicity when using the same strain.

Microscopic analysis of the morphology of crystals of a strain can provide information on its insecticidal activity (Lereclus et al., 1993; Saadoun et al., 2001; Tailor et al., 1992). All strains toxic to *H. hampei* selected in this study had the same morphology of spherical crystals, which usually identify strains toxic to Diptera, as cited by Roh et al. (2007).

Among the most toxic strains assessed in this work, the LC_{50} values of four did not differ significantly from that of the standard IPS-82. Among these strains, BR58 and BR137 exhibited high potential to control *H. hampei*, because although the molecular profile did not differ, they had lower LC_{50} values than the standard strain. Ibarra et al. (2003) observed similar results in identifying new strains of *B. thuringiensis* with toxic activity towards *A. aegypti*, with four strains that were more toxic than the standard levels of *B. thuringiensis* subsp. *israelensis* but presented a similar genetic profile.

The above results demonstrate that bioassays of strains and *cry* genes must be conducted against insects both separately and together, because even with the same gene content, they can cause different levels of toxicity. Alternatively, strains may contain genes that are the determining factor for toxicity against the pest but are not identified by PCR.

In addition to *cry* and *cyt* genes, other virulence factors in *B. thuringiensis* strains can contribute to bacteria toxicity (Vilas-Bôas et al., 2012). Moreover, synergistic interactions can occur among the Cry proteins or even among these proteins and spores. However, because of the variation in toxicity of each protein, establishing

an individual contribution to the toxic effect of a strain is difficult (Glare and O'Callaghan, 2000; Visser et al., 1990). These types of interaction could also explain the different levels of toxicity obtained by the strains tested, which although in a similar gene pool, showed different LC_{50} values for *H. hampei*.

Several groups have focused their research on searching for highly toxic strains of *B. thuringiensis* containing multiple Cry proteins with the aim to obtain specificity in the control of different insect pests and more options for the management of insects resistant to certain genes (Bobrowski et al., 2003). Thus, strains of *B. thuringiensis* toxic to *H. hampei* expressing a diversified gene content, containing more than one gene that might be toxic to the pest, are of extreme importance for the management of insect resistance to genetically modified plants.

As observed in this study, for most of the toxic strains, this gene diversity may permit the use of combined genes by adding the expression product of more than one gene to bind to different membrane receptors inserted in the same plant (Van Rie, 1999). Consequently, the probability of an insect becoming more resistant to a toxin will be reduced, which favors longer-term viability of genetically modified coffee plants (Degrande and Fernandes, 2006).

Further studies should consider the possibility of inserting one or more genes into the coffee plant to complement this work. With the results obtained to date, i.e., the selection of 11 strains virulent against *H. hampei* and identification of their gene pools, new insights will likely be yielded in future genetic investigations that examine the effect of each gene. Moreover, these surveys will help researchers understand the relationships among these strains and lead to further steps towards increasing their toxicity or even prevent the selection of resistant insects. Additionally, studies aiming at the selection of *cry* genes can be extended to include other important coffee pests, such as *Leucoptera coffeella* (Guérin Mèneville and Perrottet, 1842) (Lepidoptera: Lyonetiidae).

Conclusions

Among the 34 strains studied, 11 caused mortality to *H. hampei* larvae that exceeded 96%. From the selective bioassay, strains BR58, BR137, BR80 and BR67 had the lowest LC_{50} values and did not differ from the standard IPS82. The protein profiles produced bands with molecular masses of 28, 65, 70 and 130 kDa. The molecular characterization showed the presence of *cry1*, *cry2*, *cry3*, *cry4A*, *cry4B*, *cry10*, *cry11* and *cyt1* genes. The morphological analysis revealed that all strains had spherical crystals, very similar to those found in *B. thuringiensis* subsp. *israelensis*. As a result, these isolates have potential for biotechnological control of *H. hampei* and should be important candidates for more studies and for use as gene sources for the construction of transgenic coffee plants.

Conflicts of interest

The author declares no conflicts of interest.

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