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Potential of *Bacillus pumilus* Bp4185 and *Bacillus thuringiensis* Bt 3971 for coffee berry borer biocontrol

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ABSTRACT
Coffee berry borer (*Hypothenemus hampei*), is one of the main pests affecting coffee production worldwide. This pest feeds and reproduces inside the berry reducing its weight and quality, causing losses up to 50%. Some strains of *Bacillus* have plasmids that incorporate *cry* and *cyt* genes, whose products have toxic properties to insects. *Bacillus thuringiensis* and *Bacillus pumilus* have been evaluated as entomopathogenic. The objectives of this study was to determine the potential of two native *Bacillus* strains (*B. thuringiensis* Bt3971 and *B. pumilus* Bp4185) isolated from coffee ecosystem of Puerto Rico as biocontrol of coffee berry borer. The presence of a 12 kbp plasmid was confirmed in Bt3971 and Bp4185, and a 116 kDa protein with entomopathogenic potential from Bt3971. After 48 h of treatment, Bt3971 (A_{590}=0.8, approximately 10^{10} CFU ml^{-1}) caused 55% mortality and 82% at 72 h in a population of insects. Bp4185 strain, at 72 h caused 55% mortality (A_{590}=1.0, approximately 10^{12} CFU ml^{-1}) and 46% at 96 h (A_{590}=0.8). Both native bacterial strains, adapted to the environmental conditions of Puerto Rico, have potential for biological control of coffee berry borer.

Keywords: *cry1A*, *Hypothenemus hampei*, plasmids, toxicity

Potencial de *Bacillus pumilus* Bp4185 y *Bacillus thuringiensis* Bt3971 para el biocontrol de la broca del café

RESUMEN
La broca del café (*Hypothenemus hampei*) es un de las plagas principales del cultivo y efecta su producción a nivel mundial. El insecto crece y se reproduce dentro de la fruta y reduce su peso y calidad, lo que causa pérdidas hasta del 50%. Algunas cepas del género *Bacillus* contienen plásmidos con genes del tipo *cry* y *cyt* que codifican por proteínas tóxicas a insectos. *Bacillus thuringiensis* y *Bacillus pumilus* se han evaluado como entomopatógenas. Este estudio tuvo como objetivo determinar el potencial de *Bacillus thuringiensis* Bt3971 y *Bacillus pumilus* Bp4185 para el biocontrol de *H. hampei*. La presencia de plásmidos de 12 kbp fue confirmada en ambas cepas, así como una proteína de 116 kDa potencialmente entomopatógena en Bt3971. Después de 48 h de tratamiento, Bt3971 ocasionó 55% de mortalidad (A_{590}=0.8, aproximadamente 10^{10} UFC ml^{-1}) y 82% a las 72 h en la población de insectos. Bp4185 a las 72 h produjo 55% de mortalidad (A_{590}=1.0, aproximadamente 10^{12} UFC ml^{-1}) y 46% a las 96 horas (A_{590}=0.8). Ambas cepas nativas, adaptadas a las condiciones ambientales de Puerto Rico, tienen potencial para el control biológico de la broca del café.

Palabras clave: *cry1A*, *Hypothenemus hampei,* plásmidos, toxicidad

INTRODUCTION

Bacteria within the *Bacillus* group are an alternative of biological control against significant agricultural pests. In 1995, the *Bacillus thuringiensis* (Bt) *cry*1Ab δ-endotoxin and its genetic material were registered in the U.S. Environmental Protection Agency

(EPA) for production of Bt-corn (*Zea mays* L.) (EPA, 2014).

Bacillus thuringiensis is a spore-forming Gram + bacteria strains that produce crystalline, proteinaceous, parasporal bodies within the sporangia with insecticidal activities. Another species, *Bacillus pumilus* (Bp) is a Gram +, rod-shaped, spore-forming bacteria, found mainly in soils (Logan and De Vos, 2009). Although, some studies have shown Bp effectivity against certain insects, little is known about the toxicity of *B. pumilus* against coleopteran. Some strains of *Bacillus* have plasmids that incorporate *cry* and *cyt* genes, whose products have toxic properties to insects.

Plasmids are extrachromosomal DNA molecules of various sizes present in many bacterial species and which replicate independently of chromosomal DNA. Most are circular but there are also double or single strandlines (Snyder and Champness, 2007). Depending on their size (1-250 kbp) they can code from a few proteins to hundreds of them (Snustad and Simmons, 2006). The products of the genes they code generally provide an advantage in terms of adaptation of environments and resistance to toxic substances. Some phenotypic characteristics encoded by genes carried in plasmids provide resistance to antibiotics such as ampicillin, tetracycline and kanamycin, and the production of antibiotics such as bacteriocin active against other bacteria, degradation of complex organic compounds, production of enterotoxins, and the production of restriction and modification enzymes (Li *et al*., 2016).

B. thuringiensis acquired a long-mobile plasmid which is present in the sporulated cell altogether with millions of crystal proteins with insecticide properties. Most plasmids are not necessary for the survival of the cell in which they reside. However, in certain environmental conditions, a plasmid may be essential for survival. Phenotypes conferred by different plasmids include resistance to antibiotics, production of antibiotics, degradation of complex organic compounds, production of enterotoxins, and the production of restriction and modification enzymes (Sørensen *et al*., 2005).

The mode of action of the Cry toxins involves an osmotic imbalance of midgut epithelial cells, cell lysis, gut paralysis and death of the insect (Bravo *et al*., 2007). *B. thuringiensis* var. *tenebrionis* has been used as a biocontrol agent for insects in the order Coleoptera (Krieg *et al*., 1983), and its activity is mainly associated to the Cry 3A δ-endotoxin which is highly active against beetles. Formulations having the Cry 3A δ-endotoxin have been commercially available since the eighties, but some insects have generated resistance (Rahardja and Whalon, 1995). *B. pumilus* has antifungal properties because it produces fungicides such as bacilomicin D and fengicin (Ramarathnam *et al*., 2007).

The coffee berry borer (CBB) (*Hypothenemus hampei* Ferrari, Coleoptera: *Scolytidae*), is considered one of the most devastating pests of coffee (*Coffea* spp.) cultivation worldwide. It reduces the weight and quality of the coffee when it is reproduced and fed into the fruit (De la Rosa *et al*., 2005). The female drill pierces the fruit by the part of the crown reaching the endosperm where it lays its eggs. They hatch at the larval stage, and they cut through the cherry tissue by opening pockets to the main tunnel bordered by the mother. In this stage, it causes more damage to the fruit. The mature female leaves the seed and fertilized by a male brother and moves to another fruit (Damon, 2000). These characteristics of the pest biological cycle attempt against the effectivity of the insecticidal treatments (Méndez-López *et al*., 2003).

In Puerto Rico, *H. hampei* is the principal pest of coffee crop, too. It is distributed for all the plantations where occasioning important damages with direct impact in all the production chain. The integrated pest management program in Puerto Rico include several practices to diminish the losses and to protect the fruits for the next production cycle. Among them, one alternative is the biological control with *Beauveria bassiana,* when the infestation level are over the 7.0% and in more than the 50% of sampled fruits, the female not reach the seed (Gallardo y González, 2015). However, this pest is one of the limiting factor for coffee production and other alternative are needed.

Based on the knowledge gained from the results of different investigations, *Bacillus thuringiensis* strains would be used to control CBB. In this sense, it has been demonstrated the presence of Cry proteins with potential to CBB biological control in several *B. thuringiensis* strains (Méndez-López *et al.*, 2003; López-Pazos *et al*., 2009; Zorzetti *et al*., 2018). Besides, other proteins and secondary metabolites with insecticidal properties secreted by *Bacillus* strains has been informed (Brar *et al*., 2007; Palma *et al.,* 2014).

According to that, the characterization of native *Bacillus thuringiensis* strains is a valuable tool in order to find new insecticidal toxins. Besides, plasmid identification is useful to understand the toxicology of *Bacillus* strains with biocontrol properties against insects and it would contribute to manage resistance problems (Khorramnejad *et al.,* 2018). Therefore, this study was conducted to determine the potential of two native *Bacillus* strains (*B. thuringiensis* Bt3971 and *B. pumilus* Bp4185) isolated from coffee ecosystem of Puerto Rico as biocontrol of coffee berry borer.

MATERIALS AND METHODS

Bacterial strains

Native *B. thuringiensis* Bt3971 and *B. pumilus* Bp4185 strains isolated from coffee agro-ecosystem of Puerto Rico and conserved in the Agricultural Bacteriology Laboratory collection - Universidad De Puerto Rico Mayaguez were used.

Besides, seven strains of *Bacillus thuringiensis* subsp. *kurstaki*, two strains of the subsp. *aizawai*, and three *Escherichia coli* integrated to plasmids pKK or pTZ with *cry* genes were acquired from the *Bacillus* Genetic Stock Center - BGSC (The *Bacillus* Genetic Stock Center, Columbus, OH, http://www.bgsc.org/) and used as standards (Table 1).

Insects

Adults from *Hypothenemus hampei* were collected from field in the same coffee fruit which showed damage by the berry borer and kept for 24 hours in a wet chamber until the start of the bioassays.

Table 1. *Bacillus* strains from *Bacillus* Genetic Stock Center (http://www.bgsc.org/) used as standards.

Strains	Species / subsp.	Genes
B. thuringiensis		
4D-1	*kurstaki*	*cry1A, cry1B, cry 1C, cry 2 A*
4D-6	*kurstaki*	*cry3a, 3b, 3c*
4D-7	*kurstaki*	*cry1*
4D-8	*kurstaki*	*cry6*
4D-9	*kurstaki*	*cry7*
4D-10	*kurstaki*	*cry8*
4D-20	*kurstaki*	*cry3a, 3b, 3c*
4J-2	*aizawai*	*cry1A, cry1B, cry1C, cry1D*
4J-5	*aizawai*	*cry1A, cry1B, cry1C, cry1D*
ECE53	*E. coli* JM 103t	*cry1A* cloned in *pKK223*
ECE127	*E. coli* DH5	*cry1E* cloned in *pTZ19R*
ECE131	*E. coli* JM 103	*cry3A* cloned in *pKK223*

Screening of plasmids

All bacterial strains were cultured on nutrient agar (agar 15 g l^{-1}, meat extract 1 g l^{-1}, peptone 5 g l^{-1}, sodium chloride 5 g l^{-1}, yeast extract 2 g l^{-1}) (Sigma-Aldrich Corporation, St. Louis, MO) supplemented with ampicillin (10 mg m^{-1}). Bacterial cultures were examined for growth after 24 h of incubation at 27 ºC. Bacterial strains were inoculated in 22 ml of Luria-Bertani broth (Peptone 10 g l^{-1}, yeast extract 5 g l^{-1}, NaCl 5 g l^{-1},) (Life Technologies, Carlsbad, CA) supplemented with ampicillin (10 mg ml^{-1}), and grew overnight in an orbital shaker at 150 rpm at 27 ºC. Under these conditions, absorbance (590 nm) in the range 1.7 to 2.0 was achieved.

Three commercial protocols were used for plasmid extraction: Pure YieldTM Plasmid Miniprep System (Promega Corporation, Madison, WI), Rapid Concert mini preparation kit (Life Technologies Carlsbad, CA), and the Hi-Speed Mini Plasmid Kit (IBI Scientific, Dubuque, IA). The plasmid band size expected by these protocols is between 10 – 100 kb. After extraction, plasmid DNA was quantified in a nanophotometer P360 (VWR International, Radnor, PA) and the ratio of absorbance 260/ 280 was archived. Plasmid isolation products were loaded in agarose (1%) with 3 µl ethidium bromide and visualized in an UV trans-illuminator. A 1 kb DNA ladder (Life Technologies Carlsbad, CA) was used to compare the size of the bands.

Presence of toxic proteins

To determine the presence of toxic proteins *B. thuringiensis* Bt3971, *B. pumilus* Bp4185, subsp. *kurstaki* (4D-1, 4D-6, 4D-7, 4D-8, 4D-9, 4D-10 and 4D-20), *aizawai* / *pacificus* (4J-2 and 4J-5) and the *E. coli* clones of *B. thuringiensis* ECE-53, ECE127, and ECE131 were analyzed with SDS-PAGE.

Bacteria grew on orbital shaker overnight at 150 rpm. At the next day, bacteria were centrifuged at 10 000 rpm for 10 min. Cells were lysed using 2 µl of lisozime and 2 µl of DNase I. The cell suspension was incubated for 10 min at 30 ºC. The cell suspension was centrifuged at 14 000 rpm for 5 min. Proteins were analyzed using SDS-PAGE. A 50 µl aliquot of proteins was mixed with 20 µl of loading buffer (10% SDS, Glycerol, Mercaptoethanol, 0.1% bromo phenol blue, 0.5M Tris-HCl, pH 6.8). Acrylamide gel consisted of 30% acrylamide, 2% bis acrylamide, 1M Tris·HCl (pH 8.7), 20% SDS, H_2O, TEMED, 10% ammonium persulfate. Samples ran at 75 V for 4 h in a vertical protein gel electrophoresis chamber system (OWL Scientific), with Tris-Glycine-SDS 10X loading buffer (glycine 14.4 g, Tris base 3 g, SDS 1 g, water to complete 1 l). Dye used was 0.025% Coomassie blue and silver stain (silver nitrate 0.2 g, sodium hydroxide 5.25 ml, ammonium hydroxide 350 µl).

Bioassays

The insecticidal activity of *B. thuringiensis* Bt3971 and *B. pumilus* Bp4185 was determined in bioassay with *H. hampei*. The strains were grew in nutrient broth (D(+)-glucose 1 g l^{-1}, peptone 15 g l^{-1}, sodium chloride 6 g l^{-1} and yeast extract 3 g l^{-1}) (Sigma-Aldrich Corporation, St. Louis, MO). Strains were incubated at 28 ºC in tubes with 20 ml for 24 hours in an L.E.D agitator at 150 rpm. Standardized bacterial suspensions were prepared at 0.6, 0.8, and 1.0 of absorbance at 590 nm. Absorbance lectures of 0.6, 0.8 and 1.0 at 590 nm represented approximately 10^9, 10^{10} and 10^{12} CPU ml^{-1}, respectively in both strains. The presence of spores as assessed by microscopic preparations. Aliquots of 20 µl of the bacterial suspensions were placed in a circle inside a 10 x 15 cm Petri dish. Using a thin cell brush, an adult insect was placed in contact with the bacterial suspension for 15 minutes. Inoculated insects were placed (one insect per plate) in glass containers and covered with cheesecloth.

Mortality of insects was evaluated every 24 hours after inoculation until 96 h. A binary variable, which consisted in 0 for dead insects and 1 for live insects was used for evaluation. A randomized complete block design (RCBD) was used. Each block had a control insect (placed in contact with sterilized water) and three bacterial suspensions (0.6, 0.8, and 1.0 A, absorbance units). Each experimental treatment had 11 replicates. Mortality was corrected using the formula of Abbott Corrected mortality (%) = (%MT-%MC)/(100-%MC), where MT = mortality of CBB in treatment, and MC = CBB mortality in the control (Abbott, 1925). The efficacy of

treatment was calculated as Efficacy=100*(Ta-Ca)/100-Ca, where Ta is the mortality of the population in the treatment, and Ca is the mortality of the population in the control.

Statistical analysis

Data were analyzed with SAS software version 9.2 (SAS Inc, Cary, NC) using generalized linear mixed models (PROC GLIMMIX). Fisher's least significant difference (LSD) test at P = 0.05 was used to compare pairs of treatment means. To determine the relationship between mortality and time after inoculation linear regression analysis was conducted using PROC REG.

RESULTS AND DISCUSSION

Plasmids of the size 12 kbp were detected in the strains *B. thuringiensis* Bt3971, *B. pumilus* Bp4185 as well as in standard strains *B. thuringiensis* subsp. *aizawai* 4J-5, and *B. thuringiensis* subsp. *kurstaki* strains 4D-10, 4D-6, 4D-8 and 4D-1 (Figure 1 a).

Using the protocol Promega - Pure YieldTM Plasmid Miniprep System kit, a bigger number of plasmids were detected (Figure 1 c). Strains *B. thuringiensis* subsp. *kurstaki* 4D-9, which contains the *cry*7 gene, and *B. thuringiensis* subsp. *aizawai* 4D-20 which contains the genes *cry*3a, *cry*3b and *cry*3c, showed an additional band estimated at 30 kbp (Figure 1 e).

Plasmids of 12 kbp were consistently detected in the strains 4D-1, 4D-7, 4D-8, 4D-9, 4D-10, 4D-20, 4J-2, 4J-5, Bt3971 and *B. pumilus* Bp4185 without regard of the extraction protocol. *E. coli* cloned plasmids with *cry* genes were poorly recovered by these three methods of extraction. Additionally, in *E.coli* 53 (*cry*1Ac cloned in pKK223) plasmids were not detected with any of the assayed protocols. The use of three different kits confirmed if the expected plasmid was present or not as kits yielded different bands (Figure 1 a, c, e). *Bacillus* species have low plasmid copy numbers (approximately 40 per cell) as well as cryptic plasmids (Zhang *et al*., 2010) which can explain the complexity of plasmid profile in the strains used in this study. Additionally, Lereclus *et al.* (1982) detected variation in plasmid size (2-80 MDa) and number (1-17) in *B. thuringiensis*.

The 12 kbp plasmids detected in the native bacteria *B. thuringiensis* Bt3971 and *B. pumilus* Bp4185 were similar in size to the plasmids detected in the *Bacillus* control group obtained from the BGSC collection (strains of the subsp. *kurstaki*, and *aizawai*) that have plasmids and *cry* genes (Table 1).

In this study, plasmids of 12 kbp were consistently detected in *B. thuringiensis* Bt3971 and *B. pumilus* Bp4185. *Bacillus thuringiensis* produces endospores containing δ-endotoxin proteins toxic to insects and *cry* genes encode endotoxins. *B. thuringiensis* has several plasmids whose size varies between 2 to > 200 kbp depending on the strain. The *cry* genes are found in large (> 40 kbp) conjugative plasmids (which allow gene transfer from one cell to another) (Bravo *et al*., 1998). Plasmids of two sizes 12 kbp and 30 kbp were detected in this study. A 12 kbp plasmid was detected in the samples of the native strains of *B. thuringiensis* Bt3971 and *B. pumilus* Bp4185, and in the subsp. *kurstaki* and *aizawai* which have been characterized and identified by having Cry toxins active against coleopteran (Donovan *et al*., 1992; Ohba *et al*., 1992; Knowles, 1994). In addition, a 30 kbp plasmid was detected in the Bt subsp. *kurstaki*, which suggests the presence of more Cry toxins.

In *B. thuringiensis*, as much as three high-frequency transfer plasmids can be found, and are useful to differentiate between crystal producing (Cry+) and crystal recipient (Cry-) *B. thuringiensis*- phenotypes. Plasmids or fragments of them from *B. thuringiensis* have been cloned into commercial vectors (such as pUC, pKK, and pTZ), and transformed into *Escherichia coli*. This technique allowed locating *cry* genes, and a deeper understanding of the peptide expression as well as post-transcriptional regulators of endotoxin production (McLean and Whiteley, 1987). In this study, *E.coli* strain JM 103t containing the *cry1Ac* gene cloned in a pKK 223 plasmid did not show a band of the expected size for this plasmid (> 40 kbp) indicating a potential loss of the mobile structure. Further studies are necessary to demonstrate the presence of *cry* genes in the native strain tested. More than 900 toxin genes encoding entomopathogenic proteinaceous toxins, have been identified and characterized in the Bt strains isolated from different regions of the world (Jouzani *et al.,* 2017).

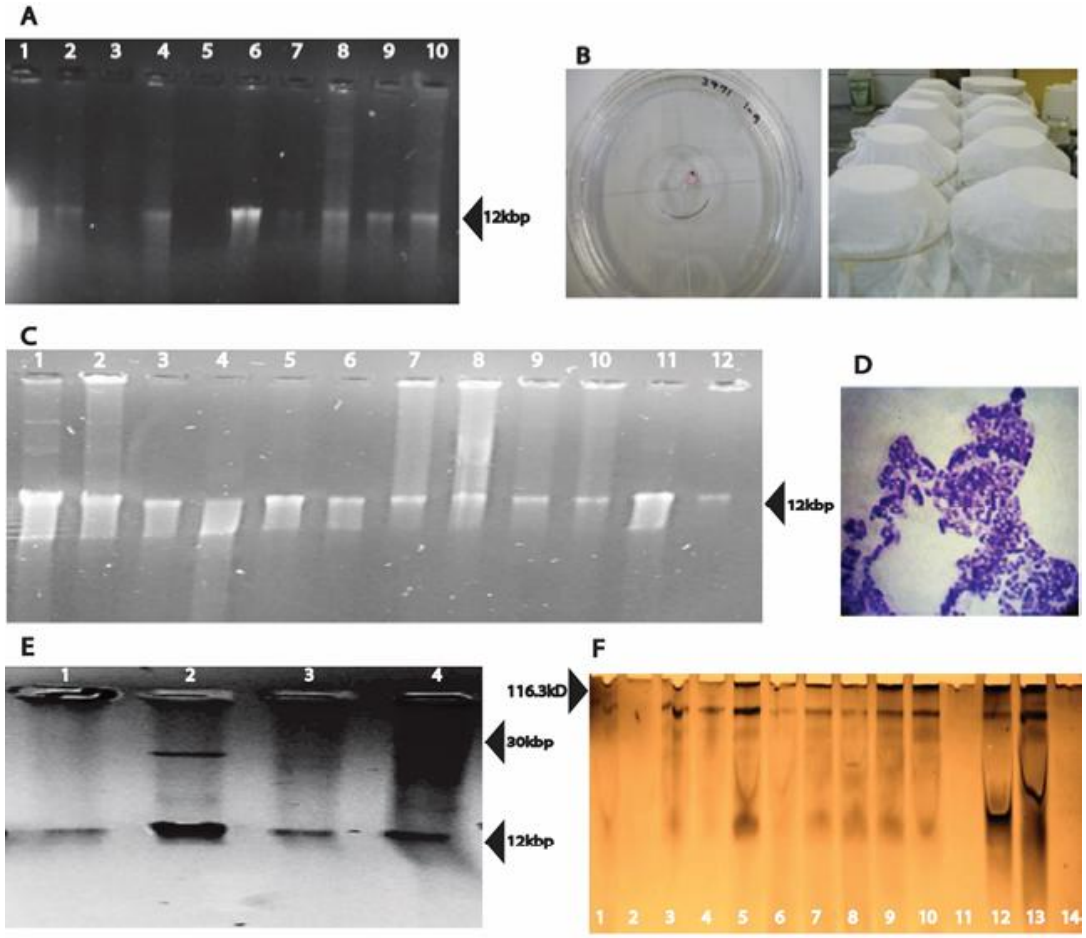

Figure 1. Plasmids from *Bacillus* strains: (A) Life-Rapid Concert mini preparation kit: 1) *Bacillus thuringiensis* Bt3971, 2) *Bacillus pumilus* Bp4185, 3) *Bt* 4J-2, 4) *Bt* 4J-5, 5) *Bt* 4D-20, 6) *Bt* 4D-10, 7) *Bt* 4D-9, 8) *Bt* 4D-6, 9) *Bt* 4D-8, 10) *Bt* 4D-1. (B) Bioassays to determine the mortality of coffee berry borer by *B. thuringiensis* Bt3971 and *B. pumilus* Bp418. (C) Extraction using the Promega YieldTM Plasmid Miniprep System: 1) *Bt* 4 D-6, 2) *Bt* 4D-20, 3) *Bt* 4D-10, 4) *E. coli* ECE-127 5), *E. coli* ECE127, 6) *Bacillus thuringiensis* Bt3971, 7) *Bt* 4J-2, 8) *Bt* 4D-8, 9) *Bt* 4D-9, 10) *Bt* 4J-5, 11) *Bacillus pumilus* Bp4185, 12) *E. coli* ECE-53. (D) Staining of *Bacillus* spores. (E) Extraction using the IBI -Hi-Speed Mini Plasmid Kit: 1) *Bacillus thuringiensis* Bt3971, 2) *Bt* 4D-9, 3) *Bt* 4J-2, 4) *Bt* 4D-20. (F) SDS-PAGE to show the protein profile of: 1) *Bacillus thuringiensis* Bt3971, 2) *Bacillus pumilus* Bp4185, 3) *Bt* 4J-2, 4) *Bt* 4J-5, 5) *Bt* 4D-1, 6) *Bt* 4D-6, 7) *Bt* 4D-8, 8) *Bt* 4D-9, 9) *Bt* 4D-10, 10) *Bt* 4D-20, 11) *Bt* 4D-7, 12) *E. coli* ECE-127, 13) *E. coli* ECE-53, 14) *E.coli* ECE-131.

Nevertheless, strains of *Bacillus* used in this study showed resistance to ampicillin. This characteristic is associated with the *RK2* gene, which provides resistance to ampicillin, tetracycline, and kanamycin (Snyder and Champness, 2007). *RK2* is the representative archetype of the incompatibility group (Inc P-1) plasmids (Li *et al*., 2016). In these plasmids, gene clusters associated to antibiotic resistance are mobile and conjugative; therefore, these elements allow bacteria to adapt to environmental stress (Norman *et al*., 2009).

In the analysis of the presence of toxic proteins by SDS-page, one band of approximately 116 kDa was detected on *B. thuringiensis* Bt3971 as well as in standard strains 4J-2, 4D-1, 4D-6, 4D-8, 4D-9, 4D-10, 4D-20 and *E. coli* clones of ECE-53, ECE127 and ECE131 (Figure 1 f). This result denoted the possible Cry proteins presence in the strain.

The δ-endotoxins are made up of two types of proteins, the Cry and Cyt proteins, which have been identified for their insecticidal activity, and δ-endotoxins have molecular

masses between 27 to 140 kDa (Ben-Dov *et al*., 2001; Pardo-López *et al.,* 2013; Jouzani *et al*., 2017). In this study, it was only possible to identify the presence of proteins in that range for *B. thuringiensis* Bt3971 bacterium, with 116 kDa. A band of similar weight was detected in *Bacillus* belonging to the subsp. *kurstaki*, *aizawai* / *pacificus*, as well as the *E. coli* clones of *B. thuringiensis* which are characterized by possessing *cry* genes with proteins toxic to beetles. *B. thuringiensis* is characterized by the formation of parasporal crystals protein called δ-endotoxins, which are formed during sporulation and are toxic against insects (Logan and De Vos, 2009).

B. thuringiensis Bt3971 and *B. pumilus* Bp4185 produced a mortality of 18% and 9% at 48 h after inoculation at A_{590}=0.6 (ca. 10^9 CPU ml^{-1}), respectively (Table 2). At the same time with A_{590}=1.0 (ca. 10^{12} CPU ml^{-1}) mortality was 36% in both strains. Therefore, the effect of concentration was significant on mortality for both strains at 48 h. At A_{590}=0.6 and A_{590}=0.8, higher mortality rates were obtained in *B. thuringiensis* Bt3971 than in *B. pumilus* Bp4185 after 96 hours of inoculation.

In *B. thuringiensis* Bt3971, mortality rates at the concentrations of A_{590}=0.8 and A_{590}=1.0 were significantly higher than at A_{590}=0.6 (LSD – Fisher; α = 0.05). In *B. pumilus* Bp4185, mortality at A_{590}=1.0 was significantly higher (LSD – Fisher; α = 0.05) than A_{590}=0.8 and A_{590}=0.6, averaged over time.

Positive linear effects ($p<0.001$) were detected in the relationship mortality and inoculation time; therefore, the higher the time after inoculation, the higher the mortality rates in both strains. The shortest time at which approximately 50% of the insects (six insects) were killed with *B. thuringiensis* Bt3971 was 48 h at A_{590}=0.8, and with *B. pumilus* Bp4185 was 72 hours at A_{590}=1.0. Mortality rates showed that *B. pumilus* Bp4185 and *B. thuringiensis* Bt3971 were effective in controlling *H. hampei*. *B. thuringiensis* reached mortality rates using smaller inoculum concentration than *B. pumilus*.

B. thuringiensis Bt3971 showed high toxicity against the coffee berry borer, expressed as high mortality rates as soon as 48 h after inoculation. Results were similar to those observed by Méndez-López *et al*. (2003) and De la Rosa *et al*. (2005) with different strains of *B. thuringiensis* for the control of the coffee borer in larval and adult stages using Bt-spore-crystal complexes. In the case of Méndez-López *et al.* (2003) these authors determined that Bt subsp. *israelensis* was highly toxic to the coffee berry borer during its larval stage. They used spore-crystal

Table 2. Susceptibility of *Hypothenemus hampei* to *Bacilllus thuringiensis* Bp3971 and *Bacillus pumilus* Bp4185.

Strains	Hours	Inoculum concentration (Absorbance 590 nm)[1]			Mean (%_ LSD-Fisher)
		0.6	0.8	1.0	
		Mortality (%)[2]			
	24	0	9	0	3.0d
B. thuringiensis	48	18	55	36	36.3c
Bt3971	72	36	82	73	63.7b
	96	82	91	100	91.0a
Mean (%)	LSD-Fisher[3]	34.0b	59.2a	52.2ab	
	24	0	9	9	6.0c
B. pumilus	48	9	9	36	18.0bc
Bp4185	72	18	9	55	27.3b
	96	36	46	100	60.6a
Mean (%)	LSD-Fisher	15.8b	18.2b	50a	

[1]Absorbance lectures of 0.6, 0.8 and 1.0 at 590 nm represented approximately 10^9, 10^{10} and 10^{12} CPU ml^{-1}, respectively in both strains. [2]Mortality rates were corrected using the formula of Abbott Corrected mortality %=(%MT-%MC)/(100-%MC), where MT = mortality of CBB in treatment, and MC = CBB mortality in the control, n= 11.[3] LSD-Fisher was done averaging hours and inoculum concentrations to determine differences among treatment means

complexes of Bt strains at different concentrations (1000-10.1 ng cm²) in an artificial diet through bioassays using larvae of the first instar of the drill. On the other hands, De la Rosa *et al*. (2005) performed bioassays using different growth stages of the coffee berry borer such as eggs, larvae and adult females. They analyzed the spore-crystal complexes of 61 strains of *Bacillus thuringiensis* mixed in an artificial diet at 1.0, 10.0 and 100 µg g^{-1} of spore-crystal complexes. Bt strains showed different degrees of toxicity in larvae of *H. hampei*, with mortality ranges from 8.3 to 83%. Similarly, Zorzetti *et al.* (2018) informed that 11 *B. thuringiensis* strains (32.3%) caused mortality to *H. hampei* larvae that exceeded 96%.

Bacillus pumilus forms ellipsoidal spores located in the central part of the cell, whose natural habitat is mostly the soil (Molina *et al.,* 2010). In the present study, *B. pumilus* Bp4185 strain showed 12 kbp plasmid. The results demonstrated that not only the Cry proteins would have toxic effect on *H. hampei* applied in the diet (Méndez-López *et al*., 2003; López-Pazos *et al.*, 2009) and other bacterial strains has potential to control this pest. In this sense, a strain of *Bacillus pumilus* has been patented for controlling corn rootworm, nematode, and armyworm infestations (Heins *et al.*, 1999) and different authors reports the use of this species for controlling other insects. For instance, Molina *et al*. (2010) using larvae of *Ceratitis capitata*, in bioassays with *B. pumilus* showed mortality between 68 to 94% after 15 days of inoculation in larvae of the first instar. Similarly, Yaman and Aslan (2010) in *Dendroctonus micans* (Coleoptera: Scolytinae) showed that *B. pumilus* caused 69% and 40% mortality in larvae and adults, respectively. *B. pumilus* Bp4185 showed a mortality of 54.5 to 100% between 72 h and 96 h after inoculation, with higher mortality and shorter inoculation time than that obtained by the authors mentioned above.

CONCLUSIONS

The Puerto Rican native strains *Bacillus thuringiensis* Bt3971 and *Bacillus pumilus* Bp4185 have the presence of plasmid (12 kbp). Besides, the presence of a protein with a molecular weight band of 116 kda in *B. thuringiensis* Bt3971 denote the possible presence of Cry proteins. This is the first study demonstrated the insecticidal activity of *B. thuringiensis* Bt3971 and *B. pumilus* Bp4185 on *H. hampei*. According to that both strains have potential for coffee berry borer biocontrol.

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Conflict of interest

The author declares no conflicts of interest.

Author contributions

Conceptualization MZ†, Investigation DV, CB, MZ†, Funding acquisition MZ†, Project administration MZ†, Formal analysis CB, Data curation CB, Writing—original draft DV, Writing, review and editing CB.

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