PERSISTENCE OF *Bacillus thuringiensis* AND *Bacillus pumilus* POTENTIAL BIOLOGICAL CONTROL AGENTS OF THE COFFEE BERRY BORER UNDER FIELD CONDITIONS OF PUERTO RICO

Persistencia De Cepas De *Bacillus thuringiensis* Y *Bacillus pumilus* Potenciales Controladoras Biológicas De La Broca Del Café En Condiciones De Campo En Puerto Rico

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ABSTRACT

Field trials were conducted in two years to evaluate persistence of *B. thuringiensis* and *Bacillus pumilus* under field conditions. Strains of *B. thuringiensis* (3971) and *B. pumilus* (4185) were isolated from coffee agro-ecosystem in Puerto Rico. Field trials consisted in plots of nine coffee trees arranged in a randomized complete block design, sprayed with Bt-3971 and Bp-4185 at 10^8, 10^9, or 10^{10} colony forming units CFU ml^{-1} or non-inoculated. In year 1, *B. thuringiensis* and *B. pumilus* survived up to six months after inoculation.

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Monitoring carried out during year 2, re-confirmed the survival of Bt-3971 and Bp-4185 inoculated in coffee trees under field conditions. Overall, Bt-3971 had significantly more recovery rates than Bp-4185. However, the higher bacterial recoveries were $1.24x10^{13}$ CFU ml^{-1} in coffee trees treated with Bt-3971 and $2.48x10^{13}$ CFU ml^{-1} in coffee trees treated with Bp-4185 in year 1 and year 2, respectively, both sprayed with 10^{10} spores ml^{-1}. *B. thuringiensis* (3971) and *B. pumilus* (4185) isolated from coffee fields of Puerto Rico are well adapted to the environmental conditions of the island, and are valuable biological control agents against coffee berry borer.

Keywords: Coffee Berry Borer; *Bacillus thuringiensis; Bacillus pumilus;* Colony forming Units.

RESUMEN

Ensayos de campo se llevaron a cabo en dos años para evaluar la persistencia de *Bacillus thuringiensis* y *Bacillus pumilus*. Las cepas *B. thuringiensis* (3971) y *B. pumilus* (4185) fueron aisladas del agro-ecosistema del cafeto en Puerto Rico. Los ensayos de campo consistieron en parcelas de nueve cafetos en diseño de bloques completamente aleatorizado. Los tratamientos fueron Bt-3971 y Bp-4185 a las dosis de 10^8, 10^9 y 10^{10} unidades formadoras de colonias por mililitro (UFC ml^{-1}) y parcelas no inoculadas. En el año 1, *B. thuringiensis* y *B. pumilus* sobrevivieron hasta seis meses después de la inoculación (marzo a septiembre). Los monitoreos realizados de marzo a septiembre del año 2, re-confirmaron la sobrevivencia de Bt-3971 y Bp-4185 bajo condiciones de campo en las unidades experimentales. En general, se recuperaron más aislados de los cafetos tratados con Bt-3971 comparado con Bp-4185. Sin embargo, las tasas de recuperación

de aislados más altas fueron 1.24 x 10^{13} UFC ml^{-1} en cafetos tratados con Bt-3971, y 2.48 x 10^{13} UFC ml^{-1} en cafetos tratados con Bp-4185 en los dos años respectivamente; ambos de cafetos tratados con la mayor concentración de esporas (10^{10} UFC ml^{-1}). *B. thuringiensis* (3971) y *B. pumilus* (4185) aisladas del agro-ecosistema de cafeto en Puerto Rico están bien adaptadas a las condiciones de la isla, y son valiosas cepas para el control biológico de la broca del cafeto.

Palabras Clave: Barrenador del fruto del café; *Bacillus thuringiensis; Bacillus pumilus;* Unidades formadoras de colonias.

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INTRODUCTION

Coffee is a crop of higher social, economic, and ecological importance of the mountains of Puerto Rico. The coffee zone is located in the central west, including 21 municipalities, with Adjuntas being the leader in coffee production (Rullan, 2006). Coffee production for the 2013-2014 cropping season was 10.31 metric Tons for a crop value of $ 26.3 million (Monroig-Ingles, 2015; Robles and Ferré-Sadurní, 2017). In September 2017, after Hurricane Maria tore through Puerto Rico, 90% of coffee plants were destroyed (Marino-Cardenas et al., 2018), being not only an economic collapse for coffee industry, but also a much wider and more serious social collapse in the mountainous region of the island.

The coffee berry borer (CBB), *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae), is a major threat to the coffee industry. CBB originated from Africa (Damon, 2000), and in America, it is found from Mexico to Brazil, including some of the countries of the Caribbean Bassin such as Cuba, Jamaica, the Dominican Republic, and Puerto Rico (Barrera and Lopez-Arroyo, 2007). The pest feeds and reproduces inside the coffee fruit, reducing its weight and quality, and in turn causing losses of up to 50% in the harvest if control measures are not carried out (De la Rosa et al., 2005). CBB is an elusive pest to control due to its cryptic nature. The whole life cycle of the insect occurs inside the fruit where the insect feeds and reproduces (Vega et al., 2009).

Bacillus sp. belongs to the *Bacillaceae* family. It is a rod-shaped, aerobic, gram-positive bacterium with the presence of endospores. The endospores have an ellipsoidal shape and are in the central part of the cell. These endospores are resistance structures that can remain active for long periods (Stahly et al., 1992). In addition to the endospores, the bacterium produces a para-sporal body or crystal proteins.

B. thuringiensis crystals have different forms during sporulation and are protein-based (De la Rosa et al., 2005). *B. thuringiensis* can synthesize some toxic proteins such as δ-endotoxin called Cry and Cyt. These proteins are toxic when ingested by susceptible insects, which include important agricultural pests (Bravo et al., 2007). The toxins produce a spore-crystal complex that possesses active insecticidal properties for the control of Lepidoptera, Diptera, and Coleoptera (Baró et al., 2009). When the crystal is ingested, the Cry protein dissolves in the mesenteron of the insect creating pores in the intestinal

membrane, which leads to the end of the intake and eventually dies of septicemia. *Bacillus* para-sporal crystals, formed in endospores, are highly commercialized as biocontrol of pests. *Bacillus thuringiensis* corresponds to 90% of the commerce of biocontrol agents (Kergunteuil et al., 2016). In the other hand, *Bacillus pumilus* is a Gram +, rod-shaped, spore-forming bacteria, found mainly in soils. *B. pumilus* has shown effectivity against certain insects (Molina et al., 2010).

The use of Bacillus for biological control is considered as environmentally safe (Allgeier et al*.,* 2019). Bacillus δ-endotoxins are part of the integrated pest management strategy and are an alternative to chemical insecticides for controlling pest. However habitat related factors in the field such as physic-chemical parameters, rainfall, relative humidity or UV-light can influence their ability to exert biological control. *B. thuringiensis* (Bt-3971) and *Bacillus pumilus* (Bp-4185) were recovered from coffee agro-ecosystem and are well adapted to the environmental conditions of Puerto Rico. In a previous study we determined the mortality of *Hypothenemus hampei* surpassed 80% with $1.80x10^{10}$ spores mL^{-1} of Bt-3971, and reached 100% after 96 hours post inoculation with 3.10×10^{12} spores mL^{-1} of Bp-4185. Also, large conjugative plasmids of 12 kbp (contain *cry* genes) were consistently detected in Bt-3971 and *Bacillus pumilus* Bp-4185, showing both strains to be lethal and antagonistic against CBB (Vazquez et al., 2020).

This research was conducted to establish the survival of two *Bacillus* strains, *B. thuringiensis* (Bt-3971) and *B. pumilus* (Bp-4185) applied to coffee trees under field conditions during two years in Puerto Rico.

MATERIALS AND METHODS

Field experiments were carried out at the Adjuntas Experimental Station, University of Puerto Rico (N18 ° 10.310; W 066 ° 47.612), during the years year 1 and year 2.

Bacterial strains

Inoculants were prepared using two species of *Bacillus*: *B. thuringiensis* (Bt-3971) and *B. pumilus* (Bp-4185). Bt-3971 and Bp-4185 were isolated from coffee agro-ecosystem, and conserved in silica-gel vials in the culture collection of the Department of Crop Protection-laboratory of bacteriology at the University of Puerto Rico – Mayaguez.

Preparation of the bacterial inoculant and spraying

Bt-3971 and Bp-4185 strains were grown in 1000 ml Wheaton® 351514 Nephelo Culture Flasks (Capitol Scientific, Austin, TX) with 80 ml of nutrient broth: D(+)-glucose, 1 g l^{-1}; peptone, 15 g l^{-1}; sodium chloride, 6 g l^{-1}; yeast extract, 3 g l^{-1}, at an initial absorbance of 0.05. Four Nephelo-flasks were used for each bacterial treatment (320 ml of inoc./treat.). Nephelo flasks were incubated at 28 ºC in an orbital shaker for 24 h, 48 h, and 96 h in Bt-3971 to reach 10^8, 10^9 and 10^{10} CFU ml^{-1}, respectively, and 9 h, 18 h, and 36 h in Bp-4185 to reach 10^8, 10^9 and 10^{10} CFU ml^{-1}, respectively.

In year 1, inoculations were made on March 13 (Bt-3971) and March 19 (Bt-4185); and March 23 (Bt-3971) and May 3 (Bp-4185). In year 2, inoculations were made on March 16 (Bt-3971) and March 30 (Bp-4185); April 6 (Bt-3971) and April 13 (Bp-4185); and June 8 (Bt-3971) and June 15 (Bp-4185).

The experimental design consisted in a randomized complete block design (RCBD) with seven treatments and six replicates. The experimental plot had nine trees, and each block was separated by a row of border trees. Measurements of viable counts of total and *Bacillus*-type bacteria were conducted in three selected trees at each plot. The treatments were Bt-3971 incubated for 96 h (10^{10} CFU ml^{-1}), 48 h (10^{9} CFU ml^{-1}), and 24 h (10^{8} CFU ml^{-1}) and; and Bp-4185 incubated for 36 h (10^{10} CFU ml^{-1}), 18 h (10^{9} CFU ml^{-1}), and 9h (10^{8} CFU ml^{-1}), and non-inoculated trees (check). Bacterial inoculants were mixed with distilled water in polypropylene bottles (Nalgene®) to reach 6 liters of diluted nutrient broth. Inoculations one and two were made with a total of 14 liters of bacterial solution applied by each treatment. The volume of application was 260 ml / tree applied for 14 seconds covering the entire surface of the tree. In the third inoculation of year 2, the amount of bacterial inoculum was increased from to 400 ml / tree for each bacterial treatment (from 14 liters to 16 liters), since the height of the trees increased.

A CO_2 backpack sprayer was used to apply the bacterial inocula. The sprayer was set at a constant pressure of 30 PSI. Bacterial inocula were placed in approx.18 liter (5-gallon) stainless steel tanks. Boom sprayer had a lance with a bronze Tee-Jet nozzle of the solid conical type (TG-2). Nine trees per replicate were selected at each experimental plot.

Monitoring and survival of aerobic endospore-forming bacteria

To determine the survival of bacteria in coffee trees under field conditions, collections of bark and fruit tissues were carried out (12 random samples, taken four from each tree) at 30, 60, 120 and 210 days after the first inoculation. Bacteria were isolated from bark

tissue of coffee trees the day before the first inoculation from a random experimental unit from each block. A 2.5 cm cut was made on the tree branch one meter above the ground using a sterile scalpel. In June and September, sampling was done taking six green fruits instead of bark tissue. Bark tissues and fruits were placed in plastic bags and transported to the laboratory on ice.

The tissues were cut into pieces of approximately 0.4 cm in diameter and placed in 9 ml tubes with nutrient broth to make dilutions. Serial dilutions were made until 10^{-10}. Then, 0.1 ml (100 µl) were transferred to plates with nutrient agar (beef extract, 1 g; yeast extract, 2 g; peptone, 5 g; NaCl 5 g; agar, 15 g) added with 2% cyclohexamide. Counts of bacterial colonies present in the plate after 72 hours were used to calculate the number of colony forming units per ml (CFU ml^{-1}).

After the first inoculation, a monitoring of bark tissues was carried out at 14 days to determine the survival of the inoculated bacteria. Two replicates of each bacterial species (*B. thuringiensis* and *B. pumilus*) were used. A total of eight samples (one sample per tree) of approximately 2.5 cm was taken at each plot. The method described above was used for dilutions and counting. For each sample, three repetitions were performed, including a laboratory control and an internal control for Bt and Bp. CFU ml^{-1} were transformed to Log_{10} to perform the ANOVA and LSD test ($\alpha = 0.05$). Non-inoculated trees were not used for ANOVA. T-test was used to determine differences between non-inoculated and bacteria-inoculated trees/plots.

Once the bacterial colonies emerged, they were purified and classified by size, morphology, configuration, margin, elevation and color. Also, biochemical tests of KOH and differential staining were performed on all white and creamy colonies with the

potential to be *Bacillus*. Bacteria were separated as aerobic endospore-forming bacteria and were contrasted against the total number of bacterial isolates detected using χ^2 chi-squared test. Different bacterial strains were isolated and placed in vials with 2.5 ml nutrient agar, and in vials with 1 ml of silica gel to preserve the strains at -20^{o}C.

RESULTS AND DISCUSSION

Total counts of bacterial isolates recovered from bark and fruits of coffee tissues were not significantly different ($P > 0.05$; χ^2). The time of incubation was a major difference between both isolates. Bt-3971 reached 10^8, 10^9 and 10^{10} CFU ml^{-1} after incubation for 24 h, 48 h, and 96 h, respectively. Meanwhile, under the same growth conditions, Bp-4185 reached 10^8, 10^9 and 10^{10} CFU ml^{-1} after 9 h, 18 h, and 36 h of incubation, respectively. This difference in growth rate was constant in the inoculations made in year 1 and year 2 in this study.

A total of 663 bacterial isolates were recovered from coffee trees inoculated with Bt-4185 and Bp-3971. These isolates were separated based on their colony color, Gram reaction, and presence of endospores. *Bacillus*-type (Gram+, KOH-, spore+) bacteria were 194 out of the 663 total isolates. At the beginning of the experiment, before the first inoculation, bacteria were isolated from bark tissue of coffee trees the day before the first inoculation. A total of 13 strains were aerobic endospore-forming bacteria. Total counts of bacteria from bark tissue before the first inoculation are summarized in Table 1.

Table 1. Total counts of bacteria isolated from bark tissue of coffee trees in experimental plots the day before the first inoculation, March year 1.

Field block	CFU ml^{-1}	CFU ml^{-1}	Logarithmic Mean

1	3.00 x10^{7}	7.48	9.15
2	2.14 x10^{9}	9.33	
3	3.00 x10^{10}	10.48	
4	2.00 x10^{10}	10.31	
5	7.00 x10^{7}	7.85	
6	3.00 x10^{9}	9.48	

In March year 1 and year 2, after the first inoculation, *Bacillus*-type bacteria were recovered from coffee trees; however, in September year 1 (six months after the last inoculation), as well as in September year 2 (after performing the third inoculation), *Bacillus*-type bacteria was higher and consistently present.

In year 1, the highest bacterial recovery was 1.24x10^{13} CFU ml^{-1} in coffee trees treated with Bt-3971 at 10^{10} spores ml^{-1}, and 4.9x10^{12} CFU ml^{-1} in coffee trees treated with Bp-4185 at 10^{9} spores ml^{-1}, both after the second inoculation in June. In year 2, the highest bacterial recovery was 1.87x10^{13} CFU ml^{-1} in coffee trees treated with Bt-3971 at 10^{8} spores ml^{-1}, after the first inoculation (March year 2), and 2.48x10^{13} CFU ml^{-1} in coffee trees treated with Bp-4185 at 10^{10} spores ml^{-1}, after the third inoculation (Table 2).

Table 2. Averages of recovery for colony forming units CFU ml^{-1} in coffee trees sprayed with *B. thuringiensis* (Bt-3971) and *B. pumilus* (Bp-4185) at a concentration of 10^8, 10^9 and 10^{10} spores ml^{-1}.

			CFU ml^{-1}							
			March		April		June		September	
Bacillus	Treatment		year 1	year 2	year 1	year 2	year 1	year 2	year 1	year 2
	hours	C	*bark*		*bark*		*fruit*		*fruit*	
Bt-3971	24	10^8	$3.00x10^{11}$	$1.87x10^{13}$	$3.00x10^{11}$	$4.40x10^{12}$	$3.43x10^{12}$	$1.06x10^{12}$	$3.35x10^{9}$	$1.20x10^{12}$
Bt-3971	48	10^9	$4.96x10^{12}$	$2.23x10^{12}$	$6.00x10^{11}$	$4.07x10^{12}$	$3.57x10^{12}$	$4.50x10^{10}$	$3.04x10^{9}$	$3.50x10^{10}$
Bt-3971	96	10^{10}	$5.00x10^{9}$	$8.00x10^{11}$	$4.00x10^{11}$	$1.50x10^{12}$	$1.24x10^{13}$	$4.35x10^{12}$	$1.48x10^{10}$	$1.70x10^{12}$
Bp-4185	9	10^8	$3.00x10^{10}$	$4.70x10^{11}$	$3.00x10^{11}$	$7.00x10^{11}$	$1.77x10^{12}$	$4.70x10^{12}$	$6.75x10^{10}$	$1.00x10^{8}$
Bp-4185	18	10^9	$4.00x10^{11}$	$3.70x10^{11}$	$1.00x10^{12}$	$1.00x10^{11}$	$4.90x10^{12}$	$5.20x10^{12}$	$4.60x10^{10}$	$3.00x10^{11}$
Bp-4185	36	10^{10}	$9.00x10^{11}$	$6.70x10^{11}$	$4.00x10^{10}$	$5.37x10^{12}$	$1.80x10^{12}$	$7.95x10^{12}$	$3.20x10^{10}$	$2.48x10^{13}$

Averages of reps = 6.

Values per experimental plot are the average of three trees

Values represent total bacterial counts in plate by the inverse of the dilution

The rates of Bp-4185 sprayed and total recovery were consistent in both years, the higher inoculum concentrations (10^{10} spores ml^{-1}), the higher bacterial recoveries. The total bacteria recovery for Bp-4185 inoculated with 10^{10} spores ml^{-1} was $4.16x10^{13}$ CFU ml^{-1}, meanwhile, with 10^{8} spores ml^{-1} the total bacteria recovered was $8.04x10^{12}$ CFU ml^{-1}. In Bp-3971, the highest recovery was detected in the 10^{8} spores ml^{-1} treatment with $2.94x10^{13}$ CFU ml^{-1}; however, this value was in the same range than the treatment 10^{10} spores ml^{-1} with $2.12x\ 10^{13}$ CFU ml^{-1}.

The highest bacterial recovery in trees treated with Bt-3971 was in June for both years (year 1 and year 2). June year 1 was exceptionally dry and hot; therefore, bacterial spores which can resist desiccation help *Bacillus*-type bacteria to strive under these conditions. *Bacillus* spores allow the bacterium to preserve and escape from harsh environmental conditions (Nicholson, 2002).

The first and second evaluations were performed in bark tissue, and the third and fourth evaluations were performed in fruit tissue. This differentiation in collected tissue was done because March and April are months of extensive vegetative growth of coffee trees. Flowering is a phenotypic response to changes in water availability, air temperature and solar brightness, a coffee flower is open after 4 to 5 months of development. In countries such as Puerto Rico with a very marked rainy season, coffee trees tend to bloom and concentrate harvest in a specific time of the year; however, it is not uncommon to find trees with blooms and grains at different points of maturity.

Logarithmic means of the *Bacillus*-type bacteria of bark and fruit coffee tissues were used to compare the population levels at each sampling time.

Non-inoculated trees were used to establish if the inoculation increases the number of CFU of Bacillus-type bacteria. In June year 1, averages of $3.02x10^{9}$ and $4.65x10^{12}$ CFU ml^{-1} were detected in non-inoculated and inoculated trees, respectively (P = 0.0632), no significant differences were detected between non-inoculated and inoculated trees in March, April, and September year 1 due to a high degree of variability in bacterial recoveries. In year 2, significant (April, T-test P = 0.0004; and May, T-test P = 0.0012) and highly significant (June, T-test P < .0001) differences were detected between non-inoculated and inoculated trees/plots. Log CFU/ml showed significantly higher number of Bacillus-type bacteria in Year 1 (June evaluation), and year 2, before the second inoculation (March), and consistently after the second inoculation in the evaluations done on April and June (Figure 1).

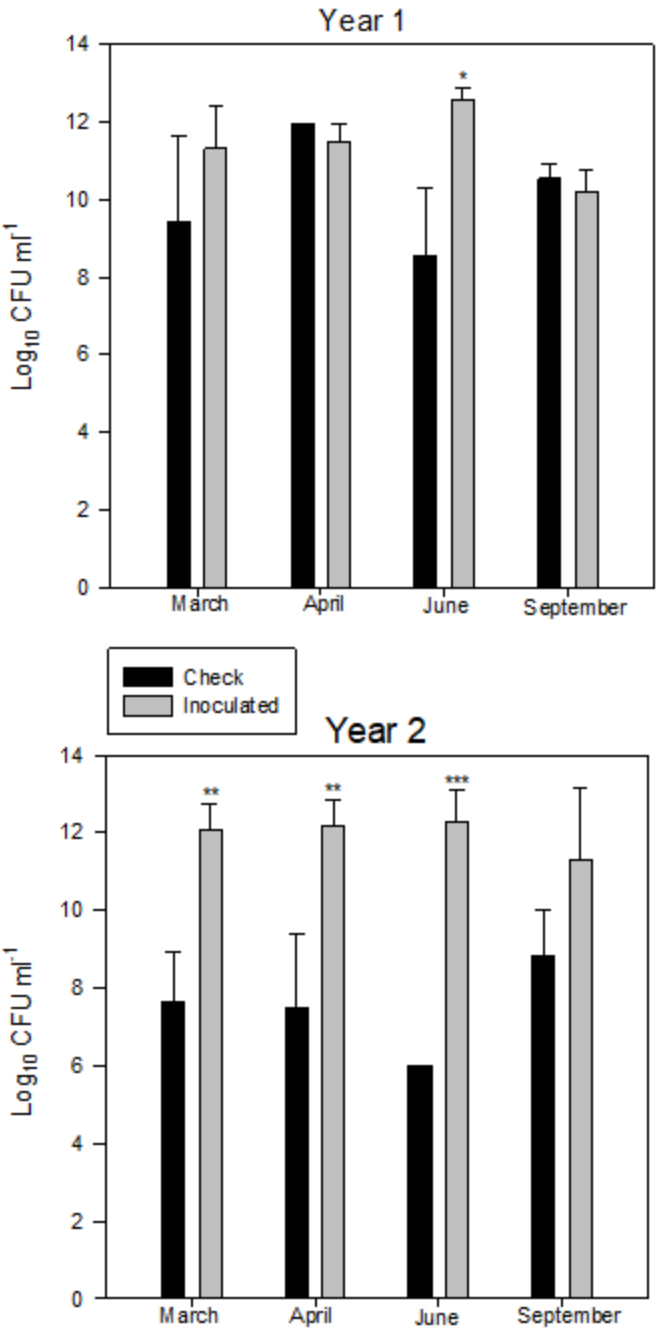

Figure 1. Means of Bacillus-type (aerobic, bacilli, endospore forming) bacteria from non-inoculated compared to Bacillus –inoculated plot/trees in year 1 and year 2 (Asterisks denote significant differences for T-test * P <0.10; ** P <0.01; *** P < 0001).

In year 1, Bt-3971 and Bp-4185 increased in total counts after the first inoculation (March). However, only in Bp-4185 a significant increase was detected (Figure 2).

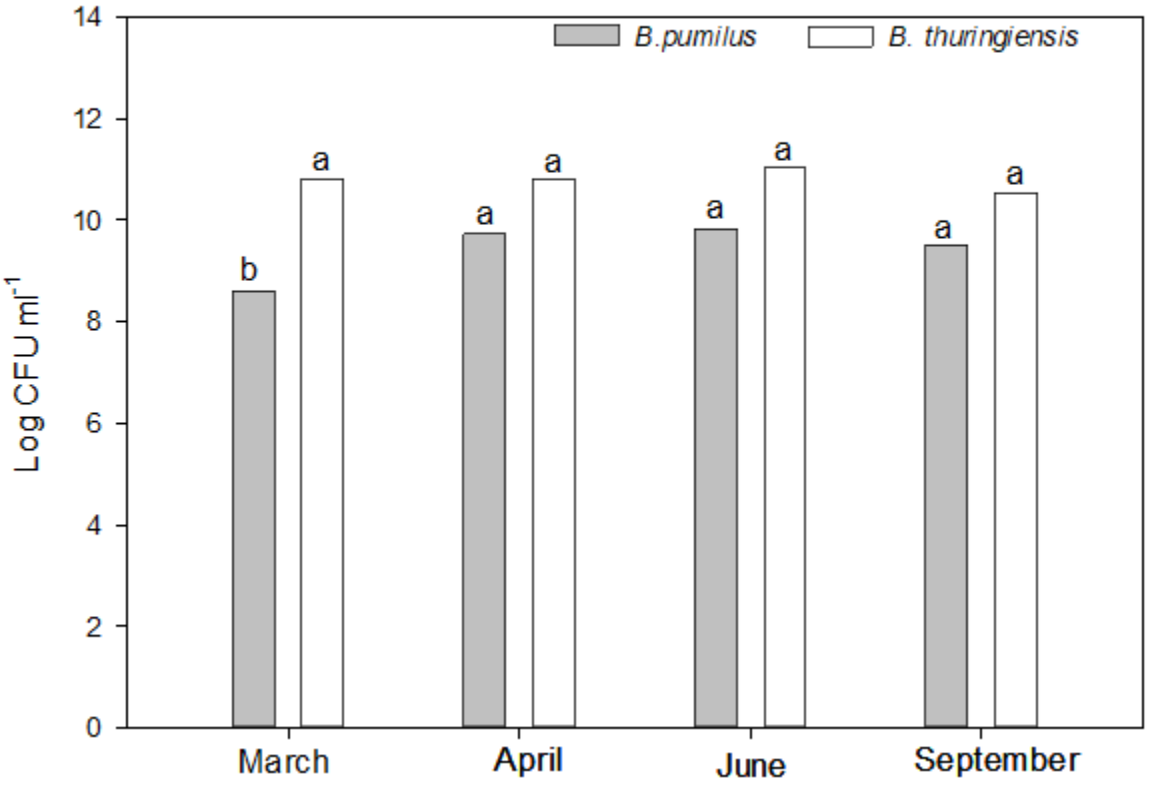

Figure 2. Bacillus-type bacterial recovery in coffee trees sprayed with *B. thuringiensis* (Bt-3971) and *B. pumilus* (Bp-4185) under filed conditions of Puerto Rico in year 1.

After the initial increase, *Bacillus* populations remained constant. Although *B. thuringiensis* had consistently higher counts than *B. pumilus*, these differences were not statistically significant, except for the first sampling conducted in March year 1 (Figure 2).

In year 1, samplings conducted from March to September determined the survival of Bt-3971 and Bp-4185 with a maximum of 6 months under field conditions in Adjuntas, P.R.

Total counts of *Bacillus*-type bacteria recovered in year 2 are summarized in Figure 3. Bt-3971 had significantly more recovery rates than Bp-4185 after the first inoculation conducted in March, and at the last monitoring conducted in September.

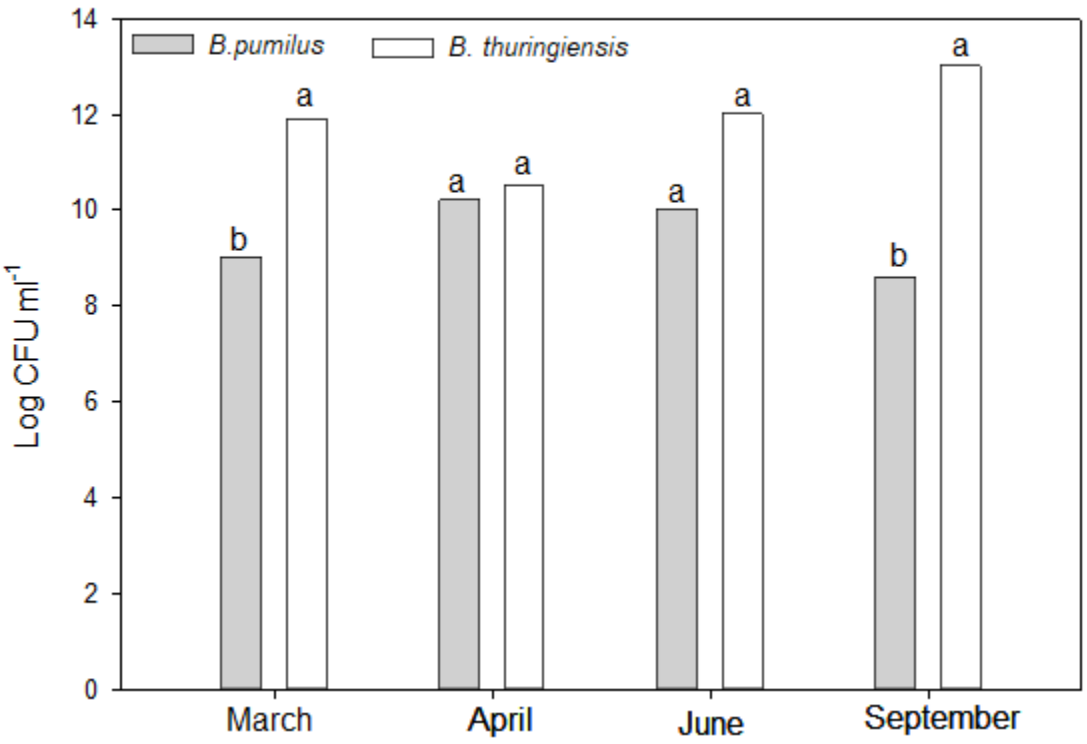

Figure 3. *Bacillus*-type bacterial recovery in coffee trees sprayed with *B. thuringiensis* (Bt-3971) and *B. pumilus* (Bp-4185) under filed conditions of Puerto Rico in year 2.

In year 2 *Bacillus*-type recoveries were consistently higher than in year 1. This difference is because in year 1 two inoculations were conducted, while in year 2 a third inoculation was made in June. It is uncertain the reason why the levels of Bp-4185 were significantly

lower in September year 2 after the third inoculation (Figure 3). This reduction could have been caused by rain registered after inoculation. In the other hand, the levels of Bt-3971 remained constant and were higher in September compared to June.

In year 1 and year 2, throughout the monitoring of *Bacillus*-type bacteria in coffee trees, we established the survival capacity of the strains of *B. thuringiensis* Bt-3971 and *B. pumilus* Bp-4185. The laboratory tests used in this study allowed us to corroborate the presence of aerobic endospore forming bacteria inoculated in coffee trees. Survival of Bt-3971 and Bp-4185 was determined by a significant increase after the second (year 1) and third inoculation (year 2). Also, according to the means reflected in the monitoring carried out between the months of March to September year 2, it could be inferred that *B. thuringiensis* and *B. pumilus* were present in the experimental plots after six months of inoculation. According to Sánchez and Peña (2000) there is little information about the ecology of *Bacillus thuringiensis*, in relation to vegetative cells and spores on plant tissues, specifically in the persistence of spores and the stability of protein crystals. This is because the viability of spores and crystals are affected by the germicidal action of visible and UV light. However, Smith and Couche (1991) reported in pine needles that *B. thuringiensis* spores can persist for more than seven months in shaded environments. Based on what was reported by Smith and Couche (1991); it is deduced in our study that both the vegetative cells and spores of *B. thuringiensis* and *B. pumilus* persisted under field conditions in coffee trees.

I. CONCLUSIONS

This study contributes with knowledge about the persistence of two *Bacillus* isolates, *B. thuringiensis* 3971 and *B. pumilus* 4185, with potential for biocontrol of coffee berry borer. Thanks to their ability to form endospores, both isolates can persist up to six months under field conditions of Puerto Rico.

The advantage of using isolates of Bacillus that are adapted to field conditions where they strive is reflected in a greater abundance which give them competitive advantage over other microbiota for biological control of pest.

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