Article

Effect of *Beauveria bassiana*-Seed Treatment on *Zea mays* L. Response against *Spodoptera frugiperda*

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Abstract: *Spodoptera frugiperda* is a widely distributed insect pest that causes major economic losses in various crops, particularly maize. On the other hand, *Beauveria bassiana* is an entomopathogenic fungus that establishes symbiotic associations with many plants and contributes to tolerance against biotic and abiotic stresses. In the present work, in laboratory experiments, the effects of the *B. bassiana* strain GHA, in addition to a native strain (PTG4), delivered via seed treatment in maize seedlings, were evaluated on *S. frugiperda* growth, development, and mortality. We inoculated maize seeds with 1×10^6 *B. bassiana* blastospores; then these seeds were germinated and grown to seedlings under growth chamber conditions. Third-instar *S. frugiperda* larvae were allowed to feed on *B. bassiana*-treated and -untreated (negative control) seedlings until reaching the sixth instar and transferred to an artificial diet until reaching adult stage. Results showed that larvae feeding on *B. bassiana* strain PTG4-treated plants prolonged their larval stage. Furthermore, feeding on plants treated with *B. bassiana* strains yielded fewer *S. frugiperda* male moths compared with feeding with the untreated control plants. Under field conditions, 1×10^6 (first trial) and 1×10^8 (second trial) of *B. bassiana* (GHA strain) blastospores were used for corn seed inoculation. In the first field trial, there were a higher number of larvae in the negative control plants compared to those in the plants treated with *B. bassiana*. No larvae were found in negative control and *B. bassiana*-treated plants in the second field trial. In conclusion, seed treatment with *B. bassiana* in maize reduced *S. frugiperda* infestation of maize plants in field trials. *S. frugiperda* development was also affected in laboratory trials.

Keywords: *Beauveria bassiana*; biological control; entomopathogenic fungi; *Spodoptera frugiperda*; *Zea mays*

1. Introduction

Worldwide, about an 18–26% reduction in crop production is due to insect pests, which mostly occurs in the fields before harvest [1]. The Fall armyworm *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae), from the tropical and subtropical zones of America [2], is a catastrophic insect pest of economic importance [3]. This voracious insect has a polyphagous feeding nature in more than 80 host species, including many commercial crops such as maize, cotton, rice, soybean, bean, and other crops from the Gramineae family [3–5]. Until 2015, damage due to *S. frugiperda* was reported only in America [6], but in the last few years, attacks have been reported in other parts of the world [5]. In late 2016 they were reported in Southern, Eastern, and Northern parts of Africa [6], which briskly expanded across the continent and, by late 2018 they were confirmed in almost 44 African countries [3]. By 2018, the presence of this insect pest was confirmed in Yemen and India,

and by 2019, devastation due to this pest was confirmed in five more Asian countries, including China [7]. The destruction generated by *S. frugiperda* relies on the geographic region, seed variety, planting time, and fundamental cultural habits in and around the field, although abiotic factors have an effect on egg and initial larval stage mortality during a rainy season, and with various predators during a dry season [4].

S. frugiperda is treated as a crucial insect pest of maize, which is the third most essential cereal crop worldwide with the highest economic value in terms of production and nutrition [6]. It causes extensive damage to maize plants by feeding on young leaf whorls, corn cobs, and tassels [3]. Younger larvae prefer epidermal leaf tissue and produce holes in them, which is the typical damage sign by these insect pests. Deadheart is caused by feeding on young plants through the whorls. Older larvae in the whorls of grown-up plants feed on cobs or kernels, which reduces yield quality and quantity [2]. The presence of various generations, their migration, and the potential to feed on a vast range of host plants make this insect one of the most difficult pests to control [2].

Synthetic/chemical insecticides or genetically modified crops have been used to control insect pests [2]. Although these control measures are very efficient, their extensive usage has caused ecological issues, environmental contamination, development of resistance, and detrimental effects on human health [8]. Since the insects have gained resistance to various chemical insecticides, farmers are compelled to use recurrent application of large amounts of insecticides, which will lead to the accumulation of chemicals in agricultural fields [2]. Taken together, scientists in different parts of the world are forced to develop more environmentally safe, cost-effective, and reliable strategies to control insect pests [1].

The Integrated Pest Management (IPM) global program for agriculture is a holistic concept for approaching the crop production system as a whole process, rather than focusing only on pest elimination. This approach combines various techniques, including the use of resistant varieties, cultural manipulation, trap cropping, and biological control [1]. The latter is one of the techniques to control insect pests with the slightest environmental impact [9]. Cost-effectiveness, high yield, not being harmful to beneficial insects, and the release of fewer chemical residues into agricultural fields make entomopathogenic microorganisms excellent alternatives to chemical pesticides [1]. At present, different bacteria (*Bacillus* spp., *Enterobacter* spp., *Pseudomonas* spp. *Paenibacillus* spp., etc.) and fungi (*Beauveria* sp., *Metarhizium* sp., *Paecilomyces* sp., *Isaria* sp., *Lecanicillium* sp., *Hirsutella* sp., etc.) are being applied as biocontrol agents [1,9]. The potential of entomopathogenic fungus to adjust to external habitats other than their original habitats has made them very efficient and adequate candidates for biological control measures [10]. Considered as facultative microorganisms that do not require arthropods as hosts to complete their life cycles, *Metarhizium anisopliae* and *Beauveria bassiana* are the best characterized and most applied entomopathogenic fungi in biological control programs [11].

The hypocrealean fungus *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) [12] has shown negative effects on 33 species of insects belonging to eight orders [13]. It implements integral protection against insect pests or constrains their growth and proliferation [12]. This fungus has also exhibited dual protection ability against *Rhizoctonia solani* and *Pythium myriotylum* in *B. bassiana*-treated tomato seedlings [11]. Distinct studies showed thant *B. bassiana* may be used as an effective biocontrol agent against *Helicoverpa armigera* in broad bean [14] and *Helicoverpa zea* in cotton and tomato [15,16]. Feeding on *B. bassiana*-treated maize plants reduced *Sitobion avenae* survival and fecundity in single aphids [13]. In corn plants, *B. bassiana* showed insecticidal activity against many lepidopterans including *Sesamia calamistis* [17] and *Ostrinia nubilalis* [18]; and when it was isolated as endophyte, against *Spodoptera frugiperda* [19]. The mortality level of target insect pests with *B. bassiana* depends on larval developmental stage [20], inoculation method [21], or fungal strains used [22]. As an endophyte, *B. bassiana* mostly does not induce direct mortality in insect pests but often reduces larval growth rate, weight, or longevity [23,24].

Taken together, the aim of the present study was to evaluate the effect of *Beauveria bassiana*-seed treatment on *Zea mays* L. against *Spodoptera frugiperda* larval activity under laboratory and field conditions.

2. Materials and Methods

2.1. Insect, Plant, and Fungal Strains for Laboratory Assays

Insect colony: *S. frugiperda* eggs were kindly donated by José Refugio Lomelí-Flores (Colegio de Postgraduados, Montecillo, Texcoco, Estado de México, México). They were carefully placed into 700 mL plastic bottles, and kept in the breeding room under controlled conditions (temperature 27 °C ± 3 °C, humidity 60% ± 5%, and photoperiod 14 h light: 10 h dark) until hatched. Neonates were then transferred to individual diet cups with 5 mL modified artificial wheat germ diet [25] as their food source (Figure S1). This diet was replaced when necessary to prevent desiccation. To perform bioassays, we used *S. frugiperda* larvae belonging to the second laboratory generation.

Plants: *Zea mays* plants were used in this study. *Zea mays* Chalqueño seeds were obtained from Centro Internacional de Mejoramiento de Maíz y Trigo (CIMMYT) and Verónica Garrocho-Villegas, Universidad Nacional Autónoma de México (UNAM), México.

Fungal strains: *B. bassiana* GHA strain, commercially obtained as Botanigard®22WP and PTG4 strain, (GenBank accession number KC759730.1, isolated from *Periplaneta americana*) kindly provided by Patricia Tamez, from Universidad Autónoma de Nuevo León (UANL), México were stored at −80 °C in a So-Low Ultra freezer (Environmental Equipment, Cincinnati, OH, USA).

2.2. Inoculation of Z. mays Seeds with B. bassiana

B. bassiana strains were activated by plating the stock cultures onto potato dextrose agar (PDA, BD Difco, México) and incubated in darkness at 25 °C ± 2 °C for 1 week. To achieve a monosporic culture, a single selected colony was inoculated into a 500 mL Erlenmeyer flask, containing 200 mL of potato dextrose broth (PDB, BD Difco) and kept at 25 °C ± 2°C on an automatic rotary shaker (Orbit1900, Labnet, México) at 120 rpm, for five days or until blastospores production. Blastospores counts were determined in a hematocytometer chamber and adjusted to a concentration of 1×10^6 spores/mL. Methyl cellulose (MC) (Sigma-Aldrich, St. Louis, MO) was mixed with the blastospores for adequate attachment to the seeds. MC was prepared by dissolving the reagent in warm distilled water at 35 °C to 40 °C to a pre-gelatinized state [26]. Seeds (20 seeds/treatment/strain) were then added, evenly mixed, and placed on a flat surface to dry at 25 °C ± 2 °C for 24 h. Controls (CC) included seeds without any treatments (nor fungi, neither adherents), and seeds without fungi but with MC (CMC). MCGHA, MCPTG4, CMC, and CC seeds were sown individually into commercial soil (Happy Flower Mexicana, S.A. de C.V, Ciudad de México, México) previously autoclaved, which was contained in 250 mL plastic cups and kept at 25 °C ± 2 °C for 10 days after germination.

2.3. Bioassays under Laboratory Conditions

Each third instar *S. frugiperda* larva (Figure S1) was carefully transferred onto a *Z. mays* plant (10 days old) for each treatment (one larva/plant, to avoid cannibalism) and then covered with a mesh bag to prevent escapes (Figure S2). Plants were replaced every 24 h. When larvae reached the sixth instar, they were returned to the artificial diet to monitor pupal stage development. Each pupa was examined under a stereoscope (Labomed Stereomicroscope, Luxeo2S, CA, USA) to determine its sex, weighed on a microbalance (A&D Company Limited, N–92, Tokyo, Japan), and measured for length with a standard, scholastic ruler. After that, pupae were transferred to individual plastic containers (7 cm diameter × 16 cm height, covered with mesh bags), separating male and female pupae. In the lower part of the container, a piece of cotton embedded in sugar syrup was provided as a food source for the adult moths. Containers were analyzed every day to check for adult emergence. Pupae were maintained under the most suitable laboratory conditions

(25 °C $\pm$ 2 °C temperature, 60% $\pm$ 5% relative humidity, and 14:12 h light and dark photoperiod). The following parameters were recorded: (a) Initial larvae numbers, (b) the number of dead larvae during the experiment, (c) the number of larvae that remained as larvae, even after reaching the sixth instar of development, (d) the number of larvae that remained as prepupa, (e) the number of larvae that reached the pupal stage at the most frequently reported time, f) larval weight before transferring back to artificial diet, g) pupal weight, h) pupal length, and i) pupal sex ratio.

2.4. Field Trial: Experiment 1

Field trials were performed in an agricultural field located in General Teran, Nuevo Leon, Mexico with a geographical location of 25°16′00.0″ N 99°41′00.0″ W. The first trial was started in mid-February 2019 as an early season corn. The main agricultural products of this region include citrus, corn, sorghum, wheat, livestock, among others. In this zone, maize crops are generally affected by *S. frugiperda*, and a phytosanitary alert program is implemented for the three corn cultivation cycles (early, intermediate and late) of the region (peaks of *S. frugiperda* expected in March, May and September) [27]. Since farmers normally used *Bacillus thuringiensis* to control them, no artificial inoculation of *S. frugiperda* was done in any of the field experiments. One-hectare field was prepared using a tractor; 38 furrows of 100 m length and 25 furrows of 80 m length were made, with a row-to-row distance of 80 cm. Using the seed inoculation procedure, mentioned in Section 2.2, 3300 *Z. mays* seeds of "criollo maize" race (kindly donated by the field owner, without any insecticides or fungicides) were inoculated with *B. bassiana* strain GHA, with a concentration of 1×10^6 blastospores/mL and methylcellulose as adherent; 2500 seeds were used as a negative control without any fungal or adherent treatment. Seeds were planted during mid-February 2019 on the furrows, with a separation of 25 cm between each seed, the distance between each row was 80 cm, and in each row, 100 seeds were planted, which were monitored every week. The first 33 furrows were used to plant *B. bassiana*-treated seeds, then five furrows were left without seeds, and the remaining 25 furrows were used to plant negative control treatments (Figure S3). In this experiment, the germination percentage at the third week after planting was recorded, and the presence of *S. frugiperda* larvae between the fifth true leaf and the tenth true leaf period was monitored by visually scouting. Other than watering every day, neither fertilizer nor pesticides were applied in the field during the whole experiment.

2.5. Field Trial: Experiment 2

The second field trial was started in mid-April 2019 as an intermediate season corn. Due to lack of farm space, negative control plants from the February experiment were cleared out, and *B. bassiana*-maize plants of the first trial were maintained aside until harvest time. We inoculated 500 "criollo maize" race seeds with *B. bassiana* GHA strain with a concentration of 1×10^8 blastospores/mL (the concentration was increased with the aim to observe major effects on *S. frugiperda* population than the observed in the first trial as an increase on insect peaks were expected for May) and methylcellulose as an adherent, and kept 500 seeds as a negative control without any fungal or adherent treatment. The seeds were planted as in the first field experiment (Figure S4). Germination percentage and the presence of *S. frugiperda* were analyzed in the whole field, whereas other parameters such as plant height and number of leaves were evaluated in the second and fourth furrows in both treatments. Germination was recorded in the third week after planting. Plant height and number of leaves were recorded in the fourth week after planting. Presence of *S. frugiperda* was monitored between the fifth and tenth true leaf period. To assess for yield effects during harvest, five corn cobs from each treatment were randomly collected, weighed (A&D Company Limited, N-92, Tokyo, Japan), and their lengths were measured with a normal scholastic ruler.

2.6. Data Analysis

Prior to statistical analysis, the values of the effect of *B. bassiana* on *Z. mays* plant germination percentage and the effect of *B. bassiana* treated plants on the percentage of each developmental stage of the larvae were arcsine transformed for normalization. Data from three biological replicates were subjected to one-way ANOVA using the software IBM SPSS Statistics, version 21. Before ANOVA, all data were tested for homogeneity of variance using Levene's test. When a significant F value was obtained after ANOVA, post-hoc Duncan's multiple range tests were performed. Considering that there were only two groups to analyze the germination data of field trial Experiment 1 and corn cob data from field trial Experiment 2, Independent sample *T*-test analysis was used. Significance levels were calculated by Levene's test for equality of variance. To interpret the putative changes in the frequency of *S. frugiperda* larva found in the first field trial, an analysis with a Generalized Linear Model (GLM), fitted with a negative binomial distribution, was used. Pearson chi-square value > 0.05 indicated goodness of fit of the data. Omnibus test with $p < 0.05$ indicated significant differences. The results of the Test of Model effects Type III were reported.

3. Results

3.1. Effect of B. bassiana on Z. mays Plant Germination

After 10 days, seedling germination percentages were determined. Compared with the absolute negative control (CC) values, the negative control with only the adherent methylcellulose (CMC) and both treatments with *B. bassiana* strains (MCPTG4 and MCGHA), showed no significant difference ($F_{(3, 11)} = 0.189$, $p = 0.901$) among them in the germination of *Z. mays* seeds (Figure 1).
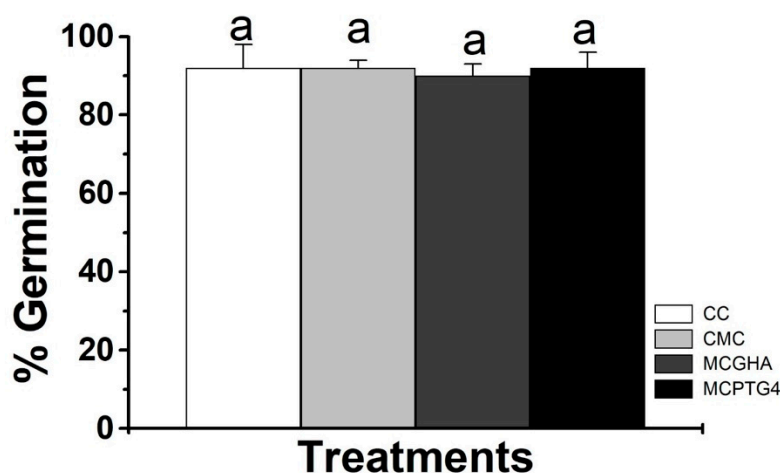

Figure 1. *Zea mays* plants germination percentage after 10 days of sowing with the different treatments. CC: Negative control, CMC: Negative control with only methylcellulose, MCPTG4: *B. bassiana* fungal strain PTG4 with methylcellulose, MCGHA: *B. bassiana* strain GHA with methylcellulose. Analysis done with Duncan's Post hoc multiple range test ($\alpha = 0.05$) after one-way ANOVA. Graphical bars with the same letters indicate that there were no significant differences among them.

3.2. Effect of B. bassiana Treated Plants on S. frugiperda Developmental Stages

The development, survival, and mortality of *S. frugiperda* larvae fed on untreated *Z. mays* plants and *B. bassiana*-treated *Z. mays* plants are shown in Table 1. Each experiment started with 10 larvae per treatment, except in CMC, which had 11 larvae. The final percentage was calculated based on the number of larvae left after some escaped during the experiments. *S. frugiperda* larvae fed on *B. bassiana* strain PTG4-treated plants markedly had changes in their life cycle. Statistical analysis with one-way ANOVA showed significant differences in the mean percentage values ($F_{(3, 11)} = 20.657$, $p < 0.001$) of larvae that remained as larva during the experiment and pupa ($F_{(3, 11)} = 5.170$, $p = 0.028$). However, there were

no significant differences among the number of dead larvae ($F_{(3, 11)}$ = 0.88, p = 0.491) and prepupa observed ($F_{(3, 11)}$ = 1.381, p = 0.317).

Table 1. Effects of feeding on *Beauveria bassiana*-treated plants on the developmental stages of *Spodoptera frugiperda*.

Stages	CC (30 Larvae)	CMC (33 Larvae)	MCPTG4 (30 Larvae)	MCGHA (30 Larvae)
Initial larva	100% [a] * (30/30)	100% [a] (33/33)	100% [a] (30/30)	100% [a] (30/30)
Dead larva	6.67% [a] (2/30)	3% [a] (1/33)	7.410% [a] (2/27)	0 [a]
Still larva	3.33% [a] (1/30)	3% [a] (1/33)	22% [b] (6/27)	0 [a]
Prepupa	6.67% [a] (2/30)	3% [a] (1/33)	7.41% [a] (2/27)	0 [a]
Pupa	83.33% [a,b] (25/30)	91% [a,b] (30/33)	62.96% [a] (17/27)	100% [b] (22/22)

* Values followed by the same letters are not significantly different and with different letters are significantly different after running Duncan's post-hoc multiple range test (p = 0.05). CC = Negative control without any treatments or adherents, CMC = Negative control with only methylcellulose, MCPTG4 = Methylcellulose with *B. bassiana* strain PTG4, MCGHA = Methylcellulose with *B. bassiana* strain GHA. Several larvae escaped: three larvae in treatment MCPTG4 and eight larvae in treatment MCGHA.

S. frugiperda sixth instar larvae fed on both *B. bassiana* treated and no-treated plants were weighed before transferring them back to the artificial diet. Data showed that larvae fed on plants treated with *B. bassiana* strain PTG4 weighed significantly ($F_{(3, 48)}$ = 4.813, p = 0.005) different than the other treatments (Figure 2). *S. frugiperda* pupal weight showed a significant ($F_{(3, 97)}$ = 3.753, p = 0.014) difference among larvae fed on GHA *B. bassiana* strain-treated plants, negative controls, and *B. bassiana* PTG4 strain fed larvae (Figure 3). *S. frugiperda* pupal length showed a significant ($F_{(3, 98)}$ = 4.491 p = 0.005) difference between the larvae fed on GHA *B. bassiana*-treated plants and all other treatments (Figure 4). Pupae sex ratio was determined by calculating the percentage of male and female pupae observed. Results showed a significant difference among treatments for pupae male ($F_{(3, 11)}$ = 7.033, p = 0.012) and female ($F_{(3, 11)}$ = 6.088, p = 0.018) developed from larvae fed on plants treated with both strains of *B. bassiana*. In addition, it was observed a lesser number of male than female pupae (Figure 5). Furthermore, we observed apparent parthenogenesis, with fertile eggs that hatched viable neonates from virgin female moths that had been feeding on *B. bassiana*-treated plants (Figure 6), indicating important changes in *S. frugiperda* physiology caused by this entomopathogen, which was not observed in virgin female moths fed on negative-control plants.
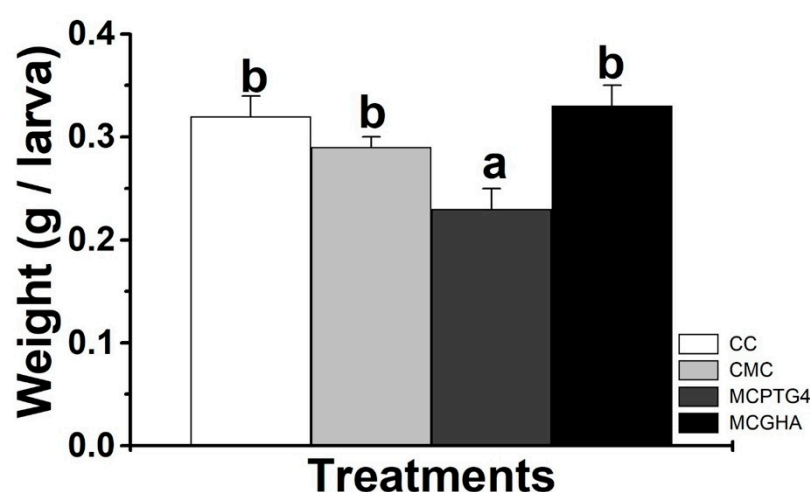

Figure 2. *S. frugiperda* sixth instar larval weight after feeding on plants from the specified treatments. CC: Negative control, CMC: Negative control with only methylcellulose, MCPTG4: *B. bassiana* fungal strain PTG4 with methylcellulose, MCGHA: *B. bassiana* fungal strain GHA with methylcellulose. Analysis done with Duncan's post-hoc multiple range test (α = 0.05) after one-way ANOVA. Graphical bars with the same letters indicate that there were no significant differences among them.
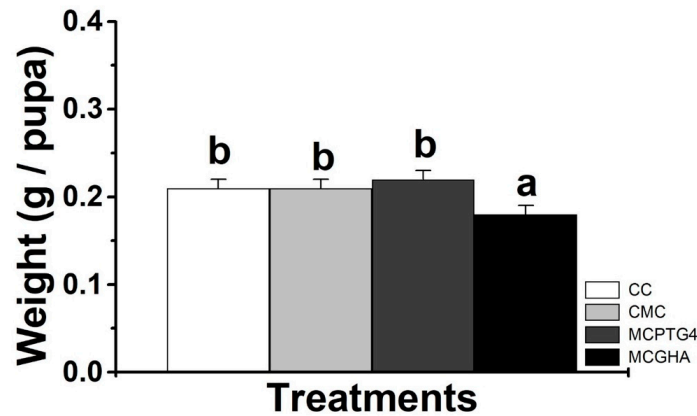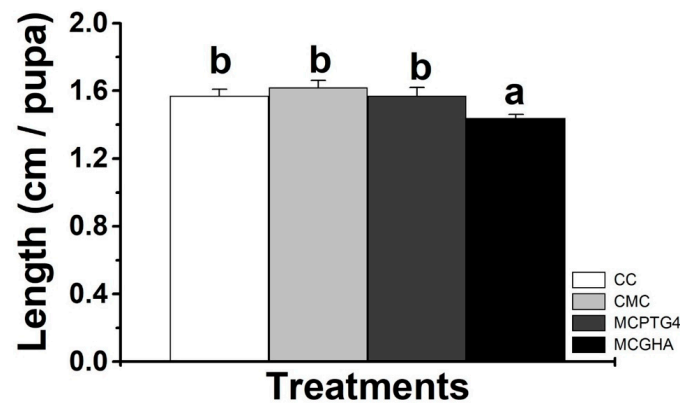
Figure 3. *S. frugiperda* pupal weight after feeding on the plants from the specified treatments. CC: Negative control, CMC: Negative control with only methylcellulose, MCPTG4: *B. bassiana* fungal strain PTG4 with methylcellulose, MCGHA: *B. bassiana* fungal strain GHA with methylcellulose. Analysis done with Duncan's post-hoc multiple range test ($\alpha = 0.05$) after one-way ANOVA. Graphical bars with the same letters indicate that there were no significant differences among them.
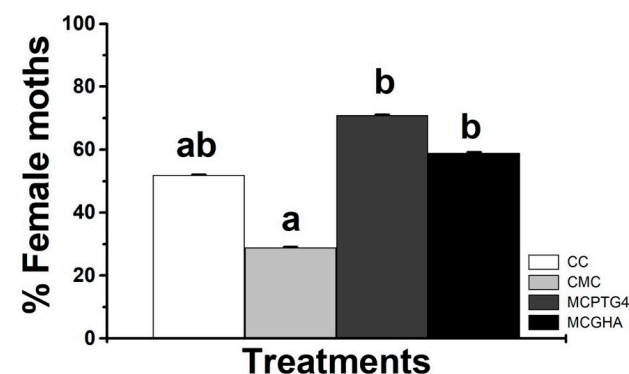

Figure 4. *S. frugiperda* pupal length after feeding on plants treated with the specified treatments. CC: Negative control, CMC: Negative control with only methylcellulose, MCPTG4: *B. bassiana* fungal strain PTG4 with methylcellulose, MCGHA: *B. bassiana* fungal strain GHA with methylcellulose. Analysis done with Duncan's post-hoc multiple range test ($\alpha = 0.05$) after one-way ANOVA. Graphical bars with the same letter indicate that there were no significant differences among them.

Figure 5. *S. frugiperda* female moths' percentage distribution after feeding on plants treated with the different treatments. CC: Negative control, CMC: Negative control with only methylcellulose, MCPTG4: *B. bassiana* fungal strain PTG4 with methylcellulose, MCGHA: *B. bassiana* fungal strain with methylcellulose. Analysis done with Duncan's post-hoc multiple range test ($\alpha = 0.05$) after one-way ANOVA. Graphical bars with the same letters indicate that there were no significant differences among them.

Figure 6. Representative virgin female moth having laid fertile eggs showing apparent parthenogenesis.

3.3. Field Trials: Experiment 1

Maize plant germination percentage in the field was low, nevertheless, there was no significant ($F_{(5, 57)} = 1.002$ and $p = 0.426$) difference among negative controls and *B. bassiana* treated plants. The average germination percentage at the third week after planting the seeds for the negative control and *B. bassiana*-treated plants were 71.5% $\pm$ 5.4% and 80.33% $\pm$ 3.49%, respectively. The number of leaves per plant at the fourth week after planting also showed no significant differences among negative control plants and *B. bassiana* treated plants with $F_{(5, 57)} = 0.928$ and $p = 0.471$. Average number of leaves per plant on the fourth week after planting the seeds for the negative control and *B. bassiana* treated plants was 3.12 $\pm$ 0.22 and 3.49 $\pm$ 0.09, respectively.

To analyze the presence of *S. frugiperda* in the experiments, we inspected each one of the plants. We did observe that *S. frugiperda* larvae between the second and third instar were found in almost all furrows of negative control plants, whereas in *B. bassiana*-treated plants, they were present in those furrows near to the negative control plants, and their number was reduced further than in the negative control plants and none were present in the last 12 furrows of *B. bassiana*-treated plants (Figure 7 and Figure S3). The analysis GLM-fitted with negative binomial distribution showed a Pearson chi-square value of 0.311, indicating goodness of fit of the data. The Omnibus test value of 0.539 indicated a non-statistically significant model. The Type III Test of Model effects showed $p = 0.342$ for the treatments (CC/GHA). Several pictures of larval instars found at the time of data collection are shown in Figure S5. Interestingly, we also observed the presence of various pathogenic and beneficial insects during the experiments that need further identification.

3.4. Field Trials: Experiment 2

Independent Samples *T*-test analysis showed no significant ($F = 0.225$ and $p = 0.648$) difference in the percentage of germination in all five furrows of negative control plants (mean 91.20% $\pm$ 2.8) and five furrows of *B. bassiana*-treated plants (mean 87.20% $\pm$ 3.5). Independent sample *T*-test analysis showed no significant difference in the number of leaves and plant height. The average number of leaves in negative controls and *B. bassiana*-treated plants at the third week after planting the seeds were 5.20 $\pm$ 0.055 and 5.23 $\pm$ 0.054, respectively. The average plant height for the negative control and *B. bassiana*-treated plants was 12.04 $\pm$ 0.16 cm and 12.36 $\pm$ 0.15 cm, respectively. In this trial, *S. frugiperda* larvae were not detected, neither in negative control plants nor in *B. bassiana* treated plants at the time of data collection. Independent Samples *T*-test analysis showed no significant difference between negative controls and *B. bassiana*-treated plants in corn cob length with $F = 0.006$ and $p = 0.937$, whereas no significant differences in corn cob weight were observed ($F = 0.048$ and $p = 0.831$) (Table 2). Although numbers were not recorded at harvesting time,

it was observed the presence of more than two corn cobs per plant in the *B. bassiana*-treated plants, compared with the negative control plants (Figure S6).
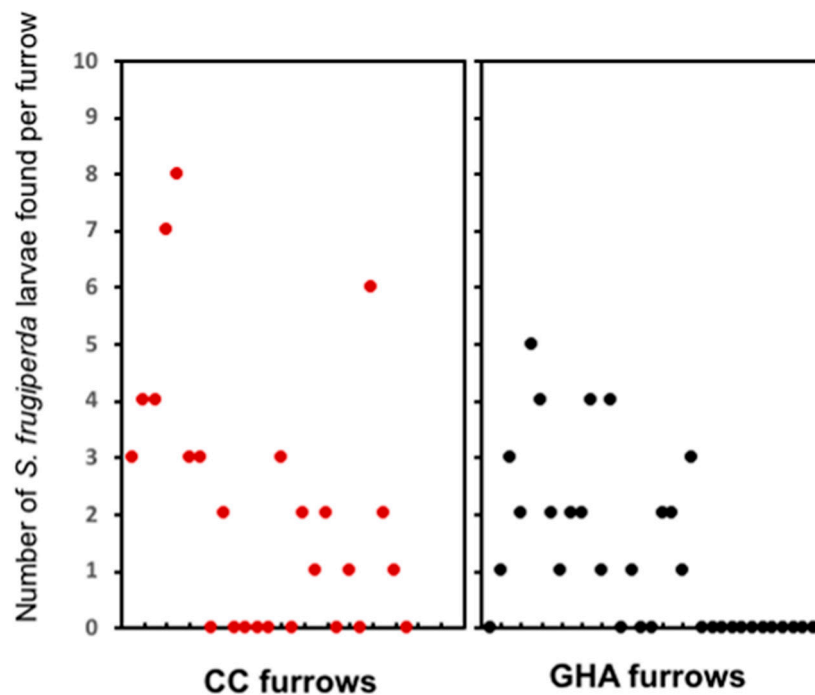

Figure 7. Presence of *S. frugiperda* in the field trial experiment 1. There were found 52 total larvae in CC furrows and 40 total larvae in GHA furrows. As insects' distribution appeared to be related to the location of the plants, the frequency of larvae found per furrow is presented. 25 CC furrows = Negative control plants. 33 GHA furrows = *B. bassiana* strain GHA with methylcellulose treated plants. The analysis GLM-fitted with negative binomial distribution showed Pearson chi-square value of 0.311, indicating the goodness of fit of the data. The Omnibus test value of 0.539 indicated a non-statistically significant model. The Type III Test of Model effects showed $p = 0.342$ for the treatments (CC/GHA).

Table 2. Average weight and length of corn cobs obtained at harvesting time.

Parameters	CC	MCGHA
Average weight of fresh corn cob in g	209.15 ± 26.11	183.43 ± 24.65
Average length of fresh corn cob in cm	18.20 ± 0.74	16.71 ± 0.75

4. Discussion

All experiments were performed using fresh cultures of *B. bassiana* strains from frozen stocks, considering former reports indicating important correlations between the number of subcultures and the stability of genetic and physiological parameters [28] of *B. bassiana* in germination, conidiation, and virulence [29].

Manufacture and formulation are the decisive elements of the success of an entomopathogenic fungus as a commercial biocontrol agent. Solid substrate fermentation for aerial-conidia and liquid culture fermentation for blastospores are typical methods for their massive production. Although aerial-conidia contain the main active ingredients as biocontrol agents, they require weeks for sporulation and fermentation, which is reduced by using blastospores. We used blastospores in our study because they tolerate drying and continue to be viable after long-term storage [30]. To assure blastospores viability and

stability during exposure to direct sunlight or ultraviolet radiation, methylcellulose can be used to make blastospore formulations [12,31].

At present, foliar spraying, plant dipping, stem injection, seed coating, and root or soil drenching methods have been used to inoculate artificially entomopathogenic fungi into different plants. McKinnon et al. [32] reported that seed coating and foliar treatments were used in most published bioassays for artificial inoculation. Some studies demonstrated that leaves are loose passages for entomopathogenic fungus entry to colonize plants [20,33]. However, effective endophytic colonization of these fungi depends on factors such as plant age, fungal species, inoculation methods, and exposure to direct sunlight and rain, among others. Diverse studies show that *B. bassiana* does not maintain its survival and viability after exposure to direct sunlight or ultraviolet radiation [31,34]. Nevertheless, various studies reported that formulation with natural substances overcomes this obstacle [31,35–37]. In the present study, we used a methylcellulose seed coating method to aim for effective colonization of *Zea mays* seeds and maintain the viability and perhaps the virulence of *B. bassiana* blastospores.

Z. mays germination percentage in all treatments, including negative controls, did not show differences under field and laboratory conditions; therefore, neither the fungus nor the adherent affected germination, which was similar to the results reported by Jaber and Enkerli [38], who showed that neither *B. bassiana* nor *M. brunneum* altered *V. faba* seed germination. In contrast, Russo et al. [8] reported an enhancement in the germination of *B. bassiana*-treated *Z. mays* seeds. Previous results in our laboratory demonstrated that *B. bassiana* and methylcellulose do not have inhibitory effects on *Z. mays* germination [26].

We analyzed the effect of maize seed treatments by *B. bassiana* GHA and a native strain (PTG4) under laboratory conditions on the physiology of *S. frugiperda*. We observed that a small percentage of larvae died, without any significant difference between larvae fed with untreated and *B. bassiana*-treated maize plants. We did not observe fungal outgrowth from *S. frugiperda* cadavers; therefore, the larvae probably were not in direct contact with *B. bassiana* blastospores. It was not possible to determine the presence of *B. bassiana* in the plant tissues that were used to feed the larvae since there was no plant material left after the larvae were fed. However, in our previous studies, we found *B. bassiana* as an endophyte in *Zea mays* plants that grew after *B. bassiana*-methylcellulose-seed treatments [26]. One perspective from this study is to analyze the microbiota of *S. frugiperda* excrement to determine if *B. bassiana* was present. On the other hand, we observed that *S. frugiperda* larvae fed on plants treated with *B. bassiana* strain PTG4 had their development considerably affected, with a prolonged larval stage, a decline in larval weight, and a smaller number of pupae. In addition, we observed a high number of escaped larvae that were fed on GHA *B. bassiana* strain-treated plants (26.7%) (Table 1), and the remaining showed a decline in pupal length and weight. Vega [39] reported that by adding cultured *B. bassiana* to the insects' diet, after removing mycelia, it was possible to reduce the percentage of pupation and the pupation time was prolonged. On the other hand, Hassan et al. [40] reported that adult malformations occurred in *B. bassiana*-treated squash beetles. Lopez and Sword [15] did not find any difference in cotton boll worm and pupal weight when the insects were fed on *B. bassiana* and *Purpureocillium lilacinum* inoculated cotton plants. In our bioassays, since they were no-choice experiments, there was not sufficient remaining plant material to analyze and determine if there were any feeding preferences of larvae between negative control plants and *B. bassiana*-treated plants. Another important observation of this study was the adult male/female ratio obtained after the development of larvae fed on *B. bassiana*-treated *Z. mays* plants. We observed a lower number of adult male moths. Interestingly Russo et al. recently reported differences in *S. frugiperda* female fertility, fecundity, and longevity using corn plants endophytically-inoculated with *B. bassiana* by foliar spray treatments [41]. In contrast, they did not find significant differences in the sex ratio of *Helicoverpa gelotopoeon* when they were fed on *B. bassiana* treated soybean plants [42]. Akutse et al. observed a higher number of emerged males in their study with different fungal strains to protect *Vicia faba* and *Phaseolus vulgaris* against *Liriomyza huidobrensis* [43].

In addition, we observed apparent parthenogenesis in female adults, but further studies are needed to determine the cause of this, in addition to the analyses of the longevity, survival capacity, eating habits, or any other changes in the life cycle of the larvae born from these eggs. Furthermore, Mahmood et al. [13] reported a reduced survival and fecundity of *Sitobion avenae* after feeding on maize plants inoculated with *B. bassiana*. Insect immunity is influenced by successive exposures to the same pathogen and has a long-term effect on its survival [44]. More studies are needed to determine whether *B. bassiana* affects *S. frugiperda* successive generations. In this regard, Bamisile et al. [10] reported that endophytic *B. bassiana* established after foliar treatment of *Citrus limon* plants acted as a growth suppressor to three successive generations of *Diaphorina citri*.

Based on our laboratory results, two preliminary field trials in 2019 were conducted. The main objective was to analyze the effect of GHA *B. basssiana*-seed treatment on natural *S. frugiperda* populations, as we observed no mortality but some effects on the insect physiology and certain avoidance to the treated plants (Table 1). Our hypothesis was that we would observe a smaller number of larvae in the treated plants. In the field trial Experiment 1, there was no significant difference between the germination of the negative control and *B. bassiana* treated plants, whereas there was a small decline in comparison with laboratory results. This decrease might be due to the uncontrolled environmental conditions and the type of seed (Criollo race) that was used; however, the percentage of *Z. mays* plants germination was not affected by the presence of *B. bassiana*. This conclusion is in contrast with the study of Russo et al. [8], who reported a 77% germination of negative control, compared with 89% germination of *B. bassiana*-treated *Z. mays* plants. Moreover, the average number of leaves per plant did not show any significant difference between the negative control and *B. bassiana*-treated plants. Despite there was a non-significant difference in the frequency of *S. frugiperda* larvae between the *B. bassiana*-treated plants and negative control plants (Figure 7), there was a slight increment in the number of larvae in the furrows of *B. bassiana*-treated plants that were immediately close to the negative control plants. This eventually decreased to zero in the last 12 rows of *B. bassiana*-treated plants, which were only 30 meters from neighboring maize fields that had *S. frugiperda* presence. Therefore, the insects showed some avoidance of feeding on the treated plants. These are preliminary results that are similar to what we observed in our laboratory bioasays. (Figure 7, Table 1). In addition, we wanted to test if an increase in the concentration of *B. bassiana* GHA strain could show any effect on plant growth or insect presence in the field, as a second peak of *S. frugiperda* insects was expected in the area of study.

In the second field trial, there were no significant differences observed in terms of germination percentage and the average number of leaves of *Z. mays* plants in both treatments, whereas a small increment in plant height was observed with *B. bassiana*-treated plants. In addition, increased germination was observed, from 71.5% (first trial) in negative control plants to 91.20% (second trial) and from 80.33% (first trial) to 87.20% (second trial) in *B. bassiana*-treated plants. In terms of the average number of leaves per plant, we observed an increase of 3.12 (first trial) to 5.20 (second trial) leaves per plant in negative control and from 3.49 (first trial) to 5.23 (second trial) leaves per plant in *B. bassiana*-treated plants. This difference might be due to the use of a higher concentration of blastospores (from 1×10^6 blastospores/mL in the first trial to 1×10^8 blastospores/mL in the second trial) or to the change in cultivation cycle conditions (intermediate). These results are similar to those by Castillo-Lopez and Sword, who indicated that *Gossypium hirsutum* height increased by establishing *B. bassiana* as an endophyte in the plants [15]. Dash et al. reported an increasing number of leaves of *P. vulgaris* after *B. bassiana* treatment [45].

We found that the length of the cob corn was slightly higher in the negative control plants than in *B. bassiana*-treated plants. However, most *B. bassiana*-treated plants yielded more than two corn cobs per plant (Figure S6). We did not find any significant difference in the corn cobs weight among the treatments, which is in agreement with Hernandez-Trejo et al. [46], who found that application of *Metarhizium robertsii* to maize plants did not show any significant difference in grain yield per hectare among the treatments tested, whereas

Russo et al. [8] found an increase in maize corn yield after applying *B. bassiana* under field conditions. Qayyum [20] reported a decrease in tomato size after colonizing the plants with *B. bassiana.* All these contradictory results might be due to the difference in the fungal strains, host plant species, and varieties, or even may be due to the geographical regions in which studies were undertaken.

We also detected a high presence of beneficial insects (honeybees) in comparison with pathogenic ones, although further taxonomic identification is needed. Therefore, this indicates that the treatments used did not affect the ecosystem present in this experimental field.

In addition, the most relevant fact in the second field trial was that we did not observe any *S. frugiperda* larvae, neither in the negative control plants nor in *B. bassiana*-treated plants, during the period of the study. These results in both trials appear to confirm our hypothesis of putative insect avoidance. Hernandez-Trejo et al., reported that *Metarhizium robertsii* decreased *S. frugiperda* incidence from 41.3% to 2.8% in the first application and 17.4% to 8.3% in the second application on maize plants [46].

However, our findings need more experiments in the field to understand the mechanisms related to these results. These two preliminary field trials need to be repeated at least in two more years. In future studies, we should consider recent reports indicating that *B. bassiana* and *Muscodor vitigenus* can produce naphthalene as a potential insect repellent [47,48]. We should also consider the effect of endophytic entomopathogens in the production of kairomones by colonized plants, as these compounds are chemical signals used by the insects to localize them [39].

5. Conclusions

We conclude that using GHA *B. bassiana*-seed treatment in *Z. mays* in two preliminary field trials, the population of *S. frugiperda* in the field was controlled, and economic damage was not observed. We obtained evidence that the environment was not affected, particularly there were no negative effects on beneficial insects, such as honeybees. In addition, using a native strain (PTG4) under laboratory conditions, we observed effects on *S. frugiperda*'s physiology and morphology that need to be further analyzed under field conditions. Therefore, the proposed technique has the potential to be applied in the future for more sustainable agriculture practices.

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