 doi:10.20944/preprints202101.0094.v1

Article

Effect of Zea mays-Beauveria bassiana seed treatment on 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, under field conditions, $1x10^6$ (first trial) and $1x10^8$ (second trial) of *B. bassiana* (GHA strain) blastospores were used for corn's seed inoculation. In the first field trial, a higher number of larvae were present in the negative control plants in comparison with those in *B. bassiana*-treated plants. No larvae were found in negative control and *B. bassiana*-treated plants in the second field trial. In further laboratory experiments, the effects of the *B. bassiana* strains GHA, in addition to a native strain (PTG4) also delivered via seed treatment in maize seedlings, on *S. frugiperda* growth, development, and mortality were evaluated. $1x10^6$ *B. bassiana* blastospores were used to inoculate maize seeds, which 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 6^{th} instar and transferred to artificial diet until reaching adult stage. Results showed that larvae feeding on *B. bassiana* strain PTG4 prolonged their larval stage. Furthermore, feeding with plants treated with *B. bassiana* strains yielded fewer *S. frugiperda* male moths and the female moths emerged with altered wings, compared with the untreated control. In conclusion, seed treatment with *B. bassiana* in maize reduced *S. frugiperda* infestation of maize plants in field trials. Besides *S. frugiperda* development was affected in laboratory trials.

Keywords: 1; Beauveria bassiana 2; Biological control 3; Entomopathogenic fungi 4; Spodoptera frugiperda 5; Zea mays

1. Introduction

Worldwide, about 18 to 26 percentage reduction in crop production is due to insect pests: out of which a great part of it occur in the fields before harvesting [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

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more than 80 host species, including many commercial crops like maize, cotton, rice, soybean, bean, and other crops from the Gramineae family [3, 4, 5]. Until 2015, damages due to *S. frugiperda* were 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 country and, by late 2018 had been confirmed in almost 44 African countries [3]. By 2018, 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, the third most essential cereal crop worldwide with a highest economic value in terms of production and nutrition [6]. It causes extensive damage to maize plants by feeding on young leaf whorls, corn cob, and tassel [3]. Younger larvae prefer epidermal leaf tissue and make holes on them, which is the typical damage symptom by these insect pests. Deadheart is formed by feeding on young plants through the whorls. Older larvae in the whorls of grown-up plants feed on cobs or kernels and can reduce the quality and quantity of the yield [2]. The existence of various generations, its migrating and feeding potential to feed on a vast range of host plants make this insect one of the biggest challenging pests to control [2].

Synthetic/chemical insecticides or genetically modified crops have been used to control insect pests [2]. Even though, these control measures are very efficient, their extensive usage has provoked ecological problems, environmental contamination, development of resistance, and ultimately negative effects on human health [8]. Since the insects have gained resistance to various chemical insecticides, farmers are compelled for recurrent application of large amounts, which will lead to the accumulation of chemicals in agricultural fields [2]. Taking 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].

Integrated Pest Management (IPM) global idea for agriculture is a holistic concept of approaching the crop production system as a whole process, rather than only pest elimination. This approach combines various techniques, like using resistant varieties, cultural manipulation, trap cropping, and biological control [1]. Biological control is one of the techniques to control insect pests with slightest environmental impact [9]. Cost-effectiveness, high yield, not harmful to beneficial insects, and release of less chemical residues in the agricultural fields make entomopathogenic microorganisms great potential 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]. Entomopathogenic fungus capacity to accommodate and sustain in external habitats other than their original habitats have made them very efficient and adequate candidates for biological control measures. [10]. Considered as facultative microorganisms that do not require arthropods as hosts to

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complete their life cycles, *Metarhizium anisopliae* and *Beauveria bassiana* are the best characterized and most employed 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]. *B. bassiana* implements integral protection against insect pests or constrains their growth and proliferation [12]. This fungus also exhibited dual protection ability against *Rhizoctonia solani* and *Pythium myriotylum* in *B. bassiana*-treated tomato seedlings [11]. Distinct studies showed that *B. bassiana* can be used as an effective biocontrol agent against *Helicoverpa armigera* in broad bean [14] *Helicoverpa zea* in cotton and tomato [15,16]. Feeding on *B. bassiana*-treated maize plants reduced *Sitobion avenae* survival and reduced the fecundity of a single aphid fed on these plants [13]. *B. bassiana* in corn plants showed insecticidal activity against many lepidopterans like *Sesamia calamistis* [17], *Ostrinia nubilalis* [18], and when isolated as endophyte, against *Spodoptera frugiperda*. [19].

Taken together, the main objective of the present study was to evaluate the tolerance of *B. bassiana*-treated *Z. mays* against *S. frugiperda* larval activity under field and laboratory conditions.

2. Materials and Methods

2.1. Insect, plant, and fungal strains for laboratory assays

Insect colony: *S. frugiperda* eggs were kindly donated by Dr. José Refugio Lomelí-Flores (Colegio de Postgraduados, Montecillo, Texcoco, Estado de México, México), 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 transferred to individual diet cups with 5 mL modified artificial wheat germ diet [20] as their food source (Fig. S6 supplementary materials). 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 Dr. Verónica Garrocho-Villegas, Universidad Nacional Autónoma de México (UNAM), México.

Fungal strains: *B. bassiana* GHA strain, commercially obtained as Botanigard® 22WP and PTG4 strain, kindly provided by Dr. 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).

2.2. Field trial: Experiment 1

Field trials were done in an agricultural field situated in General Teran, Nuevo Leon, Mexico with geographical location at 25°16'00.0"N 99°41'00.0"W. The first trial was done in mid-February 2019. 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 farmers normally use *Bacillus thuringiensis* to control them, then no artificial inoculation of *S. frugiperda* was done in any of the field experiments. One-hectare field was prepared using a tractor; 38 rows of 100 meters length and 25 rows of 80 meters length were made, with a row-to-row distance of 80 cm. Using the seed

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inoculation procedure, mentioned in section 2.4, 3300 *Z. mays* seeds of "criollo maize" race (kindly donated by the field owner, without any insecticidal or fungicidal treatment) were inoculated with *B. bassiana* strain GHA, with a concentration of $1x10^6$ blastospores/mL and methyl cellulose as adherent; 2500 seeds were used as negative control without any fungal or adherent treatment. Seeds were planted during mid-February 2019 on the rows, with a separation of 25 cm between each seed, the distance between each row was 80 cm and, in each row100 seeds were planted, which were monitored every week. First 33 rows were used to plant *B. bassiana*-treated seeds, then 5 rows were left without seeds and the remaining 25 rows were used to plant negative control treatments (Fig. S1 supplementary materials). In this experiment, germination percentage at 3rd week after planting was recorded and the presence of *S. frugiperda* larvae, between the 5th true leaf and 10th true leaf time period was monitored. To scout the whole field for the presence of *S. frugiperda* larvae, we divided the *B. bassiana*-treated plants plot into three sections. Other than watering every day, neither fertilizer nor pesticides were applied in the field during the whole experiment.

2.3. Field trial: Experiment 2

The second field trial was conducted in the same way as the first trial, but during mid-April 2019. 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 $1x10^8$ blastospores/mL and methyl cellulose as adherent and kept 500 seeds as negative control without any fungal or adherent treatment. The seeds were planted as in the first field experiment (Fig. S2 supplementary materials). 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 analyzed in the second and fourth rows in both treatments. Germination was recorded in the 3rd week after planting. Plant height and number of leaves were recorded in the 4th week after planting. Presence of *S. frugiperda* was monitored between the 5th and 10th true leaf time period. To assess for yield effects during harvest, five corn cobs from each treatment were collected, weighed (A&D Company Limited, N-92, Korea), and their length were measured with a normal scholastic ruler.

2.4. 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 a 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 Neubauer hematocytometer and adjusted to a concentration of $1x10^6$ 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. 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.

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Controls included seeds without any treatments (nor fungi, neither adherents; CC) and seeds without fungi but with MC (CMC). MCGHA, MCPTG4, CMC and CC seeds were sown into commercial soil (Happy Flower Mexicana, S.A. de C.V, México), previously autoclaved and that was contained in 250 mL plastic glasses and kept at 25 °C ± 2 °C for 10 days after germination.

2.5. Bioassays under laboratory conditions

Each third instar *S. frugiperda* larva was carefully transferred onto a 10-day old *Z. mays* plant for each treatment (1 larva/plant) and then covered with a mesh bag to prevent escapes (Fig. S7 supplementary materials). Plants were replaced every 24 hours. When larvae reached 6th instar, they were returned to artificial diet to monitor pupal stage development (Fig. S6 supplementary materials). Each pupa was examined under a stereoscope (Labomed Stereomicroscope, Luxeo2S, CA, USA) to determine its sex, weighed on a microbalance (AND, A&D Company Limited, N-92, Korea), and measured for length with a standard, scholastic ruler. After that, pupae were transferred to individual plastic containers (7 cm diameter x 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 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± 2 °C temperature, 60% ± 5% relative humidity, and 14:12 h light and dark photoperiod). The following parameters were recorded: a) Initial larvae numbers, b) number of dead larvae during the experiment, c) number of larvae that remained as larvae, even after reaching 6th instar of development, d) number of larvae that remained as prepupa, e) 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.

2.6. 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 statistics. When a significant F value was obtained after ANOVA, post-hoc Duncan's multiple range test were performed. Considering that there were only two groups to analyze in 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.

3. Results

3.1. Field trials: Experiment 1

Maize plant germination percentage in the field was low, nevertheless there was not significant ($F_{(5, 57)} = 1.002$ and $P = 0.426$) difference among negative control and *B. bassiana* treated plants. The average germination percentage on 3rd week after planting the seeds for the

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negative control and *B. bassiana*-treated plants were 71.5% ± 5.4% and 80.33% ± 3.49% respectively. Number of leaves per plant on 4th 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 4th week after planting the seeds for the negative control and *B. bassiana* treated plants was 3.12 ± 0.22 and 3.49 ± 0.09, respectively.

To analyze the presence of *S. frugiperda* in the experiments, we divided the field into three sections for both negative control and *B. bassiana*-treated plants. We did observe that *S. frugiperda* larvae between 2nd and 3rd instar were found in almost all rows of negative control plants, whereas in *B. bassiana* treated plants, they were present in those rows 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 rows of *B. bassiana*-treated plants (Fig. 1; Fig. S1 supplementary materials). A graphical representation of this distribution and several pictures of larval instars found at the time of data collection are shown in Fig. S3 supplementary materials. We also observed the presence of various pathogenic and beneficial insects during the experiments (Fig. S5 supplementary materials).

3.2. 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 rows of negative control plants (mean 91.20% ± 2.8%) and five rows of *B. bassiana*-treated plants (mean 87.20% ± 3.5%). Independent sample T-test analysis showed no significant difference in number of leaves and plant height. Average number of leaves in negative control and *B. bassiana*-treated plants on 3rd week after planting the seeds were 5.20 ± 0.055 and 5.23 ± 0.054, respectively. The average plant height for the negative control and *B. bassiana*-treated plants was 12.04 ± 0.16 and 12.36 ± 0.15, 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 a significant difference between negative control 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 1). 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*-

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treated plants, compared with the negative control plants (Fig. S4 supplementary materials).

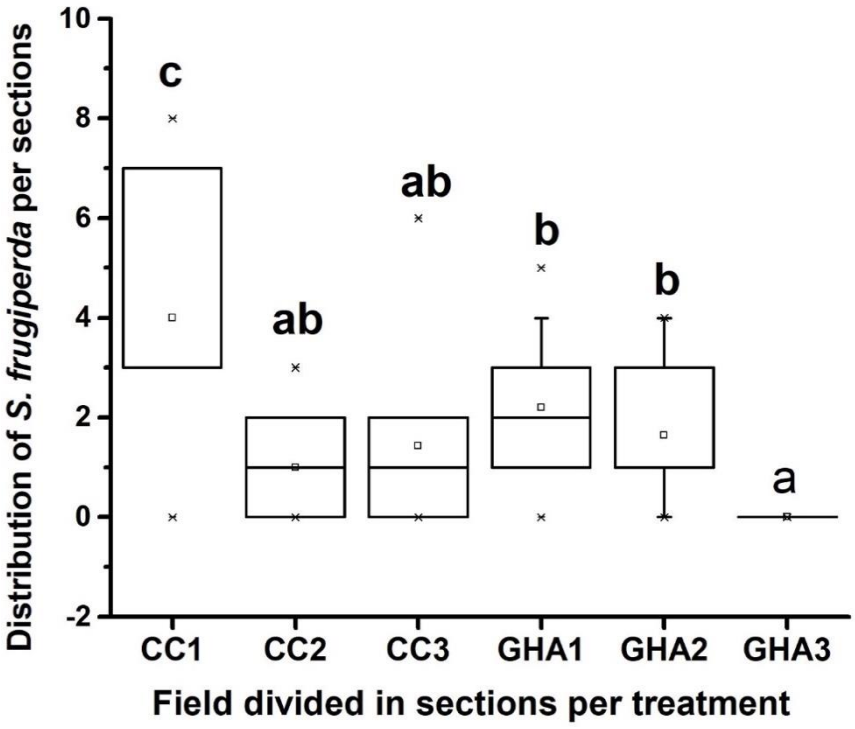

Figure 1. Presence of *S. frugiperda* in field trial experiment 1. As insects' distribution appeared to be related to location, analysis was done grouping the rows in sections. CC1, CC2, CC3 = Negative control plants. GHA1, GHA2, GHA3 = *B. bassiana* strain GHA with methyl cellulose treated plants. Post hoc analysis was done with Duncan multiple range test (α 0.05) after one-way ANOVA. Graphical bars with different letters indicate that there are significant differences among them.

Table 1. Average weight and length of corn cobs obtained at harvest 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**

3.3. Effect of B. bassiana on Z. mays plant germination

After 10 days, seedlings germination percentage was determined. Compared with the absolute negative control (CC) values, the negative control with only the adherent methyl cellulose (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 (Fig. 2).

3.4. 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

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are shown in Table 2. *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 ($F_{(3, 11)} = 20.657$, $P = 0.000$) 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 2. Effects of feeding on *B. bassiana*-treated plants on the developmental stages of *S. frugiperda*.

Stages	CC	CMC	MCPTG4	MCGHA
Initial pupa	100% ^{a*}	100% ^{a}	100% ^{a}	100% ^{a}
Dead larva	6.67% ^{a}	3% ^{a}	3.67% ^{a}	0 ^{a}
Still larva	3.33% ^{a}	3% ^{a}	**22%** ^{b}	0 ^{a}
Prepupa	6.67% ^{a}	3% ^{a}	3.67% ^{a}	0 ^{a}
Pupa	83.33% ^{ab}	91% ^{ab}	63.33% ^{a}	100% ^{b}

*Values followed by the same letters are not significantly different and with different letters are significantly different after running post hoc Duncan multiple range test ($P = 0.05$). CC= Negative control without any treatments or adherents, CMC= Negative control with only methyl cellulose, MCPTG4= Methyl cellulose with *B. bassiana* strain PTG4, MCGHA= Methyl cellulose with *B. bassiana* strain GHA.

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 3). *S. frugiperda* pupal weight showed a significant ($F_{(3, 97)} = 3.753$, $P = 0.014$) difference among larvae fed on *B. bassiana* strain GHA treated plants, negative controls, and *B. bassiana* PTG4 strain fed larvae (Figure 4). *S. frugiperda* pupal length showed a significant ($F_{(3, 98)} = 4.491$ $P = 0.005$) difference between the larvae fed on *B. bassiana* GHA treated plants and all other treatments (Figure 5). 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 (Fig. 6). Nevertheless, female adults emerged with apparent altered wings. We also observed apparent parthenogenesis among female moths (Fig. 7), indicating important changes on *S. frugiperda* physiology caused by feeding on plants treated with *B. bassiana*.

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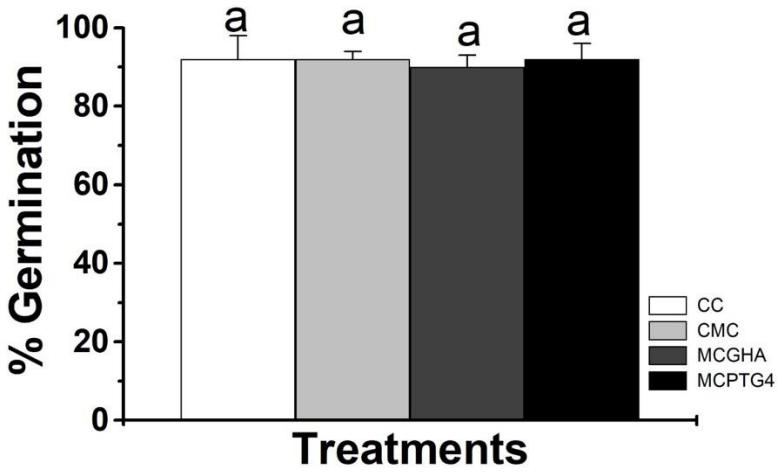

Figure 2. *Z. mays* plants germination percentage after 10 days of sowing with the different treatments. CC: Negative control, CMC: Negative control with only methyl cellulose, MCPTG4: *B. bassiana* fungal strain PTG4 with methyl cellulose, MCGHA: *B. bassiana* strain GHA with methyl cellulose. Post hoc analysis done with Duncan multiple range test ($\alpha = 0.05$) after one-way ANOVA. Graphical bars with same letters indicate that there are no significant differences among them. .

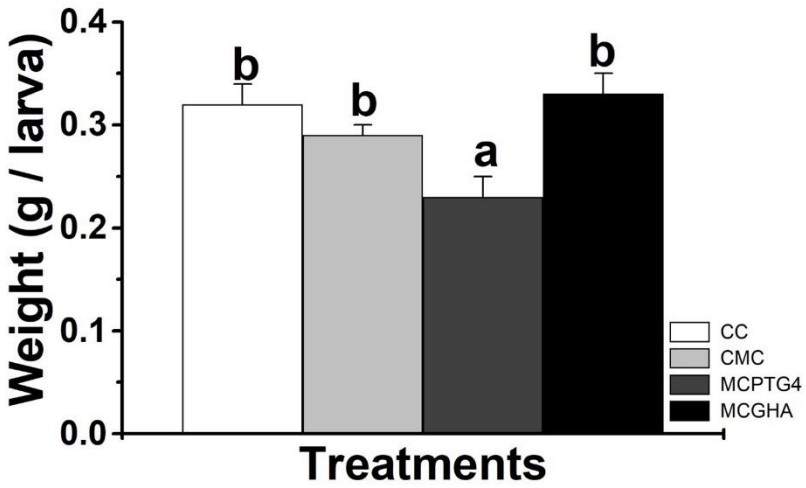

Figure 3. *S. frugiperda* 6th instar larval weight after feeding on plants from the specified treatments. CC: Negative control, CMC: Negative control with only methyl cellulose, MCPTG4: *B. bassiana* fungal strain PTG4 with methyl cellulose, MCGHA: *B. bassiana* fungal strain GHA with methyl cellulose. Post hoc analysis done with Duncan multiple range test ($\alpha = 0.05$) after one-way ANOVA. Graphical bars with same letters indicate that there are no significant differences among them. .

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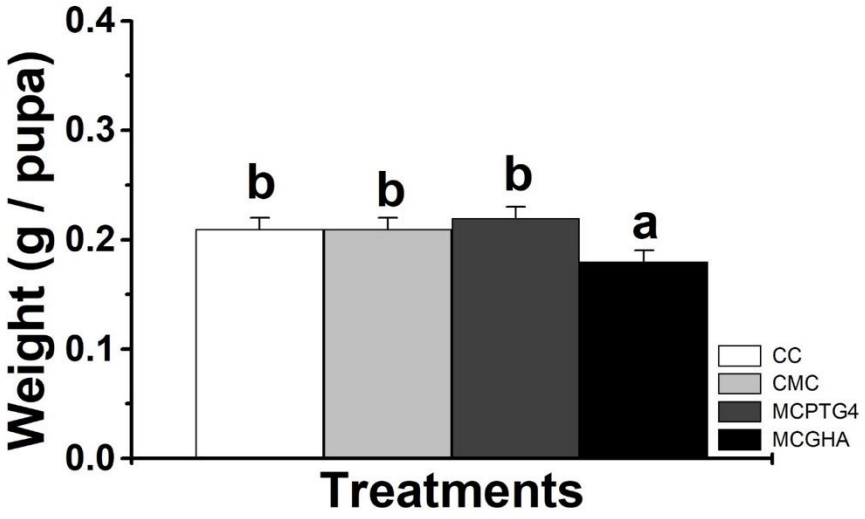

Figure 4. *S. frugiperda* pupal weight after feeding on the plants from the specified treatments. CC: Negative control, CMC: Negative control with only methyl cellulose, MCPTG4: *B. bassiana* fungal strain PTG4 with methyl cellulose, MCGHA: *B. bassiana* fungal strain GHA with methyl cellulose. Post hoc analysis done with Duncan multiple range test ($\alpha = 0.05$) after one-way ANOVA. Graphical bars with same letters indicate that there are no significant differences among them.

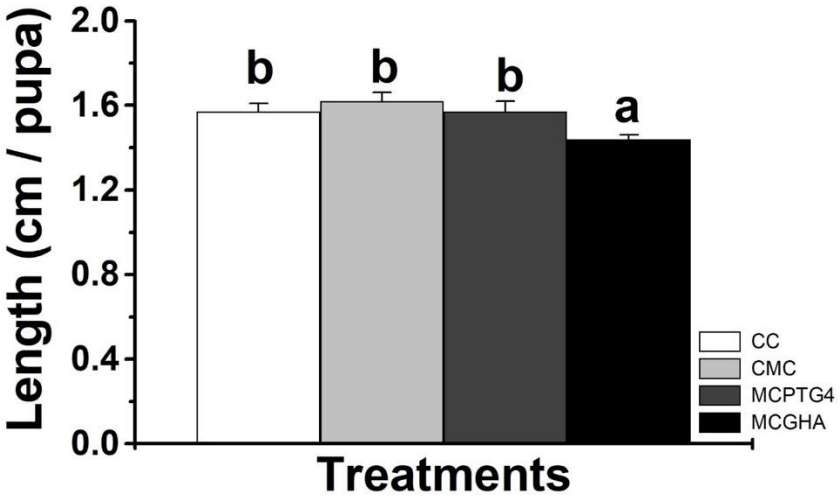

Figure 5. *S. frugiperda* pupal length after feeding on plants treated with the specified treatments. CC: Negative control, CMC: Negative control with only methyl cellulose, MCPTG4: *B. bassiana* fungal strain PTG4 with methyl cellulose, MCGHA: *B. bassiana* fungal strain GHA with methyl cellulose. Post hoc analysis done with Duncan multiple range test ($\alpha = 0.05$) after one-way ANOVA. Graphical bars with same letter indicate that there are no significant differences among them.

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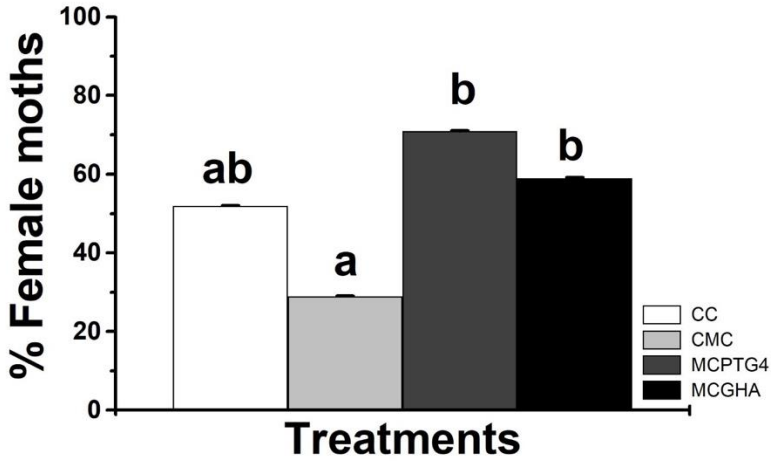

Figure 6. *S. frugiperda* female moths' percentage distribution after feeding on plants treated with the different treatments. CC: Negative control, CMC: Negative control with only methyl cellulose, MCPTG4: *B. bassiana* fungal strain PTG4 with methyl cellulose, MCGHA: *B. bassiana* fungal strain with methyl cellulose. Post hoc analysis done with Duncan multiple range test (α 0.05) after one-way ANOVA. Graphical bars with same letters indicate that there are no significant differences among them.

Figure 7. Representative adult females with altered wings and showing parthenogenesis.

4. Discussion

All experiments were performed using fresh cultures of *B. bassiana* strains from frozen stocks, considering former reports indicating important correlations between number of subcultures and the stability of genetic and physiological parameters [21] of *B. bassiana* in germination, conidiation, and virulence [22].

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. Despite aerial conidia contain the main active ingredients as biocontrol agents, they require weeks for sporulation and fermentation,

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which is reduced by using blastospores. In addition, blastospores have the potential to tolerate drying and continue to be viable after long term storage [23], therefore we used blastospores in our study. To assure blastospores viability and stability during exposure to direct sunlight or ultraviolet radiation, we used methyl cellulose to make blastospore formulations [12,24].

At present, foliar spraying, plant dipping, stem injection, seed coating, and root or soil drenching methods have been used to artificially inoculate entomopathogenic fungi into different plants. McKinnon et al. [25] 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 [26,27]. However, an 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 [24,28]. Nevertheless, various studies reported that formulation with natural substances overcomes this obstacle [24, 29–31]. In the present study, we used a methyl cellulose seed coating method to aim for an 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 is similar to the results reported by Jaber and Ownley [32], who showed that neither *B. bassiana* nor *M. brunneum* altered *V. faba* seed germination. Nonetheless, Russo et al. [33] reported an enhancement in germination of *B. bassiana*-treated *Z. mays* seeds. Previous results in our laboratory also demonstrated that *B. bassiana* and methyl cellulose do not have inhibitory effects on *Z. mays* germination.

In the field trial experiment 1, there was not a 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 agreement with Russo et al. [8] who reported a 77 % germination of negative control, compared with 89 % germination of *B. bassiana*-treated *Z. mays* plants. Furthermore, the average number of leaves per plant did not show any significant difference between the negative control and *B. bassiana*-treated plants. When we considered the whole field, there were no significant differences among the presence of *S. frugiperda* larvae in negative control plants (2.04%) and in *B. bassiana* treated plants (1.2%), but we observed that the distribution was apparently based on the proximity of the negative control treated plants to the *B. bassiana* treated plants, thus we divided the field into three sections to find out the tendency of insects' occurrence in different parts of the field. We observed there was a significant difference when those sections were compared. There was a slight increment in the number of larvae in the rows of *B. bassiana*-treated plants that were immediately after the negative control plants, which declined eventually and became zero in the final 12 rows of *B. bassiana* treated plants (Fig. S3 supplementary materials). Ramirez-Rodriguez and Sánchez -Peña observed that when *B. bassiana* was applied

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as an endophyte it was not as pathogenic as when it was directly applied against *S. frugiperda* larvae [19].

In the second field trial, same results were observed in terms of germination percentage and 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, it was observed an increased in germination, from 71.5% in negative control plants to 91.20% and from 80.33% to 87.20% in *B. bassiana*-treated plants. In the average number of leaves per plant, we observed an increase of 3.12 to 5.20 leaves per plant in negative control and from 3.49 to 5.23 leaves per plant in *B. bassiana*-treated plants. This difference might be due to the higher concentration of blastospores that was used in the second trial (from 1 x 10^6 blastospores/mL in the first trial to 1 x 10^8 blastospores/mL in the second trial). Our results agreed with 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 [43].

We found that corn cob length was slightly higher in the negative control plants than in *B. bassiana*-treated plants. One possible reason was that we found that most *B. bassiana*-treated plants yielded more than 2 corn cob plants per plant (Fig. S4 supplementary materials). We also recorded the presence of pathogenic and beneficial insects. Representative photographs are shown in Fig. S5 supplementary materials. We detected high presence of beneficial insects, in comparison with pathogenic ones. Therefore, this indicates that 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. 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 [44]. Our findings need more experiments in the field to understand the mechanisms related to these results. However, we suggest that these results might be due to volatiles that may be produced by the *B. bassiana*-treated plants, which may function as insect repellents. Plants produce an array of volatile compounds from different plant parts, including flowers, leaves, stems, fruits, and roots, which perform crucial roles in host-feeding behavior of insects in feeding, mating, egg-laying, and aggregation of conspecifics. These volatiles function in contrasting activities, including insect attractant or repellent and may activate neighboring plants defense systems [45]. For example, naphthalene used as a potential insect repellent has been found to be produced by *B. bassiana* and *Muscodor vitigenus* in various studies [46,47]. Another mechanism proposed by Vega is the disruption of the production of kairomones by endophytic entomopathogens in the plants; these compounds are chemical signals produced by the plants which are used by the insects to localize them [38].

We did not find any significant difference in the corn cobs weight among the treatments, which is in agreement with Hernandez-Trejo et al. [44] findings, who found that application of *M. robertsii* on 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.

 doi:10.20944/preprints202101.0094.v1

Qayyum [27] 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 geographical regions. More investigations under field conditions are required to confirm all these findings.

For the other hand, we analyze 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 did not observed mortality of larvae, separated by each larval instar. However, 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 fed the larvae, but it is routine in our lab to find *B. bassiana* as endophyte in *Zea mays* plants that were grown after *B. bassiana*-methyl cellulose seed treatments. Mortality level of target insect pests with entomopathogens depends on larval developmental stage [27], inoculation method [34] or fungal strain [35]. *B. bassiana* does not induce direct mortality in insect pests, but often causes reduced larval growth rate, weight or longevity [36,37]. In our study, we observed that *S. frugiperda* larvae fed on plants treated with *B. bassiana* strain PTG4 were considerably affected in their development, with a prolonged larval stage, a decline in larval weight, and a smaller number of pupas. Vega [38] reported that by adding in the insect diet liquid cultured *B. bassiana*, after removing mycelia, it was possible to reduce the percentage of pupation and the pupation time was prolonged. 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 study, we observed that larvae fed on *B. bassiana* strain GHA-treated plants showed a decline in pupal length and weight. Since this was a no-choice experiment, there were not enough left-over plant materials to analyze and determine if there were any feeding preference in larvae between negative control plants and *B. bassiana*-treated plants. One perspective of this study is to analyze the microbiota of *S. frugiperda* excrement to determine if *B. bassiana* was present. Another important observation of this study was the adult male/female ratio obtained after development of larvae fed on *B. bassiana* treated *Z. mays* plants. We observed a lesser number of adult male moths, and female moths had some apparent alterations in their wings. This result is in agreement with Hassan et al [39], who reported that malformations occurred in *B. bassiana*-treated squash beetles. Interestingly Russo et al. recently reported differences on *Spodoptera frugiperda's* female fertility, fecundity and longevity using corn plants endophytically-inoculated with *B. bassiana* by foliar spray treatments; although in contrast, they did not find significant differences in the sex ratio of *Helicoverpa gelotopoeon* when they were fed on *B. bassiana* treated soy bean plants [40]. 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* [41]. In addition, we observed apparent parthenogenesis in female adults. An explanation to this could be that because there were lesser number of male and female adults and they were born with altered wings; they were urged to do parthenogenesis. However, more detailed studies are needed to confirm these apparent alterations on *S.*

frugiperda's adult morphology and physiology. 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 [42]. 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*.

5. Conclusions

We conclude that using GHA *B. bassiana*-seed treatment in *Z. mays*, the population of *S. frugiperda* in the field was controlled and economical damage was not observed. We obtained evidence that the environment was not affected, particularly there were not negative effects on beneficial insects, including 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, this technique has potential to be applied in the future for more sustainable agriculture practices.

6. Aknowledgements

We thank Dr. Refugio Lomeli-Flores from Colegio de Postgraduados campus Montecillo, who kindly provided the *S. frugiperda* eggs to start our colony; Mr. Fernando Ferrara-Rivero's field crew, who helped to design and provided technical support for both field trials; colleagues and students from Unidad de Formulación de Biológicos, for comments and constructive discussions; and Mr. Bharathan Vellayikodath-Soumyan for his encouragement and support during both field trials data collection. This study was supported by CONACYT Ph.D. scholarship 449643 to LKP. We thank SNI-CONACYT support 16614 to PTG, 9942 to RGF, 11924 to CRP, and 54340 to MJER.

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