

Article

Fungal-Based Biopesticide Formulations to Control Nymphs and Adults of the Desert Locust, *Schistocerca gregaria* Forskål (Orthoptera: Acrididae): A Laboratory and Field Cage Study

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Abstract: This is the first field study in which we have tested the efficacy of four different entomopathogenic fungal (EPF) formulations together in single study—i.e., Green Muscle, Green Guard, *Metarhizium anisopliae*, and an isolate of *Beauveria bassiana* (isolate WG-11)—against nymphs and adults of the desert locust, *Schistocerca gregaria* Forskål (Orthoptera: Acrididae). We conducted several different studies: (a) lethal bioassay against the 3rd, 5th, and adult stages under laboratory conditions; (b) sublethal effects on the reproduction, diet consumption, fecal production, and weight gain; (c) a greenhouse trial; and (d) a field cage trial. Under laboratory conditions, all EPF formulations caused significant mortality, and the highest efficacy was observed with Green Muscle, followed by Green Guard, *B. bassiana*, and *M. anisopliae*. Susceptibility was found to be greatest in 3rd-instar nymphs, followed by 5th instars, and then adults. Along with lethal effects, sublethal doses of EPF reduced the number of egg pods per female, total eggs per pod, and egg hatching, while extending nymphal developmental time and reducing adult longevity; again, Green Muscle performed better. Sublethal doses not only retarded reproduction, but also caused behavioral changes, including reductions in food consumption, fecal production, and weight gain. All EPF formulations not only produced significant mortality in laboratory conditions, but also performed very well under the greenhouse and field conditions. The maximum mortality against 3rd-instar (81.7% and 74.0%), 5th-instar (73.3% and 65.1%), and adult locusts (67.5% and 58.9%) was observed when using Green Muscle under greenhouse and field trials, respectively. The current study showed that all of the EPF formulations have the potential to reduce pest populations, and could be used in the integrated pest management program.

Keywords: desert locust; *Metarhizium acridum*; *Metarhizium anisopliae*; *Beauveria bassiana*; lethal effect; sublethal effect; greenhouse; field efficacy



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1. Introduction

Locust plagues are considered a major constraint in sustainable agriculture production [1–3]. The desert locust, *Schistocerca gregaria* Forskål (Orthoptera: Acrididae), has produced extensive losses of different food crops in Africa and Asia [4–6], especially during locust upsurges. In 2019, *S. gregaria* entered the province of Baluchistan in Pakistan from Iran, and then spread to different parts of Sindh, and finally to all four provinces. It was estimated that Pakistan faced a loss of PKR 353 billion and 464 billion if 25% damage occurred to Rabi (sown in winter) and Kharif crops (sown in spring), respectively

(<https://www.dawn.com/news/1555487>; accessed on 12 April 2021). There was apprehension that, if left uncontrolled, the number of locusts would increase 500-fold by June 2020 in East Africa [7]—potentially the worst infestation in past 100 years [8]. Climate change aggravates this situation due to high temperatures and increased precipitation, which accelerate locust growth and reproduction [9]. *Schistocerca gregaria* has three different developmental stages: egg, nymph, and adult. Adult females lay eggs in the form of egg pods in the soil at 5–10-cm depth [10]. Egg pods are 3–4 cm long, white in color, and shaped like bananas. Egg hatching occurs in 10–14 days when it is hot, or up to 65 days when it is cool, with each egg pod consisting of 80–160 eggs [11]. Hatched eggs produce nymphs known as hoppers that have 5–6 instars, and the developmental period from the first to the last instar is 28–48 days, followed by the adult stage, which can last for several months [12,13].

Management of locust plagues depends heavily on the use of chemical insecticides [14,15]. Prior to 1980, the organochlorine insecticide dieldrin was effectively used for many years to control this plague because of its persistence. However, due to their hazardous effects on animals and the environment [4], the use of organochlorines is now banned, and locust control mainly relies on organophosphate insecticides [16] that have low persistence, making them less effective than dieldrin [17]. Between 2003 and 2005, to combat the locust outbreak, over 13 million hectares were treated using 13 million liters of chemical insecticides [18]. During the 2019–20 locust outbreak in Pakistan, the Department of Plant Protection (DPP) sprayed 150,839 L of pesticides over an area of 300,595 hectares to suppress the locust population. The effects of non-judicious, intensive, and heavy use of chemical pesticides are probably underestimated, leading to environmental problems resulting from lethal effects on the whole ecosystem [19,20].

Knowing the hazardous effects of conventional insecticides, the development of safe alternatives is essential [21,22]. The utilization of natural enemies such as insect predators and parasitoids as control methods is not likely to be effective, due to the rapid growth and highly dispersed movement of locusts [4,15]. Green technology such as biological control uses microorganisms and birds to help to reduce the locust population [9], reducing the toxic impact of pesticides on the ecosystem. Microbial biopesticides could be an effective alternative to synthetic insecticides in locust management [6,23,24]. Microbial-based control methods, including the utilization of entomopathogenic fungi (EPF), have an extra benefit in that they invade the insect host via direct contact with the insect's integument instead of ingestion of the pathogen [23,25]. The Food and Agriculture Organization (FAO) has been working for the past 20 years in different countries on the development of biopesticides based on *Metarhizium acridum* (Driver and Milner) J.F. Bisch, Rehner and Humber (Hypocreales: Clavicipitaceae) to infect grasshoppers and locusts [18]. In field conditions, locusts can be exposed to *M. acridum* conidia in three ways: direct exposure to spray application, secondary pick-up of conidia from treated vegetation, or horizontal transmission from infected individuals during copulation and during aggregation phenomena [26]. While, there have been a number of studies on the efficacy of EPF such as *M. acridum* [15,26–30] and *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) [29,31] against *S. gregaria*, ours is the first to test four different formulations against *S. gregaria* in a single study.

In addition to lethal effects, EPF have sublethal effects that can disturb overall pest status [27,32,33]. Reduction in feeding was observed among acridid insect pests following exposure to sublethal doses of entomopathogens [32,34–38]. Reduced fecundity was observed in surviving adults after being exposed to sublethal doses [27,39,40]. Similar effects have also been observed in other insect pests, such as houseflies and mosquitoes [41–43]. This is the first study to test the effects of different EPF on the survival and longevity of different developmental stages of *S. gregaria*.

Many insect pests are able to thermoregulate their body temperature across different environmental conditions [44]. Acridid insects not only change their body temperature with respect to location, but also alter their body temperature (behavioral fevers) when infected with fungal conidia under field conditions. They assume basking postures or

locations that elevate their body temperature above optimal temperatures for microbial growth [45,46]. Commonly, lower mortality was observed in the field conditions compared to the laboratory [47]. Consequently, greenhouse and field cage experiments were included in this study in order to determine whether locusts exposed to sunlight could thermoregulate and reduce EPF efficacy compared to the laboratory. The objective of this study was to evaluate the efficacy of two different commercial formulations of *M. acridum*—one of *M. anisopliae*, and one local strain of *B. bassiana* (isolate WG-11)—against 3rd- and 5th-instar nymphs and adults of *S. gregaria* in laboratory, greenhouse, and field trials.

2. Materials and Methods

2.1. Insect Collection and Rearing

Adults of *S. gregaria* were collected from a swarm in Bhakkar Punjab (Pakistan) and reared in the laboratory inside wooden cages (30 cm × 30 cm × 30 cm). The cages were made with mesh wires on three sides of cages and on the roof. The front side of each cage was fitted with a door that contained white cloth with sleeves for operations like insect introduction, diet supplementation, and cleaning. The bottom of the cage consisted of several small holes (10-cm diameter) for egg laying. Plastic cups filled with sand with appropriate moisture content were placed below the wooden cage for egg collection. The adults were provided with wheat seedlings and wheat bran as food. Three light bulbs (60 W) were placed on the top of the roof for heating, and were switched on for 6–8 h each day for thermoregulation, which enabled basking behavior, as would occur in the field. After egg laying, the small plastic cups (covered with mosquito wire mesh) were placed under the light bulb (60 W) for 6–8 h to accelerate embryonic development. Upon hatching, first-instar nymphs were released inside the cage for subsequent development [26].

2.2. Entomopathogenic Fungi and Their Culturing

The biopesticides used in this study included two commercial products based on *M. acridum*—Green Guard (BASF, CSIRO FI (=ARSEF 324)) and Green Muscle (Elephant Vert, IMI 330189 (=ARSEF7486))—one *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) (CQMCC No. 0877) from China, and a local strain of *B. bassiana* (isolate WG-11) originally isolated from soil samples collected from Lal Suhanra, Punjab, (Pakistan). Each fungal isolate was individually inoculated on a potato dextrose agar (PDA) Petri plates (100 × 20 mm), wrapped with Parafilm, and finally placed in an incubator at 25 °C with a 14:10 h (light:dark) photoperiod. After 7–10 days, fungi were harvested using a sterile scalpel, followed by the conidial suspension being placed inside a falcon tube (50 mL) containing 30 mL of 0.05% Silwet L-77 solution. Eight glass beads were added to the tube and the solution was vortexed for about 5 min for proper agitation, and the desired conidial doses were determined using hemocytometer. Conidial viability of each fungal isolate was determined by spreading 0.1 mL of solution at rate of 1×10^6 conidia/mL on Sabouraud dextrose agar with 1% yeast (SDAY) plates (two plates for each isolate) wrapped with Parafilm and placed in the incubator at 25 °C with a 14:10 h (light:dark) photoperiod for 16 h [48]. A coverslip was placed on each SDAY plate, and approximately 200 conidia were assessed for germination for one count, with a conidium considered to be germinated if the germ tube was longer than the conidia [48]. Two counts were taken from each plate, totaling four counts for each isolate [49,50].

2.3. Bioassay against 3rd- and 5th-Instar Nymphs and Adults of *S. gregaria*

The bioassays were conducted to assess the effect of different doses (1×10^5 , 1×10^6 , 1×10^7 , and 1×10^8 conidia/mL) of entomopathogenic fungi against 3rd- and 5th-instar nymphs and adults (two weeks after last molt) of *S. gregaria* at the Department of Entomology, University of Agriculture Faisalabad, Punjab (Pakistan). Different doses of each fungal isolate were prepared in 0.05% Silwet L-77 solution. Both nymphal instars and adults were treated with a 1-mL suspension of entomopathogenic fungi using a 35-mL glass atomizer [51]. Control individuals were treated in the same fashion, but with 1 mL of 0.05% Sil-

wet L-77. After application, individuals were transferred to (30 cm × 30 cm × 30 cm) cages and provided with wheat bran and wheat seedlings grown in pots [16]. Each dose of specific formulations/isolates represents a treatment, each treatment consisted of three replicates (total 45 individuals, 15/replication), and the experiment was conducted twice (using new individuals and new materials), giving a total of six replicates [16,28]. Mortality was determined at 24-h intervals, with the final count at 12 days post-application [52]. The environmental conditions were maintained at a 14:10 h (light:dark) photoperiod, 30 °C, and 60% relative humidity (RH) [51].

2.4. Effect of a Sublethal Dose on the Reproduction and Development of *S. gregaria*

The effect of a sublethal dose (1×10^4 conidia/mL) of each fungal isolate was examined against adults of *S. gregaria*. For this, a group of 30 adults (15 females and 15 males) was treated as described in the previous section. The cages were provided with 25-cm-diameter plastic cups filled with clean sand, and were provided with appropriate moisture to encourage egg laying. The total number of egg pods laid by each female, average number of eggs present per pod, and percentage of eggs hatching was determined. The egg pods were collected at the end of experiment from among the different treatments, and mean egg pods per female were determined using the number of females still alive on the day of first oviposition. The egg pods were dissected and the total number of eggs per pod assessed. Then, eggs were kept under optimal conditions of 27 °C, 60% RH, and total egg hatching was determined among the different treatments [53]. After egg hatching, a group of 30 first-instar nymphs was collected from each replicate and kept inside the cages (30 cm × 30 cm × 30 cm) and provided with fresh diet (wheat seedlings and wheat bran). The developmental duration of different stages (1st-, 2nd-, 3rd-, 4th-, and 5th-instar nymphs, and adults) was determined. Each treatment consisted of three replicates, and the whole experiment was repeated twice, with new individuals and materials for each repetition.

2.5. Effects of a Sublethal Dose on Diet Consumption, Weight Gain, and Frass Production of *S. gregaria*

The effects of a sublethal dosage (1×10^4 conidia/mL) on diet consumption, weight gain, and frass production of *S. gregaria* were determined. Fifth-instar nymphs were treated with a sublethal dose of EPF in a similar manner as in the above-mentioned bioassays. Individual 5th-instar nymphs were weighed and then transferred into a small plastic box and provided with a wheat seedling for feeding. Prior to feeding, diet weight was measured on weight balance and then transferred inside the small boxes for feeding. Daily unused diet was taken from the boxes and measured on the weight balance, and diet consumed was calculated by subtracting the remaining diet from the initial diet. During days 1–15 after inoculation, fecal pellets were collected daily from each cage (replicate). The fecal production per cage was oven-dried at 80 °C until constant weight (24 h) and then weighed. The fecal production per insect per day for each replicate was calculated by dividing the daily fecal production by the number of insects remaining alive on that date [34]. Insects were also weighed each day. Each treatment consisted of three replicates, with each replication containing 15 individuals (total 45 individuals per treatment), and the experiment was repeated twice, giving a total of six replicates.

2.6. Greenhouse Trial

The greenhouse experiment was conducted using nymphs (3rd- and 5th-instar) and adults of *S. gregaria*. The 3rd- and 5th-instar nymphs and adults were inoculated in a similar manner as in the laboratory bioassay, with 1×10^9 conidia/mL. After inoculation, each individual of each specific stage was released inside a plastic pot (12.5 cm H × 8 cm D) that contained wheat seedlings and covered with a cylindrical metal cage (30 cm H × 8 cm D). All pots with cages were kept inside trays provided with water and placed on the bench top at 1-m height. Each treatment consisted of three replicates, with 20 pots per replicate (for each stage), and each pot contained one individual of a specific stage. The experiment

was repeated twice, giving a total of six replicates. Adult mortality was observed daily for 15 days [52]. In order to avoid scavenging by live locusts, their frass and dead were removed from the cages daily. Conditions were maintained at 30 °C, 16:8 h (light:dark) photoperiod, and 60% RH.

2.7. Field Trial

The effectiveness of different fungal isolates against 3rd- and 5th-instar nymphs and adults of *S. gregaria* was evaluated in the field cages (70 cm × 70 cm × 70 cm). These wooden cages with a door on the front were placed in the field, and 120 individuals of each stage were placed in each cage [51]. Each wooden cage served as replicate, and each treatment consisted of three replicates. As is common for field applications for Green Muscle [18,26], conidia were mixed with diesel and applied at a dose of 1×10^9 conidia/mL using a knapsack sprayer. The experiment consisted of five treatments, with three replicates (three cages) in each treatment. The whole experiment was repeated twice [26], with new individuals and materials each time. The data on the locust mortality were taken daily by removing the dead from each field cage. Untreated wheat seedlings were provided in the cages in the late afternoon so that the locusts had fresh food all night. Sometimes there was mortality in the first two days due to transport and handling; to compensate for this, any mortality that occurred in the first two days, in both the treated and untreated cages, was ignored. The experiment endpoint was day 15 after the introduction of insects and treatment application.

2.8. Statistical Analysis

In the laboratory trials, the mortality among the different developmental stages (3rd- and 5th-instar nymphs, and adults) was corrected with control mortality using Abbott's formula [54]. The values were subjected to two-way analysis of variance (ANOVA), and treatment means were separated by Tukey's honestly significant difference (HSD) test [55]. The mortality among different stages and intervals was found to be less than 5%. The effects on reproduction, diet consumption, frass production, and weight gain were subjected to analysis of variance (ANOVA). The mortality in greenhouse and field trials was subjected to analysis of variance (ANOVA), and treatment means were separated by Tukey's HSD test. All of the statistical analysis was conducted using Minitab 17 software [56].

3. Results

3.1. Bioassay against 3rd- and 5th-Instar Nymphs and Adults of *S. gregaria*

Against 3rd-instar nymphs, significant differences were observed between different EPF within each dose at 5 ($F_{3,23} \leq 6.05$; $p < 0.01$), 7 ($F_{3,23} \leq 7.92$; $p < 0.01$), and 12 ($F_{3,23} \leq 7.31$; $p < 0.01$) days post-treatment. Within each EPF, a significant dose response was observed ($F_{3,23} \leq 13.6$; $p < 0.01$ at 5 days; $F_{3,23} \leq 14.3$; $p < 0.01$ at 7 days; $F_{3,23} \leq 17.2$; $p < 0.01$ at 12 days) post-treatment. The mortality of the EPF at the 10^6 dose at 12 days ranged from 46.5 to 67.5%, while the 10^8 dose resulted in 65–95% mortality. The dose response was such that increasing the dose 100-fold (from 10^6 to 10^8) meant mortality was reached 5 days earlier. Green Muscle produced mortality of 68% at the 10^6 dose after 12 days, while similar mortality was observed at day 7 for the 10^8 dose (Figure 1a–c). For *M. anisopliae*, there was 47% mortality at 12 days at the 10^6 dose, and at 7 days at the 10^8 dose (Figure 1b,c). The time taken to reach > 50% mortality was 5 days after a 10^8 dose of Green Muscle, 7 days after a 10^7 dose of Green Guard, 7 days after a 10^8 dose of *B. bassiana*, and 12 days after a 10^7 dose of *M. anisopliae* was applied. By the end of the experiment on day 12, maximum mortality was observed with Green Muscle, followed Green Guard, *B. bassiana*, and *M. anisopliae*. Overall, mortality was greatest with Green Muscle and least with *M. anisopliae* at all time intervals and at all doses (Figure 1a–c).

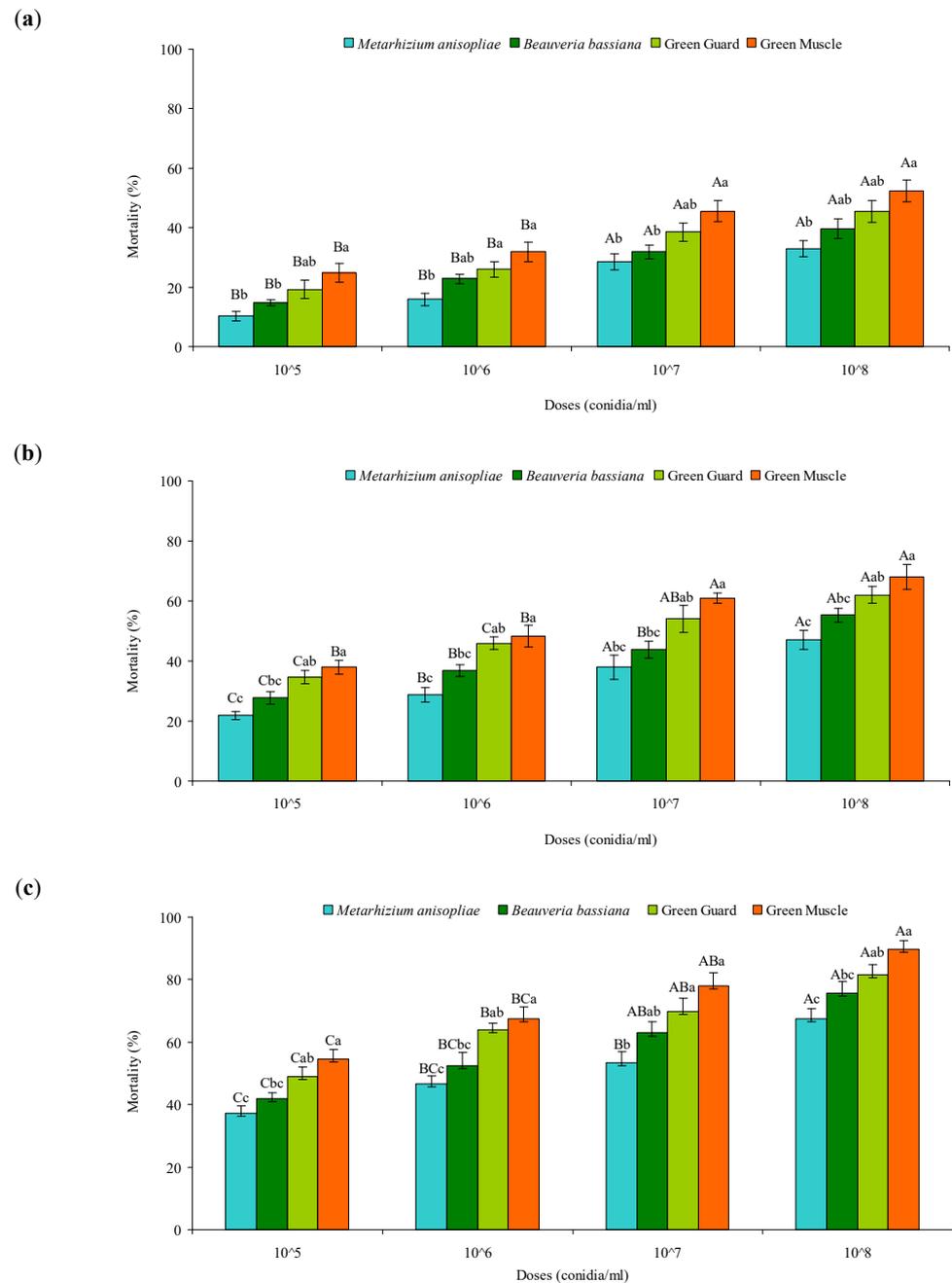


Figure 1. Mean mortality percentages (\pm SE) of 3rd-instar nymphs of *S. gregaria* after (a) 5 days, (b) 7 days, and (c) 12 days of treatment with various doses (10^5 , 10^6 , 10^7 , and 10^8 conidia/mL) of Green Muscle, Green Guard, *B. bassiana*, or *M. anisopliae*. Mortality data were corrected for control mortality, which was $<5\%$. For each subfigure: different lowercase letters indicate significant differences in mortality between each of the EPF; different uppercase letters indicate significant differences in mortality between doses (Tukey's HSD test at $p \leq 0.05$).

Clear treatment effects were observed against 5th-instar nymphs at different dose rates at 5 ($F_{3,23} \leq 15.1$; $p < 0.01$), 7 ($F_{3,23} \leq 22.4$; $p < 0.01$), and 12 ($F_{3,23} \leq 17.0$; $p < 0.01$) days post-treatment. All of the EPFs had a significant dose response ($F_{3,23} \leq 4.28$; $p < 0.01$ at 5 days; $F_{3,23} \leq 5.92$; $p < 0.01$ at 7 days; $F_{3,23} \leq 3.88$; $p < 0.01$ at 12 days). The dose response was such that increasing the dose 100-fold (from 10^6 to 10^8) meant mortality was reached just under 5 days earlier. The mortality due to the EPF at the 10^6 dose at 12 days ranged from 37.1 to 54.8%. For Green Muscle; there was 55% mortality at 12 days at a dose of 10^6 ,

similar to the 61% mortality at 7 days at a dose of 10^8 (Figure 2b,c). Until day 5, no EPF was able to kill 50% of 5th-instar nymphs, while at 7 days of exposure only two EPF (Green Muscle and Green Guard) caused > 50% mortality at a level not statistically different from one another, with the greatest mortality from Green Muscle (Figure 2a–c).

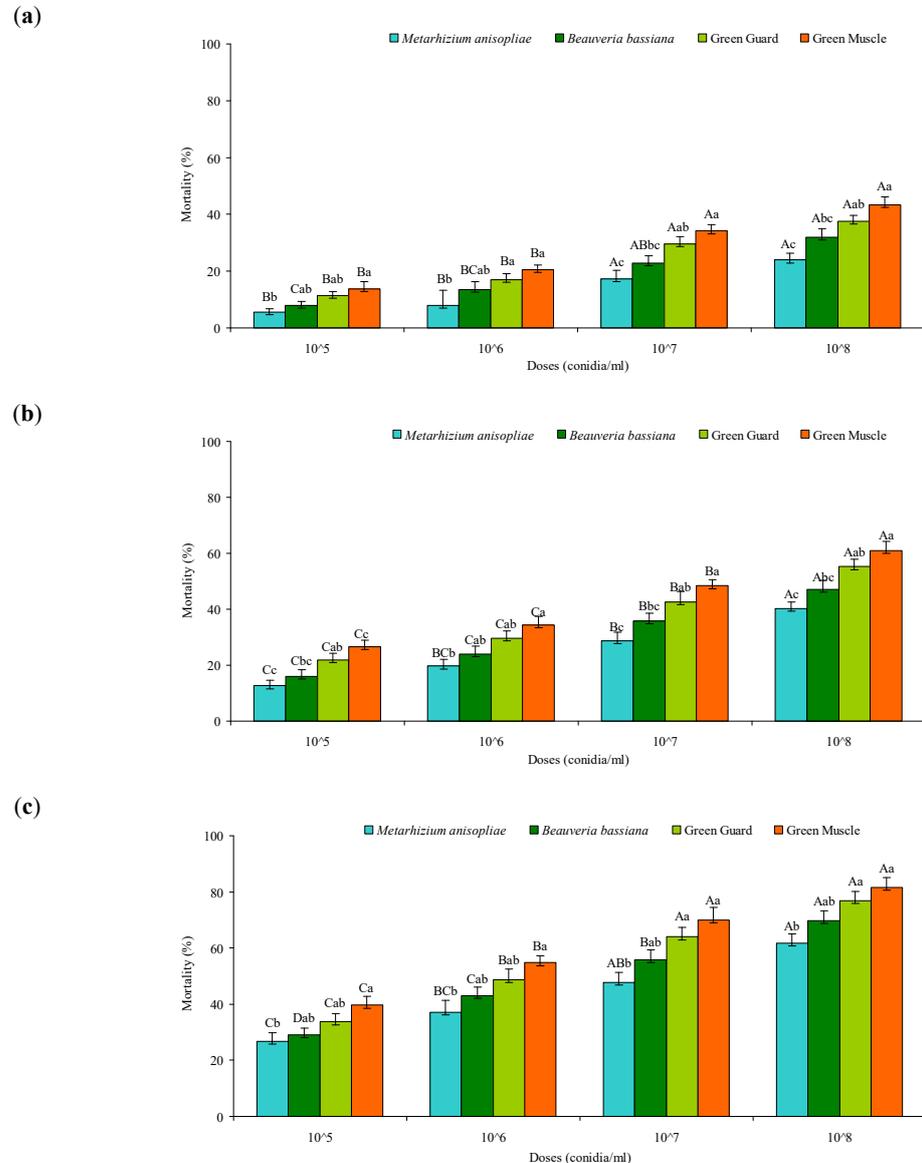


Figure 2. Mean mortality percentages (\pm SE) of 5th-instar nymphs of *S. gregaria* after (a) 5 days, (b) 7 days, and (c) 12 days of treatment with various doses (10^5 , 10^6 , 10^7 , and 10^8 conidia/mL) of Green Muscle, Green Guard, *B. bassiana*, or *M. anisopliae*. Mortality data were corrected for control mortality, which was <5%. For each subfigure: different lowercase letters indicate significant differences in mortality between each of the EPF; different uppercase letters indicate significant differences in mortality between doses (Tukey's HSD test at $p \leq 0.05$).

Against adults, each dose rate of EPF produced significantly more mortality compared to the control treatment ($F_{3,23} \leq 7.23$; $p < 0.01$), ($F_{3,23} \leq 5.53$; $p < 0.01$), and ($F_{3,23} \leq 3.17$; $p < 0.01$) at 5, 7, and 12 days after treatment, respectively. A clear dose response in terms of mortality was observed in different EPF at ($F_{3,23} \leq 16.6$; $p < 0.01$) 5 days, ($F_{3,23} \leq 17.1$; $p < 0.01$) 7 days, and ($F_{3,23} \leq 20.6$; $p < 0.01$) 12 days post-application. Once again, the dose response was such that increasing the dose 100-fold (from 10^6 to 10^8) resulted in mortality being reached just under 5 days earlier. The mortality of the EPF at the 10^6 dose at 12 days ranged from 49% for Green Muscle to 34% for *M. anisopliae*—slightly less than the levels

reached at 7 days with a 10^8 dose. No EPF caused 50% mortality at 5 days, and even at 7 days, only two EPF caused 50% mortality, and even then only at the highest conidial dose. After 12 days, all EPF caused > 50% mortality at the two highest conidial doses. As with treatments of 3rd- and 5th-instar nymphs, the greatest mortality was observed with Green Muscle, followed by Green Guard, *B. bassiana*, and *M. anisopliae* (Figure 3a–c).

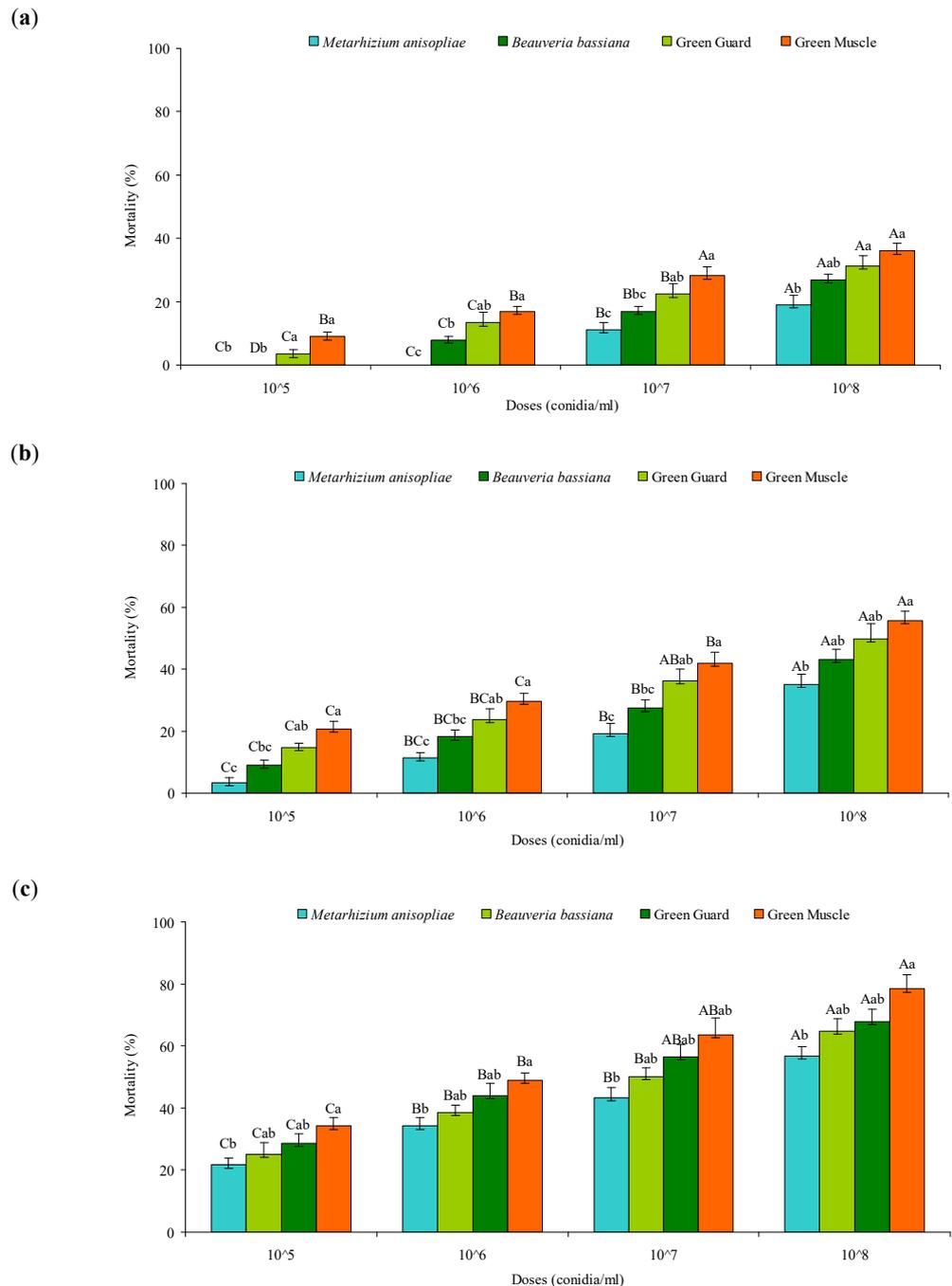


Figure 3. Mean mortality percentages (\pm SE) of *S. gregaria* adults after (a) 5 days, (b) 7 days, and (c) 12 days of treatment with various doses (10^5 , 10^6 , 10^7 , and 10^8 conidia/mL) of Green Muscle, Green Guard, *B. bassiana*, or *M. anisopliae*. Mortality data were corrected for control mortality, which was <5%. For each subfigure: different lowercase letters indicate significant differences in mortality between each of the EPF; different uppercase letters indicate significant differences in mortality between doses (Tukey's HSD test at $p \leq 0.05$).

Overall, 3rd-instar nymphs were found to be more susceptible than 5th-instar nymphs or adults.

3.2. Effects of Sublethal Doses on the Reproduction and Development of *S. gregaria*

Sublethal doses of EPF seriously affected the reproduction capability of *S. gregaria*, and also retarded the growth of their offspring. The females treated with all EPF except *M. anisopliae* produced fewer egg pods compared to the untreated controls ($F_{4,29} = 28.1$; $p < 0.01$), with no significant difference between the Green Muscle and Green Guard treatments (Table 1). In addition, there were fewer eggs per pod with EPF treatments ($F_{4,29} = 73.1$; $p < 0.01$), with Green Muscle treatments leading to the lowest number of eggs per pod (Table 1). Of the eggs laid, hatching was lowest with the Green Muscle and Green Guard treatments, with both *B. bassiana* and *M. anisopliae* resulting in intermediate levels between the other EPF and the controls. Even though the offspring themselves were uninfected, the offspring of infected females had an increase in nymphal developmental time ($F_{4,29} = 7.20$; $p < 0.01$) and a reduction in adult longevity compared to the untreated controls ($F_{4,29} = 41.2$; $p < 0.01$).

Table 1. Effects of applying one sublethal dose (1×10^4 conidia/mL) of Green Muscle, Green Guard, *B. bassiana*, or *M. anisopliae* to *S. gregaria* adults on the number of egg pods/female, number of eggs/pod, egg hatching, development time for offspring of different instars, and survival of resulting adults. Data are means \pm SE, and within each column and treatment, different letters indicate significant differences (Tukey's HSD test at $p \leq 0.05$).

Treatment	No. of Egg Pods/Female	Eggs/Pod	Egg Hatching (%)	Development Time (days)					
				L1	L2	L3	L4	L5	Adult
Green Muscle	0.76 \pm 0.11 c	23.13 \pm 1.75 d	34.44 \pm 4.99 c	8.63 \pm 0.32 a	7.31 \pm 0.17 a	7.51 \pm 0.20 a	7.90 \pm 0.26 a	10.21 \pm 0.42 a	18.65 \pm 1.17 d
Green Guard	1.06 \pm 0.12 bc	34.54 \pm 1.54 c	45.33 \pm 4.89 bc	7.96 \pm 0.23 ab	6.86 \pm 0.20 ab	6.96 \pm 0.21 ab	7.48 \pm 0.21 ab	9.64 \pm 0.32 ab	27.30 \pm 2.16 cd
<i>B. bassiana</i>	1.41 \pm 0.11 b	40.58 \pm 1.34 bc	53.33 \pm 3.56 b	7.22 \pm 0.17 bc	6.20 \pm 0.17 bc	6.71 \pm 0.17 b	7.11 \pm 0.20 abc	9.25 \pm 0.27 abc	33.20 \pm 1.81 bc
<i>M. anisopliae</i>	2.01 \pm 0.16 a	47.78 \pm 2.29 b	61.11 \pm 3.18 b	6.46 \pm 0.19 cd	5.60 \pm 0.16 cd	5.85 \pm 0.15 c	6.76 \pm 0.28 bc	8.41 \pm 0.28 bc	38.71 \pm 2.59 b
Control	2.56 \pm 0.15 a	67.36 \pm 2.44 a	85.55 \pm 4.00 a	6.04 \pm 0.16 d	5.08 \pm 0.11 d	5.01 \pm 0.13 d	6.20 \pm 0.21 c	8.06 \pm 0.28 c	55.95 \pm 2.75 a
F	28.3	73.1	21.6	22.1	28.0	30.7	7.20	7.30	41.2
p	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

3.3. Effects of a Sublethal Dose on Diet Consumption, Weight Gain, and Frass Production of *S. gregaria*

Sublethal doses induced significant behavioral changes in fifth-instar *S. gregaria*, with lower diet consumption, frass production, and weight compared to controls; most effects were already seen at 3 days after infection, and were clearly evident by 6 days (Figure 4a–c). As the days progressed, diet consumption declined in the treated groups ($F_{4,29} \leq 19.6$; $p < 0.01$), but increased in the untreated controls ($F_{4,29} \leq 6.51$; $p < 0.01$). Except for day 12, no significant difference was observed in diet consumption between Green Muscle and Green Guard. A similar trend was observed in frass production, with significantly lower frass production in the treated groups compared to the controls ($F_{4,29} \leq 104$; $p < 0.01$). Except for day 12, Green Muscle led to significantly lower frass production compared to the rest of the treatments. As days progressed, there was a decline in weight gain per day by treated locusts, while weight gain increased in the controls. Except at days 6 and 9, the effects of Green Muscle were not statistically significantly different from those of Green Guard (Figure 4a–c).

3.4. Greenhouse Trial

In the greenhouse trial, mortality in the controls was very low, and was <10% even at 15 days post-application, while mortality from all of the EPF was much higher (Figure 5). Green Muscle caused the highest mortality of third- and fifth-instar nymphs, but for adults, mortality was similar for Green Muscle and Green Guard. Mortality was less for the other two EPF, with mortality from *M. anisopliae* the lowest.

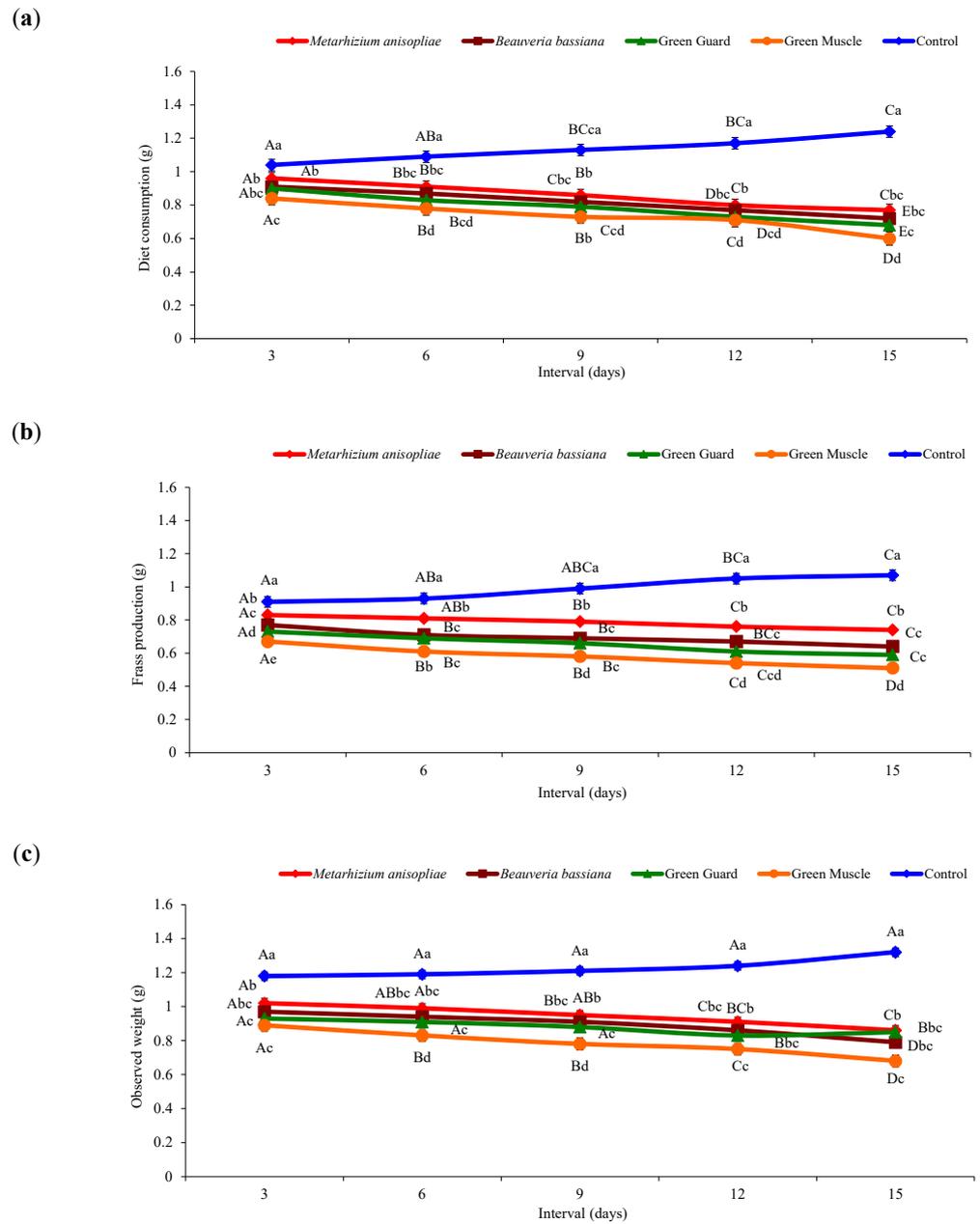


Figure 4. Means (\pm SE) for (a) diet consumption, (b) frass production, and (c) observed weight of fifth-instar *S. gregaria* treated with a sublethal dose (1×10^4 conidia/mL) of Green Muscle, Green Guard, *B. bassiana*, or *M. anisopliae*. For each subfigure: different lowercase letters indicate significant differences between different EPF; different uppercase letters indicate significant differences between different days (Tukey’s HSD test at $p \leq 0.05$).

As with the laboratory assay, the greenhouse bioassay demonstrated that 3rd-instar nymphs were most susceptible, followed by 5th-instar nymphs, and then adults (Figure 5a–c).

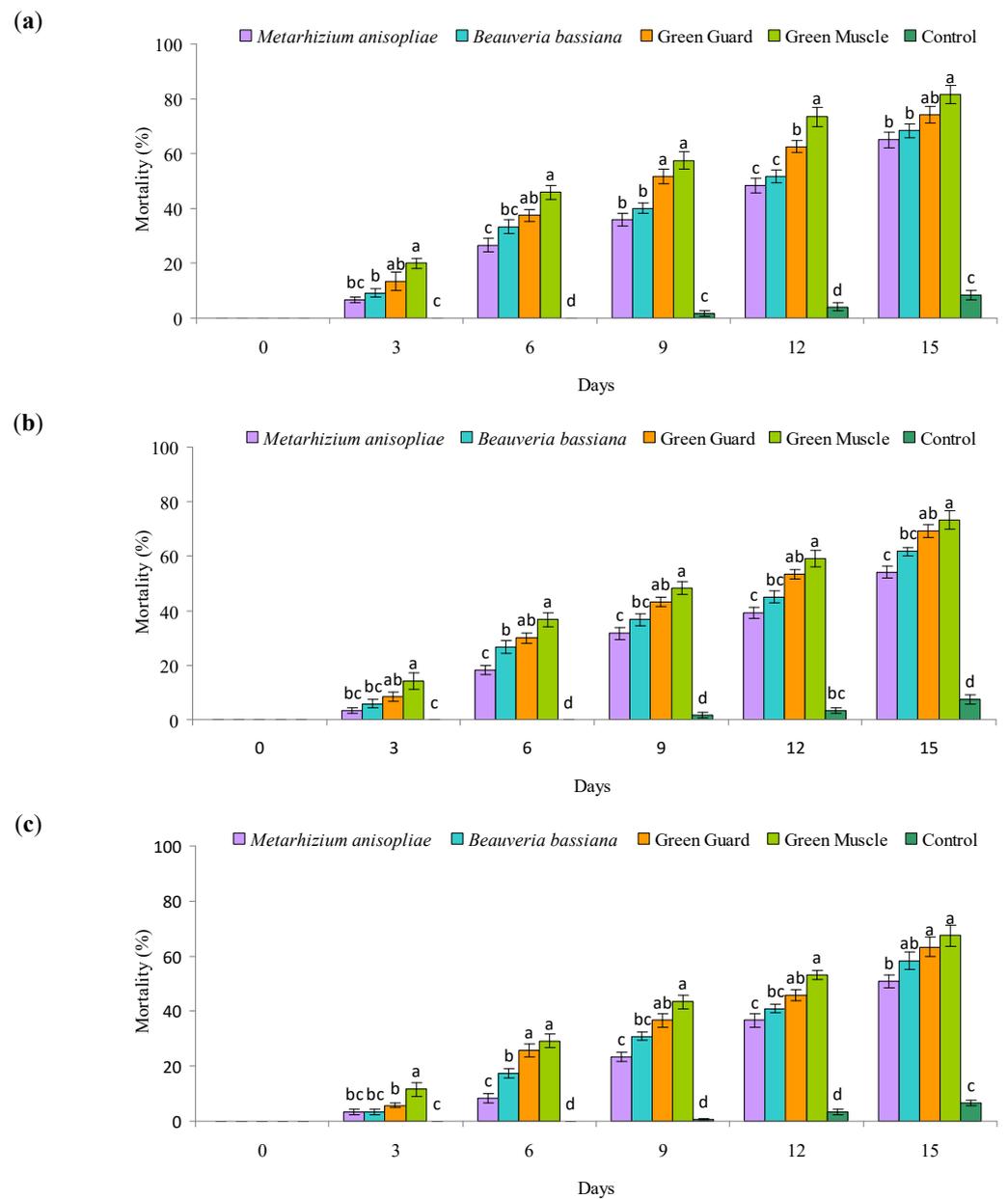


Figure 5. Mean mortality percentages (\pm SE) of (a) 3rd-instar, (b) 5th-instar, and (c) adult *S. gregaria* inoculated with 1×10^9 conidia/mL of Green Muscle, Green Guard, *B. bassiana*, or *M. anisopliae* in a greenhouse. Within each subfigure and between the treatments, different letters indicate significant differences (Tukey's HSD test at $p \leq 0.05$).

3.5. Field Trial

For EPF sprayed with a knapsack sprayer in field cages, all of the EPF produced significantly higher mortality ($F_{4,29} \leq 49.6$; $p < 0.01$ in 3rd-instar nymphs; $F_{4,29} \leq 69.9$; $p < 0.01$ in 5th-instar nymphs; and $F_{4,29} \leq 133.0$; $p < 0.01$ in adults) compared to untreated controls. Green Muscle caused the greatest mortality among all developmental stages, with mortality from Green Guard statistically slightly lower. As with the other assays, the field bioassay demonstrated that 3rd-instar nymphs were the most susceptible (Figure 6a–c).

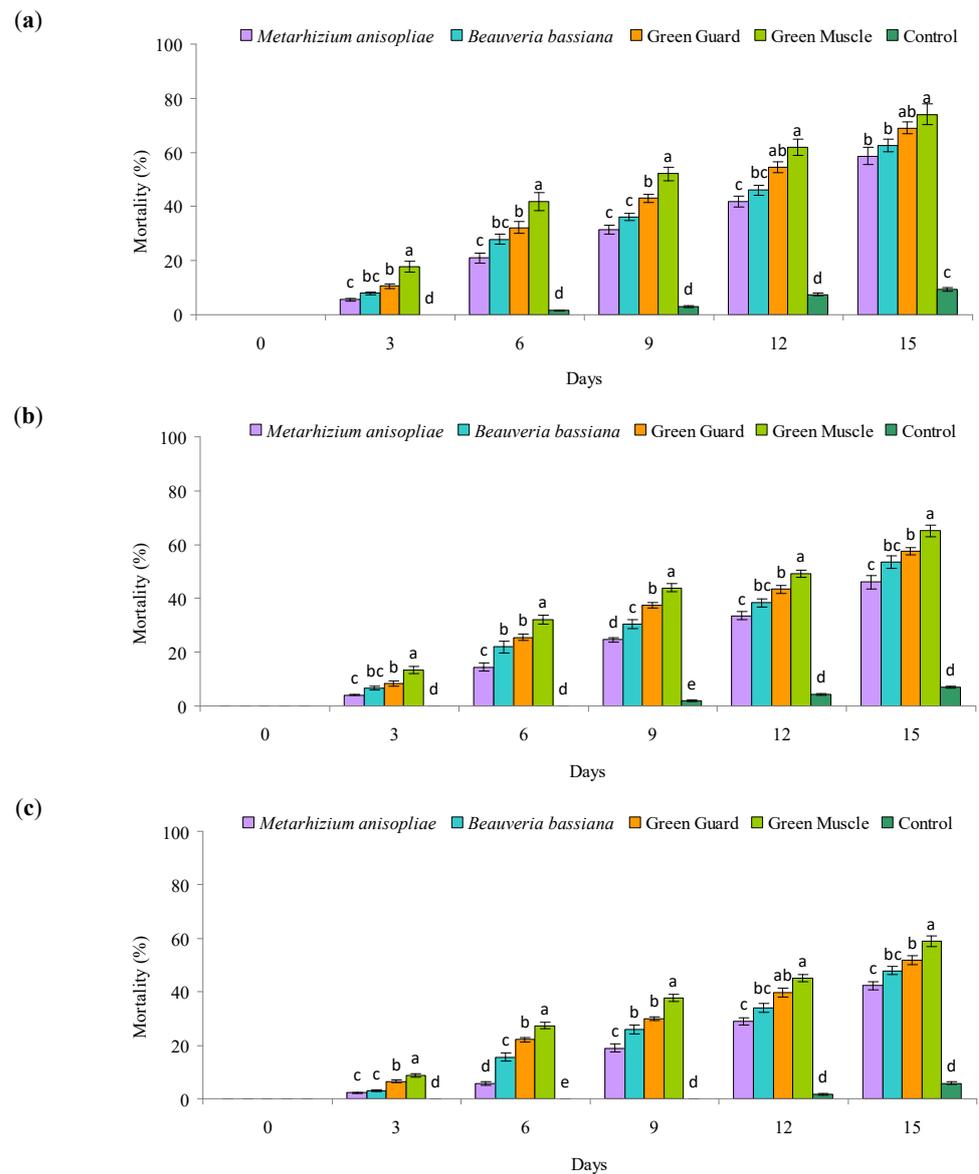


Figure 6. Mean mortality percentages (\pm SE) of (a) 3rd-instar, (b) 5th-instar and (c) adult *S. gregaria* sprayed with 1×10^9 conidia/mL of Green Muscle, Green Guard, *B. bassiana*, or *M. anisopliae* using a knapsack sprayer in field cages. Within each subfigure and between the treatments, different letters indicate significant differences (Tukey's HSD test at $p \leq 0.05$).

4. Discussion

In the present study, laboratory, greenhouse, and field trial results showed that all of the formulated products (Green Muscle, Green Guard, and *M. anisopliae* (CQMCC No. 0877)) and the local isolate of *B. bassiana* caused significant mortality of *S. gregaria*. Green Muscle performed best, with the greatest mortality, followed by Green Guard, *B. bassiana*, and *M. anisopliae*. Third-instar nymphs were more susceptible than fifth-instar nymphs or adults. Experiments in greenhouse and field conditions showed that all of the tested EPF have the potential to reduce pest populations under natural conditions. Previous studies showed significant efficacy of Green Muscle, Green Guard, and *B. bassiana* against different species of locust and grasshopper, and the present study showed that all of the EPF caused significant mortality of various stages of *S. gregaria*. As in our studies, 3rd-instar nymphs were found to be more susceptible to EPF by both Bashir and El Shafie [30], who treated desert locusts of various stages with *M. acridum*, and Youssef et al. [31], who treated desert locusts with *B. bassiana* and *Entomophthora* sp. Additive and synergistic interaction was

observed when lower and higher doses of *M. acridum* and *Paranosema locustae* were tested against 5th-instar *S. gregaria* nymphs under laboratory conditions [28].

The current study is the first report on sublethal effects of EPF on the reproduction ability, fertility, and subsequent developmental stages of *S. gregaria*. Even though the applied dose was 1/10th of a dose causing 40% mortality, and 1/10,000th of the dose causing 80% mortality, infected females produced fewer egg pods and fewer eggs per pod, and fewer of the eggs that were produced actually hatched. Increased developmental duration was observed in offspring nymphs, while reduction in adult longevity was also detected. Effects on reproduction have been found in the past, but these were at a dose that was eventually lethal. Fewer egg pods per female and a lower total number of eggs per pod were observed in *S. gregaria* and in the lamenting grasshopper *Eyprepocnemis plorans* (Charpentier) when infected with *B. bassiana* and *M. acridum* [39], and in *Dichroplus maculipennis* (Blanchard) and *Ronderosia bergi* (Stål) infected with *B. bassiana* [40]. A 21–53% reduction in egg pods per female was observed for Moroccan locusts *Dociostaurus maroccanus* (Thunberg) (Orthoptera: Acrididae) infected with *M. acridum* (IMI 380189) and *B. bassiana* [53]. Fungal infection seems to utilize host resources to such an extent that there are less resources available for egg maturation [33,57]. Contrary to our findings was a report by Blanford and Thomas [27], who did not observe any sublethal effect of *M. acridum* (IMI 330189) on *S. gregaria* reproduction. Furthermore, we have for the first time detected the effect of EPF on the fertility or hatching of eggs of this species. Lower egg fertility was observed in *D. maroccanus* when infected with fungal isolates [53].

Sublethal EPF infections not only reduced reproductive capability, but also led to behavioral changes, including reduction in food consumption, reduced fecal production, and reduced weight. Upon infection with EPF, reduction in feeding was observed, similar to the observations of Moore et al. [35] of a reduction in diet consumption in both *S. gregaria* and the Central American locust *S. piceifrons* starting three days post-infection. Likewise, Mohammadbeigi and Port [34] observed a reduction in the diet consumption of *Uvarovistia zebra* (Uvarov) (Orthoptera: Tettigoniidae) infected with fungi. In the present study, infected individuals had lower fecal production compared to controls. A reduction in fecal production in *D. maroccanus* was observed from all of the fungal treatments except EABb 90/2-Dm [53]. Reduction in weight was observed among the different EPF compared to controls but, contrary to our findings, a previous study [30] reported no significant difference in the weight of controls and individuals treated with neem oil and *M. acridum*.

In this study, we observed significant efficacy of EPF not only in the laboratory, but also in the greenhouse and field experiments, despite several environmental factors that may restrict the efficacy of these pathogens under natural conditions [47]. A most important factor is ultraviolet (UV) radiation, which directly reduces pathogen survival, and also enables locusts to increase their body temperature and produce immunity against fungal infection [58]. The current study revealed that in spite of thermoregulation, all of the EPF have the ability to cause mortality, indicating their potential as biocontrol agents in the field. Our results are consistent with [15], where it was found that Green Muscle was effective in controlling locust adults, with some added advantages compared to chemicals. As with the greenhouse experiment, in field conditions these results show that EPF have great efficacy against *S. gregaria*. Future research will focus on investigating the compatibility of EPF with other control agents, including entomopathogenic nematodes, chemical insecticides, and botanical extracts, as these have been found to be effective against a variety of insect pests [30,49,50,59–61].

5. Conclusions

The present research shows that Green Muscle, Green Guard, *B. bassiana*, and *M. anisopliae* have the potential to control locusts under field conditions. In addition, there are sublethal effects on their reproductive ability and behavior, with reduction in diet consumption, frass production, and weight observed. Future research needs to explore the combination of EPF with other control agents—including chemical insecticides, mi-

crosporidia, and botanical extracts—as a means of integrated pest management of locusts in the field.

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