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Effect of Two Strains of *Beauveria bassiana* on the Fecundity of *Nezara viridula* L. (Heteroptera: Pentatomidae)

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Abstract: The southern green stink bug (SGSB) *Nezara viridula* (L.) (Heteroptera: Pentatomidae), has become a significant pest of soybean and cotton in southern Central America and in the lower mid-southern U.S. A laboratory colony of SGSB was used to evaluate the effect of two isolates of *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Hypocreales: Cordycipitaceae) including the commercial strain GHA and the Mississippi Delta native NI8 strain on the fecundity of this pest. Water control, Tween-80, and four concentrations of each strain ($n \times 10^4$, 10^5, 10^6, and 10^7) were evaluated. Both native and commercial isolates with the highest concentrations were susceptible to the SGSB. Females, however, were much more pathogenic to both strains than males. Lethal concentration of the native strain (236 spores/mm^2) was 1.4-fold lower (326 spores/mm^2) than the GHA strain for females evaluated 20 d post-exposure. Greater concentrations (1.1×10^7 spores/mm^2, 5.2×10^6 spores/mm^2) were required to kill males with both strains native and commercial, respectively. For controls and lower concentrations, cumulative fecundity ranged from 1178 to 2082 eggs/10 couples/life reproduction, compared with 597 and 673 eggs/10 couples sprayed ($n \times 10^6$ and 10^7) with the native NI8, respectively, and 386 eggs/10 couples sprayed ($n \times 10^7$) with the commercial GHA.

Keywords: biological fitness; southern green stink bug; entomopathogenic fungus

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1. Introduction

The southern green stink bug, *Nezara viridula* (L.) (Hemiptera: Pentatomidae) is a key pest in soybean, *Glycine max* (L.) (Fabaleas: Fabaceae) and is invading this crop worldwide [1]. However, when soybean season changes and because its ability of environment adaptation and polyphagous activity, *N. viridula* shifts easily its preference from its primary host to cotton, *Gossypium hirsutum* (L.) (Malvales: Malvaceae) [2]. Both plant species are the largest crops grown throughout the southern U.S. It has been reported that 30 million ha of soybean and >5 million ha of cotton were in production in the U.S. in 2019, with a total yield loss of USD 191 million and USD 96 million, respectively, caused by the stink bug complex including *N. viridula* [3]. Those massive economic losses are caused by both adults and nymphs due to yield reduction by laceration and toxic saliva injection in developing soybean seeds and cotton fruits [4].

Despite the availability of insecticide treatments worldwide, piercing–sucking insects such as *N. viridula* have been challenging to suppress due to their highly mobile nature [3,5–8]. Nevertheless, the primary management strategy to reduce the *N. viridula* and other species of stink bug populations relies on insecticides and unfriendly environmental agronomical practices [1]. It has been reported that biological control is an alternative method for controlling *N. viridula* other than using insecticides, avoiding their hazardous effect on human health and the environment [9]. Entomopathogenic fungi are recognized as promising insect biological control agents [4]. Several genera of entomopathogen fungi such as *Beauveria*, *Metarhizium*, *Verticillium*, *Entomophara*, *Paecilomyces*, *Hirsutella*, and *Isaria*

have shown virulence to a considerable numbers of insect pest's species [10]. However, the genera *Beauveria* and *Metarhizium* have been effectively utilized on a wide scale as microbial control agents.

Beauveria bassiana (Balsamo-Crivelli) Vuillemin (Ascomycota, Hypocreales: Cordycipitaceae) is a saprobic fungus that infects and consumes insects and other arthropods. It seems to be an appropriate alternative for the control of *N. viridula* as its effectiveness has been reported by previous studies [4,9,11–15]. Although several studies have been conducted to determine the potential impact of *B. bassiana* against *N. viridula*, there is no published information on the sub-lethal effect of this entomopathogenic fungi on *N. viridula*. Accordingly, the current study was carried out to investigate the effect of *B. bassiana* on the fecundity of this pest and determine the lethal concentration (LC_{50}) for females and males of *N. viridula.* It is important to mention that there are not entomopathogenic fungi available that have been commercially developed for pentatomids as a primary target; therefore, the commercially GHA strain (BotaniGard 22WP) and the Mississippi Delta native NI8 strains isolated from *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) were used for this study. The native strain NI8 was collected and isolated by Gerald Leland (USDA-ARS, Stoneville, MS, USA) in 2003. He reported higher in vitro conidia and beauvericin production than the commercial strain GHA (10). Since 2005, the Delta native strain NI8 has been produced at USDA-ARS, Southern Insect Management Research Unit regularly for the tarnished plant bug research program.

2. Materials and Methods

2.1. Nezara Viridula Colony

Insects used in this study were adult females and males of a nineth generation (F_9) laboratory colony kept on an artificial diet at the USDA-ARS, Southern Insect Management Research Unit, Stoneville, MS, USA. The colony was originally collected in early September–late October of 2019 from crops of soybean at different sites in several counties in Mississippi. Insects were reared in a plastic rearing container of 1.5 Gal (43 × 28 × 9 cm) (https://www.fleetFarm.com (accessed on 30 November 2020) (400–500 nymphs or adults) in environmental chambers set at 27 ± 2 °C, 55 ± 10%RH, and 12:12 h (L:D) photoperiod. This method was specifically designed for medium-scale production of *N. viridula* even-aged nymphs and/or adults using artificial diet [16].

2.2. Beauveria Bassiana Spore Powder

The technical powder (spore powder) of the native strain NI8 was obtained from the USDA-ARS, Southern Insect Management Research Unit (SIMRU), Stoneville, MS, USA. The NI8 spore powder is produced at SIMRU using solid-state fermentation and the harvested hydrophobic aerial conidia are kept at −80 °C in an ultra-low temperature freezer (Fisher-brand, Isotemp, Columbia, MD, USA). The commercial strain GHA (BotaniGard 22WP) was obtained from The Biological Products Industry Alliance (BPIA) LAM International, Butte, MT, USA and was applied according to labeled instructions.

2.3. Bioassay Procedure to Determine Survival and Fecundity of N. viridula

Spore powder of the strains NI8 (1.23×10^{11} spores/g) and GHA (1.19×10^{11} spores/g) were mixed in 50 mL of 0.04% Tween-80-water solution (TWEEN-80; Sigma-Aldrich P8074, Darmstadt, Germany). After dilution, the treatment's final concentrations were 5×10^4, 5×10^5, 7×10^6, and 7×10^7 spores/mL for NI8 and 5×10^4, 5×10^5, 7×10^6 and 7×10^7 spore/mL for GHA. Both strains were assessed for spore germination following the method described in [17]. The Scepter TM 2.0 Cell Counter and Software Pro were used to quantify the number of spores/mL for each suspension [17,18]. Five aliquots of 6 mL of each dilution of both NI8 and GHA strains were assessed for spore quantification (spore/mm^2) following the procedure described in [18,19]. The final mean number of spores per mm^2 per treatment were 22, 77, 159, and 356 spores/mm^2 for 5×10^4, 5×10^5, 7×10^6, and 7×10^7 spores/mL for NI8, respectively, and 22, 82, 165, and 352 spore/mm^2

for 5×10^4, 5×10^5, 7×10^6 and 7×10^7 spore/mL for GHA, respectively. Aliquots of 6 mL of each spore suspension and two controls (water and Tween-80) were applied to each group of ten adult females and ten adult males of *N. viridula* that were 2–3 d old. Two-three days old adults are strong enough to manipulate without affecting mortality. The Image-Pro Plus 7.0.1. software (Rockville, MD, USA) was used to separate females and males. Figure 1 shows morphological differences for the recognition of both sexes. Adults were placed in a Petri dish 15 cm in diameter (Pioneer Plastic 175-C) and located in the middle of the sprayed area (38.5 cm in diameter). Both strains' suspensions were sprayed with a special designed spray tower (air-atomizing nozzle 1/4 J with a fluid cap 2850 and air cap 70) (Spraying System Co. FN5925–001A, Wheaton, IL, USA) [20]. Treatments were sprayed from the lowest to the highest concentrations. After each application, the sprayer and nozzle were rinsed by applying an aliquot of 10 mL of 10% hypochlorite solution and two aliquots of 10 mL of R.O. water (reverse-osmosis).

Figure 1. *Nezara viridula* female (**left**) and male (**right**). Notice the claspers on the terminal abdominal segment (right) (Photo by Katherine Parys USDA-ARS).

Nozzles were changed for each strain to avoid cross-contamination. After application, groups of adult females and males sprayed with similar concentration were released into an insect rearing cage (30 × 30 cm^2; BioQuip 1466A, Los Angeles, CA, USA) (10 cages: 10 couples/cage). The insects were maintained following the procedure described by [15] with some modification. Paper towel was used to cover the bottoms of the cages and a filter paper (diameter 185 mm) folded in half was placed for oviposition. *Nezara viridula* adults were fed every 4–5 d by placing one fresh corn (cob) *Zea mays* L. (Poales: Poaceae) previously disinfected by submerged 10 min in 10% hypochlorite solution. In addition to their feeding, two–three packs of stretched parafilm artificial diet were placed on top of the screening rearing insect cages [15]. The feeding process was repeated until all adults were dead. Dead adults were removed every other day and recorded. Insects were considered dead when no movement was observed after prodding. Dead insects were then placed individually into 37 mL plastic cups (T-25 SOLO-cup, Pleasant Prairie, WI, USA) containing solid media [20] and examined daily for sporulation of fungus on the cadaver. Cages were cleaned each week or when needed. Egg masses were removed every other day, and a total number of egg mases and numbers of eggs per egg mass were recorded. The Image-Pro Plus 7.0.1. Software (www.mediacy.com (accessed on 15 November 2021) was used for egg counts.

2.4. Statistical Analysis

Ten treatments included *B. bassiana* native strain NI8 (5×10^4, 5×10^5, 7×10^6, and 7×10^7 spores/mL), the commercial strain GHA (5×10^4, 5×10^5, 7×10^6 and

7×10^7 spores/mL), and the water and Tween-80 controls. Each treatment was evaluated on 10 couples of *N. viridula*. Nonparametric estimates of survival were compared between treatments using PROC LIFETEST. To calculate slopes and estimate lethal concentration (LC_{50}) of *B. bassiana* strains, mortality of females and males was recorded from control effects using Abbott's formula [21], and resistance ratio (RR_{50}) and confidence intervals were calculated using the method of Robertson and Priestler [22]. PROC PROBIT-SAS was used to analyze and correct data from the bioassays [23].

3. Results

Both isolates of *B. bassiana* tested were pathogenic to *N. viridula*. Adult females were found to be more pathogenic to native and commercial strains than that adult male. The LC_{50} values (236 spores/mm^2) of the native strain NI8 was 1.4-fold lower (326 spores/mm^2) than the commercial strain GHA for females evaluated 20 d after application (Table 1). Much higher concentrations (11,134,963 spores/mm^2, 5,206,971 spores/mm^2) were required to kill males with both strains, NI8 and GHA, respectively (Table 1). Due to *N. viridula* males had low sensitivity to both *B. bassiana* strains, the probit model did not produce a good fit of the data among concentrations. However, the overall survival of adults in the control treatments was 100% compared with the other treatment for both strains during the first 20 d of evaluation (Figure 2).

Table 1. Lethal mortality response (LC_{50}) of *Nezara viridula* female and male treated with 2 different strains of *Beauveria bassiana*, native NI8 and commercial GHA.

Concentration Response ((µg/vial or Diet Cup)								
Strain	***n***	**Slope ± SE**	**LC_{50}(95%CI) [1]**	**Probit Trend**				**RR50 (95%CI) [4]**
				Test for Slope [2]		**Test for GoF [3]**		
				X^2	***p* > X^2**	**X^2**	***p* > X^2**	
15 Days								
F [5]-NI8	60	0.79 ± 0.33	323 (163–9693)	5.81	0.0159	2.05	0.1293	6.2×10^{10} (4.2×10^{115}–9.3×10^{135})
F-GHA	60	0.21 ± 0.25	15,310 (-)	0.61	0.4350	1.22	0.2940	2.9×10^{12} (1.5×10^{-9}–2.8×10^{16})
M [6]-NI8	60	−0.04 ± 0.21	5.2×10^9(-)	0.03	0.8711	1.23	0.2927	1
M-GHA	60	0.05 ± 0.21	5.2×10^6(-)	0.05	0.8149	0.00	1.000	1.0×10^{15} (3.3×10^{117}–3.0×10^{146})
20 Days								
F [5]-NI8	60	0.97 ± 0.36	236 (148–1239)	7.17	0.0074	1.97	0.1397	1
F-GHA	60	0.52 ± 0.25	326 (102–4.6×10^9)	4.28	0.0385	1.48	0.2265	1.24 (0.26–5.97)
M [6]-NI8	60	0.05 ± 0.42	1.1×10^7(-)	0.01	0.9042	3.14	0.0429	1.1×10^4 (8.5×10^{87}–1.61×10^{96})
M-GHA	60	0.48 ± 0.21	5.2×10^5(-)	4.07	0.0437	0.44	0.6400	1.9×10^4 (1.1×10^{-36}–3.4×10^{44})

[1] LC_{50} and RR_{50} values are in conidia/mm^2, mortality was measured at 15 and 20 d after exposure. [2] Test for slope-significance indicates how dose affects mortality. [3] Test for Goodness of Fit (GoF) significance indicates if an error from probit trend is more significant than expected for simple binomial response. [4] Resistance ratio (RR_{50}) and 95% CI were calculated using a formula from Robertson and Priesler [21]. Differences among RR_{50} values are significant if 95% CI do not include 1.0. RR_{50} compare the LC_{50}s among the lowest LC_{50} as a control. [5] Female. [6] Male. (-) Values for CI not calculated.

There were statistically significant differences in female survival among concentrations for NI8 strains (Figure 2) (Table 2). High significant differences ($F_{(9, 23)} = 4.28$, $p \leq 0.0001$) in fecundity (number of eggs/mass/collection) were found on females and males exposed to the highest concentration of GHA strain (7×10^7), while couples sprayed with the native NI8 (7×10^6 and 7×10^7) were significantly higher compared with both controls and the lower concentrations (Figure 3). Figure 4 shows the cumulative fecundity for controls and lower concentrations ranged from 1178 to 2082 eggs/10 couples/life reproduction,

compared with 597 and 673 eggs/10 couples sprayed with NI8 7 × 10^6 and 10^7, respectively and 386 eggs/10 couples sprayed with GHA 7 × 10^7. In general, similar trends were observed for oviposition during the first 10 weeks except for the commercial GHA with the highest concentration; masses started lower during the 5 weeks after exposure and nearly stopped oviposition after that, while the higher concentrations of the native strain continued ovipositing until the week 13 (Figure 4). Oviposition in controls and lower concentrations for both strains were obtained until after week 22.

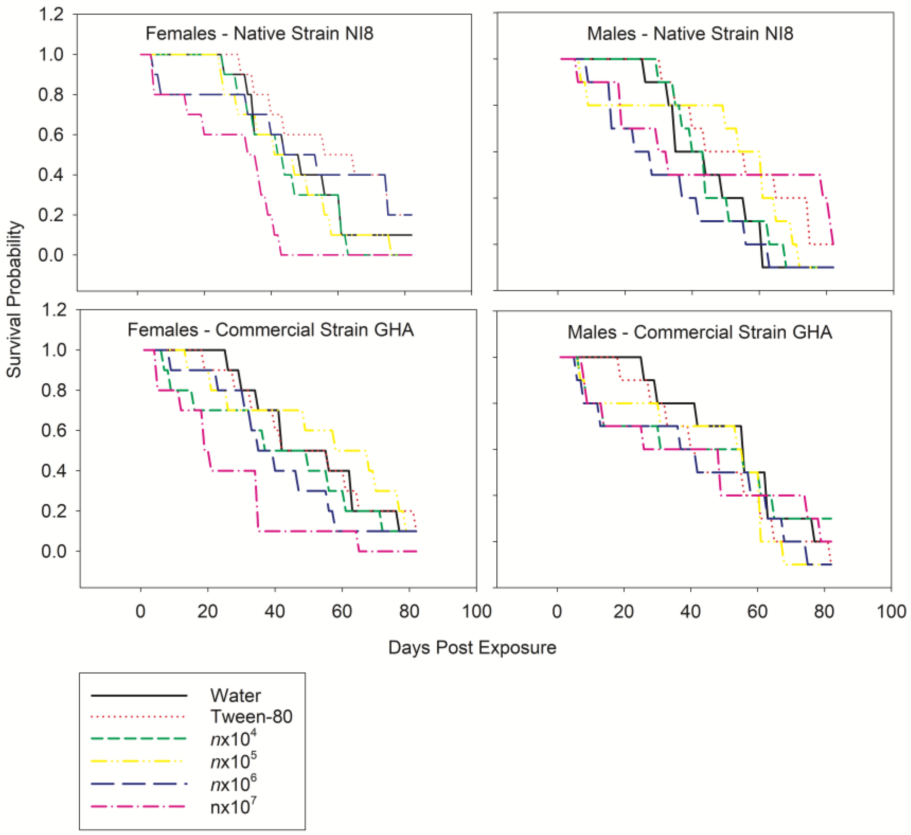

Figure 2. Product-limit survival estimates for females and males of *Nezara viridula* exposed to 2 strains of *Beauveria bassiana* NI8 and GHA at different concentrations (spores/mm^2). Survival probability at age x (p = 0.5, LIFETEST of Equality over Strata).

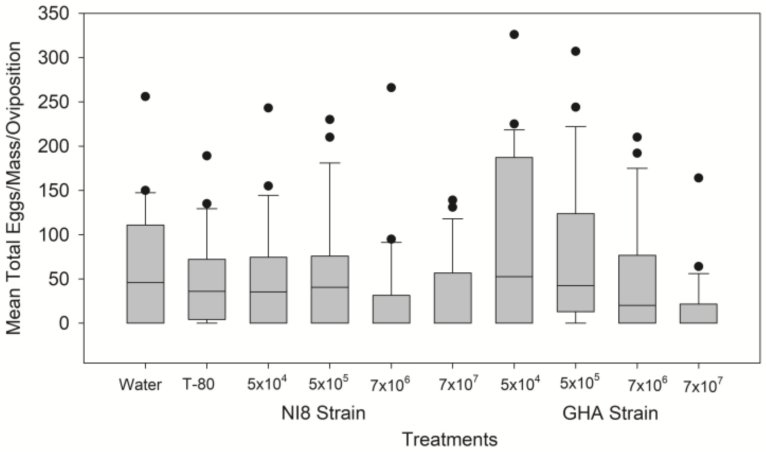

Figure 3. Mean total eggs/oviposition of *Nezara viridula* treated with 2 strains of *Beauveria bassiana* NI8 and GHA at different concentrations (one-way ANOVA and Tukey's HSD test p = 00.5; means SD).

Table 2. Test of Equality over Strata LIFETEST for *Nezara viridula* female and male treated with 2 different strains of *Beauveria bassiana*, native NI8 and commercial GHA, scored at 20 days after exposure.

Test *	Female NI8			Female GHA			Male NI8			Male GHA		
	X^2	DF	$p > X^2$	X^2	DF	$p > X^2$	X^2	DF	$p > X^2$	X^2	DF	$p > X^2$
Log-Rank	10.6448	5	0.8423	6.0283	5	0.3035	5.1194	5	0.4015	4.0621	5	0.5405
Wilcoxon	7.7946	5	0.1679	5.3465	5	0.3751	5.6568	5	0.3411	5.1611	5	0.3965
(LR)-2 Log	11.1875	5	0.0478	8.3178	5	0.1396	5.8850	5	0.3176	5.9121	5	0.3149

* Homogeneity test of survival curves ($p = 0.05$).

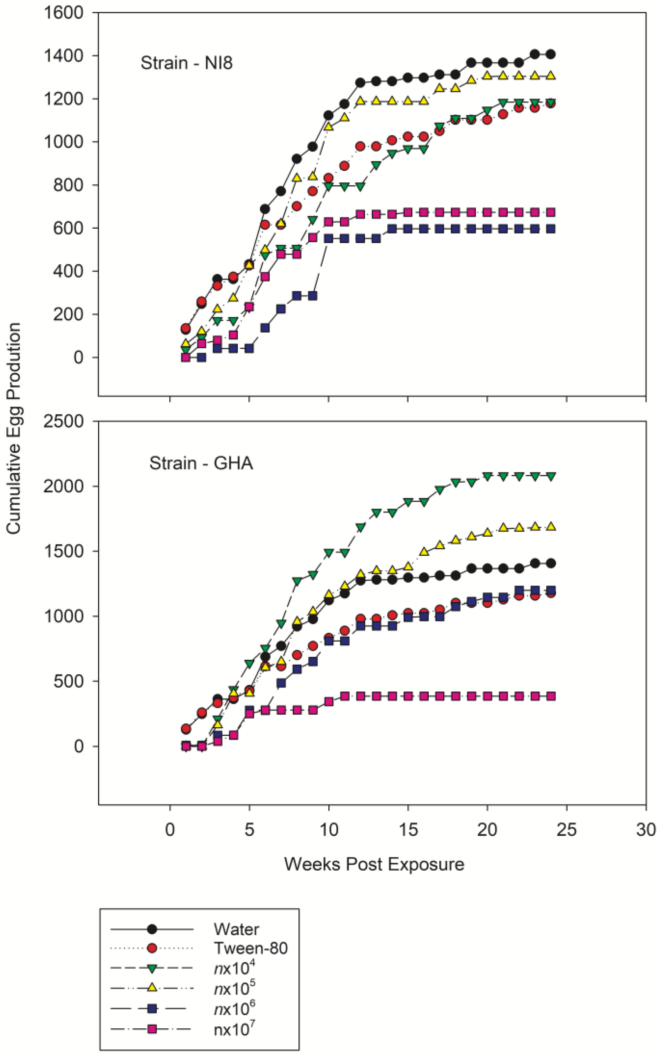

Figure 4. Cumulative egg masses production/couple/life reproduction of *Nezara viridula* exposed to 2 strains of *Beauveria bassiana* NI8 and GHA at different concentrations.

4. Discussion

The method used for the response data for *N. viridula* with *B. bassiana* concentrations and its fecundity as an explanatory variable was successfully evaluated and survived. The method was able to estimate CL_{50} values for females (spores/mm^2) in both strains 15 days after exposure. Low mortality noted for adult females and males in the water and Tween-80 controls suggested that *N. viridula* adults are susceptible to *B. bassiana* infection through direct exposure, affecting survival and oviposition. This oviposition behavior may affect crop damage and pest population growth even if insects are not killed by disease [24]. As we see in Figures 2 and 4 the infected females were ovipositing during the period before

death. However, although *B. bassiana* is a slow killer, it is essential to clarify that mortality of male and female adults treated with the highest concentration of both strains started as early as 5 days after exposure, which negatively correlated with a fecundity that declined over time (Figures 2 and 4). Although there are not reports on the impact of *B. bassiana* on the fecundity of *N. viridula*, there are previous studies investigating the effect of this pathogenic fungus and other fungi on other insects [25]. For example, some investigations corroborated our results where they found that fecundity was reduced in curculionids, chrysomelids (Coleoptera), and pyralids (Lepidoptera) that survived *B. bassiana* inoculation during their larval stage [26–28]. Identical results were reported for the green lacewing, *Chrysoperla rufilabris* Burmaister (Neuroptera: Chrysopidae) [29] where high concentrations of *B. bassiana* impacted all demographic measurements of this beneficial insect reproduction and survival. Similarly, another study also demonstrated that the oviposition rate of *Lygus hesperus* Knight (Hemiptera: Miridae) declined over time in the population that survived *B. bassiana* exposure [24]. Our results and previous investigation in other insects such as *L. hesperus* indicated that *B. bassiana* can impact the fecundity of the infected insects; however, those affected insects continue feeding on host plants, such as cotton or soybean and is unlikely to contribute to a reduction in the fruit of seed damage.

The mortality test in our study indicated that a low concentration of conidia might not be sufficient to kill female adults of *N. viridula* nor affect their fecundity, meaning that females need high concentrations of *B. bassiana* conidia and males need it even higher to successfully complete the fungus infection cycle that consists in four phases: attachment, germination, penetration, and vegetative growth [25]. Several investigations have shown that the integument composition, mainly lipids and phenols play a major role in preventing microorganisms from attaching to or penetrating the insect host's cuticle [30–32]. Research on *N. viridula's* cuticle–conidia binding has demonstrated that hydrophobic interaction occurring between the conidia wall and the epicuticle is responsible for the passive, nonspecific adhesion detected with hydrophobic conidia [33]. This can explain the low susceptibility observed in our investigation for lower concentrations of both *B. bassiana* strains, with no significant differences in mortality rates originating from no concentration-dependent patterns. Interestingly, a positive relationship was observed between the concentration rates of the decrease in fecundity mainly on the commercial strain GHA (Figure 3).

Also, the conidia showed limited attachment to the insect surface; some of our preliminary studies demonstrated that higher conidia densities can be found on infected adults and their exuvia on body regions that contain numerous setae or spines and depressions in insects treated by direct spray and by contact, indicating that conidia can be trapped between depressions or can be picked up as the insects walk on plants. Similar results were observed in previous studies on *N. viridula* treated with *Metarhizium anisopliae* [33]. In our preliminary study mortality as high as 90% was found in 2nd, 3rd, 4th, and 5th, instars and adults that were treated by direct spray. However, it is important to mention that in our preliminary study, the infected insects and their exuvia were exposed to an arena of artificial diet [18] with a regimen of 85–90% RH which differed from our present study where treated insects were maintained in cages placed in an open area under 50–60% RH, that could affect infection. It is well-known that entomopathogenic fungi are influenced by many factors, including biotic factors such as the developmental stages of the host and concentrations of the pathogens [34]. In the case of *N. viridula,* the composition of the cuticular hydrocarbons is an additional important factor that can affect infection and reproduction. Overall, it was unknown if *B. bassiana* affects the fecundity of *N. viridula*; this laboratory experiment provides information needed to better understand of how this fungus infection affects the mortality and fecundity of this insect in the laboratory. Such behavior changes can occur in the field if this microbiological agent is used for *N. viridula* management. As we mentioned, the infected insects that survive infection continue feeding, but the population growth rate is affected. On the other hand, the high concentration needed to kill this insect can affect the population of other predators and non-target arthropods. Therefore, decisions to deploy

B. bassiana whatever the strain should be based on an overall assessment of ecological and economic benefits and cost [29].

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