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Choice behavior of the generalist pentatomid predator *Podisus maculiventris* when offered lepidopteran larvae infected with an entomopathogenic fungus

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Abstract To understand the practical application of entomopathogenic fungi and predatory insects in an integrated pest management program, it is necessary to investigate the compatibility of biocontrol agents occupying the same niche. The objectives of our research were to understand the response behavior of the predatory spined soldier bug, *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae) exposed to volatiles from the entomopathogenic fungus *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae). We assessed the predator's behavior when presented with *B. bassiana*-infected lepidopteran prey and determined the volatile profile of *B. bassiana* spores. Choice behavior of *P. maculiventris* nymphs towards *B. bassiana*-infected (non-sporulating and sporulating) versus uninfected larval fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), was determined in choice arenas. The predator nymphs more frequently chose the uninfected prey and avoided fungus-infected prey. Of five *P. maculiventris* nymphs that fed on non-sporulating fungus-infected prey, four molted to

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adulthood, and the time required to molt to adult was similar to that of nymphs that fed on uninfected prey. In Y-tube olfactometer choice assays, 65% of the nymphs (26/40) avoided potato dextrose agar plugs containing *B. bassiana* and preferred clean agar plugs. Gas chromatography-mass spectrophotometry analysis showed that the primary volatile chemicals emitted from *B. bassiana* spores were 2-ethylhexanol and 2-methyl-1-butanol. Applications of *B. bassiana* combined with releases of *P. maculiventris* may be a viable option for pest management in the field.

Keywords *Podisus maculiventris* · *Beauveria bassiana* · Choice arenas · Oosporein · Volatile olfactory chemicals

Introduction

The spined soldier bug, *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae), is a generalist predator native to North America (McPherson 1982). It preys on a variety of forest and field crop pests, especially coleopteran and lepidopteran insects (McPherson 1980, 1982; Vacante et al. 1995; Desurmont and Weston 2008; Montemayor and Cave 2012). Among predatory pentatomids, *P. maculiventris* is considered the most promising agent for augmentation and conservation biological control of lepidopteran pests (Wiedenmann et al. 1994; Aldrich 1998; Oliveira et al. 2004; Szendrei et al. 2010). For example, *P. maculiventris* has been released to control the fall armyworm, *Spodoptera frugiperda* (J. E. Smith), and *S. exigua* (Hübner) on tomato and cotton with great success (Ables and McCommas 1982; De Clercq et al. 1998; Capinera 2017). The spined soldier bug is sold commercially (De Clercq and Degheele 1994) as a major predator for use in various cropping systems in the USA and Europe (Biever et al. 1992; Obrycki et al. 1997; Montemayor and Cave 2012).

Conventional and organic pest management strategies may complement the action of parasitoids and predators with applications of entomopathogenic fungi to reduce pest populations. However, little is known of the mechanisms involved and how these fungi might affect a predator's survivorship and behavior. França et al. (2006) reported that when nymphs of *Podisus nigrispinus* (Dallas) fed on larval *Alabama argillacea* (Hübner), infected with the entomopathogenic fungus *Metarhizium anisopliae* (Metsch.) (Hypocreales: Clavicipitaceae) or *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae), their reproduction as nymphs was negatively affected. However, adults that fed on infected *A. argillacea* larvae were not affected.

The susceptibility of pentatomids, including predatory species (Todorova et al. 2002; França et al. 2006; Firouzbakht et al. 2015; Araujo et al. 2020), to infection by *B. bassiana* varies according to the fungal strain, inoculum concentration, insect stadium, host plant, and abiotic conditions (Muştu et al. 2011; Erper et al. 2016). *Beauveria* species produce secondary metabolites, including oosporein (Jayaprakash and Saranraj (2017) that is toxic to erythrocyte membrane ATPases (Jeffs and Khachatourians 2007). Oosporein, a red dibenzoquinone pigment (Fan et al. 2017), is a denaturing agent and a potent antibiotic specific to Gram positive organisms. The reddish color of oosporein can be seen in the insect cadaver prior to mycosis. Fan et al. (2017) suggested that oosporein acts as an antimicrobial compound to limit microbial competition in *B. bassiana*-killed hosts, allowing the fungus to maximally use host nutrients to grow and sporulate. In addition, Abendstein et al. (2003) indicated that oosporein has no insecticidal, antifeedant, or growth inhibitory effects when ingested by lepidopterous larvae.

In an interaction study of a predator and an entomopathogenic fungus, Pell and Vandenberg (2002) observed in choice bioassays that the convergent lady beetle, *Hippodamia convergens* (Guérin-Méneville), preferred to consume uninfected live Russian wheat aphid, *Diuraphis noxia* (Kurdjumov), rather than aphid cadavers infected with *C. fumosorosea*. Ormond et al. (2011) observed that adults of the seven-spot lady beetle, *Coccinella septempunctata* (L.), avoided contact with leaf surfaces and soil inoculated with *B. bassiana* as well as aphid cadavers that were mycosed or sporulating. Alma et al. (2010) observed that adult females of the mirid *Dicyphus hesperus* (Knight) foraging for whitefly nymphs rejected *C. fumosorosea*-infected nymphs 96% of the time, compared to 39% of non-infected nymphs. Meyling and Pell (2006) observed that females of the minute pirate bug *Anthocoris nemorum* (L.) encountering aphid cadavers sporulating with *B. bassiana* rapidly withdrew and avoided contact with the dead

insects. The authors attributed this avoidance behavior to olfactory cues related to mortality risks of the adult insects or their offspring.

The behavioral response of a predator to the presence of a fungal entomopathogen will have a direct effect on the efficacy of the biological control agent (Baverstock et al. 2010). Therefore, to understand the practical application of using an entomopathogenic fungus and a predatory insect in a pest management program, it is very important to know if the two biological control agents are spatially compatible. The main objectives of the research reported herein were to: (1) measure the prey preference of *P. maculiventris* nymphs when offered *B. bassiana*-infected or uninfected prey, (2) quantify the response behavior of *P. maculiventris* nymphs exposed to *B. bassiana* spores on agar plugs in a Y-tube and assess their subsequent development to the adult stage, and (3) determine the volatile profile of *B. bassiana* spores.

Materials and methods

Insect rearing

All insects were reared in a growth chamber at 25 °C under a L:D 14:10 photoperiod. Newly hatched fall armyworm (FAW) larvae from eggs collected from a laboratory colony were reared on artificial diet (Southland Products, Inc., Lake Village, AR, USA) in Bio-Assay trays with lids (Frontier Scientific Inc., Newark, DE, USA).

For the choice arena trials, spined soldier bug (SSB) eggs were purchased from Rincon-Vitova Insectaries, Inc. (Ventura, CA, USA). Approximately 50 eggs were removed from the containers and placed into a glass Petri dish (100 × 15 mm) with filter paper and a moistened cotton ball until hatching occurred. Each group of SSB hatchlings was provided a moistened cotton ball only until they molted, at which time they were offered FAW larvae (second or third instar) to familiarize them to the prey. Once the nymphs reached the third instar, groups of five were transferred into a separate plastic Petri dish (100 × 15 mm: Thermo Fisher Scientific, Inc. Waltham, MA, USA) containing FAW larvae. Upon molting to the fourth instar, each nymph was transferred to a separate plastic Petri dish (55 × 20 mm) containing a clean filter paper (55 mm in Ø), a FAW larva, and a moistened cotton ball, and reared to the fifth instar. Due to logistical issues that made SSB eggs unavailable from Rincon-Vitova Insectaries. Inc. while the choice arena trials were on-going, nymphs were reared by Entomology Solutions (Louisville, KY, USA) and supplied directly to us as third and fourth instars for the remainder of the study. Upon arrival, the nymphs were individually fed FAW larvae in separate plastic Petri dishes until they reached the fifth instar. Fifth instars ∼ 48 h old were used in the experiments.

Inoculation of prey larvae

A suspension of *B. bassiana* strain GHA was prepared by mixing 1 ml of BotaniGard® ES (BioWorks, Rochester, NY, USA) with 100 ml of sterile distilled water. The spore concentration was counted using a C-Chip Neubauer Improved hemocytometer (Incyto DHC- NO 1, Chungnam, Korea) viewed under a Leica DM500 Brightfield microscope (Leica Microsystems, Wetzlar, Germany) at 400X magnification and adjusted to 10^7 spores ml^{-1} by adding sterile distilled water. Spore viability (i.e., percentage spore germination) was determined by spreading 100 µl of the suspension on three separate Petri dishes containing Difco™ potato dextrose agar (PDA: Becton, Dickinson and Co., Sparks, MD, USA) and incubating them at 25 °C for 16–24 h under a L:D 14:10 photoperiod. Mean percentage germination was measured by counting among 200 spores per plate (100 spores per area) the number of spores that produced a germ tube at least half the length of the spore. Counts were made at 400X magnification. Spore viability was consistently > 95%.

Twelve fourth-instar FAW were placed individually in a single well of a sterile polystyrene 12-well Costar® cell culture cluster plate (Corning Inc., Corning, NY, USA) for the fungal treatment and control. Each larva was drop-inoculated in the well with 300 µl of the *B. bassiana* suspension or water only for the control treatment. A moistened paper towel was placed over the wells, and the lid was placed on top of the plate and held closed using rubber bands. Plates were kept at 25 °C under a L:D 14:10 photoperiod for 3–4 days until the *B. bassiana*-infected larvae turned to a reddish color due to oosporein in the hemocoel (Fan et al. 2017) or the cadavers were completely covered with white spores (= mycosed). Once the larvae reached the appropriate

infection stage, reddish color or mycosed, all plates were dated and placed in a freezer at -15 °C for 14–30 days before use in the prey preference trials. Only uninfected control larvae that survived four days were frozen for later use.

Prey preference trials

Prey preference behavior of the SSB nymphs was studied by using choice arenas made from an inverted sterile Falcon® Petri dish (150 × 15 mm: Thermo Fisher Scientific, Inc. Waltham, MA, USA) lined with a round Whatman™ #5 filter paper (General Electric Healthcare, Chicago, IL, USA) trimmed to fit snug inside the lid. A diametric line was drawn in pencil on the paper. The cap from a 50 ml CentriStar™ plastic centrifuge tube (Corning, Inc., Corning, NY, USA) was used to trace a circle in the center of each paper.

Two types of infected FAW larvae (thawed mycosed, whitish color and non-mycosed, reddish color) were tested with thawed non-infected larvae (uninfected control). Prey were removed from the freezer ~ 15 min before conducting the choice trials to warm to room temperature (~23–24 °C). One infected larva and one uninfected larva were placed perpendicular to the diametric line, each 5.5 cm on opposite sides from center. A sterile camel hair paintbrush was used to place a fifth-instar SSB (starved for 24 h) under the cap of a 50 ml plastic centrifuge tube section (~ 9 cm high) with a circular hole (20 mm Ø) punched in the top, previously placed mouth down on the traced circle. After the nymph walked out of the hole onto the filter paper, the cap was removed from the arena, and the dish bottom was put in place. The dish arena was placed near the center of the laboratory bench with overhead luminescent light and room temperature of ~ 23–24 °C. Choice test observations were conducted in the morning to avoid any effect of sunlight direction on the behavior of the nymphs. However, the window blinds were closed to prevent any bias due to sunlight, and the orientation of the dish arenas as well as location of the prey were arranged for each trial. Observation time was recorded once the nymph touched the filter paper and was moving away from the cap. Each predator was allowed 15 min to roam while making a prey choice. The amount of elapsed time for each predator in the arena to make a choice by inserting its proboscis and feeding for at least 1 min on the chosen prey was recorded. Each trial was conducted in a clean Petri dish with a new filter paper circle, and the plastic cap was cleaned with 95% alcohol and air dried prior to each trial. Directional orientation of the prey types was randomly switched between trials to ensure that bias did not exist between treatment choices of the nymph.

The tested predator was removed after the 15 min allotted time and transferred to the bottom of a clean Petri dish containing clean Whatman™ #1 filter paper (General Electric Healthcare, Chicago, IL, USA), live, uninfected FAW larvae (offered as needed), and a moistened cotton ball inside a top cap excised from an Eppendorf tube. Once the nymph was in the dish, the lid was put in place, the dish labeled, dated, and placed on a cafeteria tray. Trays with dishes were transferred to a growth chamber with RH averaging 40 ± 3.2% (range 16–75%) by using plastic containers filled ~ 75% with water and refilled as needed. Nymphs were observed daily to record number of days to mycosis, death, or molt to adult. The total number of trials in which the predator chose between a mycosed, whitish prey type and a non-infected prey type was 42 (six times day^{-1} for seven days). The total number of trials in which the predator chose between a non-mycosed, reddish larva prey type and a non-infected prey type was 39 (ten times day^{-1} for three days and nine times for one day).

Y-tube olfactometer assays

For each day's series of assays, a suspension of *B. bassiana* strain GHA (10^7 spores ml^{-1}) prepared as described above was spread on ten Petri plates (100 × 15 mm) containing PDA. The plates were sealed with Parafilm™ M (Bemis Co., Inc, Neenah, WI, USA) and transferred to a growth chamber set at 25 °C under a L:D 14:10 photoperiod. After 14 days, average spore concentration per plug was determined by extracting one PDA plug from each plate with a #4 cork borer (50.3 mm^2). Each plug was placed in a separate 15 ml plastic centrifuge tube containing 1 ml of 0.01% Triton-X 100 solution and vortexed for 15 s. Suspensions were allowed to settle for 1 min. From each tube, a 10 µl aliquot of the suspension was removed from the bottom of the tube and placed into a C-Chip Neubauer hemocytometer. The number of spores ml^{-1} was counted under a Leica Brightfield microscope. Average spore concentration per plug

ranged 3–4 $\times$ 10^7 spores ml^{-1} ($\sim$ 6–7 $\times$ 10^5 spores mm^{-2}).

For each daily series of assays, 15 control plugs were extracted from untreated PDA plates and individually placed on the end of a rectangular piece ($\sim$ 22 $\times$ 7 mm) of a plastic cover slip (Fisherbrand® 22 $\times$ 22 mm; Fisher Scientific, Pittsburgh, PA, USA). The plugs were spaced apart on filter paper in a large Petri dish (150 $\times$ 15 mm) that was then sealed with Parafilm. Afterwards, 15 fungus-treated plugs were extracted from the ten 14-day-old plates. Plugs were individually placed on the end of a rectangular piece of a plastic cover slip and spaced apart on filter paper in a large Petri dish that was then sealed with Parafilm. All plugs were held at room temperature until use in the day's assays.

Preference/aversion behavior of nymphs towards plugs containing *B. bassiana* were performed as described by George et al. (2013). The Y-tube olfactometer system was a closed push/pull system, comprised of an olfactometer Clean Air Delivery System with Vacuum Control—4 Choice Push Pull (CADS: Sigma Scientific LLC, Micanopy, FL, USA) to which two Y-tubes (24 mm inner diameter $\times$ 192 mm in length) were connected, each with two external odor source chambers and one inline glass specimen chamber (21 mm inner diameter $\times$ 100 mm in length) at each of three ports on a given tube. A vacuum line was attached to the inline specimen chamber (= stem) at the downwind (pull) end of the Y-tube. Charcoal-purified, humidified air was "pushed" through each arm from the CADS to provide an upwind airflow source that would move over a treatment plug in the arm of the Y-tube. The system pushed air from each arm to move evenly into the stem with a force of 0.3 l min^{-1}, allowing the air to be gently "pulled" over the nymph's antennae and body via the vacuum line. The air push and pull system was calibrated so that the vacuum pull rate was 1.2 l min^{-1} for two Y-tubes running simultaneously with two air inflow lines each at 0.3 l min^{-1}.

A fungus-treated PDA plug was placed into a Y-tube arm, and a control plug was placed in the other arm. Separate clean forceps were used to handle control and treatment plugs to avoid cross contamination. All 58 replicates for the Y-tube assays used fifth-instar SSB that were starved for 24 h before the start of the assay. One nymph was introduced into the stem, and assay time was set for 15 min. Treatments were periodically switched between arms for each new assay to ensure that air flow rate bias did not exist between treatment choices. The Y-tube was cleaned with 70% ethanol and air-dried after each assay.

Recording of nymphal behavioral choice corresponded to one of three possible responses: fungal plug arm, control plug arm, or no choice. At the end of each assay, the time the nymph spent in the stem or treatment arms was recorded. A "choice response" was defined as the location at which a nymph settled for $\geq$ 60% of the time after making a choice. If a nymph never settled, i.e., spent $<$ 60% of the time in any part of the Y-tube, the response was no choice. After each assay, nymphs were removed from the Y-tube apparatus, transferred individually to Petri dishes prepared and incubated as described above for the choice arena tested predators. Nymphs were observed daily to record number of days to mycosis, death, or molt to adult.

Fungal volatile collection and identification

Fungal volatiles were collected with a technique using a solid-phase microextraction (SPME) fiber (50/30 μm) assembled with divinylbenzene/carboxen on polydimethylsiloxane on a StableFlex™ fiber (Supelco Inc., Bellefonte, PA, USA). A 2.5 cm^2 piece of PDA agar was cut from a 14-day-old *B. bassiana* strain GHA culture and placed in a 400 ml beaker previously washed with Sparkleen™ (Fisherbrand, Pittsburgh, PA, USA), ethanol, and acetone and baked overnight at 50 °C. The beaker was wrapped with aluminum foil. The SPME fiber, sterilized for 30 min in the injector of the gas chromatograph-mass spectrophotometer at 270 °C, punctured the aluminum foil and was maintained vertically with a SPME holder in the beaker for 30 min to collect the volatiles. The same operation was conducted with uncontaminated agar to subtract volatiles from the agar media from the fungus profiles. This procedure was replicated three times from new *B. bassiana* cultures each time.

The SPME fiber was desorbed for 2 min at 240 °C under splitless mode (i.e., all the analyte sample vaporized in the injector went onto the column), and the odor constituents were separated on a Thermo Scientific TraceGold-5MS (Thermo Fisher Scientific, Inc. Waltham, MA, USA) capillary column (30 m $\times$ 0.250 mm inner diameter; 0.25 μm film thickness) using a temperature gradient from 50 to

250 °C at 7 °C min^{-1} and held at 250 °C for 2 min. Helium was used as a gas carrier at 1 ml min^{-1}.

Identification of the compounds was performed using a Thermo Scientific™ ISQ™ QD Single quadrupole mass spectrometer and Chromeleon™ 7 software (Thermo Fisher Scientific, Inc. Waltham, MA, USA). Detection of compounds started 2 min after injection. To identify compounds, retention times and mass spectra of authentic standards were used. However, when available, mass spectra from the National Institute of Standards and Technology and the Flavors and Fragrances of Natural and Synthetic Compounds, 3rd Edition (Wiley FFNSC Library, Hoboken, NJ, USA.) databases were also used. To convert retention time values into system-independent constants, a non-isothermal Kováts retention index (*I*) using the formula described by van Den Dool and Kratz (1963) was used. This calculated *I* value allows for comparisons between different gas chromatography and mass spectrophotometry (GC–MS) materials and methods. All compounds were sorted by retention time and were confirmed by a standard. Authentic standards were purchased from Sigma-Aldrich (Saint Louis, MO, USA) and had the following purities: isobutanol (> 99%), 2-methyl-1-butanol (> 98%), α-pinene (> 99%), β-pinene (> 99%), 2-ethylhexanol (> 99.6%), limonene (> 97%), linalool (97%), and methyl salicylate (> 99%).

Statistical analysis

To determine if the feeding preference of SSB nymphs varied significantly between infected and uninfected prey, data (1 for selecting one prey type, 0 for not selecting the other prey type) for each trial were square root + 0.01 transformed to remove zeros from the data prior to analysis. Nymphs that chose no prey were excluded from the analysis. Feeding preferences of nymphs between prey types were compared using the SAS PROC TTEST computer program for Student's paired *t*-test ($\alpha \leq 0.05$). Time data (min) for each fifth instar to molt to adult were subjected to Student's *t*-test ($\alpha \leq 0.05$) to determine if there was any significant negative effect from exposure to the *B. bassiana* spores while in the choice arena and Y-tube analysis compared to those not exposed to the spores. Behavior preference data of nymphs in the Y-tube olfactometer were statistically analyzed by the χ^2 test of independence ($\alpha \leq 0.05$) in JMP (v. 10, SAS Inc, Cary, NC, USA) that gave 95% confidence intervals.

Results

Prey preference trials

Of the 42 nymphs tested, only one nymph (2%) chose to feed on the mycosed prey, 13 (31%) selected the uninfected prey, and 28 (67%) did not make a choice. Mycosed prey (1 ± 0.1 per day, $n = 7$) were attacked significantly less ($t_{12} = -2.66$, $P = 0.021$) by nymphs compared to uninfected prey (1.9 ± 0.7 per day). The single nymph that fed on the mycosed prey molted to an adult. The time for the single nymph to choose the mycosed prey was 600 s, compared to 276 ± 67 s (range 18–840 s) for the nymphs that chose the uninfected prey. Among the nymphs that made a prey choice, the percentage mortality prior to molting to adult was 11% ($n = 14$).

When given the choice between infected reddish and uninfected prey, four (10%) of the 39 nymphs chose to feed on the red prey, 18 (46%) preferred the uninfected prey, and 17 (44%) did not make a choice. Reddish prey (1.0 ± 0.0 per day, $n = 4$) were attacked significantly less ($t_6 = -4.04$, $P = 0.0068$) by nymphs compared to uninfected prey (4.5 ± 1.2 per day). Of the four nymphs that chose the reddish prey, all but one molted to adult. The mean time for the nymphs to choose the reddish prey, 198 ± 38 s (range 180–306 s), was not significantly different ($t_{20} = -1.10$, $P = 0.28$) from the time to choose the uninfected prey, 342 ± 59 s (range 16–900 s). In all prey preference trials, the mean time for nymphs to molt to adult was 8.0 days (range 7–14 days).

Y-tube olfactometer assays

In the Y-tube experiment, 69% of nymphs (40/58) made a choice towards the control plug or the fungal plug. Of the 40 nymphs that made a choice, 26 chose the control plug, whereas 14 chose the fungal plug (Fig. 1). Choice response rates were not statistically different ($\chi^2 = 3.60$; df = 1; $P = 0.057$). Of the 14 nymphs that choose the fungal plug arm, 6 contacted the fungal plug, and 8 nymphs stayed in the fungal plug arm without contacting the plug. Eighteen

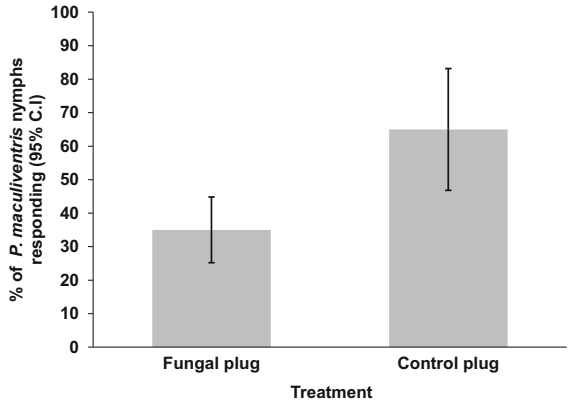

Fig. 1 Response percentages of fifth-instar *Podisus maculiventris* nymphs towards *Beauveria bassiana* fungal plugs vs. control plugs. 35% (14/40) of *P. maculiventris* nymphs chose fungal plugs *vs*. 65% (26/40) that chose control plugs. Brackets denote 95% binomial confidence intervals. There was no significant difference between attraction to treatment choice-pairs (χ^2 = 3.60; df = 1; P = 0.057)

nymphs were recorded as no choice. The overall percentage of nymphs that died prior to molting to the adult stage after exposure to the fungal plugs was 12% compared to 25% in the laboratory population not exposed to fungus.

Fungal volatiles

Nine components were confidently identified in the volatile emissions collected from *B. bassiana* (Table 1). Four components, i.e., 2-ethyl-1 hexanol, 2-methyl-1-butanol, isobutanol, and limonene, comprised more than 88% of the total blend. Eight other components, whose identifications were not confirmed by standards, were collected from the agar and the fungal headspace volatiles. These components were silanediol, diacetone alcohol, oxime methoxy-phenyl, 2-butoxyethanol, vanillin, 1,1,1,5,5,5-hexamethyl-3-((trimethylsilyl)oxy) trisiloxane, ethylene diglycol monoethyl ether, and nonanal. They are not included in Table 1.

Discussion

Our study indicates that SSB nymphs avoid FAW prey infected and sporulating with the entomopathogenic fungus *B. bassiana*. However, although a single nymph fed on a mycosed prey, it still molted to an adult at a rate similar for nymphs that fed on uninfected prey. Ullah et al. (2019) observed the reduviid *R. marginatus* survived equally well when fed with larval *S. litura* infected by *C. fumosorosea* as

Table 1 Chemical composition and characteristics of volatiles emitted by *Beauveria bassiana* strain GHA (n = 3)

Compound[a]	Retention time (min)[c]	Retention index (I)[d]	Mean percentage (± SE)
Isobutanol	2.15	604	11.37 ± 5.24
2-Methyl-1-butanol	3.31	671	21.78 ± 14.75
2,3-Butanediol[b]	4.25	724	3.48 ± 0.95
α-Pinene	7.56	896	2.80 ± 1.55
β-Pinene	8.62	951	2.96 ± 0.88
2-Ethyl-1 hexanol	9.81	1013	45.48 ± 17.47
Limonene	9.86	1016	10.13 ± 4.19
Linalool	11.47	1098	0.79 ± 0.41
Methyl salicylate	13.63	1198	1.21 ± 1.21

[a]Compounds found in volatiles emitted by pure agar (control) are excluded

[b]Identification of compound was from mass spectra using National Institute of Standards and Technology and Flavors and Fragrances of Natural and Synthetic Compounds, 3rd Edition (Wiley FFNSC Library, Hoboken, NJ, USA) databases

[c]Compounds were listed by increasing retention time and confirmed by a standard, unless stated otherwise

[d]Non-isothermal Kováts retention index (I) value for each compound were calculated using retention time following the formula from van Den Dool and Kratz (1963) and listed numerically from lowest to highest. Non-isothermal Kováts retention index (I) converts retention time into system-independent value constants that allows for comparisons between different GC–MS methods and materials

with healthy larvae. When the nymphs in our study were given a choice between oosporein-laden, reddish FAW larvae and uninfected larvae, four fed on the reddish prey compared to 18 that fed on the uninfected prey. However, only one of the four nymphs that fed on a reddish larva did not survive to adulthood, nor did it mycose. Abendstein et al. (2003) reported that oosporein absorbed in artificial medium and consumed by tortricid and noctuid larvae had no insecticidal, antifeedant, or growth inhibitory effect. In their study, only 20–25% of the larvae that ingested the oosporein died, which was not significantly different from the mortality rate of larvae that fed on untreated medium. However, the Abendstein et al. (2003) study was about herbivorous larvae. There are no published reports investigating the effect of oosporein on predators.

In our study, thawed larvae were offered to the SSB nymph in the choice arenas to remove any influence of prey movement on the predator's choice. The freezing technique allowed the predator's choice to be based solely on the infected/non-infected state of the prey as well as olfactory cues emanating from the reddish, non-sporulating or whitish, sporulating FAW cadaver. George et al. (2013) reported the use of a similar freezing technique to assure that all the caterpillar cadavers were in the same infectious stage before use in their experiment. In addition, after comparing live and frozen prey, the use of frozen larvae was recommended by Mohaghegh and Amir-Maafi (2007) to meet the nutritional needs of the predatory pentatomid *Andrallus spinidens* (F.), when live prey was scarce.

Although there was a lack of statistical significance in response rates to fungal plugs and control plugs in the Y-tube assays, nearly twice as many nymphs chose the control plug than the fungal plug (Fig. 1). Also, the testing of more new nymphs may have led to significance or greater insignificance. These results support the findings from our choice arena trials that nymphs selectively avoid fungus-infected prey. Our results concur with previous studies that showed insect predators may avoid contact with an entomopathogenic fungus while foraging (Pell and Vandenburg 2002; Meyling and Pell 2006; Alma et al. 2010; Ormond et al. 2011).

França et al. (2006) showed that *Podisus nigrispinus* (Dallas) are susceptible to infection by some isolates of *B. bassiana*. Sosa-Gómez and Moscardi (1998), Todorova et al. (2002), and Lopes et al. (2015) demonstrated the pathogenicity of *B. bassiana* to pentatomids under laboratory conditions. Sosa-Gómez and Moscardi (1998) suggested that adult pentatomids are naturally resistant to fungal infection by *B. bassiana*, and this resistance may explain the low infection rates observed in field experiments. Recently, Ugine and Thaler (2020) determined in vitro that two volatile compounds (E-2-hexenal and α-terpineol) in the adult SSBs' aggregation pheromone reduced conidial germination of *B. bassiana*. Their findings suggest that the fungistatic and fungicidal odors emitted by adult SSB may account for the pentatomids' resistance to infection and protect them against entomopathogenic fungal pathogens. Interestingly, α-terpineol is absent from the dorsal abdominal gland secretions of SSB nymphs (Aldrich et al. 1984). Therefore, the susceptibility of the SSB nymph to infection by *B. bassiana* lends support to the avoidance behavior observed in our study.

Nine volatile organic compounds emitted by *B. bassiana* strain GHA were identified, and 2-ethyl-1 hexanol, 2-methyl-1-butanol, isobutanol, and limonene were the most abundant. Our data are different from a study by Crespo et al. (2008) that found ethanol and diisopropyl naphthalene's as the major components of the volatiles emitted by *B. bassiana* strain GHA grown on a glucose-enriched medium. This discrepancy may be due to the differences in the agar media used and/or the compound extraction techniques employed. However, a recent study found that 2-methyl-1-butanol similar to our study, was the major compound released by a *B. bassiana* strain GHA (Ranger et al. 2021), whereas hexanol was the main compound of two *B. bassiana* isolates (ICIPE 276, ICIPE 278) in a study by Mburu et al. (2013). Alcohols such as 2-ethyl-1 hexanol, 2-methyl-1-butanol, and isobutanol are compounds commonly found in fungal volatiles (Nalli et al. 2006; Cale et al. 2016; Dickshat 2017). Limonene and α-pinene that we found in the *B. bassiana* profile were also detected in other fungal volatile profiles (Ahmed et al. 2018).

Further investigation needs to confirm the headspace volatile composition of our *B. bassiana* GHA - strain and determine the effect each compound has on the avoidance behavior of the SSB nymphs to fungus-infected prey. Several of the compounds identified have known activities against hemipterans. The main compound identified from *B. bassiana* headspace

volatiles, 2-ethyl-1 hexanol, has a strong affinity to the odorant binding protein of the predator *Adelphocoris lineolatus* (Goeze) (Hemiptera: Miridae) (Gu et al. 2011). However, the response (attraction or repellency) of *A. lineolatus* to 2-ethyl-1-hexanol was not investigated by the authors. 2-methyl-1-butanol is a repellent against the reduviid *Triatoma infestans* Klug (Hemiptera: Reduviidae) (Palottini and Manrique, 2016). Finally, limonene is a well-known repellent against the rice stinkbug, *Oebalus pugnax* (Fabricius) (Hemiptera: Pentatomidae) (Bhavanam et al. 2021).

Baverstock et al. (2010) indicated that there are two processes that have a significant effect on the interactions between insects and entomopathogenic fungi: (1) the ability of insects to detect and avoid fungal pathogens and (2) the transmission of fungal pathogens between host insects. In our study, we investigated the first process, and, based on our observations, SSB nymphs were not adversely affected by the presence of the fungal spores present on the surface of infected lepidopteran prey. Nearly all nymphs (93%) were able to detect the volatiles, avoid the infected prey and preferably fed on the uninfected prey. In five instances, the predator fed on infected prey. However, 80% of the nymphal predators molted to the adult stage. Therefore, strategies using applications of fungal biopesticide products containing *B. bassiana* combined with releases of the generalist predator *P. maculiventris* may be a viable option for pest management of lepidopteran pests. This integrated pest management strategy has yet to be tested under field conditions. Also, the predation behavior of this predator exposed to other entomopathogenic fungi, such as *Cordyceps* and *Metarhizium* species, should be tested in choice arenas to determine if they are compatible.

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Author contributions PBA originally formulated the idea; PBA, JG, LM, and XM conceived and designed the experiments; PBA, JG, LM, XM, ALR, REB, EBD, and JSD performed the experiments; PBA, JG, LM, and XM analyzed the data; PBA, JG, LM, and XM wrote the manuscript; PBA, XM, RLM, and RDC provided supervision during the experiments; PBA, and RDC acquired funding for this study; all authors edited and approved the manuscript.

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Declarations

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