Research Article

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Evaluation of chemical and microbial control options for *Pangaeus bilineatus* (Say) (Hemiptera: Cydnidae) infesting peanut crop

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Abstract

BACKGROUND: The peanut burrower bug, *Pangaeus bilineatus* is a major crop pest of peanuts in the southern United States. Peanuts infested by *P. bilineatus* exhibit weight and quality losses and could be discounted by ≤50% of the prevailing market price. Control of this pest is difficult because it attacks peanut pods underground, thus rendering foliar pesticide applications ineffective. Integration of entomopathogenic fungi and nematodes (EPF/EPNs) with chemical insecticides in the management of *P. bilineatus* was investigated as a potential integrated pest management containment tool.

RESULTS: The nymphs were less susceptible than adults of *P. bilineatus* to EPNs. Comparison of six strains of both *Heterorhabditis* spp. and *Steinernema* spp. demonstrated that *Steinernema carpocapsae* (All) was the most virulent EPN, causing 75.54% mortality of *P. bilineatus* adults after 7 days postinoculation (dpi), whereas the mortality generated by the application of the rest of the nematodes ranged between 17.03% (*H. bacteriophora* - Lewis) and 50% (*H. bacteriophora VS*). Application of imidacloprid by itself at ½FR (field rate) did not result in any significant mortality of *P. bilineatus* adults but application of chlorpyrifos at 1/8FR caused significant mortality (27.41–61.35%) at 7–14 dpi. However, combined applications of *S. carpocapsae* and imidacloprid resulted in significant mortality starting at 3 dpi. The interactions between *S. carpocapsae* and imidacloprid were synergistic at 3–5 dpi, but became additive at 7–14 dpi. Both chlorpyrifos and imidacloprid did not negatively impact the reproduction of *S. carpocapsae*.

CONCLUSION: The compatibility between *S. carpocapsae* and imidacloprid makes a case for the combination to be used for the management of *P. bilineatus*.

Keywords: peanuts; burrower bug; entomopathogens; infectivity; IPM; insecticides

1 INTRODUCTION

Among the six Cydnidae believed to be field pests of peanuts, *Arachis hypogaea* (L.) (Fabales: Fabaceae), the peanut burrower bug, *Pangaeus bilineatus* (Say) (Hemiptera: Cydnidae), is known to cause extensive damage to peanuts in the peanut-producing southern states of the United States that include Georgia (GA), Texas (TX), Alabama (AL) and South Carolina (SC).[1–3] Adults and nymphs of *P. bilineatus* live mostly in the soil where they feed on fully mature peanut kernels with their needle-like piercing and sucking type mouthparts.[4] *Pangaeus bilineatus* is known to undergo adult diapause in the soil during winter months and terminate diapause in early spring.[5,6] However, some active nymphs have been observed during late winter months in the state of GA, USA.[6] Populations of *P. bilineatus* used to be sporadic and remained below economic injury level, but in recent years, their numbers have surged in some crop seasons resulting in damage to peanuts in several states. For example, in southern TX, 34% of peanuts produced suffered reduced grade in an outbreak year;[7,8] in GA, infestation of peanuts by *P. bilineatus* in 2010 and 2014 resulted in downgrading several batches of peanuts.[4]

Crop damage caused by *P. bilineatus* can have severe secondary effects. For example, infestation of peanuts by this pest results in reduced oxidative stability and rises in peroxide levels.[9] Infestation of peanuts by *P. bilineatus* also is associated with aflatoxin contamination.[10] Aflatoxin, a toxin produced by the fungus *Aspergillus flavus* (Link) (Eurotiales: Trichomaceae), is acutely toxic to vertebrates but also carcinogenic to humans.[11,12] Thus, high levels of aflatoxin content in peanut induced by *P. bilineatus* infestation have food safety issues and significantly impact upon the commercial values of peanuts.[13] Losses associated with the infestation of peanuts by *P. bilineatus* warrant implementation of pest management strategies that keep the populations low.

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Cultural farming practices may impact pest populations, but little or no evidence exists that validates substantial control of *P. bilineatus* by cultural methods. For instance, it has been documented that when corn was rotated with peanuts in alternate years, peanut fields had higher incidence of F_1 *P. bilineatus* adults, especially when conservation tillage involved strip-tilling directly into corn residues.[3] *Pangaeus bilineatus* nymphal survival in corn was suspected to have been enhanced by the availability of harvest-loss peanut seeds from the previous season's crop. In addition, conservation tillage, such as strip tillage and no-till tillage, which has been shown to have some strong advantages such as reduced soil erosion, reduced time and labor, resulted in high infestation by the burrower bug,[3] thus leaving farmers no other options other than application of chemical pesticides to control this pest.

Granular formulation of the organophosphate chlorpyrifos is the only pesticide labeled for the control of *P. bilineatus* in the United States.[6] The efficacy of chlorpyrifos has not always been consistent as the application of the pesticide reduced seed damage in some seasons but failed to do so in others.[2,3] In addition, treatments of peanut crop with chlorpyrifos at-pegging (when the peanut crop is fully grown and flowering) have been documented to cause secondary outbreaks of the spotted spider mite, *Tetranychus urticae* Koch (Trombidiformes: Tetranychidae) and corn earworm, *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae).[2] Chlorpyrifos applications also triggered cutworm outbreaks.[14] The greatest challenge to peanut farmers in the southern United States is the revocation of all tolerances for chlorpyrifos in human food by the United States Environmental Protection Agency (USEPA).[15] Therefore, other pest management tools are needed to keep the populations of *P. bilineatus* below levels that cause significant losses to the peanut crop.

Entomopathogenic nematodes (EPNs) or fungi (EPF), which are soil-dwelling organisms that are harmful to insects but innocuous to vertebrates, are likely candidates for the management of *P. bilineatus* populations.[16] EPNs in the genera *Heterorhabditis* and *Steinernema* are obligate parasites that use mutualistic bacteria to kill insects, and some strains have been shown to exhibit pathogenicity toward insect pests in the order Hemiptera.[17–21] In an earlier study, we observed that *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) was not effective when applied alone, but a synergy was observed when the nematode was applied with chlorpyrifos;[4] however, as indicted above, this insecticide has been deregistered for application on foods.[15] Our earlier study[4] did not find the EPF, *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) virulent to *P. bilineatus*, although reports exist that demonstrate the efficacy of *B. bassiana* and *Metarhizium* spp. (Metschnikoff) Sorokin, and *M. brunneum* Petch (Hypocreales: Claviciptaceae) for the control of some subterranean insect pests.[16,22]

The imminent loss of chlorpyrifos to the peanut farmers has increased the urgency for effective pest management tools that can keep populations of *P. bilineatus* below levels that can cause severe crop losses. The study reported herein is an expansion of our earlier study that examined the efficacy of entomopathogens, a nematode and a fungus, when applied alone and in combination with chlorpyrifos against *P*. bilineatus.[4] We extend the study to additional entomopathogenic nematode species and strains, and an alternative pesticide to chlorpyrifos (imidacloprid). We explore the potential for synergy among the microbial agents and chemicals. Several other studies have demonstrated evidence of synergy between EPF or EPNs and chemical pesticides against pests.[16,22] For example, synergy also was observed in an experiment that evaluated the efficacy of the combined application of *M. anisopliae* (strain CIAT 224) and imidacloprid insecticide against a different burrower bug, *Cyrtomenus bergi* Froeschner (Hemiptera: Cydnidae).[23] No single control method may be adequate for the replacement of chlorpyrifos. Therefore, several integrated pest management (IPM) tools may be combined for the suppression of *P. bilineatus*. Our hypothesis is that combined applications of EPF or EPNs with a chemical pesticide other than chlorpyrifos will result in significant suppression of *P. bilineatus* populations.

The objectives of the present study were first to screen for virulence to *P. bilineatus* among a broader array of EPN than tested previously. Subsequently, the two most promising nematodes were assayed for virulence in comparison with *B. bassiana* and chemical insecticides when applied alone or in combination. Finally, based on promising results from the earlier assays, the impact of imidacloprid on EPN reproductive capacity was measured, indicating potential for recycling in the field.

2 MATERIALS AND METHODS

2.1 Insects

Insect samples used in starting and sustaining *P. bilineatus* colonies at Fort Valley State University insect rearing facility were collected either from peanut farms around the state of GA, particularly from Brooks, and Emanuel counties or from Mark Abney's laboratory at University of Georgia, Tifton, GA. *Pangaeus bilineatus* used in this study were taken from Fort Valley State University colony that was raised in a rearing room maintained at 29.4 ± 1.5 °C, 50 ± 5% relative humidity (RH) and 14 h:10 h, light:dark photoperiod, simulating summer conditions in GA. The temperature of the insect rearing room was maintained with a Goodman air conditioning and heating system GZ140181LA (Goodman, Waller, TX, USA), whereas a Vogvigo large room, 5.5-L warm and cool mist ultrasonic humidifier (TTLife, Shanghai, China) was used in maintaining the RH. A set of lamps suspended from the ceiling and connected to a timer controlled the photoperiod (light intensity 760 lx). Environmental conditions were monitored continuously with a Hobo data logger (MX 1104).

The soil used in our laboratory studies was a loamy sand [Norfolk loamy and sand (Kaolinitic, Thermic Typic Kandiudult)] with the sand:silt:clay percentages of 84:10:6, pH 6.1 and organic matter 2.8% by weight; final moisture in all containers was standardized at field capacity (14%) (the moisture level a soil will hold against gravity). The soil samples were oven-dried and autoclaved to get rid of all soil organisms. The soil was transferred to 7.5-L transparent Rubbermaid plastic container and brought to 14% moisture content by adding distilled water. Ten peanut seeds and 20 adults (10 males, 10 females) of *P. bilineatus* were placed on the soil surface. Thereafter, the container lids that were partially screened with wire netting to prevent escape of the insects and allow for ventilation were replaced. The moisture content of soil in the rearing containers was monitored with General Tools DSMM500 precision digital soil moisture meter with probe (TE Equipment, Long Branch, NJ, USA), and whenever the moisture content dropped below 14%, distilled water was sprayed at the surface of the soil to restore the moisture level.

In the current study, we evaluated the mortality of last nymphal instars and newly molted adults exposed to EPNs, EPF, chemical pesticides (chlorpyrifos and imidacloprid) or their combinations.

The external anatomy of the nymphs and the adults were used in distinguishing the stages. The dorsal thoracic section of the last nymphal instar has wing pads wherease adults at eclosion (0–4 h posteclosion) have an orange color before sclerotization.[6] Newly emerged adults were used in this study because the age range was narrow.

2.2 Microbial and chemical agents

The EPNs used in this study were obtained from the USDA-ARS Southeastern Fruit and Tree Nut Research Laboratory culture collection in Byron, GA: *H. bacteriophora* (*VS* strain), *H. bacteriophora* (Lewiston strain), *H. bacteriophora* (Oswego strain), *H. megidis* (UK211 strain) Poinar, Jackson & Klein *Steinernema feltiae* (SN strain) (Filipjev) (Rhabditida: Steinernematidae) and *S. carpocapsae* (All strain) (Weiser). The EPNs were reared as described by Woodring and Kaya[24] at ~24 °C in last instar greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae), obtained from Webster's Waxie Ranch (Webster, WI, USA). Nematodes used for experimentation were held for less than two weeks at 13 °C before being used for experiments. *Beauveria bassiana* used in the study was BotainGArd 22 WP (active ingredient: *B. bassiana*; BioWorks Inc., Victor, NY, USA). The insecticides used were chlorpyrifos (Nufos 4E; Chemnova, Research Triangle Park, NC, USA) and imidacloprid (Direct Ag Source, LLC, Eldorado, IA, USA) that were obtained commercially. All application rates described below were based on preliminary dose response assays (data not shown).

2.3 Comparative virulence of entomopathogenic nematodes to nymphs and newly emerged adults of *P. bilineatus*

Laboratory assays required for determining virulence or toxicity of chemical and EPN/EPF and their combinations to *P. bilineatus* were conducted in soil cups that have been described previously.[4,25,26] The cups (4.0 cm top diameter, 3.0 cm bottom diameter, 3.3 cm depth) held 15 g oven-dried autoclaved soil. The flat surface area for 15 g soil is ≈10 cm^2.

Adults and nymphs of *P. bilineatus* were transferred singly into experimental cups described above that contained soil and treated at the rate 2000 infective juvenile nematodes (IJs) $insect^{-1}$ (200 IJs/cm^2) with six different species or strains of the nematodes dispensed with 0.5 mL water to the soil in in each cup. The seventh treatment comprised an untreated water control. The experiment was organized as completely randomized design. Each treatment consisted of three replicates of seven insects each, and the entire experiment was repeated with a new generation of the burrower bug (hence two complete trials with a total of 42 insects per treatment). Mortality of the adults and nymphs of *P. bilineatus* was checked at 4 and 7 dpi (days postinoculation). The most virulent heterorhabditid and steinernematid were investigated further along with *B. bassiana*, chlorpyrifos and imidacloprid singly and in combination, to determine if there were interactions in the virulence to the burrower bug.

2.4 Evaluation of microbial and chemical agents for virulence and toxicity to *P. bilineatus*

The purpose of this experiment was to compare the virulence of most promising EPNs, *B. bassiana* and chemical insecticides when applied alone or in combination. Eleven treatments that included one water control, two chemical agents (chlorpyrifos and imidacloprid) and three EPF/EPNs (*B. bassiana*, *H. bacteriophora* (*VS*) and *S. carpocapsae*), and the five chemical–microbial combinations (chlorpyrifos + *S. carpocapsae*, imidacloprid + *S. carpocapsae*, *B. bassiana* + *S. carpocapsae*, imidacloprid + *H. bacteriophora*, and *B. bassiana* + *H. bacteriophora*) were investigated for virulence to *P. bilineatus* adults. A combination of chlorpyrifos + *H. bacteriophora* was not included because we reported a synergy between the agents in a previous study.[4] Bioassay arenas and parameters were as described above unless indicated otherwise. The experimental set-up was a completely randomized design with three replications of 10 soil cups for each treatment and control. Each soil cup had one adult *P. bilineatus*. The experiment was repeated once over time.

The application rates of the chemical pesticides selected following dose–response assays were CPF 4E (active ingredient: chlorpyrifos; Direct Ag Source LLC, Eldora, IA, USA) at one-eighth field rate (FR) (recommended FR is 1.9 L/4046.9 m^2), and S-Cloprid 4 AG (active ingredient imidacloprid; Direct Ag Source LLC) at ½FR (recommended FR is 236.6 mL/4046.9 m^2). Rates of pesticides provided mortalities between 25% and 40% during dose–response trials for ½FR and 1/8FR of imidacloprid and chlorpyrifos, respectively. Although we understood the problem associated with insect resistance to chemical pesticides resulting from application of suboptimal doses, the lower rates were chosen in this study to obtain mortality levels that would be intermediate and allow room for improvement when combined with other treatments. Each of the EPNs, *S. carpocapsae* or *H. bacteriophora* was applied at 2000 IJs $insect^{-1}$ (200 IJs cm^{-2}), whereas the entomopathogenic fungus *B. bassiana* was applied at FR (28.3 g/92.9 m^2, 1.3 million conidia cm^{-2}). In each combination treatment, the application rates were the same as that applied singly. We omitted the *H. bacteriophora* (*VS*) + chlorpyrifos combination because synergy has already been reported between the two agents.[4]

In the combined treatments, the application of the microbial agent was followed directly by the chemical agent, each dispensed via pipette in 0.5 mL water. In the combined applications of EPNs and EPF, the EPN was added first to the soil followed by the EPF. Following the applications of treatments, one adult of *P. bilineatus* was added to each cup and the cups were incubated at 25 °C and bug mortality in each cup was assessed 3, 5, 7, 10, 12 and 14 dpi.

2.5 Reproduction of entomopathogenic nematodes exposed to chlorpyrifos and imidacloprid

Following the evaluation of mortality of peanut burrower bugs, dead insects exposed to *H. bacteriophora* (*VS* strain) or *S. carpocapsae* (All strain) alone or in combination with the chemical agents were assessed for nematode reproductive capacity as described previously.[27] This is to determine if the chemical agents had adverse impact on nematode fecundity within the host. Cadavers of peanut burrower bug adults were placed individually onto White traps[24] and the White traps were held in an incubator at 25 °C for 12 days. At the end of incubation period, the IJs were harvested from the White traps and the reproductive yield was determined through serial dilutions.[24]

2.6 Data analysis

For the mortality data resulting from application of microbial and chemical agents, a generalized linear mixed model (GLMM) that assumed a binomial distribution was fitted to the response variable defined as the number of dead peanut burrower bug nymphs/adults from the initial number of insects (nymphs or adults) that were exposed to the treatments. A logit link function

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was used to estimate the mortality of peanut burrower bug nymphs/adults. The fixed effects in the linear predictor included trial, treatment, day after treatment application, as well as all two- and three-way interactions. To account for over-dispersion, the related random effects were identified.

No evidence of over-dispersion was determined following assessment with maximum-likelihood fit statistic Pearson chi-square/DF. The final statistical model used for inference was fitted using Residual Pseudo-likelihood. The statistical model was fitted using the PROC GLIMMIX procedure using the SAS 9.4 statistical software (SAS Institute, Cary, NC, USA). Pairwise comparisons were conducted using Bonferroni's adjustments for comparisons, to avoid inflation of type I error due to multiple comparisons.

Further analysis examined whether type of interactions between entomopathogens and chemical pesticides were synergistic, additive or antagonistic. The type of interaction was determined by comparing observed with expected mortality using an already established method.[22,26,28] Abbott's formula was used to calculate corrected mortality.[29] The expected additive proportional mortality M_E for the EPN–chemical or EPF–chemical combinations was calculated using the formula $M_E = M_C + M_M\,(1 - M_C)$, where M_E is the expected additive proportional mortality, M_C is the mortality caused by chemical agent, M_M is the mortality caused by EPN or EPF, and M_{CM} is the proportional mortalities caused by the combined agents when applied separately (or microbial-chemical agents). The X^2 values were calculated as $(M_{CM} - M_E)^2/M_E$. If the calculated values were >3.84 (1 df), a non-additive positive value indicated a synergy whereas a negative value indicated antagonistic interaction.

For the nematode reproduction, a GLMM that assumed a normal distribution was fitted to the IJ yield g^{-}insect body weight. The linear predictor included fixed effect of treatment and random effect of replication. Degrees of freedom were approximated and estimated SEs were adjusted using Kenward–Roger's procedure. Pairwise comparisons were conducted using Tukey–Kramer's adjustments for main effect comparisons, to avoid inflation of type I error resulting from multiple comparisons.

3 RESULTS

3.1 Infectivity of different entomopathogenic nematode strains to nymphs and adults of *P. bilineatus*

Three-way ANOVA showed that application of the EPNs significantly affected the mortality of nymphs ($F_{6,52}$ = 14.37; $P < 0.0001$) and adults ($F_{6,52}$ = 6.68; $P < 0.0001$) of *P. bilineatus* (Tables 1 and 2). Nymphal mortalities at 4 and 7 dpi were far lower than those of the adults [Figs 1(a), (b) and 2(a), (b)]. Nymphal mortalities at 7 dpi were higher than the control with the application of *H. bacteriophora* (*VS* strain) and *S. feltiae* (SN strain), whereas adult mortality was higher than the control with the application of *S. carpocapsae*. *H. bacteriophora* (*VS* strain) and *S. carpocapsae* (All strain) resulted in 50% and 78% adult mortality at 7 dpi, respectively [Fig. 2(b)]. Although not statistically separated from the rest of the Heterorhabditid strains, application of *H. bacteriophora* (*VS* strain) resulted in higher mortality of *P. bilineatus* adults, numerically. These two strains of nematodes, *H. bacteriophora* (*VS* strain) and *S. carpocapsae* (All strain), were investigated further in experiments reported below.

Mortality of *P. bilineatus* adults exposed to EPN/EPF (*H. bacteriophora*, *S. carpocapsae* and *B. bassiana*), chemical agents (chlorpyrifos or imidacloprid) and their combinations is shown in Fig. 3 (a, b, c, d and e). Three-way ANOVA detected differences in *P. bilineatus* mortality resulting from the treatments at 3, 5, 7, 10, 12 and 14 dpi ($F_{10,40}$ = 20.98; $P < 0.0001$) (Table 3). At 3, 5, 7, 10, 12 and 14 dpi, chlorpyrifos at 1/8FR, *S. carpocapsae*, and combinations of *S. carpocapsae* and the chemical agents, chlorpyrifos (1/8FR) or imidacloprid (½FR), resulted in mortality that was significantly higher than water control [$P < 0.005$; Fig. 3(a)–(f)]. Combined applications of *S. carpocapsae* and imidacloprid or chlorpyrifos resulted in higher mortality than combined applications of the chemical agent with *H. bacteriophora* [$P < 0.005$; Fig. 3(a)–(f)]. At 3 and 5 dpi, combined applications of *S. carpocapsae* and imidacloprid resulted in the highest *P. bilineatus* mortality compared with the rest of the treatments [$P < 0.05$, Fig. 3(a), (b)]. At 7 dpi, *S. carpocapsae* applied alone or combined with either chlorpyrifos or imidacloprid resulted in comparable mortalities of adult *P. bilineatus* that were significantly higher than the rest of the applications [$P < 0.05$; Fig. 3(c)]. At 10–14 dpi, combined application of *S. carpocapsae* and chlorpyrifos caused significantly higher mortality than combined application of *S. carpocapsae* and imidacloprid [$P < 0.05$; Fig. 3(d)–(f)]. Application of *B. bassiana* alone resulted in low mycosis and mortality of *P. bilineatus* that was not significantly different from those of the water control [Fig. 3(a)–(f)]. In addition, combined application of *S. carpocapsae* and *B. bassiana* generated significantly lower *P. bilineatus* mortality than that caused by the application of *S. carpocapsae* alone [$P < 0.05$; Fig. 3(d)–(f)].

Values of chi-square analysis of the interactions of the combined applications with respect to mortality of *P. bilineatus* are

Table 1. Three-way ANOVA table for the experiment on *Pangaeus bilineatus* nymphs exposed to one water control and six strains of entomopathogenic nematodes [*Heterorhabditis bacteriophora* (*VS* strain), *H. bacteriophora* (Lewiston strain), *H. bacteriophora* (Oswego strain), *H. megidis*, *Steinernema carpocapsae* (All strain) and *S. feltiae* (SN strain)]

Effect	*F*	df	*P*
Trial	0.00	1, 4	0.9893
Treatment	14.37	6, 52	<0.0001
Trial × Treatment	0.17	6, 52	0.8514
Day	0.00	1, 52	0.9568
Trial × Day	0.00	1, 52	0.9928
Treatment × Day	11.09	6, 52	<0.0001
Trial × Treatment × Day	0.22	6, 52	0.9523

Table 2. Three-way ANOVA table for the experiment on *Pangaeus bilineatus* adults exposed to one water control and six strains of entomopathogenic nematodes [*Heterorhabditis bacteriophora* (*VS* strain), *H. bacteriophora* (Lewiston strain), *H. bacteriophora* (Oswego strain), *H. megidis*, *Steinernema carpocapsae* (All strain) and *S. feltiae* (SN strain)]

Effect	*F*	df	*P*
Trial	9.24	1, 4	0.0384
Treatment	6.68	6, 52	<0.0001
Trial × Treatment	0.81	6, 52	0.5656
Day	29.46	1, 52	<0.0001
Trial × Day	0.00	1, 52	0.9469
Treatment × Day	0.11	6, 52	0.9953
Trial × Treatment × Day	0.19	6, 52	0.9772

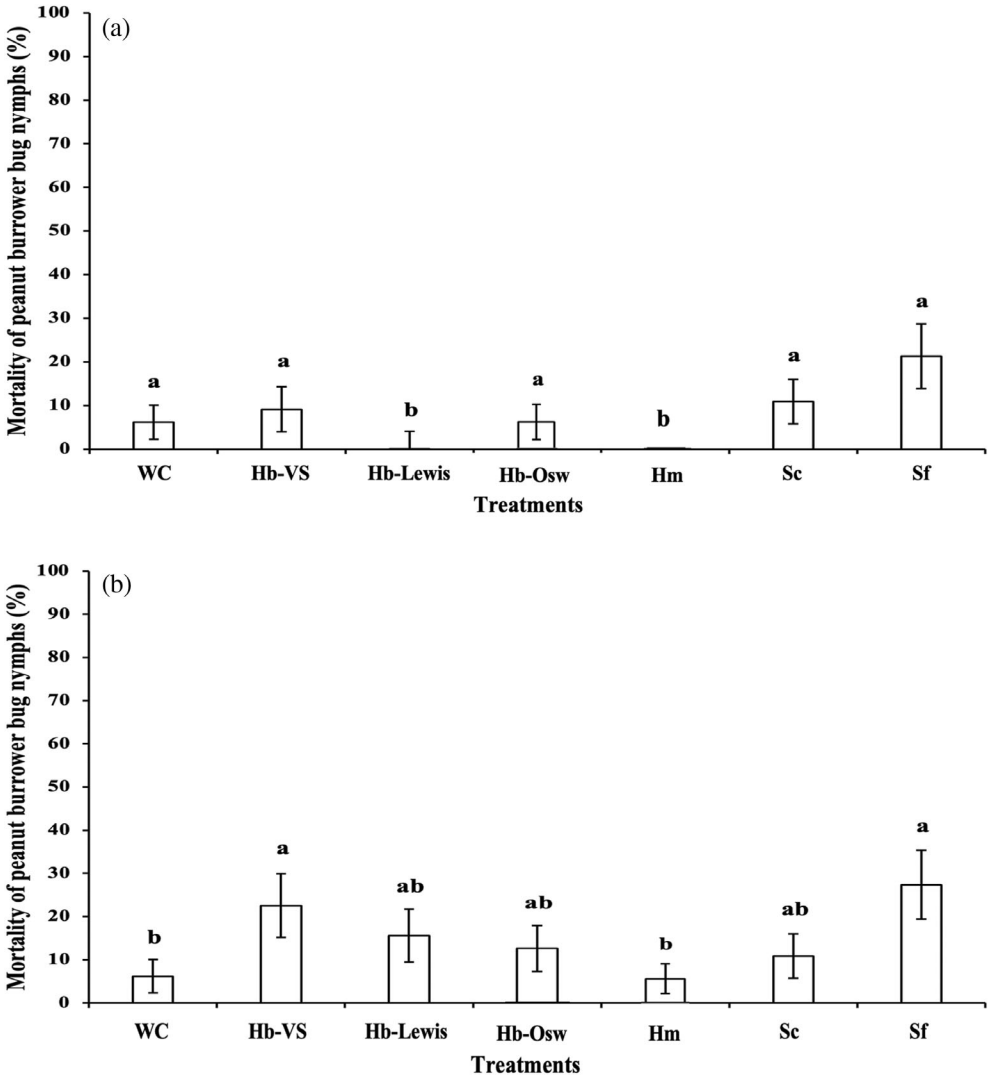

Figure 1. Mortality of *Pangaeus bilineatus* nymphs after (a) 4 and (b) 7 days postinoculation (dpi) exposure to a water control (CK) and infective juveniles of six nematodes: Hb, *Heterorhabditis bacteriophora* (*VS* strain); Lewis, *H. bacteriophora* (Lewiston strain); Meg, *H. megidis*; Oswego, *H. bacteriophora* (Oswego); Sc, *Steinernema carpocapsae* (All strain); and Sf, *Steinernema feltiae* (SN strain). Different letters above bars indicate statistical differences among treatments ($P < 0.05$) using Bonferroni's adjustments for comparisons (six replications per treatment, $N = 42$ insects per treatment).

provided for 3, 5, 7, 14 dpi (Table 4). The relationship between *H. bacteriophora* and *B. bassiana* or imidacloprid indicated additive interactions at 5, 7 and 14 dpi. Combinations of *B. bassiana* and *S. carpocapsae* indicated synergism at 3 dpi ($\chi^2 = 4.08$; $P < 0.05$), additivity at 5 dpi ($\chi^2 = 0.75$), and antagonism at 7 and 14 dpi ($\chi^2 = 5.22, 11.96$; $P < 0.05$, respectively). When imidacloprid was combined with *S. carpocapsae*, the interactions at 3 and 5 dpi were synergistic ($\chi^2 = 81.07, 10.06$; $P < 0.05$, respectively) and additive at 7 and14 dpi ($\chi^2 = 2.91, 0.88$, respectively). The interactions resulting from the combination of *S. carpocapsae* and chlorpyrifos gave additive mortality at 3, 5, 7 and 14 dpi ($\chi^2 = 0.14, 1.59, 0.06$ and 1.58, respectively).

3.2 Reproduction of nematodes in the cadavers of *P. bilineatus*

Reproduction of nematodes in *P. bilineatus* cadavers occurred in all treatments involving both nematodes, *S. carpocapsae* (Sc; Sc + Bb; Sc + Ch; Sc + Im) and *H. bacteriophora* (Hb; Hb + Bb; Hb + Ch; Hb + Im). The infective juvenile reproduction/g of *P. bilineatus* adult body weight was not significantly different among the four treatments involving *S. carpocapsae* ($F_{3,33} = 0.98$; $P = 0.41$), or the three combinations involving *H. bacteriophora* (*VS*) ($F_{2,46} = 2.48$; $P = 0.095$). The mean numbers of IJs g^{-1} of *P. bilineatus* adult body weight ranged from 414 970 to 896 930 for *S. carpocapsae*, and from 306 550 to 717 940 for *H. bacteriophora*.

4 DISCUSSION

The results presented here show that the EPNs investigated in this study varied in infectivity against the nymphs and adults of *P. bilineatus*, with the adults being more susceptible than the nymphs. Previous studies on Hemipterans had shown that there is no agreement on the impact of insect size on the infectivity of EPNs.[30–35] A more generalized study reported that EPN infection rate was lower in smaller insects, especially when EPN species are longer than the insect.[33] However, another study noted a high infectivity rate of both nymphs and adults of *Corythucha ciliata* (Say) (Hemiptera: Tingidae) by *S. carpocapsae* compared to low infectivity rate by *S. feltiae*, which is smaller than

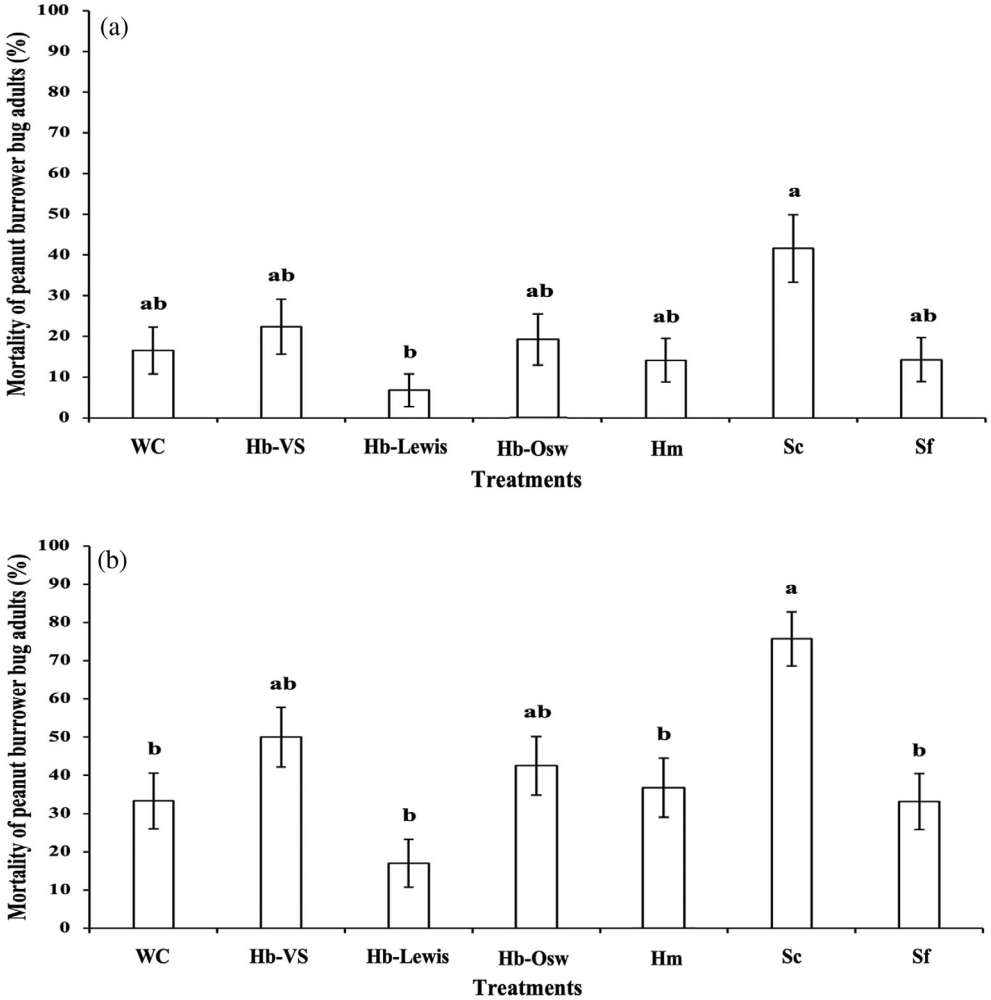

Figure 2. Mortality of *Pangaeus bilineatus* adults after (a) 4 and (b) 7 days postinoculation exposure to a water control (CK) and infective juveniles of six nematodes: Hb, *Heterorhabditis bacteriophora* (*VS* strain); Lewis, *H. bacteriophora* (Lewiston strain); Meg, *H. megidis*; Oswego, *H. bacteriophora* (Oswego); Sc, *Steinernema carpocapsae* (All strain); and Sf, *Steinernema feltiae* (SN strain). Different letters above bars indicate statistical differences among treatments ($P < 0.05$) using Bonferroni's adjustments for comparisons (six replications per treatment, $N = 42$ insects per treatment).

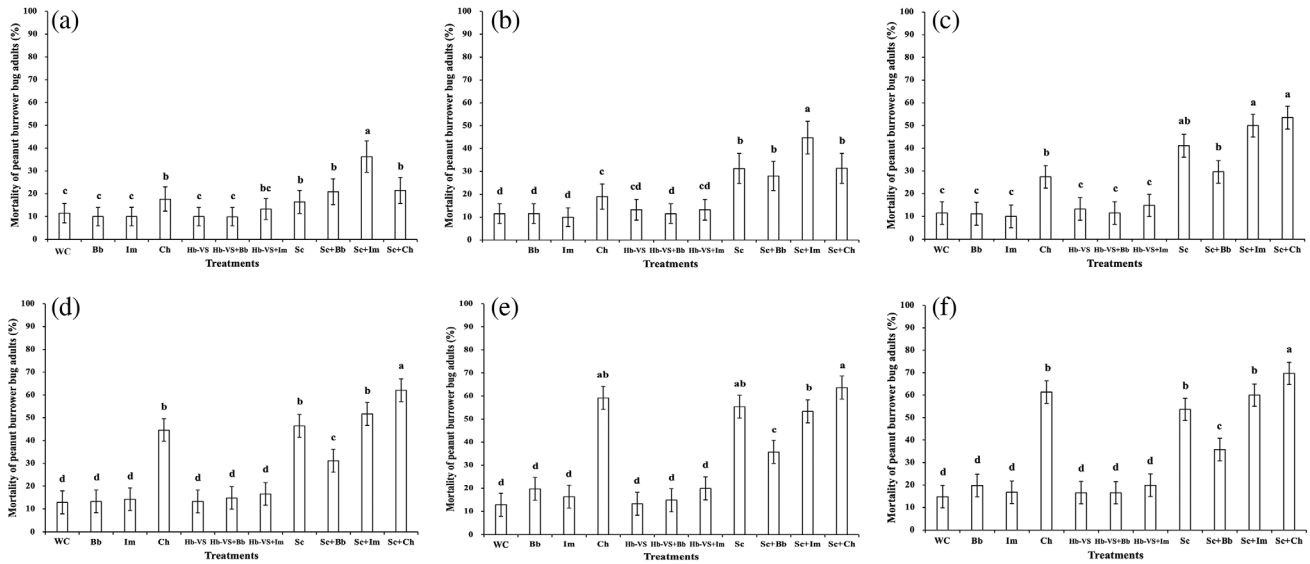

Figure 3. Mortality of *Pangaeus bilineatus* adults after (a) 3, (b) 5, (c) 7, (d) 10, (e)12 and (f) 14 days postinoculation exposure to 11 treatments, including water control (CK), FR of Bb (*Beauveria bassiana*), 1/2FR of Im (imidacloprid), 1/8FR of Ch (chlorpyrifos), 2000 IJs insect^{-1} of Hb (*Heterorhabditis bacteriophora* (*VS* strain), Hb + Bb, Hb + Im, 2000 IJs insect^{-1} of Sc (*Steinernema carpocapsae*, All strain), Sc + Bb, Sc + Im, and Sc + Ch. Different letters above error bars indicate statistical differences among the treatments ($P < 0.05$) using Bonferroni's adjustments for comparisons (six replications per treatment, $N = 60$ insects per treatment).

Table 3. Three-way ANOVA table for the mortality of *P. bilineatus* adults exposed to 11 treatments, including WC (water control), FR of Bb (*Beauveria bassiana*), ½FR of Im (imidacloprid), 1/8FR of Ch (chlorpyrifos), 2000 IJs insect^{-1} of Hb-*VS* [*Heterorhabditis bacteriophora* (*VS* strain)], Hb-*VS* + Bb, Hb-*VS* + Im, 2000 IJs insect^{-1} of Sc [*Steinernema carpocapsae* (All strain)], Sc + Bb, Sc + Im, and Sc + Ch

Effect	*F*	df	*P*
Trial	2.13	1, 4	0.2181
Treatment	20.98	10, 40	<0.0001
Trial × Treatment	2.19	10, 40	0.0589
Day	12.88	5, 220	<0.0001
Trial × Day	0.80	5, 220	0.5501
Treatment × Day	0.88	50, 220	0.6996
Trial × Treatment × Day	0.19	50, 220	1.0000

S. carpocapsae.[30] Other factors have been suggested as contributing to the differences in infectivity between nymphs and adults. For instance, a higher virulence of *S. feltiae* and *S. carpocapsae* to newly molted teneral adults of *C. ciliata* compared to the nymphs has been documented.[30,36] An additional study reported that molted nymphs of *Blattella germinica* (Dictyoptera: Blattellida) were more susceptible to EPNs than nonmolted nymphs.[37] In our study, *S. carpocapsae* was the most virulent EPN to *P. bilineatus* and the adults used were newly emerged pharate adults; it is therefore probable that a higher penetration rate of *S. carpocapsae* in the adult *P. bilineatus* resulted in higher infection compared to the nymphs.

Insect mobility and the foraging behavior of an EPN also may affect the susceptibility of an insect to an EPN penetration.[38] *Steinernema carpocapsae* is an ambusher and will more likely infect adult *P. bilineatus*, which is the dispersal stage of the insect and moves faster than the nymph. Ambush foragers tend to be well-adapted to mobile insects, whereas cruiser-type foragers such as *S. glaseri* tend to be well-adapted to sedentary hosts.[16,38] Foraging mechanism may have conferred an advantage to *S. carpocapsae* over *H. bacteriophora*, which is a typical cruiser as reported previously.[39]

Evidence of synergy was observed in the interactions resulting from the combined applications of imidacloprid and *S. carpocapsae* at 3 and 5 dpi, but at ≥7 dpi the interactions were additive. The reported synergy between *S. carpocapsae* and imidacloprid up to 5 dpi is an indication that the two can be effectively integrated for the management of *P. bilineatus*, and as a possible replacement for chlorpyrifos. Varying interactions between microbials and chemical insecticides previously have been reported including reports of synergy with neonicotinoids (such as imidacloprid) in other EPN–host systems.[4,22,40] In the study reported here, all of the combined applications involving EPNs and chemical pesticides were either additive or synergistic. Previous investigations demonstrated that insecticides with active component based on thiamethoxam, imidacloprid and chlorantraniliprole did not have adverse impact on the infectivity and viability of EPNs.[41–43]

From an applied side, recommending lower chemical pesticide rates may face regulatory hurdles resulting from insecticide resistance potential. However, we make a case that lower insecticide rates that produce overall mortality levels as a consequence of synergy, when combined with EPNs, will reduce the chances of resistance while providing superior pest control.

Table 4. Interactions between the two agents in the treatment combinations {Hb-*VS* [*Heterorhabditis bacteriophora* (*VS* strain)] + Bb (*Beauveria bassiana*); Hb-*VS* + Imidacloprid (Im); Sc [*Steinernema carpocapsae* (All strain)] + Bb; Sc + Ch (chlorpyrifos); Sc + Im} on 3, 5, 7 and 14 days postinoculation (dpi)

	3 dpi				5 dpi				7 dpi				14 dpi			
Treatments	M_{CM} (%)	M_E (%)	χ^2	Interactions	M_{CM} (%)	M_E (%)	χ^2	Interactions	M_{CM} (%)	M_E (%)	χ^2	Interactions	M_{CM} (%)	M_E (%)	χ^2	Interactions
Hb-*VS* + Bb	9.94	0	N/A	N/A	11.49	0.03	2.15	Additive	11.49	3.15	2.15	Additive	16.58	7.39	3.43	Additive
Hb-*VS* + Im	13.27	0	N/A	N/A	13.27	0.03	0	Additive	14.86	2.55	1.25	Additive	19.93	3.88	0.95	Additive
Sc + Bb	20.9	6.12	4.08	Synergistic	27.99	0.23	0.75	Additive	29.58	34.25	5.22	Antagonistic	35.79	48.55	11.96	Antagonistic
Sc + Ch	21.48	13.13	0.14	Additive	31.33	0.30	1.59	Additive	53.48	46.04	0.06	Additive	69.67	75.22	1.58	Additive
Sc + Im	36.28	6.12	81.07	Synergistic	44.77	0.23	10.06	Synergistic	49.95	33.84	2.91	Additive	60.06	46.60	0.88	Additive

Combined application of *H. bacteriophora* (*VS* strain) and imidacloprid was additive regarding the mortality of *P. bilineatus*. In an earlier investigation, *H. bacteriophora* (Oswego strain) had synergistic interaction with chlorpyrifos[4] even when the application of the nematode alone did not impact the mortality of *P. bilineatus*. This may suggest differences in infectivity among strains of *H. bacteriophora*, or differences in interaction among strains of the same EPN species with chemical pesticides.

Combined application of *S. carpocapsae* and *B. bassiana* transitioned from synergy (3 dpi) via additive (5 dpi) to antagonistic (7, 14 dpi). It is probable that toxins produced by *B. bassiana* following fungal multiplication might have resulted in the antagonistic interaction between *B. bassiana* and *S. carpocapsae*. The antagonism observed in the later stages of the infection process indicate that the combination of *S. carpocapsae* and *B. bassiana* probably would not be a viable selection for *P. bilineatus* control.

The high virulence of *S. carpocapsae* against *P. bilineatus* when applied alone is remarkable. To the best of our knowledge, *S. carpocapsae* is the first reported EPN to consistently exhibit such high virulence against *P. bilineatus* when applied alone. Application of ½FR of imidacloprid was ineffective against *P. bilineatus* but a combined application with *S. carpocapsae* resulted in interactions that were either synergistic or additive. The role of the pesticide, imidacloprid in enhancing the infectivity of *S. carpocapsae* against *P. bilineatus* may have resulted from reduction of the insect's evasive behavior.[44] Combined application of *S. carpocapsae* and imidacloprid may be the tool needed for the replacement of chlorpyrifos in the management of *P. bilineatus* by peanut farmers. Conceivably if the level of EPN efficacy needs to be improved under field conditions various techniques might be implemented such as hybridization of strains[45,46] or pheromone boosters that enhance nematode dispersal and infectivity.[47]

The next challenge will be to determine how combined application of *S. carpocapsae* and imidacloprid can be implemented in peanut farms given that *P. bilineatus* attacks peanut pods that are underground. There is no doubt that replacement of chlorpyrifos will be beneficial to human health and environment, but more studies will be needed to determine a cost-effective method for the implementation of combined application of *S. carpocapsae* and imidacloprid for the management of the peanut burrower bug, *P. bilineatus*.

In summary, of the six EPNs screened in this study, *S. carpocapsae* was the most virulent to the adults of *P. bilineatus*. Imidacloprid did not exhibit any lethal toxicity to *P. bilineatus* when applied alone but combined application with *S. carpocapsae* resulted in synergistic mortality of the adult peanut burrower bug. Combined application of *S. carpocapsae* and imidacloprid may be an integrated pest management tool for effective replacement of chlorpyrifos. Field studies will be required to validate the efficacy of combined application of *S. carpocapsae* and imidacloprid against *P. bilineatus*.

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CONFLICT OF INTEREST

We declare no conflict of interest with respect to the study reported in this manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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