



## Special Collection: Perspective on Biology and Management of Bed Bugs

# Effect of Bed Bug (Hemiptera: Cimicidae) Aldehydes on Efficacy of Fungal Biopesticides

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### Abstract

The use of the entomopathogenic fungus *Beauveria bassiana* (Bals. – Criv.) Vuill. (Hypocreales: Cordycipitaceae) has been recently incorporated in the management of bed bugs, *Cimex lectularius* L. (Hemiptera: Cimicidae). Bed bugs produce a set of aldehydes that are known to affect the growth of some fungi. Considering that bed bugs or their exuviae release these aldehydes, it was suspected that the bed bugs' aggregation sites would contain an increased level of the bed bug aldehydes. The current study examined if elevated levels of the bed bug aldehydes in the microhabitats would impact the efficacy of *B. bassiana*. Following a brief exposure to the residues of commercial products containing *B. bassiana*, the treated bed bugs were kept in a vial with or without a natural or artificial blend of bed bug aldehydes (i.e., exuviae or synthetic compounds). For a *B. bassiana* product that is not currently registered for bed bugs control, the presence of aldehydes significantly reduced 15-d mortality (61–62%) compared to the no aldehydes control (97.7%). However, when tested with a *B. bassiana* formulation designed for bed bug control, the aldehydes only caused delayed mortality for the treated bed bugs. When tested in culture, the growth rate of *B. bassiana* on a medium was significantly reduced when the bed bug aldehydes were provided in the headspace. Implications on practical bed bug management using fungal biopesticides are discussed.

**Key words:** aldehyde, bed bug, entomopathogenic fungi, antifungal, exuviae

Bed bugs (Hemiptera: Cimicidae) are among the most challenging urban pests to control. A significant challenge for bed bug control is the widespread insecticide resistance among bed bug field populations (see [Romero 2018](#) and references therein). Despite the widespread distribution of pyrethroid resistance among bed bug populations, pyrethroids remain among the most commonly used active ingredients in many commercial pesticide products against bed bugs ([Romero et al. 2007](#), [Lee et al. 2018](#)). In an attempt to address pyrethroid resistance, products containing pyrethroids are increasingly mixed with neonicotinoid insecticides ([Lee et al. 2018](#)). However, resistance to neonicotinoids has also been reported in field populations of bed bugs ([Gordon et al. 2014](#), [Romero and Anderson 2016](#)).

The use of entomopathogenic fungi as biopesticides has been considered one of the possible solutions for the control of bed bug populations resistant to synthetic insecticides ([Barbarin et al. 2017](#)). Several species of fungi have been investigated for use as control

agents against bed bugs. For example, bed bugs exposed to the fungus *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) experienced high mortality at a relative humidity (RH) of 98% ([Ulrich et al. 2014](#)). *Aspergillus tubingensis* R. Mosseray (Eurotiales: Trichocomaceae) and *Trichoderma harzianum* Rifai (Hypocreales: Hypocreaceae) have also been suggested as possible fungal species to be utilized against the tropical bed bug, *Cimex hemipterus* (Fabricius) (Hemiptera: Cimicidae). Both of these fungi were found to cause up to 90% mortality when *C. hemipterus* were placed on a surface treated with spores ([Zahran et al. 2017](#)). However, none of these fungi are currently registered for use against bed bugs. A common soil fungus, *Beauveria bassiana* (Bals. – Criv.) Vuill. (Hypocreales: Cordycipitaceae), has been used against a wide variety of insect pests ([Butt et al. 2001](#), [Meyling and Eilenberg 2007](#)). Upon adhesion to an insect cuticle, *B. bassiana* spores germinate and penetrate the cuticle to utilize nutrients from the insect and produce toxins that negatively impact the target insect

(Islam et al. 2021). Currently, only one commercial biopesticide product containing *B. bassiana* is registered for bed bug control in the United States—Aprehend ( $>2.2 \times 10^9$  spores per ml; ConidioTec LLC, Centre Hall, PA). This product is composed of a 2% suspension of *B. bassiana* spores in oil. While this product cannot be applied directly to beds and furniture, it can be used indoors as a residual treatment. This product was found to be effective for both insecticide-resistant strains of *Cimex lectularius* L. ( $>94\%$  mortality) and a susceptible strain (98–100% mortality) (Barbarin et al. 2017). *Beauveria bassiana* was also found to be highly virulent to both adult and nymphal stages of *C. lectularius*, usually resulting in 100% mortality by three to five days postexposure, and can be transferred horizontally among conspecifics from treated to untreated bed bugs within harborage sites (Barbarin et al. 2012, Aak et al. 2018). Shikano et al. (2021) found that the residues of a majority of 22 insecticides used for bed bug control resulted in reduced spore viability when Aprehend was applied to the surface. However, this reduction in spore viability caused a reduction in the effectiveness of Aprehend in only one of the insecticides tested (Shikano et al. 2021). Similarly, the use of Aprehend on a permethrin treated mattress encasement reduced the viability of *B. bassiana* spores, but did not reduce the effectiveness (Shikano et al. 2019).

Various environmental and biological factors impact the effectiveness of entomopathogenic fungi. Suitable conditions for fungal germination, such as temperature and humidity, as well as the number of spores an insect is exposed to, will impact the effectiveness of entomopathogenic fungi (Islam et al. 2021). Insects utilize various defenses against fungal infection, from the physical barrier of the cuticle to the antifungal immune response following penetration of the cuticle by fungal hyphae (Lu and St. Leger 2016). In addition to these defenses, the presence of antifungal chemicals produced by insects may impact the efficacy of fungal biopesticides. It is not uncommon to find examples of insects' semiochemicals having additional protective functions against pathogenic fungi. Alarm pheromones such as 2-heptanone and 4-methyl-3-heptanone inhibit the growth of various species of fungi (Blum 1969, Cole et al. 1975). Bojke et al. (2020) also tested the effects of various insect-associated compounds against several fungal species, including *B. bassiana*. They found that heptanal, 2,4-nonadienal, 2-decenal, and undecanal were the most effective in inhibiting fungal growth. The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), produces several quinone-containing compounds that function as defensive compounds against predators (Tschinkel 1975). These secretions have also been found to inhibit the growth of *B. bassiana* (Pedrini et al. 2015).

Bed bugs produce several volatile aldehydes [(E)-2-hexenal, 4-oxo-(E)-2-hexenal, (E)-2-octenal, and 4-oxo-(E)-2-octenal]. These chemicals are considered their alarm/defensive compound and a part of the aggregation pheromone (Siljander et al. 2008, Gries et al. 2015, Choe et al. 2016, Ulrich et al. 2016). Broad-spectrum antifungal activities of various volatile aldehydes are well known. Maruzzella et al. (1961) tested the antifungal properties of 196 compounds, including 19 aldehydes, against four species of fungi. Of these aldehydes, 73% had antifungal activity (Maruzzella et al. 1961). Similarly, Avissar et al. (1990) found that acetaldehyde inhibited the growth of the fungi *Botrytis cinerea* Pers. (Helotiales: Sclerotiniaceae) and *Rhizopus stolonifer* Vuillemin (Mucorales: Mucoraceae). Indeed, the aldehydes produced by bed bugs have also been found to inhibit some fungi. For example, (E)-2-hexenal has been investigated for use as an antifungal fumigant for use in agriculture and was found to inhibit *B. cinerea* from growing on seedless table grapes, *Vitis vinifera* L. (Vitales: Vitaceae) (Archbold et al. 1999). *Botrytis cinerea* was inhibited by (E)-2-hexenal from growing

on strawberries after harvesting (Fallik et al. 1998). In addition, synthetic (E)-2-hexenal and (E)-2-octenal were found to inhibit the growth of the fungus *M. anisopliae* on a potato dextrose agar (PDA) medium as volatiles (Ulrich et al. 2015). Bed bugs that were exposed to synthetic (E)-2-octenal 1 or 24 hr after fungal exposure (spores) had lower mortality (10 or 33.3%, respectively) compared to bed bugs that were exposed to the fungal conidia only (98.9%) (Ulrich et al. 2015). The mechanism of fungal inhibition by aldehydes has been previously investigated in several species. For example, Ma et al. (2019) found that (E)-2-hexenal reduced germination of *Aspergillus flavus* Link (Eurotiales: Trichocomaceae) and caused a disruption of mitochondrial energy metabolism and induced apoptosis during spore germination. Zhang et al. (2017) found (E)-2-hexenal caused disruptions to the integrity of the cell membrane of *Penicillium cyclopium* Westling (Eurotiales: Trichocomaceae) and resulted in the leakage of cellular components.

For bed bug control, the use of entomopathogenic fungi is geared towards residual treatments. For example, the label of Aprehend states that 'Aprehend is intended as a barrier treatment; bed bugs are exposed by crossing a surface (barrier) that has been treated with Aprehend.' This statement infers that the bed bugs would contact these fungal conidia from the treated surfaces while they emerge from their harborages in search of a bloodmeal or return to those. Since it takes several days (4–6 d) for *B. bassiana* to kill bed bugs after initial exposure, it would be reasonable to assume that most of the exposed bed bugs will have enough time to return to their harborage sites before being killed. If the harborage site contains a relatively large aggregation of bed bugs developing, it would be reasonable to assume the presence of elevated concentrations of aldehydes from bed bugs at the harborage sites. Bed bugs emit the aldehydes during unwanted mating attempts, emitted defensively or in shed exuviae accumulating at the harborage sites (Uisinger 1966, Levinson et al. 1974, Harraca et al. 2010, Kilpinen et al. 2012, Choe et al. 2016). The bed bug exuviae contain (E)-2-hexenal, 4-oxo-(E)-2-hexenal, (E)-2-octenal, and 4-oxo-(E)-2-octenal, and the aldehydes volatilize slowly from the exuviae (Feldlaufer et al. 2010, Choe et al. 2016). Thus, it was suspected that the efficacy of entomopathogenic fungal pesticides might be impacted if the treated bed bugs return to the harborages. However, most of the toxicological reports on the fungal biopesticides and bed bugs are based on laboratory experiments that did not consider these factors.

The main objective of the current study was to investigate the impact of all four primary bed bug aldehydes (as a mixture) on the efficacy of *B. bassiana*. Using two commercial fungal biopesticides products containing *B. bassiana*, the current study examined if aldehydes impact bed bug mortality. Bed bugs were first exposed to the *B. bassiana* conidia deposits and subsequently kept with or without an additional source of bed bug aldehydes for mortality monitoring. Either exuviae or a synthetic blend [based on the ratio found in 5th instar *C. lectularius* nymphs (Dery et al. 2020)] was used as a source of aldehydes. A second set of experiments was conducted to examine the impact of the bed bug aldehyde blend on the growth of *B. bassiana* in culture. We expect the current work will further expand knowledge on interactions between *B. bassiana* and *C. lectularius* and provide some important implications on practical bed bug management using the fungal biopesticides.

## Materials and Methods

### Insect

The bed bugs (*C. lectularius*) used in this study were from laboratory stock colonies started from the 'Earl' strain bed bugs purchased

from Sierra Research Laboratories (Modesto, CA, USA). The Earl strain was initially collected in Modesto, CA, in 2007. Colonies were maintained at 24–26°C and 15–30% RH, with a photoperiod of 12:12 (L:D) hr. Bed bugs were fed with defibrinated rabbit blood approximately every fourteen days.

### Fungal Biopesticides

Aprehnd and BotaniGard 22WP ( $2 \times 10^{13}$  spores per pound, Laverlam International, Butte, MT, USA) were used as commercial formulations of *B. bassiana* strain GHA. Aprehnd is formulated in a proprietary oil mixture and does not require dilution before application. This is currently the only product containing an entomopathogenic fungus registered for bed bug control. BotaniGard is a wettable powder that is designed to be mixed with water before application. BotaniGard is not registered for bed bug control. These two different formulations were included in the experiments to examine whether the impact of aldehydes on *B. bassiana* was different between them.

### Bed Bug Aldehydes

Two different sources of bed bug aldehydes were used: the natural blend from exuviae or a synthetic blend. Technical grade (*E*)-2-hexenal (98% pure) and (*E*)-2-octenal (94% pure) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and 4-oxo-(*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal were synthesized as described by [Moreira and Millar \(2005\)](#) and were verified as 97% pure using gas chromatography–mass spectrometry.

### Mortality Test

The following experiment was conducted to determine if the presence of bed bug aldehydes in the headspace affects the effectiveness of the fungal biopesticides. Groups of ten adult bed bugs ( $\approx 10$  d postbloodmeal) were collected randomly from colony vials. Each group of bed bugs was randomly assigned for one of the following four treatments: (1) fungus only, (2) fungus + exuviae, (3) fungus + synthetic aldehydes, and (4) untreated control.

The following process was used to prepare treatment dishes for fungal exposure. Filter paper discs (60 mm diameter; Whatman #1, Cytiva, Marlborough, MA) were placed into plastic culture dishes (60 mm diameter; Fisher Scientific, Waltham, MA). BotaniGard 22WP treatments were prepared by adding 0.012 g BotaniGard to 10 ml water. One milliliter of this suspension is used to treat each filter paper discs ( $3.9 \times 10^5$  spores/cm<sup>2</sup>). This represents a rate approximately the middle of the label rate for using this product to control thrips. For fungal exposures with Aprehnd, 300  $\mu$ l was placed on each filter paper disc ( $2.3 \times 10^7$  spores/cm<sup>2</sup>). This represents approximately five times the label rate and is similar to the concentration tested by [Aak et al. \(2018\)](#). As this product is not diluted before use, this larger application was used to ensure complete coverage of spores on the filter paper disc. All products were applied using a micropipette. The treatment dishes were kept uncovered in a fume hood until they dried (BotaniGard  $\approx 5$  hr; Aprehnd  $\approx 24$  hr). One milliliter of water was applied to the filter paper disc for the untreated control.

Each group of bed bugs was anesthetized with carbon dioxide ( $\approx 30$  s) and placed onto the surface of the filter paper in the treatment dish. Each dish was covered and then sealed with parafilm. After one hour, each group of bed bugs was transferred to a 20-ml scintillation vial containing a piece of clean filter paper (50  $\times$  10 mm) folded in a small tent, serving as a resting platform. For treatment

#2 (fungus + exuviae), the vial contained exuviae ( $\approx 50$ ) of mixed age and stage (3rd to 5th instar) collected from a *C. lectularius* colony vial. For treatment #3 (fungus + synthetic aldehydes), the vial contained 50 exuviae equivalent aldehyde blend (336.65  $\mu$ g total) [(*E*)-2-hexenal: 66.3  $\mu$ g; 4-oxo-(*E*)-2-hexenal: 73.6  $\mu$ g; (*E*)-2-octenal: 181.2  $\mu$ g; 4-oxo-(*E*)-2-octenal: 15.55  $\mu$ g] dissolved in 40  $\mu$ l acetone (based on the amounts found in freshly shed 5th instar exuviae of *C. lectularius*; [Dery et al. 2020](#)). The synthetic aldehyde blend was applied to absorptive matting (16 mm diameter, laboratory bench and table protector with leak-proof moisture barrier, VWR International, Radnor, PA) glued to the underside of the vial cap to prevent direct contact with the bed bugs. All vials remained sealed for the duration of the study. Mortality for each group was recorded daily for 15 d. Mortality was determined visually by counting bed bugs in vials without movement, and that could not grasp filter paper upon contact. A total of nine replications were conducted for each of the treatments and control.

### Spore Suspension

To study the effect of the bed bug aldehyde blend on the growth of *B. bassiana* in culture, a pure spore suspension of *B. bassiana* was prepared by the following process. Sterile water with 0.01% (v/v) Tween-80 (Fisher Scientific) was added to a microcentrifuge tube containing BotaniGard 22WP to obtain a spore suspension ( $9.98 \times 10^8$  spores/ml). Ten microliters of this suspension were used to inoculate a Petri dish (100  $\times$  15 mm; Fisher Scientific) containing just enough potato dextrose agar (PDA; Sigma-Aldrich, St. Louis, MO, USA) to cover the bottom of the dish. The plate was incubated at 26°C for two weeks. A small piece of mycelium was transferred to a new PDA plate from this plate. After 14 d incubation, the new PDA plate containing a pure culture of *B. bassiana* was used to obtain a spore suspension. This suspension was made by adding  $\approx 4$  ml sterile water containing 0.01% (vol/vol) Tween-80 to the plate, scraping the fungus off the media, and then filtering through a sterile Kimwipe to remove the fungal hyphae and other debris. The concentration ( $4.95 \times 10^7$  spores/ml) of spores in this suspension was determined using a hemacytometer (Bright-Line, Hausser Scientific, Horsham, PA).

### In Vitro Activity Test

To determine the rate of fungal growth as affected by the presence of aldehydes in vitro, the rate of hyphal growth was measured using a method adapted from [Inglis et al. \(2012\)](#). PDA plates, as described above, were inoculated with 1  $\mu$ l of a  $4.95 \times 10^7$  spores/ml suspension in 0.01% Tween-80 placed in the center of the plate. Each of the plates were treated with 100 [(*E*)-2-hexenal: 132.6  $\mu$ g; 4-oxo-(*E*)-2-hexenal: 147.2  $\mu$ g; (*E*)-2-octenal: 362.4  $\mu$ g; 4-oxo-(*E*)-2-octenal: 31.1  $\mu$ g] or 25 [(*E*)-2-hexenal: 33.15  $\mu$ g; 4-oxo-(*E*)-2-hexenal: 36.8  $\mu$ g; (*E*)-2-octenal: 90.6  $\mu$ g; 4-oxo-(*E*)-2-octenal: 7.78  $\mu$ g] exuviae equivalent amount of the *C. lectularius* aldehyde blend dissolved in 40  $\mu$ l acetone [based on the amount of aldehydes found in 5th instar exuviae; [Dery et al. \(2020\)](#)]. The aldehyde blend was applied to a sterile filter paper disc (16 mm diameter) placed unattached on the inner surface of the Petri dish lid. The dishes were kept inverted during the incubation process to ensure there was no physical contact between the aldehyde disc and the PDA. A positive control group received the spore suspension, and clean acetone was applied to the filter paper disc. In contrast, a negative control group was inoculated with sterile 0.01% Tween-80, and clean acetone was applied to the filter paper disc. The plates were sealed with parafilm, and each group was stored separately at 26°C in the absence of light.

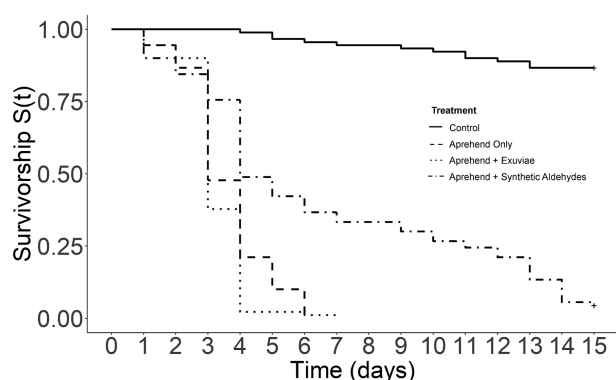


Fig. 1. Survivorship of *Cimex lectularius* treatment groups ( $n = 90$ ) following exposure to Aprehend with or without the presence of bed bug aldehydes.

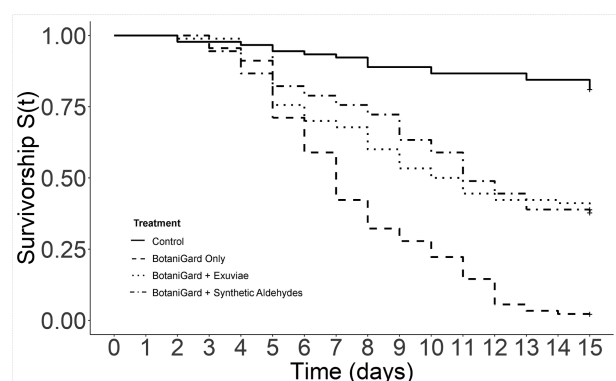


Fig. 2. Survivorship of *Cimex lectularius* treatment groups ( $n = 90$ ) following exposure to BotaniGard with or without the presence of bed bug aldehydes.

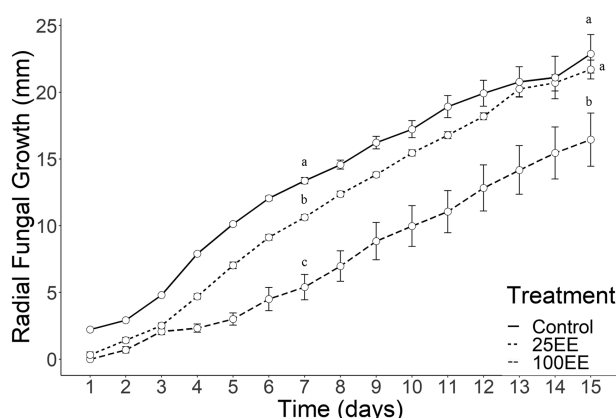


Fig. 3. Radial growth (mm, mean  $\pm$  SEM) of *Beauveria bassiana* groups ( $n = 10$ ) over time when exposed to *Cimex lectularius* aldehyde blend. Different letters indicate significant differences (Dunn's Multiple Comparisons;  $P < 0.05$ ) (EE: exuvia equivalent).

For each plate, the average diameter of visible fungal growth was determined by two perpendicular measurements. The fungus growth from the point of inoculation was measured daily for 15 d. Ten replications were conducted for each of the treatments and controls.

## Statistical Analysis

To determine if the addition of aldehydes affected bed bug mortality, survivorship curves were generated by Kaplan-Meier survival analyses with the pooled data ( $n = 90$ ), and the mortality curves were compared pairwise using the log-rank test with correction (Benjamini and Hochberg 1995) using the R package survminer (Kassambara et al. 2021). Due to instances of heteroscedasticity, the radial growth of *B. bassiana* in culture was analyzed with Kruskal-Wallis H test followed by Dunn's Multiple Comparisons. All statistical comparisons were conducted using R version 4.0.3 (R Core Team 2020).

## Results

### Mortality Test

In the test with Aprehend, there were significant differences between the four survival curves (log-rank test;  $\chi^2 = 291$ ;  $df = 3$ ;  $P < 0.001$ ). The survival curves were significantly different between 'fungus only' and 'fungus + exuviae' (log-rank test;  $P = 0.036$ ). However, both 'fungus only' and 'fungus + exuviae' groups experienced 100% mortality within a day of each other. The survival curve from 'fungus + synthetic aldehydes' was significantly different from 'fungus only' (log-rank test;  $P < 0.001$ ) (Fig. 1). The survival curves from the two groups exposed to aldehydes ('fungus + exuviae' and 'fungus + synthetic aldehydes') were significantly different (log-rank test;  $P < 0.001$ ). The median survival time of the 'fungus only' group was 3 (3–4; interquartile range, IQR) days (100% mortality at day 6) and 3 (3–4) d for the 'fungus + exuviae' group (100% mortality at day 7), while the median survival time of 'fungus + synthetic aldehydes' was 4 (4–11) d (94% mortality at day 15) (Fig. 1).

In the test with BotaniGard, there were significant differences between the four survival curves (log-rank test;  $\chi^2 = 132$ ;  $df = 3$ ;  $P < 0.001$ ). The survival curve from 'fungus only' was significantly different from those of 'fungus + exuviae' and 'fungus exposure + synthetic aldehydes' (log-rank test;  $P < 0.001$  for both). The survival curves from two groups exposed to aldehydes ('fungus + exuviae' and 'fungus + synthetic aldehydes') were similar (log-rank test;  $P = 0.69$ ). The median survival time of the 'fungus only' group was 7 (5–10, IQR) days (97.7% mortality at day 15), 10.5 (6–15) d (62.2% mortality at day 15) for the 'fungus + exuviae' group, and was 11 (8–15) d (61.1% mortality at day 15) for the 'fungus + synthetic aldehydes' group (Fig. 2).

### In Vitro Activity Test

On day 7, the radial growth of the fungus was significantly affected by the presence of aldehydes (Kruskal-Wallis test;  $H = 25.84$ ;  $df = 2$ ;  $P < 0.001$ ). The radial fungal growth distances for the control ( $13.4 \pm 0.2$  mm; mean  $\pm$  SE;  $n = 10$ ), 25 exuviae equivalent treatment ( $10.6 \pm 0.2$  mm;  $n = 10$ ), and 100 exuviae equivalent treatment ( $5.4 \pm 0.9$  mm;  $n = 10$ ) were all distinct from each other (Dunn's Multiple Comparisons;  $P < 0.05$ ) (Fig. 3).

Similarly, the radial growth of the fungus on day 15 was significantly affected by the treatment with aldehydes ( $H = 9.04$ ;  $df = 2$ ;  $P < 0.05$ ). Unlike day 7 data, the amount of growth for the control ( $22.9 \pm 1.4$  mm;  $n = 10$ ) and 25 exuviae equivalent treatment ( $21.7 \pm 0.7$  mm;  $n = 10$ ) were similar (Dunn's Multiple Comparisons;  $P = 0.559$ ). However, the growth in the 100 exuviae equivalent treatment group ( $16.5 \pm 1.9$  mm;  $n = 10$ ) was significantly less (Dunn's Multiple Comparisons;  $P < 0.05$ ) than both (Fig. 3).

## Discussion

The current study demonstrates that bed bug aldehydes can impact the speed of kill and resulting mortality of bed bugs following their brief exposure (1 hr) to fungal biopesticides. This effect was especially pronounced when bed bugs were exposed to BotaniGard, a formulation of *B. bassiana* designed to be used as an aqueous spray to control garden/agricultural pests. For example, on day 15, the fungus-treated bed bugs experienced 61–62% mortality when the aldehydes were present. In contrast, the fungus-treated bed bugs in the control group (i.e., no added aldehyde source) had 97.7% mortality. However, we observed a somewhat different outcome when the bed bugs were treated with Aprehend, an oil-based product explicitly formulated for bed bug control. Regardless of the presence of exuviae in the vials, all bed bugs treated with Aprehend were killed within 7 d. When a synthetic aldehyde blend was present in test vials, the fungus-treated bed bugs experienced delayed mortality (see Fig. 1). Bed bugs exposed to Aprehend alone reached 100% mortality on day 6, while those exposed to the synthetic aldehyde blend and Aprehend experienced 63% mortality on day 6. However, the bed bugs with synthetic aldehydes eventually reached 94% mortality on day 15. This suggests that the presence of the synthetic aldehydes resulted in delayed bed bug mortality. While there was a difference in the magnitude of the effect of the aldehydes between the two products, any direct comparison between these two products would not be feasible due to the difference in application rates used. Aprehend was applied at a higher rate than BotaniGard, potentially causing significantly more exposure to spores than BotaniGard. Despite the increased exposure to spores, the introduction of synthetic aldehydes resulted in an overall delay in mortality to bed bugs exposed to Aprehend.

In trials involving Aprehend, there was a significant difference between the two sources of bed bug aldehydes (exuviae or synthetic aldehydes). Bed bugs were killed significantly faster by the fungus when exposed to the exuviae compared with the synthetic aldehyde blend. As the synthetic aldehyde blend was based on the amount of aldehydes in freshly shed 5th instar exuviae, there were likely a larger amount of aldehydes present in the synthetic aldehyde blend compared with the exuviae, as the aldehydes from exuviae volatilize over time (Choe et al. 2016) and exuviae of random age were collected. Interestingly, no differences were observed between the two aldehyde sources when bed bugs were exposed to BotaniGard. It is difficult to say if this is a result of the two different formulations containing *B. bassiana* or different concentrations of the two products tested.

The aldehydes produced by bed bugs inhibit several species of fungi (Fallik et al. 1998, Archbold et al. 1999, Ulrich et al. 2015). Ulrich et al. (2015) reported that 1-week mortality was low (10–33%) when the bed bugs were continuously exposed to spore residues of *M. anisopliae* and volatile (E)-2-octenal within a Petri dish (60 × 15 mm) (introduced at 1 or 24 hr posttreatment). Continuous exposure to the fungal spore residue without the aldehyde resulted in 98.9% mortality during the same period. Based on an in vitro fungal growth study, Ulrich et al. (2015) found that the growth of *M. anisopliae* was entirely or partially inhibited when 0.5–20 mg (E)-2-octenal or (E)-2-hexenal was introduced in the fungal culture media plates either via direct contact or fumigation (Ulrich et al. 2015). While Ulrich et al. (2015) tested the individual inhibitory effects of (E)-2-octenal or (E)-2-hexenal on *M. anisopliae*, the present study tested all four major bed bug aldehydes as a mixture to determine their overall inhibitory effects against *B. bassiana*. Within a natural aggregation site, all four aldehyde compounds will be present (e.g., emitted during

unwanted mating attempts, emitted defensively, or in shed exuviae) (Usinger 1966, Levinson et al. 1974, Harraca et al. 2010, Kilpinen et al. 2012, Choe et al. 2016). In particular, it is likely that two ketoaldehydes produced exclusively by nymphs (and exuviae) may also play a significant role in fungal inhibition. Noge et al. (2012) found that one of the bed bug ketoaldehydes, 4-oxo-(E)-2-hexenal, inhibited bacterial growth more strongly than (E)-2-octenal or (E)-2-hexenal.

The present results found that the growth of *B. bassiana* in culture was reduced/delayed by the presence of bed bug aldehydes. However, the growth of the fungus in the lower dose aldehyde treatment (0.168 mg; 25 exuvia equivalents) had reached an equal level with the control group by day 15, while the fungal growth was still significantly reduced in higher dose aldehyde treatment (0.673 mg; 100 exuvia equivalents) relative to the control. This finding suggests that the antifungal effect of the aldehydes is concentration-dependent. Ulrich et al. (2015) reported that the growth of *M. anisopliae* in culture was completely inhibited when large amounts (>0.5 mg) of (E)-2-octenal or (E)-2-hexenal was introduced via direct contact or fumigation within a Petri dish (60 × 15 mm). In contrast, fungal growth occurred at lower aldehyde concentrations (<0.5 mg) (Ulrich et al. 2015). The lack of complete inhibition of *B. bassiana* also suggests that the aldehydes do not prevent spores from germinating. Sosa-Gomez et al. (1997) found that (E)-2-decenal did not inhibit the germination of *B. bassiana* spores in artificial media.

It is unknown how much fungal infection risk might exist for bed bugs in structural settings (e.g., bedroom). However, it is clear that various pathogens, including bacteria and fungi, are associated with bed bugs (Strand 1977). One natural fungal pathogen of bed bugs is *A. flavus*, which has been repeatedly found to infect both wild and laboratory colonies of cimicids. For example, *A. flavus* is known to infect the eastern bat bug, *Cimex adjunctus* Barber (Hemiptera: Cimicidae), in both laboratory and wild colonies in the caves where the bat bugs naturally occur (Reeves 2001). Usinger (1966) also reported that *A. flavus* infected a laboratory colony of *Paracimex* (Hemiptera: Cimicidae). Further, *A. flavus* was found to infect both adult and second instar nymphs of *C. lectularius* when placed at 30°C and 90% relative humidity (RH); laboratory colonies established under these conditions were destroyed by the fungus within 18 d (Cookbain and Hastie 1961). *Aspergillus flavus* is a common soil fungus and pathogen of some crops and is also among the most commonly found indoor fungus (Li et al. 1995, Shelton et al. 2002, Klich 2007, Hedayati et al. 2010).

In addition to their role in mediating aggregation and alarm responses, bed bug aldehydes may also function as a defense to these various fungal pathogens. The antifungal activity of aldehydes in bed bugs and other related cimicids might be more critical when the insects are living under a larger amount of fungal infection pressure, such as in the nests of birds or caves where bats roost. In these settings, temperature and humidity may be elevated relative to indoor environments. For example, the microclimate of the refugia of the bat bug *Afrocinex constrictus* Ferris and Usinger (Hemiptera: Cimicidae), was determined in two caves in the Mt. Elgon area of Kenya by Reinhardt et al. (2008). The temperature in these locations was variable depending on the location of *A. constrictus* within the cave, with daytime temperatures ranging from ~18–25°C, with relative humidity generally over 70% (Reinhardt et al. 2008). Future research is warranted to investigate the risk of fungal infection and the potential antifungal action of any aldehyde pheromone for these cave-dwelling cimicids.

The current study might provide valuable insights into some of the earlier reports on fungal biopesticides and bed bugs. Aak

et al. (2018) found a lack of correlation between the level of aggregation and bed bug mortality caused by the horizontal transfer of *B. bassiana*. Aak et al. (2018) suspected that this might be due to a higher concentration of aldehydes in aggregations, offsetting the effect of a greater chance of fungal transfer between individual insects within an aggregation. However, some caution is warranted when interpreting these current results. The mortality assay in the present study was conducted in a closed system (i.e., capped vial). In contrast, bed bugs in a natural harborage will be in an open system where aldehydes from exuviae and bed bugs could constantly diffuse and dissipate. As a result, more realistic concentrations of aldehydes in bed bug aggregations could be lower than the concentrations of the aldehydes tested in the present study. However, Eom et al. (2012) has reported the presence of detectable amounts of (*E*)-2-hexenal and (*E*)-2-octenal from indoor air of rooms infested with 200–1,000 bed bugs. The compounds were not detected from control samples (no bed bug infestation). Considering live bed bugs can also serve as a source of these aldehydes along with their exuviae (e.g., defensive emission of aldehydes against unwanted mating attempts; Harraca et al. 2010, Kilpinen et al. 2012), it might be reasonable to assume that aldehydes would be present in the microhabitats of bed bugs (e.g., cracks and crevices) where the bed bugs aggregate and develop.

Our results show that aldehydes associated with bed bugs can impact the effect of *B. bassiana* on bed bugs. The presence of bed bug aldehydes in the headspace reduced or delayed the resulting mortality after the exposure to the *B. bassiana*. The varied impact of bed bug aldehydes on *B. bassiana* was dependent on how the fungus was formulated. Furthermore, the presence of the aldehydes in the headspace slowed the growth of the fungus in culture. These findings might have the following practical implications in bed bug management. The presence of bed bug aldehydes is a possible factor that might, in some circumstances, suppress or interfere with the effectiveness of *B. bassiana* in the field. The impact of aldehydes on *B. bassiana* also may be a plausible pathway to creating resistance if the use of this fungus for bed bug control is widely adopted. Any potential effects of the aldehydes on fungal biopesticides could be largely mitigated if used before large infestations are established. The early detection and management of bed bugs would prevent the formation of larger aggregations and the production/accumulation of high amounts of antifungal aldehydes in the harborage sites. Current findings also suggest that fungal biopesticide applications might work better if most of the existing exuviae are removed (i.e., removal of the source of antifungal aldehydes) from bed bugs' harborage sites before making an application. In any case, field experiments are warranted to test the significance of bed bug aldehydes on the performance of fungal biopesticide products in a realistic setting.

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