BioControl (2015) 60:517–526
DOI 10.1007/s10526-015-9667-2

Inhibition of the entomopathogenic fungus *Metarhizium anisopliae* sensu lato in vitro by the bed bug defensive secretions (*E*)-2-hexenal and (*E*)-2-octenal

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Received: 20 September 2014 / Accepted: 26 March 2015 / Published online: 2 April 2015

Abstract The two major aldehydes (*E*)-2-hexenal and (*E*)-2-octenal emitted as defensive secretions by bed bugs *Cimex lectularius* L. (Hemiptera: Cimicidae), inhibit the in vitro growth of an isolate of *Metarhizium anisopliae* sensu lato (*s.l.*) (Metsch.) Sokorin (Hypocreales: Clavicipitaceae) (ARSEF 1548). These chemicals inhibit fungal growth by direct contact and via indirect exposure ("fumigation"). Fumigation with (*E*)-2-octenal for as little as 0.5 h was sufficient to inhibit all fungal growth. Bed bugs placed on filter paper treated with an isolate of *M. anisopliae s.l.* conidia in the absence of (*E*)-2-octenal exhibited 99 % mortality after one week. However, bed bugs placed on fungal-treated filter paper and exposed to (*E*)-2-octenal at 1 h experienced 10 % mortality. The inhibition of fungal growth by bed bug aldehydes is discussed in the context of other biotic and abiotic barriers to infection.

Keywords *Cimex lectularius* (Hemiptera: Cimicidae) · Aldehydes · Antifungal activity · Volatiles · Defensive chemistry · Glandular secretions

Handling Editor: Nicolai Meyling.

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Introduction

Entomopathogenic fungi have been used against a variety of insect species (Shah and Pell 2003), with *Beauveria bassiana* (Bals.) Vuillemin (Hypocreales: Clavicipitaceae) and *Metarhizium anisopliae* (Metsch.) Sokorin (Hypocreales: Clavicipitaceae) representing the active biopesticide in the majority of commercially-developed products for use against most agricultural insect and arthropod pests (de Faria and Wraigt 2007; Hajek and Delalibera 2010; Vega et al. 2012). In addition to plant pests, entomopathogenic fungi are being developed for control of blood-sucking arthropods, including ticks and mosquitoes (Blanford et al. 2005; Bukhari et al. 2011; Fernandes et al. 2012). *B. bassiana* was shown to be pathogenic to the bed bug *Cimex lectularius* L. (Hemiptera: Cimicidae) (Barbarin et al. 2012). Not only was the fungus efficacious, but due to the gregarious nature of bed bugs, horizontal transmission also was observed, as infected individuals could transfer the fungus to uninfected bed bugs.

Various abiotic factors such as temperature, humidity, and sunlight can pose a challenge to the use of entomopathogenic fungi under field conditions (Jaronski 2010). The insect cuticle likewise can pose a physical and chemical barrier to infection by entomopathogenic fungi (Gołębiowski et al. 2008; Hajek and St Leger 1994; Ortiz-Urquiza and Keyhani 2013; Wilson et al. 2001). In this study, we examine another biotic factor, namely chemical defensive secretions, which may impact the ability of an isolate of *M. anisopliae* sensu lato (*s.l*) to infect bed bugs. (*E*)-2-hexenal and (*E*)-2-octenal are the major defensive secretions of immature and adult bed bugs (Collins 1968; Levinson et al. 1974; Schildknecht et al. 1964). These aldehydes also are considered to act as pheromones, affecting bed bug behavior (Benoit et al. 2009; Harraca et al. 2010; Levinson et al. 1974; Levinson and Bar Ilan 1971; Siljander et al. 2008). Kilpinen et al. (2012) reported that one bed bug could release as much as 40 µg of these aldehydes in a single emission. One or both of these aldehydes are found in several other heteropteran species (Aldrich 1988), where they act not only as pheromones, but as kairomones, attracting parasitoids (Vieira et al. 2014). (*E*)-2-hexenal and (*E*)-2-octenal also are found in some plants, where they have been shown to exhibit antibacterial and antifungal activity (Battinelli et al. 2006; Bisignano et al. 2001; Cleveland et al. 2009; Kubo and Kubo 1995; Trombetta et al. 2002). In this study, we report that (*E*)-2-hexenal and (*E*)-2-octenal, can prevent the in vitro growth and development of an isolate of entomopathogenic fungus *M. anisopliae s.l*, and may be a barrier to infection of bed bugs with this fungus.

Materials and methods

Chemicals

(*E*)-2-Hexenal and (*E*)-2-octenal were obtained from Bedoukian Research, Inc. (Danbury CT, USA). Their purity by gas chromatography was 99.7 and 97.6 %, respectively. Tween® 80 was from Sigma-Aldrich (St. Louis MO, USA), and spectral grade acetone (Honeywell Burdick & Jackson, Morristown NJ, USA) was used for all dilutions of aldehydes.

Fungal isolates

An isolate of wild-type *M. anisopliae s.l.* (ARSEF 1548) was obtained from the United States Department of Agriculture Entompathogenic Fungus Collection in Ithaca, NY, USA. This strain originally was isolated from the rice black bug *Scotinophara coarctata* (Hemiptera: Pentatomidae). Fungal cultures were maintained on Difco™ potato dextrose agar (= PDA; Becton–Dickinson, Sparks MD, USA) and incubated at room temperature for 14 days. Conidia were harvested by scraping colonies with a sterile spatula and suspending in distilled water containing 0.01 % Tween 80. Spore suspensions were determined using a hemocytometer (Spencer, Buffalo NY, USA) and adjusted to a concentration of 1×10^7conidia ml^{-1}. Conidial viability was determined to be above 90 % for all bioassay and germination studies.

In vitro fungal growth when exposed to (*E*)-2-hexenal and (*E*)-2-octenal

To determine the effect of bed bug aldehydes on fungal growth in vitro, sterile 6 mm disks (Whatman, Grade AA) were impregnated with dilutions of either (*E*)-2-hexenal or (*E*)-2-octenal in acetone to yield 0.1, 0.25, 0.33, 0.5, 1, 5, 10, or 20 mg of aldehyde per disk. After drying, disks were placed in the center of a PDA plate previously inoculated with 25 µl of fungal suspension (10^7 conidia ml^{-1}). To determine any fumigant action these aldehydes might have, filter paper disks were prepared as above, and then placed on the inner lid that covered fungal-inoculated PDA plates. In this way, aldehyde-treated disks did not come into direct contact with the inoculated plate. Controls consisted of inoculated plates exposed to untreated (acetone) disks, either by direct contact or by fumigation. All cultures were kept at room temperature and humidity (25 ± 2 °C and 30 ± 5 % RH) for 72 h at which time fungal growth was visually assessed by measuring any zone of inhibition in the inoculated plates. Each experiment was replicated four times.

To determine the minimum exposure time required for *M. anisopliae s.l.* inhibition by fumigation, sterile 6 mm disks were impregnated with 0.5 mg (*E*)-2-octenal, the minimal amount needed to suppress all *M. anisopliae s.l.* growth (from the preceding experiment). After drying, the treated disks were placed

on the inner lid that was used to cover a series of PDA plates that had each been inoculated with 25 µl of fungal suspension (10^7 conidia ml^{-1}). Exposure to octenal was terminated at various times (0.25, 0.5, 1, 2, and 3 and 24 h) post-exposure, by removing the lids containing the treated disks and replacing them with lids containing no disk. Plates were incubated at room temperature for 72 h and fungal growth assessed, as above. Controls consisted of inoculated plates exposed to untreated (acetone) disks. Each experiment was replicated four times, and kept at the same conditions as above.

Plates inoculated with *M. anisopliae s.l.* also were exposed to 0.5 mg octenal at various times post-inoculation, to determine fungal growth, if any, when not exposed to octenal for a prescribed period of time. This was accomplished by inoculating plates with 25 µl of fungal suspension (10^7 conidia ml^{-1}), covering these plates with lids containing no disk, and then replacing these lids with lids containing octenal-treated (0.5 mg) disks at 0.5, 1, 2, 3, 4, 6, 12, 24, and 48 h post-inoculation. Each experiment was repeated four times and plates were kept and assessed as noted above.

Effects of (*E*)-2-octenal on *M. anisopliae s.l.* germination

To determine conidial germination in the presence of octenal, plates were inoculated with 2.5×10^4 *M. anisopliae s.l.* conidia and exposed to 0.5 mg octenal at 6 or 12 h post-inoculation by placing lids containing octenal-treated disks (0.5 mg) at either 6 or 12 h post-inoculation. Germination was observed under ×400 magnification at 6, 12, 24, and 48 h from the time the conidia were placed on medium. Conidia with germ tubes larger than twice the diameter of the conidium were considered to have germinated (Inglis et al. 2012). Each treatment was repeated four times. 100 conidia per replicate were counted, and mean germ tube lengths were determined for each treatment. Controls consisted of inoculated plates exposed to untreated (acetone) disks.

Bed bugs

A colony of *C. lectularius* was established from bed bugs originally obtained from Harold Harlan (Crownsville, MD, USA). The colony was kept at ambient conditions (25 ± 2 °C and 30 ± 5 % RH) and fed weekly on expired, human red blood cells and plasma using an artificial (in vitro) feeding system (Feldlaufer et al. 2014). Adult males and females were used for the experiments and had not been fed for eight days prior to use.

Exposure of bed bugs to *M. anisopliae s.l.* and (*E*)-2-octenal

Bed bugs were exposed to *M. anisopliae s.l.* by being placed on filter paper previously treated with conidia. In these experiments, conidial suspensions in sterile water containing 0.01 % Tween 80 were applied to 47 mm (diam.) filter paper disks (Whatman No. 1) to yield a final concentration of 1×10^5 conidia cm^{-2}. Filter papers were allowed to dry for 120 min before being placed in glass Petri dishes (60 × 15 mm). Fifteen adult male and female bed bugs were then placed on the dried, treated surface. One treatment group remained as such (fungal-exposed control). Two additional treatment groups of fungal-exposed bed bugs had a disk containing 0.5 mg octenal added to the dish at 1 and 24 h post-exposure, respectively. Additional control groups consisted of bed bugs placed on 0.01 % Tween 80-treated filter paper (untreated), and another fungal treatment group was removed to an untreated surface 24 h post-exposure. To determine toxicity, if any, to octenal, bed bugs were placed on untreated filter paper to which a disk containing 0.5 mg octenal was added. Each treatment consisted of three replicates and the experiment was repeated twice by preparing new aldehyde dilutions and new fungal suspensions from separate fungal culture plates.

In these experiments, bed bugs were kept in glass desiccators (150 mm i.d.; Fischer Scientific, Pittsburgh, PA, USA) over distilled water (98 ± 1 % RH) at 25 ± 2 °C, and mortality was assessed at one week post-treatment. We had previously shown (Ulrich et al. 2014) that this humidity was necessary to cause a >98 % infection rate in fungal-treated bed bugs. Mycosis was confirmed in all treated bugs that died, by maintaining them at 100 % RH for an additional week and viewing them under a stereomicroscope (see Lacey and Solter 2012). Temperature and RH were verified by traceable relative humidity-temperature meters (Fisher, Pittsburgh, PA, USA).

Statistical analysis

For treatment comparisons for the mortality and germination data, R statistical software (R Core Team 2014) was used to fit a generalized linear model (Bates et al. 2014), where we assumed the sampling distribution was an over-dispersed binomial (quasi-binomial). The over-dispersion scale factor was estimated at 1.48 for the mortality data and 1.93 for the germination data. Germination tube length was approximately Gaussian following log transformation and a linear model was fit, which included a time × treatment, and time by treatment interaction effect. Mean separations were done using the "multcomp" package (Hothorn et al. 2008) using "single-step". Adjusted p values were based on the joint normal or t distribution of the linear function.

Results

In vitro fungal growth when exposed to (*E*)-2-hexenal and (*E*)-2-octenal

The two aldehydes inhibited in vitro growth of *M. anisopliae s.l.* conidia by both direct contact (when a treated disk was placed on the growth media) and by fumigation (treated disk placed on the inner cover, not directly contacting the conidia) (Table 1). At all exposures, there was either total inhibition of fungal growth (no evidence of hyphal growth) or unrestricted fungal growth (no apparent inhibition or zone of inhibition), when the plates were examined 72 h post-exposure (Fig. 1a, b). (*E*)-2-hexenal inhibited all fungal growth either by contact or fumigation at amounts of 1.0 mg and above, while (*E*)-2-octenal appeared to be somewhat more active, inhibiting all fungal growth at 0.5 mg and above. Control dishes treated directly or indirectly with acetone-treated disks showed no inhibition of fungal growth.

Using the lowest amount of (*E*)-2-octenal that exhibited total inhibition (0.5 mg per disk) of *M. anisopliae s.l.* by fumigation, we found that all exposure times greater than 0.25 h resulted in total inhibition of fungal growth (Table 2). However, when conidia were allowed to propagate prior to the addition of octenal disks (0.5 mg per disk), fungal growth was only observed when the disks were added at 12 h or later. Interestingly, addition of (*E*)-2-octenal to inoculated plates 12 h post-inoculation was the only instance where we observed zones of inhibition (Fig. 1c). As stated above, in all other experiments, regardless of (*E*)-2-octenal amounts or exposure times, only total inhibition or unrestricted growth was observed.

Effects of (*E*)-2-octenal on *M. anisopliae s.l.* germination

We observed that exposure of *M. anisopliae s.l.* conidia to octenal affected germination (Fig. 2). Germination was significantly delayed (GLM based on quasi-binomial distribution: $\chi^2 = 2551.4$; df = 6; $P < 0.001$) in our trials (Table 3). Conidia exposed to octenal 6 h after initial inoculation exhibited no germination after 12 h compared to controls that had 80 % germination. When exposed to octenal at 12 h post-inoculation, conidia had significantly less

Table 1 Inhibition of *M. anisopliae s.l.* (ARSEF 1548) by (E)-2-hexenal and (*E*)-2-octenal

Aldehyde	Amount[a] (mg)								
	20	10	5.0	1.0	0.5	0.33	0.25	0.1	Control
(*E*)-2-hexenal									
Contact	−	−	−	−	+	+	+	+	+
Fumigation	−	−	−	−	+	+	+	+	+
(*E*)-2-octenal									
Contact	−	−	−	−	−	+	+	+	+
Fumigation	−	−	−	−	−	+	+	+	+

Plates inoculated with 25 µl of a fungal suspension containing 10^7 conidia ml^{-1}

− inhibition, + growth

[a] 6 mm disk treated with either (E)-2-hexenal or (*E*)-2-octenal in acetone

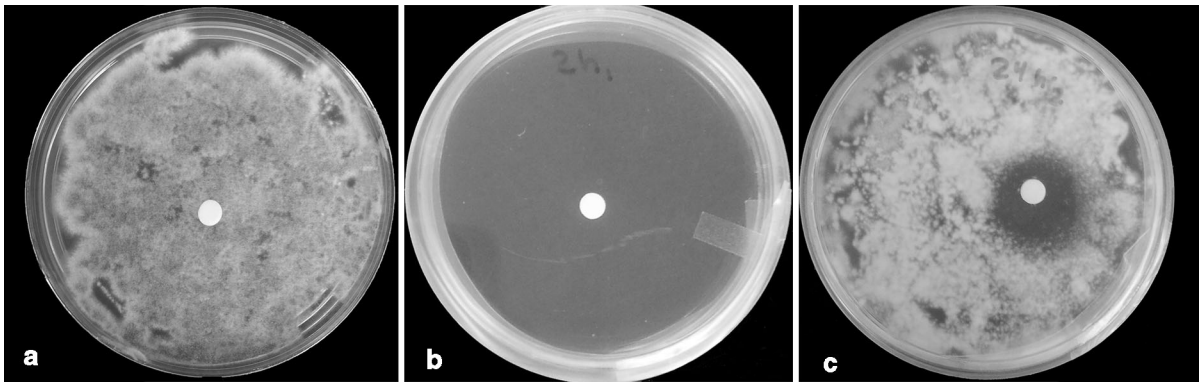

Fig. 1 Representative fungal growth on agar plates exposed to either (*E*)-2-hexenal or (*E*)-2-octenal. **a** Unrestricted growth; **b** total inhibition of growth. Partial inhibition of growth **c** only occurred when an octenal-treated (0.5 mg) disk was added to a plate inoculated with *M. anisopliae s.l.* (ARSEF 1548) conidia 12 h previously

Table 2 Suppression of *M. anisopliae s.l.* (ARSEF 1548) exposed to (*E*)-2-octenal at various times

	Disk removed at[a]	Disk added at[b]
Time (h)		
0.0	+	nd
0.25	+	nd
0.5	−	−
1	−	−
2	−	−
3	−	−
4	nd	−
6	nd	−
12	nd	±[c]
24	−	+
48	nd	+

Agar plates inoculated with 25 µl of a *M. anisopliae s.l.* suspension containing 10^7 conidia ml^{-1}

+ growth, − inhibition, nd: not determined

[a] 6 mm disks treated with 0.5 mg (*E*)-2-octenal were placed on the inside lids of agar plates at the time of inoculation (0.0 h), and removed at various times post-exposure by replacing the lid containing the octenal-treated disk with a lid containing no disk

[b] 6 mm Disks treated with 0.5 mg (*E*)-2-octenal were placed on the inside lids of agar plates at various times post-inoculation

[c] The only time a zone of inhibition was observed (see Fig. 1)

germination at 24 h than controls (85.2 vs. 98.0 %, respectively). Additionally, aldehyde-exposed conidia had shorter germ tube lengths compared to controls at 24 and 48 h (GLM based on Gaussian distribution: $F = 3534.6$; $df = 4, 495$; $P < 0.001$) (Table 4).

Exposure of bed bugs to *M. anisopliae s.l.* in the presence of octenal

There was a significant relationship between mortality of bed bugs exposed by contact to *M. anisopliae s.l.* and the presence (*E*)-2-octenal (GLM based on quasi-binomial distribution: $\chi^2 = 407.0$; $df = 5$; $P < 0.001$). Bed bugs contacting fungal-treated surfaces in the absence of octenal experienced high mortalities, depending on whether they were removed from the treated surfaces at 24 h (79 %) or remained on the treated surface for one week (99 %) (Table 5). However, we observed lower mortalities in bed bugs exposed to *M. anisopliae s.l.* in the presence of (*E*)-2-octenal. Mortality was 10 % when a (*E*)-2-octenal-treated disk was added to fungal-exposed bed bugs at 1 h, while mortality rose to about 33 % when the octenal-treated disk was added at 24 h. Little or no mortality was observed in bed bugs not exposed to fungal-treated filter paper, even when (*E*)-2-octenal was added to the Petri dish.

Discussion

In addition to the barriers posed by the cuticle and insect immune system (Vilcinskas and Götz 1999; Wang and St Leger 2005), other biotic factors can limit the effectiveness of pathogens. For instance, termites show

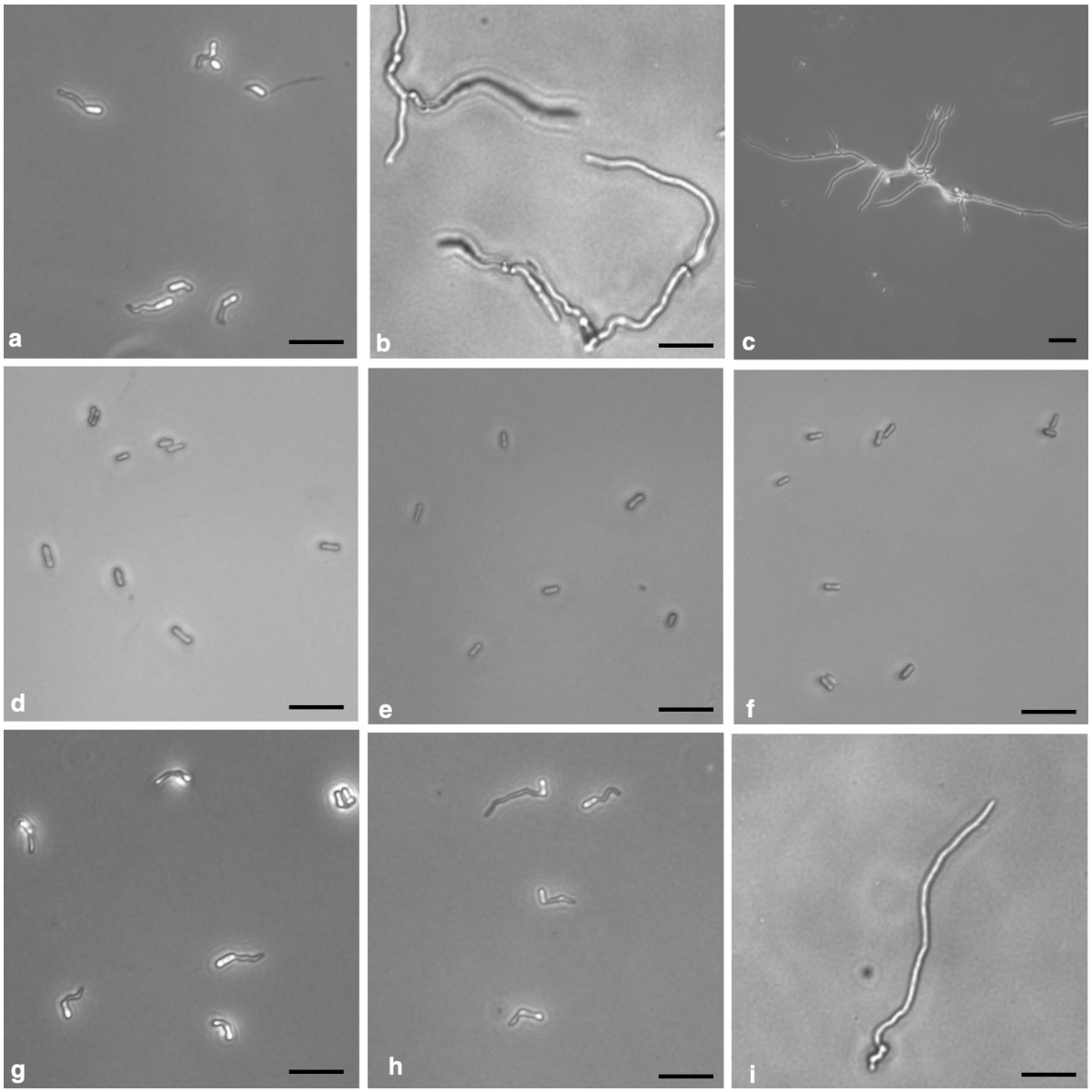

Fig. 2 Germination of *M. anisopliae s.l.* (ARSEF 1548) conidia in the presence of (*E*)-2-octenal. **a–c** Germination of controls (no aldehyde) at 12, 24 and 48 h respectively; **d–f** germination of conidia exposed to aldehyde at 6 h post-inoculation at 12, 24 and 48 h after initial inoculation respectively; **g–i** germination of conidia exposed to aldehyde at 12 h post-inoculation after 12, 24 and 48 h after initial inoculation respectively. *Bar* 25 µm

an array of behavioral and biochemical defenses to thwart *M. anisopliae* infection (Chouvenc and Su 2010; Myles 2002). Likewise, insect defensive secretions previously have been shown to inhibit both bacterial and fungal growth in vitro. Salicylaldehyde, released by larvae of the brassy willow leaf beetle, is toxic to the entomopathogenic bacteria *Bacillus thuringiensis* (Gross et al. 2008), and (*E*)-2-decenal, a primary aldehyde component of the stink bug *Nezara viridula* scent gland, is fungistatic toward *M. anisopliae* (Sosa-Gomez et al. 1997). Our results indicate that these two, structurally-related aldehydes (*E*)-2-hexenal and (*E*)-2-octenal, considered the primary defensive secretions of bed bugs, inhibit the in vitro growth of *M. anisopliae s.l.* (ARSEF 1548), and may play a part in disinfecting a bed bug's microenvironment consistent with the action of a

Table 3 Percent mean germination of *M. anisopliae s.l.* (ARSEF 1548) observed after 6, 12, and 24 h of incubation after exposure to (*E*)-2-octenal at 6 or 12 h after initial inoculation

	Total hours incubated		
	6 h	12 h	24 h
Exposure to aldehyde added at time (h)[1]			
Control	1.0 (0.2, 3.0)[a]	80.0 (74.2, 85.1)[b]	98.0 (95.5, 99.4)[c]
6 h	0	0	1.0 (0.2, 3.0)[a]
12 h	0.8 (0.1, 2.6)[a]	80.8 (75.0, 85.7)[b]	85.3 (80.0, 89.6)[b]

Each mean percentage represents the average of four replicates, with 100 conidia evaluated per replicate. Conidia with germ tubes larger than the diameter of the conidium were considered to have germinated. 95 % confidence intervals are shown in parentheses

PDA plates inoculated with 25 µl of a *M. anisopliae s.l.* suspension containing 10^6 conidia ml $^{-1}$

Different superscripts (for both rows and columns) indicate means that differ significantly at $P \leq 0.05$

[1] 6 mm Disks treated with 0.5 mg (*E*)-2-octenal were placed on the inside lids of agar plates at various times post-inoculation

Table 4 Mean germ tube length of *M. anisopliae s.l.* (ARSEF 1548) observed after 12, 24, and 48 h of incubation after exposure to (*E*)-2-octenal at 6 or 12 h after initial inoculation

	Total hours incubated		
	12 h	24 h	48 h
Exposure to aldehyde added at time (h)[1]			
Control	2.2 (2.1, 2.3)[a]	4.2 (4.1, 4.3)[c]	nd
6 h	0	0	0
12 h	2.0 (1.9, 2.1)[a]	2.0 (1.9, 2.1)[a]	3.6 (3.5, 3.7)[b]

100 germinated conidia evaluated per treatment. Data were subjected to log transformation before statistically analyzed. Values in table are expressed in log µm for germ tube lengths. 95 % confidence intervals are shown in parentheses. PDA plates inoculated with 25 µl of a *M. anisopliae s.l.* suspension containing 10^6 conidia ml $^{-1}$

nd: not determined

Different superscripts (for both rows and columns) indicate means that differ significantly at $p \leq 0.05$

[1] 6 mm disks treated with 0.5 mg (*E*)-2-octenal were placed on the inside lids of agar plates at various times (in hours) post-inoculation

defensive secretion. Interestingly, Sosa-Gomez et al. (1997) demonstrated that (*E*)-2-decenal was fungistatic against *M. anisopliae*, but not against *B. bassiana*. This finding may account for the differential susceptibility of bed bugs when comparing our results with those of Barbarin et al. (2012).

Our microscopy results indicate that germinated conidia require a period of time to recover from aldehyde exposure before the germination process can resume. Aldehydes are common disinfectants, able to alkylate fungal proteins and DNA (Fernandes et al. 2012). Mutations caused by alkylating agents can induce DNA repair responses delaying germination, similar to what was reported regarding delayed germination after exposure to UV radiation (Braga et al. 2001). Compared to dormant conidia, metabolically active, germinating conidia may be better able to repair cellular damage more rapidly. This would account for why conidia were able to grow when exposed to octenal 12 h after inoculation but not at 6 h. Our observations on in vivo conidial growth of *M. anisopliae s.l.* indicate that the 12–24 h time frame is critical with regard to exposure to (*E*)-2-octenal.

While exposure of bed bugs to an isolate of *M. anisopliae s.l.* for 24 h is sufficient to cause 99 % mortality under our experimental conditions, we observed significantly lower mortality when octenal-treated disks were added to fungal-exposed bed bugs at 1 h post-exposure. In addition, while mortality in bed bugs increased when the octenal-treated disk was added at 24 h, the level was still significantly lower than in fungal-treated control bed bugs, receiving no aldehyde exposure. In research with two species of termites, and four species of beetles, *M. anisopliae* enters the conidial germination phase of host colonization at approximately 12–24 h post-inoculation (Hänel 1982; McCauley and Zacharuk 1968; Moino et al. 2002). Zimmermann (2007) also states that germination of *M. anisopliae* conidia generally takes place about 20 h after contacting the cuticle of a susceptible insect. Whether these observations with other insect

Table 5 Mortality (%) of bed bugs exposed to *M. anisopliae s.l.* (ARSEF 1548) by contact in the presence of octenal

	Treatment		
	Fungus (continuous)	Fungus (24 h only)	No fungus
Octenal disk			
Added at 1 h	10.0 (4.6, 20.4)[a]	nd	2.2 (0.4, 11.1)[a]
Added at 24 h	33.3 (22.7, 46.0)[b]	nd	nd
Not added	98.9 (89.0, 99.9)[c]	78.9 (66.9, 87.4)[c]	1.1 (0.1, 11.0)[a]

Bed bugs (n = 15 bugs per group; six replicates pooled from two experiments) were placed on filter paper treated with *M. anisopliae s.l.* conidia (10^5 conidia cm^{-2}) for either 168 h (one week; 'continuous') or 24 h ('24 h only'). Disks containing octenal (0.5 mg octenal per disk) were added at the prescribed times. Mortality assessed at one week post-exposure. All bed bugs kept at 25 ± 2 °C and 98 ± 1 % RH

nd: not determined

Different superscripts (for both rows and columns) indicate means that differ significantly at $P \leq 0.05$. 95 % confidence intervals are shown in parentheses

species also apply to bed bugs is speculative. We likewise recognize that attempts to correlate germination timing of our in vitro results with in vivo applications to bed bugs can be problematic, as insect host cuticle can present a very different substrate, as pointed out in aphid studies with entomopathogens (Yeo et al. 2003). Regardless, our study demonstrates that exposure of conidia from an isolate of *M. anisopliae s.l.* (ARSEF 1548) to bed bug defensive secretions inhibits fungal development both in vitro and in vivo, and these chemicals may play a role in an insects' defense to fungal biopesticides.

Acknowledgments We thank Barbara Thorne (University of Maryland, College Park, USA) for guidance and reviewing the manuscript. We also thank members of the Armed Forces Pest Management Board (Silver Spring MD, USA), and personnel at the Walter Reed National Military Medical Center (Bethesda MD, USA) for their help in obtaining the blood products used to maintain insect colonies. This project was funded in part by the University of Maryland's Department of Entomology's Gahan Fellowship. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employee.

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