Fungal volatile organic compounds show promise as potent molluscicides

Salim Khoja,a Khalifa M Eltayef,a Ian Baxter,c James C Bull,a Edric Joel Loveridgeb and Tariq Buttana

Abstract

BACKGROUND: Slugs and snails constitute major crop pests. Withdrawal of metaldehyde has prompted a search for more environmentally friendly yet fast acting molluscicides. This study investigated the response of representative molluscs to conidia and volatile organic compounds (VOCs) of the insect pathogenic fungus Metarhizium brunneum Petch.

RESULTS: Conidia of M. brunneum had antifeedant/repellent properties with repellency being dependent upon the fungal strain and conidia concentration. Three commonly produced fungal VOCs, 1-octene, 3-octanone and 1-octen-3-ol, were repellent at low doses (1–5 μL) but could kill slugs and snails on contact or fumigation. At the highest dose tested (10 μL), 100% mortality was achieved for Cornu aspersum Muller (garden snail) and Derocerus reticulatum Muller (grey field slug) within 1 h post-treatment with the first deaths being recorded in <11 min. Aqueous formulations (20% v/v) of the most potent VOCs, 3-octanone and 1-octen-3-ol, could be sprayed onto plants to kill or drive the pest of the crop with no phytotoxic effects.

CONCLUSION: The sensitivity of terrestrial molluscs to 3-octanone and 1-octen-3-ol and the ephemeral nature of these compounds make these excellent candidates for development as mollusc repellers or molluscicides.

© 2019 The Authors. Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

Keywords: slugs; snails; fungal volatiles; Metarhizium; repellents; molluscicides

1 INTRODUCTION

Snails and slugs are serious pests of agricultural and horticultural crops. They cause feeding damage to both aerial and subterranean parts of the plant, including leaves, shoots, roots, tubers, corms, bulbs, flowers and seed. Besides reducing plant stand and crop yield they increase the risk of infection by opportunistic plant pathogens.1 Feeding damage can significantly reduce the aesthetic appearance of plants (e.g. nursery stock, root crops, salads) and hence their marketability. Slug and snail population densities have increased over the years due to changes in crop management such as minimum tillage, direct drilling, and over-wintering arable crops.2,3 Their pest status is set to increase further due to legislative changes to pestide usage and climate change with weather patterns favoring their population growth.3,4 The problem is often exacerbated by the accidental introduction of highly damaging invasive molluscs species such as the golden apple snails (Pomacea canaliculate Lamarc, Pomacea maculata Perry), which have devastated rice crops in Asia and pose a serious threat to food security in the region.5–7

Current mollusc control is still heavily dependent upon the use of chemical pesticides such as metaldehyde, ferric phosphate, methiocarb and thiodicarb.8 Metaldehyde-based products by far dominate the molluscicide bait market worldwide but have corresponding ecotoxicological effects on non-target species and lead to contamination of drinking water.5,6 However, metaldehyde will be withdrawn in the UK by 2020 in order to protect wildlife and to comply with the EC drinking water Directive 98/83/EC.9 The nematode Phasmarhabditis hermaphrodita Schneider has been developed as a biological molluscicide but is considered expensive and ineffective against mature molluscs.10,11 Live nematodes have been shown to elicit avoidance behavior in some slug species.12 Various plant-derived products (e.g. caffeine, neem, terpenoids, isothiocyanates) show promise as molluscicides and mollusc repellents.13–17 Whereas some compounds act as feeding deterrents others exhibit fumigant or contact toxicity.13,17,18 Although many plant species have been screened to identify potential molluscicides very little attention has been given to fungi as a source of such compounds. Of the limited number of studies, it is clear that slugs avoid or taste and reject the mushroom sporocarp. Wood et al.,19 investigated the feeding behavior of the banana slug, Arion limax columbianus Gould, on the sweetbread mushroom, Clitopilus prunulus Scop, and discovered that 1-octen-3-ol released by this mushroom acted as an antifeedant.

Many fungal species produce low molecular weight volatile organic compounds (VOCs) with insect behavior modifying properties.20 In the case of fungi ecolocalization is a major function of VOCs and fungivores have been shown to respond to the VOCs of fungi feeding on their food supply.21,22 In the case of Metarhizium the fungus uses VOCs to arrest feeding by herbivorous insects thereby promoting fungal infection.23,24 These VOCs are synthesized primarily by the hyphae of the fungus and released into the surrounding environment for an ephemeral period. Thus the attractive or repulsive nature of the VOCs may be used to manipulate pest populations.25,26

© 2019 The Authors. Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.
2 METHODS

2.1 Collection and maintenance of molluscs

The following slugs and snails were collected from local gardens and parks in Swansea: garden snail (Cornu aspersum), great grey or leopard slug (Limax maximus Linnaeus), black slug (Arion ater Linnaeus), large red slug (Arion rufus Linnaeus), grey field slug (Deroceras reticulatum) and Yellow slug (Limax flavus Linnaeus). The animals were collected at dawn or early evening and maintained in ventilated plastic containers at room temperature (21 ± 2°C) until required. They were fed diverse plant materials (turf, flowers, lettuce leaves) and grouped according to body weight since it was required. They were fed diverse plant materials (turf, flowers, lettuce leaves) and grouped according to body weight since it was required. Studies were conducted on immature D. reticulatum and C. aspersum, which weighed 0.5–1.0 g and 1–3 g, respectively.

2.2 Maintenance of M. brunneum cultures and preparation of conidia

The origin and growth of M. brunneum strains ARSEF4556 and V275 on Sabouraud Dextrose Ager (SDA) and broken rice grain is described by Ansari and Butt.10 Conidia were harvested from sporulating SDA cultures by gently scraping the surface with a sterile spatula. Conidia were harvested from the surface of rice grain using a MycoHarvester.31 Harvested conidia were air dried sterile spatula. Conidia were harvested from the surface of rice grain using a MycoHarvester.31 Harvested conidia were air dried overnight and weighed before use. Conidia had more than 95% viability as determined by the plate count technique.32

2.3 Snail avoidance of M. brunneum conidia

Choice and no-choice assays were conducted to see if molluscs avoided M. brunneum. In no choice assays, the garden snail was provided lettuce leaves (4 g wet weight) pre-coated with different doses (0.4, 0.2, 0.04 g) of dry conidia of M. brunneum strains V275 and ARSEF4556. Controls consisted of leaves not treated with conidia. Two snails, starved for 24 h, were placed in each test arena 24 h after inoculating the leaves with the fungal inoculum. The assays were performed in a 300 mL cylindrical plastic container at room temperature with leaf consumption based on residual weight being measured 24 and 48 h post-treatment. There were five replicates per treatment and the whole study repeated twice. Choice assays were as above with the following modifications, the test arena consisted of a 2.5 L ventilated plastic box (16 cm x 10 cm x 16 cm) and lettuce leaves in Petri dishes were placed next to each other with one treated with different doses of M. brunneum conidia and the other left untreated (control). Leaf consumption based on residual weight was measured 24 and 48 h post-treatment. There were three replicates per treatment and the whole study repeated twice.

2.4 Contact toxicity studies

Individual D. reticulatum and C. aspersum were placed in 9 cm diameter Petri dishes lined with moist Whatman filter paper number 1. The molluscs were exposed to 1, 5 and 10 μL of 1-octene, 1-octen-3-ol and 3-octanone, which were applied to the fleshy dorsal surface of the molluscs using an Accumax PRO Micropipette. Control molluscs were left untreated. The Petri dishes were sealed with Parafilm to prevent the escape of animals and VOCs. Mortality was recorded 0.5, 1, 3, and 24 h post-treatment. Slugs and snails were considered dead if they did not respond to probing with a needle.10 There were five replicates per treatment with the whole experiment being repeated twice. Assays were conducted at room temperature (21 ± 2°C).

To determine the mean time to death the above experiments were repeated but mortality was recorded continuously.

2.5 Fumigation assay

Individual D. reticulatum and C. aspersum were exposed to different doses (1, 5 and 10 μL) of 1-octene, 1-octen-3-ol and 3-octanone dispensed from a 5 mm diameter filter paper placed on a glass coverslip attached to the lid of a 9 cm diameter Petri dish. Control animals were left untreated. The Petri dishes were sealed with Parafilm to prevent the escape of animals and VOCs. Mortality was recorded 0.5, 1, 3 and 24 h post-treatment. Slugs and snails were considered dead if they did not respond to probing with a needle.10 There were five replicates per treatment with the whole experiment being repeated twice. Assays were conducted at laboratory temperature (21 ± 2°C).

2.6 Repellence assay

Repellence of 1-octene, 1-octen-3-ol and 3-octanone was investigated using ventilated plastic containers (30 x 13 x 13 cm). Each container was partitioned in the middle by a piece of cardboard with a hole at the base of the partition allowed free movement of the molluscs. Each chamber contained a Petri dish lined with filter paper with one being treated with different amounts of the active (5, 10, 25, and 50 μL) and the other treated with water only (control). Four animals (D. reticulatum or C. aspersum) were released from a Petri dish placed in the middle of the arena and their location recorded at 10 min, 0.5, 1, 3, and 24 h post-treatment.

Figure 1. Test arena to evaluate mollusc repellent properties of Metarhizium brunneum volatile organic compounds (VOCs). The ventilated plastic container (30 x 13 x 13 cm) was split in two equal halves by a cardboard partition. A hole at the base of the partition allowed free movement of the molluscs. Each chamber contained a Petri dish lined with filter paper with one being treated with different amounts of the active (5, 10, 25, and 50 μL) and the other treated with water only (control). Four animals (D. reticulatum or C. aspersum) were released from a Petri dish placed in the middle of the arena and their location recorded at 10 min, 0.5, 1, 3, and 24 h post-treatment.
Figure 2. Avoidance behavior of the garden snail to lettuce leaves inoculated with different amounts of conidia of *Metarhizium brunneum* strains V275 and ARESF4556. The amount of food consumed was lower for treated than untreated (control) leaves. Antifeedant activity increased with dose, with conidia of V275 being marginally more repellent. Antifeedant properties of the conidia was generally more pronounced 24 than 48 h post-treatment. (a = No choice and b = Choice studies).

chamber recorded at 10, 30, 60, and 180 min and then every 24 h post-treatment.

The above study was repeated using *C. aspersum* only. In these assays, 4 g of fresh oilseed rape leaves were placed in each half of the test arena. Four snails starved for 24 h were released in the middle and feeding damage assessed. Leaf damage, based on wet residual weight of the leaves, was determined 24 and 48 h post-treatment. Both assays were conducted under the same laboratory conditions (21 ± 2 °C).

2.7 Antifeedant assay
To determine if leaves exposed to VOCs were avoided by molluscs, 4 g of fresh oilseed rape leaves were fumigated inside a cylindrical plastic chamber (approximately 6 cm height, 9 cm diameter) lined with a Whatman filter paper. The leaves were exposed to different doses (1, 5, and 10 μL) of 1-octen-3-ol, 3-octanone or water (control) dispensed from Sharrow King size (7.1 mm diameter) filter tips (Wilson's and Co Ltd, UK). An additional control of no treatment was also included. Exposure to higher doses (>20 μL) caused the leaves to become dehydrated and necrotic. The chamber lid was removed 24 h later, allowing any gases to escape before introducing two healthy snails (starved for 24 h). At this stage VOCs had dispersed and the filter was exhausted. There were five replicates per treatment with the whole study being repeated twice. Snail mortality and leaf damage (based on wet residual weight) were recorded 24 h and 48 h post-treatment. The study was repeated with the lettuce being replaced with 4 g fresh oilseed rape leaves. Studies were conducted at 21 ± 2 °C.

2.8 Evaluation of aqueous formulations of VOCs
Since highly diluted, aqueous formulations of the VOCs may be required to protect crops on a large scale, it was important to establish if these could kill the pest on contact or drive them off the crop. In the first of two studies, individual *D. reticulatum* and *C. aspersum* were exposed to different volumes (10, 20, 50, 100 μL) of 3-octanone and 1-octen-3-ol diluted in water to 5%, 10%, and 20% (v/v). Water was used as a control. The chemicals were applied using an Accumax PRO Micropipette to the fleshy dorsal surface of the animals. Individuals were placed in a 9 cm diameter Petri dish and sealed with Parafilm. Assays were conducted at room temperature (21 ± 2 °C) and the percentage mortality recorded 0.5, 1, 3, and 24 h post-treatment. There were five replicates per treatment.
Figure 3. Behavior of slugs and snails when exposed to the volatile organic compounds (VOCs) 1-octene, 3-octanone and 1-octen-3-ol. Slugs occasionally vomited a brownish fluid (top left) or produced a brownish froth which readily stained the underlying moist filter paper (top middle). Dead or dying animals were immobile and appeared shriveled (top left and middle) unlike the healthy slug (top right). Snails exposed to VOCs produced either pale yellow (bottom left) or clear frothy secretions (bottom middle) unlike the healthy control snails (bottom right).

| VOCs         | Doses (μL) | D. reticulatum | C. aspersum |
|--------------|------------|----------------|-------------|
|              | Time to death (min) ± SE | Mortality (%) ± SE | Time to death (min) ± SE | Mortality (%) ± SE |
| 1-octene     | 1          | All Alive      | 0           | All Alive      | 0           |
|              | 5          | 516.5 ± 302.6  | 40 ± 24.5   | 247.6 ± 121.3 | 100         |
|              | 10         | 252.4 ± 76.2   | 100         | 42.2 ± 9.2    | 100         |
| 1-octen-3ol  | 1          | 79.2 ± 3       | 100         | 37 ± 9.6      | 100         |
|              | 5          | 42 ± 10.1      | 100         | 27.4 ± 6.2    | 100         |
|              | 10         | 29.6 ± 0       | 100         | 29.6 ± 0      | 100         |
| 3-octanone   | 1          | 43 ± 9.7       | 20 ± 20     | 55.5 ± 10.4   | 40 ± 25.8   |
|              | 5          | 37 ± 4.7       | 100         | 36 ± 17.4     | 100         |
|              | 10         | 31.2 ± 8.9     | 100         | 10.2 ± 0.7    | 100         |
| Control      | 0          | All Alive      | 0           | All Alive      | 0           |

In the second study aqueous formulations were applied to plants infested with the molluscs using a 0.5 L hand-held sprayer (Screwfix, Loughborough, UK). Briefly, 30 mL of 5% and 20% (v/v) aqueous 1 octen-3-ol and 3-octanone were sprayed onto 25 (30-day old) corn plants (Zea mais variety Madlen, Maisadour Semences, Marsan, France) grown in plastic trays (35 × 20 × 15 cm). Each tray was placed in a Bugdorm. Twenty D. reticulatum or C. aspersum were released onto the tray a few minutes before spraying. Mortality was recorded 3 and 24 h post-treatment.

2.9 Statistical methods
For M. brunneum conidia avoidance studies, the mean and standard error were calculated. Survival was modelled using Generalized Linear Models (GLMs) with logit link functions and binomial error distributions. Since mortality after different time points was observed using different individuals, there was no issue of temporal correlation or censoring typically associated with longitudinal bioassays. Repellence was also modelled as binomial data (repelled vs. not repelled). Percentage weight changes were arcsine-transformed and modelled using Normal linear models. The effects of dose, time post-treatment, and concentration where included, were modelled as categorical fixed effects and included as main effects and all interactions. In the case of binomial GLMs, over-dispersion was assessed (and found to be ≪2 in all cases) by comparing residual deviance to residual degrees of freedom. Statistical significance of explanatory variables was tested using likelihood ratio tests assuming a $\chi^2$ null distribution for binomial
Figure 4. Volatile organic compound (VOC) contact effect on survival of the slug *D. reticulatum*. Slug mortality (means with SE error bars) over 24 h, under varying concentrations of 1-octene, 1-octen-3-ol, and 3-octanone.

Figure 5. Volatile organic compound (VOC) contact effect on survival of the snail, *C. aspersum*. Snail mortality (means with SE error bars) over 24 h, under varying concentrations of 1-octene, 1-octen-3-ol, and 3-octanone.

GLMs, and F-ratios for Normal models. All statistical analysis was performed using R v3.5.0.33

3 RESULTS

3.1 Antifeedant properties of *M. bruneum* conidia

In no choice assays, *Cornu aspersum* consumption of lettuce leaves treated with conidia of *M. bruneum* was dependent upon the fungal dose and strain (Fig. 2(a)). It was more pronounced for strain V275 especially at the highest dose. The percentage lettuce leaf consumed was lower 24 h than 48 h post-treatment for both strains (Fig. 2). In choice assays, the snails tended to move towards the treated leaves before moving to the untreated leaves. This was particularly obvious at the low doses of both strains and in the first hour of the experiment. The snails spent only a few minutes at the treated leaf with insignificant feeding before moving towards the untreated leaf. The snails consumed most of the untreated leaf material before returning to the treated leaf or wandered in the test arena. Leaf consumption of treated leaves was linked with dose, strain and time (Fig. 2(b)). Far less was consumed 24 h than 48 h post-treatment and consumption was generally lower for leaves treated with conidia of V275 (Fig. 2(b)).

3.2 Contact toxicity assays

Our attention focused on the ubiquitous fungal VOCs, 1-octene, 1-octen-3-ol and 3-octanone. These compounds were toxic on contact, causing 100% mortality of adult and juvenile slugs and snails including *Cornu aspersum*, *Limax maximus*, *Arion ater*, *Arion rufus*, *Deroceras reticulatum* and *Limax flavus* (data not presented). In-depth studies conducted on *D. reticulatum* and *C. aspersum* showed that they exhibited various behaviors depending on the chemical, dose and method of exposure. Slugs exposed to the
Fungal volatiles kill slugs and snails

VOCs vomited a brown liquid or exhibited contorted body movements, producing yellow or brown staining mucus before shrinking and dying (Fig. 3). Snails exposed to the VOCs withdrew into the shell or produced a yellow watery mucus before dying often with their head exposed. Treating snails with diluted aqueous formulations triggered production of clear, frothy mucus (Fig. 3).

Mortality of *D. reticulatum* and *C. aspersum* was dependent upon the VOC, dose and time post-treatment (Table 1, Figs. 4 and 5). The statistical interaction between dose and time post-treatment was not significant for either species (slugs: $P > 0.05$ for all VOCs; snails: $P > 0.95$ for all VOCs), with any of the three VOCs. Overall, mortality generally increased with increasing dose (slugs: 1-octene, $\chi^2 = 22.6$, $P < 0.001$, 1-octen-3-ol, $\chi^2 = 9.38$, $P = 0.009$, 3-octanone, $\chi^2 = 33.1$, $P < 0.001$; snails: 1-octene, $\chi^2 = 20.6$, $P < 0.001$, 1-octen-3-ol, $\chi^2 = 0.797$, $P = 0.67$, 3-octanone, $\chi^2 = 36.0$, $P < 0.001$) and time (slugs: 1-octene, $\chi^2 < 0.01$, $P > 0.99$, 1-octen-3-ol, $\chi^2 = 10.2$, $P = 0.017$, 3-octanone, $\chi^2 < 0.01$, $P > 0.99$; snails: 1-octene, $\chi^2 = 20.6$, $P < 0.001$, 1-octen-3-ol, $\chi^2 = 18.5$, $P < 0.001$, 3-octanone, $\chi^2 = 5.39$, $P = 0.15$), but mortality was higher with 1-octen-3-ol or 3-octanone than with 1-octene.

### 3.3 Fumigation assays

Fumigation of slugs and snails also proved lethal (Figs. 6 and 7) but marginally less potent than direct contact. As with direct contact, the statistical interaction between dose and time post-treatment was not significant for either species (slugs: $P > 0.16$ for all VOCs; snails: $P < 0.68$ for all VOCs), with any of the three VOCs. Similarly, there were typically increases in mortality with both dose (slugs: 1-octene, $\chi^2 = 14.1$, $P < 0.001$, 1-octen-3-ol, $\chi^2 = 8.95$, $P = 0.010$, 3-octanone, $\chi^2 = 36.0$, $P < 0.001$; snails: 1-octene, $\chi^2 = 2.64$, $P = 0.039$, 1-octen-3-ol, $\chi^2 = 1.05$, $P = 0.30$).

**Figure 6.** Volatile organic compound (VOC) fumigation effect on survival of the slug *D. reticulatum*. Slug mortality (means with SE error bars) over 24 h, under varying concentrations of 1-octene, 1-octen-3-ol, and 3-octanone.

**Figure 7.** Volatile organic compound (VOC) fumigation effect on survival of the snail, *C. aspersum*. Snail mortality (means with SE error bars) over 24 h, under varying concentrations of 1-octene, 1-octen-3-ol, and 3-octanone.
Figure 8. Slug repellence (means with SE error bars) over 24 h, under varying concentrations of 1-octene, 1-octen-3-ol, and 3-octanone.

3-\(\chi^2\) = 5.71, \(P = 0.058\), 3-octanone, \(\chi^2 = 17.9\), \(P < 0.001\) and time (slugs: 1-octene, \(\chi^2 = 3.19\), \(P > 0.36\), 1-octen-3-ol, \(\chi^2 = 12.2\), \(P = 0.007\), 3-octanone, \(\chi^2 = 14.6\), \(P = 0.002\); snails: 1-octene, \(\chi^2 = 8.30\), \(P = 0.040\), 1-octen-3-ol, \(\chi^2 = 45.6\), \(P = 0.001\), 3-octanone, \(\chi^2 = 19.4\), \(P < 0.001\), and mortality was higher with 1-octen-3-ol or 3-octanone than with 1-octene.

3.4 Repellence assays

Slugs were repelled by 1-octene but the statistical interaction between dose and time post-treatment was not significant (\(\chi^2_{df = 12} = 2.99\), \(P > 0.99\)) (Fig. 8). However, repellence increased with increasing time (\(\chi^2_{df = 4} = 22.4\), \(P < 0.001\)) but not dose (\(\chi^2_{df = 3} = 3.50\), \(P = 0.32\)) (Fig. 8). Where 1-octen-3-ol was applied, dose was not found to be statistically significant, either in its interaction with time (\(\chi^2_{df = 12} = 19.02\), \(P = 0.09\)) or as a main effect (\(\chi^2_{df = 3} = 7.64\), \(P = 0.054\)) but mortality significantly increased with time post-treatment (\(\chi^2_{df = 4} = 29.8\), \(P < 0.001\)) (Fig. 8). Where 3-octanone was applied, the statistical interaction between dose and time post-treatment was not significant (\(\chi^2_{df = 12} = 11.9\), \(P = 0.45\)) but repellence increased with both time (\(\chi^2_{df = 4} = 42.6\), \(P < 0.001\)) and dose (\(\chi^2_{df = 3} = 26.3\), \(P < 0.001\)) (Fig. 8).

Snails were repelled by 1-octene but the statistical interaction between dose and time post-treatment was not significant (\(\chi^2_{df = 12} = 1.22\), \(P > 0.99\)) (Fig. 9). Furthermore, neither time (\(\chi^2_{df = 4} = 0.52\), \(P = 0.97\)) nor dose (\(\chi^2_{df = 3} = 2.24\), \(P = 0.52\)) increased repellence as a main effect. This finding was repeated with 1-octen-3-ol: the statistical interaction between dose and time post-treatment was not significant (\(\chi^2_{df = 12} = 11.9\), \(P = 0.45\)) but repellence increased with both time (\(\chi^2_{df = 4} = 42.6\), \(P < 0.001\)) and dose (\(\chi^2_{df = 3} = 26.3\), \(P < 0.001\)) (Fig. 8).

Where 3-octanone was applied, dose was not found to be statistically significant, either in its interaction with time (\(\chi^2_{df = 12} = 9.80\), \(P = 0.63\)) or as a main effect (\(\chi^2_{df = 3} = 1.78\), \(P = 0.62\)). However, repellence did increase with increasing time after treatment (\(\chi^2_{df = 4} = 11.7\), \(P = 0.02\)) (Fig. 9).
Fungal volatiles kill slugs and snails

3.5 Antifeedant assays

Snail feeding damage was reduced if leaves were fumigated with VOCs. With 1-octen-3-ol the statistical interaction between dose and time post-treatment was non-significant ($\chi^2_{df=3} = 23.0, P > 0.99$). Leaf weight change increased with time ($F_{1,75} = 22.5, P < 0.001$) but decreased with dose ($F_{3,76} = 19.5, P < 0.001$) (Fig. 10). Where 3-octanone was applied, dose was not found to be statistically significant, either in its interaction with time ($F_{3,72} = 0.575, P = 0.63$) or as a main effect ($F_{3,76} = 1.43, P = 0.24$). However, weight change did increase with increasing time after treatment ($F_{1,75} = 22.2, P < 0.002$) (Fig. 10). It should be noted, however, that snails survived all treatments except at the highest dose (10 μL) of 1-octen-3-ol which resulted in 20% mortality 24 h post-treatment.

3.6 Aqueous formulation assays

Application of aqueous formulations of VOCs to snails showed that with 1-octen-3-ol none of the statistical interactions between dose, time post-treatment, and concentration were significant ($\chi^2_{df=27} = 20.3, P > 0.99$) but mortality increased with dose ($\chi^2_{df=3} = 35.6, P < 0.001$), time ($\chi^2_{df=3} = 24.8, P < 0.001$), and concentration ($\chi^2_{df=3} = 69.2, P < 0.001$) (Fig. 13). Where 3-octanone was applied, the three-way interaction between dose, time, and concentration was not significant ($\chi^2_{df=27} = 13.1, P = 0.99$) (Fig. 14). The only significant two-way interaction was a synergistic effect on mortality between dose and concentration ($\chi^2_{df=9} = 20.8, P = 0.014$). As main effects, mortality increased with dose ($\chi^2_{df=3} = 12.1, P = 0.007$), time ($\chi^2_{df=3} = 48.7, P < 0.001$), and concentration ($\chi^2_{df=3} = 138, P < 0.001$) (Fig. 14).

4 DISCUSSION

This is the first study to show that conidia of insect pathogenic fungi possess mollusc antifeedant properties. The fact that conidia of *M. brunneum* reduced feeding of starved snails suggests that the spores possess irritants or antifeedant compounds that protect against grazing by fungivores in the soil, the natural habitat of this fungus. Most surprising was the movement of the snails to the treated leaves, which suggest the spores were also emitting an attractant. We postulate that the attractants helped with spore dispersal as reported for collembola, which respond to 1-octen-3-ol and other VOCs produced by *Metarhizium conidia*. *Metarhizium* infected insects produce copious conidia at the surface of the mycosed cadaver, which would result in a concentration of the antifeedant or irritant compounds and may explain why there are no reports of molluscs feeding on mycosed cadavers. Conidia of *M. brunneum* strain V275 appeared to have greater antifeedant
or irritant properties suggesting that the type and quantity of irritant/antifeedant compounds vary between strains. It is possible that the irritant/antifeedant compounds are labile since the snails returned to the treated leaves after they had consumed the untreated leaves.

Although *Metarhizium* species produce a wide range of VOCs, the eight-carbon volatiles, 1-octene, 1-octen-3-ol and 3-octenone, are of particular interest because these ubiquitous fungal compounds which are known to play a major role in fungal-invertebrate interactions. These VOCs have been shown to influence the behavior of a wide range of insects, acting as either attractants or repellents. One notable study is that of Yanagawa et al., who reported that termite aversion of the insect pathogenic fungus *Isaria fumosorosea* Wize was due to 3-octanone and 1-octen-3-ol present in small amounts (0.01 ng per 10⁷ conidia) on the conidial surface. At such small quantities, these compounds may not repel molluscs but may act as irritants or antifeedants. If this is the case, then it would explain why the snails in the current study avoided feeding on leaves treated with *M. brunneum* conidia.

Very few VOCs have been shown to have pesticidal properties. The current study is the first to show that the fungal VOCs 1-octene, 1-octen-3-ol and 3-octenone are toxic to the terrestrial molluscs *D. reticulatum* and *C. aspersum* on contact or when deployed as fumigants. Both *D. reticulatum* and *C. aspersum* try to avoid direct exposure or food tainted by these compounds. Both molluscs produced secretions in response to the volatiles, presumably as part of a defence and repair response. The secretions could also be an innate stress response to neutralize the fungal VOCs or prevent secondary infections via damaged tissue since the mucus is supposed to have antimicrobial properties.

The VOCs of *Metarhizium* and other fungi are repellents for several invertebrates including store grain pests (*Sitophilus zeamais* Motschulsky), and termites (*Coptotermes formosanus* Shiraki). Repellency is dependent upon the target since some insects are tolerant of higher quantities than others. For example, mushroom pests such as the phorid fly, *Megaselia halterata* Wood, are tolerant of relatively high levels of 1-octen-3-ol. In the current study, the VOCs killed both *D. reticulatum* and *C. aspersum* on contact even at relatively low doses. Based on their behaviour and time to
Fungal volatiles kill slugs and snails

Figure 12. Evaluation of different volumes of aqueous formulations of 3-octanone applied to slug mantle. Slug mortality (means with SE error bars) over 24 h, under varying concentrations of 3-octanone. The three-way interaction between dose, time, and concentration was not significant ($\chi^2 < 0.001$, df = 27, P > 0.99). The only significant two-way interaction was a synergistic effect on mortality between dose and concentration ($\chi^2 = 19.2$, df = 9, P = 0.023) (Fig. 12). As main effects, mortality increased with dose ($\chi^2 = 143$, df = 3, P < 0.001), time ($\chi^2 = 51.5$, df = 3, P < 0.001), and concentration ($\chi^2 = 158$, df = 3, P < 0.001).

dead the compounds were toxic irritants. The snail was more tolerant than the slug in the fumigation assays, presumably because it could withdraw into the impervious shell.

Both *D. reticulatum* and *C. aspersum* avoided 1-octen-3-ol and 3-octenone environments including food exposed to these compounds, presumably the VOCs inform the pests of food quality since many molds producing these VOCs also produce harmful toxins. Gebauer recorded high mortality in *D. reticulatum* fed wheat grain contaminated with *Fusarium*, a ubiquitous toxigenic fungus. Avoidance behavior has been reported for beetle and weevil pests of stored grain and parasitoids of the grain beetle larva. For example, females of the parasitoid *Lariophagus distinguendus* Forster are strongly attracted to the odor of the feces of the granary weevil (*Sitophilus granarius* Linnaeus) larval host but avoid feces which has absorbed 1-octen-3-ol emitted by the toxigenic grain mold *Aspergillus* since this reduces host fitness.

Entomopathogenic fungi belonging to the genera *Beauveria* and *Metarhizium* are known to produce VOCs that influence the behavior of many insect species. Most insects exhibit avoidance behavior, presumably to avoid infection since avoidance is often linked to virulence. Repellency has been reported for termites, seven-spot ladybirds (*Coccinella septempunctata* Linnaeus) and poultry red mites (*Demansus gallinae* deGeer). Volatiles emitted by inoculum of *Metarhizium* species may account for the ovipositional deterrence behavior of several insects including sweet potato weevil (*Cylas formicarius* Fabricius), house flies (*Musca domestica* Linnaeus) and stable flies (*Stomoxys calcitrans* *Pest Manag Sci* 2019; 75:3392–3404 © 2019 The Authors. wileyonlinelibrary.com/journal/ps Pest Management Science published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.
Linnaeus).\textsuperscript{43,46} Presumably, adults recognize environments that may be hostile to the eggs or emergent larvae since *Metarhizium* is capable of infecting both eggs and larvae.\textsuperscript{47} In contrast, very little is known about the susceptibility of slugs and snails to fungal infection. Laboratory studies show that entomopathogenic and nematophagous fungi are able to infect molluscan eggs. Ovicial activity has been reported for the aquatic snail, *Biomphalaria glabrata* Say, inoculated with *M. anisopliae* (Metchnikoff) Sorokin and the grey field slug, *Agriolimax agrestis* Linnaeus, infected by *Pochonia chlamydospora* Goddard.\textsuperscript{48,49} It is possible that molluscs use the VOC cue to avoid sites where pathogenic fungi are present to ensure their eggs are not attacked.

Application of aqueous formulations of 1-octen-3-ol and 3-octanone complemented studies using neat compounds. They showed that mortality was dose-related and that slugs were more susceptible to the VOCs than the snail. The fact that significant mortality was observed 24 h post-treatment suggests that either the VOCs continue to cause damage or substantial irreparable damage from which the animal could not recover. Indeed, fungal VOCs are known to have neurotoxic or cytotoxic properties with some of the toxicity mediated through the generation of reactive oxygen species.\textsuperscript{50} The VOCs have much potential as replacement products for metaldehyde with many ecological benefits. For example, the natural VOCs are ephemeral so leave no residues. In a crop, the VOCs would deter molluscs entering the treated area. Exactly how long the repellency would last would depend on the formulation and whether the molluscs could be conditioned to avoid VOC-treated crops. Molluscs are supposed to have the ability to acquire a long-term olfactory memory after a single conditioning session.\textsuperscript{51} To ensure the molluscs do not migrate back to the treated crop, it would be imperative to include a trap crop. This ‘push-pull’ strategy would allow for more targeted control of the pest with concomitant reduced pesticide input. Another commercial use of the VOCs would be as fumigants, to treat produce which may be infested with exotic pests such as golden apple snails.\textsuperscript{7} Ephemeral fungal VOCs would pose less of a risk than conventional chemical molluscicides to non-target animals including predators and decomposers.\textsuperscript{52}

**Figure 13.** Evaluation of different volumes of aqueous formulations of 1-octen-3-ol applied to snail mantle. Snail mortality (means with SE error bars) over 24 h, under varying concentrations of 1-octen-3-ol. None of the statistical interactions between dose, time post-treatment, and concentration were significant ($\chi^2_{df = 54} = 20.3, P > 0.99$) but mortality increased with dose ($\chi^2_{df = 3} = 35.6, P < 0.001$), time ($\chi^2_{df = 3} = 24.8, P < 0.001$), and concentration ($\chi^2_{df = 3} = 69.2, df = 3, P < 0.001$).
Figure 14. Contact toxicity of different volumes of aqueous formulations of 3-octanone applied to snail mantle. Snail mortality (means with SE error bars) recorded over 24 h, under varying concentrations of 3-octanone. The three-way interaction between dose, time, and concentration was not significant ($\chi^2 = 13.1$, df = 27, $P = 0.99$). The only significant two-way interaction was a synergistic effect on mortality between dose and concentration ($\chi^2 = 20.8$, df = 9, $P = 0.014$). As main effects, mortality increased with dose ($\chi^2 = 12.1$, df = 3, $P = 0.007$), time ($\chi^2 = 48.7$, df = 3, $P < 0.001$), and concentration ($\chi^2 = 138$, df = 3, $P < 0.001$).

ACKNOWLEDGEMENTS
This work was supported by a BBSRC NPRONET grant BV021 and the Impact Acceleration Account grant EP/R511614/1 for Swansea University from the UK Engineering and Physical Sciences Research Council (EPSRC). The authors also thank Certis Europe BV for partially funding the research.

REFERENCES
1 Hoffman GD and Rao S, Association of slugs with the fungal pathogen Epichloë typhina: potential role in stroma fertilisation and disease spread. Ann Appl Biol 162:324--334 (2013).
2 Douglas MR and Tooker JF, Slug (Mollusca: Agriolimacidae, Arionidae) ecology and management in no-till field crops, with an emphasis on the mid-Atlantic region. J Integr Pest Manage 3:C1--C9 (2012).
3 Dewar AM, Ferguson A, Pell JK, Nicholls C and Watts J, A Review of Pest Management Needs in Cereals and Oilseed Rape in the UK. Agriculture and Horticulture Development Board, Kenilworth, UK (AHDB) Research Review No. 86, pp. 1--248 (2016).
4 Willis JC, Bohan DA, Choi YH, Conrad KF and Semenov MA, Use of an individual-based model to forecast the effect of climate change on the dynamics, abundance and geographical range of the pest slug Deroceras reticulatum in the UK. Glob Chang Biol 12:1643--1657 (2006).
5 Bergey EA, Figueroa LL, Mather CM, Martin RJ, Ray EJ, Kurien JT et al., Trading in snails: plant nurseries as transport hubs for non-native species. Biol Invasions 16:1441--1451 (2014).
6 Cowie RH, Dillon RT Jr, Robinson DG and Smith JW, Alien non-marine snails and slugs of priority quarantine importance in the United States: a preliminary risk assessment. Am Malacol Bull 27:113--132 (2009).
7 Schneiker J, Weisser WW, Settele J, Bustamante JV, Marquez L, Vil lareal S et al., Is there hope for sustainable management of golden apple snails, a major invasive pest in irrigated rice? NUS-Wageningen J Life Sci 79:11--21 (2016).
8 Castle GD, Mills GA, Gravell A, Jones L, Townsend I, Cameron DG et al., Review of the molluscicide metaldehyde in the environment. Environ. Sci Water Res. Technol. 3:415--428 (2017).
9 Castle GD, Mills GA, Gravell A, Leggatt A, Stubbs J, Davis R et al., Comparison of different monitoring methods for the measurement of metaldehyde in surface waters. Environ Monit Assess 191:75 (2019).
10 Rae R, Verdun C, Grewal PS, Robertson JF and Wilson MJ, Biological control of terrestrial molluscs using Phasmarhabditis.
hermaphroditic – progress and prospects. Pest Manag Sci 63:1153–1164 (2007).

11 Speiser B, Zaller JG and Neudecker A, Size-specific susceptibility of the pest slugs Deroceras reticulatum and Arion lusitanicus to the nematode biocontrol agent Phasmarhabditis hermaphrodita. BioControl 46:111–120 (2001).

12 Wynne R, Morris A and Rae R, Behavioural avoidance by slugs and snails of the parasitic nematode Phasmarhabditis hermaphrodita. Biocontrol Sci Technol 26:1129–1138 (2016).

13 Abdelgaleil SA, Molluscicidal and insecticidal potential of monoterpenoids. J. Stored Prod. Res. 62:54(2016).

14 Ebenso IE, Molluscicidal effect of neem components. Adv. Environ. Biol. 5:1215–1219 (2011).

15 Herrera JM, Pizzolitto RP, Zunino MP, Dambolena JS and Zygadlo et al., Size-specific susceptibility of the egg masses to fungal infection. J. Invertebr. Pathol 125:1–3(2015).

16 Kozlowski J, Ch Atlas and Kozlowska M, Initial evaluation of the effectiveness of selected active substances in reducing damage to rape plants caused by Arion Lusitanicus. J. Plant Prot. Res. 10:520–526 (2010).

17 Combet E, Henderson J, Eastwood DC and Burton KS, Eight-carbon aldehydes and nucleotides from two pathogenic fungus affect the direction of motion of termites, Phasmarhabditis hermaphrodita. J. Chem Ecol 38:28–36 (2019).

18 Bojke A, Tkaczuk C, Stepnowski P and Golbiowski M, Comparison of volatile compounds released by entomopathogenic fungi. Microbiol Res 214:129–136 (2018).

19 Wood WF, Archer CL and Largent DL, 1-Octen-3-ol, a banana slug antifeedant from mushrooms. Biochem Syst Ecol 29:531–533 (2001).

20 Butt TM, Coates CJ, Dubovskiy IM and Ratcliffe NA, Entomopathogenic fungi: new insights into host–pathogen interactions, in Advances in Genetics, Amsterdam, The Netherlands, Vol. 94, Academic Press, pp. 307 – 364 (2016).

21 Herrera JM, Pizzolitto RP, Zunino MP, Dambolena JS and Zygadlo et al., Effect of fungal volatile organic compounds on a fungus and an insect that damage stored maize. J. Stored Prod. Res. 62:74–80 (2015).

22 Holighaus G and Rohlf M, Fungal allelochemicals in insect pest management. Appl Microbiol Biotechnol 100:5681–5689 (2016).

23 Holighaus G and Rohlf M, Volatiles and non-volatile fungal oxylipins in fungus-invertebrate interactions. Fungal Ecol 38:28–36 (2019).

24 Bojke A, Tkaczuk C, Stepnowski P and Golbiowski M, Comparison of volatile compounds released by entomopathogenic fungi. Microbiol Res 214:129–136 (2018).

25 Combet E, Henderson J, Eastwood DC and Burton KS, Eight-carbon aldehydes in mushrooms and fungi: properties, analysis, and biosynthesis. Mycoscience 47:317–326 (2006).

26 Kaminski E, Stawicki S and Wasowicz E, Volatile flavor compounds produced by molds of Aspergillus, Penicillium, and fungi imperfect. Appl Environ Microbiol 27:1001–1004 (1974).

27 Yanagawa A, Fujiwara-Tsujii N, Akino T, Yoshimura T, Yanagawa T and Shimizu S, Musty odor of entomopathogens enhances disease-prevention behaviors in the termite Coptotermes formosanus. J. Chem Ecol 41:1118–1126 (2015).

28 Yanagawa A, Imai T, Akino T, Toh Y and Yoshimura T, Offactory cues from pathogenic fungus affect the direction of movement of termites, Coptotermes formosanus. J. Chem Ecol 41:1118–1126 (2015).

29 Mburu DM, Maniania NK and Hassanali A, Comparison of volatile blends and nucleotide sequences of two Beauveria bassiana isolates of different virulence and repellency towards the termite Macrotermes michaelseni. J. Chem Ecol 39:101–108 (2013).

30 Ansari MA and Butt TM, Effects of successive succubating on stability, virulence, conidial yield, germination and shelf-life of entomopathogenic fungi. J. Appl Microbiol 110:1460–1469 (2011).

31 Bateman R, The MycoHarvester: cleaning up locust control. Int Pest Control 45:76–77 (2003).

32 Goettel S and Inglis GD, Chapter V-3-fungi: Hyphomycetes, in Manual of Techniques in Insect Pathology, ed. by Lacey LA. Academic Press, London, pp. 213–249 (1997).

33 R Core Team R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria (2018). Available: https://www.R-project.org/.

34 Schenck JW and Butler TM, Natural and released inoculum levels of entomopathogenic fungal biocontrol agents in soil in relation to risk assessment and in accordance with EU regulations. Biocontrol Sci Technol 20:503–552 (2010).

35 Dromph KM, Collemboles as vectors of entomopathogenic fungi. Pedobiologia 47:245–256 (2003).

36 Holighaus G, WeißBecker B, von Fragramin M and Schütz S, Ubiquitous eight-carbon volatiles of fungi are infochemicals for a specialist fungivore. Chemoecology 24:57–66 (2014).

37 Werner S, Polle A and Brinkmann N, Belowground communication: impacts of volatile organic compounds (VOCs) from soil fungi on other soil-inhabiting organisms. Appl Microbiol Biotechnol 89:8651–8665 (2016).

38 Newar J and Ghatak A, Studies on the adhesive property of snail adhesive mucus. Langmuir 31:12155–12160 (2015).

39 Cilia G and Fratini F, Antimicrobial properties of terrestrials snail and slug mucus. J. Complementary Integr. Med 15:1 – 10 (2018).

40 Pfeil RM and Mumma RO, Bioassay for evaluating attraction of the phorid fly, Megaselia halterata to compost colonized by the commercial mushroom, Agaricus bisporus and to 1-octen-3-ol and 3-octanone. Entomol Exp Appl 69:137–144 (2011).

41 Gebauer J, Survival and food choice of the grey field slug (Deroceras reticulatum) on three different seed types under laboratory conditions. Anzeiger für Schädlingskunde 75:1–5 (2002).

42 Steiner S, Emdmann D, Steidle JL and Ruther J, Host habitat assessment by a parasitoid using fungal volatiles. Front. Zool. 4:1–3 (2007).

43 Dotaona R, Wilson BA, Ash GJ, Holloway J and Rother J, Relationship between virulence and repellency of entomopathogenic isolates of Metarhizium anisopliae isolate is present. J. Invertebr. Pathol 148:67–72 (2017).

44 Mburu DM, Ochola L, Maniania NK, Njagi PGN, Gitonga LM, Ndung’u MW et al., Relationship between virulence and repellency of entomopathogenic isolates of Metarhizium anisopliae and Beauveria bassiana to the termite Macrotermes michaelseni. J. Insect Physiol 55:774–780 (2009).

45 Klippen O and Steenberg T, Repellent activity of desiccant dusts and conidia of the entomopathogenic fungus Beauveria bassiana when tested against poultry red mites (Dermanyssus gallinae) in laboratory experiments. Exp Appl Acarol 70:329–341 (2016).

46 Machtinger ET, Weeks ENI and Geden CJ, Oviposition deterrence and immature survival of filth flies when exposed to commercial fungal products. J. Insect Sci 16:54 (2016).

47 Aydin T, Branco M, Güven Ö, Gonçalves H, Lima A, Karaça İ et al., Significant mortality of eggs and young larvae of two pine processionary moth species due to the entomopathogenic fungus Metarhizium brunneum. Biocontrol Sci Technol 28:317–331 (2018).

48 Duarte GF, Rodrigues J, Fernandes ÉK, Humber RA and Luz C, New insights into the amphibious life of Blomphalaria glabrata and susceptibility of its egg masses to fungal infection. J. Invertebr. Pathol 125:31–36 (2015).

49 Trevet IW and Esslemont JM, A fungus parasite of the eggs of the gray field slug. J. Quecket Microeco Club 1:1–3 (1938).

50 Inamdar AA, Masurekar P and Bennett JW, Neurotoxicity of fungal volatile organic compounds in Drosophila melanogaster. Toxicol Sci 117:418–426 (2010).

51 Matsuo R, Consolidation and reconciliation of olfactory memory in the terrestrial pulmonaryes, in Memory consolidation. Nova, New York, pp. 53 – 64 (2015).

52 Horgan FG, Bernal CC, Letana S, Naredo Al, Ramp D and Almazan MLP, Reduced efficiency of tropical flies (Diptera) in the decomposition of snail cadavers following molluscicide poisoning. Appl Soil Ecol 129:61–71 (2018).