A simple in vitro method to study interactions between soil insects, entomopathogenic fungi, and plant extracts

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Abstract

A combined application of a biological control agent, such as an entomopathogenic fungus (EPF), with other means of pest control may lead to enhanced or more reliable efficacy of the biocontrol agent. Ideally, the combined components would interact synergistically, yielding significantly higher mortality of the target pest. The mode of such interactions is often unclear and particularly difficult to elucidate for soil-dwelling pests. Here, an efficient image analysis protocol was developed to study behavioural responses of soil-dwelling insects to application of control measures in two-dimensional terraria. The interactions between the EPF *Metarhizium brunneum* Petch (Hypocreales), a supposedly repellent extract of garlic, *Allium sativum* L. (Amaryllidaceae), and wireworms, *Agriotes obscurus* (L.) (Coleoptera: Elateridae), as target hosts were investigated. Tunnelling activity and mortality of wireworms were studied under choice and no-choice situations of various combinations of the control agents. A treatment with *Metarhizium* spores resulted in wireworm mortalities of about 80%. Contrary to expectations, a combination of the EPF with the garlic extract did not increase, but slightly decrease wireworm mortality. The data gained from the image analysis revealed that the tunnelling activity of wireworms was clearly reduced in the presence of garlic, which, together with a reduced germination rate of spores, is a possible explanation for the antagonism detected in the combined treatment. The methodological approach developed here can be used to study the interactions among control agents and soil insects over several days and weeks.

Introduction

Entomopathogenic fungi (EPF) used for biological control encounter diverse environmental conditions in the field, which influence the EPFs' capability to infect and control the target pest. Abiotic factors, such as adverse temperatures or humidity conditions, may impede the efficacy of an EPF treatment. This contributes to the fact that EPFs are often considered less successful than conventional pesticides (reviewed in Jaronski, 2010). In addition, other control means interact with released biocontrol agents and may influence their efficacy. These measures are not limited to synthetic chemicals only (e.g., fertilisers, pesticides; Samson et al., 2005; Jaronski,

2007) but also include natural compounds (e.g., secondary plant metabolites; Shah et al., 2008), and other macro- and microorganisms (Friberg et al., 2005; Acevedo et al., 2007; Jaronski, 2010).

Interactions may be positive or negative for the growth of EPFs and their performance and efficacy in pest control. Unsterilised soil often shows fungistatic properties towards EPFs (e.g., Pereira et al., 1993) and numerous secondary plant metabolites, among them many essential oils, are known for their antimicrobial effects (Hammer et al., 1999). Antagonistic interactions were also observed between *Metarhizium anisopliae* (Metschn.) Sorokin and the insect-parasitic nematode *Heterorhabditis bacterio-phora* Poinar against the sugar cane borer, *Diatraea saccharalis* Fabricius (Acevedo et al., 2007). In contrast, interactions can also be synergistic and may lead to an increased performance of the EPF, as was demonstrated for *M. anisopliae* in combination with the insecticide spinosad against *Agriotes* spp. wireworms in the laboratory

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(Ericsson et al., 2007) or in combination with insect-parasitic nematodes against grubs and vine weevils in the field (Ansari et al., 2004, 2010).

The mechanisms behind both types of interaction are poorly understood. Shah et al. (2008) detected a synergistic interaction between *M. anisopliae* and neem seed cake (*Azadirachta indica* A. Juss.) applied against black vine weevils, *Otiorhynchus sulcatus* (Fabricius). They hypothesised that the repellent and antifeedant properties of neem forced the weevil larvae to cover greater distances in the substrate when foraging for food. As a result, the state of starvation and the enhanced activity of the insects, which may have resulted in an accumulation of EPF conidiospores on the cuticle, could have increased the chances for successful fungal infection.

A second potential mechanism of synergy may be that additional control agents or bioactive compounds help an EPF to surmount the host's defence mechanisms (Shah et al., 2007, 2008). In laboratory trials, low-dosed insecticides resulted in lethargic, moribund wireworms (Vernon et al., 2008; van Herk et al., 2013), and combined treatments of low-dosed insecticides and an EPF led to increased wireworm mortality (Ericsson et al., 2007). Sublethal doses of *Bacillus thuringiensis* Berliner spores had the same efficiency-enhancing effect on an EPF applied against diamondback moths in the laboratory (Nian et al., 2015).

The main objective of the present study was to investigate potential synergistic interactions between an EPF and a plant extract with repellent properties, when applied together for wireworm control. Wireworms are the root-feeding larvae of click beetles (Coleoptera: Elateridae), and can cause severe damage to many agricultural crops worldwide (reviewed in Ritter & Richter, 2013; Traugott et al., 2015). Crop protection against wireworms is difficult, because effective soil insecticides have been withdrawn from the market in recent years due to environmental concerns including long term persistence of insecticides' active ingredients or their metabolites in the soil and side effects on non-target organisms (e.g., Vernon et al., 2008). More recently, it was shown that the latter may include sublethal effects, which can be disastrous especially for social insects (reviewed in Desneux et al., 2007).

As a consequence, interest in biological control methods has increased and stimulated research on natural antagonists of wireworms. Among these, EPF play an important role with a history of successful biocontrol products (Butt et al., 2001; Ekesi & Maniania, 2007). Numerous EPF are in application against agricultural pests today, e.g., *Beauveria bassiana* (Balsamo) Vuillemin, *Isaria fumosorosea* Wize, *Lecanicillium lecanii* (Zimm.) Zare & W. Gams (2001), and *Metarhizium anisopliae* (Metschn.) Sorokin. Particularly *Metarhizium* spp. (Hypocreales) cause

naturally disease in elaterid larvae (Kleespies et al., 2013) and turned out to provide promising EPF strains against wireworms in studies under laboratory conditions (Kabaluk et al., 2005; Kölliker et al., 2011).

In our study, we used an isolate of *Metarhizium brunneum* Petch, which has already successfully been tested for virulence against wireworms (Eckard et al., 2014), and examined its efficacy in combination with garlic extract. Garlic [*Allium sativum* L. (Amaryllidaceae)] was shown to produce various metabolites which have repellent and even toxic effects on above-ground insects, e.g., *Sitophilus zeamais* Motschulsky and *Tribolium castaneum* (Herbst) (Huang et al., 2000), and *Megalurothrips sjostedti* (Trybom) (Oparaeke et al., 2006). Preliminary tests with garlic extract in our laboratory confirmed a repellent effect on wireworms (S. Eckard, unpubl.). We hypothesised that a treatment with garlic extract will have an effect on wireworms, which is similar to a treatment with sublethal doses of an insecticide. It follows that the combination of the EPF and the plant extract could interact synergistically and enhance the efficacy of the biocontrol measure.

The second aim of the study was to monitor wireworm tunnelling activity as behavioural response to treatments. For this purpose, wireworm activity was assessed under choice and no-choice situations with single and combined treatments of the EPF and the garlic extract. This should help understand the mechanisms behind potential interactions between the control agents. Recent studies have shown that the failure of wireworm control measures are attributable to the ignorance of the behavioural responses of the pest insects to treatments. Application of neonicotinoids as soil insecticides against wireworms resulted in temporary crop stand protection without reliably killing wireworms (Vernon et al., 2013). Later, it was found out that neonicotinoid and bifenthrin (pyrethroid) treatments did not cause significant mortality in wireworms, but resulted in 'moribund' larvae that recovered and presumably continued to forage and damage following crops (van Herk et al., 2013, 2015). Studying such mechanisms in soil pests is particularly difficult due to their cryptic lifestyle. Nevertheless, attempts have been made to get insight into soil pest insects' behaviour and ecology (van Herk & Vernon, 2007; Johnson et al., 2007; Mankin et al., 2008; Schumann et al., 2013). All of these set-ups have their advantages and drawbacks. For instance, so called 'destructive set-ups' are comparatively straightforward but the observation device gets destroyed by the measurement and no further record beyond one census date is possible (Dawson & Byers, 2008). On the other hand, non-destructive set-ups have the advantage of allowing observations of the same individuals for several census dates. However, they often rely on special,

usually expensive, technical equipment and training (Mankin et al., 2008).

In the present study, we used a two-dimensional terrarium set-up as described by Klingler (1957) and Schumann et al. (2013). Instead of recording positions of larvae at a certain time point, we used the set-up for monitoring of wireworm behaviour over a period of several days, by taking photographs and recording the lengths of tunnels in the substrate as a measurement of their burrowing activity. This required development of a novel yet simple and cheap image analysis method, which enabled us to detect changes in wireworm behaviour occurring in between application date and the eventual death of the treated insect. In particular, the set-up was used to answer the following questions. Does the combined application of *Metarhizium* and garlic lead to increased wireworm mortality? If yes, what are the mechanisms underlying this interaction? Do wireworms try to avoid garlic-treated substrates shown by an increased tunnelling activity ('repel' hypothesis)? And/or does the garlic treatment lead to sublethal toxic effects, which would be indicated by a decreased tunnelling activity of moribund wireworms ('stress' hypothesis)? Interactions were tested with combinations of untreated, EPF-treated, or repellent-treated substrates, applied either to the entire terrarium (no-choice conditions) or only one side of it, allowing wireworms to move between substrates with different treatments (choice conditions).

On the basis of mortality data and results of the tunnelling activity monitoring, a test of the hypotheses was possible. An increase of wireworm mortality in treatments with EPFs and garlic combined (RF_RF), accompanied by an increase of tunnelling activity in no-choice treatment combinations would support the 'repel' hypothesis, whereas increased mortality accompanied by decreased activity would favour the 'stress' hypothesis.

Materials and methods

Terraria for assessment of wireworm activity

We constructed slim, earth-filled terraria between two vertical glass plates from Klingler (1957) and Schumann et al. (2013) to observe wireworm tunnelling activity. Glass plates measured 51 × 41 cm (width × height), leaving an experimental arena of 200 cm^2. The glass plates were separated with 5-mm plastic spacers along the sides and bottom, the top remained open. Bottom spacers had six mesh-sealed holes (2 mm diameter) for drainage. The space between the glass plates was filled with 1 l of 1:1 (vol/vol) moist peat substrate and sand. The humidity of the substrate was adjusted gravimetrically to 8% (wt/wt) at the beginning of the experiment. Glass plates were held together with four 41-mm foldback clips and terraria were mounted vertically in wooden racks (Figure 1A). This arrangement allowed movements of wireworms in a horizontal (50 cm) and vertical (40 cm) dimension. The 5-mm soil-layer gave wireworms enough space to move, while repeated observation of their position and tunnels in the substrate was possible from the outside without opening the terraria. Carrot slices were inserted into the substrate at both upper corners of the terraria to provide food for the wireworms (Figure 1B).
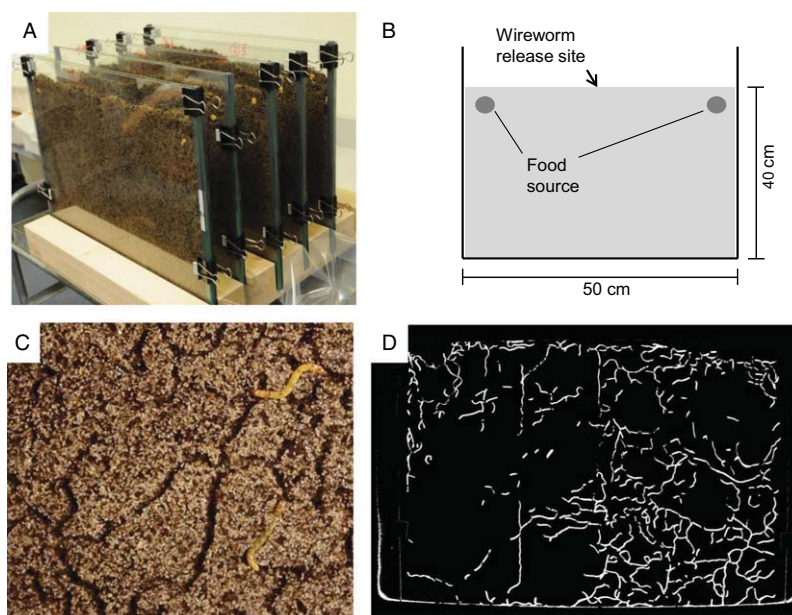

Figure 1 (A) Two-dimensional terraria in a rack. (B) Schematic set-up of a terrarium. (C) Wireworm tunnels visible in the substrate. (D) Black and white picture of a terrarium with garlic-treated substrate on the left side after conversion, ready for quantitative assessment of tunnel traces.

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Wireworms, fungal isolate, and garlic extract

Agriotes obscurus L. larvae originated from a laboratory livestock, established and maintained according to the method of Kölliker et al. (2009). In spring time, adult click beetles were collected from natural grassland habitats and identified using morphological characters. Single-species cohorts were transferred to oviposition cages, consisting of pots filled with about 10 l of 6:1 (vol/vol) sterile field soil and sand, and sealed with a mesh-bag on top. A grass mixture of *Festuca rubra* L., *Festuca pratensis* Huds., *Poa pratensis* L., and *Lolium perenne* L. was grown in the pots to provide a suitable oviposition environment for adults and a food source for emerging larvae. Wireworms passed through their first larval stages in the pots and were collected approximately half a year later. They were subsequently stored in plastic containers filled with ca. 6 l of humid peat substrate and carrots as food at 7 °C in the dark. Two weeks prior to experimental use, late instars (ca. L6) were transferred individually to little cups (ca. 100 ml) and kept with a wet piece of paper towel without food supply at 23 °C. Only starved but healthy and active specimens were used for the experiments.

Metarhizium brunneum strain ART2825 was used as fungal pathogen. The strain was isolated from an *A. obscurus* cadaver in Switzerland and revealed high virulence against two major wireworm species (Eckard et al., 2014). Prior to experiments, the isolate was passed through wireworm hosts and re-isolated on selective medium (Strasser et al., 1996). Conidia of the second subculture were washed from the agar plate with 0.1% aqueous Tween80 solution and conidia concentration was adjusted to 7.95×10^8 conidia ml^{-1}. A volume of 0.88 ml conidia suspension was thoroughly mixed with 1 l of terrarium substrate. The final concentration in the terrarium substrate was 1.75×10^8 conidia l^{-1}, which is adapted from experiences with unpublished field trials.

A powder formulation of garlic extract, containing 6% (wt/wt) garlic oil on a carbohydrate carrier substance (Neem Biotech, Cardiff, UK) was used as repellent. Preliminary tests with various concentrations of this garlic formulation in Petri dishes made wireworms avoid treated areas, whereas the carrier substance alone did not affect wireworm locomotion or mortality (S. Eckard, unpubl.). In these tests, 0.7 g garlic extract per l of moist peat substrate was determined as the lowest dose exhibiting visible repellent effects on wireworm behaviour. The same dosage was applied in the present experiment.

Experimental design

The experimental space in the terraria was split in two parts to allow two kinds of experimental approaches (in six replicates). The first approach represented a 'no-choice' situation where the same treatment was applied to both halves of the terraria and wireworms were not given the chance to avoid direct contact with the treatments. Four substrate treatment combinations were assessed: untreated control (0_0, untreated substrate on both sides), repellent treatment (R_R, garlic-treated substrate on both sides), EPF treatment (F_F, EPF-treated substrate on both sides), and a combination of the latter two (RF_RF, garlic and EPF mixed into the substrate and mixture applied to both sides) (Figure 2). Besides mortality, increased or decreased wireworm tunnelling activity as a response to these treatments was assessed by measuring the total length of wireworm tunnels (see below).

In the second approach, 'choice' situations were provided by applying different treatments to the two sides of the terraria. Wireworms were given the chance to move between the sides and avoid contact with one of the treatments. In addition, the set-up allowed assessment of tunnelling activity in one side of the terrarium, depending on treatments in the neighbouring side. Choice treatment combinations included terraria with one side untreated and the second side treated with repellent (0_R) or fungal spores (0_F), and terraria with one side treated with repellent and the other side treated with fungus (R_F) (Figure 2).

Treatments in choice and no-choice situations were applied to terrarium substrates prior to the release of wireworms. At the beginning of each experiment, 10 late instars of *A. obscurus* were released into the centre of the top opening of each terrarium. The experiments were performed at 23 °C in the dark. Every treatment combination was replicated 3× simultaneously and the whole experiment was carried out twice (i.e., two experimental cohorts). This resulted in a total of six replicates per combination.

Assessment of wireworm mortality and mycosis

Dead and live larvae were counted weekly by visual inspection with a desk lamp through the glass plates of the terrarium. Dead specimens were removed by carefully opening the terrarium without disturbing the wireworm tunnels in the soil matrix. After 3 weeks, one glass plate was removed for assessment of wireworm activity (see below) and the remaining larvae were collected from the terrarium substrate. All larvae were subsequently maintained individually in small cups (100 ml) filled with wet peat substrate and incubated in a dark climate chamber at 23 °C and 65% r.h. Besides mortality, mycosis (i.e., formation of mycelium and spore layers of the EPF on cadavers) was assessed at 4, 5, 6, and 9 weeks after application of treatments and release of wireworms.
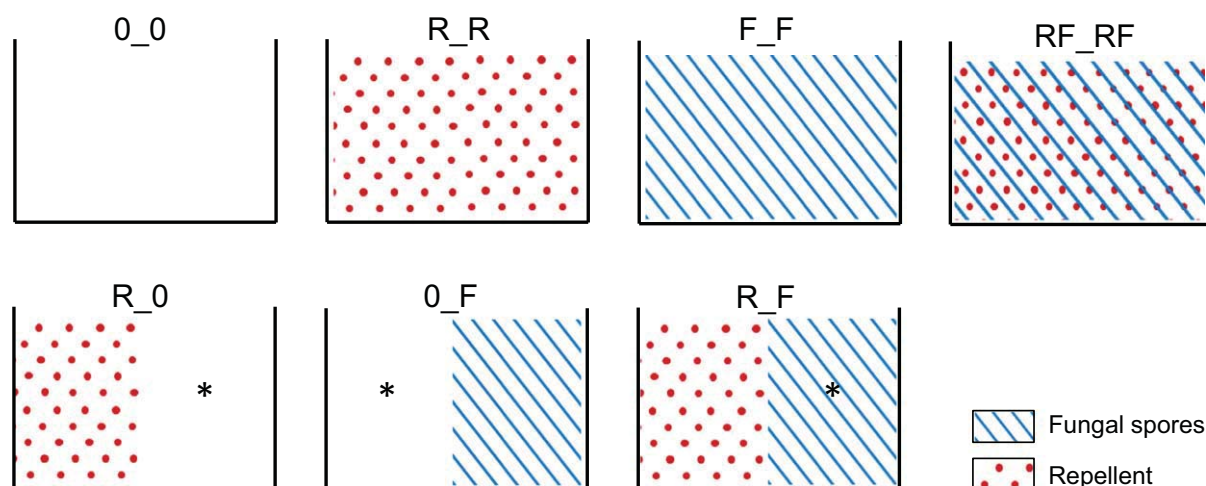
Figure 2 Experimental set-up of (top row) no-choice and (bottom row) choice treatments: untreated control (0_0), pure repellent (R_R), pure fungus (F_F), repellent and fungus mixed in the entire terrarium substrate (RF_RF), one side repellent treated and the other side untreated (R_0), one side untreated and the other side treated with fungus (0_F), repellent and fungus treatment on separate sides of one terrarium (R_F). Asterisk indicates the side used as response variable in the linear model of pixel counts (choice treatments). One randomly chosen side of the 0_0 treatments was added to the analysis as control (n = 6).

Measurement of wireworm activity

Wireworms moving through the substrate in the terraria left tunnels, which were traceable through the glass plates (Figure 1C). These tunnels were used to quantify behavioural activity. One week after release of the wireworms, tunnels were retraced on one glass plate of each terrarium with a waterproof red felt tip pen (point diameter 2 mm). After 3 weeks, the glass plates were removed from the terraria, cleaned, and the tunnel traces marked after the 1st week were photographed in front of a homogeneous, bright background. Resulting photographs were converted to black (background) and white (tunnels) pictures using the free graphic software Gimp 2.6 (GNU Image Manipulation Program by Kimball et al., 2008) (Figure 1D). Finally, a histogram of brightness of the picture was produced and pixels representing the colour white (tunnels) in the histogram were counted. A step by step description of the image analysis is given in the Appendix I.

Measurement of fungal germination rates

Germination rates of *Metarhizium* conidia were assessed in untreated terrarium substrates and substrates treated with either the garlic extract or the unloaded carrier substance of the garlic extract. Therefore, 400-ml samples of terrarium substrate were thoroughly mixed with treatments or left untreated. Afterwards, 1 ml of spore suspension (9×10^8 spores ml^{-1}) was added and the substrate was again thoroughly mixed. Six 9-cm glass Petri dishes were each filled with 40 g of substrate and incubated for 7 days at 23 °C in the dark. Then, 20 g substrate per Petri dish were transferred into a 200-ml Erlenmeyer flask and suspended in 100 ml tetrabasic sodium pyrophosphate solution (Riedel-de Haën, Seelze, Germany; 1.8 g l^{-1} tap water). After 3 h of shaking horizontally at 120 r.p.m., 100 μl of the suspension was plated on selective medium and incubated for another 4 days at 23 °C in the dark. Finally, ca. 30 spores of three fields of view, respectively, were inspected with a stereo microscope at 200× magnification. Spores with a protruding germination tube were classified as germinated.

Statistical analysis

Statistical analyses were performed with the free software R v.3.1.0 (R Development Core Team, 2013). In a first step, effects of single and combined treatments with EPF and garlic on wireworm mortality were assessed. A disproportionate increase or decrease in wireworm mortality in the combined treatments (RF_RF, R_F) would suggest a positive (synergistic) or negative (antagonistic) interaction between the EPF and the garlic extract, which would be confirmed by a significant interaction between the two factors (see below). Treatment effects were analysed with generalised linear models for no-choice treatment combinations (0_0, R_R, F_F, RF_RF) and choice treatment combinations (R_0, 0_F, R_F) including the untreated control (0_0), respectively. Mortality was defined as a binary response variable (dead/alive) with a binomial error distribution and a logit link function was applied. The application of fungal spores and the repellent including their interactions and the experimental cohort

were treated as independent variables. The analysis was performed separately for each individual census date. The analysis was run first using wireworm mycosis caused by *Metarhizium* (mycosed/not mycosed) as binary response variable and then repeated with total mortality (dead/alive) as response variable. However, as both analyses produced similar results, only results for total mortality are shown.

In a second step, effects of treatments on wireworm tunnelling activity in no-choice set-ups were analysed. Tunnels of both sides of the terraria were used for analyses. An increase of tunnelling activity in treated substrates would indicate escaping behaviour and, consequently, a 'repellent' effect of the treatment. On the contrary, decreased tunnelling activity would indicate sublethal toxic effects supporting a 'stressing' effect. Treatment effects on tunnelling activity were analysed with linear models with pixel counts of the wireworm tunnels as response variable. The presence of fungal spores and the repellent including their interaction and the experimental cohort were treated as independent variables. As reduced tunnelling activity may also have been a direct consequence of wireworm mortality, we tested an alternative model where the response variable 'pixel counts' was standardised by the number of viable larvae present at the evaluation date.

Tunnelling activity was also analysed in choice treatment combinations. An increase of tunnelling activity in untreated or EPF-treated sides neighbouring garlic-treated sides (R_F, R_0) would underpin the repellent effect of the latter. Such a behaviour accompanied by increased mortality of the wireworms in the combined treatment (R_F) would support the 'repel' hypothesis. By contrast, increased mortality linked with decreased tunnelling activity would favour the 'stress' hypothesis. The response variables for these analyses were the pixel counts of the garlic-free side of the terrarium (either untreated or EPF treated). Terraria with an untreated and an EPF-treated side (0_F) were also included in the analyses to check for a possible influence of the EPF on wireworm behaviour. Terraria with both sides untreated (0_0) were included as control and one side was randomly chosen for measuring tunnelling activity. The presence of the repellent or the EPF on the neighbouring side of the terrarium, respectively, and the experimental cohort were considered as independent variables. Again, an alternative model was generated with pixel counts per live larva as response variable in order to account for possible reduced tunnelling activity due to dead larvae.

Treatment effects on fungal germination were analysed by a generalised linear mixed effect model (glmer) from the r-package 'lme4' (v.1.1-11; Bates et al., 2015) with germination considered as binary response variable (positive/negative). A binomial error distribution and a logit link function were assumed. The presence of the carrier substance and the garlic extract were treated as independent variable. The identity of the glass Petri dish was included as random factor.

Results

In no-choice situations with treatments uniformly applied to the whole terrarium substrate, application of *M. brunneum* conidia significantly increased wireworm mortality (Table 1). About 80% of the larvae in EPF-treated terraria died within 2 months (Figure 3). The presence of garlic alone did not induce mortality during the experimental period. However, garlic antagonistically decreased fungal-induced mortality in combined treatments at all census dates, as shown by the negative interaction coefficients (Table 1). The experimental cohorts differed in mortality after 3 and 6 weeks, but not after 9 weeks (Table 1).

Wireworms' tunnelling activity in no-choice set-ups was significantly reduced in terraria treated with the repellent (R_R, RF_RF) compared to repellent-free treatments (0_0, F_F) (Figure 4, Table 2). Tunnelling activity was also reduced in the presence of the EPF alone, but this effect was not significant. The same is true for the interaction between the EPF and the repellent. There was no difference in tunnelling activity between the two cohorts (Table 2). In the alternative model, which accounted for dead larvae in no-choice treatments, effects pointed towards the same direction, but were no longer significant due to reduced statistical power (Table S1).

Table 1 Coefficients, standard errors of the mean, and P-values of fitted generalised linear models on wireworm mortality in no-choice treatment combinations (0_0, R_R, F_F, RF_RF; see Figure 2 for explanation) at 3, 6, and 9 weeks

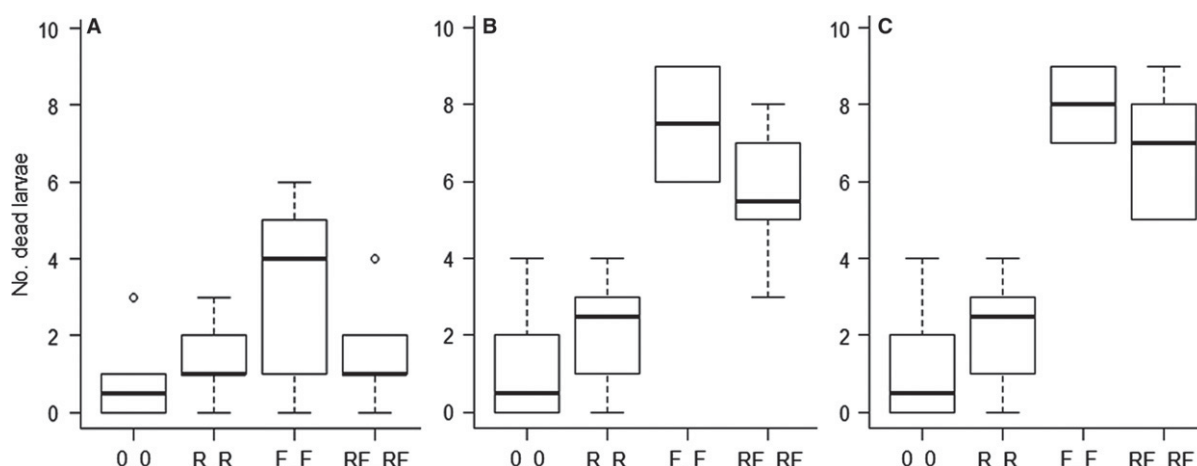| Time (weeks)	Factor	Estimate	SEM	P
3	Intercept	−3.49	0.68	<0.001
	Fungus	0.52	0.20	0.009
	Repellent	−0.15	0.20	0.44
	Fungus*repellent	−0.44	0.20	0.029
	Cohort	1.25	0.39	0.001
6	Intercept	−1.46	0.56	0.009
	Fungus	1.44	0.19	<0.001
	Repellent	−0.15	0.18	0.42
	Fungus*repellent	−0.54	0.18	0.004
	Cohort	0.87	0.35	0.014
9	Intercept	−0.57	0.59	0.33
	Fungus	1.73	0.21	<0.001
	Repellent	−0.15	0.21	0.46
	Fungus*repellent	−0.53	0.21	0.011
	Cohort	0.48	0.37	0.20

Figure 3 Boxplots of wireworm mortality (number of dead *Agriotes obscurus* larvae out of 10 released ones, n = 6) in no-choice treatments (see Figure 2 for explanation) after (A) 3, (B) 6, and (C) 9 weeks. Boxes show the 1st (bottom line), 2nd (median, bold line), and 3rd (top line) quartiles of the data distribution, whisker ends represent the lowest and the highest data point within the 1.5 interquartile range (IQR) of the lower and the upper quartile, respectively. Circles represent outliers between 1.5 and 3 IQR.

Wireworm mortality reached the same level of about 80% after 9 weeks in choice situations with only one side of the terrarium treated with *Metarhizium* (0_F, R_F; Figure 5). Again, the presence of the EPF in any of the treated sides was crucial for wireworm mortality (Table 3). There was, however, no interaction (neither synergistic nor antagonistic) between the repellent and the EPF when treatments were applied on separate terrarium sides (Table 3).

The presence of the repellent on one side of the terrarium led to decreased tunnelling activity in neighbouring sides of choice set-ups (Table 4, Figure 6). The EPF reduced tunnelling activity in the EPF-treated side and this effect was increased, if the neighbouring side contained the repellent, showing a positive interaction of EPF and repellent. A significant difference in tunnelling activity between the two experimental cohorts was not observed.

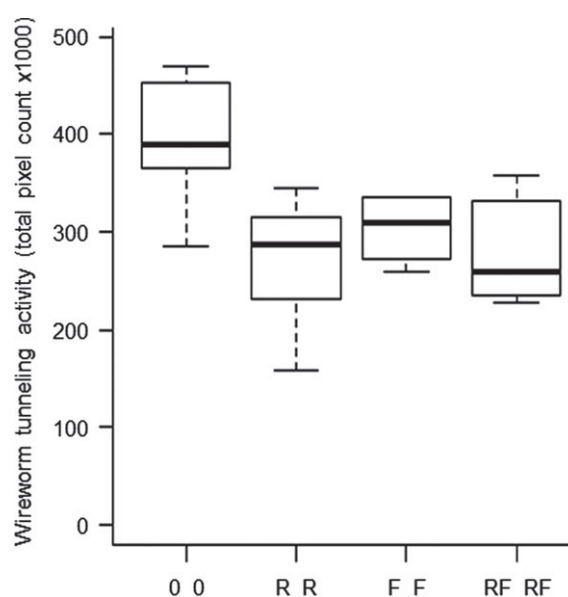Overall, there was a similar reduction caused by the repellent in wireworm activity in choice and no-choice

Table 2 Coefficients, standard errors of the mean, and P-values of the fitted linear model on wireworm tunnelling in no-choice treatment combinations (0_0, R_R, F_F, RF_RF; see Figure 2 for explanation) and the untreated control (0_0)

Factor	Estimate	SEM	P
Intercept	348699	36815	<0.001
Fungus	−20737	11892	0.097
Repellent	−37722	11892	0.005
Fungus*repellent	25253	12148	0.051
Cohort	−25090	23284	0.30

Figure 4 Wireworm tunnelling activity in no-choice treatment combinations (see Figure 2 for explanation) as sum of pixel counts from both sides (n = 6). Boxes show the 1st (bottom line), 2nd (median, bold line), and 3rd (top line) quartiles of the data distribution, whisker ends represent the lowest and the highest data point within the 1.5 interquartile range of the lower and the upper quartile, respectively.

set-ups, respectively. However, in no-choice but not in choice set-ups, this decrease in activity was accompanied by antagonistic interactions of the EPF and the repellent on wireworm mortality. The alternative model accounting for dead larvae yielded similar results except that the effect

Table 3 Coefficients, standard errors of the mean, and P-values of fitted generalised linear models on wireworm mortality in choice treatment combinations (R_0, 0_F, R_F; see Figure 2 for explanation) and the untreated control (0_0) at 3, 6, and 9 weeks

Time (weeks)	Factor	Estimate	SEM	P
3	Intercept	−2.32	0.60	<0.001
	Fungus	0.93	0.22	<0.001
	Repellent	−0.09	0.22	0.69
	Fungus*repellent	0.17	0.23	0.44
	Cohort	0.46	0.36	0.21
6	Intercept	−0.91	0.59	0.12
	Fungus	1.69	0.20	<0.001
	Repellent	−0.01	0.19	0.95
	Fungus*repellent	−0.03	0.20	0.88
	Cohort	0.46	0.38	0.23
9	Intercept	−0.77	0.61	0.21
	Fungus	1.80	0.20	<0.001
	Repellent	−0.12	0.20	0.54
	Fungus*repellent	−0.14	0.20	0.49
	Cohort	0.43	0.39	0.27

Table 4 Coefficients, standard errors of the mean, and P-values of the fitted the linear model on wireworm tunnelling in choice treatment combinations (R_F, 0_F, R_0; see Figure 2 for explanation) and the untreated control (0_0)

Factor	Estimate	SEM	P
Intercept	200182	23234	<0.001
Fungus	−18884	7507	0.021
Repellent	−32424	7507	<0.001
Fungus*repellent	−21979	7667	0.010
Cohort	−27793	14695	0.074

of the EPF alone was no longer significant, although the estimate showed the same trend to reduced activity (Table S2).

Conidia germination was affected by garlic extract. Only (mean ± SD =) 60 ± 10% of spores germinated in garlic-treated substrates. This germination rate was significantly lower than that from untreated substrates (86 ± 4%). Spores in the treatment with the carrier substance alone reached a germination rate of 81 ± 4%, which was similar to that in untreated substrates (Table 5).

Discussion

Interactions between wireworms, EPF, and garlic extract

The overall challenge for successful application of microbial control agents is to enhance efficiency under field conditions and/or to reduce its dose to a level providing cost-efficiency. This applies also for the *M. brunneum* strain and its dose used here, and the presented terrarium assay was to serve the investigation of interactions between this EPF and garlic extract to gain a mechanistic understanding for this objective.

Wireworm mortality in our laboratory experiments was increased by the presence of the EPF, whereas the treatment with the garlic extract alone did not cause mortality. When treatments were applied to substrates on both sides of terraria (no-choice), the interaction between EPF and garlic turned out to be antagonistic. Furthermore, this antagonism came along with reduced wireworm activity, which was measurable as a
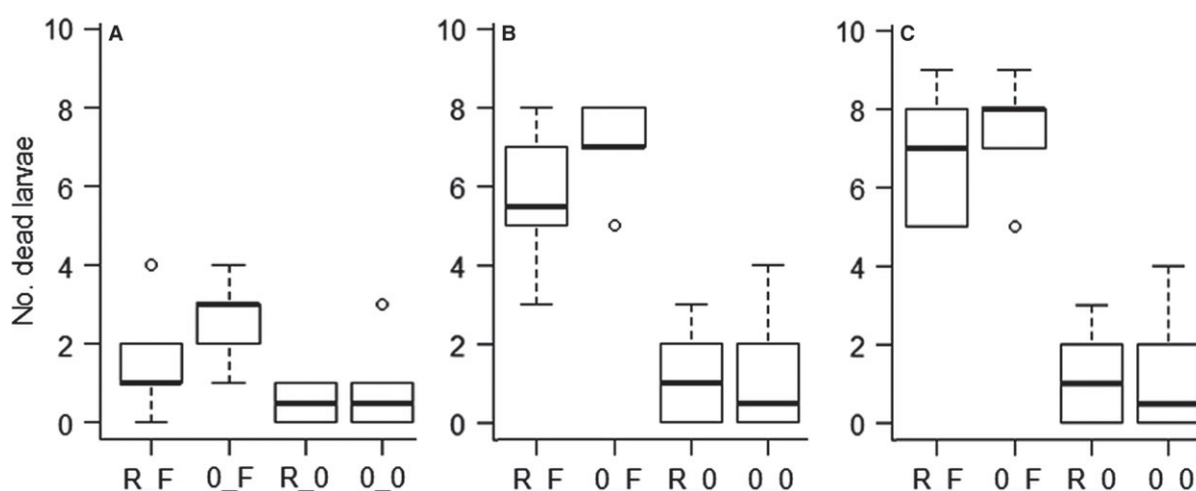

Figure 5 Boxplots of wireworm mortality (number of dead *Agriotes obscurus* larvae out of 10 released ones, n = 6) in choice treatments (see Figure 2 for explanation) and the untreated control (0_0) after (A) 3, (B) 6, and (C) 9 weeks. Boxes show the 1st (bottom line), 2nd (median, bold line), and 3rd (top line) quartiles of the data distribution, whisker ends represent the lowest and the highest data point within the 1.5 interquartile range (IQR) of the lower and the upper quartile, respectively. Circles represent outliers between 1.5 and 3 IQR.
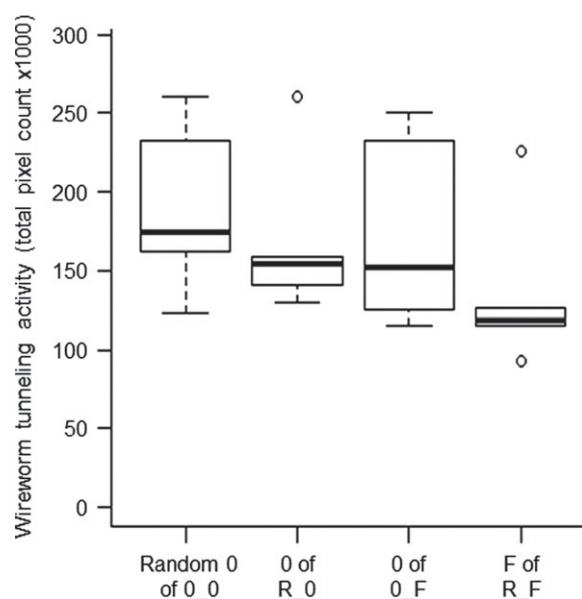
Figure 6 Wireworm tunnelling activity in choice treatment combinations as pixel counts from sites as described in Figure 2 (n = 6). Boxes show the 1st (bottom line), 2nd (median, bold line), and 3rd (top line) quartiles of the data distribution, whisker ends represent the lowest and the highest data point within the 1.5 interquartile range (IQR) of the lower and the upper quartile, respectively. Circles represent outliers between 1.5 and 3 IQR.

decrease in the number and/or length of tunnels burrowed by the wireworms.

Based on these results, it seems that reduced wireworm mortality was related to reduced activity, which would support the 'repel'-hypothesis. Shah et al. (2008) detected a synergistic interaction between neem seed cake and *Metarhizium*, leading to increased mortality in vine weevil larvae. They hypothesised that repellent effects of neem elicited escape behaviour of their insects, leading to increased movement in EPF-contaminated substrates and hence accumulation of spores on the insects' cuticle. In our experiment, the exact opposite was observed. The garlic extract caused a decrease in wireworm activity, which may have resulted in fewer spores accumulating on the wireworms' cuticle, which could explain the antagonism found in the no-choice set-up. This conclusion, however, is questioned by a lack of antagonism in choice treatments (see below).

Wireworm mortality in no-choice treatment combinations differed significantly between both experimental cohorts at 3 and 6 weeks, but not 9 weeks at the end of the experiment. This can be explained by differences in the EPF's killing speed. In a bioassay with dip inoculation, the median lethal time of the used strain *M. brunneum*

Table 5 Coefficients, standard errors of the mean, and P-values of the fitted generalised linear model on fungal germination rates in substrates treated with garlic extract (repellent) or the unloaded carrier substance (without garlic extract)

Factor	Estimate	SEM	P
Intercept	1.245	0.079	<0.001
Carrier substance	−0.154	0.096	0.085
Repellent	−0.498	0.088	<0.001

ART2825 in *A. obscurus* was determined to be 14 days with 11–16 days as upper and lower confidence interval (Eckard et al., 2014). In the present experiment, wireworms were not dipped into a spore suspension but eventually picked up inoculum when burrowing through EPF-treated substrate. This was probably adding even more variance to speed of kill, since infection depended on individual wireworm behaviour. Nevertheless, the final mortality, which was achieved at the latest at 9 weeks, was equal in both cohorts.

In choice set-ups, with treatments applied to different sides of the terraria, EPF application was again the only one eliciting wireworm mortality. In contrast to no-choice experiments, in the choice experiments there was no interaction detected between the EPF and the garlic treatment, despite the fact that wireworm activity was decreased in the EPF-treated sides of the terraria, when garlic was applied to the neighbouring sides. A major difference between the choice and the no-choice experiment was that *Metarhizium* spores were spatially separated from the garlic extract in the former, whereas both treatments were in direct contact in the latter. It follows that there may have been a direct antagonistic interaction between the EPF and the garlic extract in no-choice treatments. Extracts of *Allium* spp., like many other plant essentials, have been shown to contain antimicrobial and antifungal compounds (Yoshida et al., 1987; Pai & Platt, 1995; Bakkali et al., 2008). Spore germination experiments from substrate samples of no-choice terraria pointed in the same direction. Samples from untreated substrates or substrates containing the carrier material without garlic extract showed average spore germination rates of 81 and 86%, respectively, whereas garlic-treated substrates yielded significantly lower average germination rates of 60%. Reduced wireworm mortality in combined treatments from no-choice set-ups may therefore simply be a consequence of a lower number of viable *Metarhizium* spores. This would explain why the antagonism was not found in treatment combinations of choice set-ups, where *Metarhizium* spores and the garlic extract had very limited direct contact.

The observed decrease in tunneling activity may be explained as a behavioural response to stress. Wireworms react to stress with reduced foraging activity (Vernon et al., 2008; van Herk & Vernon, 2013), and the combined treatment of wireworms with sublethal insecticide concentrations and an EPF was already shown to increase overall mortality in a previous study (Ericsson et al., 2007). According to Shah et al. (2008), EPFs might surmount a host insect's defence mechanisms more easily, if the insect is already struggling with a stress factor exhibiting repellent, antifeedant, or other sublethal toxic effects. With the application of the garlic extract to the entire terrarium substrate (no-choice), we intended to induce a similar 'stress' situation. However, as this did not lead to increased, but decreased wireworm mortality, our data do not support the 'stress' hypothesis in the sense of Shah et al. (2008). Still, we cannot rule out that 'stressed' wireworms in the treatment combination succumbed to mycosis after infection with fewer spores than in the fungal treatment alone. If at all, however, such a synergistic effect must have been weak and was probably levelled out by the reduced germination rate of *Metarhizium* spores in the presence of the garlic extract.

The combination of the EPF and the garlic treatment on separate sides of the terraria (choice set-up) had a negative effect on wireworm activity in the EPF-treated side. One explanation is that reduced tunnelling in EPF-treated sides neighbouring a garlic treatment may be due to the volatile nature of some of the compounds of the garlic extract. Repellent compounds may have diffused from the garlic treated into the EPF-treated side. If so, mobility of the garlic extract compounds repelling wireworms and those inhibiting conidia germination must be different, as the latter was not affected in choice treatments.

Another possible explanation is that wireworms were not only able to sense the garlic extract but also the EPF, and tried to avoid garlic-treated substrates as well as those contaminated with fungal spores. Evidence for that was already found by Kabaluk et al. (2005), who reported that wireworms emigrated from plots treated with high concentrations of *Metarhizium* spores. In our no-choice set-up, there was no significant decrease, but at least a tendency to decreased wireworm activity in pure EPF treatments. Whether or not this was an indication of avoidance behaviour remains to be tested in further studies. It is clear, however, from the results presented that less tunnelling activity did not generally save wireworms from mycosis and that their avoidance behaviour, if present at all, seems far from being effective. It is important to note that tunnelling activity was only assessed once, 1 week after application. At this time, most of the wireworms were still alive and mycosis was not visible from the outside,

although many of them have probably been infected. Therefore, a third explanation for reduced tunnelling in EPF-treated sides is that wireworms did not reduce their activity in advance, to avoid contact with infectious substrates, but only after being infected by the EPF, as a first symptom of infection. In no-choice set-ups, wireworm activity tended to be lower in treatments where only EPF but not garlic is involved, which supports this explanation. Lethargy as a symptom of recent infections of host insects was described earlier (Madelin, 1963). In addition, it is known from other soil insects that they try to get rid of spores by moulting before the EPF is able to penetrate the cuticle, which also implies a reduction of foraging behaviour and, hence, movement (Ferron, 1967).

In future experiments, the observation device and image analysis protocol presented here may be employed to test these hypotheses in more details. For example, the method may easily be adapted to differentiate between behavioural changes at different time points in the course of a longer observation period. This may be done by mapping tunnels with different colours at multiple census dates. These colours may then be selected and pixel counts assessed individually in the course of the image analysis process, and help unveil behavioural changes over time or transient effects during shorter periods.

Evaluation of the image analysis procedure

The big advantage of the presented image analysis protocol is its flexibility to quantify activity of soil insects at various time periods, spanning hours, days, or even weeks. Data collected with this approach quantify soil insect activity during entire observation periods rather than providing snapshot evidence of an insect's position at a certain census date, as given in earlier studies. Schumann et al. (2013) examined the attractivity of CO_2-emitting beads for western corn rootworm (WCR) larvae, *Diabrotica virgifera virgifera* LeConte. Their approach revealed larval position at a defined moment but left uncertainty about the activity in between census dates. van Herk & Vernon (2007) used a similar approach and tracked wireworm movements in a horizontal 2D-arena. Their data gave a consistent picture of wireworm activity for a period of 2 h; however, this approach did not allow observation over larger time periods like days and weeks. In addition, we regard a vertical set-up as more appropriate, since it takes into account the natural escaping behaviour of wireworms (Furlan, 2004). There are methods available which are even better suited to reflect the natural situation in soil, as they allow to observe soil insect behaviour in three dimensional spaces, like x-ray (Mankin et al., 2008) or acoustic techniques (Johnson et al., 2007). The main drawback of these techniques, however, is that both require special equipment

and training. The more natural habitat in these techniques therefore clearly comes at the cost of ease of use and affordability under conventional laboratory conditions. Additionally, x-ray use may be harmful to insects under observation, especially when large arenas or high resolution require high doses of radiation (Johnson et al., 2007).

Assessing the efficacy and the mode of action of treatments against soil insects – whether applied singly or in combination – may be difficult when results are based on mortality data alone. The terrarium set-up combined with the image analysis protocol used in this study provides a simple and efficient approach to study the behaviour of soil insects on a larger and flexible temporal scale after application has taken place. This can help to unveil the nature of interactions, which are otherwise difficult to observe in a below-ground, concealed environment. In the present study, the method was used successfully to understand the mechanisms behind the antagonistic interaction between the EPF and the garlic treatment against wireworms. However, it can easily be adapted to study the behaviour of other soil-dwelling insects and to elucidate their interactions with natural antagonists or chemical compounds with attractant, repellent, antifeedant, or sublethal toxic effects.

Acknowledgements

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Appendix I

Step by step description of image analysis using Gimp 2.6 (GNU Image Manipulation Program; Kimball et al., 2008), to quantify tunnel traces re-drawn and photographed from front terrarium glass plates:

- Select red areas representing the wireworm tunnels with the 'Select by color' tool, balance the threshold in order to select a maximum of tunnel pixels, and exclude artefacts from the background.
- Copy-paste the selection into a new picture (640 × 400 px) with white background.
- Select the background and set its default colour to black. After deleting the selected background, red tunnels on black background are shown.
- Select red tunnels with the command 'invert selection' ([ctrl] + [i]).
- Set the default background colour to white and delete the selected red tunnels, which consequently turn white.
- Select the area of the image to be analysed (treated/untreated terrarium halves, or whole terrarium) with the tool 'rectangle', or – if necessary to exclude artefacts – manually with the selection tool 'Free/Lasso'.
- Chose the menu 'Colors', then 'Info', and finally 'Histogram' in order to measure the white pixels in the selected area as follows:
  - Leave the channel in the default setting 'Value'.
  - Set the left colour threshold to 128 in order to retrieve all white pixels which range from 128–255 (128 > black) and to avoid compression artefacts in both directions.
  - The program displays the white pixels, representing tunnel traces, as 'Count'.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Coefficients, standard errors of the mean, and P-values of the fitted linear models on wireworm tunnelling in no-choice treatment combinations (0_0, R_R, F_F, RF_RF; see Figure 2 for explanation) and the untreated control (0_0). Pixel counts per live wireworm were used as dependent variable to account for the effect of wireworm mortality on tunnelling activity within the 1st week.

Table S2. Coefficients, standard errors of the mean, and P-values of the fitted alternative linear models on wireworm tunnelling in choice treatment combinations (R_F, 0_F, R_0; see Figure 2 for explanation) and the untreated control (0_0). Pixel counts per live wireworm were used as dependent variable to account for the effect of wireworm mortality on tunnelling activity in the 1st week.