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Mortality risk from entomopathogenic fungi affects oviposition behavior in the parasitoid wasp *Trybliographa rapae*



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ABSTRACT

Biological control of pests in agroecosystems could be enhanced by combining multiple natural enemies. However, this approach might also compromise the control efficacy through intraguild predation (IGP) among the natural enemies. Parasitoids may be able to avoid the risk of unidirectional IGP posed by entomopathogenic fungi through selective oviposition behavior during host foraging. *Trybliographa rapae* is a larval parasitoid of the cabbage root fly, *Delia radicum*. Here we evaluated the susceptibility of *D. radicum* and *T. rapae* to two species of generalist entomopathogenic fungi, *Metarhizium brunneum* isolate KVL 04-57 and *Beauveria bassiana* isolate KVL 03-90. Furthermore, *T. rapae* oviposition behavior was assessed in the presence of these entomopathogenic fungi either as infected hosts or as infective propagules in the environment. Both fungi were pathogenic to *D. radicum* larvae and *T. rapae* adults, but with variable virulence. When host patches were inoculated with *M. brunneum* conidia in a no-choice situation, more eggs were laid by *T. rapae* in hosts of those patches compared to control and *B. bassiana* treated patches. Females that later succumbed to mycosis from either fungus laid significantly more eggs than non-mycosid females, indicating that resources were allocated to increased oviposition due to perceived decreased life expectancy. When presented with a choice between healthy and fungal infected hosts, *T. rapae* females laid more eggs in healthy larvae than in *M. brunneum* infected larvae. This was less pronounced for *B. bassiana*. Based on our results we propose that *T. rapae* can perceive and react towards IGP risk posed by *M. brunneum* but not *B. bassiana* to the foraging female herself and her offspring. Thus, *M. brunneum* has the potential to be used for biological control against *D. radicum* with a limited risk to *T. rapae* populations.

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1. Introduction

Combining multiple natural enemy species may result in a more efficient biological control of pest insects in agroecosystems (Cardinale et al., 2003; Letourneau et al., 2009; Snyder et al., 2006, 2008; Stiling and Cornelissen, 2005). However, natural enemies can interact unintentionally disrupting biocontrol efficiency. Identification of the mechanisms underlying such interactions is thus vital to mitigate potentially adverse effects (Straub et al., 2008).

Enhanced regulation of pest populations through a conservation biological control strategy (Eilenberg et al., 2001) targeting the indigenous natural enemy community could be complemented by inoculation with commercialized biological control agents such

as entomopathogenic fungi (de Faria and Wraight, 2007). However, combining multiple natural enemies against the same pest species could compromise control through intraguild predation (IGP) (Straub et al., 2008). IGP is evident when both competition and predation (including the actions of predators, parasitoids and pathogens) occur between species which share a common prey or host resource (Rosenheim et al., 1995). Chemical cues emanating from the host and its environment guide parasitoids during host foraging (Afsheen et al., 2008; Girling et al., 2011; Mills and Wajnberg, 2008; Vet and Dicke, 1992) to identify suitable host patches and high quality hosts in order to maximize offspring survival and thus increase parasitoid fitness. Snyder and Ives (2008) argued that by exhibiting anti-predator behavior at foraging, such as selective oviposition behavior, IGP may be less disruptive to parasitoids. Thus, the mortality risk perceived by the parasitoid may affect e.g. the decision to oviposit and egg allocation to a specific patch. It has been demonstrated that parasitoids avoid foraging in host patches with predators (e.g. Petersen et al., 2000;

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Nakashima et al., 2004), and that discrimination between healthy and fungal infected hosts does occur in some parasitoids (Brobyn et al., 1988; Fransen and van Lenteren, 1993; Mesquita and Lacey, 2001).

The cabbage root fly, *Delia radicum* L. (Diptera: Anthomyiidae) is a noxious pest on cruciferous crops in temperate climates throughout the Holarctic region. The female fly oviposits close to the stem base and the larva feeds by burrowing into the roots, causing crop damage (Finch, 1989).

Natural enemies of *D. radicum* include parasitoids, such as the larval specialist *Trybliographa rapae* Westwood (Hymenoptera: Figitidae), and the pupal specialists *Aleochara bipustulata* L. and *A. bilineata* Gyllenhal (Coleoptera: Staphylinidae) (Fuldner, 1960; Wishart and Monteith, 1954). Important egg predators of *D. radicum* are *Bembidion* spp. and *Agonum* spp. (Coleoptera: Carabidae) (Prasad and Snyder, 2004), while adults of *Aleochara* spp. also serve as predators on immature stages (Fuldner, 1960; Hartfield and Finch, 2003). Entomopathogenic fungi including the generalist genera *Beauveria* and *Metarhizium* (Ascomycota: Hypocreales) (Bruck et al., 2005) and the specialist species *Entomophthora muscae* (Cohn) Fresenius (Entomophthoromycota: Entomophthorales) (Klingen et al., 2000) can together target all stages in the life cycle of *D. radicum*.

Eilenberg and Meadow (2003) suggested that inundation biological control with a highly virulent isolate of *M. anisopliae* (Metsch.) Sorokin *sensu lato* or *B. bassiana* (Balsamo) Vuillemin *sensu lato* would be an efficient strategy against the immature stages of *D. radicum*. Several isolates of these two genera have been screened through laboratory, greenhouse and field trials for their efficacy to control *D. radicum*, targeting larvae, pupae (Bruck et al., 2005; Chandler and Davidson, 2005; Vänninen et al., 1999a,b), and adults (Meadow et al., 2000).

Females of *T. rapae* attack all three larval instars of *D. radicum* and the parasitization rate in production fields varies from a few percent up to >50% (Hemachandra et al., 2007a; Meyling et al., 2013; Wishart and Monteith, 1954). Host patch choice by *T. rapae* is based on volatile cues released from plants infested with *D. radicum* larvae (Brown and Anderson, 1999; Neveu et al., 2002; Nilsson et al., 2012), informing about e.g. host density (Hemachandra et al., 2007b; Jones and Hassell, 1988) and attack from other herbivores (Pierre et al., 2011). However, it is unknown whether *T. rapae* can evaluate the suitability of host patches inoculated with generalist entomopathogenic fungi or fungal infected hosts and how oviposition behavior is affected.

We hypothesize that there is a risk for foraging *T. rapae* females, through unidirectional IGP, by introducing generalist entomopathogenic fungi such as *Metarhizium* spp. and *Beauveria* spp. to the agroecosystem. The aims of this study thus were (1) to evaluate the susceptibility of *D. radicum* and *T. rapae* to two species of entomopathogenic fungi and (2) to investigate *T. rapae* oviposition behavior during host foraging when entomopathogenic fungi were present either as infected hosts or as infective propagules in the environment.

2. Materials and methods

2.1. Insects

Cabbage root flies *D. radicum* and their parasitoid *T. rapae* were continuously reared under L:D 16:8 h on Swedish turnips cultivar 'Vige' as described by Nilsson et al. (2011) which was modified from Finch and Coaker (1969) and Neveu et al. (1996).

D. radicum larvae for bioassays were reared in polystyrene boxes (173 × 112 × 40 mm) prepared with 1 cm sand (0.8–1.2 mm, Rådasand, Sweden) in the bottom and 3 mm moistened

vermiculite (2–5 mm, Weibulls Horto, Sweden) spread on top of the sand. Newly laid eggs (opaque white, <24 h old) were taken from the continuous rearing and placed on the sand-vermiculite in the boxes. A 1.5–2 cm thick turnip slice with peel was carefully placed on top of the eggs. Small incisions in the peel had been prepared to facilitate larvae penetration.

The boxes with *D. radicum* were placed 2–4 together inside a larger plastic box (400 × 300 × 190 mm) with moistened tissue paper in the bottom and the lid slightly opened to allow aeration, and incubated at 20 ± 1 °C in darkness. After 13 days the *D. radicum* larvae were picked with a soft forceps and placed in a petri dish with moist filter paper until used within two hours. The mean size (±SD) of the larvae used (length 4.9 ± 0.78 mm, width 1.50 ± 0.23, n = 123) corresponded to early third instar, the stage preferred by *T. rapae* (Neveu et al., 2000).

Adults of *T. rapae* were used for dose–response infection assays 1–2 days after emergence, with equal numbers of males and females. In the choice and non-choice bioassays 2–4 day old females were used, corresponding to the age of maximum egg laying (Jones, 1986). All bioassays included medium sized specimens (≈2 mg).

2.2. Fungi

Isolates of two generalist entomopathogenic fungal species were used for the experiments; *Metarhizium brunneum* Petch (isolate KVL 04-57) and *Beauveria bassiana* (isolate KVL 03-90), which are stored at –80 °C at the University of Copenhagen, Department of Plant and Environmental Sciences. The *M. brunneum* isolate has the same genotype as the commercial biological control agents F52/Met52 (Novozymes) and GranMet/Bipesco 5 (Samen Schwarzenberger, Austria) (Nielsen et al., 2006) which were found to show relatively high virulence against *D. radicum* larvae (Bruck et al., 2005). Both fungal species occur naturally in agricultural soil and *B. bassiana* was found to naturally infect adult *T. rapae* (Meyling et al., 2011).

Stock cultures of the isolates were grown on 4% Sabouraud dextrose agar (SDA; Merck, Sweden) in vented petri dishes and then stored at 8 °C for up to six months. Subcultures were prepared by transferring conidia from stock culture plates onto new SDA plates and incubating at 20 ± 1 °C for 20 days before use in the experiments. Conidia were harvested by flooding the cultures with sterile 0.05% Triton-X 100 (VWR, Sweden), and scraping with a sterile L-shaped spreader (VWR, Sweden) and the resulting suspensions transferred to 50 ml centrifuge tubes (Sarstedt, Sweden). The suspensions were then centrifuged twice for 3 min at 3000 rpm (Eppendorf Centrifuge 5702) and supernatant with hyphal fragments discarded and replaced by sterile 0.05% Triton-X 100. Concentrations of the resulting stock suspension were established in a haemocytometer (Fuchs–Rosenthal 0.0625 mm², depth 0.200 mm, VWR, Sweden). To assess conidial viability, germination tests were prepared by plating 100 µl of 10^{–2} dilutions onto SDA and incubating at 20 ± 1 °C for 24 h. Germination was evaluated under 400X magnification (Leitz Wetzlar Dialux 20) under three separate cover slips (24 × 40 mm, Chance propper Ltd., England) per plate on three individual plates. A conidium was considered germinated when the germ tube extended beyond the width of the conidium (Inglis et al., 2012). The mean (±SD) germination for all assays was 98.9 ± 0.81% for *M. brunneum* and 92.3 ± 4.39% for *B. bassiana*. Stock suspensions of conidia were refrigerated and used the day after preparation, at which time the germination tests were evaluated.

2.3. *D. radicum* dose–mortality and time–mortality bioassays

The objectives of this experiment were to (a) determine the virulence (median lethal concentration, i.e. LC₅₀) of the two fungal

isolates against early third instar *D. radicum* larvae, (b) estimate the LC₉₀ for use in the *T. rapae* dual-choice bioassays (see Section 2.5.2 below) and (c) determine the time–mortality response at different concentrations.

From the stock conidia suspensions the following concentrations were prepared; 1×10^4 , 1×10^5 , 1×10^6 , 1×10^7 , 1×10^8 and 1×10^9 conidia ml⁻¹ and a control with sterile 0.05% Triton-X 100. Separate batches of 10 third instar larvae were immersed in 5 ml of the respective suspensions by placing them on the edge of a test tube and carefully pushing them into the suspension with a sterile inoculation loop moistened by the suspension. The test tube was vortexed for 1 s, after which the larvae were left in the suspension for 20 s, and then poured onto a filter paper in a Büchner funnel and left to air dry for 1 min. The larvae were transferred individually to separate 30 ml medicine cups (Hammarplast Medical AB, Sweden) with 20 × 20 mm filter papers, moistened by deionized water, placed on the wall of the cup. The cups were incubated in darkness at 20 ± 1 °C. After 24 h the filter paper was removed and a thin slice of turnip (15 × 15 × 3 mm) was provided to each larva allowing for observation of larval condition with minimum disturbance. Avoiding placement of items in the cups during the first 24 h minimized the opportunity for larvae to mechanically remove conidia from the cuticle. The turnip slice was replaced every five days. The larvae were checked daily for mortality for 7 days, since pupation started after this period. Dead larvae were surface sterilized in 10% sodiumhypochlorite (Sigma–Aldrich, Sweden) for 5 s, then rinsed in deionized water for an additional 5 s after which they were incubated in sealed medicine cups under moist conditions.

As a criterion of mycosis the color of infected larvae, subsequent mycelial protrusion and the formation of distinctive conidia was used. Infected larvae usually turned characteristically hard and cream-colored for *M. brunneum* and pinkish purple for *B. bassiana* prior to emergence of mycelia. Mycelia protrusion usually occurred from mycosed larvae the day after death with subsequent formation of conidia.

The treatments were arranged in a completely randomized design on trays (270 × 197 mm, Hammarplast Medical AB, Sweden) in polystyrene boxes (310 × 225 × 126 mm, COFA, Sweden). The experiment was replicated on four different occasions, each time with 10 larvae for each concentration and fungal isolate.

2.4. *T. rapae* dose–mortality and time–mortality bioassays

Bioassays with the two fungal isolates were performed in order to (a) determine the virulence (LC₅₀) to adult *T. rapae*, and (b) determine the time–mortality relationships at different concentrations.

The concentrations prepared were: 1×10^5 , 1×10^6 , 1×10^7 , 1×10^8 and 1×10^9 conidia ml⁻¹ and a control with 0.05% Triton-X 100. For each concentration 10 adult individuals (5 males and 5 females) were individually inoculated by applying 1 µl of the suspension on the thorax with a pipette, resulting in expected exposure rates of 10², 10³, 10⁴, 10⁵ and 10⁶ conidia per individual. After inoculation each individual was put singly in a sealed medicine cup under moist conditions. After 24 h incubation in the moist chambers, the wasps were provided with a cotton wick soaked in 0.3 M sucrose as food, in a new medicine cup. The food was renewed weekly. The wasps were incubated in L:D 16:8 h and monitored daily for 14 days. Dead wasps were surface sterilized as described in Section 2.3 and transferred to moist chambers. A wasp was considered to be mycosed if mycelia protruded through the cuticle after death and subsequently formed distinctive conidiation. The experiment was repeated on four different occasions, each time with 10 individuals for each concentration and fungal

isolate. The treatments were arranged in a completely randomized design in polystyrene boxes as for *D. radicum*.

2.5. *T. rapae* choice bioassays; experimental arena and post assay procedure

The experimental arena ('patch') consisted of a 55 mm petri dish (VWR, Sweden), containing a 50 mm filter paper (quality 1701, Munktell Filter AB, Sweden) and a 35 × 35 × 6 mm piece of turnip. Larvae of *D. radicum*, treated as described for the respective choice bioassays in Sections 2.5.1 and 2.5.2 below, were distributed on the turnip. The thickness of the turnip allowed free probing access for the parasitoid, since the ovipositor length is 2.9 mm (Brown and Anderson, 1998). Around the turnip 2 ml of dry vermiculite (2–5 mm) was evenly distributed. The petri dishes were sealed with parafilm and incubated for 18 h in darkness at 20 ± 1 °C for *D. radicum* larvae to establish in the turnip. The following day, the parafilm and lids were removed. For the respective choice bioassay, the vermiculite was then inoculated as described below in Sections 2.5.1 and 2.5.2. Just before the onset of the choice bioassays a 20 mm high cylindrical metal barrier (mesh width 0.8 mm, Sintab, Sweden) with 5 mm inward overhang was placed around the outside of each petri dish to prevent larvae from leaving the arena. Two arenas were placed inside a transparent plastic box (185 × 185 × 115 mm). At the onset of the choice bioassays, a 2–4 day old mated and sugar-fed female *T. rapae* was introduced into each box. The parasitoid had access to water and 0.3 M sucrose in 30 ml cups through a 4 cm piece of dental cotton roll throughout the experiment. The bioassays were performed in a climate cabinet for 24 h at 20 ± 1 °C and L:D 16:8 h with illumination provided by white fluorescent lamps (Long Life T8 Ultimate 36 W/830 3000 K, Aura Light, Sweden) reaching ca 4200 lux inside the boxes.

After termination of the experiments described in Sections 2.5.1 and 2.5.2, the females of *T. rapae* were incubated individually in medicine cups at 20 ± 1 °C in L:D 16:8 h, provided with a cotton wick soaked in 0.3 M sucrose, and monitored daily during 14 days for mortality. Dead wasps were treated and mycosis assessed as described in Section 2.4.

Larvae of *D. radicum* from each host patch arena were placed in glass vials and frozen overnight. The larvae were subsequently dissected and observed for parasitoid eggs in dilutions of a few drops of green food coloring dye (Ekströms, Sweden) in 10 ml distilled water. Two separate drops of the mixture were pipetted on a glass slide, one larva was placed in one drop, and the head cut off with a scalpel. With the blunt end of the scalpel the content of the larva was then pressed and scraped out into the drop. The head and the larval integument were transferred to the other drop. Cover slips were placed over the drops, pressed gently and the content inspected for parasitoid eggs (Jones, 1986) under 60X magnification (Wild Heerbrugg, 195672).

2.5.1. Choice bioassays on host patch quality under no-choice and dual choice situations

The objectives of these experiments were to evaluate the oviposition behavior of *T. rapae* females when infective fungal propagules were present in the host patch in (a) a no-choice situation and in (b) a dual choice situation.

Thirteen day old *D. radicum* larvae were inoculated with Triton-X 100 and treated as described in Section 2.3. After 24 h incubation 10 larvae were randomly selected and transferred to an experimental arena, where they were left to feed for 18 h.

For the no-choice bioassay the host patches were inoculated by pipetting either 1.5 ml 0.05% Triton-X 100 (Control), 1.5 ml *M. brunneum* 1×10^8 conidia ml⁻¹ suspension, or 1.5 ml *B. bassiana* 1×10^8 conidia ml⁻¹ suspension, to the vermiculite around the turnip piece. Two arenas of the same treatment were placed in the

experimental box, and a female *T. rapae* introduced. The boxes were placed in a randomized block design, and the experiment was replicated on eight occasions with two blocks each time ($n = 16$).

In the dual choice situation, each *T. rapae* female was offered the choice between two host patches where the vermiculite was inoculated with 1.5 ml Triton-X 100 (Control) or 1.5 ml of a 1×10^8 conidia ml^{-1} suspension of either *M. brunneum* or *B. bassiana*. The position of the treatments (left or right) within the box was randomized. The experiments were replicated on three occasions with six boxes per fungal isolate each time ($n = 18$).

2.5.2. Dual choice bioassays on host quality

The objective of this experiment was to reveal whether ovipositing *T. rapae* females are able to discriminate between healthy and fungal infected hosts.

A surplus of 13 day old *D. radicum* larvae were treated as described in Section 2.3, and inoculated with either; a suspension of 1×10^8 conidia ml^{-1} of *M. brunneum*, or 1×10^9 conidia ml^{-1} of *B. bassiana*, or 0.05% Triton-X 100 (Control). The previous dose-mortality bioassays of *D. radicum* revealed that at these concentrations all exposed *D. radicum* larvae could be expected to become infected ($>LC_{90}$; Table 1). After 24 h incubation 10 larvae were randomly selected from each treatment and transferred to an experimental arena, and left to feed for 18 h. At the start of the experiment, post-inoculation time of the larvae was 42 h (24 + 18 h). This was considered sufficient time for the fungi to germinate, penetrate the cuticle and start to proliferate in internal tissues. The vermiculite around the turnip of each host patch was subsequently moistened with 1.5 ml of sterile deionized water.

Two host patch arenas, one with 10 fungal infected larvae and one with 10 control larvae, were placed in opposite corners in a plastic box, and the female *T. rapae* introduced. The position of the treatments (left or right) within the box was randomized. The experiments were replicated on four occasions with six boxes per fungal isolate each time ($n = 24$).

2.6. Statistical analysis

Data were analyzed in R statistical software version 3.1.0. (R Core Team, 2012), whereas Survival Analysis was performed with the software SPSS Statistics Version 20.0 (IBM Corp., 2011). For the dose-mortality bioassays the mortalities were corrected for control mortality using Abbott's formula (Abbott, 1925). Control mortalities were always less than 5% and 10% for *T. rapae* and *D. radicum*, respectively. The effect of increasing concentrations of the fungal isolates on the proportional number of mycosed insects was analyzed using a Probit analysis of binomial proportions, and the lethal concentrations for 50% mortality (LC_{50}) and 90% mortality (LC_{90}) calculated, including their 95% fiducial limits (Finney, 1952). For *T. rapae* the response at day 7 was chosen, since investigations on the lifetime oviposition pattern showed that the mean

daily fecundity is highest during the first six days after emergence (Jones, 1986). For *D. radicum*, day 7 was also chosen, since after this time the larvae started to pupate. Assumptions of homogeneity of variance between the blocks were met, and the data sets were thus pooled for each experimental treatment.

A Cox proportional-hazards regression model (Cox, 1972) was used for analyzing the time-mortality response (i.e. survival) of all fungal concentration compared to baselines, for *D. radicum* over 7 days and for *T. rapae* over 14 days. The Cox proportional hazard is expressed as the hazard ratio (relative average daily risk of death), which is assumed to remain constant over time. The event was defined as mycosis, i.e. death from fungal infection. Specimens that died from other causes were omitted from further analysis. There were no incidence of mycosis in the controls (hence no variance), thus the lowest fungal concentrations resulting in mycosis were chosen as the baseline for comparison of hazard ratios. Furthermore, preliminary analysis showed no significant difference in hazard ratio between the control and the lower concentrations. Factors were block and fungal concentration for both species and additionally sex for *T. rapae*. The proportional cumulative survival of 50% of the population, i.e. median survival time (MST), of the insect populations at the various concentrations and their 95% confidence intervals were calculated and pairwise compared with a log-rank χ^2 test (Bewick et al., 2004).

The egg count data of the no-choice bioassay were assessed using a generalized linear mixed model (GLMM) with binomial error distribution and log-link function to compare the ovipositing and non-ovipositing females (1/0), and a GLMM with Poisson error distribution and log-link function was used to evaluate the egg count data with the treatment as fixed factor. In a second Poisson GLMM model, the egg counts in the no-choice bioassays were evaluated with the incidence of mycosed females (infected/non-infected) and their longevity (up to 14 day s) as fixed factors. The number of eggs laid in the dual-choice bioassays of host and host patch quality were analyzed using a binomial GLMM for proportions. The Linear Mixed Effects "lme4" package was used to perform all GLMM including block as random effect. Data overdispersion was checked in all the models, but all values were below 2.

3. Results

3.1. *D. radicum* and *T. rapae* dose-mortality bioassays

Both fungal isolates were pathogenic to *D. radicum* larvae and *T. rapae* adults and increasing fungal concentrations resulted in an increase in mortality (Table 1). For *D. radicum* larvae exposed to *M. brunneum* or *B. bassiana*, the LC_{50} values were 2.44×10^6 and 1.08×10^7 conidia ml^{-1} while the LC_{90} values were 7.54×10^7 and 4.84×10^8 conidia ml^{-1} , respectively. Inoculation of adult *T. rapae* with *M. brunneum* or *B. bassiana* resulted in LC_{50} values of 1.57×10^7 and 1.83×10^7 conidia ml^{-1} and LC_{90} values of 1.78×10^8 and 2.42×10^8 conidia ml^{-1} , respectively (Table 1).

Table 1

Results of probit analysis of binomial proportions and calculated lethal concentration values of dose-mortality response assays 7 days post-inoculation of *D. radicum* and *T. rapae*. The concentrations presented are conidia ml^{-1} .

Insect host	Fungal isolate	n	df ^a	Slope ± SE	Z ^b	P(Z)	χ^2 ^c	P(χ^2)	LC50 (95% FL)	LC90 (95% FL)
<i>Delia radicum</i>	<i>M. brunneum</i> KVL 04-57	224	4	0.86 ± 0.093	9.21	<0.001	152.30	<0.001	2.44×10^6 (1.35×10^6 to 4.42×10^6)	7.54×10^7 (3.00×10^7 to 1.89×10^8)
	<i>B. bassiana</i> KVL 03-90	234	4	0.78 ± 0.084	9.25	<0.001	139.78	<0.001	1.08×10^7 (5.76×10^6 to 2.02×10^7)	4.84×10^8 (1.68×10^8 to 1.40×10^9)
<i>Trybliographa rapae</i>	<i>M. brunneum</i> KVL 04-57	196	3	1.22 ± 0.15	8.29	<0.001	156.43	<0.001	1.57×10^7 (9.66×10^6 to 2.56×10^7)	1.78×10^8 (8.38×10^7 to 3.77×10^8)
	<i>B. bassiana</i> KVL 03-90	198	3	1.14 ± 0.14	8.44	<0.001	147.54	<0.001	1.83×10^7 (1.11×10^7 to 3.01×10^7)	2.42×10^8 (1.10×10^8 to 5.32×10^8)

FL = fiducial limits.

^a df = number of terms (i.e. concentrations) used for the regression minus 2 (Finney, 1952).

^b Z is the ratio of the coefficients to the standard error of the fungal concentrations.

^c likelihood ratio χ^2 test statistic indicates a satisfactory goodness-of-fit of empirical data compared to estimated regression line.

3.2. *D. radicum* time–mortality bioassays

In the Cox model for survival of *D. radicum* larvae treated with different fungal concentrations no statistically significant differences were observed between the blocks, neither for *M. brunneum* nor *B. bassiana* (Table 2). The concentrations of both fungal species had effects on larval survival. With the concentration 1×10^6 conidia ml^{-1} as the Cox model baseline, there were significant differences compared to the concentrations 1×10^8 and 1×10^9 conidia ml^{-1} for both fungi (Table 2). The hazard ratios (HR) increased with increasing fungal concentration while the MST of *D. radicum* decreased with increasing fungal concentrations (Table 2). At the highest concentration (1×10^9 conidia ml^{-1}) the MST was 4 days for *M. brunneum* compared to 5 days for *B. bassiana*.

3.3. *T. rapae* time–mortality bioassays

Survival of *T. rapae* adults treated with different concentration of *M. brunneum* was not affected by experimental blocks or sex of parasitoids while there was a significant effect of fungal concentration (Table 3). All concentrations were significant different from 1×10^5 conidia ml^{-1} as the Cox model baseline (Table 3).

For *B. bassiana*, no differences were observed between the blocks, but there was a significant difference between males and females (Table 3). The life span over all fungal concentrations was (mean \pm SD) for females 8.8 ± 2.5 days and for males 8.1 ± 2.7 days. Fungal concentration had significant effect on survival with all concentrations compared to 1×10^5 conidia ml^{-1} as the Cox model baseline (Table 3).

With increasing fungal concentration, the MST of the *T. rapae* population decreased and the hazard ratios increased, indicating faster speed of kill by *M. brunneum* compared to *B. bassiana* (Table 3). At the highest concentration (1×10^9 conidia ml^{-1}) the MST was 4 days for *M. brunneum* and 6 days for *B. bassiana*.

3.4. Choice bioassays on host patch quality, no-choice and dual choice situations

In the no-choice situation, the treatment had no significant effect on the proportion of non-ovipositing females (binomial GLMM: likelihood ratio test (LRT) = 3.6306, $df = 2$, $P = 0.1648$). The number of eggs laid by *T. rapae* was found to be significantly dependent on the treatment (Poisson GLMM: LRT = 9.834, $df = 2$, $P = 0.00732$; Fig. 1). More eggs were laid in hosts in *M. brunneum* inoculated patches compared to the control patches (Poisson

GLMM: $Z = -2.555$, $df = 1$, $P = 0.01063$) and compared to host patches inoculated with *B. bassiana* (Poisson GLMM: $Z = -2.755$, $df = 1$, $P = 0.00587$). The numbers of eggs found in *D. radicum* larvae did not differ for those in control and *B. bassiana* inoculated host patches (Poisson GLMM: $Z = 0.213$, $df = 1$, $P = 0.832$; Fig. 1). Females that later died from mycosis from either of the fungi laid significantly more eggs than non-mycosed females (Poisson GLMM: $Z = 4.856$, $df = 1$, $P < 0.001$), but no effect was found between number of eggs laid and female longevity (Poisson GLMM: $Z = -0.886$, $df = 1$, $P = 0.3755$). For *M. brunneum* treatments, the proportion of mycosed *T. rapae* was 0.81, and their mean (\pm SD) longevity post-experiment was 5.9 (± 1.1) days ($n = 13$). The proportion of mycosed *T. rapae* due to *B. bassiana* was 0.56 ($n = 9$) and they showed a mean (\pm SD) longevity of 7.4 (± 2.8) days.

In the choice between fungal inoculated and non-inoculated host patches, *T. rapae* females did not discriminate between either *M. brunneum* and control (binomial GLMM: $Z = 0.915$, $df = 1$, $P = 0.360$), or *B. bassiana* and control (binomial GLMM: $Z = 0.918$, $df = 1$, $P = 0.359$). In the dual choice experiment, the proportion of mycosed *T. rapae* due to *M. brunneum* was 0.39 ($n = 7$), and their mean (\pm SD) longevity post-experiment was 9.1 (± 2.9) days while for *B. bassiana* the proportion of mycosed *T. rapae* was 0.44 ($n = 8$) with longevity of 7.6 (± 1.4) days.

3.5. Dual choice bioassays on host quality

When offered a choice between healthy host larvae and *M. brunneum* infected ones, *T. rapae* laid significantly more eggs in the healthy larvae (binomial GLMM: $Z = -3.283$, $df = 1$, $P = 0.00103$; Fig. 2A). However the proportions of eggs laid in healthy host larvae and those infected by *B. bassiana* were not significantly different (binomial GLMM: $Z = -1.321$, $df = 1$, $P = 0.187$; Fig. 2B). No parasitoids succumbed to mycosis by *M. brunneum* and the majority (79%, $n = 19$) survived until 14 days post-experiment, while the proportion of mycosed *T. rapae* due to *B. bassiana* was 0.48 ($n = 11$) with a mean (\pm SD) longevity of 10.1 (± 2.6) days.

4. Discussion

In this study *D. radicum* larvae were susceptible to both *M. brunneum* and *B. bassiana*. Compared to *B. bassiana*, a lower inoculum level was required for *M. brunneum* to kill half of the larvae; in addition *M. brunneum* appeared to kill faster at the highest concentration. An isolate of *M. brunneum* of similar origin as the one used here was also found by Bruck et al. (2005) to infect *D. radicum*,

Table 2
Survival analysis results of time–mortality response of *D. radicum* 7 days post-inoculation at the various concentrations for both fungal species.

Fungal isolate	Factors	MST ^a (95% CI)	HR ^b (95% CI)	Z (HR)	P (HR)	df	n
<i>Metarhizium brunneum</i> KVL 04-57	Treatment	–	–	62.56	<0.001	3	4
	Block	–	–	2.18	0.536	3	4
	1.0×10^6	–	–	–	–	–	38
	1.0×10^7	–	1.71 (0.88–3.33)	2.52	0.112	1	38
	1.0×10^8	6 (5–6)B	4.98 (2.67–9.30)	25.42	<0.001	1	40
	1.0×10^9	4 (3–5)A	9.03 (4.77–17.08)	45.74	<0.001	1	39
<i>Beauveria bassiana</i> KVL 03-90	Treatment	–	–	54.18	<0.001	3	4
	Block	–	–	2.49	0.476	3	4
	1.0×10^6	–	–	–	–	–	39
	1.0×10^7	–	1.06 (0.44–2.55)	0.016	0.899	1	39
	1.0×10^8	6 (5–7)b	5.04 (2.46–10.35)	19.43	<0.001	1	39
	1.0×10^9	5 (5–6)a	8.27 (4.05–16.86)	33.75	<0.001	1	39

The hazard ratios (HR) indicate the relative average daily risk of death compared to the baseline, which was set to 1×10^6 conidia ml^{-1} . The median survival time (MST) gives the proportional cumulative survival of 50% of the population. MST values followed by different upper or lower case letters within a column are significantly different (log rank χ^2 test, $\chi^2 \geq 6.23$, $P < 0.05$).

^a MST = median survival time, given in days.

^b HR = hazard ratio, compared to the baseline 1×10^6 conidia ml^{-1} .

Table 3Survival analysis results of time–mortality response of *T. rapae* 14 days post-inoculation at the various concentrations for both fungal species.

Fungal isolate	Factors	MST ^a (95% CI)	HR ^b (95% CI)	Z (HR)	P (HR)	df	n
<i>Metarhizium brunneum</i> KVL 04-57	Treatment	–	–	169.71	<0.001	4	5
	Block	–	–	6.73	0.081	3	4
	Sex (female)	–	0.83 (0.61–1.14)	1.32	0.250	1	2
	1.0 × 10 ⁵	–	–	–	–	–	38
	1.0 × 10 ⁶	10.5 (10–12)D	3.76 (2.00–7.08)	16.85	<0.001	1	40
	1.0 × 10 ⁷	9 (8–9)C	9.11 (4.73–17.53)	43.70	<0.001	1	39
	1.0 × 10 ⁸	6 (5–6)B	54.72 (24.95–120.03)	99.78	<0.001	1	40
	1.0 × 10 ⁹	4 (3–4)A	238.92 (101.26–563.69)	156.34	<0.001	1	40
<i>Beauveria bassiana</i> KVL 03-90	Treatment	–	–	147.78	<0.001	4	5
	Block	–	–	0.082	0.994	3	4
	Sex (female)	–	0.57 (0.42–0.77)	13.19	<0.001	1	2
	1.0 × 10 ⁵	12 (11–14)e	–	–	–	–	40
	1.0 × 10 ⁶	9 (9–10)d	3.49 (2.06–5.91)	21.63	<0.001	1	39
	1.0 × 10 ⁷	8 (8–8)c	7.64 (4.26–13.70)	46.61	<0.001	1	40
	1.0 × 10 ⁸	7 (6–7)b	25.77 (13.40–49.57)	94.77	<0.001	1	40
	1.0 × 10 ⁹	6 (5–6)a	64.03 (32.00–128.13)	138.13	<0.001	1	39

The hazard ratios (HR) indicate the relative average daily risk of death compared to the baseline, which was set to 1×10^5 conidia ml⁻¹. The median survival time (MST) gives the proportional cumulative survival of 50% of the population. MST values followed by different upper or lower case letters within a column are significantly different (log rank χ^2 test, $\chi^2 \geq 15.36$, $P < 0.05$).

^a MST = median survival time, given in days.

^b HR = hazard ratio, compared to the baseline 1×10^5 conidia ml⁻¹.

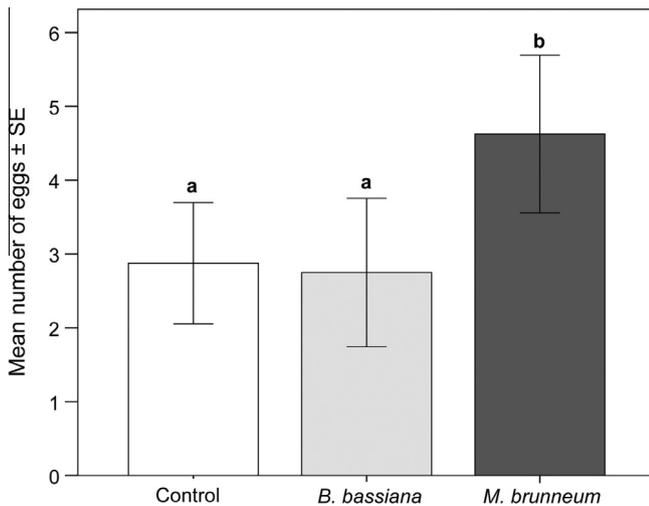


Fig. 1. Mean numbers of eggs (\pm SE) laid per female of *Trybliographa rapae* in 24 h in no-choice bioassays on host patch quality ($n = 16$). Bars followed by different letters are significantly different (Poisson GLMM, $P < 0.05$).

indicating the isolate's potential in biological control against this pest. However, the important natural enemy of *D. radicum*, the parasitoid *T. rapae*, was also susceptible to infections by the tested fungal isolates. The current study demonstrated that *T. rapae* can experience foraging time constraints at different fungal concentrations, particularly when exposed to *M. brunneum*. This study thus highlights that there is a risk associated with host foraging in fungal contaminated host patches for *T. rapae*.

Jones (1986) observed that the first 6 days after emergence is the most fecund period for *T. rapae*. In the current study the median survival time for this proovigenic wasp at the lower fungal concentrations tested was greater than 6 days. If a *T. rapae* female becomes infected, while emerging from soil contaminated with high levels of fungal inoculum, its fitness (i.e. reproductive success) is severely reduced if death occurs within the first 6 days. However, if the female has sufficient time to oviposit in high quality hosts before it dies, its fitness may not be significantly affected by the fungal inoculum. Applying a minimum dose required for adequate biological control of *D. radicum* in cruciferous crops will

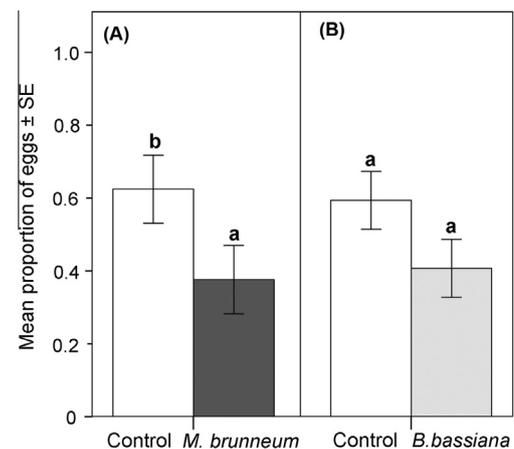


Fig. 2. Mean proportions of eggs (\pm SE) laid per female of *Trybliographa rapae* in 24 h in the dual-choice bioassays on host quality. (A) Control versus *M. brunneum* infected larvae ($n = 24$). (B) Control versus *B. bassiana* infected larvae ($n = 24$). Bars followed by different letters are significantly different (binomial GLMM, $P < 0.05$).

likely reduce the infection risk on *T. rapae* and allow the parasitoid population to persist. In a field situation the ecological susceptibility (Roy and Pell, 2000) would probably be different due to e.g. abiotic factors and local habitat differences. Surviving a fungal infection may have fitness consequences (i.e. reduced lifetime fecundity). This needs to be investigated for *T. rapae* (e.g. Alix et al., 2001), since sublethal effects of entomopathogenic fungi on reproduction have been observed for other insects (Baverstock et al., 2006; Roy et al., 2008; Seiedy et al., 2012).

Since both of the fungi tested are pathogenic to *T. rapae* it would be beneficial to the foraging parasitoid to evaluate the risk of infection in the host patch environment to reduce or avoid interaction with the fungus. However, no behavioral responses towards IGP risk posed to adult *T. rapae* were observed when either *M. brunneum* or *B. bassiana* were present in the choice situation. This inability to avoid either of the two fungi was counterintuitive since an IGP threat exists. Free conidia in arenas simulating natural habitats of other insects, including natural enemies, have been found to be deterrents. For example, termites were found to avoid the odours from dry conidia in sawdust, and the magnitude of

response was related to the virulence of the fungal isolate (Mburu et al., 2009). Meyling and Pell (2006) found that a predatory bug avoided *B. bassiana* (isolate KVL 03–90 same as in this study) conidia on leaves but not on the soil surface, which is a non-preferred habitat. In contrast, adult ladybirds avoided *B. bassiana* on both leaves and soil (Ormond et al., 2011). Our results imply that *T. rapae* may be unable to identify host patches containing free conidia.

When given no choice between host patches, a higher oviposition rate in *M. brunneum* inoculated patches compared to either control or *B. bassiana* was observed. Realized time limitation, e.g. due to a lethal infection, can influence the decisions on whether to oviposit or not and how many eggs to lay (Deas and Hunter, 2014; Javois and Tammaru, 2004; Roitberg et al., 1993). Furthermore, when life expectancy decreases, insects become less selective with the host quality and may oviposit on or in lower quality hosts than normal (Fletcher et al., 1994; Javois and Tammaru, 2004; Vet and Dicke, 1992). Our observations could therefore indicate that the female *T. rapae* perceived decreased life expectancy and thus allocated resources to oviposition rather than retaining eggs before succumbing to mycosis. Indeed, higher oviposition rates were seen from individuals that later were mycosed.

Upon finding and accepting the host patch by *T. rapae*, subsequent host location is mediated by larval movement and larva related cues, perceived by sensory organs on the antenna and ovipositor upon probing (Butterfield and Anderson, 1994; Brown and Anderson, 1998). Acceptance of suitable hosts follows the evaluation of quality traits such as instar (Neveu et al., 2000) and feeding status (Brown and Anderson, 1999) presumably through gustatory responsiveness of the ovipositor to factors present in host hemolymph (Brown and Anderson, 1998). The reduced oviposition observed in *M. brunneum* infected *D. radicum* larvae compared to the control may have been caused by perceiving the host quality as inferior for offspring development. After initiation of the fungal infection process, physical and chemical alteration of the larval cuticle and subsequently the hemocoel and hemolymph (Gillespie et al., 2000) may have been detected by sensory organs on the ovipositor of *T. rapae* females. Presence of an IG predator inside the host, such as pathogens or other parasitoids, could pose a significant threat to the offspring and thus result in rejection. Furthermore, the ability of parasitoids to avoid fungus infected hosts has been observed to be related to the stage of disease progression (Brobyn et al., 1988; Fransén and van Lenteren, 1993; Mesquita and Lacey, 2001). A more pronounced reduction in oviposition by *T. rapae* in fungal infected hosts may thus be evident in more advanced stages of disease development.

In the dual choice experiment on host quality no *T. rapae* foraging among *M. brunneum* infected larvae succumbed to mycosis, as opposed to parasitoids exposed to *B. bassiana* infected larvae. This could be caused by reduced foraging time with *M. brunneum* infected larvae compared to *B. bassiana* infected hosts, but further direct behavioral observation studies are necessary.

The perception of IGP risk by *T. rapae* from *M. brunneum* but not from *B. bassiana* may relate to differences in cues emitted by the two fungi. However, these cues may be dependent on the context and complexity of the tested system which may not have been reflected by our experimental arenas. Additional studies should expand on the complexity of our system in order to provide a more complete volatile exposure.

For vegetable cruciferous crops, mixing entomopathogenic fungi into the substrate when raising plantlets in the greenhouse for subsequent transplanting to the field would be a convenient method for control of the inoculum levels applied. Chandler and Davidson (2005) and Razinger et al. (2014) found that this method provided some control of *D. radicum* using *Metarhizium* sp. Seed treatment may be another approach since Keyser et al. (2014)

found that seed treatment by *M. brunneum* (isolate KVL 04–57 as in this study) resulted in infection in insects exposed to the growing roots. These two methods would also take advantage of the endophytic and rhizosphere competent property of *Metarhizium* sp. (Sasan and Bidochka, 2012; Razinger et al., 2014; Wyrebek et al., 2011) in order for the fungi to preestablish before *D. radicum* attack.

5. Conclusions

This study demonstrated that the tested *M. brunneum* isolate is a promising biological control candidate against *D. radicum* larvae. Furthermore, *T. rapae* showed an ability to perceive and react to the IGP risk posed by *M. brunneum* while *B. bassiana* was not avoided to the same extent. Thus *M. brunneum* has the potential to be used for biological control against *D. radicum* with a low expected risk to *T. rapae* populations. The potentially complementary biological control effect against immature *D. radicum* by conservation biological control targeting *T. rapae* populations in combination with inoculation with *M. brunneum* must be studied under field conditions.

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